

Ethological foundations of measuring obsessive-compulsive phenotypes in the deer mouse: A methodological perspective

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Ethological foundations of measuring obsessive-compulsive phenotypes in the deer mouse: A methodological perspective

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This thesis is dedicated to **everyone** who helped me along the way.

Arina Fick-van der Merwe (17 January 1993 - 3 February 2019)

We met here at pharmacology and I even had the pleasure of sharing a publication with you.

Taken too soon.

May you rest in peace with your husband, Christiaan.

“One thing only I know, and that is that I know nothing.”

-Socrates

Abstract

Pre-clinical models which leverage the study of animal behavior are invaluable tools to advance our understanding of a variety of psychological constructs. Such ethological investigations allow us to gain insight into abnormal biological processes which may contribute to the provocation of abnormal cognitive patterns which in turn may develop into a variety of 'abnormal' behaviors. Such behaviors, if allowed to proceed unchecked, typically progress into pathological states which interfere with the typical functioning of individuals, then termed psychiatric disorders. An example of such a disorder is obsessive-compulsive disorder (OCD), the focal point around which the investigations in this thesis were conceptualized. OCD is typically characterized by disruptions in both cognition (obsessions) and behavior (compulsions) leading to significant impairment in both executive function and quality of life.

From an ethological perspective, OCD has been modelled in a handful of species applying several experimental frameworks and behavioral tests. Broadly speaking, these typically rely on the observation of repetitive behaviors and how they respond to established or prospective treatment strategies. As models become more established, investigations into the neurobiology of these modeled compulsive-like behaviors are initiated. Encouragingly, many analogous neurotransmission systems, receptors, brain regions and sub-circuits have been implicated in the expression of OCD-like behavior in these animal models, highlighting their translational relevance for the study of intricate psychiatric disorders such as OCD. However, a key short coming of such models remains the demonstration of the affective components of OCD, i.e. compulsions, within them.

The species employed in the current investigation is the deer mouse (*Peromyscus maniculatus bairdii*), a validated but still rapidly developing model of OCD which offers select key advantages. Firstly, the compulsive-like behaviors exhibited by these animals are entirely naturalistic, not requiring any specific breeding, genetic knockout, pharmacological or behavioral induction. This exemplifies an ideal model, since it permits the careful study of abnormal behaviors in a context which is as similar as possible to the human condition, since the behaviors in question presumably originate naturally due to some unique underlying disturbance. Furthermore, deer mice naturally present with three different types of compulsive-like behaviors. These include excessive, highly stereotypical movements such as back flipping, the building of excessively large nests (LNB), and occurrence of high marble-burying (HMB). The two former behaviors have been validated by showing a response to chronic escitalopram (an SSRI; 50 mg/kg/day) treatment. As such, the current investigation set out to continue the development of the deer mouse model of OCD, by studying HMB, albeit indirectly, by dissecting the marble burying test (MBT), the behavioral test widely applied to

test anti-compulsive and anti-anxiety drug action. This was done by means of an investigation into the methodological parameters of the test as well as a review of relevant literature pertaining to the application of the test. Secondly, LNB which represents a highly goal-directed behavior, ideal for the study of the motivational factors underlying compulsive murine behavior, was investigated in a novel experimental paradigm.

The dissection of the MBT presented here represents a 'back-to-basics' approach concerning ethological studies. The test itself is relatively straight-forward, requiring a number of marbles to be placed on a flattened layer of bedding material (typically wood shavings) inside a test cage. Rodents are then introduced to these prepared cages and allowed a certain amount of time to interact. The number of marbles which are buried beneath the surface after the test session are tallied as an index of compulsive-like behavior. While being widely applied in the scientific literature, the MBT is striking in terms of the lack of methodological congruence between different laboratories. Resultantly, we argue here that special attention should be paid to even the most seemingly insignificant details of even well-established behavioral tests. Indeed, by investigating the use of several different burying substrates of differing densities in the MBT, we show that such a manipulation can robustly influence the outcomes of the test. Furthermore, we highlight that the subjective manner in which the test is scored is indeed subject to inter-observer variability.

Following from the findings above, we performed a review of investigations in which the MBT was applied as the primary behavioral assay. Once again, it was highlighted that the test is employed with a wide range of experimental parameters in terms of the size of test cages, number of test repetitions, test duration, number and size of marbles, burying substrate and scoring criteria used. In terms of treatment response, the MBT reports anti-compulsive effects for a wide variety of drugs, some of which are not typically effective in the clinical setting. These findings are further complicated by the realization that many of these drugs, which require lengthy periods for onset of action, are administered acutely, often minutes before the test. Taking all of these findings into account, we argue for the standardization of many of the aforementioned variables, so as to improve the practical utility of the MBT as a screening test for anti-compulsive drug action.

Another behavior exhibited by certain species such as mice and rabbits, concerns the excessive, diligent building of nests. As is the case with the MBT, the concept underlying LNB as a test for compulsive-like behavior is relatively simple. In brief, examining LNB involves the provision of an excess of nesting material to the housing cages of mice and recording the quantity of material that is used by each subject over the course of several nights. Animals that consistently use excessive quantities of material as appraised against the behavior of the

larger population, are then selected for further investigation. Indeed, it has been shown that deer mice selected in this fashion show a reduction in nesting size following SSRI treatment. Since all mice that express LNB are housed under identical conditions to their normal counterparts, it stands to reason that there is some unique underlying dysfunction motivating the disinhibited building of nests.

Towards investigating this premise, cohorts of normal and LNB were selected and tested in a novel testing environment. Here, the mice were placed into cages containing automated nesting material dispensers. To acquire nesting material, mice would have to execute lever-presses. After mice had learned this action-outcome association, their behavior was tested under two unique circumstances. Firstly, the nesting material was withdrawn from the dispensers so that lever-presses no longer resulted in any outcome. In the second instance, the levers delivered a mild electric shock each time a lever-press was executed. Interestingly, in this novel environment, LNB mice generally executed more lever presses than their NNB counterparts, particularly during the two unique phases described above. Mice were retested under identical circumstances following 28-days of chronic escitalopram treatment, showing preliminary evidence that the motivational drive to engage in nesting behavior abates, as was evinced by the lower number of lever-presses that were executed during the punishment phase.

The study design above was intended to test whether LNB is characterized by unique cognition, inspired by the finding that OCD patients often show difficulty in changing their obsessive-compulsive behavioral routines, and that they are prone to endure negative consequences so that their compulsive rituals may be given attention. This represents a shift in ethological investigations, which in many cases rely primarily on appraising behavior at face-value, without attempting to divulge the cognitive factors which may play a role in the presence of such behaviors which are considered abnormal in the first place.

In conclusion, this thesis argues that ethological studies should be continually refined to improve the validity of the findings made, regardless of how simple the core premise of the given model or behavioral test may be. Furthermore, the foundational basis of ethological studies should be conceptualized bearing in mind that animal behavior is often more conscientious than many behavioral tests take into account, and that careful study design can leverage this to carefully investigate behaviors at a deeper level, which in turn can add to our knowledge of psychiatric disorders.

Keywords: Obsessive-compulsive disorder, deer mouse, ethology, marble-burying test, lever-pressing

Results from the current investigation were presented as follows:

- a) GEOFFREY DE BROUWER, DE WET WOLMARANS, BRIAN H. HARVEY (2018). Contextual lever-pressing in the deer mouse model of obsessive-compulsive disorder (OCD). World Congress of Pharmacology 2018 (WCP 2018), Kyoto, Kyoto Prefecture, Japan (1 - 6 July 2018).

Publications

- a) DE BROUWER, G., WOLMARANS, D., (2018). Back to basics: A methodological perspective on marble-burying behavior as a screening test for psychiatric illness. *Behavioural Processes*, 157:590-600.
- b) DE BROUWER, G., FICK, A., HARVEY, B.H., WOLMARANS, D., (2019). A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: Mapping the way forward. *Cognitive, Affective, & Behavioral Neuroscience*, 19(1):1-39.
- c) DE BROUWER, G., HARVEY, B.H., WOLMARANS, D., (2019). Naturalistic operant responses in deer mice (*Peromyscus maniculatus bairdii*) and its response to outcome manipulation and serotonergic intervention. *Behavioural Pharmacology*, Accepted for publication.

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1. Introduction

Abbreviations used in Chapter 1 (in order of appearance)

<i>OCD</i>	–	<i>obsessive-compulsive disorder</i>
<i>DSM</i>	–	<i>Diagnostic and Statistical Manual of Mental Disorders</i>
<i>O/C(s)</i>	–	<i>obsessive-compulsive/obsessions and compulsions</i>
<i>CSTC</i>	–	<i>cortico-striatal-thalamic-cortical</i>
<i>SRI(s)</i>	–	<i>serotonin reuptake inhibitor(s)</i>
<i>SSRI(s)</i>	–	<i>selective serotonin reuptake inhibitor(s)</i>
<i>CBT</i>	–	<i>cognitive behavioral therapy</i>
<i>HMB</i>	–	<i>high marble-burying</i>
<i>LNB</i>	–	<i>large nest building</i>
<i>NNB</i>	–	<i>normal nest building</i>
<i>NB</i>	–	<i>nest building</i>
<i>MBT</i>	–	<i>marble-burying test</i>

1.1. Thesis Layout

The current thesis is compiled in article format, as prescribed by the North-West University guidelines. As such, the main body of the thesis is presented as 3 manuscripts, two of which have already been published in accredited international journals and one of which is preliminarily accepted for publication.

Chapter 1 will present a summary of the problem statement in broad terms, study questions and aims, expected outcomes and a basic framework for the study progression. Chapter 2 will contain the literature study conducted to support the entirety of the study. Chapters 3, 4 and 5 will present key findings of the project in three separate published articles (Chapters 3 and 4) or as a scientific manuscript (Chapter 5). These were formatted according to the 'Instructions to Authors' provided by each of the relevant journals. Chapter 6 comprises a summary of the key findings and conclusions made from the study as a whole.

Addendum A contains letters of approval from co-authors for manuscripts A – C to appear in this thesis. Addendum B contains confirmation letters pertaining to the publication of Manuscripts A – C. Preliminary results of an ongoing 5-HT_{1A} receptor-binding study are contained in Addendum C. Addenda D – F contains the reviewer comments and the candidate's detailed responses with respect to the publication of Manuscripts A – C respectively. Addenda G and H contain two additional papers that the candidate co-authored during the time he was working on this thesis and are provided as supplementary evidence of the broad scientific experience of the candidate. Thus, Addenda C – H are not intended for examination purposes per se but serve as evidence of the activity of the candidate during the degree and the experience he has gained.

Since each of the three primary manuscripts were submitted to three unique journals, each is prepared and presented according to the prescriptions of each journal. Chapters 1, 2 and 6 are prepared according to the referencing style of *Behavioural Processes*, the journal in which the first article (Chapter 3) was published. The additional articles presented in Addenda G and H are formatted according to the journals where they are or will be submitted.

Since all three manuscripts were prepared using US English, the entire thesis was formatted as such.

* * *

1.2. Problem Statement

Animal models of human psychiatric conditions are essential tools that can potentially allow for a deeper insight into the underlying pathophysiology and cognitive abnormalities observed in the clinical conditions (Willner, 1986; Nestler and Hyman, 2010; Campos et al., 2013). The current study continues to build upon and critically dissect the translational application of the deer mouse (*Peromyscus maniculatus bairdii*) model of obsessive-compulsive disorder (OCD) and therefore stems from a number of prior studies undertaken in our laboratory (Korff et al., 2008, 2009; Guldenpfennig et al., 2011; Wolmarans et al., 2013; Wolmarans et al., 2016a, 2016b, 2017).

Obsessions can be described as intrusive, repetitive thoughts occupying the mind of the patient. This often causes context-specific anxieties that can mostly be directly related to the content of the obsession (Abramowitz and Jacoby, 2015; Gillan and Sahakian, 2015). Such obsessions often, but not always, result in compulsions, i.e. repetitive motor or cognitive patterns, purportedly aimed at relieving the level of anxiety experienced. In fact, OCD was previously classified as an anxiety disorder (American Psychiatric Association, 1994) prior to the publishing of the latest version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), in which it is currently included as the archetypal disorder in a new group of 'obsessive-compulsive and related disorders' alongside trichotillomania (hair-pulling), body dysmorphic disorder, hoarding and excoriation (skin-picking) disorder (American Psychiatric Association, 2013).

With respect to the framework in which the current investigation was conducted, it is important to note that OCD is clinically heterogeneous (Abramowitz et al., 2009; Markarian et al., 2010; Rowsell and Francis, 2015) and that OCD patients often present with obsessions and/or compulsions relating to highly specific themes, accounting for the various subtypes of the disorder. As such, various classifications have been developed which best describe the major symptom subtypes observed in OCD. The most widely accepted classification system identifies five distinct obsessive-compulsive (O/C) subtypes, i.e. 1) harm prevention obsessions with checking and reassurance seeking as the associated compulsions, 2) symmetry obsessions associated with ordering and/or counting compulsions, 3) contamination obsessions associated with washing compulsions, 4) repugnant obsessions associated with covert themes of violence, sex and religion, and 5) hoarding obsessions associated with 'collecting' compulsions (Abramowitz et al., 2009; Markarian et al., 2010); however, with respect to the latter, hoarding has, since the publication of the DSM-V, been afforded unique illness status (American Psychiatric Association, 2013).

OCD neurodysfunction is thought to primarily arise due to disruption in cortico-striatal-thalamic-cortical (CSTC) circuit function (Wood and Ahmari, 2015). This is noted by abnormal activation of certain components of the CSTC circuit during symptom provocation and subsequent restoration to baseline levels following successful treatment (Menziés et al., 2008; Harrison et al., 2013). The CSTC circuits and their various constituents regulate a number of neurocognitive domains and processes, including but not limited to error processing (Milad and Rauch, 2012), reward processing (Jung et al., 2013), punishment learning (Morein-Zamir et al., 2012; Palminteri et al., 2012), motor pattern expression (Nambu, 2008), learning and cognition (Di Filippo et al., 2009), all of which are believed to contribute to the expression of OCD-like behaviors to some extent (Nambu, 2008; Gillan et al., 2016; Gruner and Pittenger, 2017; Vaghi et al., 2017) and some of which will be examined in Chapter 5. Considering that the CSTC circuitry is well supplied with serotonergic and dopaminergic input, originating primarily from the raphe nuclei (Nolfe et al., 1998; Hornung, 2003) and substantia nigra respectively (Nicola et al., 2000; Nambu, 2008) and that OCD is characterized by hyposerotonergic and hyperdopaminergic signaling (Westenberg et al., 2007; Markarian et al., 2010), the rationale for targeted pharmacotherapy is revealed. Indeed, focal concepts of treatment are directed towards facilitating serotonergic neurotransmission (Hirschtritt et al., 2017), which over time modifies the expression of specific serotonin receptors, such as 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} which are able to modify the outflow of serotonin and dopamine (Barnes and Sharp, 1999; Goddard et al., 2008). The hyperdopaminergic state is also addressed by the use of dopamine receptor antagonists, particularly in treatment resistant cases (Atmaca, 2016; Hirschtritt et al., 2017). Both of these treatments are thought to correct unbalanced neurotransmission which restores appropriate functioning to the CSTC circuitry (Goddard et al., 2008; Nambu, 2008), thereby correcting the abnormal cognition, feedback learning and behavioral control which potentially cause OCD-like behaviors.

With respect to specifics of pharmacological intervention, patients predominantly respond to chronic high dose treatment with selective serotonin reuptake inhibitors (SSRIs; Pampaloni et al., 2010; Albert et al., 2013; Stewart, 2016). Indeed, the therapeutic response of OCD to any class of serotonin reuptake inhibitor (SRI) requires up to eight or more weeks of uninterrupted treatment (Fineberg et al., 2013; Hirschtritt et al., 2017). Such a slow onset of action is thought to entail long term changes in post-synaptic receptor function in response to prevailing increases of synaptic serotonin concentrations (El Mansari and Blier, 2006; Goddard et al., 2008). This increased activity of serotonin is also responsible for moderating hyperactive dopaminergic pathways, another keystone issue, albeit highly debated, within the context of OCD (Westenberg et al., 2007; Koo et al., 2010). Although a number of case reports indicate that some phenotypes of OCD may respond better to diverse treatments including agents that

modulate excitatory amino acid, e.g. glutamate, signaling (Coric et al., 2003; Coric et al., 2005; Grant et al., 2007; Hirschtritt et al., 2017), SSRIs remain the mainstay of pharmacological therapy. In addition to this, SSRIs also are seemingly able to improve learning from punishing feedback, a cognitive failure which is thought to be central to the development of OCD-like behavior (Palminteri et al., 2012) as will be investigated in Chapter 5. That said, treatment response remains inadequate, with up to 30 - 40% of patients remaining refractory to SSRIs *and* all other available treatment modalities (Albert et al., 2013; Atmaca, 2016), including augmentation of SSRI therapy with low dose anti-dopaminergic drugs (Albert et al., 2013; Fineberg et al., 2013) and cognitive behavioral therapy (CBT; Whittal et al., 2005; Anand et al., 2011; Simpson et al., 2013). Nevertheless, this is understandable, as a significant body of information pertaining to the manifestation of OCD remains to be elucidated. This includes elucidation in terms of the neurobiological etiology (Milad and Rauch, 2012; Wood and Ahmari, 2015) and neuropsychological mechanisms (Shin et al., 2014; Gruner and Pittenger, 2017) underlying the disorder.

As alluded to earlier, animal models of OCD are valuable frameworks in which to investigate the phenomenology of OCD. Indeed, a number of animal models of OCD at different stages of development exist; these have been reviewed elsewhere (Albelda and Joel, 2012; Alonso et al., 2015). Although each is representative of a unique behavioral phenotype, they have in common their reflection of mostly singular compulsive-like behavioral traits which are often borne from some behavior-provoking intervention or training component and which normally responds to drugs known to be effective in clinical OCD (Albelda and Joel, 2012; Alonso et al., 2015; Ahmari, 2016). While it remains difficult to demonstrate the cognitive, i.e. motivational/obsessional components of O/C-like animal behavior, accurate models that may also be representative of the neurocognitive constructs underlying OCD, may provide greater insight into the association between obsessions and compulsions, whether they be related to causality or manifesting as different expression of the same underlying disturbances.

Considering the deer mouse as a species in which to study O/C-like phenotypes, previous studies undertaken in this laboratory collectively aimed to appraise and validate various distinct, but equally repetitive, persistent and seemingly purposeless behaviors, i.e. deviating from the norm as displayed by the majority of subjects in any specific laboratory-housed deer mouse colony. Said behaviors can all be regarded as spontaneously occurring, yet extreme, manifestations of otherwise normal rodent behaviors and include spontaneous motor stereotypy (Powell et al., 1999; Korff et al., 2008, 2009; Guldenpfennig et al., 2011; Wolmarans et al., 2013), high marble-burying (HMB) as a result of excessive digging (Weber and Hoekstra, 2009; Wolmarans et al., 2016b), and excessively large nest-building (LNB; Wolmarans et al., 2016a; Lewarch and Hoekstra, 2018). Whereas stereotypy better

resembles the compulsive symptoms of OCD, i.e. the seemingly purposeless repetition of motor patterns (American Psychiatric Association, 2013; Wolmarans et al., 2013), HMB and LNB, given their goal-directed preoccupation with seemingly functional outcomes, may, by virtue of their goal-directed nature, potentially reflect the neurocognitive abnormalities that are proposed to underlie the clinical illness (Hoffman and Morales, 2009; Gillan and Robbins, 2014; Banca et al., 2015; Scheepers et al., 2018). Nevertheless, putative evidence for unique cognition (which is a key focus of the current thesis) in low and high stereotypical deer mice respectively was also recently presented as evinced by differences in the social appraisal of and interactions with animals of a different stereotypical cohort (Wolmarans et al., 2017). Therefore, stereotypy in itself cannot be regarded as a phenotype borne exclusively from and only related to aberrant motor disturbances.

That said, the current investigation aims to elaborate on HMB (indirectly, by focusing on the *methodology* of the test used to assess such behavior, the marble-burying test; MBT) and LNB as O/C-like phenotypes which *may* be founded in aberrant cognition, i.e. the obsession-related motivational drive to be preoccupied with non-reactive objects on the one side, and to build excessively large nests on the other (Greene-Schloesser et al., 2011; Wolmarans et al., 2016b). Indeed, 11 – 15% of the deer mouse breeding colony express HMB (Wolmarans et al., 2016b), while 30% of the animals express LNB behavior (Wolmarans et al., 2016a). Moreover, both phenotypes manifest irrespective of sex. Hence, following the initial characterization of HMB and LNB, the current thesis is also orientated towards the questions of ethological relevance and methodological integrity that are, or should be, pivotal to pre-clinical investigations. In fact, as both digging or burying and nest building (NB) are part of the normal behavioral repertoire of rodents, the question arises: How should these behaviors then be applied, as they often are, as frameworks in which to study *abnormal* behaviors which may be emblematic of an underlying psychopathology? In other words, based on which principles can HMB and LNB be regarded as behaviors that constitute deviations from the norm? This question is important as it relates to our perspectives on animal behaviors that are put forward as ‘valid’ paradigms in which to study abnormal psychobiological processes, which if studied *appropriately*, should allow us to broaden our insight into psychiatric illness. This will be the core focus of the current project.

The MBT has been used extensively as both a model for O/C- or anxiety-like behavior *and* as a screening test for anti-compulsive (Egashira et al., 2007; Egashira et al., 2013; Taylor et al., 2017; Egashira et al., 2018) and anxiolytic (Broekkamp et al., 1986; Kedia and Chattarji, 2014) drug action. As generally published, the test generally only relies on the quantification of digging or ‘burying’ activity that is purportedly directed towards non-reactive glass marbles. Briefly, animals which are naive with respect to previous marble exposure and which have

typically not been subjected to any preceding intervention apart from treatment, are introduced to test cages prepared with a 5 cm-thick layer of bedding material on which any number of marbles are arranged. Subjects are then allowed to explore the cage and interact with the marbles where after the number of marbles that have been 'buried' is quantified as a measure anti-compulsive or anxiolytic drug action. However, a core focus of the current thesis is the methodological variance, which up until this point in time has pervaded studies of MB behavior, ultimately undermining its value, usefulness, and relevance as an appropriate pre-clinical framework. In fact, while it can be expected that MBT results will differ as a function of strain (Thomas et al., 2009), genetic knockout (Burne et al., 2005), behavioral intervention (Kedia and Chattarji, 2014) and treatment (Taylor et al., 2017), it is its variance in delivering results when applied *within what is supposed to be the same context* (Çaliskan et al., 2017) that is worrisome; such variance is afforded great attention in the current work. In fact, while a number of O/C- and anxiety-related conclusions are often drawn from MB investigations, the present thesis will show and argue that in general, the MBT as historically reported, is an inappropriate and inadequate measure of both O/C- and anxiety-like behavior or as a screening test for anti-compulsive and anxiolytic drug action. While digging, burrowing and burying form part of the normal behavioral repertoire of rodents, research in general, except works which investigate MB activity after some form of compulsive-inducing or anxiogenic intervention, do not distinguish truly compulsive- or anxiety-like burying phenotypes from normal behavior. Further, we will demonstrate the influence of methodological inconsistencies which have been intrinsic to the test on scoring outcomes (**Chapter 3**), while arguing that a contextually valid and standardized approach with respect to future investigations employing the MBT, will be paramount in our efforts to further our understanding of OCD (**Chapter 4**).

Apart from appraising the MBT as tool for the appropriate investigation of HMB, the current investigation will also focus on LNB as an abnormal manifestation of an otherwise normal rodent behavior. In fact, while it is known that rodents build nests of various sizes for reasons that include safety and temperature control (Jirkof, 2014; Stewart and McAdam, 2017), various authors have hypothesized that the building of nests notably excessive in size may resemble O/C-like behavior (Hoffman and Morales, 2009; Greene-Schloesser et al., 2011; Wolmarans et al., 2016a). Indeed, from a teleonomic perspective (Thornhill, 1996), LNB can be regarded as a maladaptation in a specific component of the normal behavioral repertoire of deer mice as excessively large nests expressed in the laboratory serve no unique purpose. Appraised against the background of evolution, nesting size and quality play a major role in mate choice and reproductive success in many species, e.g. birds (Holveck and Riebel, 2009), fish (Jamieson, 1995), and frogs (Felton et al., 2006). However, this is not true for mice, which generally exercise mate choice by random or on the basis of amongst others, dominance,

probability of genetic success, overall health, or patterns of ultrasonic vocalization (for a detailed review, see Latham and Mason (2004). Further, that both male and female deer mice engage in LNB (Wolmarans et al., 2016a), also excludes the likelihood of sex-related differences in nesting phenotype. Therefore, it is likely that excessive nest-building is expressed at the cost of other functions for which effort, time and energy is required, and may thus be regarded as a naturalistic maladaptation (Crespi, 2000). From an O/C perspective, it follows to note that LNB is sensitive to chronic (4-week), high dose (50 mg/kg/day) escitalopram (an SSRI) treatment (Wolmarans et al., 2016a), and manifests in a persistent manner. Therefore, while LNB is, like OCD (Stein, 2000; Westenberg et al., 2007; Koo et al., 2010), associated with aberrant serotonergic signaling (Winter et al., 2018), it may also potentially be explanatory of the associations between compulsive-like motor patterns and cognitive involvement, such as inappropriate punishment learning (see section 2.3.3; Morein-Zamir et al., 2012; Palminteri et al., 2012). Indeed, considering the fundamental motivational constructs for nesting alluded to above, LNB may well be related to *abnormal* concerns about safety or perfectionism that may in fact not underlie other forms of compulsive-like behavior, e.g. spontaneous stereotypy; such thematic differences are in fact in line with the clinical picture of OCD (Exner et al., 2014; Wang and Zhanjiang, 2017). For example, OCD in humans is often typically characterized by an internal feeling that specific environmental stimuli are “*not just right*”, resulting in repetitious corrective actions until this feeling is diminished (Coles et al., 2003; Ghisi et al., 2010). Furthermore, obsessions and compulsions in general are thought to arise from a discrepancy between what is perceived and what is accepted as impossibly strict personally held standards (Wu and Cortesi, 2009). As such, patients often engage in excessive repetitious actions to balance this discrepancy in the pursuit of perfection or even the *perfect feeling* of accomplishing a task. Such engagement often transpires without a clear endpoint since patients are overwhelmed by the aforementioned sense of unease, which due to its implicit, inflated and intrusive nature, is unlikely to be moderated by ‘corrective’ action (Abramowitz and Jacoby, 2015). Apart from being a putative murine behavioral correlate for perfectionism, LNB may also be reflective of threat over estimation and executive bias towards threatening stimuli that are often seen in clinical OCD (Exner et al., 2014; Wang and Zhanjiang, 2017). In this regard, patients are typically overwhelmed by thoughts that their actions may result in catastrophic outcomes leading to profound emotional responses and neutralizing strategies (Rachman, 2002; Exner et al., 2014), e.g. engaging in excessive checking or harm-prevention compulsive routines (Rachman, 2002). With respect to the focus of the current thesis on the ethological and methodological relevance of studying otherwise normal rodent behaviors with the intent of broadening our understanding of psychiatric illness, it is important to emphasize that such compulsions are triggered in highly specific contexts only and that, in the absence of such triggers and compulsions, patients often demonstrate

normal executive functioning (Olley et al., 2007; Exner et al., 2014; Morein-Zamir et al., 2014). As such, the current study will, in addition to its critical appraisal of the MBT, aim to elaborate on LNB as a unique O/C-like behavioral phenotype in the deer mouse model of OCD that may be reflective of the unique cognitive processes underlying clinically heterogeneous OCD.

* * *

Therefore, in working towards a better understanding of clinically heterogeneous OCD, we will 1) investigate (**Chapter 3**) and critically evaluate (**Chapter 4**) a current framework and method that is employed to probe O/C-like behaviors, i.e. the MBT, 2) investigate whether LNB expressing animals present with a unique motivational drive to execute such behaviors, compared to animals that build nests of 'normal' sizes, viz. normal nest building (NNB) animals, by means of applying contextual punishment (**Chapter 5**).

* * *

1.3. Study Questions

Drawing from the above, the current study aims to answer the following questions:

Study Questions		Key References
<u>Manuscript A – Experimental Paper</u>		
1.1	How does the marble burying performance of deer mice and the quantification thereof respond when tested repeatedly under circumstances of methodological manipulation?	(Thomas et al., 2009; Wolmarans et al., 2016b)
1.2	How is MBT scoring influenced by observer bias?	(Angoa-Pérez et al., 2013; Egashira et al., 2018)
1.3	What constitutes <i>abnormal</i> behaviors beyond a <i>normal</i> behavioral repertoire?	(De Boer and Koolhaas, 2003; Eilam et al., 2006)
<u>Manuscript B – Critical Review</u>		
2.1	Critical review of MBT literature across <u>all</u> years up until 2017 containing the words <i>marble</i> and/or <i>burying</i> , in the title with respect to the reported methodology, pharmacological interventions and conclusions.	(Broekkamp et al., 1986; Nicolas et al., 2006; van der Staay, 2006; Thomas et al., 2009; Drucker, 2016; Çaliskan et al., 2017)
2.2	Is the MBT as reported adequate, relevant and accurate for studies into O/C- or anxiety-like behavior? If not, what are the major aspects that undermine its usefulness?	(Njung'e and Handley, 1991; Nicolas et al., 2006; Thomas et al., 2009; Taylor et al., 2017)
2.3	How should studies that apply the MBT be conceptualized, applied and interpreted in future in order to elaborate on our understanding of OCD and anxiety?	(Schneider and Popik, 2007; Llaneza and Frye, 2009; Umathe et al., 2012; Egashira et al., 2018)

Table and study questions continue on the next page...

<u>Manuscript C – Experimental Paper</u>	
3.1 Will differences in lever-pressing behavior directed at the acquisition of nesting material exist between animals selected for NNB and LNB?	(Szechtman and Woody, 2004; Joel, 2006; Hoffman and Morales, 2009; Nielen et al., 2009; Morein-Zamir et al., 2012; Palminteri et al., 2012)
3.2 Will the lever-pressing behavior of NNB and LNB expressing animals differ under circumstances of reward (nesting material) withdrawal and contextual punishment (by means of footshock upon lever-press)?	(Nielen et al., 2009; Morein-Zamir et al., 2012; Exner et al., 2014; Winter et al., 2018)
3.3 Will chronic high dose oral escitalopram modify the behavioral responses of LNB, but not NNB animals as potentially highlighted in 3.1 and 3.2?	(Joel, 2006; Hoffman and Morales, 2009; Greene-Schloesser et al., 2011; Wolmarans et al., 2016a)

* * *

1.4. Project Aims

To address study questions 1.1 – 1.3, we aimed to:

- Dissect the MBT with respect to all aspects of its methodology, i.e. burying substrate, zone configuration, number of marbles used, repeated exposure, scoring (observer) procedure configuration;
- Identify and describe appropriate conditions for the execution of the MBT that will be representative of a preoccupied burying phenotype, thereby preventing misinterpretation and dubious conclusions made from MB data with respect to its resemblance of compulsive-like behaviors.
- **Importantly:** As study question 1 concerned a methodological dissection of the MBT as a valid screening tool to highlight high burying behavior, we did not include mice based on what we *believed* to be normal or HMB phenotypes. Instead, all deer mice used in this phase of the study, irrespective of sex, were screened for burying behavior. In fact, the inclusion of animals which we believed to express HMB only, would be counterproductive and undermine the purpose of this specific aim.

To address study questions 2.1 – 2.3, we aimed to:

- Examine available literature up until 2017 that applied the MBT to screen for compulsive- or anxiety-like behavior or for anti-compulsive and anxiolytic drug action as the primary objective of the investigation, with respect to 1) the methods applied, 2) the pharmacological interventions employed and 3) the conclusions made;
- Highlight key issues for consideration when performing the MBT and provide a recommended framework for future MB investigations, based on the pitfalls and possible experimental inconsistencies highlighted earlier.

To address study questions 3.1 – 3.3, we aimed to:

- Classify deer mice of both sexes into NNB and LNB cohorts;
- Conceptualize and design and testing of an automated nesting material dispenser that can be inserted individually into deer mouse housing cages (see also Addendum F);
- Determine whether NNB and LNB animals present with inherent differences in learning the associations between lever-pressing and obtaining nesting material;
- Characterizing and quantifying the lever-press responses executed by LNB animals compared to their NNB counterparts;

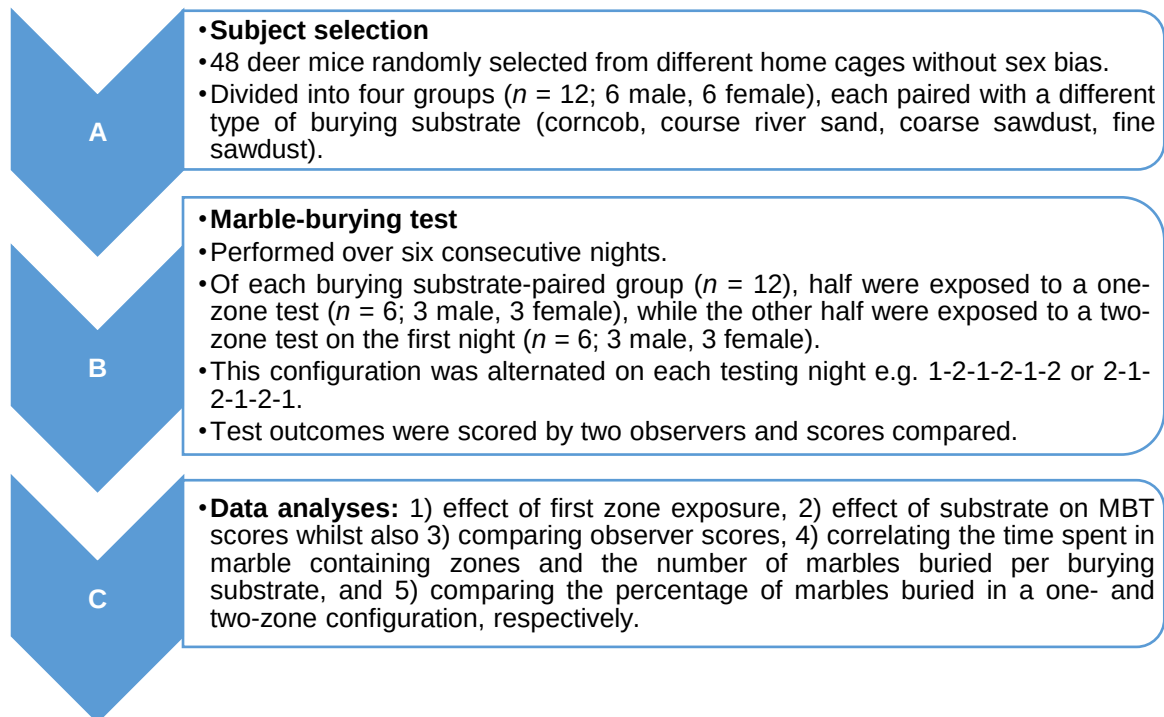
- Determine whether LNB, but not NNB animals will persistently execute lever-press responses following the sudden removal of the rewarding outcome, viz. the nesting material, after they had acquired the response-outcome contingency between lever-presses and nesting material;
- Determine whether the number of lever-presses executed by LNB animals are differentially influenced by contextual punishment, viz. mild electric foot shock upon pressing for nesting material, when compared to the number executed by NNB animals and
- To establish whether lever-pressing, and its response to outcome withdrawal and punishment are sensitive to chronic (28-day) high dose oral escitalopram treatment, i.e. 50 mg/kg/day.

1.5. Project Layout

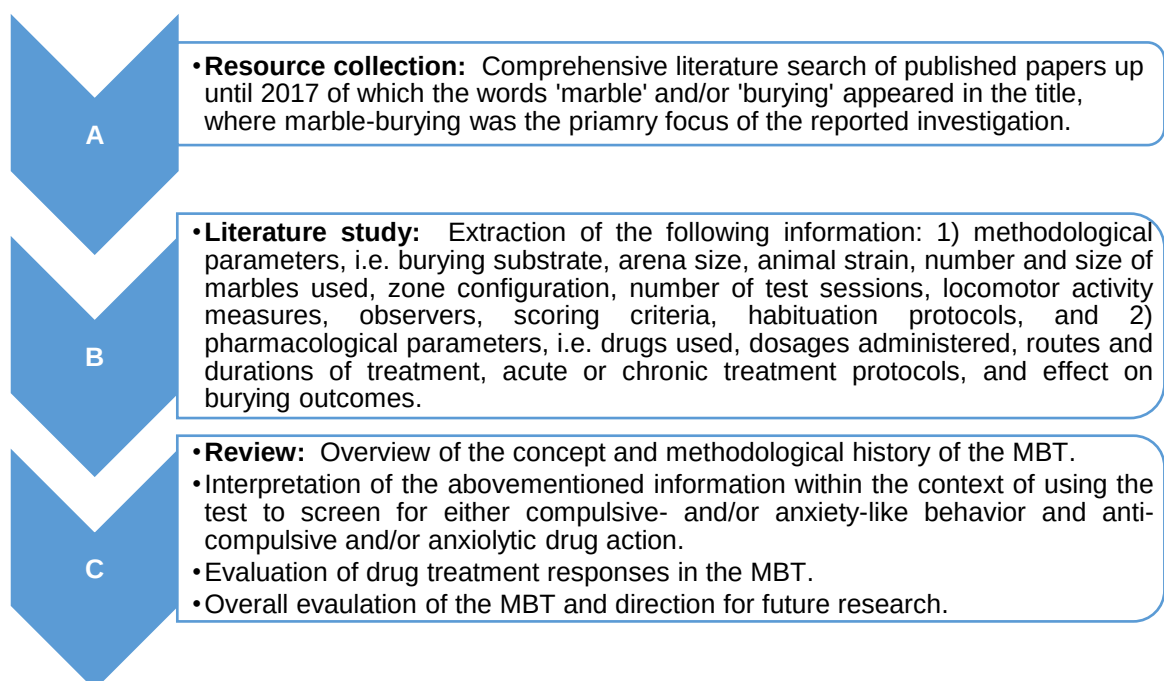
To answer the study questions as described in paragraph 1.3, the current project was divided into three major phases, namely:

- 1) A behavioral investigation to scrutinize and dissect the MBT as it is influenced by significant and elaborate methodological manipulation;
- 2) A literature review of the MBT and critically evaluating its usefulness as a screening tool for compulsive- and anxiety-like behaviors or as a screening test that may be reflective of anti-compulsive and anxiolytic drug action;
- 3) A behavioral investigation to assess the response of NNB and LNB animals in a contextually designed response-outcome scenario where lever-presses result in the dispensing of nesting material. Further, this phase will also characterize the response of NNB and LNB animals to reward withdrawal as well as self-administered punishment, viz. electrical foot-shock, upon the retrieval of nesting material and how such responses adapt as a function of chronic high dose oral escitalopram;

1.5.1. A Methodological Investigation of the Marble-Burying Test (Manuscript A)

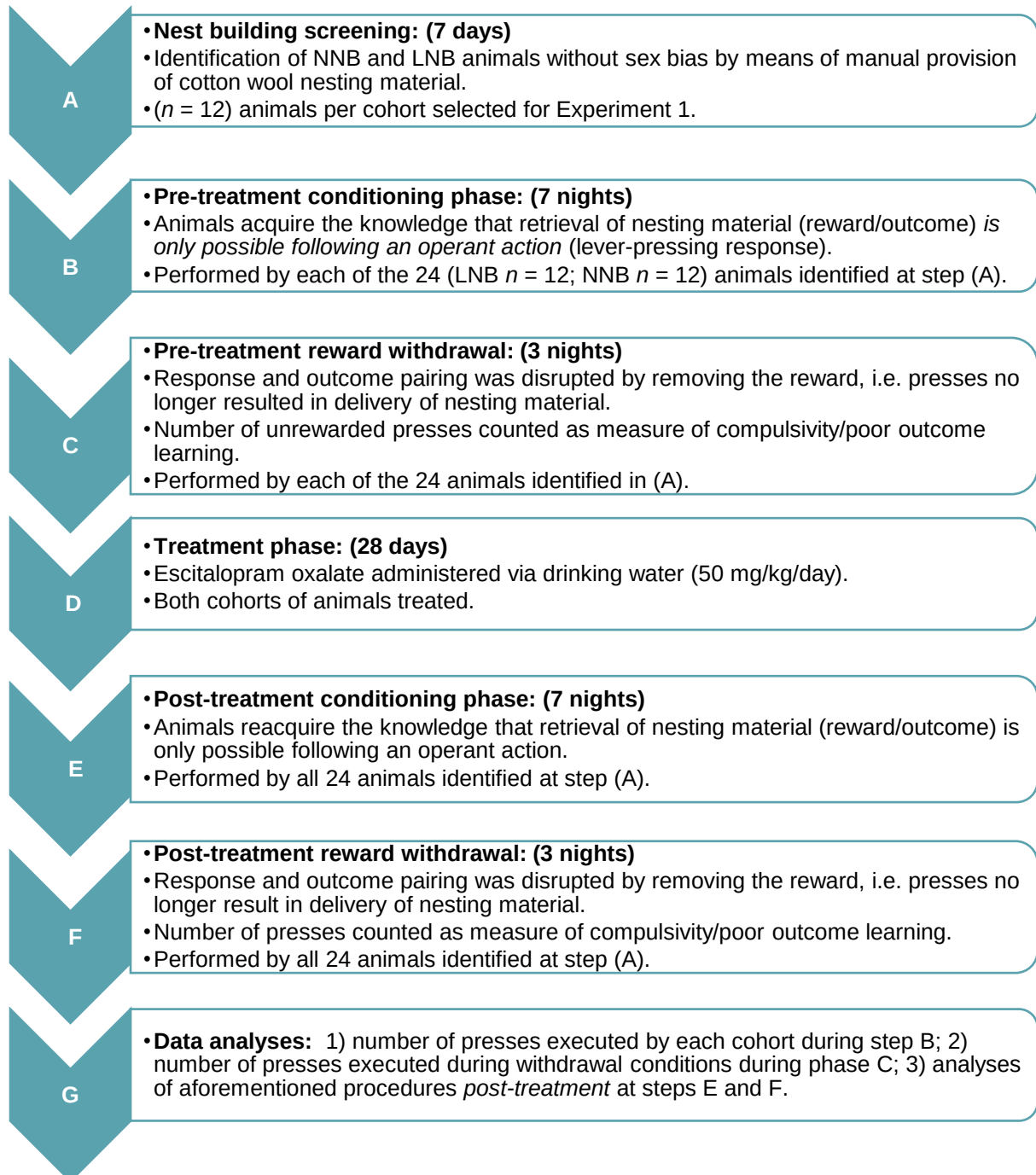


1.5.2. A Critical Review of Current Literature Employing the Marble-Burying Test (Manuscript B)

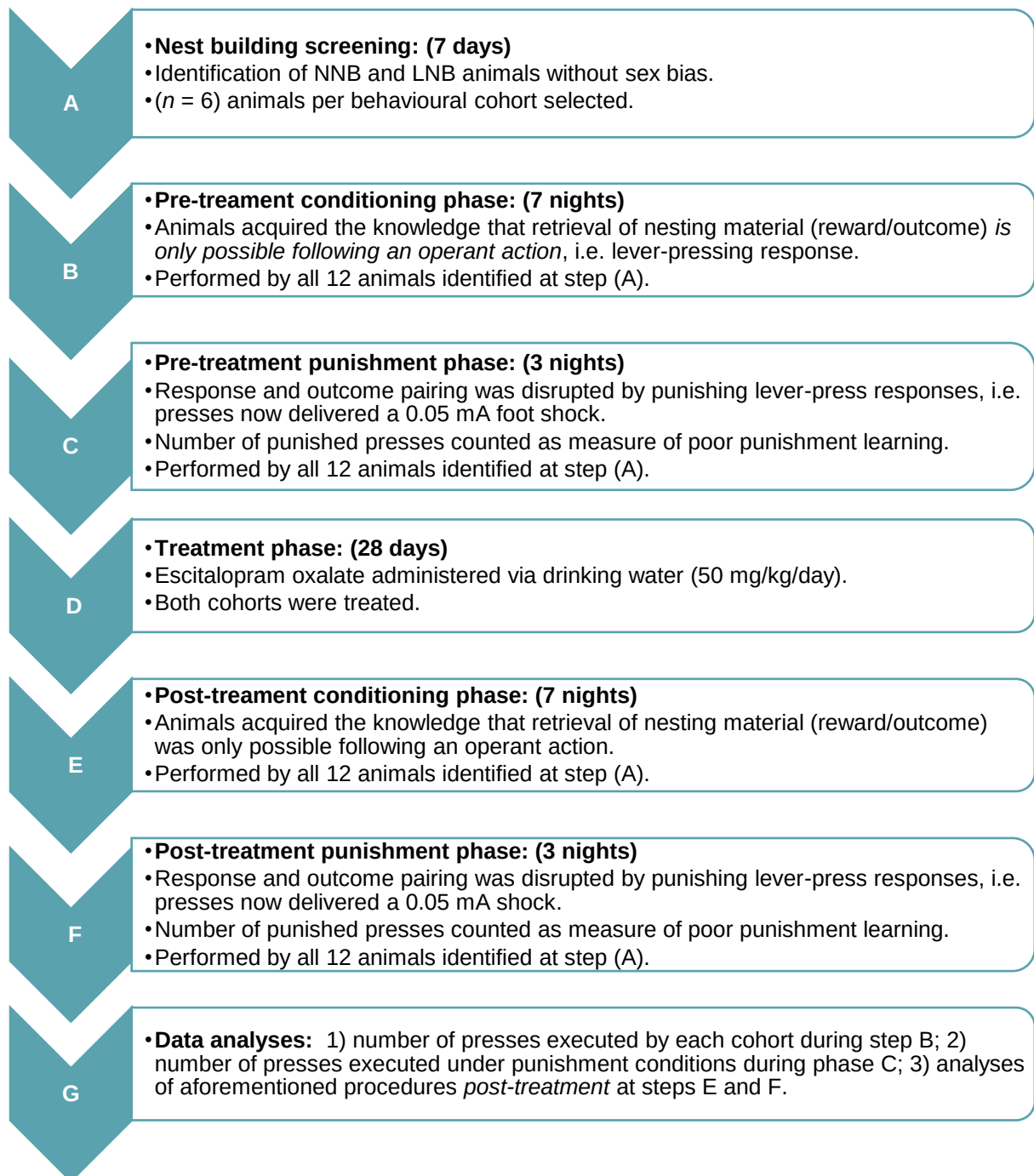


1.5.3. Lever-Pressing Nest Building Study (Manuscript C)

1.5.3.1. Compulsivity Investigation (Experiment 1)



1.5.3.2. Punishment Learning Study (Experiment 2)



1.6. Expected Outcomes

Study Question	Expected Outcome
1.1	Based on a thorough review of the literature, we expect that under circumstances of methodological manipulation, the MBT as applied in deer mice over the course of six successive sessions, will yield different results as a function of the various manipulations. We therefore expect to highlight the influence of within- and between-laboratory methodological variance on burying outcomes, and hence also on the conclusions drawn from MB investigations.
1.2	Based on the fact that substrates of various density and particle size may influence perceptions of what constitutes a buried marble, we expect observer scores to vary between different observers over various MB trials and as a function of burying substrate.
1.3	In terms of what would constitute <i>abnormal</i> burying behaviors with respect to OCD, we hypothesize that, since digging, burrowing and burying are natural rodent behaviors, all subjects in the current investigation will express burying behavior to a certain extent. We further hypothesize, that in the case of truly compulsive-like burying behavior, such animals will persistently engage in excessive burying activity, relative to what is expressed by the larger population over successive trials as identified against the background of the normal distribution of burying scores.
2.1 – 2.3	In this critical review of the MBT as it has been and still is applied, we aim to describe the experimental and conceptual caveats that are characteristic of MB investigations and how these relate to compulsive- and anxiety-like behaviors. Further, we will conclude by identifying a number of methodological shortcomings as they appear in literature, which collectively undermine the value of the test for application in preclinical neuropsychiatric research. Further, based on this literature review, we will provide a directive and recommendations for future research employing the MBT.

3.1	It is expected that while both NNB and LNB animals will acquire suitable knowledge of the response (lever-press)-outcome (nesting material) contingency, LNB animals will demonstrate a superior motivational drive for excessive nesting behavior as reflected by their persistent expression of lever-pressing.
3.2	We further expect that animals expressing LNB behavior will be slower reacting to nesting material withdrawal and contextual punishment viz. electrical foot shock, compared to animals expressing NNB, which are expected to terminate nesting activity once nesting material is withdrawn and punishment is inflicted, hence reflecting a persistent and intrusive drive to satisfy the obsession to engage in nesting behavior, irrespective of the lack of outcome or negative consequences of such behavior.
3.3	We expect SSRI therapy to improve goal-directed feedback and punishment-based learning, i.e. animals expressing LNB will adjust to nesting material withdrawal and punishment quicker than before treatment. As such, animals will either stop engaging in lever-pressing behavior in the case of withdrawal or adapt their behavior to build smaller nests in the case of punishment. We do not expect escitalopram to modify the behavior of NNB animals.

1.7. Ethical Approval

The current investigation was approved by the AnimCare Research Ethics Committee (NHREC registration number AREC-130913-015) of the NWU, approval number: NWU-00261-16-A5. The ARRIVE-guidelines for animal experimentation were followed as closely as possible (Kilkenny et al., 2010).

All animals were bred and housed at the PCDDP Vivarium of the North-West University, Potchefstroom Campus (SAVC registration number FR15/13458; SANAS GLP compliance number G0019). All procedures performed were done so in accordance with the code of ethics and complied with national legislation (South African National Standard for the Care and Use of Animals for Scientific Purposes; SANS 10386:2008).

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2. Literature Review

Abbreviations used in Chapter 2 (in order of appearance)

OCD	–	<i>obsessive-compulsive disorder</i>
O/C(s)	–	<i>obsessions and compulsions/obsessive-compulsive</i>
DSM-V	–	<i>Diagnostic and Statistical Manual of Mental Disorders</i>
Y-BOCS	–	<i>Yale-Brown Obsessive-Compulsive Scale</i>
QOL	–	<i>quality of life</i>
5-HT	–	<i>serotonin/5-hydroxytryptamine</i>
SERT	–	<i>serotonin reuptake transporters</i>
SSRI(s)	–	<i>selective serotonin reuptake inhibitor(s)</i>
DA	–	<i>dopamine</i>
LNB	–	<i>large nest building</i>
NNB	–	<i>normal nest building</i>
CSTC	–	<i>cortico-striatal-thalamo-cortical</i>
ACC	–	<i>anterior cingulate cortex</i>
PFC	–	<i>prefrontal cortex</i>
STR	–	<i>striatum</i>
OFC	–	<i>orbitofrontal cortex</i>
NAc	–	<i>nucleus accumbens</i>
CN	–	<i>caudate nucleus</i>
SN	–	<i>substantia nigra</i>
SNr	–	<i>substantia nigra pars reticulata</i>
SNC	–	<i>substantia nigra pars compacta</i>
GABA	–	<i>gamma-aminobutyric acid</i>
GPI	–	<i>globus pallidus interna</i>
GPe	–	<i>globus pallidus externa</i>
STN	–	<i>subthalamic nucleus</i>
dACC	–	<i>dorsal aspect of the anterior cingulate cortex</i>
mOFC	–	<i>medial orbitofrontal cortex</i>
lOFC	–	<i>lateral orbitofrontal cortex</i>
cAMP	–	<i>cyclic adenosine monophosphate</i>
PLC	–	<i>phospholipase C</i>
HMB	–	<i>high marble-burying</i>
RL	–	<i>reward learning</i>
PL	–	<i>punishment learning</i>
RI	–	<i>response inhibition</i>
JRE(s)	–	<i>just right experience(s)</i>
NJRE(s)	–	<i>not just right experience(s)</i>

- H* – *highly stereotypical cohort*
N – *no to low stereotypical cohort*

2.1. Obsessive-Compulsive Disorder

2.1.1. Classification, Description, Diagnosis and Comorbidity

Obsessive-compulsive disorder (OCD) is a neuropsychiatric disorder rooted in genetic predisposition and recognizable by alterations in neurotransmission as well as deviations in neuroanatomical, neuropsychological and cognitive processes (Goddard et al., 2008; Markarian et al., 2010; Wood and Ahmari, 2015; Gruner and Pittenger, 2017). At a most fundamental level, these perturbations manifest as obsessions and/or compulsions (O/Cs). Obsessions are defined as repetitive, prolonged thoughts of an intrusive nature, or any type of mental intrusion which is considered unwanted by the sufferer (Julien et al., 2007; American Psychiatric Association, 2013; Seli et al., 2016; Stewart, 2016). These thoughts or mental images are often accompanied by anxiety and general feelings of unease (American Psychiatric Association, 2013; Gillan and Sahakian, 2015). It must be noted that obsessive and intrusive thoughts are universal in occurrence, existing in clinical and non-clinical populations (Rassin et al., 2007; Julien et al., 2009) and across disease borders (Hollander and Benzaquen, 1997; Lawrence et al., 2017). However, those arising from OCD may be unique in terms of content, triggers and frequency (Julien et al., 2007; Rassin et al., 2007; Julien et al., 2009; Najmi et al., 2009) and that greater levels of importance are ascribed to OCD-related obsessions, compared to those occurring in other conditions or under typical conditions (Taylor et al., 2006; Miegel et al., 2019). For example, a commonly encountered obsession involves recurring thoughts of uncleanness or contamination. These obsessive thoughts elicit a certain level of distress in the sufferer, ultimately resulting in excessive washing compulsions in response (Rachman, 2002; Taylor et al., 2006; Robinson and Freeston, 2014). As such, compulsions are defined as ritualistic repetitions of certain cognitive or motor patterns, e.g. excessive mental counting and hand washing that in many cases are aimed at reducing anxiety (Taylor et al., 2006; American Psychiatric Association, 2013; Gillan and Sahakian, 2015; Stewart, 2016). An important factor for consideration regarding this compulsion-relief cycle is that the 'reprieve' is often temporary, which results in a negatively reinforced pattern of obsession-anxiety-compulsion-relief (Deacon and Abramowitz, 2005; Overduin and Furnham, 2012; Robinson and Freeston, 2014; Gillan and Sahakian, 2015), ultimately creating a worsening cycle of compulsive behavior. In many cases, as opposed to delusions, patients retain insight and are aware of the irrationality of their compulsive rituals but are unable to control them in spite of this awareness (Najmi et al., 2009; Robbins et al., 2012).

Following the recent publication of the fifth (and latest) edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V; American Psychiatric Association, 2013), OCD was reclassified under a newly created group of disorders, i.e. 'obsessive-compulsive and related disorders' along with body dysmorphic disorder, trichotillomania (hair pulling), excoriation (skin picking), and hoarding disorder. All are commonly diagnosed in patients with OCD (Chamberlain et al., 2005) and share certain common features such as intrusive/obsessive thoughts and/or perseverative behavioral patterns (Hollander and Benzaquen, 1997; American Psychiatric Association, 2013; Woods and Houghton, 2014; Abramowitz and Jacoby, 2015). This represents a phenomenological shift in the classification of OCD, as in earlier versions of the DSM, OCD was classified as an anxiety disorder (American Psychiatric Association, 1994). This change occurred mainly based on the clinical conundrum of whether anxiety was always an instigating factor promoting the expression of OCD (Bartz and Hollander, 2006; Nutt and Malizia, 2006; Hollander et al., 2008; Stein et al., 2010). Indeed, while anxiety may be a common symptom of OCD, behavioral and cognitive inflexibility, together with behavioral action motivation, which are also core characteristics of the O/C spectrum disorders, are believed to be central to OCD symptomology (Gu et al., 2007; Fineberg et al., 2013b; Grassi et al., 2015; Gruner and Pittenger, 2017). Despite this reclassification, doubt remains regarding the grouping of these disorders, given that the underlying cognitive processes of OCD are distinct from the other grouped disorders, with the exception of body dysmorphic disorder (Abramowitz and Jacoby, 2015).

While the aforementioned O/Cs form the primary diagnostic features of OCD, there are a number of important conditions that must be met for a conclusive OCD diagnosis. The criteria below are all adapted from the DSM-V (American Psychiatric Association, 2013) with additional references provided to support these. First, OCD can be diagnosed based on presence of either obsessions or compulsions, or a combination of both, as is most commonly found (Stewart, 2016). Secondly, patients should be aware of the fact that the O/Cs are excessive and senseless (Marazziti et al., 2002; Jacob et al., 2014), and that such O/Cs must be considered non-pleasurable and mostly unwanted (Najmi et al., 2009; Seli et al., 2016). Thirdly, patients must generally be unable to suppress the obsessive thoughts or images, since typical, non-O/C intrusive thoughts can be rather easily ignored (Julien et al., 2007; Allen et al., 2016). Furthermore, O/Cs must be time consuming, i.e. consuming more than 1 hour per day, and must significantly interfere with the normal functional, occupational and social routines of the patient (Eilam et al., 2006; Ruscio et al., 2010). Lastly, O/C symptoms must not be attributable to any other mental disorder or the action of any drug or substance (Stewart, 2016).

Taking a step further into clinical presentation, it is important to note that OCD is a clinically heterogeneous condition, with the O/Cs of patients typically relating to a discrete number of themes which have been analyzed and organized into a series of symptom subtypes (Mataix-Cols et al., 2005; Abramowitz et al., 2009; Vorstenbosch et al., 2012; Rowsell and Francis, 2015). Patients tend to maintain their specific OCD subtype over extended periods of time, rarely changing from one to another (Fullana et al., 2004; Fullana et al., 2009). Furthermore, it is evident that OCD patients develop O/Cs relating to highly specific concepts and that certain O/Cs may be triggered and/or exacerbated by relevant contextual environments or cues (Markarian et al., 2010; Exner et al., 2014). There is emergent evidence that OCD subtypes may even be *etiopathologically* heterogeneous, implying that unique differences in physiology may underlie the different subtype presentations (Mataix-Cols et al., 2004; McKay et al., 2004; Harrison et al., 2013; Thorsen et al., 2018). Taking all of the above into account, O/C symptom subtypes have been clustered into various subgroup classifications, of which the following five-member classification is quite well representative, although classifications do indeed differ between publications (Hodgson and Rachman, 1977; Calamari et al., 1999; Calamari et al., 2004; Pinto et al., 2008; Zhang et al., 2013; Rowsell and Francis, 2015), viz.:

- harm prevention O/Cs manifesting as checking and reassurance seeking,
- symmetry obsessions and ordering and/or counting compulsions,
- contamination obsessions and washing compulsions,
- repugnant obsessions associated with themes of violence, sex and religion, and
- hoarding obsessions related to concerns about losing possessions, and ‘collecting’ compulsions, which in severe cases is classified as a unique disorder, i.e. hoarding disorder.

Given the often-abstract nature of OCD presentation, various clinical scales and assessment tools have been developed to diagnose, classify and evaluate symptom severity. These are important tools as most are used as benchmark assessments to quantify clinical improvements in human drug and psychological trials (Deacon and Abramowitz, 2005). Generally, these tools consist of comprehensive questionnaires coupled to numerical rating scales to categorize the severity of the condition (Grabill et al., 2008; Overduin and Furnham, 2012). The Maudsley Obsessional Compulsive Inventory, which revealed three symptom subtypes only, was one of the first psychometric tools to be validated (Hodgson and Rachman, 1977). Over time, a number of similar rating scales have been developed and validated, each suggesting various phenomenological symptom clusters (see Grabill et al. (2008) for review). One such scale, i.e., the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS; Goodman et al., 1989a; Goodman et al., 1989b), is a widely applied diagnostic tool which not only assesses

obsessions and compulsions separately but additionally quantifies the functional impact and severity of O/C symptoms to provide a robust clinical picture of the condition. As opposed to the Maudsley Obsessional Compulsive Inventory, the Y-BOCS has been validated extensively and reveals up to seven symptom dimensions (McKay et al., 2004). Indeed, symptom subtypes derived from the Y-BOCS have been identified by factor (Leckman et al., 1997; Mataix-Cols et al., 1999) and cluster (Calamari et al., 1999; Calamari et al., 2004) analyses, each revealing at least four corresponding symptom dimensions, i.e. contamination, harming, hoarding, and symmetry that suggest their clinical dominance. This being said, it is important to note that a lack of professional consensus regarding the validity of symptom subtype classifications still exists (Rowell and Francis, 2015). In an extensive review of clinical literature pertaining to the classification of OCD based on symptomology, neuroimaging, comorbidity and responses to treatment, McKay et al. (2004) aimed to identify unique neurocognitive profiles for various phenotypes of O/C subtypes. Considering the broad scope of said review, it is interesting to note that at the time, limited conclusive evidence was provided to support most purported subtype classifications. However, in congruence with clinical data obtained using the Y-BOCS, the authors found consistent evidence that support hypotheses of unique neurocognitive foundations underlying contamination/washing, checking, hoarding, and symmetry/ordering O/Cs respectively and concluded that such findings warranted further investigation. Indeed, recent findings have supported the ideas of McKay et al. (2004) by reporting unique neuropsychological, neurobiological and treatment response profiles per symptom subtype (Besiroglu et al., 2007; Grados and Riddle, 2008; Leckman et al., 2010; Hashimoto et al., 2011; Hashimoto et al., 2014; Lennertz et al., 2014; Wong et al., 2015; Gruner and Pittenger, 2017; Thorsen et al., 2018) substantiating the idea that the various symptom dimensions may represent wholly unique dysfunctions.

Therefore, as most current classifications are largely based on qualitative observations of symptomology which can be observed or self-reported, a need exists for a more comprehensive and holistic approach to O/C subtype classification that will also take into account specific neurocognitive deviations and possible unique responses to treatment together with respective comorbidities which may be present. Indeed, accurate subtype classifications are essential as unique etiologies of OCD may necessitate tailored treatment approaches (Mataix-Cols et al., 2004; Van Den Heuvel et al., 2008; Harrison et al., 2013; Thorsen et al., 2018). With respect to the current investigation, the concept of subtype validity is important as we will review and investigate the relevance of two commonly proposed phenotypes of compulsive-like behavior as they may be interrogated in a single species model of naturalistic, but symptom heterogeneous OCD, viz. the deer mouse.

In addition to the aforementioned diagnostic features, OCD is a highly comorbid condition with up to 90% of patients with lifetime OCD also presenting with another DSM-V disorder (Nestadt et al., 2001; Ruscio et al., 2010; de Mathis et al., 2013). In fact, 76% of OCD patients are also diagnosed with anxiety disorders, while 63% suffer from depressive or bi-polar episodes (Ruscio et al., 2010; Goodwin, 2015). Autism spectrum disorders are also commonly diagnosed alongside childhood OCD, at a rate of 30% - 40% (Leyfer et al., 2006). Up to 59% of schizophrenic patients experience some O/C symptoms with 33% meeting the criteria for an OCD diagnosis (Poyurovsky et al., 2004). With respect to the recent classification status of OCD as an O/C spectrum disorder, it is interesting to note that OCD demonstrates lower (yet still highly relevant) comorbidity rates with other conditions included in the O/C spectrum e.g. tic disorder (39%), trichotillomania (6%) and non-specific somatoform conditions including body dysmorphic disorder (3 - 16%), than with anxiety and mood disorders as alluded to earlier (Jaisoorya et al., 2003).

Taking all of this into account, OCD is a highly complex disorder, especially given its close interplay with a number of other affective disorders, the heterogeneous nature of its symptom presentation, and the diverse cognitive and biological foundations proposed to underlie the condition, the latter of which will be discussed in section 2.2. As such, much work has been done to elucidate and dissect the epidemiological presentation of the disorder along with the paired neuropsychological and cognitive irregularities, some of which will be discussed here to provide a clearer picture of the condition.

2.1.2. Epidemiology and Impact

OCD affects a significant percentage of the global population, with the DSM-V reporting twelve month prevalence rates (i.e. the percentage of the global population who have experienced OCD symptoms within the last 12 months) ranging between 1.1% and 1.8% (Kessler et al., 2005; Kessler et al., 2009; Ruscio et al., 2010; American Psychiatric Association, 2013). However, Ruscio et al. (2010) reported lifetime prevalence of 2.3%, in accordance with older large-scale studies (Karno et al., 1988). Interestingly, in contrast to patients with lifetime OCD, Ruscio et al. (2010) noted that most respondents who reported O/Cs only at some point in their lives, reported only one OCD subtype. The mean age of onset is 19.5 years, while onset of symptoms after the age of 30 is rare (Ruscio et al., 2010). OCD does indeed demonstrate sex differences in presentation. Males generally present with the condition at an earlier age (Lochner et al., 2004; Jaisoorya et al., 2009), while symptom dimensions and comorbidity patterns tend to differ as well (Lochner et al., 2004; Jaisoorya et al., 2009). More specifically, men more often present with O/Cs related to sexual themes and religion, while women typically present with O/Cs related to contamination and cleansing (Labad et al., 2008).

Furthermore, OCD symptoms appear to be modulated by significant events in the female hormonal cycle such as onset of menstruation, pregnancy, and menopause (Lochner et al., 2004; Guglielmi et al., 2014) of which there is putative evidence in pre-clinical models as well (Schneider and Popik, 2007; Hoffman and Morales, 2009; Mitra et al., 2016; Mitra et al., 2017). There is also strong evidence that OCD is a familial disorder (Samuels et al., 2001; Taylor, 2013), with heritability rates estimated to be between 26% and 65%, although further investigation is certainly required (Markarian et al., 2010; Taylor, 2013).

Along with the relatively high prevalence of the condition, OCD presents with notable functional impairment and reductions in patient quality of life (QOL; Hollander et al., 1996; Masellis et al., 2003; Fontenelle et al., 2010; Coluccia et al., 2016). As OCD had been implicated as the tenth largest cause of functional disability in the industrialized world (Murray and Lopez, 1996), Eisen et al. (2006) studied the effects of OCD on patient QOL using both administered surveys as well as self-report measures to deliver conclusive insight into this issue. Not only were significant reductions in QOL reported with respect to various aspects of daily life, including work, personal relationships, perceptions of wellbeing, relaxation, and domestic functioning, but positive correlations between OCD severity and QOL impairment were also found (Eisen et al., 2006; Fontenelle et al., 2010; Ruscio et al., 2010). Such functional impairments are easy to conceptualize given that survey data have indicated that OCD patients can spend in excess of 6 hours *per day* entertaining their O/Cs in one fashion or another (Eilam et al., 2006; Ruscio et al., 2010). Interestingly, obsessions were found to have a more severe impact on QOL compared to compulsions (Masellis et al., 2003; Eisen et al., 2006). These findings have since been replicated in a more recent study (Huppert et al., 2009) that also demonstrated even greater impairments in QOL in patients diagnosed with other comorbid psychopathologies. This data, also supported by epidemiological studies (Ruscio et al., 2010; Grant et al., 2012) collectively highlight the burden of OCD in terms of personal functioning and fulfilment as well as the economic impact of symptoms. In addition to the tremendous burden patients experience due to OCD, the significant negative effects on caregivers (Grover and Dutt, 2011) together with the burden on healthcare systems (Markarian et al., 2010) and even economies (Kessler et al., 2009; Vorstenbosch et al., 2012), highlights the continued need for safe, effective and tolerable treatment which will arise from better understanding of the condition.

2.1.3. Treatment of OCD

2.1.3.1. Pharmacological Therapy

Currently, the primary aim of *pharmacological* treatment of OCD is focused on increasing serotonergic (5-hydroxytryptamine/serotonin; 5-HT) neurotransmission (El Mansari and Blier, 2006; Goddard et al., 2008; Fineberg et al., 2013a; refer also to section 2.2). As such, drugs that inhibit serotonin reuptake transporters (SERT), e.g. the serotonin reuptake inhibitor (SRI), clomipramine (Piccinelli et al., 1995), and various selective serotonin reuptake inhibitors (SSRIs), e.g. escitalopram (Fineberg et al., 2007), paroxetine (Dunbar et al., 1995; Abudy et al., 2011), fluoxetine (Abudy et al., 2011; Diniz et al., 2011), and sertraline (Rasmussen et al., 1997a; Rasmussen et al., 1997b), constitute the first line of pharmacological therapy (Bandelow et al., 2008; Abudy et al., 2011; Fineberg et al., 2013b; Hirschtritt et al., 2017). Importantly, although there is little evidence that SSRIs are more effective than SRIs in the treatment of OCD (Geller et al., 2003), the relatively more tolerable side-effect profile of SSRIs has resulted in their preferential application (Blier and de Montigny, 1999; Fineberg and Gale, 2005; Fineberg et al., 2013a). However, while treatment response largely remains dependent on enhanced serotonergic signaling, such treatments must be administered *chronically* and in *higher doses* than those typically employed for depression (Goddard et al., 2008; Spijker and Nolen, 2010; Fineberg et al., 2013a; Stewart, 2016; Hirschtritt et al., 2017). For example, paroxetine administered in doses of 40 and 60 mg, and not 20 mg per day, was found to be highly effective in the treatment of OCD (Dunbar et al., 1995; Hollander et al., 2003a).

As alluded to earlier, both SRIs and SSRIs facilitate serotonergic neurotransmission by inhibiting SERT on presynaptic serotonergic neurons, thereby increasing synaptic concentrations of 5-HT. Not only does this increase allow for improved post-synaptic receptor binding and neural activation (Bergqvist et al., 1999; Blier and de Montigny, 1999; Nutt et al., 1999; Goddard et al., 2008), but also results in the *sustained* activation of auto-inhibitory 5-HT_{1A/B/D} receptors located on presynaptic neurons (Barnes and Sharp, 1999; Bergqvist et al., 1999; Blier and de Montigny, 1999). Initially, this action decreases the release of 5-HT at the synapse via negative feedback (Barnes and Sharp, 1999; Blier and de Montigny, 1999). However, after a period of continued dosing, down-regulation of these presynaptic receptors begins to occur, reducing the negative feedback, ultimately resulting in elevated 5-HT release (Goddard et al., 2008). This may possibly explain the relatively slow onset of action of drug treatment in OCD compared to other psychiatric disorders (Spijker and Nolen, 2010; Hirschtritt et al., 2017). Considering that both depression and OCD are treated with drugs inhibiting SERT, and that the onset of action against depression is usually quicker, an interesting distinction can be made between the actions of SSRIs in the treatment of depression versus

OCD. It has been demonstrated that *depressed* patients stabilized on SSRIs may experience recurrent depressive episodes upon depletion of L-tryptophan, the dietary precursor of 5-HT, highlighting the importance of an increased synaptic 5-HT concentration in the mechanistic response to depression (Smith et al., 1997). However, 5-HT depletion does not seem to influence the response to treatment in OCD, indicating a more pronounced role of *altered post synaptic receptor interactions* induced by SSRIs (Smeraldi et al., 1996). These differences in therapeutic mechanisms may also contribute to the disparate onset of action durations observed in the treatment of depression (4 – 6 weeks; Dougherty et al., 2004; Spijker and Nolen, 2010) and OCD (8 – 12 weeks; Albert et al., 2013; Fineberg et al., 2013a), all of which points to a much more slowly-reacting pathology and/or therapeutic mechanism than that which is observed in depression. For a closer look at the neurobiology of OCD and the serotonergic system, see section 2.2.

Although OCD demonstrates preferential response to drugs that alter serotonergic neurotransmission, it remains a clinical challenge that approximately 40 - 60% of patients remain refractory to SSRI monotherapy (Erzegovesi et al., 2001; Albert et al., 2013; Fineberg et al., 2013a; Atmaca, 2016) as shown by insufficient reductions in psychometric test scores such as the Y-BOCS following typical first-line treatment (Abudy et al., 2011; Albert et al., 2013). In such cases, different strategies are employed, e.g. switching to an alternative SRI/SSRI, administration of SRI/SSRI drugs intravenously or increasing the SRI/SSRI dose (Abudy et al., 2011; Albert et al., 2013; Fineberg et al., 2013a). However, augmentation of SRI/SSRI therapy with drugs targeting different neuronal mechanisms are most commonly employed (Bloch et al., 2006; Albert et al., 2013; Fineberg et al., 2013a; Veale et al., 2014). In this regard, augmentation with low dose anti-psychotic drugs, e.g. haloperidol (McDougle et al., 1994; Bloch et al., 2006), risperidone (McDougle et al., 2000; Hollander et al., 2003b; Veale et al., 2014), and possibly quetiapine (Denys et al., 2004a; Carey et al., 2005), which are, to varying extents antagonists of dopamine (DA) $D_{1/2}$ and 5-HT $_{2A/2C}$ receptors amongst others (Richelson and Souder, 2000; Westenberg et al., 2007) has indeed shown the most promise (Maina et al., 2003; Fineberg et al., 2006; Komossa et al., 2010; Dold et al., 2013; Fineberg et al., 2013a; Dold et al., 2015). However, it has been found that cessation of successful DA antagonist augmentation therapy (or indeed any successfully stabilizing protocol) within twelve months of commencement can result in relapse rates of up to 80%, reinforcing the need for sustained treatment periods in the effective control of the condition (Maina et al., 2003; Fineberg et al., 2013a). Taken together, these findings implicate dopaminergic involvement in the pathology of OCD. However, considering that dopaminergic antagonists administered as monotherapy are ineffective against or even exacerbate OCD (Westenberg et al., 2007; Schirmbeck and Zink, 2012; Ulhaq and Abba-Aji, 2012), and that

they often antagonize a diverse range of receptors also, i.e. adrenergic $\alpha_{1/2}$, 5-HT_{1A/2A/2C/7}, histamine H₁, and muscarinic M_{1/3} (Richelson and Souder, 2000), the contribution of dopaminergic modulation to the treatment response of OCD is not as readily evident as is the case regarding 5-HT; yet it is receiving increased interest (Denys et al., 2004b; Hesse et al., 2005; Koo et al., 2010). That said, while often effective, such dual-treatment strategies may also induce severe extrapyramidal movement disruptions in patients, making them a far from optimal solution (Koo et al., 2010; Fineberg et al., 2013a).

Considering the controversy revolving around the previous classification status of OCD as an anxiety disorder (Abramowitz and Jacoby, 2015), it is interesting to note that drugs commonly used for anxiety and related disorders, i.e. the benzodiazepines, monoamine oxidase inhibitors, and mood stabilizers, are largely ineffective for the management of OCD (Fineberg, 2004; Bandelow et al., 2008; Goddard et al., 2008), suggesting that OCD and anxiety disorders are indeed founded in different neurocognitive constructs that extend beyond conventional anxiety.

2.1.3.2. Psychological Treatments

Various psychological interventions which are focused on different components of symptom presentation and that are addressed via cognitive and behavioral approaches respectively (McFall and Wollersheim, 1979; Whittal et al., 2005), are employed to treat OCD. Cognitive behavioral therapy describes psychological interventions which aim to weaken distorted cognitive patterns associated with obsessions without any confrontation with or habituation to anxiety provoking conditions (Whittal et al., 2005). Alternatively, therapies focusing on the behavioral aspects (behavioral therapy) of OCD involve repeated, therapist-guided interactions with obsession-provoking conditions (in actuality or via imagined scenarios) whilst inhibiting patients from carrying out any typical compulsive response (Abramowitz, 2006). The latter approach, aptly named exposure-and-response-prevention, was pioneered by the work of Meyer (1966) and has since been regarded as a major triumph in modern psychiatric medicine (Abramowitz, 2006). Exposure-and-response-prevention is often regarded as the superior psychological intervention for OCD (Whittal et al., 2005; Abramowitz, 2006).

Nevertheless, such treatments do have their drawbacks, since exposure-and-response-prevention often results in residual symptoms and can induce significant distress in some cases due to its inherent anxiogenic component (Whittal et al., 2005; Abramowitz, 2006). Regardless, psychological treatments have been found to be as effective as drug therapy (O'Kearney et al., 2006), although the outcomes seem to differ as a function of OCD symptom subtype (Rufer et al., 2006). As such, psychological interventions are highly important for managing OCD (March et al., 1994; Ivarsson et al., 2015) or drug refractory cases (Tolin et

al., 2004; Anand et al., 2011; Simpson et al., 2013). Cognitive behavioral therapy in particular has recently been reiterated as a first-line treatment, with or without the addition of SSRIs (Hirschtritt et al., 2017).

Cognitive behavioral therapy and the concepts behind it are of some interest in the current investigation as we will expose different cohorts of deer mice, viz. large and normal nest-builders (LNB/NNB) to contextual punishment in a specific phase of the study (results reported in Chapter 5). Given our hypothesis that LNB animals express such behavior as a direct result of obsessive cognition, it will be interesting to establish how LNB, when compared to NNB animals, respond to contextual punishment and whether LNB expressing animals will learn over time that the drive to build large nests for whichever reason, is unfounded in a 'safe' home cage environment. Although we will not apply cognitive behavioral therapy to treat the symptoms of LNB animals directly, the psychological construct of the interventions made in the current investigation, overlaps to some extent with the fundamentals of cognitive behavioral therapy and will hopefully provide more insight into the possible aberrant neuropsychology in LNB deer mice (*Peromyscus maniculatus bairdii*).

Regardless of the widening number of treatment modalities available, a large proportion of patients, up to 30-40%, remain refractory to the aforementioned pharmacological and psychological interventions *in addition to* surgical approaches (Atmaca, 2016; Hirschtritt et al., 2017), reinforcing the need for improved understanding of and targeting of dysfunctional neurobiological and neuropsychological dysfunctions occurring during OCD.

2.2. Neurobiology of OCD

Although the neurobiology of OCD has been studied extensively, no definitive and clear causal relationships have yet been established between specific constructs of pathophysiology and symptomology. This being said, experimental work has identified the involvement of distinct brain regions, multiple neurotransmission systems and neural networks in the genesis of OCD. In this section, we will aim to provide the reader with a concise overview of the present understanding of the neurobiology underlying OCD and the subsequent implications for treatment strategies, both current and prospective. This section begins with a look at the proposed neuroanatomy of OCD, followed by an examination of the global neurotransmission systems and how they are distributed throughout the implicated circuitry, which will finally be followed by a summary of how all this information fits together to constitute a neurobiological and conceptual roadmap of OCD.

2.2.1. The Cortico-Striatal-Thalamo-Cortical Circuit and Beyond

Pioneering theories proposing a unifying neurobiological origin of OCD have implicated the involvement of a hyperactive cortico-striatal-thalamo-cortical (CSTC) circuit (Insel, 1992; Rauch and Baxter, 1998; Saxena et al., 1998; Groenewegen, 2003; Saxena, 2003).

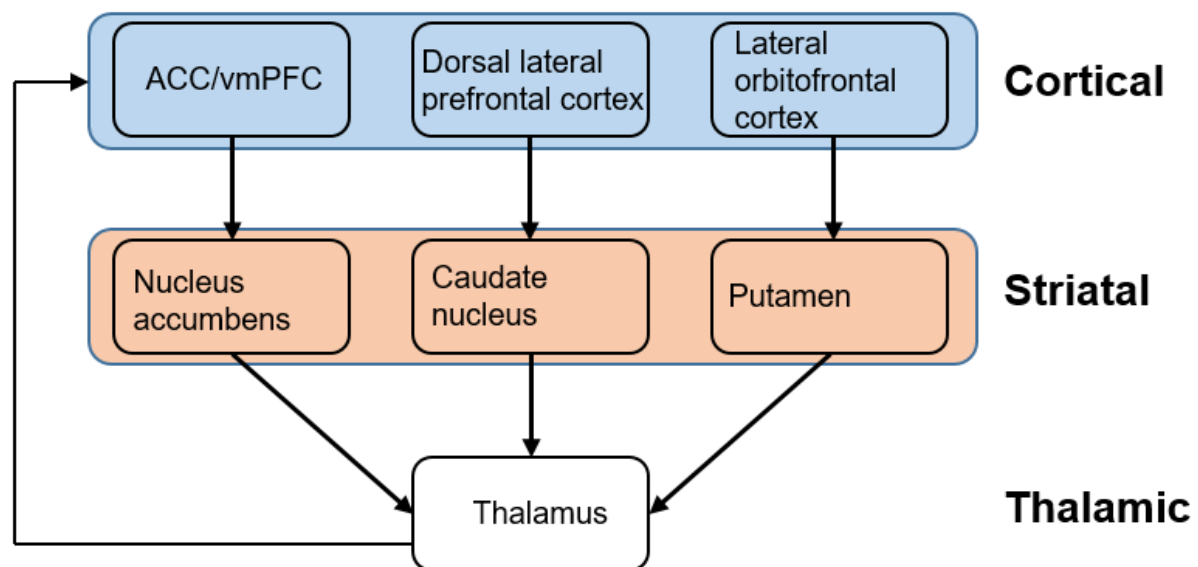


Figure 1: Broad schematic representation of the CSTC circuit. ACC - anterior cingulate cortex; vmPFC - ventro-medial prefrontal cortex. Adapted from (Milad and Rauch, 2012).

Briefly, the CSTC circuit (**Figure 1**) comprises functional substructures of the cortex, i.e. the anterior cingulate cortex (ACC), prefrontal cortex (PFC), and orbitofrontal cortex (OFC), which are respectively linked to the thalamus via unique functional units in the striatum (STR), i.e. nucleus accumbens (NAc), caudate nucleus (CN) and putamen (Nambu, 2008; Di Filippo et

al., 2009; Milad and Rauch, 2012; Harrison et al., 2013). Also important are the two major populations of dopaminergic neurons, i.e. the mesolimbic and nigrostriatal pathways which are broadly involved in reward and movement functions (Wise, 2009). The substantia nigra (SN) and its subdivisions, the pars reticulata (SNr) and pars compacta (SNc), the latter of which is *indirectly* involved in motor planning and reward seeking via integration with the CSTC circuit by supplying it with dopaminergic input, are also implicated (Nicola et al., 2000; Nambu, 2008; Wise, 2009; **Figure 2**). As a behavioral and regulatory opponent to DA, the CSTC circuitry is also significantly innervated by *serotonergic* neurons originating in the raphe nuclei, particularly those of the dorsal bundle (Nolfe et al., 1998; Cools et al., 2008). The neurotransmission of OCD is discussed further in section 2.2.2. Broadly speaking, the STR integrates and processes a number of reward-related cognitive processes including motivation, motor-planning, decision making and motivation, all of which are altered to a certain extent in OCD as will become evident throughout this literature study (Nambu, 2008; Harrison et al., 2013; Yager et al., 2015; Gruner and Pittenger, 2017). The three distinct striatal domains exert direct input to the thalamus via a mixture of gamma-aminobutyric acid (GABA)-ergic and glutamatergic efferents, which in turn feed back to the cortex via glutamatergic signaling, completing the CSTC circuit (Di Filippo et al., 2009; Milad and Rauch, 2012). It is important to note that the STR projects to the thalamus via two pathways, i.e. the direct and indirect pathways (**Figure 2**).

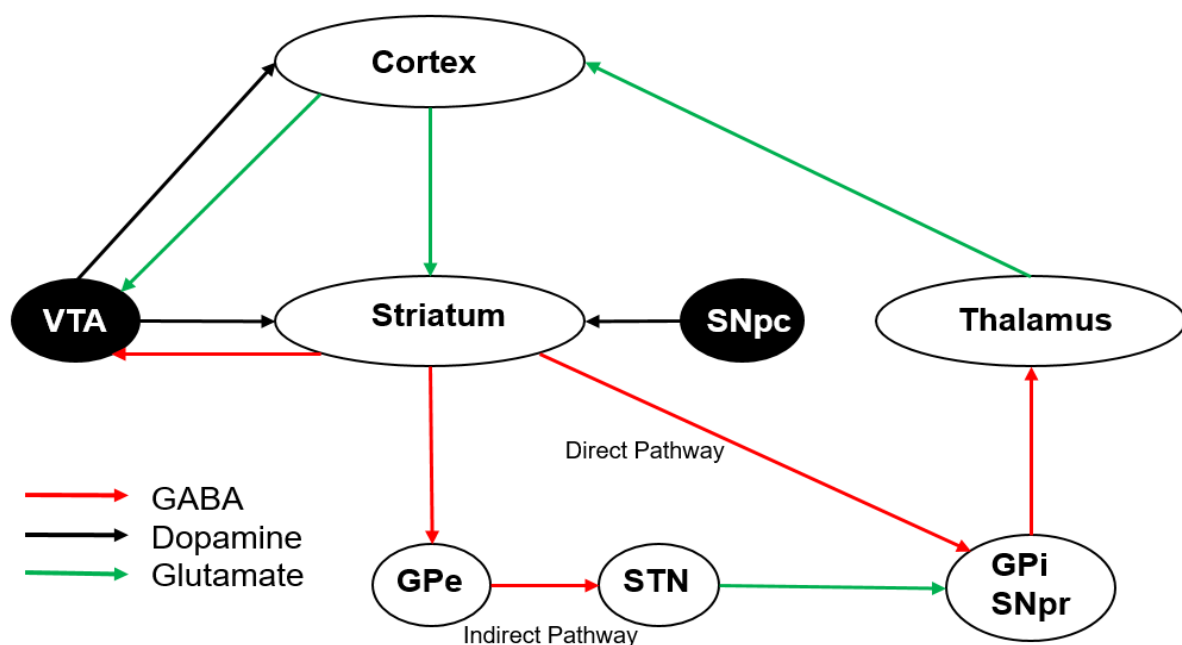


Figure 2: Simplified representation of the direct and indirect pathways within the CSTC circuit. VTA - ventral tegmental area; SNpc - substantia nigra pars compacta; GPe - globus pallidus externa; STN - subthalamic nucleus; GPi - globus pallidus interna; SNpr - substantia nigra pars reticulata. Adapted from (Di Filippo et al., 2009).

The direct pathway travels via the globus pallidus interna (GPi) and SNr by means of GABAergic interneurons to the thalamus; these normally, i.e. in the absence of activation, prevent activation of the thalamus, since the GABAergic neurons extending from both the GPi and globus pallidus externa (GPe) are electrophysiologically tonically active. This in turn results in a net inhibitory effect on the thalamus (Groenewegen, 2003; Gernert et al., 2004) and thereby also complex motor behavior including amongst others, motor tics and compulsive routines. The indirect pathway travels to the subthalamic nucleus (STN) via the GPe by means of GABAergic interneurons. From here the pathway travels to the GPi/SNr via glutamatergic efferents, and finally to the thalamus via GABAergic neurons, inhibiting thalamic activity and reducing the expression of motor behavior (Stocco et al., 2010; Yager et al., 2015).

Thus, the GPi/SNr receives inputs from both pathways and serves as the integration point for the two pathways. During *activation*, the direct pathway results in increased GABAergic outflow to the GPi/SNr, slowing *subsequent* GABAergic currents to the thalamus, thereby *increasing* the excitability of thalamic neurons by reducing the amount of *tonic* inhibitory GABAergic signaling; the net result of direct pathway activation is therefore also activation of the CSTC circuitry to facilitate motor patterns and cognitive action (Groenewegen, 2003; Stocco et al., 2010; Milad and Rauch, 2012). This is only achieved when the SNc simultaneously exerts excitatory DA D₁ receptor-mediated feedback to the striatum (**Figure 2**), which causes the striatum to release GABA onto the GPi, promoting signal propagation via the *entire* behaviorally-activating direct pathway as described above (Leisman and Melillo, 2013). By contrast, the indirect pathway, when activated, results in elevated glutamatergic outflow to the GPi/SNr, *stimulating* the inhibitory activity of GABA projections to the thalamus, thereby lowering thalamic output.

However, an important secondary level of control exists. As explained, the neurons of the direct pathway express D₁ receptors which, when activated by the SNc serve to elevate the activity of the pathway (Hodge and Butcher, 1980; Nicola et al., 2000; Groenewegen, 2003; Stocco et al., 2010). However, the indirect pathway expresses D₂ receptors which suppress activity of the pathway when activated, which as described above is inhibitory in nature, thus causing a stimulating effect on output to the thalamus (Hodge and Butcher, 1980; Groenewegen, 2003; Nambu, 2008). To close the circuit, the thalamus projects back to the cortex by means of glutamatergic monosynaptic projections (Nambu, 2008). It is believed that patients with OCD present with a bias in favor of the direct pathway, resulting in compulsive repetition of certain motor patterns due to deficient inhibitory control exerted by the circuitry. Important cognitive functions, including fear learning, sensorimotor gating and response inhibition, which are also thought to be coordinated by the CSTC circuits, have also been shown to be abnormal in clinical populations (see section 2.3; Ahmari et al., 2013; Marek et

al., 2013; Wood and Ahmari, 2015). This influence of DA on the CSTC circuit is further complicated by the fact that dopaminergic activity can be extensively modulated by 5-HT itself (see section 2.2.2.1; Barnes and Sharp, 1999; Amargós-Bosch et al., 2004; Westenberg et al., 2007).

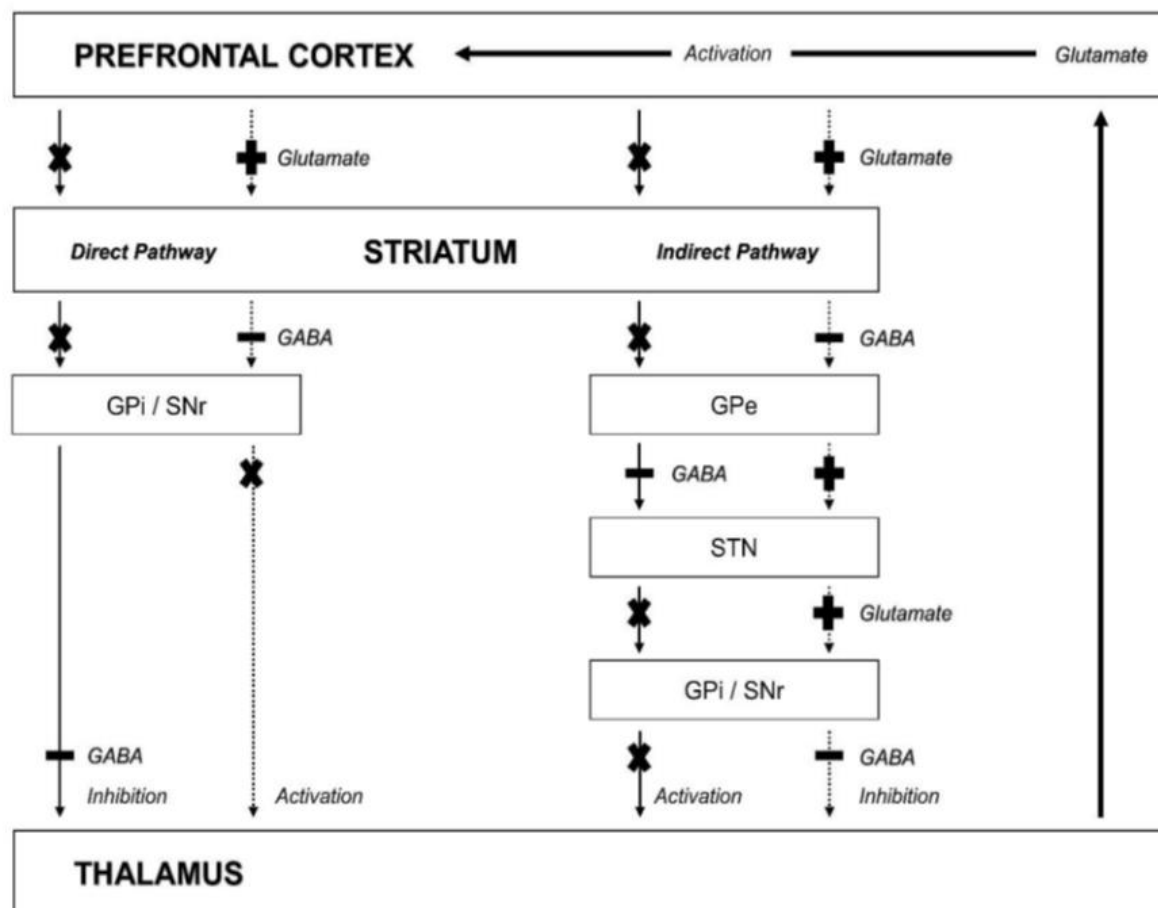


Figure 3: Schematic representation of the GABAergic and glutamatergic connections in the direct (left) and indirect (right) pathways in the CSTC circuits. Solid lines: thalamo-cortical inhibition; Dotted lines: thalamo-cortical activation (own figure).

From a translational perspective, the CSTC circuit has also been demonstrated to play an extensive role in animal models of repetitive, compulsive-like behaviors (Ting and Feng, 2011; Ahmari, 2016; Monteiro and Feng, 2016). Pre-clinical models have shown that direct stimulation of components of the circuit, either electrically or pharmacologically, can induce long lasting, repetitive behaviors including excessive grooming behaviors, reduced spatial alternation, and excessive checking (see discussion on models, section 2.5; Yadin et al., 1991; Szechtman et al., 1998; Ahmari et al., 2013). Furthermore, numerous lines of evidence arising from neuroimaging, cognitive and pharmacological studies have implicated multiple role-players of the aforementioned CSTC circuit in the pathology of the condition that came to the

fore in terms of altered connectivity, altered basal and provoked activity levels and even physical size of the brain regions (see Menzies et al., 2008; Milad and Rauch, 2012; Ahmari, 2016; Vaghi et al., 2017). That being said, perturbations exclusively founded on CSTC circuit dysfunction do not provide an all-encompassing explanation for the neurological dysfunctions underlying OCD since the hippocampus and amygdala are also implicated (see 2.2.2.4; Menzies et al., 2008; Milad and Rauch, 2012). Thus, while the demonstration of true O/Cs in animal models remains challenging, focus remains on aspects of repetitive behaviors with superficial similarities to OCD which can be modelled, including many of the same neuroanatomical constructs. Progression in the field of neuroscience has allowed for more intricate analysis of the CSTC circuits, revealing that it can further be divided into three ‘sub-circuits’, all originating at the cortex. To round off the discussion of the proposed neurological abnormalities putatively involved in OCD, a closer look at the function of the CSTC circuit components in the context of these discrete circuits as well as evidence for its dysfunction in OCD, follows. Lastly, brain regions distinct from the CSTC circuitry thought to be involved in the pathophysiology, will be briefly explored.

2.2.1.1. The Affective Circuit

This circuit, originating in the ACC and ventromedial PFC and then traveling through the NAc to the thalamus, is primarily involved in the control of affective states, cognitive conflict, error detection and reward valuation processes (Milad and Rauch, 2012). The ACC, specifically the dorsal aspect (dACC), has been found to be hyper-activated in patients with OCD during the processing of incongruent information (Milad and Rauch, 2012; Wood and Ahmari, 2015). There is also evidence for abnormally elevated activity between the dACC and the dorso-lateral PFC of OCD patients, which purportedly results in the defective decision making characteristics seen in OCD (Menzies et al., 2008; Milad and Rauch, 2012). Further, reductions in ACC-volume have also been found in patients with OCD (Menzies et al., 2008; Milad and Rauch, 2012; Wood and Ahmari, 2015).

2.2.1.2. The Ventral Cognitive Circuit

The OFC is divided into two functional units, namely the medial OFC (mOFC) and lateral OFC (lOFC), both of which regulate cognitive processes closely associated with OCD in one form or another (Milad and Rauch, 2012). The mOFC, which is critically involved in the motivational aspects of behavior and the processing of reward value, is often hypoactive in OCD (Menzies et al., 2008; Milad and Rauch, 2012; Wood and Ahmari, 2015). The lOFC is involved in the processing of responses to punishment and the expression of excessive repetitive behavioral rituals and is considered to be hyperactive in OCD (O'Doherty et al., 2003; Milad and Rauch, 2012). That being said, many clinical studies do not report distinction between these two

regions (Wood and Ahmari, 2015). The second sub-circuit, the ventral cognitive circuit which extends from the IOFC to the putamen and finally the thalamus, processes motor planning and response inhibition (Milad and Rauch, 2012). Lesions to the OFC cause changes to reward-related behavior and importantly, inhibit reversal learning processes (Cools et al., 2002; Menzies et al., 2008). Impaired reversal learning, which describes a bias towards repetitive behaviors and difficulty in adjusting behaviors in the light of changed circumstances, a key feature of OCD that shows improvement following serotonergic facilitation, is founded in OFC dysfunction (Chamberlain et al., 2008). Inaccurate reward valuation or an inability to adapt to changing reward circumstances can lead to poor learning and inappropriate decision making, resulting in repetitive behaviors due to a lack of behavioral inhibition exerted by the OFC (O'Doherty et al., 2003). As with the ACC, changes in OFC volume are also evident in OCD patients (Menzies et al., 2008).

2.2.1.3. The Dorsal Cognitive Circuit

This circuit, projecting from the dorsal lateral PFC to the caudate nucleus of the striatum and on to the thalamus, is anatomically associated with the mOFC and is involved in the control of fear-response behaviors (Milad and Rauch, 2012). It may be of value for future studies to interrogate potential dysfunctions within this circuit, as OCD has also been proposed as a condition of dysfunctional fear processing (Melli et al., 2015; Nikodijevic et al., 2015; Raines et al., 2015).

2.2.1.4. The Amygdala, Hippocampus and Beyond

The amygdala (together with the hippocampus, mPFC, and OFC) is critically involved in the processing of reward, fear and anxiety (Maroun and Richter-Levin, 2003; Seymour et al., 2007; Marek et al., 2013; Wood and Ahmari, 2015) and represents a distinct target for investigations into OCD pathophysiology, deviating from the regions already discussed which form the core theories of OCD neurodysfunction (Milad and Rauch, 2012; Simon et al., 2014; Via et al., 2014). Normal functioning of the amygdala is crucial for fear extinction, i.e. preventing excessive and inflated behaviors that may be triggered by previously experienced anxiogenic stimuli (Marek et al., 2013). Indeed, as alluded to in the previous paragraph, abnormal processes of fear extinction are evocative of an OCD patient failing to experience comfort after performing anxiety neutralizing rituals (Abramowitz et al., 2009). The amygdala and mPFC are directly connected in a bi-directional fashion and indirectly connected through the hippocampus via a series of excitatory synaptic connections (Marek et al., 2013). Further, the PFC is seemingly able to influence activity of the amygdala, since lesions to the former inhibit activity of the latter, allowing a point of interaction between the CSTC circuit and the fear circuit (Maroun and Richter-Levin, 2003). Indeed, OCD patients show hyper activation of the

amygdala when faced with obsession specific stimuli (Breiter et al., 1996; Simon et al., 2010; Simon et al., 2014). Additionally, severity-dependent, detectable differences in amygdala activation when processing emotionally provoking (although not O/C specific) stimuli in a manner which is functionally distinct from any other anxiety disorders, have been observed in OCD (Cannistraro et al., 2004; Cardoner et al., 2011; Via et al., 2014).

There is also interest in the amygdala and its role in reward processing (O'Doherty et al., 2002; Schott et al., 2008) via its extensive connections to the ventral tegmental area (VTA) and other members of the CSTC circuit such as the NAcc shell and mOFC in the so called corticolimbic-ventral striatal networks (Kelley, 2004; Nieh et al., 2013). The amygdala and VTA relay information to the ventral striatum, and given their roles in processing affect and reward respectively (O'Doherty et al., 2002; Lammel et al., 2012), are thought to contribute to the aberrant cognitive processing of OCD; this taking into account that OCD is often underpinned by anxiety and distinct disruptions in reward processing (Nestadt et al., 2001; Angst et al., 2005; Figuee et al., 2011; Nieh et al., 2013).

2.2.2. The Neurotransmission of OCD

Broadly, it has been demonstrated that dysfunctional neurotransmission especially in the serotonergic, dopaminergic and glutamatergic systems (Stein, 2000; Goddard et al., 2008; Koo et al., 2010; Wu et al., 2012; Fineberg et al., 2013a) underlies the neuropathology of OCD. The latter system will not be discussed here as it falls beyond the conceptual scope of this thesis and because experimental work regarding glutamatergic neurobiology and treatment in OCD, remains limited. As such, the receptor subclasses of the serotonergic and dopaminergic systems that are of greatest importance in OCD are described below.

2.2.2.1. The Serotonergic System

Serotonergic involvement in OCD is *implied* by the broad use of SRIs/SSRIs and their various modulatory effects. Generally speaking, OCD is characterized by hypoactive serotonergic signaling which *permits* excessive dopaminergic tone to exist (Barnes and Sharp, 1999; Amargós-Bosch et al., 2004; Westenberg et al., 2007; Goddard et al., 2008; Koo et al., 2010; Nikolaus et al., 2010). Furthermore, there is accumulating evidence that 5-HT, in concert with DA (Daw et al., 2002; Boureau and Dayan, 2011; Cools et al., 2011), plays an important role in positive and negative reinforcement learning procedures, both of which are abnormal in OCD (see section 2.3). As alluded to earlier, the majority of central serotonergic neurons arise at the raphe nuclei (particularly the dorsal bundle) which projects to and innervates the PFC, STR, thalamus, amygdala and hippocampus (Nolfe et al., 1998; Hornung, 2003; Cools et al., 2008).

This tryptophan-derived neurotransmitter exerts its effects at a number of receptors of which 14 subtypes have been identified. They are categorized into seven families (5-HT₁₋₇) based on pharmacological and structural similarity. Most of these families are G-protein-coupled receptors which function via second messenger systems with the exception of 5-HT₃ receptors, which are ligand-gated ion channels. A detailed discussion of all 5-HT receptors does not fall within the scope of the current thesis and will therefore not be provided here. While the current literature review will focus on those 5-HT receptors commonly implicated in the pathology of OCD, a full review of all 5-HT receptors is provided by Barnes and Sharp (1999).

* * *

The 5-HT₁ receptor family

The G_i-coupled 5-HT_{1A} receptor, when located pre-synaptically, functions to reduce serotonergic outflow, particularly from the raphe nuclei (Corradetti et al., 1996; Blier and de Montigny, 1998; Barnes and Sharp, 1999; Millan et al., 2008). This occurs because the receptor is coupled to G_i effector proteins, and activation thereof induces a reduction in cellular production of cyclic adenosine monophosphate (cAMP), ultimately resulting in cellular hyperpolarization and decreased downstream neuronal activity (Barnes and Sharp, 1999). Importantly, through interactions with dopaminergic neurons (as heteroreceptors) or when located on tonically active GABAergic interneurons (Fink and Göthert, 2007), 5-HT_{1A} receptors can reduce dopaminergic activity (Amargós-Bosch et al., 2004; Fink and Göthert, 2007; Westenberg et al., 2007). Normally, 5-HT receptors function as inhibitors of complex motor behavior and cognitive processing in the frontal cortex (most notably the ACC), limbic structures and hippocampus (Deakin and Graeff, 1991; Barnes and Sharp, 1999; Westenberg et al., 2007). It is therefore argued that an increased stimulation or over-expression of pre-synaptic 5-HT_{1A} receptors can result in an inhibited serotonergic, and therefore altered (brain region dependent) dopaminergic outflow to the brain areas which typically malfunction in psychiatric conditions such as OCD. It is within this framework that SSRIs presumably act to improve OCD symptoms; the initial decrease of serotonergic firing due to increased pre-synaptic 5-HT_{1A} receptor activation is eventually overcome following weeks of treatment, due to down-regulated expression of the receptor in response to elevated 5-HT concentrations in synaptic spaces (Blier and de Montigny, 1999; Nutt et al., 1999; Goddard et al., 2008), ultimately elevating 5-HT release and indirectly inhibiting DA release. This latter function presumably occurs via down-regulation of 5-HT_{1A} receptors located on inhibitory, tonically active GABAergic interneurons, thereby facilitating (by means of a decrease in 5-HT_{1A}-mediated neuronal hyperpolarization) their release of GABA, slowing the discharge rate of

downstream dopaminergic neurons (Fink and Göthert, 2007). Indeed, the actions of 5-HT on the 5-HT_{1A} receptors have been demonstrated in clinical (Harrison and Markou, 2001) and pre-clinical (Yadin et al., 1991; Seibill et al., 2003) studies that described OC-like behavioral phenomena following 5-HT_{1A} agonism with low doses of 8-hydroxy-2-(dipropylamino)-tetralin hydrobromide (8-OH-DPAT). There is also evidence that co-administration of pindolol, a 5-HT_{1A} antagonist can augment the effect of SSRIs in treatment resistant OCD by preventing the auto-inhibition instigated by increased 5-HT availability and its subsequent actions of binding to 5-HT_{1A} (Dannon et al., 2000).

There is also interest in the 5-HT_{1B/D} receptors as potential therapeutic targets. As is true of the 5-HT_{1A} receptor, these G_i coupled receptors inhibit adenylate cyclase and reduce the availability and downstream signaling actions of cAMP (Barnes and Sharp, 1999; Millan et al., 2008), resulting in neuronal hyperpolarization. 5-HT_{1B} receptors are distributed widely in structures of the CSTC circuit basal ganglia, particularly the SNr, GPI/GPe and ventral pallidum (Johnson et al., 1992; Barnes and Sharp, 1999). Similar distribution patterns of 5-HT_{1D} are found, particularly in the GPI/GPe, SNr, caudate putamen, hippocampus and cortex (Barnes and Sharp, 1999). Furthermore, the 5-HT_{1B/D} receptors are not only responsible for the regulation of 5-HT outflow and activity, but also for that of different neurotransmitters, e.g. DA, GABA and glutamate via their actions as auto- and hetero-pre-synaptic receptors, critically modulating a number of role players in OCD circuitry (Blier and de Montigny, 1999; Goddard et al., 2008; Koo et al., 2010). 5-HT_{1B} receptors in particular are involved in the modulation of dopaminergic outflow, since activation thereof has been shown to *suppress* GABA-mediated inhibitory action potentials in DA-releasing neurons in the rat brain (Johnson et al., 1992). A lack of serotonergic action on these receptors can critically feed into CSTC circuit hyperactivity. The 5-HT_{1D} agonist, sumatriptan, has for example, resulted in modest improvements in severe treatment resistant depression (Stern et al., 1998; Koran et al., 2001). Furthermore, the involvement of 5-HT_{1B/D} receptors has also been associated with OCD in genetic studies (Mundo et al., 2002).

The 5-HT₂ receptor family

The 5-HT_{2A/C} receptors have received special attention with respect to their role in OCD. Indeed, they are acted upon to varying degrees by the anti-dopaminergic drugs used in augmentation strategies, potentially explaining the efficacy of these second-line treatments (Goddard et al., 2008; Brakoulias and Stockings, 2019).

The 5-HT_{2A} receptor exerts its actions via activation of phospholipase C (PLC) by coupling to the G_q protein, subsequently modifying cellular calcium and inositol phosphate metabolism, causing a net neuronal excitation and excitatory synaptic potentials (Barnes and Sharp, 1999).

The receptor is distributed widely in the cortical regions, NAc and CN, all of which are commonly associated with OCD (see section 2.2.1). OCD patients have shown abnormal 5-HT_{2A} distribution in the CN (Adams et al., 2005) as well as sexually dimorphic polymorphisms of genes related to 5-HT_{2A/C} receptor expression (Enoch et al., 1998). Experimental work has shown that 5-HT_{2A} agonists bolster outflow from dopaminergic neurons (Amargós-Bosch et al., 2004). In line with this, drugs antagonizing the 5-HT_{2A} receptor such as risperidone (McDougle et al., 2000; Hollander et al., 2003b; Goddard et al., 2008), clozapine (Goddard et al., 2008) and olanzapine (Koran et al., 2000; Albert et al., 2013), have shown therapeutic efficacy in OCD, although primarily so in an augmenting role only (Bloch et al., 2006; Albert et al., 2013; Atmaca, 2016). Furthermore, the effects of these drugs on multiple receptors, including DA receptors (see below), may make specific deductions about receptor effects tenuous (Richelson and Souder, 2000; Koo et al., 2010). There is also evidence that 5-HT_{2A} receptors possess constitutive activity since they may be downregulated by the presence of antagonists (Barnes and Sharp, 1999), providing another possible explanation for the synergistic interaction of 5-HT_{2A} antagonism with some SSRIs. Goddard et al. (2008) suggest that low-dose antagonism of 5-HT_{2A} is key in OCD treatment response, since this selectively targets the mPFC (Blair et al., 2006), whereas higher doses produce antagonism in other OCD-specific brain regions which may in fact be counterproductive to the desired effect.

Interestingly, the 5-HT_{2C} receptor, another G_q-coupled receptor (like 5-HT_{2A}) which also stimulates PLC (Millan et al., 2008), has also been associated with O/C-like behavior expression in animal models. In fact, 5-HT_{2C} knockout mice express thoughtful, patterned chewing routines when presented with non-nutritive plastic screens. This behavior is thought to resemble the goal-directed and planned motor routines in OCD (Chou-Green et al., 2003b). In a different investigation, the same authors demonstrated that 5-HT_{2C} knockout mice also consume food excessively and that they become overweight only under certain stressful conditions. These findings suggest a role of stress-sensitization and impulse control deficits related to the absence of 5-HT_{2C} receptors (Chou-Green et al., 2003a). However, in the signal attenuation model of OCD, 5-HT_{2C} receptor antagonism reduced the expression of compulsive behaviors (Flaisher-Grinberg et al., 2008; Fineberg et al., 2010). Agonists of the receptor have been shown to induce a compulsive-like grooming behavior (Graf, 2006) whereas other studies have downplayed the role of 5-HT_{2A/C} receptors completely in the context of pre-clinical studies (Tucci et al., 2015; Majić et al., 2017). Human studies have shown limited evidence that *activation* by means of the non-specific 5-HT_{1A/2A/2C} agonist, psilocybin, reduces obsessive symptoms (Moreno et al., 2006). Despite this apparent ambiguity of the overall collection of results, it is interesting that 5-HT_{2A} and 5-HT_{2C} elicit diverging responses with respect to compulsive behavior. This possibly indicates that they govern unique subpopulations of

neurons which at some level regulate the behavioral expression of repetitive behavior. In line with this, Goddard et al. (2008) suggested that 5-HT_{2A} antagonists and 5-HT_{2C} agonists may play a beneficial augmenting role in the treatment of OCD when co-administered with SSRIs. Importantly, these two receptors may also play diverging roles in the expression of impulsive vs. compulsive behaviors as elaborated in section 2.3.5. One last interesting trait shared by both 5-HT₂ subtype receptors is that they are notably downregulated following chronic exposure to agonists *and* antagonists (Barnes and Sharp, 1999), which may also play an interesting role with respect to the treatment response of the augmentation strategy.

The 5-HT transporter

Since the SERT protein is the target of SRI/SSRI therapy, aberrant expression thereof is also possibly involved in the pathology of OCD, particularly by permitting a hyperdopaminergic state (Westenberg et al., 2007; Koo et al., 2010). Many clinical investigations have implicated altered SERT expression in OCD patients compared to controls and in response to SRI treatment (Bengel et al., 1999; Stengler-Wenzke et al., 2004; Hesse et al., 2005; Hasselbalch et al., 2007; Reimold et al., 2007; Zitterl et al., 2008; Matsumoto et al., 2010; Atmaca et al., 2011). Although not without doubt, these findings possibly indicate either genetic insufficiency or degenerative SERT-related pathology in OCD sufferers. Indeed, genetic studies have identified a possible link between SERT polymorphism and OCD (Taylor, 2013). Similar findings have been made in animal studies of OCD (Wolmarans et al., 2013), where it has been demonstrated that deer mice presenting with O/C-like behaviors present with lower striatal SERT concentrations compared to normal controls. Activation of the previously discussed 5-HT receptors can also influence the membrane expression of SERT proteins indirectly (Millan et al., 2008).

2.2.2.2. The Dopaminergic System

Considering the role of DA in the functioning of the CSTC circuitry (Denys et al., 2004b; Pessiglione et al., 2006; Cools et al., 2009; Di Filippo et al., 2009; Koo et al., 2010), as well as in the processing of reward appraisal, reward-based learning and behavioral feedback processing (Schultz, 2002; Phillips et al., 2003; Wise, 2004; Morris et al., 2006; Pessiglione et al., 2006; Schultz, 2007; Schott et al., 2008; Di Filippo et al., 2009; Schultz, 2013, 2017), and that OCD is postulated to be founded in altered CSTC circuit functioning (see paragraph 2.2.1; Nambu, 2008; Markarian et al., 2010; Manning, 2016; Monteiro and Feng, 2016) and deficient reward processing (see paragraph 2.3.4; Szechtman and Woody, 2004; Brown et al., 2011; Figuee et al., 2011; Palminteri et al., 2012; Pinto et al., 2014), dysfunctional dopaminergic signaling is believed to be a key role player the pathology of OCD (Denys et al., 2004b; Koo et al., 2010). Dopaminergic neurons of the nigrostriatal pathway and their connections to the

SNr/SNc and subsequent projections to the CSTC circuit members are implicated in OCD pathology (Wise, 2004; Nambu, 2008; Wise, 2009; Milad and Rauch, 2012) by virtue of their role in motor and cognition activation (Wise, 2004, 2009). Furthermore, mesolimbic DA pathways which connect the amygdala/MTA with the striatum and other reward and memory processing circuits, are also implicated (Holden, 2001; Schott et al., 2008; Di Filippo et al., 2009; Marek et al., 2013).

The involvement of dopaminergic neurotransmission in OCD is supported at a neurocognitive level by demonstrations that 5-HT acts as a behavioral opponent to the actions of DA (Daw et al., 2002; Boureau and Dayan, 2011; Cools et al., 2011; Palminteri et al., 2015) and that OCD is purported to be a condition characterized by hyposerotonergic functioning. Further, as alluded to earlier, a lack of significant serotonergic input to the SNr (Moukhles et al., 1997), thereby permitting a hyperdopaminergic tone to exist in the CSTC circuit, is believed to underlie OCD (Westenberg et al., 2007; Koo et al., 2010; Nikolaus et al., 2010). Pharmacological studies showing that augmentation of SSRI therapy with low-dose antipsychotics may prove valuable in treating SSRI refractory OCD (Bloch et al., 2006; Komossa et al., 2010; Veale et al., 2014), and that down-regulation of DA transporters (Hesse et al., 2005) in OCD may be reversed by SSRI therapy (Pogarell et al., 2005), convincingly implicate DA in the pathophysiology of the condition. Furthermore, a wealth of evidence demonstrates that manipulation of dopaminergic receptors may aggravate OCD symptoms in both clinical and pre-clinical investigations (Lykouras et al., 2003; Ondo and Lai, 2008; Schirmbeck and Zink, 2012; Sevincok et al., 2014; Kim et al., 2019), supporting the concept of perturbations in dopaminergic signaling in OCD.

DA receptors, like the majority of 5-HT receptors are a family of G-protein coupled receptors, with 5 major subtypes identified (Missale et al., 1998). DA D₁ and D₂ receptors are the subtypes primarily investigated in OCD due to their status as targets of many of the adjunctive therapies and their distribution throughout the various structures innervating the CSTC circuits and other O/C-related role players. However, investigations into the involvement of other receptors such as D₄, due to their *apparent* structural and functional similarity (D₁ and D₅ are highly alike, as are D₂ and D₄; Missale et al., 1998), are also emerging (Camarena et al., 2007). D₁ and D₂ receptors respectively stimulate and inhibit neurotransmission by the action of their second messengers (G_s and G_i respectively) and their subsequent influences on cAMP concentrations (Missale et al., 1998; Di Filippo et al., 2009). While dysfunction in serotonergic neurotransmission remains the most prominent focus relating to OCD neuropathology, the role of DA is becoming increasingly clear and important for our understanding of future therapeutic strategies (Denys et al., 2004b; Koo et al., 2010). Considering the core focus of the current project, as well as that the SSRI escitalopram will be employed in this work, a

detailed review of the dopaminergic involvement in OCD, falls outside the scope of this overview. For this, the reader is referred to (Denys et al., 2004b; Sesia et al., 2013; Hauser et al., 2017).

2.2.3. In summary of the proposed neurodysfunction characteristic of OCD

Despite the inherent complexity of its underlying pathology, OCD is mainly characterized by a relative *hyperactivity of* dopaminergic neurotransmission, which is permitted by a relative downward shift in serotonergic signaling. OCD patients therefore theoretically exhibit increased activation of the direct CSTC circuit pathway, priming them for excessive and abnormal cognitive processing, behavioral activation, and altered learning processes which describe many of the aberrancies characteristic of OCD (see section 2.3). In line with this, serotonergic agents like SRIs are believed to improve serotonergic signaling, bringing balance to the functioning of dopaminergic pathways that regulate the direct and indirect CSTC circuit pathways.

The therapeutic effects seen in OCD following the administration of SSRIs are partly modulated via 5-HT_{1A/B/D} auto-receptors which can slow both serotonergic and dopaminergic outflow dependent on their specific neural localization. Theoretically speaking, blocking 5-HT_{1A} would eventually improve serotonergic tone (as explained in 2.2.1), whilst activating 5-HT_{1A/B/D} receptors would curb dopaminergic activity. Furthermore, antagonism of the post-synaptic 5-HT_{2A/C} and D₂ receptors would seem important, since these receptors are the primary targets of the atypical antipsychotic medications (Goddard et al., 2008). Theoretically, non-selective antagonism of D₁ and D₂ would represent the ideal avenue for the treatment of OCD. Blocking the behaviorally activating effects of the direct pathway via D₁ receptor antagonism is thus only a partial solution which is indeed reflected in the clinical treatment response (Westenberg et al., 2007; Fineberg et al., 2013a). The indirect pathway functions to inhibit behavior; however, D₂ receptor activation normally *suppresses* activity of this pathway, resulting in a behaviorally *activating* effect (Groenewegen, 2003). Blockade of D₂ will therefore theoretically prevent inhibition of the pathway, allowing it to exert inhibitory control.

In light of this, the current work lays the foundation to explore the involvement of serotonergic functioning, as indicated by frontal-cortical 5-HT_{1A} receptor expression density, in two unique, but equally persistent and repetitive compulsive-like behavioral phenotypes in the deer mouse model of OCD. More specifically, we would like to establish whether different phenotypes of the same putative condition, may be founded in distinct 5-HT_{1A} receptor involvement. The first steps have been taken to investigate this (see preliminary results, addendum C).

* * *

2.3. Cognitive Theories of Obsessive-Compulsive Behavior

A number of studies have investigated possible cognitive abnormalities in OCD patients that may underlie the expression of O/Cs. Given that the present investigation is aimed toward a better approach to assessing animal behavior from an ethological perspective in order to better understand *abnormal* behavioral phenotypes as they reflect extreme manifestations of otherwise normal behavior, such cognitive disturbances will now be discussed as a theoretical basis for the design of the current study. In the current context, HMB and LNB behavior in deer mice are postulated to represent two distinct O/C-like phenotypes, and will be considered here. Indeed, taken from clinical literature, it is evident that OCD is founded in a complex interplay between genetic, physiological and environmental factors (Chamberlain et al., 2005; Markarian et al., 2010; Taylor, 2013; Brander et al., 2016; Gruner and Pittenger, 2017). This section will attempt to illuminate the atypical cognition that underlies OCD which, together with the aforementioned neurobiological factors, may contribute on a psychological level to the symptomology of the condition.

2.3.1. Dysfunctional Beliefs in the Development of Obsessions and Compulsions

In an attempt to better understand altered thought processes underlying psychopathological conditions such as OCD, putative cognitive theories have been proposed. One such theory involves the occurrence and dysfunctional interpretation of intrusive thoughts (Rachman, 1997) and is based on previous work investigating OCD (Salkovskis, 1989; Salkovskis, 1996). As alluded to earlier, obsessions can be defined as ‘repetitive, prolonged thoughts of an intrusive nature, or any type of mental image which is considered unwanted by the sufferer and generally found to be unpleasant’ (Najmi et al., 2009; American Psychiatric Association, 2013; Seli et al., 2016). Congruent with this definition, it is accepted that all persons experience intrusive thoughts to some extent, but that such intrusions, in the context of OCD, are distinguishable by virtue of their duration, degree of mental interference and the level of distress elicited (Rachman, 1971; Rachman and de Silva, 1978; Julien et al., 2007; Rassin et al., 2007). The dysfunctional belief theory of Rachman (1997) argues that O/Cs arise from the ‘catastrophic misinterpretation’ of intrusive thoughts. This assertion is founded on two core concepts, i.e. 1) that the inappropriate interpretation of any sensation or stimulus can cause anxiety, and 2) that OCD patients often overestimate the practical importance of their obsessions; this is associated with a decreased ability to suppress or control these thoughts (Abramowitz et al., 2003; Julien et al., 2007; Najmi et al., 2009), permitting them to develop into truly all-consuming obsessions. Further, it is theorized that once these thoughts have been misinterpreted, anxiety is aroused that ultimately results in *either* avoidance behavior or

compulsive attempts to neutralize the alarming stimulus. This concept is of major importance in the current investigation, as both of these coping styles have been hypothesized to underlie the marble-burying response (De Boer and Koolhaas, 2003). In line with the theory of Rachman (1997), other authors have also proposed related cognitive models based on the appraisal of dysfunctional beliefs (Frost and Steketee, 2002; Clark, 2004). In an extensive review by Julien et al. (2007), the authors presented evidence to support the notion that intrusive thoughts occur in *all* persons. However, congruence between intrusive thoughts and actual obsessions was also demonstrated by significant correlations between the content of intrusions and the nature of the O/Cs being expressed. To investigate whether dysfunctional beliefs were related to all subtypes of OCD or to certain O/C constructs only, Taylor et al. (2006) presented findings that somewhat support the appraisal theories of OCD. The authors identified two distinct cohorts of OCD patients, viz. those who demonstrated increased measures of perfectionism as well as an inflated appraisal of O/C beliefs, and those who measured similar to healthy controls. Interestingly, O/C constructs diagnosed in patients of the former cohort include thoughts related to harm, anxiety, and depression. It can therefore be proposed that patients experiencing fear of harm (another cognitive propensity of OCD sufferers, see 2.3.2), may demonstrate elevated anxiety and thus ascribe inflated importance to these thoughts, ultimately progressing to safety-seeking O/Cs. Taylor et al. (2006) have since suggested that 3 key features of pathological belief, i.e. 1) increased personal importance ascribed to OCD-related thoughts, 2) intolerance of uncertainty and 3) an elevated sense of personal responsibility to overestimated threat, play a central role in both the *development* and *maintenance* of aberrant thought processes in OCD.

Taken together, it is hypothesized that OCD patients may be unusually sensitive to certain stimuli, possibly due to an unrealistic appraisal of the importance of thoughts and feelings arising from interacting with highly particular contextual stimuli. The theory of dysfunctional beliefs in OCD provides some theoretical basis for the current investigation as we hypothesize that while HMB may be driven by fears of contamination or foreign objects, LNB behavior may be motivated by inflated feelings of perfectionism and/or insecurity.

2.3.2. Overestimation of Threat

Closely related to the theories of Salkovskis (1989) and Rachman (1997) is the tendency of OCD patients to overestimate threat as suggested by the Compulsive Cognitions Working Group (2001) and supported by others (Muller and Roberts, 2005; Taylor et al., 2006; Julien et al., 2007; Cisler and Koster, 2010; Robinson and Freeston, 2014; Wang and Zhanjiang, 2017). Applying the obsessional beliefs questionnaire, the OCCWG assessed a range of factors related to the cognitive theories underlying OCD, i.e. dysfunctional beliefs pertaining

to *personal responsibility* towards feelings of *threat to self or others*. OCD patients, but not healthy controls have previously been shown to demonstrate aberrant cognitive processing manifesting as 1) an overestimation of threat, 2) an inflated sense of responsibility, 3) intolerance of uncertainty, 4) perfectionism, 5) over-emphasizing the importance of intrusive thoughts, and 6) an exaggerated need to exert control over such thoughts (Obsessive Compulsive Cognitions Working Group, 2001). These findings constituted early evidence that OCD patients present with altered prevailing cognitive patterns compared to the non-clinical sample.

To elaborate on the concepts provided by (Obsessive Compulsive Cognitions Working Group, 2001), Exner et al. (2014) aimed to elucidate the cognitive nature of threat over-estimation in OCD. This investigation was based on the aforementioned findings suggesting that OCD patients may demonstrate attentional bias towards perceived environmental threats and that they may experience an increased sense of vulnerability to these threats (Sookman and Pinard, 2002; Moritz and Jelinek, 2009). As such, Exner et al. (2014) employed a cognitive task (probabilistic cue learning) comprising two unique role-play scenarios. Whereas the first scenario was designed to be emotionally non-provocative (predicting weather), the second was intentionally emotionally arousing and involved predicting the possible outcomes of a global epidemic-scale pathogen outbreak. Interestingly, the two groups performed similarly in the neutral task, while the OCD cohort showed a decreased prediction accuracy compared to the control group in the emotional task. When applying the 6 dimensions of the obsessional beliefs questionnaire alluded to above, only patients who over-estimated threat, demonstrated deficits in the risk prediction task. Taken from the Exner et al. (2014) investigation, three important putative conclusions can be drawn, viz. 1) the functional capacity of OCD patients remains unaffected in the absence of contextual reminders, e.g. washing compulsions will often only be worsened in the presence of possible contamination cues and not otherwise, 2) context-specific circumstances may allow maladaptive learning processes in OCD patients, and 3) over-estimation of threat was the only dimension of the obsessional beliefs questionnaire related to deficits in contextual learning. While this study demonstrated that maladaptations in context-specific learning are observable in OCD, several studies have indicated reduced memory function in a number of general memory tasks which are postulated to result from disrupted CSTC circuit functioning as discussed earlier (Savage et al., 2000; Joel et al., 2005b; Muller and Roberts, 2005). Feedback learning under rewarding and punishing circumstances is also abnormal in OCD, as will be elaborated upon in section 2.3.3. The underlying principle in the ritualistic performance of compulsions to avoid future harm is characteristic of OCD (Grabill et al., 2008; Grassi et al., 2015); *more importantly* however, is that this is often only true in terms of avoiding irrational, over-estimated, attention-biased

threats (Muller and Roberts, 2005; Cisler and Koster, 2010) since patients often incur and endure various other negative outcomes, while neglecting other important life processes in order to attempt to protect themselves from these (Hollander et al., 1996; Mancebo et al., 2008; Huppert et al., 2009; American Psychiatric Association, 2013; Mavrogiorgou et al., 2015).

As is true for the theory of dysfunctional belief (Rachman, 1997), the possibility that OCD patients over-estimate threat also has relevance for the current study as we hypothesize that LNB as expressed by deer mice, may be related to such a form of aberrant cognition (Jirkof, 2014). Similarly, HMB, if characterized appropriately, may also be a behavior aimed at removing stimuli (marbles) that could potentially be considered threatening.

2.3.3. Deficits in Reward and Punishment Related Learning

Before we consider deficits in reward and punishment related processing, it is important to place the concept of ‘feedback’ within the motivational framework directing the actions of organisms. It is suggested that a significant proportion of the repertoire of human and animal behavior (and therefore learning) is aimed at either obtaining a specific reward (or a desired outcome), behaviors which are guided by reward learning (RL; Schultz, 1998; Hollerman et al., 2000; Schultz, 2007; Wise, 2009) or avoiding negative consequences that are guided by punishment learning (PL; Harvey et al., 1975; Seymour et al., 2007; Cools et al., 2008; Faulkner and Deakin, 2014). Further, abnormalities in both RL (Figeet et al., 2011; Milad and Rauch, 2012; Pinto et al., 2014; Hauser et al., 2017) and PL (Fullana et al., 2004; Morein-Zamir et al., 2012; Endrass et al., 2013) have been implicated in the manifestation of certain O/C phenotypes. Within this context, it is believed that specific populations of serotonergic and dopaminergic neurons are linked to the processing of PL and RL respectively and in an oppositional configuration (Daw et al., 2002; Seymour et al., 2007; Wise, 2009; Boureau and Dayan, 2011; Cools et al., 2011). However, both SRI/SSRI (McCabe et al., 2010; Palminteri et al., 2012) and dopamine modulating (Missale et al., 1998; Schultz, 2002; Pessiglione et al., 2006; Schultz, 2013) drugs appear to directly modulate the cognitive processing of both RL and PL, presenting interesting implications for their possible cognition-altering mechanisms in O/C symptom attenuation.

Considering the role of dopaminergic signaling in the pathology of OCD as alluded to in paragraph 2.2.2.2, it is noteworthy that studies into cognitive functioning have revealed its involvement in the processing of reward related feedback, contextual learning and approach behavior (Schultz, 2002; Wise, 2004; Cools et al., 2009). In short, the term ‘reward’ within the current context denotes any type of experience which elicits a sense of pleasure or gain, e.g.

the satisfactory completion of a goal-directed action or tasting appetizing food (Schultz, 2002; Wise, 2004). Increased dopaminergic signaling has been measured during such rewarding experiences. Not only is DA released in *response* to rewarding circumstances (Schultz, 1998; Phillips et al., 2003; Brown et al., 2011), but also begins to be released during the *anticipation* of such rewards as associations are made, which can prime behavior towards attaining previously obtained rewards (O'Doherty et al., 2002; Schultz, 2002; Schott et al., 2008). Furthermore, phasic reductions in dopaminergic signaling are evident when rewards are expected, but *not* obtained (Schultz, 2002; Wise, 2009; Ting et al., 2015; Hauser et al., 2017). These differences between dopaminergic responses during rewarding and non-rewarding outcomes are believed to be fundamental to consolidate response-outcome learning. In fact, differences in dopaminergic signaling in response to reward (increased spikes of activity) or punishment (suppressed activity), constitute a code to guide future behavior by associating different levels of dopaminergic activity to the expected outcomes of actions (Schultz, 2002; Wise, 2004; Morris et al., 2006; Schultz, 2013, 2017). As such, increased dopaminergic signaling during the experience of reward - for instance during the administration of psychoactive drugs - will induce future reward seeking behavior (Wise, 2009; Ting et al., 2015), whereas a decline in DA release experienced during anticipated (but unrewarded) situations, might induce a reduction in attentional arousal and motivational drive towards such scenarios over repeated presentation (Schultz et al., 1997; Hollerman and Schultz, 1998). However, DA release which remains unaltered during *repetitive but similar* outcomes, e.g. locking doors, washing hands, and brushing teeth, will normally maintain habitual, but also goal directed behavior, *viz.* not inducing either excessive outcome-seeking or avoidance behavior. In summary, dopaminergic signaling appears to be paramount in learning from rewarding experiences, reinforcing associations between certain actions and said rewards, activating behavior to acquire learned rewards, and arousing attention to potentially rewarding scenarios. Changes to dopaminergic activity are also significant with respect to the unlearning of previously conditioned rewards (reversal learning), a process which becomes impaired in patients stabilized on drugs which manipulate dopaminergic activity and is once again closely correlated with CSTC circuit function (Cools et al., 2002).

That said, as explained earlier, OCD has been linked to fear and anxiety, which may result in behavioral rituals to avoid feared outcomes (Markarian et al., 2010). Once again, a definition for punishment is required for the context of this discussion, which can be simplified as: "*stimuli which motivate avoidance behavior*" (Deakin and Graeff, 1991). According to the opponency theories which describe the functional relationship between DA and 5-HT (Daw et al., 2002; Boureau and Dayan, 2011; Cools et al., 2011), serotonergic neurotransmission mediates distinct cognitive processes relating to PL (Deakin and Graeff, 1991) particularly within the

amygdala and OFC (Seymour et al., 2007). While evidence for the role of DA in RL is quite significant and well accepted, the role of 5-HT with regard to PL is far more ambiguous (Seymour et al., 2007; Crockett et al., 2009; Crockett et al., 2012; Faulkner and Deakin, 2014). Evidence of serotonergic activation during aversive events underlies these findings, particularly via 5-HT_{1A} receptors of the dorsal raphe nuclei (Deakin and Graeff, 1991). The role of 5-HT has also been further investigated in numerous 5-HT depletion studies (see Faulkner et al., 2014 for review); these yielded largely inconclusive findings.

In simplistic terms, in a manner similar (but opposed) to DA, serotonergic signaling demonstrates phasic increases during the experience of aversive stimuli and thus is involved in processing the *negative valence* associated with an event (Deakin and Graeff, 1991; Cools et al., 2011; Geurts et al., 2013). Therefore, whereas DA is closely linked to reward processing and the motivational drive to acquire rewards (Wise, 2004; da Silva et al., 2018), 5-HT inversely encodes *aversion* and promotes behavioral *inhibition* to avoid punishment (Boureau and Dayan, 2011; Cools et al., 2011). As such, certain paradoxical situations will arise where behavioral *activation* will be necessary to avoid punishment. These are proposed to be resolved by the brain's ability to calculate differences in the values of both the aversive and rewarding outcomes (Cools et al., 2011). Hence, opponency theories place greater emphasis on the *differences* between positive and negative valence in the ultimate decision-making process. Logically, disruptions one way or another (since OCD shows disruptions in dopaminergic and serotonergic functioning) could result in unnaturally biased reward and punishment valuation, leading to abnormalities in learning and action motivation. By extension, said processing is involved in behavioral switching, i.e. changes of responses according to feedback, which can be as basic as inhibiting a behavior that elicits any kind of aversive affect or motivating another totally different behavior. Incidentally, behavioral task switching is also affected in OCD (Gu et al., 2007; Gruner and Pittenger, 2017).

The crucial final piece of the puzzle is that of task completion. The successful completion of any goal directed action should ultimately result in a sense of completion which feeds back and inhibits any excessive and unwarranted behavior. Indeed, loss of such inhibitory control is characteristic of conditions exhibiting repetitive behaviors such as substance abuse, TM, pathological gambling and OCD (Fineberg et al., 2010; Dalley et al., 2011; Hirschtritt et al., 2017). As explained above, 5-HT and DA play prominent roles in the inhibition of excessive behavior by means of calculating the value thereof. Indeed, excessive dopaminergic activity can facilitate excessive reward seeking (Arias-Carrión and Pöppel, 2007; Adamantidis et al., 2011; da Silva et al., 2018), whilst a lack of 5-HT activity can cause a lack of behavioral inhibition (Clarke et al., 2006; Crockett et al., 2009; Faulkner and Deakin, 2014). Both cases lead to behavioral loops without any clear endpoint and which are subsequently performed

relentlessly, once again highlighting the importance of a balanced dopaminergic and serotonergic function, particularly taking into account that such dysfunctions are typical of OCD. Demonstrable deficits in serotonergic function in OCD appear to play a role here, since patients exhibit abnormalities in task completion. Hence, the condition has even been proposed to be a “pathology of stopping” (Szechtman and Woody, 2004; Hoffman and Morales, 2009; Hinds et al., 2012).

Applying these principles to patients with OCD, it can be assumed that a patient expressing washing compulsions may feel compelled to engage in stereotyped rituals in response to an increased perceived threat of being contaminated and an inflated sense of importance ascribed to dealing with this perceived threat (according to sections 2.3.1 and 2.3.2). Based on the DA/5-HT response-outcome learning theory, the completion of a typical hand washing cycle should provide appropriate feedback and a sense of task completion, while further hand washing rituals, eliciting the same repetitive rewarding response, should be prevented. However, since OCD patients persist in the repetition of specific cognitive (i.e. mental counting) and motor (i.e. hand washing) routines, it can be hypothesized that the interplay between reward anticipation, valuation, subsequent action motivation and task completion, all of which are dependent on dopaminergic and serotonergic signaling, is disrupted.

The question thus arises whether the repetitive motor patterns observed in patients with OCD can in fact be related to deficient reward processing at one of the aforementioned levels. This idea culminated in a bridging collective theory that O/Cs may be founded in abnormal reward processing related to the completion of what are *proposed to be anxiety reducing compulsions* (Holden, 2001; Kalanthroff et al., 2016). Nielen et al. (2009) aimed to test this theory by confronting two groups of participants (non-clinical and OCD cohorts, respectively) with two possible outcomes in an associative learning task, i.e. either gaining money following correct responses or losing money due to inappropriate actions. In short, participants were required to learn to respond to 10 cues, half of which required a button press, while the other half required the participants to withhold their input. Each cue and input (or lack of input) was immediately followed up with feedback, allowing patients to learn and update their behavior. The task was carried out under either the rewarding (monetary gain) or punishing (monetary loss) paradigms. Findings from this investigation demonstrated that OCD patients were not only slower to learn the correct associations in both paradigms, but that they learned even slower following punishing compared to rewarding feedback. Moreover, participants from the OCD cohort demonstrated significantly decreased response times compared to the non-clinical cohort. Interestingly, Y-BOCS scores were inversely correlated with learning difficulty, suggesting that the severity of OCD symptoms may differentially influence learning ability. This study was however limited by the fact that punishment was non-contextual (i.e. not related

to any particular O/C symptom dimension), but it did highlight *possible* learning abnormalities nonetheless.

To further elucidate the mechanisms underlying the proposed deficits in punishment learning in OCD patients, Palminteri et al. (2012) designed a study based on the opponency theory of Daw et al. (2002). To test this hypothesis, the investigation compared the associative learning abilities of healthy participants, as well as untreated and SSRI-treated OCD patients. Interestingly, SSRI treatment improved instrumental learning deficits associated with both reward and punishment feedback. These findings are juxtaposed relative to the theory of Daw et al. (2002), in revealing a role of 5-HT in both modalities of instrumental learning. Given that OCD patients often do not experience the reward of task completion (Reed, 1977, 1985; Hinds et al., 2012), and considering the role of anti-dopaminergic agents in the treatment of OCD, the findings of Palminteri et al. (2012) do not undermine the hypothesis that a hyperdopaminergic state may interfere with the normal coding of outcome-related feedback (Schultz, 2002; Wise, 2004; Cools et al., 2011). Indeed, the results from said investigation support both the Schultz (2002) and Daw (2002) theories in demonstrating that by virtue of its therapeutic effect in OCD, SSRIs may indeed oppose the purported role of DA in the pathology of OCD. Furthermore, recent publications have confirmed the crucial role of 5-HT in reward (Iigaya et al., 2018) and the role of DA in a defensive behavior, historically ascribed to 5-HT (Faulkner and Deakin, 2014; Lloyd and Dayan, 2016). Taken together, the findings summarized in this paragraph are as a whole congruent in confirming significant impairments in reward and punishment processing in patients with OCD. As such, the current investigation will attempt to elucidate the nature of such deficits in an established and robust animal model of OCD that purportedly resembles more than one O/C behavioral phenotype.

2.3.4. The Role of Habit in Normal Behavior and OCD

Closely related to reward circuitry is the relatively novel concept of ‘behavioral addictions’ and how habitual behavior may become a form of addiction (Holden, 2001; de Wit and Dickinson, 2009). According to Holden (2001), the classic description of addiction was limited to the compulsive use of addictive substances. However, it is argued that certain *habitual* behaviors, e.g. gambling, food and sex addictions and excessive internet use may resemble substance addiction in terms of its persistence, susceptibility to tolerance, clinical withdrawal symptoms following the cessation thereof, and propensity to evolve into impulsive behaviors, all of which are founded in a lack of CSTC circuit mediated control (Sun et al., 2009; Nederkoorn et al., 2010; Dalley et al., 2011; Griffiths, 2012). Pathological habits such as excessive gambling have been shown to persist due to habit formation and *not* due to satisfaction of the activity itself, possibly indicating RL disruption (Jolley et al., 2006). Such observations have prompted

a review of brain activity and cognitive function in patients diagnosed with gambling addiction revealing that patients demonstrate a decrease in reward sensitivity, diminished sensitivity to punishment, and an increased attention bias toward gambling related cues (van Holst et al., 2010). In addition, activation of the ACC, PFC, OFC, and amygdala is elevated in gambling addicts faced with contextual cues (van Holst et al., 2010). These findings are remarkably similar to those observed in OCD and are congruent with theories pertaining to the aberrant cognition and neurobiology proposed to underlie O/C behavior. Other findings revealed gambling-addicted individuals show activation of brain regions analogous to those of individuals addicted to alcohol and cocaine (Goudriaan et al., 2006), highlighting similar neurological involvement underlying behavioral and substance addictions. Indeed, OCD patients are hampered in another related cognitive domain known as response inhibition (RI), i.e. the ability to inhibit pre-potent actions (Chamberlain et al., 2007; Page et al., 2009; Haber and Heilbronner, 2013; Rasmussen et al., 2016). RI deficits are evident in the development of addictive conditions like substance abuse, gambling and overeating (Lawrence et al., 2009; Nederkoorn et al., 2010). Therefore, given that habit formation is related to RI deficits, and that OCD patients typically experience difficulty exerting RI, it is possible that OCD rituals may constitute some type of behavioral addiction, a concept which is gaining increased interest as an explanation of the cognitive genesis for O/Cs (Evans et al., 2004; Page et al., 2009; Gillan et al., 2011; Banca et al., 2015; Rasmussen et al., 2016; Vaghi et al., 2017).

Habitual behavior is an important aspect of daily life and is not per se linked to purposelessness. To further elaborate on how habit modulates normal behavior, the dual-system theory of behavior (de Wit and Dickinson, 2009) proposes that two main motivational systems drive voluntary and planned action, i.e. the goal-driven and habitual systems. Simply stated, goal-directed motivation primes an individual to take certain steps to achieve a specific outcome, e.g. the washing of hands (Dickinson and Balleine, 1993; Balleine and Dickinson, 1998; de Wit and Dickinson, 2009). Habitual responses however take over when the same action (often involving making a decision which rarely changes) is performed in a repetitive fashion and the outcome is well expected. In other words, actions are primed by association with a particular expected outcome, learned from prior experience (Adams and Dickinson, 1981; Adams, 1982; Dickinson and Balleine, 1993; Gillan et al., 2016; Smith and Graybiel, 2016). Functionally, habitual routines are important in the daily activities of humans and aid in facilitating repetitive but normal actions, e.g. the path to walk to work or locking doors which are no longer explicitly rewarding. Although habitual behavior is inflexible by nature, it remains purposeful and necessary, since it allows otherwise complex motor plans to be executed at a far lower cognitive cost, sparing precious cognitive processing capacity for other tasks (Gillan et al., 2016). However, an example of habitual action without explicit functional purpose is

provided by the 'slip of action' phenomenon, where a habitual action interferes with a goal directed action e.g. washing hands after entering the bathroom when the goal was simply to retrieve an object left behind there. Habits become pathological when these automated responses occur excessively or inappropriately, or when behaviors fail to change to promote superior outcomes (Banca et al., 2015; Gillan et al., 2016).

Considering the possible role of abnormal habit formation in OCD, Gillan et al. (2011) provided evidence of decreased sensitivity to the devaluation of goal-directed outcome-of-action in OCD sufferers and hence proposed that compulsive actions may be performed ritualistically due to the lack of 'decreasing reward returns' (and subsequent RI failures) on actions. This in turn develops into habitually-controlled responses which are triggered by specific stimuli (Balleine and Dickinson, 1998) and cannot be inhibited, possibly accounting for the fact that compulsions only develop in the framework of the particular symptom subtypes that are observed in OCD patients. In line with previous findings (Holden, 2001; Denys et al., 2004b), the authors conclude that this overreliance on habit instead of outcome, coupled with a decreased sense of 'closure' after completing tasks, may underlie certain phenotypes of O/C behavior. The same authors followed up on these findings by demonstrating that OCD patients are also prone to forming avoidance habits in response to negative outcomes which accompany their compulsions (Gillan et al., 2014; Gillan and Sahakian, 2015; Gillan et al., 2016).

2.3.5. Impulsivity and OCD - The Other Side of the Same Coin

Impulsivity and compulsivity both refer to cognitive constructs that ultimately lead to a lack of behavioral control, which at face value may resemble one another, but are quite distinct in terms of the cognitive patterns underlying them. Impulsivity is a trait purportedly linked to OCD and which is founded in the disruption of cortico-striatal function, similarly to OCD (Dalley et al., 2011). Impulsivity is also studied in relation to a number of other psychological disorders such as substance abuse, pathological gambling, and attention deficit hyperactivity disorder (Fineberg et al., 2010; Robbins et al., 2012; Grassi et al., 2015). While OCD is characterized by the compulsive performance of tasks, a possible link between pure impulsivity and OCD has been investigated (Sohn et al., 2014; Grassi et al., 2015). More specifically, distinct circuits within the CSTC may be involved in governing impulsivity and compulsivity, respectively (see Fineberg et al. (2010) for review). However, both constructs are founded in a loss of 5-HT dependent inhibitory control (Cools et al., 2011; Dalley et al., 2011). That being said, the two constructs can be differentiated based on findings from several investigational approaches *viz.* response to pharmacological interferences (5-HT_{2A} vs 5-HT_{2C} cause opposing effects on impulsive/compulsive tasks), neuropsychological task performance, and

neurocognitive imaging. That said, impulsivity does share certain traits with OCD such as a distinct implication of aberrant reward processing (Fineberg et al., 2010). At a glance, impulsive and compulsive behaviors may phenotypically resemble each other, e.g. with respect to behaviors performed thoughtlessly and excessively in a manner which may be perceived as irrational. However, where the two concepts diverge from one another, is in the fact that they result from unique deficits in response control (Summerfeldt et al., 2004; Dalley et al., 2011). By fusing the definitions of the two terms from various authors, the following descriptions can be offered: Compulsivity is purportedly rooted in stereotypical behavior which is resistant to diminishing reward as well as punishing outcomes and is rigidly performed according to pre-formulated plans in a ritualistic fashion which may be considered unpleasant by the individual (Summerfeldt et al., 2004; Fineberg et al., 2010; Robbins et al., 2012). Impulsivity goes hand-in-hand with erratic behavior which is founded in inadequate decision making processes, a disregard for reward and an inability to delay task performance resulting in rapid repetitious behavior without any particular outcome in mind (Durana et al., 1993; Fineberg et al., 2010; Robbins et al., 2012). The distinction between the two conditions can be better conceptualized within certain conditions such as substance abuse. Impulsivity may characterize the initial drug-seeking behaviors, which are performed without thought of consequences in a risky, inappropriate fashion. Over time, progression to compulsive substance seeking occurs, where the next dose is sought in rigid fashion in spite of negative outcomes (Robbins et al., 2012).

While the association between OCD and impulsivity is not clear, there is evidence that OCD patients do indeed exhibit increased impulsivity (Sohn et al., 2014; Grassi et al., 2015). In line with this, the current investigation tested in a specific phase, the excessive performance of a simple task (lever-pressing) following withdrawal of reward, which could also be construed to be impulsive task performance (within the boundaries of an animal model, wherein cognitive processes cannot be readily quantified or examined). Subsequent experiments assessing nest building under punishing circumstances could arguably measure compulsivity, since classical views have positioned impulsivity as reward/risk seeking-related behavior, while compulsivity was linked to harm avoidance. This may be a gross oversimplification but provides a proof-of-concept for investigation in an animal model.

In light of the discussion in section 2.3, we propose that certain forms of O/C behaviors (and by extension, similar animal behaviors, e.g. HMB and LNB) may be related to either absent or insufficient reward feedback processing, resulting in cognitive and/or motor routines aimed at seeking an ever elusive sense of closure following task completion. Together with the concept of habitual addictions as proposed in paragraph 2.3.4 and increased sensitivity to threat and

prominence of inflated beliefs or increased impulsivity, a possible framework of cognitive abnormalities in the deer mouse model of OCD is proposed.

2.3.6. Obsessive-Compulsive Phenotypes as Viewed in Light of the Cognitive Theories of OCD

2.3.6.1. Perfectionism-Driven OCD

As alluded to earlier, perfectionism has been suggested as an underlying factor in OCD symptomology for some time (McFall and Wollersheim, 1979). While typically not considered a symptom subtype in OCD classifications (Abramowitz et al., 2009; Rowsell and Francis, 2015), perfectionism is a behavioral trait often associated with the disorder as well as a number of others including anxiety, depression and eating disorders (Wu and Cortesi, 2009). OCD patients often experience intrusive thoughts pertaining to one or another stimulus being 'not just right'. These 'not just right experiences' (NJREs) may often result in distress and compel patients to undertake corrective actions directed toward the precipitating factor until a sense of perfectionism or completion is achieved (Coles et al., 2003; Julien et al., 2007). This type of experience goes hand-in-hand with symmetry and ordering O/Cs typically experienced by certain OCD patients and which are frequently related to a personal need for circumstances to be 'just right' (Radomsky and Rachman, 2004). NJREs may be conceptualized as obsessions when they become increasingly frequent, distressing and disabling (Coles et al., 2003). Although these findings were made in a non-clinical sample, the authors impress that the *interpretation* of these obsessions is the discriminating factor between normal everyday obsessions and those related to OCD. This is in line with the cognitive model of OCD proposed by Rachman (1997) and Frost and Steketee (2002) which proposes that intrusive thoughts are experienced by *all* persons, but due to their relentless and persistent nature, are ascribed an inflated sense of importance by the OCD sufferer due to the influence of the cognitive and biological malfunctions already discussed. Indeed, OCD patients have been shown to score highly on measures of perfectionism, even after controlling for co-morbid conditions such as depression (Coles et al., 2003; Ghisi et al., 2010). Sequentially viewed, a scenario may arise beginning with a person experiencing a particular NJRE in response to his/her surroundings. This intrusive thought may persist and be bothersome to such an extent that it develops into a true obsession, at which point corrective compulsions may be instituted to suppress the distress and regain control. However, since the person may have unrealistic standards for the completion of said corrective action due to highly perfectionistic traits coupled with an inherent insensitivity to task completion signals, no clear end point would be present, leading to an exacerbating behavioral loop.

Attempts have since been made to model such NJREs within animal models. Evidence of stereotyped nest-building behavior in rabbits was presented by (Hoffman and Morales, 2009). Here, the researchers presented evidence that rabbits will continue to transport nesting material to a nesting box if the researchers covertly removed any accumulated material. In contrast, undisturbed individuals would cease accumulating nesting material once their nests had been completed. That said, nesting material accumulation persisted in rabbits given *prebuilt* nests in the nest boxes, indicating that the building process itself was necessary to generate a stop signal to the straw carrying procedure. In other animal nesting models, aberrant nest building occurs despite any experimental manipulation (Greene-Schloesser et al., 2011; Wolmarans et al., 2016b). While the exact cause of excessive nesting behavior is unknown, it is possible that it may arise from a perfectionism/NJRE-driven construct as described above when considering that nesting is a behavior aimed at providing general protection and temperature control (Jirkof, 2014; Stewart and McAdam, 2017) and that the behavior is expressed when the animals are housed alone, which may increase the desirability of these.

2.3.6.2. Safety-related Obsessions and Compulsions

The second applicable symptom dimension is safety/checking (Rowell and Francis, 2015) which aligns with the theory of threat-overestimation. Often, OCD patients present with an attention bias towards threatening aspects of their surroundings (Moritz and Jelinek, 2009; Cisler and Koster, 2010; Exner et al., 2014). In a similar fashion to the proposed sequence of events describing OCD progression (from intrusive thoughts to obsessions to compulsions), patients with these biases may quickly develop intrusive thoughts. Whereas the aforementioned perfectionism-driven compulsions result in patients feeling uneasy or incomplete (Markarian et al., 2010), patients with safety related O/Cs experience sincere distress due to thoughts that they may be harming themselves or others through their own actions or inaction. Real life examples include an individual compulsively re-driving routes to ensure he did not harm anyone whilst commuting, or a teacher relentlessly flattening floor rugs to ensure that no one trips over them (Rachman, 2002).

To further illustrate the cognitive separation between different OCD constructs, one can consider contamination OCD reviewed above, which may appear to overlap with safety related concerns (Melli et al., 2015) to some extent; interestingly though, the neural activation patterns of ‘washers’ versus ‘checkers’ seem to be distinct (Phillips et al., 2000). In fact, contamination OCD is possibly motivated by two distinct cognitive processes (Melli et al., 2015). Evidence has also been presented that OCD patients suffering from contamination O/Cs present with impaired decision-making ability when exposed to contextually distressing scenarios, such as

role play tasks concerning pathogen outbreaks, but not in neutrally challenging scenarios (Exner et al., 2014). It should therefore be evident that, while the central concept of fear and harm avoidance is a common facilitator for O/Cs related to safety and contamination concerns, such fears follow distinct cognitive (and likely neural) pathways. This possibly explains why certain O/Cs are only precipitated in certain patients under certain circumstances (Mataix-Cols et al., 2004; McKay et al., 2004; Besiroglu et al., 2007).

From a translational perspective, based on the fact that various animals build nests for safety and protection amongst other purposes (Smithers, 1983; Jirkof, 2014; Stewart and McAdam, 2017), the construction of large nests and other stereotypical nesting-associated behaviors have been proposed as a model of compulsive behavior (Hoffman and Morales, 2009; Greene-Schloesser et al., 2011; Wolmarans et al., 2016b). While promising, more work is required to elaborate possible underlying neuropsychological processes associated with this occasionally aberrant behavior.

* * *

2.4. Developing Animal Models of OCD

Animal models of psychiatric conditions are employed to further our understanding of the etiology, pathology and symptom manifestation of the human condition. Accurate models also provide us with frameworks in which the testing of novel treatments can be performed in a pre-clinical setting (Alonso et al., 2015). According to van der Staay (2006), the definition of a robust animal model can be summarized as follows:

“An animal model with biological and/or clinical relevance in the behavioral neurosciences is a living organism used to study brain–behavior relations under controlled conditions, with the final goal to gain insight into, and to enable predictions about these relations in humans and/or a species other than the one studied, or in the same species under conditions different from those under which the study was performed.”

Before looking closer at developing animal models of psychiatric conditions, distinction between an animal model and a test of animal behavior should also be made. Animal models refer to naturally occurring or induced behavioral phenotypes which mimic those seen in humans, and that occur consistently in a specific species either naturally or due to external induction. An animal model should therefore provide an accurate and robust framework for investigating the human illness in as many facets as possible. On the other hand, tests of animal behavior refer to the tasks experimental animals are subjected to in order to quantify and characterize the core traits of the model. For example, the Flinders Sensitive Line (FSL) rat refers to a genetic line of rats that express depressive behaviors and distinct changes in neurotransmission. Whereas the FSL rat constitutes the model in which depressive-like phenotypes can be investigated, accurate tests that can assess and describe such phenotypes, will include among others, the forced swim test (Overstreet, 1993). It is with respect to this distinction that approaches to research are often vague. For example, in studies that investigate marble-burying behavior as an abnormal behavioral phenotype, some investigations regard the behavior in itself as a model of OCD, while in others, quantifications of marble-burying behavior are only used as a measure of a possible anxiety- or compulsive-like phenotype. Although such dichotomous approaches may be useful in certain scenarios, it is most often counterproductive, as will be explained in Chapter 4. When designing animal models of psychiatric conditions, three forms of validity are generally advocated to which such models must adhere. These three levels of validation criteria are termed face, predictive and construct validity, respectively (Willner, 1986; van der Staay, 2006; Fineberg et al., 2010; Fineberg et al., 2011; Albelda and Joel, 2012a, 2012b; Alonso et al., 2015), and together constitute the basis of robust, reliable, and advantageous animal models of psychiatric disease that allow for accurate comparisons between the modelled behavior and the human condition.

However, throughout Chapters 3 and 4, we will also argue for a fourth type of validity, i.e. methodological validity. As will be explained there, methodologically valid preclinical neuropsychiatric disease models should follow *contextual and appropriate* experimental design which is highly standardized to allow for appropriate reproducibility and which can deliver relevant, applicable and translatable results that can be repeated across laboratories. Although this may be regarded as a fundamental principle for all preclinical investigations, it is remarkable that only a handful of investigations of which the findings pertain to a singular theme, e.g. aberrant burying behavior, indeed adhere to this criterion, as we will show in Chapters 3 and 4. As the concept of methodological validity will be addressed in detail throughout this thesis, we will only summarize the three main criteria for the validation of psychiatric animal models as they have been established over time from the OCD-centric perspective, below.

2.4.1. Face Validity

In the context of psychiatric disorders, face validity pertains to the visible behavioral or symptomological similarities between the animal and the human patient (van der Staay, 2006; Albelda and Joel, 2012a). As explained earlier, OCD is partially characterized by repetitive behaviors that are *seemingly* senseless (Abramowitz et al., 2009). While motor repetition is characteristic of a number of animal models (Yadin et al., 1991; Hoffman and Morales, 2009; Wolmarans et al., 2013; Tucci, 2015), it remains challenging to describe such behavior in animals as O/C-like. As described in section 2.3, compulsions in OCD are believed to be associated with aberrant cognitive processes, which are difficult to demonstrate in animals. Furthermore, repetitive behaviors are a symptom of a number of psychiatric disorders, e.g. autism spectrum disorders (Leekam et al., 2011); Tourette's syndrome (Worbe et al., 2010), tic disorder (Kerbeshian et al., 2009), trichotillomania (Woods and Houghton, 2014) and Parkinson's disease (Stoffers et al., 2001) and therefore cannot be regarded as conclusive evidence to establish robust face validity in animal models of OCD. That said, a large number of these repetitive behaviors are fostered in the same neurobiological constructs and may thus represent varied expressions of the same maladaptive biological and the consequent cognitive failures induced thereby (Dalley et al., 2011; van Velzen et al., 2014).

However, by applying our current understanding of OCD, we can attempt to examine repetitive behaviors from different perspectives to establish associations between such behaviors and aberrant cognition. For instance, as alluded to earlier in this work, it is accepted that compulsions in humans are often performed in an attempt to avoid negative outcomes or to *temporarily* relieve some of the anxiety arising from obsessions (Rapoport, 1989; Gillan and Sahakian, 2015; Kalanthroff et al., 2016). It can therefore be hypothesized that should such

anxiety be the trigger of repetitive behavior, it could be possible that during the normal wake cycle of an animal, truly O/C-like behaviors will be expressed in bouts of varying intensity, followed by periods of reduced compulsive behavior until such time that the anxiety begins to intensify once more. Also, O/Cs are commonly triggered by certain environmental contexts or stimuli, e.g. if a patient expressing fears of contamination touches a foreign object and subsequently develops anxiety or feelings of disgust, this may ultimately result in cleaning compulsions (McKay et al., 2004; Markarian et al., 2010). Thus, if the repetitive behavior observed in animals are subject to being triggered by a specific *contextual* stimulus, such behavior would possibly be more reminiscent of OCD. Lastly, the motivational drive to carry out compulsions may be very overpowering, as evinced by the fact that preventing compulsions from being expressed, is often anxiogenic (Eilam et al., 2006; Abramovitch et al., 2013) and that compulsions are often carried out in spite of negative social and occupational consequences (Mancebo et al., 2008; American Psychiatric Association, 2013; Mavrogiorgou et al., 2015; Coluccia et al., 2016). Accordingly, compulsive-like behaviors in animals that voluntarily persist despite aversive feedback, may strengthen the face validity of an animal model of OCD as the inflated sense of importance underlying the expression of such behaviors overrides the punishment-related feedback. This concept is of major importance in the current study, most notably for the work presented in Chapter 5.

2.4.2. Construct Validity

Construct validity refers to similarities between the underlying pathophysiology induced or observed in the model animal to those typical of the human patient (van der Staay, 2006; Albelda and Joel, 2012b; Alonso et al., 2015). However, in psychiatry, the concepts of face and construct validity may often overlap in fundamental ways. Indeed, while repetitious behavior for example may be a characteristic of numerous models, such behaviors may be borne from unique underlying neurocognitive constructs and thus be representative of unique psychobiological processes. Thus, models of specific conditions will only be strengthened if both the symptom and its neuro-psychobiological foundation agrees with that observed in the human condition. In the case of OCD, this would involve *inter alia* demonstration of abnormal functioning of the serotonergic, dopaminergic, and glutamatergic systems coupled with involvement of the CSTC circuitry that together, in some manner, give rise to neuropsychological processes which are abnormal compared to the norm (Abramowitz et al., 2009; Fineberg et al., 2011; Albelda and Joel, 2012a; Gruner and Pittenger, 2017).

More specifically, animal models of OCD that are founded on robust construct validity may further demonstrate involvement of the OFC, ACC and basal ganglia (Coetzer, 2004; Albelda and Joel, 2012a; Manning, 2016), while demonstrable involvement of the 5-HT_{1A} (Lesch et al.,

1991; El Mansari and Blier, 2006), 5-HT_{1B/D} (Mundo et al., 2002; El Mansari and Blier, 2006; Moreno et al., 2006), and 5-HT_{2A/C} receptors (El Mansari and Blier, 2006; Goddard et al., 2008) will also highlight serotonergic mechanisms underlying the observed behavior.

In terms of dopaminergic involvement, altered activity or receptor expression density of striatal D₁/D₂ receptors could relate to O/C-like behavior as evinced by the modest therapeutic efficacy of DA antagonists in patients that remain refractory to monotherapy with SSRIs (McDougle et al., 1990; McDougle et al., 1994; McDougle et al., 2000; Denys et al., 2004b; Perani et al., 2008; Nikolaus et al., 2010). That said, although the effect of most atypical antipsychotics, e.g. risperidone and quetiapine, may be confounded by their 5-HT_{2A} antagonistic effects (McDougle et al., 2000; Westenberg et al., 2007; Goddard et al., 2008), previous findings have indicated the importance of both serotonergic and dopaminergic mechanisms in the therapeutic effects of such drugs (Adams et al., 2005; Flaisher-Grinberg et al., 2008; Perani et al., 2008).

Taking the above into consideration, we further propose that, as far as animal models of O/C-like behavior are concerned, the concept of construct validity must be expanded to include as far as possible, the demonstration of aberrant psychological processes that may underlie or drive the manifestation of motor routines. This is important as, taken from section 2.3, dysfunctional perceptions of otherwise normal circumstances or scenarios, often result in the expression of O/C behavior. If such anomalous cognition contributes to the development of OCD, it must be considered in association with abnormal pathophysiology as a unique, but equally important factor contributing to O/C-like behavior in animals.

2.4.3. Predictive validity

Robust predictive validity within the context of pharmacological studies involves the successful treatment of symptoms in the animal model using agents known to be effective in human therapy (van der Staay, 2006; Albelda and Joel, 2012a; Alonso et al., 2015). Conversely, agents and regimens tested and found *ineffective* to treat the human condition should *also* be ineffective in the model, as this implies similarity between the model and the actual condition (Fineberg et al., 2011). Predictive validity in this regard may demonstrate some overlap with construct validity, as successful treatment may implicate pathophysiology in the target system i.e. successful use of SSRIs/SRIs would indicate that serotonergic modulation to some extent at least, is involved in the manipulation of the observed behavior. However, from another non-pharmacological perspective, brain components and their role in the manifestation of psychological disorders are often elucidated by means of selectively damaging key components of implicated neural circuits and studying the behavioral effects thereof (Yin et

al., 2004; Joel et al., 2005a; Schilman et al., 2010). By providing an accurate framework in which to study the pathological or therapeutic responses of such interferences, the model would also be found on robust predictive validity.

Due to the fact that successful OCD treatment requires a notable onset period, i.e. 8 – 12 weeks, to evince therapeutic effect (Nutt et al., 1999; Goddard et al., 2008; Albert et al., 2013), it is important that chronic, but *not* sub-chronic or acute treatment is effective in an animal model of OCD. Although it can be argued that responses following acute treatment may highlight the involvement of relevant neurotransmitter systems and that a response only following chronic treatment is therefore not paramount, such findings may be of more relevance for the construct validity of the model. In fact, it would not be of much usefulness in terms of elucidating the mechanisms underlying the slow treatment response in clinical OCD. Similarly, OCD treatment typically requires higher SSRI doses than those normally employed to treat depression (Dunbar et al., 1995; Hollander et al., 2003b; Fineberg et al., 2011). Thus, higher doses *should* demonstrate more pronounced attenuation of O/C behavior in animals. On a psychological level, behavioral interference that are used in clinical OCD, e.g. exposure and response prevention and cognitive behavioral therapy, will significantly contribute to our understanding of psychobiological crosstalk in the manifestation of O/C behavior. In fact, this is an area of little interest in current preclinical work. With respect to the current investigation, certain behavioral interventions i.e. punishment learning using electrical shocks, will be employed in an attempt to modify obsessive-like behaviors which can possibly contribute to both the face and predictive validity of the model investigated.

Predictive validity in an OCD model will thus comprise signs of reduced O/C-like behavior in at least 40 – 60% of the affected individuals following high dose, chronic SSRI treatment (Fineberg, 2004; Abramowitz et al., 2009; Albert et al., 2013). However, resistance to punishment feedback processing, may also be regarded as strongpoints of an animal model of OCD.

* * *

2.5. Overview of Current Animal Models of OCD

In this section, we will provide the reader with a concise overview of the pharmaco-behavioral animal models of OCD currently in use. A number of models founded on genetic manipulation also exist, but as the current investigation concerns a *naturalistic* animal model of OCD and its response to pharmacological and behavioral intervention, genetic models are considered beyond the scope of the current work. However, for a comprehensive review of animal models of OCD, please refer to (Fineberg et al., 2011; Albelda and Joel, 2012a, 2012b; Alonso et al., 2015). To date, several successful models have been designed and validated in various respects. However, a key shortcoming of the entire body of work remains the difficulty in demonstrating an obsessive component underlying the compulsive behaviors, the latter of which has been extensively demonstrated according to the reviews presented above.

2.5.1. Pharmacologically Induced Animal Models

As suggested by the name, these studies are founded on elicitation of abnormal behavior following the administration of specific receptor-targeting drugs. As such, these animal models are employed in an attempt to derive the role of particular receptors or neurotransmitter systems in the expression of O/C behavior.

2.5.1.1. Decreased Spontaneous Alternation in Rats

Decreased spontaneous alternation in a T-maze by rats (Yadin et al., 1991) relies on the manipulation of the natural tendency of rodents to alternate their choice of two arms when exploring a T-shaped maze, i.e. traveling down alternating arms during consecutive trials (Dennis, 1935). However, following the administration of 8-OH-DPAT, a 5-HT_{1A} agonist, this natural tendency to alternate is reduced, as animals notably make more repeated and persistent choices. The persistent behavior observed constitutes the basis of the face validity, while strong predictive validity was shown by a positive response to chronic SSRI treatment. That 5-HT_{1A} agonist involvement underlies persistent arm choice is also in line with the neurobiological O/C theory as stimulation of presynaptic 5-HT_{1A} receptors, decreases serotonergic outflow to the basal ganglia and frontal cortex. The findings of Yadin et al. (1991) have since been replicated (Fernandez-Guasti et al., 2003; Tsaltas et al., 2005).

2.5.1.2. Quinpirole Induced Compulsive Checking

Another model of induced behavior focuses on dopaminergic manipulation and was described by (Szechtman et al., 1998; Szechtman et al., 2001). In this model the DA D₂/D₃ agonist, quinpirole, is administered to precipitate repetitious motor patterns resembling compulsive-like checking at specific 'preferred locations'. Briefly, quinpirole treated rats approach these

preferred locations persistently, even if allowed to explore the arena freely. Further, treated rats are also quicker to return to these locations after exploring other non-preferred locations, while distinct motor rituals were also often carried out in proximity of a particular site (Szechtman et al., 1998; Szechtman et al., 2001). Recently, it has also been shown that checking behavior is exacerbated under conditions of uncertainty, a finding that is congruent with clinical literature (Sookman and Pinard, 2002; Sarawgi et al., 2013). As such, the authors argue that the aforementioned criteria qualified the behavior as sufficiently conscientious and excessively repetitive to be considered as resembling human safety related checking compulsions. Once again, the effect of treatment was assessed by the administration of clomipramine (an SRI), which produced mildly attenuating effects (Szechtman et al., 1998; Szechtman et al., 2001). This model continues to remain relevant and is being used to examine specific receptor effects on the compulsive checking behavior (Tucci et al., 2013; Tucci et al., 2015).

2.5.2. Models Based on Naturally Occurring Compulsive-like Behavior

These models investigate behaviors that occur seemingly spontaneously when animals are housed under laboratory conditions. No genetic, pharmacological or training component is involved in these models. While the deer mouse model that will be employed in this investigation is also a naturalistic model of OCD, it will be reviewed separately and in greater depth in Section 2.6.

2.5.2.1. Large Nest Building in Rodents and Rabbits

In brief, nest building is a naturally occurring behavior that, being expressed in the laboratory, is aimed at protection from human handling and harsh environmental factors, e.g. bright light and temperature fluctuations (Smithers, 1983; Jirkof, 2014; Stewart and McAdam, 2017). However, previous investigations into the nature of nest building have revealed notable within-species variation, viz. LNB in mice (Greene-Schloesser et al., 2011) and rabbits (Hoffman and Morales, 2009). This behavior serves no seeming purpose as LNB expressing animals are maintained and cared for under the same conditions as control animals. As such, the building of excessively large nests constitutes useful face validity to model O/C-like behavior as it may closely resemble the symptomology and aberrant cognition central to clinical compulsive behavior (Hoffman and Morales, 2009). Further, promising predictive validity has been established by the demonstration that LNB in both house mice (*Mus musculus*; Greene-Schloesser et al., 2011) and deer mice (Wolmarans et al., 2016b) demonstrates attenuation following treatment with high dose SSRIs (Greene-Schloesser et al., 2011). Further, the abnormal nesting behavior in rabbits is also attenuated by DA antagonists (Hoffman and

Morales, 2012). That said, apart from its modest face and predictive validity, very little has subsequently been done to determine the construct foundations of the model. In this regard, the current investigation will aim to elaborate the construct validity of LNB as it pertains to the cognitive triggers of such behavior. A detailed review and dissection of LNB behavior as a phenotype of compulsive-like behavior and how it has been applied in this investigation will be provided in Chapter 5.

2.5.2.2. Marble-burying

From the outset, it is important to emphasize that MB is *not a definitive*, if not also a highly controversial, model of any specific neuropsychological construct. In fact, observations of MB behavior as it is broadly applied, can at most be used to test for certain anxiety- or compulsive-like behavioral symptomologies following external interference (See Chapter 4 for a complete review). That said, a number of investigations do apply the analysis of MB behavior as an animal model of either anxiety or OCD, whether it is appropriate to do so or not. However, in this work, we will investigate the MB response within different experimental paradigms and show that, *if performed under appropriate conditions, aberrant* MB behavior, e.g. HMB, may be both a valuable framework in which to study anxiety- or compulsive-like behavioral phenotypes and a useful test for such constructs in any rodent. For the purpose of reviewing MB as a model, we will briefly summarize the current literature here.

The marble-burying test (MBT) for compulsivity was founded on observations that rodents often bury small foreign objects, either noxious or non-noxious beneath the bedding material of their cages in a manner that may resemble hoarding- or contamination-like compulsive behavior. Initially, the MB response was proposed as a model of anxiety (Poling et al., 1981; Hadley et al., 2006) and was used to investigate the effect of anxiolytic drugs (Broekkamp et al., 1986). However, subsequent findings demonstrating that marbles do not induce avoidance behavior, that burying often persists over time, and that no correlation between marble-burying and other measures of anxiety could be found (Gyertyan, 1995; Nicolas et al., 2006; Thomas et al., 2009), suggest a behavior more evocative of OCD (Thomas et al., 2009; Albelda and Joel, 2012a; Alonso et al., 2015). Since these assertions, the MBT has been increasingly applied as a measure of anti-compulsive drug action (Egashira et al., 2013; Wolmarans et al., 2016a; Taylor et al., 2017; Egashira et al., 2018). However, while persistent MB may present with some face validity as a ‘model’ of O/C-like behavior, it lacks any form of predictive validity as currently applied; in fact, it demonstrates response to more or less every intervention tested. In Chapters 3 and 4, we will not only provide a methodological dissection of the MBT, but also argue that such behaviors, as it is currently reported, is far from ideal when considered from a pre-clinical perspective. This is mainly due to a significant lack of

standardization of the test parameters, which is crucial to the modelling of psychiatric conditions in translational models (van der Staay, 2006).

2.5.3. Models Based on Behavioral Training

2.5.3.1. The Signal Attenuation Model

The signal attenuation model of Joel and Avisar (2001) was designed to assess whether rats trained to execute a response before receiving a positive outcome, demonstrate sensitivity to a decreasing return on action and therefore present with deficits in reward related feedback (Szechtman and Woody, 2004; Joel, 2006). This model is of particular interest for the current investigation as the methodology and rationale underlying this work by Joel and Avisar (2001) has been adapted and applied to the design of the current lever-pressing investigation (Chapter 5).

In short, rats are trained in various stages to perform successful lever-presses before attaining a positive goal, i.e. food pellets. First, 'classical conditioning' is performed by the presentation of cues, *viz.* light and audible tones, whenever a food pellet is presented to the test subjects. This step is performed to ensure that the animal associates the cue with the presentation of food. Secondly, rats are trained to press a lever for food delivery. During this stage a successful lever-press is followed by the cues *and* food delivery and thus the subject acquires knowledge relating to the response-outcome contingency, with the cues introduced as contextual reminders that the goal has been reached. Subsequently, the signal attenuation stage involves devaluing the cue stimulus by repeatedly presenting the cue *without* the delivery of food. This process occurs without the presentation of levers and, as was true during the first stage, the subject has no control over the situation. In the end, the final stage again requires subjects to press levers, receiving only a stimulus but no food, measuring performance on the lever-press under extinction conditions. As subjects should acquire knowledge that the reward on task completion is devaluated and therefore that continuously engaging in lever-pressing is senseless, the number and frequency of lever-presses are counted to determine the level of compulsivity exhibited by the subject (Joel, 2006; Goltseker et al., 2015). The degree to which such conditioning/reinforcement is able to drive compulsive lever-pressing is dependent on the motivational value ascribed to the reinforcer (Schultz, 2002; Wise, 2004; Schultz, 2013). Whereas *food* is used in the classical model of (Joel and Avisar, 2001) to fulfil this role, mice involved in the current investigation will have to lever-press in order to acquire nesting material as the reward. This should allow us to characterize the *importance* placed upon the acquisition of nesting material, since it has already been established that aberrant nesting behavior is an apparent and treatment-sensitive compulsive-

like behavioral phenotype. Considering the predictive validity of the signal attenuation model, it demonstrates sensitivity to paroxetine and fluvoxamine, following acute intraperitoneal injections either 30 or 60 minutes prior to testing. While still implicating a role for serotonergic neurotransmission in compulsive-like lever-pressing, an acute response to SSRIs, for the reasons explained earlier, undermines the predictive and construct validity of compulsive behavior models to a certain extent. Further, confounding pharmacological results have also been presented in that both desipramine (a tricyclic antidepressant) and diazepam also attenuated lever-press behavior, albeit at high doses only (Joel et al., 2004), despite being typically ineffective in the treatment of clinical OCD.

The model also possesses some construct validity. Lever-pressing responses in the absence of reward are bolstered by lesions to the STN (Winter et al., 2009) and OFC in a manner reversible by paroxetine in the latter region (Joel et al., 2005a; Schilman et al., 2010). These OFC lesions were shown to significantly reduce the concentrations of 5-HT to levels well below normal in both the anterior and posterior striatum, implicating aberrant signal propagation in the CSTC circuitry in lever-pressing behavior (Schilman et al., 2010). Specific receptors of the serotonergic system, namely 5-HT_{2A} and 5-HT_{2C} have also been investigated in this paradigm of which 5-HT_{2C} antagonists appeared to demonstrate anti-compulsive effects (Flaisher-Grinberg et al., 2008). Further, deficits in reward valuation and altered perceptions of task completion, which are evidently also important factors within this model as well as in OCD, are also implicitly involved in CSTC circuit-dysfunction underlying O/C pathology (Fineberg et al., 2011; Wood and Ahmari, 2015).

* * *

2.6. The Deer Mouse Model of OCD – Past, Present and Future

The deer mouse model is a relatively unique animal model of OCD that was originally examined from the perspective of stereotypical movement disorder (Handley, 1991). However, such stereotypical expression has since been validated as a naturalistic animal model of OCD based on the time-consuming and varying expression of repetitive motor patterns by cage-reared mice (Korff and Harvey, 2006; Korff et al., 2008, 2009) which transpires without sex bias. Also, such behavior only occurs in 40 – 45% of the deer mouse colony (Wolmarans et al., 2013). Since 2016, the face validity of the model has evolved to include an additional two O/C-like phenotypes, viz. LNB behavior (occurring in 30% of the population; Wolmarans et al., 2016b) and HMB (occurring in 11 – 15% of the population only; Wolmarans et al., 2016a). Whereas both stereotypy and LNB demonstrate a modest to fair response to chronic high dose oral escitalopram (Wolmarans et al., 2013; Wolmarans et al., 2016b), high marble-burying remains treatment refractory, in spite of being just as persistent, repetitive and seemingly purposeless (Wolmarans et al., 2016a). Indeed, given that clinical OCD is a phenotypically heterogeneous condition of which different phenotypes demonstrates varying responses to treatment (McKay et al., 2004), the current study will, in addition to dissecting the MBT and appraising its role in preclinical literature, also aim to further our understanding of LNB behavior as an additional, unique, cognition-driven behavioral phenotype within the deer mouse model of OCD, thereby strengthening our understanding of symptom heterogeneous OCD. As such, this section will summarize the current status of the deer mouse model of OCD and subsequently elaborate on the focus of the current investigation, i.e. analyzing HMB in a manner appropriate to the context of OCD and elucidating the underlying cognitive processes which may promote LNB.

2.6.1. Spontaneous Stereotypy as an Animal Model of OCD

Motor stereotypy is typically defined as a behavior that is “repetitive, invariant and that has no goal or function” (Mason, 1991). This definition describes stereotypy in the context of bouts of recurring movements which are remarkably sustained and not easily inhibited. Furthermore, stereotypy is recognized by conforming to three core characteristics viz. 1) rigidity or resistance to change, 2) occurring even in the absence of an initial stimulus, if applicable, and 3) having no evident functional end-point or outcome (Mason, 1991).

Pioneering studies concerning motor stereotypy in the deer mouse began with the work of Powell et al. (1999). The authors observed how cage-reared deer mice develop stereotypical behaviors in the form of repetitive jumping, backward somersaulting and pattern running. They

also demonstrated that such behavior is not as rigid as per the definition of Mason (1991) and that it is subject to modulation, albeit not complete attenuation, with environmental enrichment.

Subsequently, Korff et al. (2008) explored deer mouse stereotypy as a putative model of OCD-like behavior. In congruence with previous findings (Powell et al., 1999), they demonstrated that deer mice can be separated into two stereotypical cohorts, viz., highly stereotypical (H) and low to non-stereotypical (N) cohorts. At that time, the seemingly purposeless and rigid motor patterns constituted the putative face validity of the model as the authors argued that such behavior resembles the inflexible motor routines often observed in the human condition (Markarian et al., 2010). Pharmacological drug challenges were employed to establish the predictive validity of the model and it has been demonstrated that the SSRI, fluoxetine, significantly reduced the expression of stereotypy. Moreover, further evidence for serotonergic and dopaminergic involvement in stereotypy was demonstrated in that both *meta*-chlorophenylpiperazine (mCPP; a serotonergic 5-HT_{2A/C} agonist) as well as quinpirole (a dopaminergic D_{2/3} agonist) reduced the number of stereotypical movements (Korff et al., 2008). However, desipramine (a noradrenalin reuptake inhibitor) was ineffective in modifying stereotypical behavior. As such, the model demonstrated response to pharmacological agents shown to be clinically effective in OCD, while remaining refractory to a treatment modality that is also ineffective in treating the clinical condition. These results provided putative predictive validity to the deer mouse model.

To investigate whether the deer mouse model of OCD can also be founded on construct, subsequent investigations analyzed frontal-cortical and striatal cAMP concentrations and phosphodiesterase type 4 (PDE₄) activity in the N and H cohorts, respectively (Korff et al., 2009). The effect of chronic fluoxetine treatment on these variables was also examined. Indeed, H animals were found to express elevated frontal cortical cAMP concentrations compared to N animals, whilst PDE₄ activity was reduced in the PFC and striatum. Moreover, chronic fluoxetine decreased both cAMP and PDE₄ activity in the PFC of H animals (Korff et al., 2009). Indeed, PDE₄ is involved in the metabolism of cAMP which is an important second messenger involved in the signal transduction pathways of 5-HT_{1A} receptors (Barnes and Sharp, 1999; Houslay et al, 2003).

To elaborate on the above, and based on O/Cs being time-consuming and interfering with the normal routines of patients (Eilam et al., 2006; Ruscio et al., 2010), our research group performed a detailed analysis of the phenotypical nature of stereotypy as it occurs during the normal wake cycle of the animals (Korff et al., 2008; Guldenpfennig et al., 2011; Wolmarans et al., 2013). These studies contributed significantly to our knowledge of the assessment of *compulsive-like* phenotypes in animals, by showing the time-consuming nature of such

phenotypes and demonstrating its lack of response to sub-chronic treatment with escitalopram. However, not only did *chronic* treatment with high dose oral escitalopram (50 mg/kg/day) reduce the number of bouts of H activity in stereotypical animals, but it also modified such behavior to bouts of normal activity, thus indicating an attenuating effect on the time-consuming nature of compulsive-like behavior and emulating the response of clinical OCD to SSRI treatment (Wolmarans et al., 2013). The construct validity of the model was further strengthened by findings which indicated that hyposerotonergic striatal function (evidenced by altered SERT expression) in the behavior of H, but not N animals (Guldenpfennig et al., 2011).

2.6.2. High Marble Burying as a Unique, Treatment Resistant Phenotype in the Deer Mouse Model

Previous work employing the MBT carried out in this laboratory (Wolmarans et al., 2016a)—which prompted the follow up investigations presented here (Chapters 3 and 4)—revealed some important findings regarding the MBT and its utility with respect to modelling OCD in animal models. This study reported for the first time (to the best of our knowledge) the use of deer mice in the MBT as part of an ongoing validation of the deer mouse model of OCD (Hadley et al., 2006; Korff et al., 2008, 2009; Guldenpfennig et al., 2011; Wolmarans et al., 2013; Wolmarans et al., 2016b, 2016a, 2017). Further, the study design tested the behavior *repeatedly* before and after *chronic* treatment in the same subjects, constituting a more robust framework in which to study burying behavior than typically applied (see Chapter 4).

First, the authors selected two cohorts without sex bias, one non-stereotypical and one highly stereotypical (as discussed above, 2.6.1). These groups were tested in the MBT, revealing no statistically significant differences between the cohorts. Further, 28 days of high dose oral escitalopram treatment (50 mg/kg/day) also failed to modify the average burying behavior of either group. Consequently, the MB data of the 36 animals involved in the study were then analyzed as a whole irrespective of stereotypical cohort. Interestingly, of these, 4 animals (~11%)—again including both male and female—were identified which buried *significantly* more marbles in a *consistent* and *persistent* manner compared to the behavior of the larger sample. However, as opposed to stereotypy and LNB, the MB scores of these 4 animals were not significantly affected by chronic escitalopram treatment. Therefore, at face value, the behavior of the four HMB mice resembled a truly compulsive-like behavioral phenotype, whereas the erratic burying of the remaining animals was hypothesized to resemble the inherent and natural behavioral tendency of rodents to bury objects (De Boer and Koolhaas, 2003; Wolmarans et al., 2016a).

These findings must be viewed in line with the clinical picture of treatment-refractory OCD, which apart from sharing the typical phenotypic characteristics of treatment-sensitive OCD, typically occurs in 30–40% of affected individuals (Ruscio et al., 2010; Albert et al., 2013). The four animals identified above, apart from meeting all of the characteristic compulsive-like criteria in as far as animal models are concerned, further highlight the importance of taking a closer look at individual behavior in (particularly in naturalistic models) after examining inter-cohort differences, as truly compulsive behavioral phenotypes may emerge. Indeed, if examining the behavior based on cohort pooling, individual variances with respect to potentially important behavioral phenotypes may be overlooked. Currently, investigations in our laboratory are underway to determine if HMB may be a potential framework in which to study treatment-refractory OCD.

2.6.3. Towards Large Nest Building as a Unique Phenotype of Compulsive-like Behavior

Given that OCD can be considered as a clinically heterogeneous condition that presents with different symptom phenotypes (Abramowitz et al., 2009; Rowsell and Francis, 2015) an animal model that may naturally emulate such phenomenology, would be a valuable tool to examine the mechanisms underlying symptom heterogeneity. To this end, Wolmarans, Stein, and Harvey (2016a) endeavored to analyze nest-building behavior in deer mice as a possible unique phenotype of O/C-like behavior in addition to high motor stereotypy and high marble-burying.

As alluded to earlier, rodents housed under laboratory conditions build nests in response to a range of environmental stimuli. Indeed, experimental determinations of nesting size using 24 male and 26 female deer mice subjects revealed average daily nesting sizes of 1.7g and 2.1g of cotton wool, respectively (King et al., 1964). However, recent work applying LNB as a measure of O/C-like, and not normal behavior was conducted by Greene-Schloesser et al. (2011). This study was performed using two genetic lines of common house mice (*Mus musculus*), bred over 55 generations to express normal and LNB behavior, designated SMALL and BIG respectively, and followed on the work of (Lynch, 1980) and (Bult and Lynch, 1996, 1997, 2000). Considering the response of LNB to pharmacological drug challenges, i.e. the SSRI, fluoxetine and SRI, clomipramine, both resulted in a reduction of LNB following chronic treatment. Based on these findings, Wolmarans et al. (2016b) assessed nest-building behavior in the deer mouse colony housed. Departing from the methodology of Greene-Schloesser et al. (2011) that employed in-bred strains of mice, they demonstrated that *aberrant LNB* is naturally expressed in approximately 30% of the population across both sexes and both stereotypical cohorts. Further, such behavior persisted over several consecutive

trials and responded to chronic, high-dose oral escitalopram treatment, while NNB behavior remained unaltered. Some construct validity of LNB as a compulsive-like phenotype has recently been shown in data that implicated serotonergic deficiencies in the mPFC of LNB mice, albeit in a different species viz. *Mus musculus* (Winter et al., 2018). Therefore, by virtue of its persistence and seemingly purposeless nature within the context of identical housing conditions as that which apply for the rest of the population, and by demonstrating response to pharmacological agents effective in clinical OCD, LNB presents with sufficient face and predictive validity to be regarded as an additional O/C-like behavioral phenotype.

2.6.4. Applying LNB to Establish a Cognitive Foundation for Compulsive-like Behavior in the Deer Mouse Model of OCD

The background and literature presented in this paragraph aims to provide the reader with the theoretical and conceptual framework that underlies the rationale of the work presented in Chapter 5. Since LNB already presents with good face and predictive validity as previously described, the current investigation will aim to further our understanding of its cognitive construct.

2.6.4.1. Potential Motivators of LNB – Safety or Perfectionism

Given that LNB is expressed in a third of deer mice only (Wolmarans et al., 2016b) and that it occurs in both sexes, the question arises as to why such behavior only develops in certain individuals and not in others? In order to answer this question, the reader is referred back to the cognitive abnormalities pertaining to OCD that are discussed in section 2.3. In fact, since the current animal model seemingly presents with phenotypical similarity to clinical OCD, we hypothesize that given its phenotypical presentation, LNB may be a consequence of inflated concerns about safety (Hoffman and Morales, 2009; Jirkof, 2014). Since it is argued that rodents build nests for protection in both natural (Smithers, 1983) and laboratory settings (Jirkof, 2014), we can assert that the building of large nests may be an exaggerated form of safety seeking behavior, which is central to an important construct in OCD theory, viz. the security motivation system (Szechtman & Woody, 2004). Indeed, since other potential motivators for LNB, e.g. pregnancy (Hoffman and Morales, 2009) and temperature fluctuations (Gaskill et al., 2012; Stewart and McAdam, 2017), have been corrected for by same sex co-habitation and constant temperatures, it can be asserted with confidence that LNB may arise from a unique psychobiological construct, e.g. *inflated* concerns relating to safety. However, it cannot be excluded that LNB may also occur as a result of perfectionistic obsessions related to feelings of “just right experiences”, similar to the NJREs discussed earlier (JREs; Hoffman and Morales, 2009). Indeed, with respect to OCD, a succinct definition of perfectionism has

been proposed by (Pitman, 1987) and later reiterated by Coles et al. (2003) as “*attempts to match sensations with expectations*”. Perfectionistic compulsions could be driven by the need to have strict control over certain facets of everyday life (Leckman et al., 1994); it has been demonstrated that OCD patients often engage in compulsive behavior in an attempt to achieve an elusive sense of perfection. As alluded to earlier, these obsessions have been viewed in a slightly different, yet similar light – that OCD patients experience NJREs (Coles et al., 2003). Conceptually ‘just right experience’ (JRE)-compulsions can be envisaged as the need to make small changes to a specific behavioral program to obtain perfection, while NJREs would imply that changes are made to remove perceived imperfections from the subject of the underlying obsession.

The study of NJREs provided conclusive evidence that these experiences are typical of OCD in showing that NJREs are correlated with measures of perfectionism, checking, and ordering compulsions, and that it is particularly specific to OCD compared to other psychiatric conditions (Coles et al., 2003). Furthermore, it is proposed that the motivation to carry out NJRE-driven compulsions could differ from the classical view of O/Cs, *viz.* compulsion being carried out in an attempt to prevent feared future consequences (American Psychiatric Association, 2013). Instead, NJRE-driven compulsions are proposed to reduce anxiety *without* expressing fear of negative consequences. Lastly Coles et al. (2003) recommended the inclusion of questions that aim to differentiate between feared consequences and NJREs in diagnostic questionnaires. Albeit being investigated in an animal model, this distinction will be under scrutiny in the current work. As can also be true for safety-related LNB, actual compulsive-like nest-building behavior may be related to factors such as altered reward feedback, overreliance on habitual action, or deficits in task completion.

2.6.4.2. The Role of Insensitivity to Punishment and Habitual Responses in LNB

Insensitivity to punishment and negative outcomes seem to be a characteristic feature of OCD as demonstrated and alluded to before (see section 2.3.3). Direct observation of OCD patients has shown that their symptoms are time-consuming (Rasmussen and Eisen, 1991; Eilam et al., 2006) with patients often spending in excess of 6 hours per day in engaging in obsessive rituals and compulsions. As mentioned before, the time spent engaging in ritualistic O/C behavior is also an important diagnostic factor considered in OCD (American Psychiatric Association, 2013). These characteristics of OCD translate to the daily lives of patients as they will perform O/C rituals in spite of the drastic negative outcomes of such behavior on QOL (Hollander et al., 1996; Huppert et al., 2009; Coluccia et al., 2016). Subsequent studies (Nielen et al., 2009; Endrass et al., 2013) revealed that OCD patients performed poorly when

required to update their behavior following punishing feedback, strengthening our assertion that they may become insensitive to the negative outcomes transpiring from their behavior. In the current investigation, we will aim to directly test the proposed insensitivity to punishment and deficits in punishment learning in the model by introducing a contextual punishment upon performing excessive operant actions to acquire nesting material (Chapter 5). Based on this hypothesis and considering the supporting literature summarized in this chapter, we expect the highly compulsive cohort to endure self-administered punishment to a greater extent compared to NNB controls.

However, to assess whether LNB deer mice are also more intrinsically prone to develop habitual rituals instead of being flexible with respect to changes in goal-directed outcomes, *viz.* not only possibly being insensitive to a *negative* outcome, but also to *no* outcome, similar methodology to that of Joel and Avisar (2001) will be followed. In this regard, mice of both normal and LNB cohorts will be allowed time to acquire knowledge of the response-outcome contingency required for the provision of nesting material (outcome) which will then suddenly be withdrawn. Should excessive lever-presses be executed during this test following reward withdrawal, insights into the motivational value ascribed to the act of obtaining nesting material can arguably be derived.

Collectively, this phase of the investigation can potentially deliver insight into whether some of the cognitive deficits of OCD, namely impaired reward appraisal, feedback learning and an overreliance on habitual responses, are demonstrable in a cohort of animals selected for an O/C behavioral phenotype.

* * *

2.7. Conclusion to Chapter 2

The modelling of OCD in translational animal models has shown tremendous progression over the recent decades and several models are available. While all of these models employ repetitive behaviors as the cornerstone for validity, it is fair to impose that the time for refining these available methods (as far as realistically possible) has arrived by translating our understanding of the clinical presentation of OCD in humans back to the available translational models, i.e. going from the bedside to the bench, instead of the other way round. Indeed, if the available methods at our disposal are not applied in an appropriate manner, our collective understanding of neuropsychiatric research, more specifically with respect to OCD, will be undermined.

Considering Chapters 3 and 4 of this thesis, there are significant obstacles regarding the replication of translational animal studies, most of which are believed to arise from inadequate application and reporting of behavioral methods (Drucker, 2016; Kafkafi et al., 2018). These can range from differences in basic aspects such as housing conditions (light/dark cycles, water and food supply, background noise, light levels) to insufficient detail provision regarding specific experimental procedures, ultimately preventing the accurate recreation of test conditions and clouding the conclusions drawn from the current body of work. As will be discussed here, inadequately designed methods (Chapter 3) or insufficiently reported studies (Chapter 4) undermine the translational value of what may even be the simplest of behavioral tests such as the MBT. In this work, we will argue that authors should strive to include as much information as is reasonably possible regarding experimental configurations to address these issues, and that every effort should be made to replicate experimental conditions as closely as possible across laboratories to aid in the reproducibility and comparability of data. In the end, such an approach will benefit the collective body of research into specific topics, and hence also our understanding of OCD.

Regarding the seemingly aberrant behaviors itself as they relate to OCD, emphasis should be placed on the specificity and persistence of ritualistic behaviors over extended periods of time, following observations that O/Cs are expressed in a highly context specific manner while being stable over an extended timeframe. We also argue that naturally occurring behaviors, provided they be extensively validated, may constitute highly useful, relevant and superior models in which to test potential novel therapeutic solutions, given the complex interplay of neurotransmitter dysfunction involved in the etiology of OCD. By extension, behaviors which involve no form of training or conditioning procedures are also favorable, since these inherent naturalistic behaviors may allow an opportunity to dissect *potentially* abnormal

physiological/cognitive processes in test subjects which may ultimately drive expression of aberrant behaviors.

Indeed, one of the most monumental challenges when modelling O/Cs pertains to the demonstration of the highly abstract nature of obsession-associated symptoms. Given that we cannot question or in any other manner directly examine the cognitive processes accompanying O/C-like behaviors in animals, we propose that careful experimental design (detailed under section 2.6.4) can allow better insight into such processes by means of indirect implication, as is the primary focus of Chapter 5. To better illustrate this point, consider the previously discussed signal attenuation model. While compulsive lever-press responses for food following a substantial training program resemble OCD at face value, the *meaning* of the behavior must be considered. Excessive lever-pressing in this context may indeed provide insight into RL deficits, but since the behavior was induced using Pavlovian-style conditioning, it could be argued that the response of animals in this context is habitual rather than obsessive in nature, and therefore not linked to any type of compulsion-evoking cognitive pattern. In contrast, untrained, exploration-driven lever-pressing to obtain nesting material instead presents a direct link to normal functional routines of rodents, and since nest building is predominantly aimed at the provision of safety and security, aberrations measured in this behavior of a persistent and resistant fashion can likely indicate increased threat perception or anxieties regarding safety in individual animals or groups.

In addition to exploring the importance of appropriate methodologies (Chapters 3 and 4) and assessing seemingly abnormal behaviors within a relevant context (Chapter 5), the current work will also aim to establish whether two unique phenotypes, i.e. HMB and LNB, which share common O/C-traits, e.g. persistence, repetitiveness and seemingly purposelessness, but differ in terms of treatment response to SSRI-intervention, are associated with unique serotonergic involvement.

In summary, the work contained in the current thesis will not only regard seemingly abnormal behavior from a ethological and methodological perspective, but it will also aim to elaborate on our understanding of the cognitive and neurobiological triggers of normal behaviors that may become pathological manifestations in some individuals. As such, we hope to elucidate our current understanding of what the modelling of OCD in animals should involve, and how symptom heterogeneous OCD may be founded in abnormal cognitive and neurobiological constructs.

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3. Manuscript A

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- *De Wet Wolmarans* as the study leader, assisted in the conceptualization of the study together with *Geoffrey de Brouwer*, performed the behavioral analyses, processed the data, interpreted the statistical analyses and assisted in the writing of the manuscript.

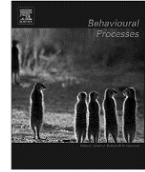
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Back to basics: A methodological perspective on marble-burying behavior as a screening test for psychiatric illness

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ABSTRACT

Animal models of human psychiatric illness are valuable frameworks to investigate the etiology and neurobiology underlying the human conditions. Accurate behavioral measures that can be used to characterize animal behavior, thereby contributing to a model's validity, are crucial. One such measure, i.e. the rodent marble-burying test (MBT), is often applied as a measure of anxiety- and compulsive-like behaviors. However, the test is characterized by noteworthy between-laboratory methodological differences and demonstrates positive treatment responses to an array of pharmacotherapies that are often of little translational value. Therefore, using a naturalistic animal model of obsessive-compulsive disorder, i.e. the deer mouse (*Peromyscus maniculatus bairdii*), the current investigation attempted to illuminate the discrepancies reported in literature by means of a methodological approach to the MBT. Five key aspects of the test that vary between laboratories, viz. observer/scoring, burying substrate, optional avoidance, the use of repeated testing, and determinations of locomotor activity, have been investigated. Following repeated MB tests in four different burying substrates and in two zone configurations, we have demonstrated that 1) observer bias may contribute to the significant differences in findings reported, 2) MB seems to be a natural exploratory response to a novel environment, rather than being triggered by aberrant cognition, 3) burying substrates with a small particle size and higher density deliver the most accurate results with respect to the burying phenotype, and 4) to exclude the influence of normal exploratory behavior on the number of marbles being covered, assessments of marble-burying should be based on pre-occupation with the objects itself.

1. Introduction

Studies of animal behavior play an important role in elucidating the etiopathology, disease mechanisms and treatment response underlying psychiatric illness (van der Staay, 2006). However, accurate and reliable behavioral screening tests constitute the foundation of such investigations. Indeed, analyses of animal behavior can only bring about useful conclusions if the choice and execution of the assessment tool is appropriate and valid. One such screening paradigm, i.e. marble-burying (MB), is regularly applied as a measure of anxiety- (Shimazaki et al., 2004; Nicolas et al., 2006) and compulsive-like (Egashira et al., 2008a; Angoa-Pérez et al., 2013) behavior. While findings that rodents often express MB in the absence of anxiety (Thomas et al., 2009) suggest a behavior that is more compatible with inherent – viz. the natural tendency to bury objects (Smithers, 1983) – rather than defensive or neophobic burying, an abundance of literature also demonstrate that rats and mice will often *persist* in burying harmless objects (Londei et al., 1998; Thomas et al., 2009) and that this seemingly non-

functional repetitive behavior may reflect the behavioral symptoms of obsessive-compulsive disorder (OCD). However, it has also been suggested that the non-reactive nature of harmless stimuli such as glass marbles may elicit a form of anxiety in the animal (Diamant et al., 1991) and it remains arguable whether this form of burying is entirely devoid of a stress-related construct.

Apart from being a screening-test for putative agents used in the treatment of OCD (Gyertyan, 1995; Millan et al., 2002; Egashira et al., 2008a), MB has also been used to model treatment response relating to impulsiveness (Schneider and Popik, 2007), autism (Angoa-Pérez et al., 2013) and co-morbid behavioral and psychological symptoms of dementia (Torres-Lista et al., 2015). Considering the fact that conclusions drawn from the MB test are, with few exceptions (see work by Torres-Lista et al. (2015)) exclusively based on the number of marbles buried (albeit after correcting for changes in locomotor behavior), it is intriguing that a diverse range of pharmacological agents has been shown to modify MB behavior. That said, behavioral tests used to establish the effects of pharmacotherapeutic interference need not naturally

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represent the complete scope of neurobehavioral aberrancies present in one or even more than one specific clinical condition. For instance, with respect to the marble burying test, it can provide valuable insight into anxiety-related neurobiology if such behavior for instance responds accurately to known anxiogenic (or anxiolytic) interference (Schneider and Popik, 2007) and is subsequently known to resemble a state akin to anxiety. However, MB is commonly applied as a rapid baseline ‘treat-expose-observe’ measurement of anxiety-like and compulsive-like behavior, i.e. during a single post-treatment exposure of animals to marbles within an unconditioned – or novel – framework. Although it is true that most drugs acting in the central nervous system can also manipulate normal emotional and cognitive processes, both in humans (Curran et al., 1998; Hindmarch, 2004) and animals (Eilam and Szechtman, 2005), numerous conflicting findings indicate that without prior within-laboratory validation, MB behavior, whether applied as a screening test for neophobic, compulsive or inherent burying, varies significantly with respect to treatment response and as a function of methodological manipulation. It has shown sensitivity for traditional anxiolytics i.e., benzodiazepines (Nicolas et al., 2006), various classes of noradrenergic and serotonergic antidepressants i.e., desipramine, imipramine, citalopram, paroxetine, and fluvoxamine (Broekkamp et al., 1986; Abe et al., 1998; Millan et al., 2002; Schneider and Popik, 2007), agents that interfere with glutamatergic signaling (Shimazaki et al., 2004; Egashira et al., 2008a; Iijima et al., 2010) and both traditional (Nicolas et al., 2006; Bruins Slot et al., 2008) and atypical antipsychotics (Egashira et al., 2008b). Although these agents are linked by their broad interference in major central neurotransmitter systems, viz. noradrenergic, serotonergic, dopaminergic, GABAergic and, glutamatergic signaling, MB is also modified by a number of miscellaneous agents, i.e. progesterone (Schneider and Popik, 2007), leuprolide (Uday et al., 2007), nitric oxide synthase inhibitors (Krass et al., 2010), tiptidine (Honda et al., 2011), and anti-oxidants (de Almeida et al., 2014). Notwithstanding, the usefulness of burying behavior as a screening test for anxiety- and/or compulsive-like behavior is complicated by contradictory findings, e.g. attenuation by anxiogenic drugs (Nicolas et al., 2006) as well as attenuation of burying by both serotonin (5HT)-2_{AVC} agonists and antagonists (Njung’e and Handley, 1991). Moreover, pharmacological studies mostly use acute single-dose treatments administered between 90 and 30 min before recording burying behavior (Nicolas et al., 2006; Uday et al., 2007); this in spite of certain classes of drugs, e.g., antidepressants, only being effective in the clinical treatment of anxiety and OCD following chronic administration (Fineberg and Craig, 2007; Huh et al., 2011).

Considering the phenotypic expression of MB, irrespective of it being employed in investigations of anxiety- or compulsive-like behavior, and that it responds to agents that interfere with multiple neurobiological mechanisms, the question arises whether between-laboratory methodological differences may contribute to the sundry of findings reported. Indeed, between laboratory standardization of experimental methodology is not only important to yield consistent and reliable results, it also ensures the use of robust behavioral measures that are accurate in determining putative targets for future investigation (Wahlsten, 2001; Richter et al., 2009). In fact, as opposed to the largely standardized approaches followed with respect to various other behavioral assessments, e.g. the forced swim test (Bogdanova et al., 2013), elevated-plus maze test (Rodgers and Dalvi, 1997) and open field tests (Stanford, 2007), MB investigations differ with respect to five important constructs, viz. means of quantification and the number of observers used (Egashira et al., 2008a; Angoa-Pérez et al., 2013), nature of the burying substrate (Egashira et al., 2008a; Krass et al., 2010; Kedia and Chattarji, 2014), whether avoidance behavior is allowed for (Broekkamp et al., 1986; Nicolas et al., 2006; Torres-Lista et al., 2015), singular or repeated testing (Uday et al., 2007; Torres-Lista et al., 2015), and determinations of locomotor activity (Nicolas et al., 2006; Angoa-Pérez et al., 2013). With respect to quantification, most investigations regard a marble as being buried if it is either two thirds

(Saadat et al., 2006; Egashira et al., 2008a,b) or half covered (Schneider and Popik, 2007) by bedding material; however, some records exist that reported quantification of completely covered marbles (Torres-Lista et al., 2015). The majority of investigations employ a single blind observer to score burying activity, irrespective of the burying criteria applied. Considering burying substrate, sawdust (Egashira et al., 2007; Umathe et al., 2008), husk (Kedia and Chattarji, 2014), corncob (Thomas et al., 2009), and wood chips (Saadat et al., 2006; Llana and Frye, 2009) among others have been used, with rare reference to particle size or substrate weight per volume. The question of whether the MB test must allow for avoidance behavior in analyses of anxiety-like symptomatology is addressed differently; some authors employ a single-zone configuration, i.e. marbles being evenly spaced throughout the cage (Egashira et al., 2008b), while others use a two-zone layout, with marbles either along the border (Llana and Frye, 2009), or in one half of the arena only (Schneider and Popik, 2007). Investigations relating to both OCD and anxiety, approach the matter of repeated testing differently with some employing a single exposure only (Egashira et al., 2008a), while others expose animals to marbles repeatedly over the course of a given time period (Thomas et al., 2009). As MB performance depends on normal inherent locomotor ability, MB data should be explained in terms of the effects of administered compounds on the general locomotor activity of test subjects. However, investigations into MB activity, differ with respect to this aspect as well (Nicolas et al., 2006; Angoa-Pérez et al., 2013). As such, we investigated how modification of these parameters may influence the conclusions drawn from the MB test by exposing wild type deer mice (*Peromyscus maniculatus bairdii*) to marbles in different experimental paradigms. Although burying behavior demonstrates between species variance (Smithers, 1983; Nicolas et al., 2006), deer mice have been employed in marble-burying trials before (Wolmarans et al., 2016), while their behavior has extensively been characterized with respect to its resemblance of obsessive-compulsive symptomatology (Korff et al., 2008; Güldenpfennig et al., 2011; Wolmarans et al., 2013, 2016, 2017). Specifically, the current work aimed to address the following questions:

- Will the MB scores of two blind observers correspond?
- Is MB activity subject to modification by burying substrate and will such behavior persist or abate over time? Further, will initial exposure to a one- or two-zone configuration respectively, influence MB behavior during subsequent MB trials, i.e. will the opportunity to avoid marbles in the two-zone configuration, as opposed to exposure in the one-zone configuration, differentially affect future MB activity?
- Will the average expression of MB behavior differ as a function of zone-configuration during the first, and subsequent exposure to marbles?
- Will any of the burying substrates be superior in revealing the burying phenotype (as opposed to other forms of behavior, e.g. burrowing) and ensuring consistent and accurate scoring?
- Will the number of buried marbles correlate with an altered index of locomotor activity as evinced by shorter distances travelled?

2. Materials and methods

2.1. Animals

48 deer mice (males and females) were obtained from the deer mouse colony maintained and housed at the vivarium of North-West University (NWU), Potchefstroom, South Africa. Both sexes have been included as they present with analogous psycho-behavioral performance, notably also in the marble-burying test (Supplementary Fig. 1; Hadley et al., 2006; Korff et al., 2008; Güldenpfennig et al., 2011; Wolmarans et al., 2013, 2016). Mice were randomly chosen from different litters, without weight bias and housed in groups of 6 same-sex animals per cage. One week before the onset of marble-burying

assessment, each animal was allocated individually to an automatic climate-controlled laboratory cage (35 cm (l) × 20 cm (w) × 13 cm (h); Techniplast® S.P.A., Varese, Italy) and maintained at 23 °C on a 12 h light/dark cycle (lights on at 06h00 and off at 18h00). Bedding material consisted of ground corncob (particle ϕ 3–4 mm), while nesting material was provided in the form of paper towel sheets. Food and water were available ad lib for the duration of the study. Cages were cleaned and new bedding and nesting material added weekly. Ethical approval for the current investigation was granted by the North-West University AnimCare Research Ethics Office (Number - NWU-00262-16-A5).

2.2. The marble-burying test

2.2.1. Experimental layout

Animals were divided into four groups ($n = 12$), each group being challenged with the MB test in the presence of a different burying substrate, viz. fine river sand (obtained from Sandman Pty. Ltd., Potchefstroom, South Africa; particle $\phi = 0.2$ – 0.4 mm; 1.65 g/cm³), fine sawdust (obtained from SA Vaccine Producers, Sandringham, South Africa; pine, particle $\phi = < 0.1$ mm, 0.17 g/cm³), coarse sawdust/wood shavings (obtained from SA Vaccine Producers, Sandringham, South Africa; pine, particle $\phi = 5$ – 10 mm, particle thickness 0.5 – 1 mm, 0.07 g/cm³), and ground corncob (obtained from the Pre-Clinical Drug Development Platform, NWU; particle $\phi = 3$ – 4 mm, 0.38 g/cm³). To divulge the influence of neophobia on burying behavior, each group was further sub-divided into two clusters ($n = 6$). During the first trial one cluster was challenged in a one-zone (1Z) configuration, while the other was introduced to a two-zone (2Z) configuration. In the 1Z setup, 15 marbles (see below) were evenly spaced throughout the arena. In the 2Z setup, 6 marbles were evenly

spaced in one half of the arena only. However, following the first screen, both clusters in each group alternated for a further five trials between the different zone configurations, resulting in three one-zone, and three two-zone MB challenges having been completed by each individual.

2.2.2. Experimental procedure

On each respective testing day, the MB trials were carried out between 19h00 and 23h00 under dim red light in exact replicas of the home cages, albeit prepared with burying substrates corresponding to the study layout. As behavior in the presence of marbles, and not under novel circumstances per se was the focus of the current investigation, animals were habituated in their home cages containing the relevant burying substrate as per the study design for 24 h immediately before the onset of the first experimental procedure, and remained housed on the same substrates for the duration of the study. Habituation to the substrates occurred in a housing room separate from the testing room. Animals were moved to the testing room in their home cages 15 min prior to placement in the test cages. 15 (1Z) or 6 (2Z) glass marbles ($\phi = 1.5$ cm) of the same transparent color were evenly spaced on a 5 cm thick layer of initially autoclaved burying substrate. Each mouse was allocated to a marble-containing cage and allowed 30 min to explore. To avoid the impact of residual odors on behavior, each mouse was tested in the same assigned test cage throughout the study. To distinguish burying from burrowing and other forms of locomotor activity, a video recording of each trial was made. After returning the animals to their home cages, the marbles were counted by two separate observers. According to the broadly applied criteria (Broekkamp et al., 1986; Egashira et al., 2008a; Umathe et al., 2008), a marble was considered buried when two thirds or more of its size was covered with

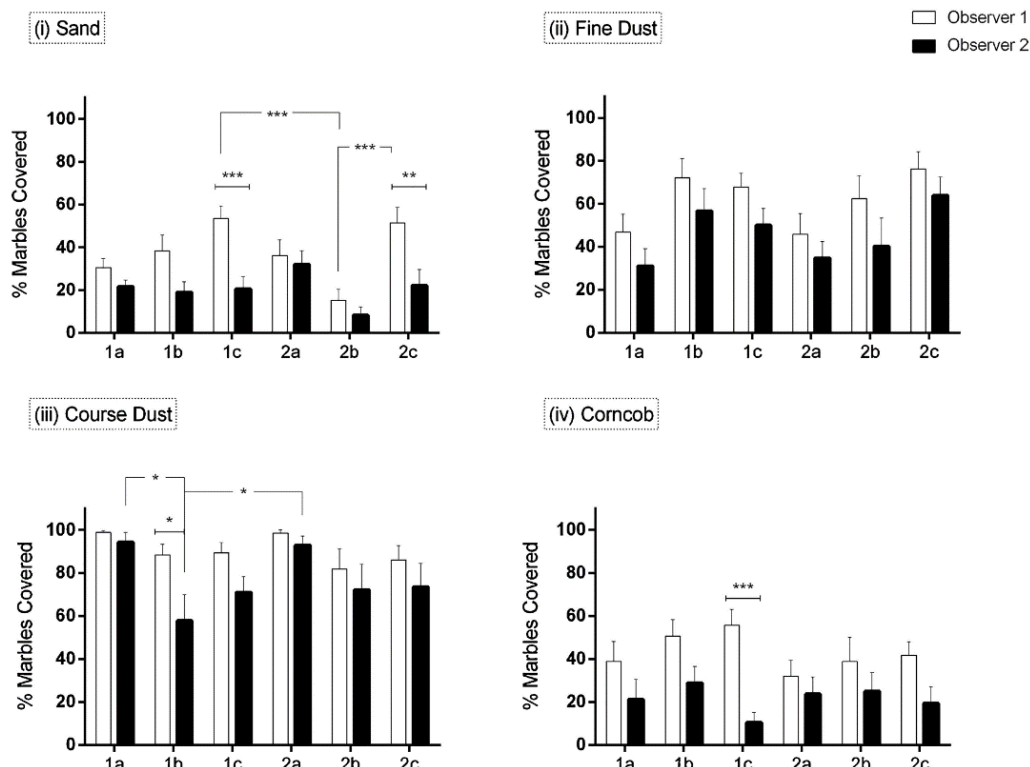


Fig. 1. Observer scores across trials in all burying substrates.

1a–1c: Three respective one-zone trials; 2a–2c: Three respective two-zone trials; Two-way ANOVA followed by Bonferroni post-hoc analyses. Descriptive statistics are provided in Supplementary Table 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

burying substrate. Zone analyses (% time spent in marble-containing zone with respect to the two-zone setup) were performed using Ethovision XT[®] 12 (Noldus[®] Information Technologies). Marble-burying behavior was measured in the same animals during six separate trials on six consecutive evenings.

2.3. Statistics

Statistical analyses were performed using IBM SPSS[®] Statistics 24 and GraphPad Prism[®] 6. Ordinary two-way analyses of variance (ANOVA) followed by Bonferroni post-tests were applied to compare the MB scores of two different observers as recorded across the different trials (Fig. 1). For this purpose, scores have been collated according to zone configuration and not time, and as such, repeated measures were not employed. Burying scores were set as within subject (dependent) factor and zone configuration and observer as between (independent) subject factors. Statistical significance was set at $p < 0.05$. However, to determine whether burying behavior demonstrates time-dependent modification, two-way repeated measures (RM) ANOVA followed by Bonferroni post-tests were applied for animals initially exposed to the 1Z and 2Z configurations, respectively (as time/trial demonstrated no main effect on the data presented irrespective of zone configuration, the results were collated according to zone-configuration; Fig. 2A and B). In this case, the average burying scores of both observers were set as within subject factor, while trial and burying substrate were set as between subject factors. Statistical significance was set at $p < 0.05$. To determine the influence of zone configuration during the first MB trial, viz. one-zone vs. two-zone, on subsequent burying activity, and to establish whether the average expression of MB behavior (as calculated by the scores of both observers) persist or abate over time, mixed three-way ANOVAs (BBW) were applied (also Fig. 2A and B). Zone configuration during the first trial (i.e. one-zone/two-zone) and burying substrate (sand/fine sawdust/course sawdust/ground corncob) were set as between-subject factors and MB trial as within-subject factor. Statistical significance of three-way interactions was set at $p < 0.05$. Where applicable, simple two-way interactions were established of which statistical significance was accepted at a Bonferroni-adjusted alpha level of 0.025. This criterion was also applied when significant simple two-way interactions were followed up with analyses of simple main effects (Gamst et al., 2008). To compare the average MB scores generated during the first trial in the one-zone and two-zone configurations respectively (Fig. 3), as well as the average one-zone and two-zone expression of MB behavior in the respective burying substrates (data not illustrated), ordinary two-way ANOVAs followed by Bonferroni post-hoc tests were applied. Burying scores were set as within subject factor and zone configuration and burying substrate as between subject factors. Statistical significance was set at $p < 0.05$. Finally, with respect to MB analyses in the two-zone configuration, Spearman's rank order correlations were run for each burying substrate to explore the relationships between the number of covered marbles and time spent in the marble-containing zone (Fig. 4) and total distance travelled (data not illustrated), respectively. All data are mean $\pm$ standard error (SEM) unless otherwise stated, while specific p -values and Cohen's d effect sizes are reported in Supplementary Tables 1–6.

3. Results

3.1. Fig. 1: Comparisons between the MB scores recorded by two separate observers

The data depicted in Fig. 1 has been grouped per burying substrate and collated according to zone configuration. As such, the X-axis labels, i.e. '1a', '1b', and '1c' represent the first, second and third one-zone MB trials for both clusters of animals, irrespective of the zone-exposure during the first trial. Similarly, the labels '2a', '2b', and '2c', represent the first, second and third two-zone MB trials of the same animals. This

configuration has been followed for all burying substrates (Fig. 1i–iv).

Two-way ANOVA failed to reveal significant interactions between zone configuration and observer with respect to MB scores recorded in any of the burying substrates [Fig. 1i: $F(5,132) = 2.145$, $p = 0.06$; Fig. 1ii: $F(5,132) = 0.09$, $p = 0.9$; Fig. 1iii: $F(5,132) = 0.8392$, $p = 0.52$; Fig. 1iv: $F(5,132) = 1.219$, $p = 0.52$]. As such, analyses of the main effects for observer were performed, which demonstrated significance with respect to all four burying substrates [Fig. 1i: $F(1,132) = 24.81$, $p < 0.0001$; Fig. 1ii: $F(1,132) = 8.882$, $p = 0.0034$; Fig. 1iii: $F(1,132) = 9.653$, $p = 0.0023$; Fig. 1iv: $F(1,132) = 20.99$, $p < 0.0001$]. Subsequent pairwise comparisons followed by Bonferroni post-hoc analyses revealed significant differences between the MB scores recorded by the two observers with respect to burying activity in sand, course sawdust and corncob, but not in fine sawdust (Fig. 1i, iii, and iv; Supplementary Table 1).

3.2. Fig. 2A – Comparisons between the average one-zone MB scores generated in the different burying substrates by animals initially exposed to either the one- or two-zone configurations

The data depicted in Fig. 2A only represents the average MB scores (as calculated by the scores of both observers) generated in the three respective one-zone MB trials for all animals. However, in order to divulge the habitative influence of voluntary avoidance during the first exposure, burying substrate and time on MB behavior, data was collated according to the zone-configuration that was applied at the first trial and a three-way mixed ANOVA was run.

A statistically significant three-way interaction was demonstrated between first exposure zone configuration, burying substrate and trial [Fig. 2A: $F(6,176) = 3.317$, $p = 0.004$]. There was a statistically significant simple two-way interaction between first exposure zone configuration and burying substrate at the level of the first trial [Fig. 2A, '1ai and 1aai'; $F(3,88) = 9.247$, $p < 0.0001$], but not at the levels of second [Fig. 2A, '1bi and 1bii'; $F(3,88) = 0.835$, $p = 0.478$] and third [Fig. 2A, '1ci and 1cii'; $F(3,88) = 0.594$, $p = 0.620$] trials. Furthermore, a significant simple main effect of first exposure zone configuration on burying behavior was demonstrated during the first trial in fine sawdust [$F(1,88) = 32.479$, $p < 0.0001$] and corncob [$F(1,88) = 32.479$, $p < 0.0001$], but not in sand [$F(1,88) = 0.021$, $p = 0.884$] and course sawdust [$F(1,88) = 0.567$, $p = 0.453$]. Also, a simple main effect of burying substrate on burying behavior was demonstrated with respect to both levels of first exposure zone configuration [1Z: $F(3,88) = 54.773$, $p < 0.0001$; 2Z: $F(3,88) = 33.248$, $p < 0.0001$]. Therefore, pairwise comparisons were performed for said significant simple main effects of which the Bonferroni adjusted p -values are reported in Supplementary Table 2.

3.3. Fig. 2B – Comparisons between the average two-zone MB scores generated in the different burying substrates by animals initially exposed to either the one- or two-zone configurations

The same statistical analysis as performed for the one-zone data, viz. three-way mixed ANOVA, was applied to the data depicted in Fig. 2B. In this case, albeit also collated according to first exposure zone configuration, only data from the three respective two-zone trials are represented.

A statistically significant three-way interaction was demonstrated between first exposure zone configuration, burying substrate and trial [Fig. 2B: $F(6,176) = 3.818$, $p = 0.001$]. There was a statistically significant simple two-way interaction between first exposure zone configuration and burying substrate at the level of the third trial [Fig. 2B, 'Figure 2ci and Figure 2cii'; $F(3,88) = 3.638$, $p = 0.016$], but not at the levels of first [Fig. 2B, 'Figure 2ai and Figure 2aai'; $F(3,88) = 0.977$, $p = 0.408$] and second [Fig. 2B, 'Figure 2bi and Figure 2bii'; $F(3,88) = 1.684$, $p = 0.176$] trials. Furthermore, a significant simple main effect of first exposure zone configuration on burying

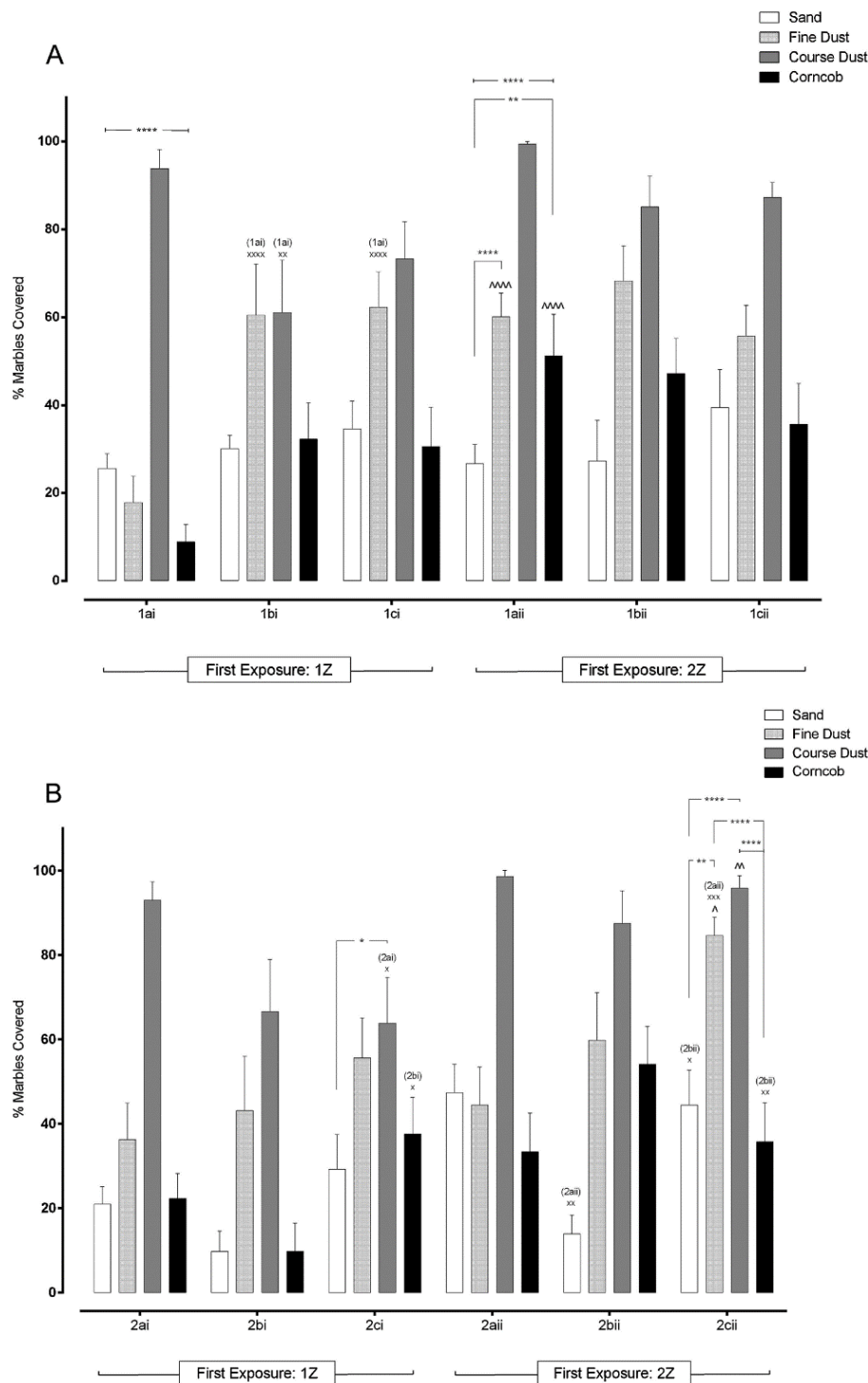


Fig. 2. (A) Average burying scores of two observers recorded during the respective one-zone trials in animals first exposed in either a one- or two-zone configuration, respectively.

1ai – 1ci: Three respective one-zone trials of animals exposed in a one-zone configuration during the first trial; 1aii–1cii: Three respective one-zone trials of animals exposed in a two-zone configuration during the first trial; *Three-way mixed ANOVA followed by Bonferroni post-hoc analyses – within-trial comparisons; †Three-way mixed ANOVA followed by Bonferroni post-hoc analyses – comparisons of burying behavior in the respective burying substrates between trial 1ai and 1aii. ‡Two-Way Repeated Measures ANOVA of burying scores over time in the respective burying substrates during the one-zone trials within each respective first-exposure cohort (viz. 1Z and 2Z), followed by Bonferroni post-hoc analyses. Descriptive statistics are provided in Supplementary Tables 2 and 4. $xx/x/^{*}p < 0.01$, $xxxx/^{*}p < 0.0001$. (B) Average burying scores of two observers recorded during the respective two-zone trials in animals first exposed in either a one- or two-zone configuration, respectively.

2ai – 2ci: Three respective two-zone trials of animals exposed in a two-zone configuration during the first trial; 2aii – 2cii: Three respective two-zone trials of animals exposed in a two-zone configuration during the first trial; *Three-way mixed ANOVA followed by Bonferroni post-hoc analyses – within-trial comparisons; †Three-way mixed ANOVA followed by Bonferroni post-hoc analyses – comparisons of burying behavior in the respective burying substrates between trial 2ci and 2cii. ‡Two-way Repeated Measures ANOVA of burying scores over time in the respective burying substrates during the two-zone trials within each respective first-exposure cohort (viz. 1Z and 2Z), followed by Bonferroni post-hoc analyses. Descriptive statistics are provided in Supplementary Tables 3 and 4. $x/^{*}p < 0.05$, $xx/^{*}p < 0.01$, $xxx/^{*}p < 0.001$, $xxxx/^{*}p < 0.0001$.

behavior was demonstrated during the last trial in fine sawdust [$F(1,88) = 6.980$, $p = 0.01$] and course sawdust [$F(1,88) = 8.499$, $p = 0.005$], but not in sand [$F(1,88) = 1.930$, $p = 0.168$] and corncob [$F(1,88) = 1.588$, $p = 0.211$]. Also, a simple main effect of burying substrate on burying behavior was demonstrated with respect

to both levels of first exposure zone configuration [1Z: $F(3,88) = 4.229$, $p = 0.008$; 2Z: $F(3,88) = 18.996$, $p < 0.0001$]. Therefore, pairwise comparisons were performed for said significant simple main effects of which the Bonferroni adjusted p -values are reported in Supplementary Table 3.

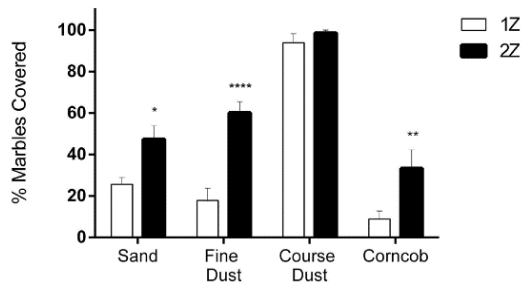


Fig. 3. Average burying scores generated during the first trial by animals exposed in the one- or two-zone configurations, respectively.

1Z: Average burying scores during the first trial generated by animals exposed in a one-zone configuration; 2Z: Average burying scores during the first trial generated by animals exposed in a two-zone configuration. *Two-way ANOVA followed by Bonferroni post-hoc analyses. Descriptive statistics are provided in Supplementary Table 5. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

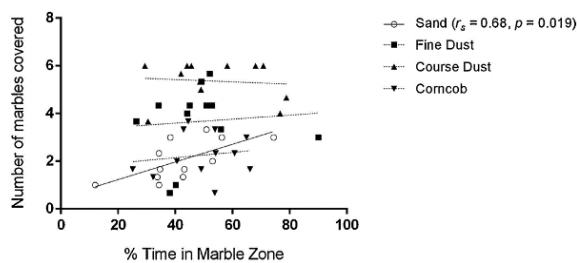


Fig. 4. Spearman's correlations between the average burying scores and the % time spent in the marble containing zone (data represents the two-zone trials only).

3.4. Fig. 2 – Comparisons of between-trial burying activity

In order to establish whether the average burying scores generated in the two different MB zone configurations demonstrate time-dependent modification, two-way RM ANOVA was run for each cluster. As trial had no overall significant effect on burying behavior, irrespective of zone-configuration (data not shown), a separate analysis was performed for animals first exposed in a one-zone and two-zone configuration, respectively. For illustrative purposes, the one-zone and two-zone data of each cluster have been split and are represented in Fig. 2A and B, respectively.

Two-way RM ANOVA revealed a significant interaction between trial and burying substrate in the burying activity of animals that were first exposed in a one-zone configuration [$F(15,220) = 4.490$, $p < 0.0001$]. Furthermore, now both trial [$F(5,220) = 4.472$, $p = 0.0007$] and burying substrate [$F(3,44) = 18.42$, $p < 0.0001$] demonstrated significant main effects on burying behavior. Post-hoc comparisons revealed significant differences between burying scores generated over the three different one-zone trials in fine sawdust and course sawdust (Supplementary Table 4).

A significant two-way interaction between trial and burying substrate was also demonstrated in the burying activity of animals that were first exposed in a two-zone configuration [$F(15,220) = 3.721$, $p < 0.0001$]. However, only burying substrate [$F(3,44) = 43.87$, $p < 0.0001$] and not trial [$F(5,220) = 0.9071$, $p = 0.477$] as well, was the source of the interaction that resulted in significant differences between the inter-trial burying activity with respect to the two-zone configuration only. Descriptive statistics of the relevant post-hoc comparisons are also represented in Supplementary Table 4.

3.5. Fig. 3 – Comparisons of the average burying scores generated in the one- and two-zone configurations

With respect to burying behavior during the first trial, two-way ANOVA revealed a significant interaction between zone configuration and burying substrate [$F(3,88) = 3.910$, $p = 0.013$]. Further, simple main effects was demonstrated for both zone configuration [$F(1, 88) = 36.01$, $p < 0.0001$] and burying substrate [$F(3,88) = 72.16$, $p < 0.0001$] and pairwise comparisons were run for each. Animals buried a significantly greater proportion of marbles in the first two-zone, compared to the first one-zone configuration in all substrates, except course sawdust (Fig. 3; sand, 25.58 ± 11.55 vs $47.33 \pm 23.36\%$, $p = 0.025$; fine sawdust, 17.83 ± 20.79 vs $60.08 \pm 18.69\%$, $p < 0.0001$; course sawdust, 93.83 ± 15.07 vs $98.58 \pm 4.90\%$, $p > 0.999$; corncob, 8.91 ± 13.46 vs $33.33 \pm 31.65\%$, $p = 0.009$). Descriptive statistics for the between-substrate comparisons are not indicated on Fig. 3, but are provided in Supplementary Table 5.

Considering the average burying activity across both the first and subsequent trials, two-way ANOVA of the respective one-zone and two-zone data of animals first exposed to either the one- or two-zone configurations (data not illustrated; however, see Supplementary Fig. 2 and Supplementary Table 6), failed to reveal a significant interaction in either of the groups [first exposure 1Z: $F(3,280) = 0.3899$, $p = 0.76$; first exposure 2Z: $F(3,280) = 0.7609$, $p = 0.52$]. A main effect of burying substrate was observed in both instances [first exposure 1Z: $F(3,280) = 47.17$, $p < 0.0001$; first exposure 2Z: $F(3,280) = 72.22$, $p < 0.0001$] and was followed up with pairwise comparisons between the burying scores in the respective substrates.

3.6. Fig. 4 – Spearman's correlations between the number of marbles covered in the two-zone configuration, and time spent in the marble-containing zone

Spearman's rank order correlations were run to assess the relationship between the number of marbles covered in the two-zone configuration and the time spent in the marble-containing zone (Fig. 4). A positive correlation was demonstrated for burying activity in sand [$r_s(10) = 0.68$, $p = 0.019$; CI: 0.15–0.90], but not in other substrates [fine sawdust, $r_s(10) = 0.18$, $p = 0.578$; course sawdust, $r_s(10) = -0.14$, $p = 0.502$; corncob, $r_s(10) = 0.18$, $p = 0.573$].

3.7. Spearman's correlations between the number of marbles covered in the two-zone configuration, and total distance travelled

Spearman's rank order correlations failed to reveal any significant associations between the number of marbles covered and the total distance travelled in any of the burying substrates [data not illustrated, however see Supplementary Fig. 3; sand, $r_s(10) = 0.29$, $p = 0.357$; fine sawdust, $r_s(10) = 0.24$, $p = 0.459$; course sawdust, $r_s(10) = -0.15$, $p = 0.495$; corncob, $r_s(10) = -0.38$, $p = 0.211$].

4. Discussion

The MB test is a widely-applied screening tool used to assess the behavioral manifestations of a number of putative neurocognitive constructs in rodents, i.e. compulsiveness (Egashira et al., 2008a), anxiety (Kedia and Chattarji, 2014), behavioral symptoms of circadian hormone changes (Schneider and Popik, 2007), symptom phenotypes of dementia (Torres-Lista et al., 2015), and motor perseverance (Thomas et al., 2009). Although the burying of marbles, irrespective of the construct it is intended to mimic, may seemingly resemble some of the said clinical symptomatology, the purported mono-dimensional architecture of the action and its quantification deserve consideration. Indeed, MB has been reported to respond to various pharmacological agents that together constitute a therapeutic framework that interfere

in most aspects of central nervous system functioning (Broekkamp et al., 1986; Ichimaru et al., 1995; Abe et al., 1998; Millan et al., 2002; Takeuchi et al., 2002; Yamada et al., 2002; Shimazaki et al., 2004; Nicolas et al., 2006; Egashira et al., 2007; Hedlund and Sutcliffe, 2007; Schneider and Popik, 2007; Uday et al., 2007; Bruins Slot et al., 2008; Egashira et al., 2008b; Umathe et al., 2008, 2009; Iijima et al., 2010; Krass et al., 2010; Gomes et al., 2011; Honda et al., 2011; Umathe et al., 2012; de Almeida et al., 2014; Dixit et al., 2014). Taken these findings into consideration, two questions arise. First, how can one behavioral phenotype be sensitive to an array of drugs altering a number of divergent neurobiological constructs? Consequently, if such therapeutic sensitivity is accurate, can MB truly be applied as a behavioral measure of specific neuropsychological symptomatology? Indeed, although these questions have been raised previously (Gyertyan, 1995; De Boer and Koolhaas, 2003; Thomas et al., 2009), and considering that burying is a natural response within the normal behavioral repository of rodents (Smithers, 1983; Jirkof, 2014), translational research that apply the MB test as a measure of specific psychiatric constructs, e.g. anxiety and OCD, continue.

However, the relevance of the MB test as a screening tool for behavioral modification cannot simply be disregarded. Previous investigations that applied the MB test with careful consideration of the purported construct it is to measure, are proof of this (Li et al., 2006; Kedia and Chattarji, 2014; Torres-Lista et al., 2015). It is therefore not burying behavior per se that prove to be the root of skepticism, but rather its manner of application. If standardized and targeted MB protocols for the assessment of the various behavioral symptoms of different neuropsychological constructs have existed, the questions asked here would have been addressed – this is not the case. For instance, in investigations of neophobia, the MB test is applied both in one- (Kinsey et al., 2011) and two-zone (Nicolas et al., 2006) configurations. Whereas a one-zone configuration does not allow the animal to avoid exposure to marbles, a two-zone configuration is sensitive to the natural tendency of animals to evade or actively engage with anxiogenic stimuli. However, conclusions drawn from these investigations are often equally applied to an anxiogenic construct.

The current investigation aimed to elucidate the factors that may manipulate the manifestation of MB behavior and thus reveal possible implications of between-laboratory methodological differences on translational conclusions. To this end, we manipulated a number of methodological aspects of the MB test, most of which often demonstrate inter-laboratory variance. Using 48 treatment-naïve deer mice that have never been exposed to marbles before, we investigated the influence of 1) manner of quantification and observer scoring accuracy, 2) burying substrate, 3) zone configuration and optional avoidance, 4) repeated exposure, and 5) assessment of locomotor performance on MB assessments. As most investigations do not use specific mouse lines selected for high burying activity, we employed deer mice of both sexes for two reasons. Deer mice have extensively been analyzed within translational frameworks, specifically relevant to OCD (Korff et al., 2008, 2009; Güldenpfennig et al., 2011; Wolmarans et al., 2013; Stein and Harvey, 2016; Wolmarans et al., 2016) and motor stereotypy (Powell et al., 1999; Hadley et al., 2006). Furthermore, both sexes have been included as they present with equal behavioral performance in all modalities previously tested, including marble-burying behavior (Supplementary Fig. 1; Wolmarans et al., 2016). Also, the fact that obsessive-compulsive disorder demonstrates near equal distribution across both sexes (Ruscio et al., 2010), albeit males befalling the condition earlier (Kessler et al., 2007), rationalizes the choice of adult deer mice of both sexes in the current investigation. Further, animals have been distributed equally and without sex bias across all groups in the current investigation.

4.1. A matter of counting

Irrespective of the zone-configuration or burying substrate

employed, quantification in the MB test is exclusively based on the counting of covered marbles, usually by a single observer that is blind to the experimental grouping (Millan et al., 2002; Egashira et al., 2007, 2008a,b; Krass et al., 2010). Furthermore, without methodological manipulation (see Torres-Lista et al., 2015), a marble is regarded 'buried' if two-thirds of its size is covered in burying substrate. Employing two blind observers and four different burying substrates (Fig. 1), findings from the current investigation demonstrate a significant main effect of observer in all burying substrates. The robust within- and between-trial differences between the scores of observers 1 and 2 demonstrated in sand (Fig. 1i) in all probability resulted from more accurate counting (albeit being observer specific) that was made possible by the small particle size of sand as opposed to the other burying substrates (also see Supplementary photographs 1a-c vs. 2a-c, 3a-d and 4a-c). As such, a differential approach to the scoring criterion, i.e. two-thirds covered, between the two observers is evident. Although such differences also reached statistical significance in course sawdust (Fig. 1iii) and corncob (Fig. 1iv), the recorded scores of the two observers in fine sawdust (Fig. 1ii) remained insignificant. However, given that the main effect of observer in the fine sawdust data reported is meaningful, the insignificant between-observer differences can perhaps be explained by challenging scoring conditions in fine sawdust (see also paragraph 4.2). Importantly, the differences reported in Fig. 1 and Supplementary Table 1 have several implications for conclusions drawn from the MB test. By virtue of its quantification, the MB test is susceptible to observer bias. Furthermore, the within-trial differences reported here resulted in significant between-trial results in sand and course sawdust (Fig. 1i, observer 1; Fig. 1iii, observer 2). These differences have implications for both single and multiple trial MB tests, as they can undoubtedly contribute to reporting bias. Still, it could be argued that by employing one observer only, the standard error will remain constant over several trials. However, although this may be true for most experimental investigations, a fundamental aspect of the MB test makes such a deduction irrelevant. Conclusions drawn from the MB test are intrinsically linked to the actual number of marbles covered. Given that the current MB test was carried out using between 6 and 15 marbles, one marble may modify the reported data with 7–17%. The significant between-observer differences in the scores reported here (some as much as 30% e.g. Fig. 1i, Figure 1c) demonstrate that such errors may often be greater than a one-marble difference. Taken together and considering that none of the observers in the current investigation intentionally scored MB behavior differently, observer bias can in fact not be regarded as a constant error.

4.2. Effect of first-trial configuration, time and burying substrate on repeated testing

Whereas one-zone configurations are often introduced as a measure of compulsive-like activity, albeit with a single or multiple trials (Thomas et al., 2009), a two-zone configuration is hypothesized to more closely mimic anxiety-like constructs (Nicolas et al., 2006). However, we demonstrate here that behavior in both configurations are modifiable by experimental planning (first trial conditions; where first exposure in a two-zone trial bolsters burying activity in the subsequent one-zone experiments), procedure (burying substrate; where substrates with a smaller particle size and higher density, e.g. sand, better reflect marble covering activity) and repetition (where burying activity is either subject to habituation, as observed in the one-zone data of animals first exposed in a one-zone paradigm, or where such activity demonstrates time-dependent alteration, as observed in the two-zone paradigm). Indeed, results from one-zone trials only (Fig. 1A) demonstrate that animals having been first exposed in a two-zone configuration, covered significantly more marbles during their first one-zone trial compared those first exposed in a one-zone configuration, although only in fine sawdust and corncob (Fig. 2A, Figure 1aii vs. Figure 1ai; fine sawdust). It would therefore seem that having the ability to avoid

marbles during the first trial, intensifies burying behavior during subsequent trials. We considered that the ability to avoid marbles during the first trial can instigate aggravated burying behavior in a subsequent one-zone trial, as exposure could not be evaded. However, we could not account for this possibility, as all animals, irrespective of the first trial zone configuration, covered a greater percentage of marbles during the first two-zone, compared to the first one-zone trial (Fig. 3). Further, this effect was not observed during subsequent one-zone trials, which suggests that data obtained during a single one-zone trial may be subject to experimental interference, and not reflect an aberrant cognitive construct. This conclusion is supported by within-group (i.e. first-exposure cohort), repeated measures data demonstrating that animals that were first exposed in a one-zone configuration, habituate to the presence of marbles by the second and third one-zone trials (Fig. 2A, fine sawdust, 1bi and 1ci vs 1ai). Further, that these effects were only demonstrated in fine sawdust and corncob, introduce the influence of a third variable on the nature of the conclusions drawn. Taken together, it seems that MB in a one-zone configuration, either performed as a single trial or during repeated testing, cannot be regarded as an accurate measure of compulsive- or anxiety-like behavior, but more closely resemble investigative behavior subject to experimental modification and habituation.

A somewhat different trend was demonstrated in the data from the respective two-zone trials (Fig. 2B). Again, first-trial exposure, burying substrate, and time interacted to modify burying behavior. Further, the main influence of first exposure only became significant during the third, and not the first or second, two-zone trials (Fig. 2B, Figure 2cii vs Figure 2ci). However, our data suggest that animals first exposed in a two-zone configuration are less prone to avoid marbles as time progress compared to animals that were first exposed in a one zone configuration (Fig. 2B, Figure 2cii vs Figure 2ci, fine sawdust and course sawdust). It could be argued that the inability to avoid marbles in the first trial, resulted in subsequent avoidance behavior in the two-zone trials (Fig. 2B, Fig. 2ai–Figure 2ci). That said, this finding cannot be attributed to an anxiety-related construct as evinced by repeated measures data that indicate the varying nature of burying behavior across different trials (Fig. 2B, Figure 2ci vs. Figure 2ai, course sawdust; Figure 2ci vs. Figure 2bi, corncob; Figure 2bii vs. Figure 2aii, sand; Figure 2cii vs Figure 2bii, sand and corncob; Figure 2cii vs Figure 2aii, fine sawdust). Furthermore, if this finding resulted from the effects of anxiety, it could be expected that the average burying behavior in the two-zone trials would be significantly lower than MB in one-zone trials. We say this for the following reason. If MB resulted from an anxiety-like construct, it could be expected that animals will either avoid or, if avoidance is not an option, bury marbles. However, animals subjected to a two-zone configuration did not avoid the marble-containing zone and covered at least the same percentage of marbles by choice as when subjected to the one-zone configuration (Supplementary Fig. 2) irrespective of whether they were first exposed in a one- or two-zone configuration. In fact, the average burying scores over all trials were analogous between the one- and two-zone configurations. Importantly, from this it is evident that time-dependent modifications in burying behavior are masked in analyses of average burying behaviors. In fact, comparisons of average behaviors across trials, neither reflect the changes in burying behavior that occurred over time (Fig. 2A and B), nor provide indication that zone-configuration may modify first-exposure behavior (Fig. 3). Further, comparisons of average behavior over time also fail to divulge the influence of different burying substrates on the observed changes in burying behavior, as indicated by Figs. 1 and 2A,B (as opposed to the data reflected in Supplementary Fig. 2). This is important, as demonstrations of inter-trial variance in burying activity may divulge more of the underlying neurobiological constructs triggering burying behavior.

The fact that no significant differences in behavior in course sawdust between animals first exposed in a one- or two-zone configuration respectively could be demonstrated, can possibly be attributed to the

nature of activity recorded in said substrate. As demonstrated in Fig. 2A and B, the average ‘burying’ percentages in course sawdust are significantly higher compared to the other burying substrates (Fig. 2A, Figure 1ai, iaii; Fig. 2B, Figure 2ci, Figure 2cii). The ‘burying’ behavior observed in course sawdust is indeed not related to burying, but rather to digging and burrowing, resulting in the displacement of marbles underneath the burying substrate (Supplementary video file 1). In this regard, our findings reported with respect to the two-zone data (Fig. 2B) in sand and fine dust, highlight the important role of burying substrate, as these seem to be the most sensitive to changes in burying activity (however, refer also to paragraph 4.3).

Collectively, data from Figs. 2A,B and Figure 3 and Supplementary Fig. 2 indicate that while a single trial of MB may not reflect an aberrant cognitive construct, repeated testing is also subject to modification by experimental procedure and habituation (Fig. 2A, Figure 1ai–ci and Figure 1aii–cii). However, from the reported two-zone data (Fig. 2B), it seems that the choice to voluntarily engage in investigative or burying activity, modifies burying behavior to a greater extent, compared to exposure in a one-zone paradigm. In fact, animals exposed in the two-zone paradigm, irrespective of zone configuration in the first trial, demonstrated greater variance in burying activity over three trials. As such, it may be valuable to apply a two-zone paradigm over repeated trials in all investigations of marble-burying activity, as it more closely reflect the goal-directed choice to engage in burying behavior. We therefore conclude that with respect to the marble-burying test, the majority of behaviors observed in one-zone paradigms are related to exploration and investigation of a novel environment and that behaviors in a two-zone paradigm more appropriately reflect goal-directed interaction. Further, it is possible that should these experiments have been carried out separately and in different laboratories, viz. either in a one- or two-zone configuration and with one burying substrate only, they would have yielded distinctly different results. As such, it is likely that conclusions drawn from MB tests carried out under different circumstances, bare little relevance for aberrant cognitive constructs, but elucidate more of how animals respond in specific novel spatial environments.

4.3. Where and how to bury my marbles? The importance of preoccupation, observation, and appropriate burying substrates

In an attempt to distinguish between actual burying behavior and other forms of behavior not directly related to the purpose of covering marbles, we ran a Spearman’s correlation between the percentage of time spent in the marble containing zone (only two-zone data was analyzed) and the number of marbles covered (Fig. 4). Interestingly, a positive correlation could be demonstrated in sand only. This finding is of importance for future investigations as it may provide insight into findings obtained from burying behavior in sawdust. The sand used in the current investigation was not only characterized by the smallest particle size (0.2–0.4 mm), but was also the heaviest and most dense substrate (1.65 g/cm³). This is important for two reasons. First, quantifying the number of marbles covered is more accurate due to the small particle size of sand, viz. it is easier to determine whether two-thirds of a marble is covered in the burying substrate (see Supplementary photographs 1a–c vs. 2a–c, 3a–d and 4a–c), compared to the other burying substrates. Secondly, due to the heavy and dense nature of the substrate, burrowing is not possible while accidentally covering marbles during normal behavioral routines, is unlikely (Supplementary video file 2 vs. 3). This argument is supported by the data in Fig. 4 that indicate sand to be the only substrate in which the number of marbles being covered, increased as a function of time spent in the marble-containing zone. Burying behavior in the other substrates is therefore not related to the preoccupation of mice with marbles, but rather results from normal motor routines and exploratory behavior. Although such behavior is also expressed in sand, the substrate is resistant to its coincidental effects on the displacement of marbles.

Importantly, we do not exclude preoccupied burying behavior in the other substrates. As demonstrated in the Supplementary video files (video files 4–6), animals do indeed respond to marbles in all substrates. However, the difference between a ‘buried’ marble and a ‘covered’ marble is founded on preoccupation. In fact, here we demonstrate that it is inappropriate to score burying behavior after a 30-min exposure session without simultaneous observation. Further, based on our findings in course sawdust, we are in congruence with Gyertyan (1995) who argued the MB test to be a quantifiable measure of digging, rather than burying behavior. Whereas this argument might be applied to justify the use of the MB test to measure anxiety or compulsivity, one important fact must be highlighted. Digging and burrowing are also related to normal exploration and are therefore not exclusively related to any of these constructs (Smithers, 1983; De Boer and Koolhaas, 2003). Importantly, we elaborate on the conclusion of Gyertyan (1995); MB cannot only be reflective of digging or burrowing behavior, but must be stratified based on preoccupation. By demonstrating preoccupation, normal rodent behavior can be distinguished from the goal-directed action of burying marbles which will contribute to the validity of the test as a measure of aberrant cognition.

4.4. Burying activity and locomotor capability

Normal locomotor ability is an important prerequisite for marble-burying behavior, as drug induced locomotor suppression may confound MB data. However, whereas some authors record locomotor and MB activity simultaneously, others use separate experiments. We anticipated that, in treatment naïve animals, the total number of marbles covered will demonstrate either a positive or a negative correlation with the total distance travelled. By analyzing behavior in the two-zone configurations, we hypothesized that high burying behavior will be associated with a decrease in the total distance travelled as animals will be preoccupied with the objects; they would therefore explore the arena to a lesser extent and travel shorter distances (negative correlation). On the other hand, we believed that avoidance of marbles will be associated with a decrease in the locomotion measured due to stationary behavior, resulting in a positive correlation between the number of marbles covered and the total distance travelled.

However, our data indicate that neither point of departure was accurate (Supplementary Fig. 3). Indeed, data presented in this investigation indicates that locomotor activity in treatment naïve animals is not associated with either an increase or decrease in burying behavior and that it is possible to perform simultaneous MB and locomotion analyses. In effect, comparing the data presented in Fig. 4 and Supplementary Fig. 3, it seems as if burying behavior manifests as an orthogonal construct to normal locomotor activity. Animals burying an increased number of marbles in sand and spending significantly more time in the marble-containing zone, i.e. apparently showing preoccupation and decreased exploratory behavior (Fig. 4), would be expected to demonstrate a decrease in locomotor activity compared to animals that generated lower burying scores, purportedly exploring the arena more. This could not be substantiated by the locomotor data presented in Supplementary Fig. 3. Based on reasonable and logical arguments, it remains important to study the behavior of animals performing the MB task as it may be possible that anxiety-related avoidance can be confused with an inhibited locomotor response. However, in congruence with previous findings (Nicolas et al., 2006), we conclude that simultaneous recording of locomotor and MB activity is appropriate and valid, provided that the results be scrutinized to distinguish between findings relating to aberrant cognition, and those that undeniably result from locomotor modification.

5. Conclusion

The current investigation attempted to provide insight into the disparity between findings related to the MB test used as a measure of

either anxiety or compulsivity, as well as its broad response to pharmacological interference. Further, we aimed to conceptualize an appropriate methodological framework for measuring MB behavior. In order to address these goals, we included all of the major methodological differences as reported in studies of MB behavior. We further performed the MB test repeatedly in the same animals, but in alternating zone-configurations to divulge the influence of avoidance behavior. As such, the following major conclusions can be drawn:

- The quantification of MB behavior is susceptible to observer bias and are modified by the nature of burying substrate;
- MB as quantified in most investigations that employ sparse substrates, e.g. husk, sawdust, or wood shavings, is neither a measure of compulsivity, or anxiety, but possibly results from normal exploration that is subject to habituation;
- Although results from the MB test are modified by zone-configuration, unconditioned behavior in a one-zone configuration is more reflective of normal exploration than of aberrant cognition. Hence, we propose that the application of MB without any pre-test anxiogenic or compulsive-inducing interference, be performed applying a two-zone paradigm as this ensures 1) that burying activity is a result of voluntary engagement and 2) that subjects have the opportunity to avoid exposure to marbles completely. Indeed, voluntary, but persistent and repetitive burying activity would thus accurately resemble a compulsive phenotype, while marble avoidance or habituation towards marble-directed activity over time, would more accurately indicate either passive- or active anxiety-related responses;
- Burying substrates with a small (0.2–0.4 mm) particle size and high weight/volume ratio, e.g. sand, are resistant to the coincidental effects of normal exploration or burrowing and are therefore appropriate substrates to distinguish burying from other forms of behavior;
- Although simultaneous recordings of locomotor activity is warranted, careful analyses of data is necessary to exclude the probability of either false positive or negative data; and
- Quantification of MB as often reported, is inappropriate. Irrespective of its susceptibility to observer bias, MB can only be distinguished from other forms of normal behavior based on whether preoccupation with a marble will result in the object being buried.

We conclude that MB, having been proposed as a measure of anxiety- and/or compulsive-like behavior and executed as reported, is often inadequate to measure either construct as findings reported here demonstrate that endpoint MB quantification is subject to manipulation by experimental design as far as observer bias, burying substrate, zone configuration and trial repetition are concerned. In this regard, we propose that analogous between-laboratory methodology is followed with respect to such parameters of the experimental design, especially with respect to its differential application in investigations relating to anxiety and compulsivity, respectively. Moreover, current conclusions drawn from the MB test related to pharmacological interference and its subsequent neurobiological and translational relevance for anxiety and/or OCD, must be viewed in light of the findings reported in this investigation. In fact, if no single procedure is followed across all laboratories with respect to certain conditions, and if MB is not stratified based on preoccupation, the findings from one investigation cannot support, contradict or elucidate that which has been reported elsewhere.

Conflicts of interest

The current paper was funded by the Medical Research Council of South Africa. The authors declare no conflicts of interest.

Author contributions

DWW and GdB conceptualized the current investigation, performed all literature and practical research and statistics and wrote the manuscript.

Data accessibility

Our data will be available for investigation following justifiable and reasonable request.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.beproc.2018.04.011>.

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4. Manuscript B

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- *Geoffrey de Brouwer* assisted in the conceptualization of the review with *De Wet Wolmarans*, performed the literature search, data extraction, conceptualized and wrote the manuscript.
- *Arina Fick* assisted in the literature search and information extraction.
- *De Wet Wolmarans* as the study leader, assisted in the conceptualization of the review together with *Geoffrey de Brouwer*, assisted with the writing and editing of the manuscript.
- *Brian Harvey* proofread the manuscript and provided professional insight and guidance with respect to the overall direction of the review.

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A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive–compulsive disorder: Mapping the way forward

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Abstract

Rodent marble-burying behavior in the marble-burying test (MBT) is employed as a model or measure to study anxiety- and compulsive-like behaviors or anxiolytic and anticomulsive drug action. However, the test responds variably to a range of pharmacological interventions, and little consensus exists regarding specific methodologies for its execution. Regardless, the test is widely applied to investigate the effects of pharmacological, genetic, and behavioral manipulations on purported behaviors related to the said neuropsychiatric constructs. Therefore, in the present review we attempt to expound the collective translational significance of the MBT. We do this by (1) reviewing burying behavior as a natural behavioral phenotype, (2) highlighting key aspects of anxiety and obsessive–compulsive disorder from a translational perspective, (3) reviewing the history and proof of concept of the MBT, (4) critically appraising potential methodological confounds in execution of the MBT, and (5) dissecting responses of the MBT to various pharmacological interventions. We conclude by underlining that the collective translational value of the MBT will be strengthened by contextually valid experimental designs and objective reporting of data.

Keywords Marble-burying test · Validity · Methodology · Review · Anxiety · Obsessive–compulsive disorder · Animal model

Introduction

From a translational point of view, preclinical screening tests can broadly be applied on two levels with respect to neuropsychiatric states: namely, (1) as frameworks with which to study the underlying etiology, neurobiology, and cognitive deficits of specific human conditions, and/or (2) as a means to characterize and quantify the effects of novel pharmacological or behavioral manipulations that are proposed to be of possible benefit in the modeled clinical condition. The accurate and reliable application of such tests relies mainly on three forms of validity—that is, face, construct, and predictive

(Albelda & Joel, 2012a, 2012b; Alonso, Lopez-Sola, Real, Segalas, & Menchon, 2015; Monteiro & Feng, 2016; Willner, 1984). Briefly, *face validity* refers to observable behaviors or signs that seemingly resemble those present in the modeled condition. A screening test or model with robust *construct validity* demonstrates the involvement of neurobiological and/or neurocognitive systems known to be implicit in the modeled condition, whereas *predictive validity* refers to the model's sensitivity to analogous pharmacological or behavioral interventions that are effective in the human condition or to its insensitivity to those that have no demonstrable clinical efficacy. Although few screening tests for psychiatric conditions are founded equally in all levels of validity, a certain degree of overlap between the preclinical and clinical states of being needs to be established, at one or more of the different levels, before the test can be regarded as an instrument appropriate for conducting investigations (Wolmarans, Stein, & Harvey, 2017).

The analysis of marble-burying behavior by rodents is often applied as a preclinical screening test for anxiolytic (Broekkamp, Rijk, Joly-Gelouin, & Lloyd, 1986; Nicolas, Kolb, & Prinssen, 2006; Thomas et al., 2009) and anticomulsive (Egashira et al., 2018; Taylor, Lerch, &

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Chourbaji, 2017; Uday, Pravinkumar, Manish, & Sudhir, 2007; Umathe, Manna, & Jain, 2012) drug action. It is also used as a model to characterize and quantify the purported behavioral manifestations of neuropsychological constructs related to, *inter alia*, posttraumatic stress disorder (PTSD; Kedia & Chattarji, 2014) and Alzheimer's disease (Torres-Lista, López-Pousa, & Giménez-Llort, 2015). However, the marble-burying test (MBT) is characterized by variable responses to a range of pharmacological compounds administered both acutely and chronically (Millan, Girardon, Mullo, Brocco, & Dekeyne, 2002; Sugimoto, Tagawa, Kobayashi, Hotta, & Yamada, 2007; Taylor et al., 2017; Umathe et al., 2012), and little consensus exists regarding the specific methodology to be followed in its execution (Çalışkan et al., 2017), even when the test is applied with respect to a specific condition (Jimenez-Gomez, Osentoski, & Woods, 2011; Sugimoto et al., 2007). Indeed, methodological congruence between different investigations into marble-burying is found only in the application of the number of marbles buried as an indicator of behavioral severity. Considering the unique neurocognitive constructs underlying different mental disorders, such as general anxiety (Mathew, Price, & Charney, 2008), PTSD (Yehuda, 2002), and obsessive-compulsive disorder (OCD; Westenberg, Fineberg, & Denys, 2007), it is perplexing that a seemingly mono-dimensional behavioral phenotype can be applied to mimic and assess the behavioral symptomatology of such diverse conditions, albeit within a highly specific context. Although it is not uncommon to apply a specific behavioral measurement in the analysis of behaviors that are believed to mimic different mental disorders—for example, the open field test, in assessments of anxiety (Prut & Belzung, 2003), aggression (Lewis, Gariépy, Gendreau, Nichols, & Mailman, 1994), and social interaction (File & Seth, 2003)—the use of the MBT differs from these tests in that, given the different approaches taken to its application, no clear hypothesis seems to underlie its use within the conceptual and contextual boundaries of a specific disorder. In fact, the realities that the test is applied as a screening tool for specific conditions and drug responses without methodological congruence between laboratories (Dey, Chatterjee, & Kumar, 2016; Gawali et al., 2016) and that it demonstrates inconsistent responses to different treatments (Li, Morrow, & Witkin, 2006; Njung'e & Handley, 1991a) complicate comparisons of the published findings and cloud the translational usefulness of marble-burying as a measure of anxiolytic or anticomulsive drug action.

The purpose of the present article is to provide a critical review of the marble-burying test, first as it is commonly applied as a model in rodents to elucidate the underlying mechanisms that apparently relate to anxiety- and obsessive-compulsive (OC)-like behaviors, and second as a screening test for novel anxiolytic or anti-OC therapies. Because marble-burying is closely related to natural animal behavior (Jirkof, 2014), we will first summarize and appraise the relevant

aspects of normal digging, burrowing, and burying behavior from an ethological perspective. Next we will briefly review key aspects of anxiety and OCD that are of relevance for preclinical research. We will then elaborate on the history and proof of concept of the marble-burying test, followed by a dissection of its experimental application and responses to different treatments. Finally, we will conclude by arguing that in general the marble-burying test—as it has been applied, executed, and reported to this point in time—is of no translational value, and that a contextually standardized approach to the analysis of *aberrant*, but not *inherent* (i.e., occurring as a natural phenotype within the larger population), burying behavior, performed within the context of the preclinical psychiatric research described here, will be pivotal to furthering our understanding of the neurobiological mechanisms underlying clinical anxiety and OCD and their responses to treatment.

Digging, burrowing, and burying as natural behaviors

Digging, burrowing, and burying are core components of the normal behavioral repertoire of rodents (Jirkof, 2014; Layne & Ehrhart, 1970; Pisano & Storer, 1948; Poling et al., 1981; Weber & Hoekstra, 2009). Digging can be regarded as the primary action by which more complex tasks—namely, burrowing or the burying of objects—are achieved, and it refers to the displacement of a substrate using mostly the forepaws (Layne & Ehrhart, 1970). Burrowing—that is, the construction of tunnels for habitation (Adams & Boice, 1981; Sherwin, Haug, Terkelsen, & Vadgama, 2004)—and burying—that is, the displacement of either aversive (De Boer & Koolhaas, 2003) or nonaversive (Poling, Cleary, & Monaghan, 1981) objects beneath any available substrate—thus represent the application of digging to a more specific outcome.

Research into the origins of ethological and laboratory digging has confirmed that digging as a naturally occurring behavior is unaffected by age or sex (Deacon, 2006; Masuda, Ishigooka, & Matsuda, 2000). However, strain variation in digging, burrowing, and burying behavior is common (Deacon, Thomas, Rawlins, & Morley, 2007; Layne & Ehrhart, 1970; Weber & Hoekstra, 2009; Webster, Williams, Owens, Geiger, & Dewsbury, 1981). Furthermore, there is evidence that laboratory-reared rats and mice show comparable burrowing activity to their wild-type counterparts when they are afforded living conditions that allow for the complete expression of digging activity (Adams & Boice, 1981; Boice, 1977; Jirkof, 2014; Weber & Hoekstra, 2009). Indeed, digging and burrowing serve analogous purposes across both natural and laboratory settings and are central to rodent survival and social structure (Deacon, 2006; Ebensperger & Blumstein, 2006). Fundamentally, these behaviors are motivationally driven by the needs to store food (Fleming & Brown, 1975;

Jenkins & Breck, 1998), control temperature (Ellison, 1995; Tracy & Walsberg, 2002), facilitate social interaction, nurture and protect young (Denenberg, Taylor, & Zarrow, 1969), and avoid predation (Ebensperger & Blumstein, 2006; Ruffer, 1965; Tracy & Walsberg, 2002). That said, digging and burrowing can also be regarded as a *mandatory behavioral need*, as was demonstrated by Sherwin et al. (2004). Indeed, when studying digging and burrowing behavior in laboratory mice, it has been demonstrated that such behaviors persist in cages that already contain extensive burrow networks. However, although digging and burrowing are natural and persistent under laboratory conditions, even in the offspring of captive-bred animals (Adams & Boice, 1981; Weber & Hoekstra, 2009), such behaviors are subject to modification by a number of factors, including preexposure to the burying substrate—which results in decreased, albeit persistent, burrowing activity (Schultz, 1972)—as well as the burying substrate itself, which can influence the number of burying episodes and the overall measurable digging activity (Layne & Ehrhart, 1970; Webster et al., 1981). Furthermore, gross digging behavior can also be influenced by genetics, even in closely related species, which can exhibit notably different burrow architecture and digging activity (Dudek, Adams, Boice, & Abbott, 1983; Layne & Ehrhart, 1970; Weber & Hoekstra, 2009; Webster et al., 1981). Considering that the common standard housing conditions in the majority of rodent housing facilities provide only a thin layer of any given form of bedding material—for example, corn cob (Jimenez-Gomez et al., 2011), wood chips (Deacon et al., 2007), or paper (Burn, Peters, & Mason, 2006)—digging and burrowing often cannot be readily expressed. It can therefore be expected that such behaviors will be expressed to a greater extent following the provision of ample bedding or burrowing substrate (Adams & Boice, 1981; Webster et al., 1981). Furthermore, although the majority of studies that have investigated burying behavior from a novelty—or *neophobic*—perspective have confirmed that anxiety or related concepts of threat/danger both provoke and exacerbate the digging/burying response (De Boer & Koolhaas, 2003; Njung'e & Handley, 1991b; Pinel & Treit, 1978), it is important to emphasize that such behaviors still occur in the absence of such triggers, although possibly they manifest differently (Deacon et al., 2007; Jirkof, 2014; Layne & Ehrhart, 1970; Sherwin et al., 2004; Weber & Hoekstra, 2009).

Although burying behavior, as an intentional outcome of digging, may bear face resemblance to digging or burrowing, burying refers to the *concerted* effort to either cover a particular object with substrate (De Almeida, De Carvalho, Silva, De Sousa, & De Freitas, 2014; De Boer & Koolhaas, 2003; Kinsey, O'Neal, Long, Cravatt, & Lichtman, 2011; Pinel & Treit, 1978; Poling et al., 1981) or displace an object beneath any available substrate by means of *digging in proximity* to it (de Brouwer & Wolmarans, 2018; Gyertyán, 1995). Rodents

have been shown to bury a number of objects, including food (Jenkins & Breck, 1998), marbles (Taylor et al., 2017), live scorpions (Londei, Valentini, & Leone, 1998), rodent chow (either contaminated or uncontaminated; Poling et al., 1981), electrified probes (Treit, 1990), mouse traps (Linfoot et al., 2009), flashing cubes, and air-blasting tubes (Terlecki, Pinel, & Treit, 1979). In light of theories of goal-directed behavior (de Wit & Dickinson, 2009), it can be hypothesized that harmful or noxious objects would elicit a unique burying response, as compared to nonreactive and nonharmful objects. However, investigations into phenotypic differences between the burying behaviors elicited by nonaversive and aversive objects have yielded inconsistent results (Londei et al., 1998; Poling et al., 1981; Terlecki et al., 1979). Collectively, the findings demonstrate that although rodents may initially express increased burying behavior toward potentially harmful, as compared to nonharmful, objects (De Boer & Koolhaas, 2003; Londei et al., 1998; Poling et al., 1981), burying behavior in general is primarily triggered by an investigative drive that is subject to habituation (Broekkamp et al., 1986; de Brouwer & Wolmarans, 2018; Poling et al., 1981; Thomas et al., 2009). It can therefore be concluded that the burying activity engaged in by rodents is a robust, naturally occurring behavior that, although initially modifiable by the nature of the stimulus, is displayed by all animals and is not *dependent* on a contextual trigger.

Fundamentals of anxiety and OCD from a preclinical perspective

Clinical anxiety: a state of abnormal fear processing

Anxiety can be described as a negative state of emotion experienced in anticipation of a forthcoming threat (American Psychiatric Association, 2013; Cryan & Holmes, 2005). However, in contrast to the previous clustering of most anxiety-related conditions into a single diagnostic category based on the presence of anxiety as a common factor (American Psychiatric Association, 2000), the fifth and most recent edition of the *Diagnostic and Statistical Manual of Mental Disorders* separates these conditions into three categories—that is, anxiety disorders, such as generalized anxiety disorder (GAD); trauma and stress-related disorders, such as PTSD; and OC and related disorders, such as OCD and trichotillomania (American Psychiatric Association, 2013). These categories diverge from one another mainly in terms of the underlying fear-provoking trigger, the magnitude of the distress experienced, and the degree of phenomenological and epidemiological overlap between conditions. Although some controversy remains regarding the updated diagnostic categories (Abramowitz & Jacoby, 2015), anxiogenic threats, irrespective of the condition, are of either an internal nature—that

is, born from within the patient, as from concerns about occupational or academic performance—or an external nature—such as confrontation with specific environments or situations, including wide-open spaces or forced social interaction (American Psychiatric Association, 2013). Anxiety is often characterized by transient or long-lasting somatic (e.g., muscle stiffness/weakness, peripheral nervous system activation, fatigue), cognitive (e.g., mental distress that impairs normal function, decreased focus, irritability), and behavioral (e.g., avoidance behavior, disturbed sleep, altered food intake, restlessness) symptoms, which frequently occur in anticipation of possible future threats (American Psychiatric Association, 2013; Nuss, 2015; Ohl, 2003). Furthermore, states of anxiety have the potential to interfere significantly with the daily functioning of a patient. That being said, anxiety *can also be regarded as a normal human response* to threatening or stressing situations, in that short-lived states of anxiety in response to tangible threats are normal (American Psychiatric Association, 2013; Cryan & Holmes, 2005).

Briefly, anxiety arises from the abnormal processing of fear- or stress-provoking stimuli in the limbic system of the brain, which comprises the limbic cortex (insular and cingulate), hippocampus, and amygdala (Martin, Ressler, Binder, & Nemeroff, 2009; Nuss, 2015). Furthermore, the amygdala, as the area responsible for fear expression, aggression, and defensive behavior, consolidates fear memory and receives neuronal and hormonal input from the hippocampus, thalamus, and hypothalamus (Martin et al., 2009). Several neuroendocrine, neurotransmitter, and neuropeptide alterations in these brain regions have been shown to play a role in the manifestation of anxiety. These include attenuated γ -amino butyric acid (GABA)-mediated signaling and bolstered glutamate activity (Meldrum, 1984), and serotonin (5-HT), dopamine (DA), and noradrenalin (NA) have also been implicated (Bandelow et al., 2008). Furthermore, a number of neuropeptides—including cholecystokinin (CCK; Bandelow et al., 2008), neuropeptide-Y (NPY; Tasan et al., 2009), vasopressin (AVP) and oxytocin (Neumann & Landgraf, 2012), Substance P (Santarelli et al., 2001; Tillisch et al., 2012), and corticotropin releasing hormone (CRH; Mathew et al., 2008)—also contribute to the psychopathology of anxiety.

The treatment of anxiety is complicated and is often influenced by the anxiety subtype diagnosed. Although some debate exists, the mainstay approach to the acute treatment of moderate to severe states of anxiety includes the use of compounds that potentiate the effects of GABA—for example, benzodiazepines and barbiturates (Bandelow et al., 2012; Bandelow et al., 2008). However, the focus of long-term management involves chronic administration of antidepressants that target mainly serotonergic and noradrenergic signaling, including selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), serotonin–noradrenalin reuptake inhibitors (SNRIs), and monoamine oxidase inhibitors

(Bandelow et al., 2012; Bandelow et al., 2008). Miscellaneous agents, such as 5-HT_{1A} agonists, antihistamines, atypical antipsychotics, antiepileptic drugs, and beta receptor antagonists, are also used in various instances (Bandelow et al., 2012; Bandelow et al., 2008). Nevertheless, as is also true for OCD (see paragraph 3.2), the efficacy of pharmacotherapeutic intervention in anxiety is, to say the least, suboptimal (Bandelow et al., 2012).

From a preclinical perspective, a distinction needs to be made between the animal models of and behavioral tests for specific conditions. In the case of preclinical neuropsychiatric investigations, animal models refer to robust, valid frameworks that as a whole emulate as closely as possible a holistic picture of the modeled human condition (Geyer & Markou, 1995; van der Staay, 2006). With respect to anxiety, a valid example would be the learned helplessness model, in which rodents not only display behavioral traits akin to those observed in anxiety, but also present with a neurobiological picture resembling that of the clinical condition (Ohl, 2005). On the other hand, a valid test for possible anxiety-like manifestations in any animal, normal or seemingly diseased, refers to a behavioral measure that can accurately identify the phenotype—for example, the elevated-plus maze (EPM; Ohl, 2003). Therefore, animal models of anxiety are dependent on showing that animals are experiencing a certain level of anxiety (Cryan & Holmes, 2005; Ohl, 2003), while the behavioral tests used to measure and characterize such anxiety-like traits should be accurate in doing so. Since rodents do not present with cognitive processing analogous to that of humans, the cognitive aspects of anxiety are often difficult to demonstrate in preclinical models. Therefore, current behavioral tests focus on rodent-specific behavioral parameters in order to recognize, characterize, and assess anxiety-like states—for example, *seemingly abnormal* manifestations of typical rodent behaviors, such as exploration and the avoidance of open and illuminated areas (Cryan & Holmes, 2005; Ohl, 2003), food intake (Ohl, 2003), vigilance and risk assessment (including freezing, stretching, and carefully sniffing unknown objects; Ohl, 2003), and sociability, as well as changes in peripheral nervous system activity (Ohl, 2003). Considering the nonreactive nature of glass marbles, which can be introduced as a novel, unconditioned stimulus (Egashira et al., 2018), it can be hypothesized that the MBT, when applied as a screening test for anxiolytic drug action, could possibly be accurate in measuring anxiety-like manifestations that are best related to a *specific phobia*—that is, neophobia or novelty-induced apprehension (Bruins Slot, Bardin, Auclair, Depoortere, & Newman-Tancredi, 2008; Kedia & Chattarji, 2014; Wolmarans, Stein, & Harvey, 2016). Indeed, phobias are characterized by a disproportionate fear of a specific object or situation that often occurs upon confrontation within the specific fear-related context, resulting in marked anxious responses in human subjects (American Psychiatric Association, 2013). However, as is true

for the MBT's use in research into compulsive-like behavior (Egashira et al., 2018), the differential application of marble-burying both as a model (Njung'e & Handley, 1991b; Jimenez-Gomez et al., 2011) and as a screening test (Kedia & Chattarji, 2014) for anxiety renders its translational usefulness clouded. In fact, neither of these approaches, as currently reported, is valid or appropriate, as we will argue throughout this article.

Apart from the MBT, the shock probe burying test (SPBT) also employs digging behavior as a phenotype of anxious behavior (De Boer & Koolhaas, 2003). As is true for marble-burying (Broekkamp et al., 1986; Jimenez-Gomez et al., 2011; Thomas et al., 2009), shock probe burying also responds to anxiolytic treatment (De Boer & Koolhaas, 2003; Pinel, Treit, Ladak, & MacLennan, 1980). In a comprehensive review of defensive burying (De Boer & Koolhaas, 2003), the authors concluded that along with fight, flight, and freezing, the burying of *aversive* stimuli forms part of the unconditioned defensive repertoire of rodents, representing a form of "active avoidance." Other commonly used, and well validated, tests for anxiety-like manifestations in rodents include the open field test (OFT; Cryan & Holmes, 2005; Ohl, 2003), EPM (Cryan & Holmes, 2005; Ohl, 2003), elevated zero maze (E0M; Cryan & Holmes, 2005), and light-dark test (LDT; Cryan & Holmes, 2005; Ohl, 2003), all four of which *rely on the natural tendency of animals to explore* a novel environment. Here, anxiolytic-like drug properties are indicated by bolstered exploration of central (OFT), open (EPM and E0M), or illuminated (LDT) areas of an arena, as opposed to time spent in the relatively "safe" corner (OFT), enclosed (EPM and E0M), or dark (LDT) spaces. In addition, the EPM and E0M also assess other aspects of anxiety-like behavior—for example, risk-taking behavior—and *specific fears* for an anxiogenic scenario—that is, height. Finally, the social interaction test (SIT; File & Seth, 2003) is used to mimic scenarios of forced social interaction, thereby measuring behaviors related to *fear of and apprehension toward* forced or undesirable social interaction. As such, changes to a number of behavioral parameters, including avoidance, voluntary peer interaction, and investigative social behaviors—for example, following, sniffing, and body contact—are used to recognize anxiolytic-like drug action (Cryan & Holmes, 2005; File & Seth, 2003; Ohl, 2003). Importantly, tests like the SIT provide a platform for testing anxiety-like states by demonstrating sensitivity to anxiolytic drug effects, where the administration of anxiogenic compounds manifests as *reduced* sociability (File & Seth, 2003), thereby adding to the face and predictive validity of the SIT with respect to anxiety. In summary, valid rodent tests for anxiety are accurate in highlighting changes in exploration; avoidance of aversive stimuli, in terms of distal positioning to or covering of the stimulus; reduced risk assessment behavior; decreased sociability; and decreased general activity levels—that is, increased freezing and stationary positioning. With respect to actual anxiety-like behaviors born

from fear of novelty, such behaviors have been shown to abate as a function of repeated exposure (Savy et al., 2015), or even over the course of a single continuous test session (Choleris, Thomas, Kavaliers, & Prato, 2001). Apart from possible changes in exploratory behavior, the MBT does not seem to resemble any such manifestation of anxiety-like behavior, as we will explain later.

Obsessions and compulsions: repetitious, persistent and intrusive

Whereas *obsessions* are described as persistent unwanted images, doubts, or intrusive thoughts that can cause distress (Abramowitz, Taylor, & McKay, 2009), *compulsions* can be understood as repetitive overt or covert behavioral routines that are often expressed in an attempt to alleviate the obsession-driven anxiety (Abramowitz & Jacoby, 2015; American Psychiatric Association, 2013; Veale & Roberts, 2014). However, both obsessions and compulsions can also be diagnosed in the absence of the other characteristic. Indeed, although OCD is no longer considered an anxiety disorder in the most recent version of the *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, 2013), anxiety, manifesting as a form of psychoneurosis, is accepted to play an important role in OC symptom manifestation (Abramowitz & Jacoby, 2015). However, the anxiolytic relief arising from carrying out compulsions is typically brief and is thought to contribute, via processes of negative reinforcement, to symptom exacerbation (Ahmari, 2016; American Psychiatric Association, 2013). Fundamentally, obsessions and compulsions are attributable to dysfunctional beliefs—that is, a disproportionate sense of importance attached to specific thoughts and feelings (American Psychiatric Association, 2013). These can broadly be grouped within five main symptom clusters: namely, (1) obsessions about contamination and washing compulsions; (2) obsessions concerning harm, together with checking compulsions; (3) obsessions with symmetry, ordering, and counting; (4) repugnant obsessions involving themes of sex, religion, and violence; and (5) hoarding and associated collecting compulsions, recently awarded the status of a separate disorder (Abramowitz et al., 2009; American Psychiatric Association, 2013; Rowsell & Francis, 2015). Importantly, although patients may present with more than one symptom cluster, it is important to note that obsessions and subsequent compulsions mostly develop with respect and in response to specific sets of conditions/stimuli only (American Psychiatric Association, 2013; Rowsell & Francis, 2015). For example, whereas safety-related obsessions are paired with persistent checking compulsions, symmetry obsessions will be associated with excessive ordering compulsions. Patients with OCD therefore function normally under circumstances and in contexts that are unrelated to the triggers of their OC symptoms, and in many cases

they actively avoid their triggers (American Psychiatric Association, 2013). That being said, patients diagnosed with only one symptom dimension are rare (Mataix-Cols, do Rosario-Campos, & Leckman, 2005). Irrespective of the OC cluster, the symptoms can have a negative influence on the normal daily routine of a patient, interfering with the academic, social, and occupational functions of patients, as well as affecting their overall quality of life (Sørensen, Kirkeby, & Thomsen, 2004).

Although different hypotheses have been put forward to explain the symptomology of OCD (Husted, Shapira, & Goodman, 2006), it seems like abnormal regulation of goal-directed behavior may be central to the symptomology of OCD. Thus, the brain areas implicated in OCD are those that translate cognitive planning and experiences into motor behavior, and subsequently mediate goal-directed (viz. reward-related) behavior. These brain areas include the prefrontal cortex, striatum and thalamic nuclei that communicate with each other via different pathways (Evans, Lewis, & Tobst, 2004; Nambu et al., 2000; van den Heuvel et al., 2010). The term “CSTC circuit” (i.e., cortico-striatal-thalamic-cortical circuit) denotes the functional organization of these structures (Stocco, Lebiere, & Anderson, 2010) and consists of both direct (*behaviorally activating*) and indirect (*behaviorally inactivating*) pathways. Although evidence indicates some degree of neuroanatomic variation underlying the various symptom clusters (Rowell & Francis, 2015; Stein et al., 2010)—which may explain some of the variation in treatment response of the different OC phenotypes (McKay et al., 2004)—the CSTC circuit is fundamental in the planning, execution and termination of complex motor behavior and reward-based learning—two major processes that are hypothesized to be dysfunctional in patients with OCD (Stocco et al., 2010). Furthermore, it is believed that there is a bias in favor of the direct thalamus-activating pathway over the indirect thalamus-inhibiting pathway in the basal ganglia of OCD patients, as compared to healthy controls (Saxena & Rauch, 2000). This not only results in an overactive orbitofrontal cortex (OFC), but also increases the activities of both the caudate nucleus and thalamus. The subsequent hyperactivity in the CSTC circuit is believed to be central to the pathological presentation of OCD (Bartz & Hollander, 2006; Saxena & Rauch, 2000).

Certain neurotransmitters have been identified as playing a central role in the pathogenesis of OCD, of which glutamate, GABA, and the monoamines DA and 5-HT have been the most well studied (El Mansari & Blier, 2006; Markarian et al., 2010; Pittenger, Krystal, & Coric, 2006; Sareen et al., 2004). The pharmacological treatment of OCD is based primarily on the use of SSRIs (Abramowitz et al., 2009; Fineberg, 2004; Goddard, Shekhar, Whiteman, & McDougall, 2008), which are typically administered in high doses for extended periods of time (Dougherty, Rauch, & Jenike, 2004; Fineberg, 2004). In fact, the therapeutic onset

of SSRI action can take up to 10 weeks (Goddard et al., 2008). Despite the demonstrable efficacy and tolerability of SSRIs in the treatment of OCD, up to 30% of patients remain treatment-resistant (Fineberg, 2004). In such cases, low-dose antipsychotics, such as risperidone and quetiapine, are often combined with ongoing SSRI treatment in an augmentation strategy (Abramowitz et al., 2009; Dougherty et al., 2004; Fineberg, 2004); however, antipsychotics are typically ineffective when administered as monotherapy (Fineberg, 2004).

Given the abstract and internal nature of obsessions, the presence thereof remains difficult to demonstrate in animal models of OCD (Albelda & Joel, 2012a, 2012b). However, because overt compulsions are often easily observed and characterized, persistent repetitive behaviors form the foundation of animal models of OCD with respect to face validity. In fact, a number of putative and validated animal models of OCD have been described (see Albelda & Joel, 2012a, 2012b; Alonso et al., 2015, for comprehensive reviews in this regard). These include behavioral models, such as spontaneous stereotypy in the deer mouse (Korff, Stein, & Harvey, 2008; Powell, Newman, Pendergast, & Lewis, 1999), excessive nest building (Greene-Schloesser et al., 2011), and compulsive lever pressing (Joel, 2006); pharmacological models, such as quinpirole-induced compulsive-like checking (Szechtman, Sulis, & Eilam, 1998) and 8-OH-DPAT-induced persistent arm preference in a T-maze (Yadin, Friedman, & Bridger, 1991); and genetic models, such as *Sapap3* knockout (KO) mice, which demonstrate excessive self-grooming (Welch et al., 2007). All of these models are, although they differ with respect to the nature of the observable behavior, are congruent with respect to the presentation of persistent and repetitive motor rituals.

With respect to mimicking the human illness as accurately as possible, an animal model of OCD should ideally adhere to a number of criteria. These include repetitive, perseverative, and seemingly inappropriate behaviors that are resistant to change (Alonso et al., 2015; Bechara & Lewis, 2012; Szechtman et al., 2017). Furthermore, cognitive inflexibility, characterized by behaviors without a clear endpoint and that cannot be suppressed indefinitely, such as failure to adapt to altered reward conditions in the signal attenuation test (Joel, 2006), should ideally be present (Joel, 2006; Sohn, Kang, Namkoong, & Kim, 2014). The involvement of abnormal learning processes (Nielen, den Boer, & Smid, 2009) and reliance on habitual rather than goal-directed task performance (Alonso et al., 2015) are also of value. Considering that the expression of compulsions is time-consuming and that it results in significant functional impairment (American Psychiatric Association, 2013; Sohn et al., 2014), animal models of OCD demonstrating such alterations in the normal behavioral routines of subjects may also contribute to our understanding of the intrusive nature of compulsive routines (Wolmarans et al., 2017).

In line with the discussion above, and considering that compulsions are mostly *directed* toward reducing the distress caused by intrusive thoughts related to specific scenarios, we propose that the MBT should be appreciated as characterizing a truly aberrant behavioral phenotype that accurately highlights an animal's persistent, repetitive, and behaviorally inflexible preoccupation and direct interaction with marbles (Njung'e & Handley, 1991b; Wolmarans et al., 2016, 2017), and that incidental or "normal" behaviors that result in the covering of marbles must at all costs be excluded in the assessment of marble-burying behavior. Whether this distinction is appreciated and applied by all investigators is dubious, yet it has major implications for interpreting the presenting behavioral data and the test's translational relevance for human OCD.

In summary, whereas anxiety disorders and OCD are often diagnosed as co-morbid disorders (American Psychiatric Association, 2013; de Mathis et al., 2013), an important distinction between OCD and anxiety disorders is that anxiety-related disorders are typically related to real-world, everyday situations or concerns, whereas obsessions are often of a more abstract and irrational nature (Abramowitz et al., 2009; American Psychiatric Association, 2013). Given the phenotypic presentation of the two conditions alluded to above, it can be hypothesized that an anxious animal will either actively engage to neutralize the anxiety-provoking stimulus or present with passive avoidance and hypervigilance under unfamiliar and/or unconditioned circumstances. However, given the non-reactive and nonharmful nature of the marble stimulus, habituation with respect to both scenarios should develop over time. On the other hand, since OCD is best described by persistent, repetitive, and rigid behaviors, particularly with respect to a specific context, we propose that murine marble-burying behavior, when applied as a screening test for anticomulsive drug action, should preferably be characterized by persistent, repetitive, and voluntary direct interaction with the objects. We will elaborate on this below.

The MBT: a brief history and proof of concept

As we explained earlier, although rodents bury both nonharmful and noxious objects (De Boer & Koolhaas, 2003; Pinel et al., 1980; Pinel & Treit, 1978), burying responses toward the latter group of objects are initially more robust, thus confirming defensive burying as an integral component of the rodent defensive repertoire (for a comprehensive review on the topic, see De Boer & Koolhaas, 2003). Marble-burying, first described within the context of the defensive-burying test, purportedly represents a simplified application of defensive burying, and simply involves the burying of nonaversive marbles in the absence of prior conditioning or training (Poling et al., 1981). However, as is true for defensive burying, the action of marble-burying involves pushing

bedding material toward the marbles with the snout or forepaws or spraying burying substrate over the objects while facing away from the marbles (Gyertyán, 1995). Also, in some cases holes are dug into which marbles are rolled (Gyertyán, 1995). Based on the goal-directed and direct interaction with marbles, such behaviors are typically unique and do not represent normal cage exploration (Gyertyán, 1995; Njung'e & Handley, 1991b).

Marble-burying was initially applied as a screening test for anxiolytic compounds (Broekkamp et al., 1986; Treit, Pinel, & Fibiger, 1981); indeed, the test illustrated accurate predictive validity in that meprobamate, clonazepam and flunitrazepam reduced MB without affecting self-grooming or locomotor responses (normal, nonanxious behaviors; Broekkamp et al., 1986). However, on the basis of observations that rodents will persist in burying nonreactive objects (Gyertyán, 1995; Njung'e & Handley, 1991b), including familiar food pellets (Poling et al., 1981; Thomas et al., 2009), the proposed anxiety-like involvement in marble-burying behavior, and thus also the fundamental value of the MBT, has been questioned. Although it is true that the drug challenges introduced in numerous investigations have yielded results that largely agree with anxiolytic responses (Broekkamp et al., 1986; Bruins Slot et al., 2008; Kinsey et al., 2011), additional behavioral investigations have indicated a clear lack of anxiety-like involvement in the marble-burying response, whereas a number of nonanxiolytic compounds, such as haloperidol, also reduce marble-burying behavior (Bruins Slot et al., 2008; Matsushita et al., 2005; Nicolas et al., 2006). In fact, it has been shown that marble-burying persists over repeated exposure, even when avoidance of the marbles is possible and after animals have been habituated to the presence of marbles in their home cage environments for extended periods (Njung'e & Handley, 1991b; Thomas et al., 2009). Moreover, the facts that marble-burying correlates poorly with the outcomes of other classical experimental tests of anxiety (Sanathara et al., 2018; Savy et al., 2015; Thomas et al., 2009; although see also Greene-Schloesser et al., 2011) and that it is subject to significant between-strain variation (Angoa-Pérez, Kane, Briggs, Francescutti, & Kuhn, 2013; Egashira et al., 2013; Nicolas et al., 2006; Thomas et al., 2009) further suggest that marble-burying as it is normally carried out in the lab—that is, not based on individual differences in behavior, but rather to characterize group differences in *supposed* abnormal burying behavior, mostly following drug intervention—represents an inherent, rather than a neophobic or anxiety-related, behavioral phenotype. As such, the MBT has since been employed to model the purported behavioral manifestations of OCD—that is, seemingly purposeless repetition (Egashira, Harada, et al., 2007; Gaikwad, Parle, Kumar, & Gaikwad, 2010; Iijima, Kurosu, & Chaki, 2010; Taylor et al., 2017; Umathe, Bhutada, Dixit, & Shende, 2008; Umathe et al., 2012); in most cases, however, this approach

is also unjustified, as will be explained. However, it must be emphasized that it is possible to induce excessive marble-burying behavior by using known anxiogenic interventions, such as restraint-induced stress (Kedia & Chattarji, 2014). In this regard, putative anxiolytics and anticomulsive compounds can be identified on the basis of their effects on *induced* burying behavior; this is because a direct association can be made between an elicited anxiogenic or compulsive-like response and favorable drug action. That said, most anxiety- and compulsivity-related investigations that have employed marble-burying as a measure of behavioral severity have applied naturalistic, noninduced burying activity in its own right as a means to identify and characterize anxiolytic and anticomulsive drug action (Broekkamp et al., 1986; Bruins Slot et al., 2008; Londei et al., 1998; Umathe et al., 2012), an approach that is inherently flawed.

A methodological review of the MBT

The oft-reported methodology for the MBT is simple and based largely on the study design employed by Broekkamp et al. (1986). Briefly, the test involves the placement of any number of marbles (usually between 4 and 25, depending on the zone configuration of the marble-burying arena; see paragraphs 5.2 and 5.5; Çalişkan et al., 2017) gently onto the surface of a layer of bedding material (normally no thicker than 5 cm). However, whereas Broekkamp et al. (1986) placed the marbles in close contact with one another centrally in the cage, most recent investigations have employed an experimental configuration in which marbles were spaced evenly throughout the arena (one-zone; 1Z; Egashira et al., 2018; Gawali et al., 2016; Millan et al., 2002) or in one section of the arena only (two-zone; 2Z; Gyertyán, 1995; Nicolas et al., 2006; Njung'e & Handley, 1991b; Torres-Lista et al., 2015). Occasionally, marbles are placed around the perimeter of the burying cage, as well (Chaki et al., 2003; Taylor et al., 2017; Young, Batkai, Dukat, & Glennon, 2006), resembling another example of a two-zone setup. Subsequently, subjects are introduced to the marble-containing arenas for up to 30 min (Çalişkan et al., 2017) and are allowed voluntary interaction with the environment. The number of marbles that have been buried is then counted by an observer, who is often blind to the treatment status of the subjects (Angoa-Pérez et al., 2013; Egashira et al., 2018; Millan et al., 2002). Importantly, since the majority of investigations do not distinguish between the specific behavioral patterns resulting in marbles being lowered into the burying substrate (Egashira, Okuno, Abe, et al., 2008; Gaikwad & Parle, 2011; Gomes, Casarotto, Resstel, & Guimarães, 2011; Harasawa, Ago, Itoh, Baba, & Matsuda, 2006; Honda, Kawaura, Soeda, Shirasaki, & Takahama, 2011; Li et al., 2006; Matsushima, Shiota, Kikura-Hanajiri, Goda, & Eguchi, 2009; Shimazaki, Iijima,

& Chaki, 2004; Umathe et al., 2012; Yamada et al., 2002), the term “buried” is generally applied to indicate the number of *covered* marbles. Although the reported methodologies always refer to the duration of exposure as well as the number of marbles and the burying substrate used, several experimental variables are subject to modification, either intentionally or not. Such interinvestigation differences, although they may often have meaningful implications for interpretation of the findings, are not always declared. Therefore, the following paragraphs will summarize key aspects of these variables and explain their fundamental meanings within the context of behavioral investigations.

Burying substrate and its relation to marble size

Substrates commonly used include corn cob (Supplementary Fig. 1a; Angoa-Pérez et al., 2013; Jimenez-Gomez et al., 2011; Thomas et al., 2009), sawdust (Supplementary Fig. 1b; Bruins Slot et al., 2008; Dixit et al., 2014; Harasawa et al., 2006; Krass, Rünkorg, Wegener, & Volke, 2010), wood chips (Supplementary Fig. 1c; Llanaez & Frye, 2009; Londei et al., 1998; Saadat, Elliott, Colado, & Gree, 2006; Thomas et al., 2009), wood shavings (Supplementary Fig. 1d; Poling et al., 1981), river sand (Supplementary Fig. 1e; de Brouwer & Wolmarans, 2018), and Sani-chips (Supplementary Fig. 1f; Kinsey et al., 2011; Thomas et al., 2009; Young et al., 2006). However, detailed descriptions of the respective burying substrates, including mass per volume, particle size, and manner of placement—namely, compacted or not—are almost never reported (Casarotto, Gomes, Resstel, & Guimarães, 2010; Egashira, Okuno, Abe, et al., 2008; Egashira, Okuno, Harada, et al., 2008; Iijima et al., 2010; Umathe et al., 2012). Indeed, such details are important for a number of reasons. Due to the sparse and light nature of burying substrates such as sawdust (pine, weighed and calculated at 0.17 g/cm³; de Brouwer & Wolmarans, 2018) and wood shavings (pine, weighed and calculated at 0.07 g/cm³; de Brouwer & Wolmarans, 2018), marbles simply placed gently on the surface of these substrates may appear from the outset to be covered to a depth of at least two-thirds of their size, when compared to more dense substrates (de Brouwer & Wolmarans, 2018; Supplementary Fig. 2a; see also Paragraph 5.6). Even if marbles are placed with care, any disturbance of the surrounding bedding material by any form of exploratory activity by the animal may result in marbles settling deeper, or even beneath, the substrate. Since most investigations do not report the use of video tracking, except for purposes of locomotor activity (LMA) tracking (Nicolas et al., 2006), endpoint quantification of the number of marbles buried may be a caveat in the interpretation of data. In contrast, denser substrates with a higher mass-per-volume ratio—for example, corncob (weighed and calculated at 0.38 g/cm³; de Brouwer & Wolmarans, 2018) or fine river sand (Supplementary Fig. 2b; weighed and

calculated at 1.65 g/cm^3 ; de Brouwer & Wolmarans, 2018)—are generally more resistant to the effects of normal exploration, and therefore may be better suited as appropriate substrates in which to carry out the MBT (Supplementary Fig. 3a vs. 3b; Angoa-Pérez et al., 2013; de Brouwer & Wolmarans, 2018; Jimenez-Gomez et al., 2011).

Moreover, considering that the number of marbles used in the execution of the MBT typically varies from 4 to 25 (Çalışkan et al., 2017; Gaikwad et al., 2010; Krass et al., 2010; Schneider & Popik, 2007; Sugimoto et al., 2007; Uday et al., 2007; Umathe et al., 2008), and that the most common criterion applied to quantify buried marbles is equated to two-thirds of the marble being covered, the particle size of the burying substrates is an equally important variable. For instance, whereas a 15-mm marble used in our laboratory weighs 5.6 g, a 20-mm or 23-mm glass marble of similar density would weigh approximately 13.26 or 20.16 g, respectively. This is important for two reasons. First, it is evident that marbles of a greater mass would settle to the bottom of the testing arena more quickly than marbles of a lower mass, especially when placed in cages containing burying substrates of a sparse nature—for example, sawdust (Supplementary Fig. 1b) or wood shavings (Supplementary Fig. 2a)—which are subject to disturbance by any routine movement of the animals during the test session (de Brouwer & Wolmarans, 2018). Second, smaller marbles (e.g., 10 mm) introduced into an arena prepared with burying substrates of a larger particle size—for example, wood shavings (Supplementary Fig. 2a; average diameter of 8 mm)—may complicate the quantification of the number of marbles buried, as compared to cages prepared with larger marbles in relation to the substrate particle diameter (Supplementary Fig. 2b). This may include scenarios in which either smaller marbles are placed in cages with burying substrates with a small particle diameter (e.g., 10-mm marbles in cages prepared with corncob; particle $\phi = 4 \text{ mm}$; Supplementary Fig. 1a) or larger marbles are placed in cages containing burying substrates with a larger particle size (e.g., 15-mm marbles in cages fitted with sawdust; particle $\phi = 8 \text{ mm}$). This is true because the commonly applied two-thirds-covered criterion to quantify the number of “buried” marbles is based on visual observation of the testing arena. In line with this argument, Gyertyán (1995) investigated the effect of marbles on observable digging behavior. The findings there indicated that digging behavior in lighter and sparser substrates—for example, sawdust—occurred similarly in cages containing and not containing marbles. These findings were replicated by Thomas et al. (2009). Importantly, Gyertyán also demonstrated that the covering of marbles with bedding material from digging bouts occurred incidentally because of general digging activity, rather than as a result of marble-directed activity, and that such digging behavior was by no means either triggered or bolstered by the presence of the marbles. This finding possibly elucidates why marbles spaced evenly throughout the

cage (Njung’e & Handley, 1991b), instead of in close proximity of one another in the center of the cage (Broekkamp et al., 1986), seem to bolster burying activity findings.

Zone configuration—a question of choice

As we alluded to earlier, the MBT can be carried out with marbles spaced evenly throughout the entire arena (Badgujar & Surana, 2010; Bruins Slot et al., 2008; Kinsey et al., 2011) or placed in one section of the arena only (Llaneza & Fryc, 2009; Nicolas et al., 2006; Njung’e & Handley, 1991b; Savy et al., 2015; Schneider & Popik, 2007; Thomas et al., 2009). A core concept of the MBT when applied as a measure of anxiety is that animals unconditioned to the testing paradigm may experience neophobia-related anxiety when confronted with marbles for the first time, thereby invoking either active burying or passive avoidance behavior (Bruins Slot et al., 2008; Kinsey et al., 2011). This idea was mostly strengthened by early findings demonstrating the efficacy of anxiolytic drugs in reducing marble-burying activity (MBA) without affecting other behaviors (Broekkamp et al., 1986), whereas numerous investigations have subsequently aimed to examine whether novelty-induced anxiety is indeed a trigger for marble-burying behavior (Gyertyán, 1995; Nicolas et al., 2006; Njung’e & Handley, 1991b; Thomas et al., 2009). To appropriately characterize anxiety-like responses in the MBT, a 2Z paradigm is ideal, since the majority of behaviors observed in the 1Z paradigm are related to exploration and investigation of a novel environment (de Brouwer & Wolmarans, 2018). Furthermore, as opposed to 1Z setups, the behaviors in a 2Z paradigm more appropriately reflect goal-directed interaction while also being reflective of definite avoidance behavior. Interestingly, with few exceptions (see, e.g., Schneider & Popik, 2007), most 2Z investigations have failed to demonstrate passive avoidance of marbles (Broekkamp et al., 1986; de Brouwer & Wolmarans, 2018; Kachler, Singewald, Sinner, & Philippu, 1999; Nicolas et al., 2006; Njung’e & Handley, 1991b; Thomas et al., 2009). That said, these findings also do not indicate active neutralizing interaction with marbles, in that animals often spend equal time in both zones of the arena, regardless of the duration of the experiment, even if the two zones are separated by a divider (Nicolas et al., 2006; Njung’e & Handley, 1991b; Savy et al., 2015; Thomas et al., 2009; Torres-Lista et al., 2015).

However, considering that *externally provoked* marble-burying behavior may be a valid means to assess putative anxiolytic responses, Kedia and Chattarji (2014) showed that mice stressed by acute immobilization buried more marbles following experience of the stressor than did nonstressed counterparts, whereas Llaneza and Frye (2009) demonstrated a positive correlation between increased MBA and increased vigilance/immobility behavior in a shock-induced conditioned fear paradigm. Furthermore, significantly bolstered marble-

burying was also found in prior-stressed animals (Dey et al., 2016). Increased marble-burying has also been reported in animals injected with diphtheria toxin (Sanathara et al., 2018). Since the investigation by Kedia and Chattarji employed MBA to measure the manifestations of *induced* anxiety (that is to say, as a measure of induced anxiety) in animals, it cannot be concluded that the presence of marbles in test arenas per se contributed to the experienced level of anxiety; rather, it may be an accurate predictor of anxiogenic manipulation, albeit variably so (cf. Kedia & Chattarji, 2014, vs. Nicolas et al., 2006; Njung'e & Handley, 1991b). It can therefore be considered that although evidence indicates that the anxious state of an animal may affect its response toward the presence of marbles, such association remains unlikely where the marbles are introduced as anxiogenic stimuli. In fact, many researchers have concluded that anxiety and associated concepts of novelty are *not driving or reinforcing factors* for marble-burying behavior, but that such behaviors *in the majority of animals* are born from a need to investigate novel surroundings (Gyertyán, 1995; Masuda et al., 2000; Nicolas et al., 2006; Njung'e & Handley, 1991b; Poling et al., 1981; Thomas et al., 2009; Wolmarans et al., 2016).

Although the literature concerning animal models of OCD has since repositioned the MBT more as a measure of compulsive-like, instead of anxiety-like, behavior (Albelda & Joel, 2012a, 2012b; Alonso et al., 2015; Thomas et al., 2009), investigations applying the MBT in anxiety-related studies are still performed (Nicolas et al., 2006; Saadat et al., 2006); these may also benefit from employing a 2Z configuration. With respect to the MBT, and in line with OC theory (Alonso et al., 2015), an animal expressing compulsive-like behavior should repetitively and persistently engage in burying behavior, even if presented with a choice not to engage in such activity. If an animal therefore chooses to avoid exposure to the marbles, compulsive-like repetition should be excluded, hence undermining the application of the test under such specific circumstances as a measure of anticomulsive drug action (Wolmarans et al., 2016).

To habituate or not

Since marble-burying behavior may partly be driven by an inherent need for investigation, the novelty of burying substrates may trigger natural exploratory activity, in the form of digging and burrowing, and may therefore influence the number of marbles being covered (Gyertyán, 1995; Thomas et al., 2009). Although bolstered burying activity under novel circumstances has been demonstrated previously (Schultz, 1972), an influence of novelty-induced anxiety with respect to unfamiliar bedding or marbles has also largely been excluded (Gyertyán, 1995; Thomas et al., 2009), providing further support that marbles are often covered as a coincidental effect of normal exploratory behaviors (de Brouwer & Wolmarans,

2018; Gyertyán, 1995; Njung'e & Handley, 1991b; Thomas et al., 2009).

However, to exclude the possible effects of novel cage exploration on burying outcomes, it is important to consider adequate habituation with the burying substrates before the onset of behavioral analysis. This may be more applicable for anxiety than for compulsivity-related studies. This is because in compulsivity studies, animals should be exposed over the course of repetitive trials instead of a single trial (de Brouwer & Wolmarans, 2018; Gyertyán, 1995; Njung'e & Handley, 1991b; Taylor et al., 2017; Thomas et al., 2009; Wolmarans et al., 2016); hence, habituation is introduced coincidentally in the experimental design. In fact, when the test was repeated on up to five consecutive days with the same subjects, no significant differences in MBA were found across several different investigations (Gyertyán, 1995; Njung'e & Handley, 1991b; Poling et al., 1981; Thomas et al., 2009; Wolmarans et al., 2016). Also, it has been shown that marble-burying behavior remains unaltered even when the test is repeated many times over the course of a single day (Njung'e & Handley, 1991b; Thomas et al., 2009). In line with this, performance on the MBT is unaltered even after habituating the subjects to marbles in a home cage environment for a 5- to 21-day period (Njung'e & Handley, 1991b; Poling et al., 1981; Thomas et al., 2009). Furthermore, in studies relating to anxiety, the point of departure is to introduce a novel, unconditioned stimulus in the form of marbles. It is important, therefore, to exclude the possible effects of other novelty factors, such as the burying substrate, on marble-burying performance (Casarotto et al., 2010; Gawali et al., 2016; Taylor et al., 2017; Umathe et al., 2012).

Locomotor performance

Since the burying of marbles entails physical motor activity, an important aspect of the MBT that needs consideration in the study design and the interpretation of findings is the inherent LMA of the subjects being tested. Although this is frequently reported alongside MBT results, there are examples in which LMA assessments have not been discussed (Angoa-Pérez et al., 2013; Kedia & Chattarji, 2014). Due to the central acting agents often modifying the normal locomotor abilities of subjects (Kinsey et al., 2011; Lynch, Castagné, Moser, & Mittelstadt, 2011), it is important to distinguish purported anxiolytic and anticomulsive drug action from incidental inhibition of LMA, which may manifest in practical terms as altered MBA (Nicolas et al., 2006). In fact, findings showing drug-decreased MBA paired with markedly suppressed LMA are often discarded as false-positive data (Kinsey et al., 2011; Njung'e & Handley, 1991a). Investigations into MBA that have also reported results from locomotor assessments have employed several approaches, including separate analyses of LMA (Krass et al., 2010; Millan et al., 2002; Saadat et al., 2006) and simultaneous recording of marble-burying and LMA (Egashira et al., 2018;

Egashira, Okuno, Matsushita, et al., 2008; Jimenez-Gomez et al., 2011; Matsushita et al., 2005; Nicolas et al., 2006). Furthermore, LMA assessments may employ either the same subjects tested for marble-burying behavior (Egashira et al., 2013; Schneider & Popik, 2007; Umathe, Vaghasiya, Jain, & Dixit, 2009) or a separate group exposed to treatment regimens analogous to that of the group tested for burying behavior (Gaikwad et al., 2010; Saadat et al., 2006; Uday et al., 2007). Considering that marble-burying behavior may involve preoccupation with objects, it is possible that simultaneous measurements of burying and locomotion may yield inappropriate results, since animals engaging in burying activity may travel shorter overall distances than animals engaging in normal exploratory routines (de Brouwer & Wolmarans, 2018). Although this remains to be established, it is an aspect that needs careful consideration in the interpretation of data obtained from marble-burying investigations.

Arena size

Significant variation exists across investigations with respect to the size of the arena employed (Broekkamp et al., 1986; Casarotto et al., 2010; Egashira, Harada, et al., 2007; Millan et al., 2002; Njung'e & Handley, 1991b; Thomas et al., 2009; Uday et al., 2007; Umathe et al., 2008; Wolmarans et al., 2016). Furthermore, since both rats and mice are employed in marble-burying investigations, it would be expected that appropriately larger arenas would be employed for rats, as compared to those used in mouse studies. However, no clear guidelines exist, with rat arenas ranging from $35 \times 25 \times 19$ cm (Poling et al., 1981) to $45 \times 24 \times 21$ cm (Llaneza & Frye, 2009) and $47 \times 27 \times 15$ cm (Schneider & Popik, 2007). Important to note is that the latter two studies employed the two-zone test, effectively utilizing half of the floor space for marble-burying. It is perhaps with respect to the arenas used for studies relating to mouse burying behavior that the greatest variation is observed; in some cases, the arenas employed have been of similar dimensions, or even larger than, those employed in rat studies. These sizes range from $23 \times 17 \times 14$ cm (25 marbles; Broekkamp et al., 1986) and $38 \times 32 \times 28$ cm (25 marbles; Casarotto et al., 2010) to $45 \times 60 \times 25$ cm (24 marbles; Badgujar & Surana, 2010). Arena size is important because the “density” of marbles spread across the bedding material surface, depending on the number of marbles used, would differ between smaller and larger arenas. Between-laboratory differences in this regard may significantly influence behavioral performance and the subsequent interpretation of data, especially in experimental paradigms employing a 1Z setup (Badgujar & Surana, 2010; Bruins Slot et al., 2008; Dixit et al., 2014). In experiments in which the possibility to avoid exposure to marbles is undesirable (1Z condition), large between-marble spaces may undermine the purpose of the investigation, as is evident when comparing the ratios of floor space to marbles reported in the literature. Higher marble

densities of $21.2 \text{ cm}^2/\text{marble}$ (Bruins Slot et al., 2008) or $23.8 \text{ cm}^2/\text{marble}$ (Dixit et al., 2014) stand in stark contrast to lower densities of $112.5 \text{ cm}^2/\text{marble}$ (Badgujar & Surana, 2010) or $75 \text{ cm}^2/\text{marble}$ (Saadat et al., 2006), all of which have been employed in a 1Z test configuration. Furthermore, a densely spaced marble grid may be overly subject to incidental covering of the marbles, resulting from indiscriminate disturbances of the burying substrate during nonspecific behavioral routines (de Brouwer & Wolmarans, 2018; Gyertyán, 1995; Njung'e & Handley, 1991b). That said, whereas arena size is an important factor for consideration in the defensive-burying test, in which the extent of shock probe burying is *reduced* by larger rectangular arenas (Cueto-Escobedo, Contreras, Bernal-Morales, Guillen-Ruiz, & Rodríguez-Landa, 2013) and *induced* by circular arenas (Cueto-Escobedo et al., 2013; Davis, Moore, Cowen, Thurston, & Maggio, 1982), few investigations have explicitly analyzed the influence of arena size in the MBT. In one such investigation, Poling et al. (1981) hypothesized that a restricted test arena could potentially force subjects to interact with marbles or other “bury”-inducing objects. They tested this hypothesis by examining the burying of regular, uncontaminated food pellets and marbles in a smaller- as well as a larger-than-home-cage-sized arena. However, objects were buried equally in both paradigms, suggesting that restricting the arena size, and thereby forcing interaction between the subject and the marbles, neither triggers nor bolsters burying activity.

A matter of counting—observers and scoring criteria

Because scoring in the MBT is *always* performed manually, three important between-laboratory variables that differ in assessments of burying activity are (1) the number observers (either blind or nonblind); (2) the criteria to determine what constitutes a buried marble, be it one-half (Kinsey et al., 2011; Schneider & Popik, 2007; Taylor et al., 2017; Thomas et al., 2009), two-thirds (Broekkamp et al., 1986; Egashira, Harada, et al., 2007; Egashira, Okuno, Harada, et al., 2008; Gawali et al., 2016; Harasawa et al., 2006; Iijima et al., 2010; Jimenez-Gomez et al., 2011; Kedia & Chattarji, 2014; Millan et al., 2002; Nicolas et al., 2006; Takeuchi, Yatsugi, & Yamaguchi, 2002; Uday et al., 2007), or completely (Ichimaru, Egawa, & Sawa, 1995; Torres-Lista et al., 2015) covered; and (3) whether video recordings of the test sessions are made, to verify the counting results and examine specific behaviors (Jimenez-Gomez et al., 2011; Kedia & Chattarji, 2014; Umathe et al., 2012). Since the counting of marbles remains a subjective visual inspection of the test cage, there exists room for observers to make interpretations of what may or may not constitute a buried marble according to the chosen criteria, which may further be complicated by substrates of a sparse nature (see paragraph 5.1; de Brouwer & Wolmarans, 2018). To this end, methods to eliminate such inherent biases may be useful, as in the studies of Angoa-Pérez et al. (2013)

and Kinsey et al. (2011), in which two observers counted marbles and data were accepted only when they met a certain agreement criterion or scores were averaged between observers. Such improvements to the interpretation of experimental results have been highlighted in initiatives such as the ARRIVE guidelines, which aim to improve the quality of animal research reporting while simultaneously attempting to optimize ethical concerns (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010).

Effect of sex, species, strain, and genes

Sex is an important factor for consideration regarding marble-burying performance. Two-thirds of studies have employed male subjects (Çalışkan et al., 2017), 8% of the marble-burying investigations to date have employed only females, and the remainder have operated without sex bias, or with no reference to sex at all (Badgujar & Surana, 2010; Çalışkan et al., 2017; Njung'e & Handley, 1991a, 1991b; Taylor et al., 2017; Yamada et al., 2002). With respect to the possible influences of sex on marble-burying behavior, the oestrous cycle has been shown to have at least some influence on burying activity. In fact, it has been demonstrated that marble-burying is bolstered during the rat metoestrus and reduced during proestrus, which correlate with suppressed and elevated sex hormone levels, respectively (Schneider & Popik, 2007). However, this effect was not observed in *all* subjects in that investigation. Furthermore, Llana and Frye (2009) found that both MBA and vigilant/avoidant behaviors were minimized during the oestrous phase, in line with the observations of Schneider and Popik. In addition, it was shown that the administration of exogenous progesterone and estradiol to noncycling ovariectomized dams attenuated anxiety and MBA in a fashion similar to cycling dams during the oestrous phase (Schneider & Popik, 2007). Also, Schneider and Popik demonstrated that the naturally increased MBA that occurred during metoestrus was attenuated by progesterone administration. It is therefore entirely possible that the ovarian cycle is a potential confounder of marble-burying results. That said, the importance of using both sexes in OCD studies has been highlighted (Albelda & Joel, 2012b; Taylor et al., 2017), whereas the rationale for a study to exclude either of the sexes needs careful consideration and motivation, as is noted in the ARRIVE guidelines (Kilkenny et al., 2010). This, by extension, also applies to anxiety testing, since ovarian cycling has been shown to modulate test results (Frye, Petralia, & Rhodes, 2000), and even drug response in anxiety investigations (Regenass, Möller, & Harvey, 2018).

The defensive-burying response, from which the MBT is methodologically derived (Poling et al., 1981), was first demonstrated in rats. Since the publishing of the first articles in which marble-burying was applied in rodents, a large number of mouse and rat strains have been employed, including Swiss Albino (Gaikwad et al., 2010; Jimenez-Gomez et al., 2011;

Uday et al., 2007; Umathe et al., 2008; Umathe et al., 2012), C57BL/6J (Casarotto et al., 2010; Gomes et al., 2011; Kedia & Chattarji, 2014; Kinsey et al., 2011; Krass et al., 2010; Nicolas et al., 2006; Thomas et al., 2009), ICR (Egashira, Harada, et al., 2007; Egashira, Okuno, Abe, et al., 2008; Egashira, Okuno, Harada, et al., 2008; Egashira, Okuno, Matsushita, et al., 2008; Iijima et al., 2010; Matsushita et al., 2005; Shimazaki et al., 2004; Sugimoto et al., 2007), NMRI (Bruins Slot et al., 2008; Gyertyán, 1995; Millan et al., 2002), MF1 (Njung'e & Handley, 1991a, 1991b), ddY (Abe, Nakai, Tabata, Saito, & Egawa, 1998; Egashira et al., 2013; Honda et al., 2011), and most recently, the deer mouse (*Peromyscus maniculatus*, 2018; Wolmarans et al., 2016). Wistar (Schneider & Popik, 2007) and Long-Evans/hooded (Llana & Frye, 2009; Poling et al., 1981) rats have also been used, as well as a number of miscellaneous strains (Çalışkan et al., 2017; please also refer to the supplementary tables). Variations in digging activity based on genetics and the typical natural environments of species have extensively been reported in the literature (Dudek et al., 1983; Layne & Ehrhart, 1970; Weber & Hoekstra, 2009; Webster et al., 1981). To investigate the influence that strain may have on marble-burying and avoidance behaviors, Nicolas et al. (2006) investigated the burying-related activities of three strains of mice—that is, C57BL/6J, BALB/c, and CBA/J. Indeed, it was reported that BALB/c mice presented with the lowest level, and CBA/J and C57BL/6J with higher levels of burying activity, congruent with and supporting earlier findings (Dudek et al., 1983). Subsequently, Nicolas et al. selected C57BL/6J and CBA/J for further study with respect to neophobic responses in a 2Z setup. Here, no significant difference in the numbers of marbles buried between the two strains was found; however, C57BL/6J mice spent markedly less time on the marble-containing side than did CBA/J mice. Since the C57BL/6J mice, and not the other two strains, presented with both avoidance anxiety and superior MBA, their behavior potentially presents with robust face validity for modeling anxiety-like manifestations in the MBT (Nicolas et al., 2006). Unfortunately, the test was carried out only once in all subjects, and it is therefore not clear whether the seemingly neophobic response in C57BL/6J mice would have habituated over time. Thomas et al. (2009) later expanded on strain differences by testing ten strains of mice with respect to a number of anxiety-related behaviors, including marble-burying behavior in a 1Z paradigm. However, in this study, although the findings concerning CBA/J mice were largely congruent with those of Nicolas et al., C57BL/6J mice buried fewer marbles than did many of the other strains (Thomas et al., 2009). When considering how rodent strain effects may relate to OCD, it is interesting that Korff et al. (2008) demonstrated significantly greater stereotypic behavior in deer mice, a recognized animal model of OCD, than in C57BL mice, which also concurs with the aforementioned findings

of different expressions of purported OC-like behaviors by different strains. The differences in evinced anxiety and stereotypic behavior observed in these two strains further highlight the importance of separating anxiety- and OC-related behaviors in a test or model. This is, of course, where the MBT receives its harshest criticism. However, it is important to note that Nicolas et al. (2006) first assessed burying activity in a 1Z paradigm, whereby interaction with the marbles was forced, and that they subsequently observed avoidance behavior in a 2Z paradigm. The findings of Thomas and Nicolas are therefore congruent with respect to the neophobic trait reported in C57BL/6J mice, highlighting the presented evidence that the MBA observed in most strains, except for C57BL/6J, is poorly correlated with other conventional tests of anxiety, such as open field exploration and the light–dark avoidance test (Cryan & Holmes, 2005; Ohl, 2003). Although these results contradict some findings from other laboratories (Greene-Schloesser et al., 2011), they highlight the significance of between-laboratory methodological differences and the influence they may have on the reporting and interpretation of data.

In addition to different strains being used, a number of genetically modified strains have also been characterized with respect to burying behavior. Since these studies are numerous and mostly included the MBT only as one of a number of behavioral experiments (Angoa-Pérez et al., 2013; Balemans et al., 2010; Burne, McGrath, Eyles, & Mackay-Sim, 2005; Duangdao, Clark, Okamura, & Reinscheid, 2009; Egashira, Tanoue, et al., 2007; Gavioli, Rizzi, Marzola, Zucchini, & Regoli, 2007; Lähdesmäki et al., 2002; Mosienko et al., 2012; Sanathara et al., 2018; Shmelkov et al., 2010; Tasan et al., 2009; Yamada et al., 2002), we will only highlight some key findings here. Tryptophan hydroxylase 2 (TPH2) knock-out (KO) mice, which present with no detectable brain levels of 5-HT, demonstrate bolstered MBA in a 1Z paradigm as compared to their wild-type counterparts (Angoa-Pérez et al., 2013). In contrast, mice lacking the vitamin D (VDR; Burne et al., 2005) and vasopressin-1a (V1a; Egashira, Tanoue, et al., 2007) receptors have been demonstrated as burying fewer marbles than their respective wild-type controls, indicating that MB performance is indeed affected by genetic manipulation in addition to the more common pharmacological manipulations carried out in the test. In this regard, the adrenergic system has also been implicated, since α_2 -adrenergic receptor KO mice bury markedly fewer marbles than their wild-type counterparts (Lähdesmäki et al., 2002). Interestingly, and considering that the MBT may be accurate in mimicking anxiogenic responses, knockout of the nociception/orphanin FQ peptide receptor (NOP), which normally produces anxiolytic effects when stimulated, caused an anxious behavioral phenotype in the EPM and light–dark tests, but not in marble-burying and several other classic tests of anxiety. This led the authors to conclude that the NOP

receptor possibly plays a role in the *modulation* of anxious behaviors, albeit on an exceedingly complex level (Gavioli et al., 2007). Finally, neuropeptide Y receptors, specifically the Y2 and Y4 receptors, seem to modulate anxiety-like behavior, in that Y2- and Y4-KO mice have been demonstrated to present with anxiolytic responses in the MBT as well as in other anxiety tests (Tasan et al., 2009). Mice lacking the melanin-concentrating hormone receptor (MCHR) also display increased MBA (Sanathara et al., 2018), findings that were further corroborated by pharmacological interventions in the same study. Together, these findings suggest that genetic KO models do indeed provide an interesting avenue for further research, but that the aforementioned genetic KO investigations are subject to many of the methodological constraints highlighted throughout this review.

As we have illustrated in the preceding paragraphs, sex, species, strain, and genes all have profound effects on digging behavior as a whole, and thus, each of these parameters requires careful consideration with respect to analyses of marble-burying performance. Indeed, findings reported following the use of different sexes or strains may not be comparable between laboratories, due to apparent differences in the inherent behaviors of the respective subjects. Depending on the application of the MBT, strains that exhibit robust burying/digging behavior and/or neophobic behavior may be ideal.

To summarize this section, it is evident that a number of methodological differences characterize the execution of the MBT. Furthermore, as we explained in the preceding paragraphs, the importance of these differences in the presentation, analysis, and interpretation of findings related to anxiety- and compulsive-like activity cannot be overstated. Thus, the question arises of how such an apparently mono-dimensional and often inherent behavior can be applied and appraised as the core symptom of a number of cognitive constructs, without consideration of the methodological confounds discussed above. This question will now be discussed from a pharmacological perspective.

Responses of marble-burying to pharmacological manipulation

Given its application in a wide range of translational frameworks, the MBT has been challenged with a wide range of pharmacological compounds. Indeed, if we consider Tables 1, 2, and 3, marble-burying has demonstrated varying responses to a number of interventions, including agents that target the nor-adrenergic, dopaminergic, serotonergic, cholinergic, glutamatergic, and GABAergic systems. Furthermore, several miscellaneous receptors have also been targeted, including neurokinin (NK), imidazoline, calcium channel, and endocannabinoid receptors, and genetic studies have also considered a number of putative anxiety-linked receptor targets, including the α_2 , NOP,

Table 1 Drugs effective in the MBT

Drug	Mechanism of action	Dose range	ED	LMA	Reference
Drugs targeting the serotonergic system					
5-MeO-DMT	5-HT _{1A} & 5-HT ₂ agonist	0.25, 1, 2.5, 5 mg/kg i.p. 20 min pre	2.5, 5	—	(Njung'e & Handley, 1991a)
8-OH-DPAT	5-HT _{1A} agonist	0.3, 1, 3, 10 mg/kg i.p. 10 min pre	10	—	(Njung'e & Handley, 1991a)
		1, 3, 10 mg/kg i.p. 30 min pre	3, 10	#	(Ichimaru et al., 1995)
		0.1, 1, 3 mg/kg 30 min pre	3	/	(Matsushita et al., 2005)
		0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	0.63, 2.5	#	(Bruins Slot et al., 2008)
		3 mg/kg i.p. 30 min pre	3	/	(Egashira, Okuno, Matsushita, et al., 2008)
Bupirone	5-HT _{1A} agonist	1, 5, 10, 20 mg/kg i.p. 30 min pre	20	—	(Njung'e & Handley, 1991a)
		15, 30, 60 mg/kg p.o. 60 min pre	30, 60	/	(Ichimaru et al., 1995)
		10, 20, 40, 80, 160 mg/kg p.o. 60 min pre	80, 160	—	(Abe et al., 1998)
		1, 3, 10, 30 mg/kg i.p. 30 min pre	3, 10, 30	—	(Nicolas et al., 2006)
		1, 3, 10, 30 mg/kg i.p. 30 min pre	10, 30	—	(Young et al., 2006)
Citalopram	Selective serotonin reuptake inhibitor	1, 5, 10, 20 mg/kg i.p. 30 min pre	20	/	(Njung'e & Handley, 1991a)
		5, 10, 15 mg/kg i.p. 20 min pre	5, 10, 15	/	(Takeuchi et al., 2002)
		1–3 mg/kg i.p. 30 min pre	1–3	#	(Li et al., 2006)
		0.3, 1, 3, 10, 30 mg/kg i.p. 30 min pre	3, 10, 30	—	(Nicolas et al., 2006)
		2.5, 10, 40 mg/kg s.c. 60 min pre	2.5, 40	/	(Bruins Slot et al., 2008)
		10 mg/kg i.p. 30 min pre	10	/	(Krass et al., 2010)
Clomipramine	Serotonin noradrenalin reuptake inhibitor	15, 30, 60 mg/kg p.o. 60 min pre	60	#	(Ichimaru et al., 1995)
		0.16, 0.63, 2.5, 10, 40 mg/kg i.p. 30 min pre	10, 40	—	(Millan et al., 2002)
		3–30 mg/kg i.p. 30 min pre	30	#	(Li et al., 2006)
		3, 10, 30 mg/kg i.p. 30 min pre	10, 30	—	(Nicolas et al., 2006)
DOI	5-HT _{2A/C} agonist	0.01, 0.02, 0.05, 0.1, 0.5, 1, 2.5, 5 mg/kg i.p. 30 min pre	0.1–5	/	(Njung'e & Handley, 1991a)
		0.3, 1 mg/kg i.p. 30 min pre	1	/	(Egashira, Okuno, et al., 2012)
Duloxetine	Serotonin–noradrenalin reuptake inhibitor	1, 3, 10, 30 mg/kg i.p. 30 min pre	10, 30	—	(Nicolas et al., 2006)
Escitalopram	Selective serotonin reuptake inhibitor	2 mg/kg s.c. 11 days	2	/	(Taylor et al., 2017)
Fenfluramine	5-HT releaser	1, 2.5, 5, 10 mg/kg i.p. 30 min pre	2.5, 5, 10	/	(Njung'e & Handley, 1991a)
Fluoxetine	Selective serotonin reuptake inhibitor	5, 10, 20, 40, 80, 160, 320 mg/kg p.o. 60 min pre	40, 80, 160	—	(Abe et al., 1998)
		5, 10, 15 mg/kg i.p. 20 min pre	15	/	(Takeuchi et al., 2002)
		3–30 mg/kg s.c. 30 min pre	30	/	(Li et al., 2006)
		3, 10, 30 mg/kg i.p. 30 min pre	10, 30	—	(Nicolas et al., 2006)
		5, 10, 15 mg/kg i.p. 30 min pre (acute)	10–15	/	(Uday et al., 2007)
		0.16, 0.63, 2.5, 10 mg/kg s.c. 60 min pre	2.5, 10	#	(Bruins Slot et al., 2008)
		5, 10, 15, 30 mg/kg s.c. 30 min pre (acute)	15, 30	/	(Umathe et al., 2008)
		2.5, 5 mg/kg s.c. twice daily 7 days (chronic)	5	/	(Umathe et al., 2008)
		1.5, 5, 10 mg/kg i.p. 30 min pre	10	/	(Umathe et al., 2009)
		10 mg/kg p.o. 60 min pre	10	#	(Badgular & Surana, 2010)
		10 mg/kg i.p. 30 min pre	10	#	(Prajapati et al., 2011)
		2.5, 5, 10 mg/kg i.p. 30 min pre	5, 10	/	(Umathe et al., 2012)
		1, 3, 10 mg/kg i.p. 30 min pre	10	#	(Nardo et al., 2014)
		5 mg/kg i.p. 30 min pre	5	#	(Kalariya et al., 2015)
Fluvoxamine	Selective serotonin reuptake inhibitor	1, 5, 10, 20 mg/kg i.p. 30 min pre	10, 20	/	(Njung'e & Handley, 1991a)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
		15, 30, 60 mg/kg p.o. 60 min pre	30, 60	/	(Ichimaru et al., 1995)
		15, 30, 60 mg/kg/day p.o.	60	—	(Ichimaru et al., 1995)
		0.16, 0.63, 2.5, 10, 40 mg/kg s.c. 30 min pre	2.5, 10, 40	+	(Millan et al., 2002)
		3, 10, 30 mg/kg s.c. 30 min pre	10, 30	/	(Shimazaki et al., 2004)
		10, 30, 60 mg/kg i.p. 30 min pre	30, 60	/	(Harasawa et al., 2006)
		30 mg/kg p.o. 60 min pre	30	/	(Egashira, Harada, et al., 2007)
		2.5, 5, 10 mg/kg i.p. 4 h pre	5, 10	—	(Schneider & Popik, 2007)
		1, 5, 10 mg/kg i.p. 30 min pre	5, 10	/	(Sugimoto et al., 2007)
		30 mg/kg i.p. 30 min pre	30	/	(Matsushima et al., 2009)
		10, 30 mg/kg p.o. 60 min pre	30	/	(Egashira, Shirakawa, et al., 2012)
		30 mg/kg p.o. 60 min pre	30	/	(Egashira, Okuno, et al., 2012)
Gepirone	5-HT _{1A} agonist	5, 10, 20 mg/kg i.p. 30 min pre	10, 20	—	(Njung'e & Handley, 1991a)
Imipramine	Tricyclic antidepressant	4.6, 10, 22 mg/kg s.c. 30 min pre	10, 22	#	(Brockkamp et al., 1986)
		5–30 mg/kg i.p. 30 min pre	15–30	#	(Li et al., 2006)
Indalpine	Selective serotonin reuptake inhibitor	1, 5, 10, 20 mg/kg i.p. 30 min pre	5, 20	/	(Njung'e & Handley, 1991a)
Ketanserin	5-HT ₂ antagonist	1 mg/kg i.p. 30 min pre	1	—	(Njung'e & Handley, 1991a)
mCPP	Non-specific 5-HT agonist	1, 2.5, 5, 10, 20 mg/kg i.p. 30 min pre	2.5, 5, 10, 20	/	(Njung'e & Handley, 1991a)
		0.3, 1, 3 mg/kg i.p. 30 min pre	1, 3	—	(Nicolas et al., 2006)
		0.1, 0.3, 1 mg/kg i.p. 30 min pre	1	/	(Nardo et al., 2014)
MDMA	5-HT reuptake inhibitor/releaser	2.2, 4.4, 11, 22 µmol/kg i.p. 30 min pre	4.4–22	/	(Saadat et al., 2006)
Methysergide	5-HT _{1A} agonist; 5-HT ₂ antagonist	1, 5 mg/kg i.p. 30 min pre	5	—	(Njung'e & Handley, 1991a)
Mirtazepine	5-HT _{2/3} antagonist	1, 3 mg/kg i.p. 30 min pre	3	/	(Egashira, Shirakawa, et al., 2012)
MKC-242	5-HT _{1A} agonist	0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 mg/kg p.o. 60 min pre	3.2, 6.4	—	(Abe et al., 1998)
Paroxetine	Selective serotonin reuptake inhibitor	0.1, 0.3, 1, 3 mg/kg i.p. 30 min pre	0.3, 1, 3	—	(Nicolas et al., 2006)
		2.6, 6.7, 13.6 µmol/kg i.p. 30 min pre	13.5	/	(Saadat et al., 2006)
		3 mg/kg p.o. 60 min pre	3	/	(Egashira, Harada, et al., 2007)
		10 mg/kg i.p.	10	+	(Casarotto et al., 2010)
		10 mg/kg i.p. 30 min pre	10	/	(Krass et al., 2010)
		3 mg/kg p.o. 60 min pre	3	/	(Egashira, Okuno, et al., 2012)
		3 mg/kg p.o. 60 min pre	3	/	(Egashira et al., 2018)
<i>Psilocybe argentipes</i> (raw plant material)	Non-specific 5-HT agonist	0.05, 0.1, 0.5, 1, 1.5, 2 g/kg p.o. 30 min pre	0.1, 0.5, 1	/	(Matsushima et al., 2009)
Psilocybin	Non-specific 5-HT agonist	0.025, 0.125, 0.25, 0.5, 1, 1.5 mg/kg p.o. 30 min pre	1.5	/	(Matsushima et al., 2009)
Ritanserin	5-HT _{2A/C} antagonist	1, 5, 10, 20 mg/kg i.p. 30 min pre	20	—	(Njung'e & Handley, 1991a)
		0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	0.63, 2.5	#	(Bruins Slot et al., 2008)
RU-24,969	5-HT _{1A/B} agonist	0.1, 1, 2.5, 5, 10 mg/kg i.p. 30 min pre	2.5, 5, 10	+	(Njung'e & Handley, 1991a)
Tandospirone	5-HT _{1A} agonist	20, 40, 80, 160, 320 mg/kg p.o. 60 min pre	80, 160, 320	—	(Abe et al., 1998)
TFMPP	Non-specific 5-HT agonist	1, 5, 10, 20 mg/kg i.p. 30 min pre	5, 10, 20	—	(Njung'e & Handley, 1991a)
WAY-161503	5-HT _{2C} agonist	1, 3 mg/kg i.p. 30 min pre	3	/	(Egashira, Okuno, et al., 2012)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
YM992	SSRI/5-HT _{2A} antagonist	5, 10, 15 mg/kg i.p. 20 min pre	15	/	(Takeuchi et al., 2002)
Zimeldine	Selective serotonin reuptake inhibitor	10 mg/kg i.p. 30 min pre	10	/	(Njung'e & Handley, 1991b)
		1, 3, 10, 15, 30 mg/kg i.p. 30 min pre	10, 15, 30	/	(Njung'e & Handley, 1991a)
		10 mg/kg p.o. 21 days	10	#	(Njung'e & Handley, 1991a)
Drugs targeting the dopaminergic system					
Amantidine	Dopamine reuptake inhibitor and releaser	3, 10, 30 mg/kg, i.p. 30 min pre test	30	/	(Egashira, Okuno, Harada, et al., 2008)
Amisulpride	D _{2/3} antagonist	0.63, 2.5, 10, 40 mg/kg s.c. 60 min pre	10, 40	#	(Bruins Slot et al., 2008)
Apomorphine	D ₂ agonist	0.046, 0.1, 0.22, 0.46 mg/kg s.c. 10 min pre	0.22, 0.46	#	(Broekkamp et al., 1986)
Aripiprazole	D ₂ antagonist/partial agonist; 5-HT _{1A} partial agonist	0.04, 0.16, 0.63, 2.5, 10 mg/kg i.p. 60 min pre	10	#	(Bruins Slot et al., 2008)
		0.3, 1 mg/kg i.p. 30 min pre	10	/	(Egashira, Okuno, Matsushita, et al., 2008)
Aripiprazole + Ethanol	D ₂ antagonist/partial agonist; 5-HT _{1A} partial agonist	0.1, 0.5, 4, 6 i.p. 30 min pre	0.5, 4, 6	/	(Gaikwad & Parle, 2011)
		0.1 mg/kg i.p. and 0.1% w/v i.p. (sub-effective doses)	0.1 + 0.1	/	(Gaikwad & Parle, 2011)
Bifeprunox	D _{2/3} -HT _{1A} partial agonist	0.001, 0.0025, 0.01, 0.04, 0.16, 0.63, 2.5 mg/kg i.p. 60 min pre	0.001–0.01, 0.16 2.5	/	(Bruins Slot et al., 2008)
Bupropion	Dopamine reuptake inhibitor and releaser	20 mg/kg s.c. 30 min pre	20	+	(Honda et al., 2011)
Carbidopa	DOPA decarboxylase inhibitor	9 mg/kg i.p. 30 min pre	9	/	(Njung'e & Handley, 1991a)
Carbidopa + 5-Hydroxytryptophan	DOPA decarboxylase inhibitor + Serotonin precursor	9 mg/kg + 5, 10, 20 mg/kg i.p. 30 min pre	9 + 5, 10, 20	–	(Njung'e & Handley, 1991a)
Clozapine	Dopamine antagonist/ 5-HT ₂ antagonist	0.46, 1, 2.2, 4.6 mg/kg s.c. 30 min pre	2.2, 4.6	#	(Broekkamp et al., 1986)
		0.04, 0.16, 0.63, 2.5, 10 mg/kg s.c. 60 min pre	0.16–10	/	(Bruins Slot et al., 2008)
GBR-12909	DA reuptake inhibitor	1.9, 4.8, 9.7, 19.4 µmol/kg i.p. 30 min pre	9.7, 19.4	/	(Saadat et al., 2006)
Haloperidol	D ₂ antagonist	0.046, 0.1, 0.22, 0.46, 1 mg/kg s.c. 30 min pre	0.46, 1	#	(Broekkamp et al., 1986)
		0.01, 0.03, 0.1 mg/kg i.p. 30 min pre	0.1	–	(Matsushita et al., 2005)
		0.03, 0.1, 0.3, 1 mg/kg i.p. 30 min pre	0.3, 1	–	(Nicolas et al., 2006)
		0.0025, 0.01, 0.04, 0.16, 0.63 mg/kg s.c. 60 min pre	0.0025, 0.04–0.63	/	(Bruins Slot et al., 2008)
L-741,626	D ₂ antagonist	3, 10 mg/kg s.c. 30 min pre	10	–	(Egashira, Okuno, Matsushita, et al., 2008)
Olanzapine	D _{1/5} antagonist	0.3, 1, 3 mg/kg i.p. 30 min pre	3	/	(Egashira, Okuno, Matsushita, et al., 2008)
		0.0025, 0.01, 0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	2.5	#	(Bruins Slot et al., 2008)
PD-168,077	D ₄ agonist	2.5, 10, 40 mg/kg s.c. 60 min pre	40	#	(Bruins Slot et al., 2008)
Perospirone	D ₂ antagonist	3, 10 mg/kg p.o. 60 min pre	10	/	(Matsushita et al., 2005)
Perphenazine	D _{2/3} agonist	0.1, 0.22, 0.46, 1, 2.2 mg/kg s.c. 30 min pre	1, 2.2	#	(Broekkamp et al., 1986)
Pramipexole	D _{2/3} agonist	0.05, 0.5 mg/kg i.p.	0.5	–	(Jimenez-Gomez et al., 2011)
Quetiapine	D ₂ antagonist	10, 30, 100 mg/kg p.o. 60 min pre	100	–	(Egashira, Okuno, Matsushita, et al., 2008)
Quinpirole	D ₂ agonist	0.1, 0.3, 1 mg/kg i.p. 30 min pre	1	–	(Egashira, Okuno, Matsushita, et al., 2008)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
Remoxipride	D _{2/3} antagonist	0.04, 0.16, 0.63, 2.5, 10, 40 mg/kg s.c. 60 min pre	40	#	(Bruins Slot et al., 2008)
Risperidone	D ₂ antagonist	0.1, 0.3, 1 mg/kg p.o. 60 min pre 0.03–1 mg/kg i.p. 30 min pre 0.01, 0.04, 0.16, 0.63 mg/kg s.c. 60 min pre	1 1 0.16, 0.63	— # —	(Matsushita et al., 2005) (Li et al., 2006) (Bruins Slot et al., 2008)
SLV-313	D ₂ antagonist/HT _{1A} agonist	1 mg/kg/day s.c. 21 days 0.01, 0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	1 2.5	# #	(Torres-Lista et al., 2015) (Bruins Slot et al., 2008)
Thiordazine	D ₂ /D ₃ antagonist	4.6, 10, 14, 46 mg/kg s.c. 30 min pre	10–46	#	(Brockkamp et al., 1986)
Trifluoperidol	D ₂ antagonist	0.1, 0.32, 1 mg/kg s.c. 30 min pre	0.32, 1	#	(Brockkamp et al., 1986)
Trifluoperazine	D ₁ /D ₂ antagonist	0.3, 1, 3 mg/kg p.o. 30 min pre	3	/	(Egashira et al., 2018)
Drugs targeting the adrenergic system					
Clonidine	α ₂ agonist; imidazoline I ₁ agonist	0.01, 0.03, 0.3 mg/kg i.p. 30 min pre 15, 30, 60 mg/kg i.p. 30 min pre	0.03, 0.3 60	— #	(Young et al., 2006) (Dixit et al., 2014)
d-Amphetamine	Monoamine releaser/reuptake inhibitor* Also affects DA, 5-HT	0.1–10 mg/kg i.p. 10 min pre 0.1, 0.3, 1, 3 mg/kg i.p. 30 min pre 0.5, 1, 5 mg/kg i.p.	1–10 1, 3 1.5	+ + +	(Li et al., 2006) (Nicolas et al., 2006) (Jimenez-Gomez et al., 2011)
Desipramine	Noradrenalin reuptake inhibitor	3–30 mg/kg i.p. 30 min pre 10 mg/kg i.p. 4 h pre	30 10	# —	(Li et al., 2006) (Schneider & Popik, 2007)
Methamphetamine	Monoamine releaser/reuptake inhibitor* Also affects DA, 5-HT	0.5, 2.7, 4.8 μmol/kg i.p. 30 min pre	2.7, 4.8	/	(Saadat et al., 2006)
Methylphenidate	Monoamine releaser/reuptake inhibitor* Also affects DA, 5-HT	9.3, 18.5, 37 μmol/kg i.p. 30 min pre	37	/	(Saadat et al., 2006)
Mianserin	Noradrenalin reuptake inhibitor; anti-serotonergic	3.2, 10, 32 mg/kg s.c. 30 min pre	10, 32	#	(Brockkamp et al., 1986)
Milnacipran	Serotonin-noradrenalin reuptake inhibitor	5, 10, 20 mg/kg i.p. 30 min pre 20 mg/kg s.c. 30 min pre	10, 20 20	/ /	(Sugimoto et al., 2007) (Honda et al., 2011)
Nisoxetine	Noradrenalin reuptake inhibitor	3–30 mg/kg i.p. 30 min pre	30	#	(Li et al., 2006)
Nomifensine	Noradrenalin reuptake inhibitor	10 mg/kg i.p. 4 h pre	10	+	(Schneider & Popik, 2007)
para-chloroamphetamine (PCA)	Monoamine releaser/reuptake inhibitor* Also affects DA, 5-HT	0.0025, 0.01, 0.04, 0.16, 0.63, 2.5 mg/kg 15 mg/kg 2 doses 6 hours apart (neurotoxic dose, day 28 post) 2.4, 3.9, 4.8, 9.7 μmol/kg i.p. 30 min pre	2.5 15 4.8–9.7	# # /	(Bruins Slot et al., 2008) (Saadat et al., 2006) (Saadat et al., 2006)
Prazosin	α ₁ antagonist	0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	2.5	#	(Bruins Slot et al., 2008)
TDIQ	α ₂ partial agonist	0.03, 0.3, 1, 3, 10, 17 mg/kg i.p. 30 min pre	1, 3, 10, 17	/	(Young et al., 2006)
Venlafaxine	Serotonin-noradrenalin reuptake inhibitor	10–60 mg/kg i.p. 30 min pre	30–60	#	(Li et al., 2006)
Yohimbine	α ₂ antagonist; anxiogenic	1, 5, 10 mg/kg i.p. 30 min pre 0.3, 1, 3, 10 mg/kg i.p. 30 min pre	5, 10 3, 10	— —	(Njung'e & Handley, 1991b) (Nicolas et al., 2006)
Drugs targeting the GABAergic system					
(+)-limonene epoxide	GABA potentiator	25, 50, 75 mg/kg p.o. 30 min pre 25, 50, 75 mg/kg/day p.o. 14 days	25, 50, 75 25, 50, 75	# #	(De Almeida et al., 2014) (De Almeida et al., 2014)
Allopregnanolone	GABA _A potentiator	0.1, 0.2, 0.5, 1 μg/kg i.c.v. 15 min pre	1	/	(Umathe et al., 2009)
Alprazolam	Benzodiazepine receptor agonist	0.03, 0.1, 0.3, 1 mg/kg i.p. 30 min pre	0.3, 1	—	(Nicolas et al., 2006)
Baclofen	GABA _B agonist	3, 10 mg/kg i.p. 30 min pre	10	—	(Egashira et al., 2013)
βCCM (anxiogenic)	Benzodiazepine receptor inverse agonist	0.1, 1 mg/kg i.p. 0 min pre	1	—	(Jimenez-Gomez et al., 2011)
Chlordiazepoxide	Benzodiazepine receptor agonist	4.6, 10, 22, 46 mg/kg s.c. 30 min pre 3–30 mg/kg i.p. 30 min pre 10, 30, 100 mg/kg i.p. 30 min pre	10, 22 30 30, 100	# # —	(Brockkamp et al., 1986) (Li et al., 2006) (Nicolas et al., 2006)
Clonazepam	Benzodiazepine receptor agonist	0.22, 0.46, 1 mg/kg s.c. 30 min pre	0.22–1	#	(Brockkamp et al., 1986)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
Diazepam	Benzodiazepine receptor agonist	0.46, 1, 2.2, 4.6, 10 mg/kg s.c.	2.2, 4.6, 10	#	(Broekkamp et al., 1986)
		30 min pre			
		0.05, 0.1, 0.25, 1, 5 mg/kg i.p.	1, 5	+	(Njung'e & Handley, 1991b)
		30 min pre			
		1, 3, 10 mg/kg p.o. 60 min pre	10	/	(Ichimaru et al., 1995)
		0.25–32 mg/kg p.o. 60 min pre	4, 8	—	(Abe et al., 1998)
		1, 3, 10 mg/kg i.p. 30 min pre	3, 10	/	(Nicolas et al., 2006)
		0.03, 0.3, 1, 3, 10, 30 mg/kg i.p.	3, 10, 30	—	(Young et al., 2006)
		30 min pre			
		2 mg/kg i.p. 1 h pre	2	—	(Schneider & Popik, 2007)
		0.16, 0.63, 2.5, 10 mg/kg s.c. 60 min pre	2.5, 10	+	(Bruins Slot et al., 2008)
		2.5 mg/kg i.p. 30 min pre	2.5	/	(Casarotto et al., 2010)
		0.5, 1, 5 mg/kg i.p.	1, 5	/	(Jimenez-Gomez et al., 2011)
		0.3, 1, 3 mg/kg i.p. 60 min pre	1, 3	—	(Kinsey et al., 2011)
		0.5, 2 mg/kg i.p. 30 min pre	2	?	(Umathe et al., 2012)
Ethanol	GABA potentiator; NMDA receptor antagonist	2 mg/kg p.o. 30 min pre	2	#	(De Almeida et al., 2014)
		2 mg/kg/day p.o. 14 days	2	#	(De Almeida et al., 2014)
		2 mg/kg s.c. 11 days	2	/	(Taylor et al., 2017)
		460, 1000, 2200, 3200 mg/kg s.c. 30 min pre	1,100–3,200	#	(Broekkamp et al., 1986)
		Withdrawal from addiction schedule	—	—	(Umathe et al., 2008)
Flunitrazepam	Benzodiazepine receptor agonist	0.1, 1.2, 2.4, 4.8% w/v solution i.p.	1.2, 2.4, 4.8	/	(Gaikwad & Parle, 2011)
		30 min pre			
Flunitrazepam	Benzodiazepine receptor agonist	0.046, 0.1, 0.46 mg/kg s.c. 30 min pre	0.1, 0.46	#	(Broekkamp et al., 1986)
Meprobamate	GABA receptor potentiator	22, 46, 100, 150, 220 mg/kg s.c.	150, 220	#	(Broekkamp et al., 1986)
		30 min pre			
Muscimol	GABA _A agonist	0.3, 1 mg/kg i.p. 40 min pre	1	/	(Egashira et al., 2013)
Tiagabine	GABA reuptake inhibitor	1, 3, 10 mg/kg i.p. 30 min pre	3, 10	—	(Nicolas et al., 2006)
Drugs targeting the glutamatergic system					
CX-546	AMPA receptor potentiator	3, 10, 30 mg/kg s.c. 30 min pre	30	/	(Iijima et al., 2010)
Fenobam	mGluR ₅ antagonist	30, 100, 300 mg/kg i.p. 30 min pre	300	+	(Nicolas et al., 2006)
LY341495	mGluR ₂ antagonist	1, 3, 10 mg/kg i.p. 60 min pre	1, 3, 10	#	(Shimazaki et al., 2004)
Memantine	NMDA antagonist	1, 3, 10 mg/kg i.p. 30 min pre test	10	/	(Egashira, Okuno, Harada, et al., 2008)
MGS0039	mGluR _{2/3} antagonist	1, 3, 10 mg/kg i.p. 60 min pre	3, 10	/	(Shimazaki et al., 2004)
MK-801	NMDA antagonist	0.03, 0.1, 0.3 mg/kg i.p. 30 min pre test	0.3	+	(Egashira, Okuno, Harada, et al., 2008)
MTEP	mGluR ₅ antagonist	1, 3, 10, 30 mg/kg i.p. 30 min pre	30	/	(Nicolas et al., 2006)
Ro 25-6981	NMDA antagonist	0.3, 1, 3 mg/kg s.c. 30 min pre	3	/	(Iijima et al., 2010)
Drugs targeting the endocannabinoid system					
AM404	Endocannabinoid reuptake inhibitor	0.3, 1, 3 mg/kg i.p. 30 min pre	0.3, 1, 3	/	(Gomes et al., 2011)
		0.05, 0.1, 1, 5, 10, 20 µg/mouse i.c.v.	0.1, 1, 5	/	(Umathe et al., 2012)
		2 min pre			
Anandamide	Endocannabinoid CB ₁ agonist	0.1, 0.5, 1, 5, 10, 20, 40 µg/mouse i.c.v.	1, 5, 10	/	(Umathe et al., 2012)
		2 min pre			
Anandamide + AM404	Endocannabinoid CB ₁ agonist	0.5 µg/mouse + 0.05 µg/mouse i.c.v. (sub-effective doses)	0.5 + 0.05	/	(Umathe et al., 2012)
Anandamide + URB597	Endocannabinoid CB ₁ agonist	0.5 µg/mouse + 0.01 µg/mouse i.c.v. (sub-effective doses)	0.5 + 0.01	/	(Umathe et al., 2012)
Cannabidiol	CB ₁ antagonist	5, 15, 30, 60 mg/kg i.p. 30 min pre	15, 30, 60	/	(Casarotto et al., 2010)
		30 mg/kg/day 7 days (chronic)	30	#	(Casarotto et al., 2010)
		5, 15, 30 mg/kg i.p. 30 min pre	30	#	(Nardo et al., 2014)
Cannabidiol + Fluoxetine	CB ₁ antagonist + SSRI	3 mg/kg + 15 mg/kg i.p. 30 min pre (sub-effective doses)	3 + 15	#	(Nardo et al., 2014)
Capsazepine	TRPV1 antagonist	0.1, 1, 10, 100 µg/mouse i.c.v. 2 min pre	100	/	(Umathe et al., 2012)
JZL184	MAGL inhibitor; cannabinoid potentiator	4, 16, 40 mg/kg i.p. 2 h pre	16, 40	—	(Kinsey et al., 2011)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
PF-3845	FAAH Inhibitor; cannabinoid potentiator	1, 3, 10 mg/kg i.p. 2h pre	10	/	(Kinsey et al., 2011)
Tetrahydrocannabinol (THC)	Cannabinoid CB ₁ agonist	0.03, 0.1, 0.3, 1, 3, 10, 30 mg/kg i.p. 60 min pre	0.3, 10, 30	–	(Kinsey et al., 2011)
URB597	FAAH inhibitor; cannabinoid potentiator	0.1, 0.3, 1 mg/kg i.p. 30 min pre 0.01, 0.05, 0.1, 1, 5, 10, 20 µg/mouse i.c.v. 2 min pre	0.1, 0.3, 1 0.05–5	/	(Gomes et al., 2011) (Umathe et al., 2012)
WIN 55,212-2	Cannabinoid CB ₁ agonist	0.3, 1, 3 mg/kg i.p. 30 min pre	1, 3	–	(Gomes et al., 2011)
Drugs targeting miscellaneous targets					
(+)-SKF 10047	Sigma σ_1 agonist	3, 10 mg/kg i.p. 30 min pre	10	/	(Egashira, Harada, et al., 2007)
2-BFI	Imidazoline I ₂ agonist	5, 10, 15 mg/kg i.p. 30 min pre	15	#	(Dixit et al., 2014)
7-Nitroindazole (7-NI)	Nitric oxide synthase inhibitor	20, 50 mg/kg i.p. 30 min pre 10, 20, 40 mg/kg i.p. 30 min pre	50 20, 40	– /	(Krass et al., 2010) (Gawali et al., 2016)
Agmatine	Imidazoline receptor agonist; Nitric oxide synthesis inhibitor	10, 50 mg/kg 30 i.p. min pre 10, 20, 40 mg/kg i.p. 30 min pre 10, 20, 40 mg/kg i.p. 30 min pre	10, 50 20, 40 20, 40	/	(Krass et al., 2010) (Dixit et al., 2014)
Agmatine + clonidine	Imidazoline I ₁ / α_2 agonist	10 mg/kg + 30 mg/kg (subeffective doses)	10 + 30	#	(Dixit et al., 2014)
Agmatine + moxonidine	Imidazoline I ₁ / α_2 agonist	10 mg/kg + 0.25 mg/kg (subeffective doses)	10 + 0.25	#	(Dixit et al., 2014)
Agmatine + 2-BFI	Imidazoline I ₂ agonist	10 mg/kg + 10 mg/kg (subeffective doses)	10 + 10	#	(Dixit et al., 2014)
Agmatine + 7-NI	Nitric oxide synthesis inhibitor	10 mg/kg + 10 mg/kg (subeffective doses)	10 + 10	/	(Gawali et al., 2016)
Amlodipine	Calcium channel blocker	3, 10 mg/kg i.p. 30 min pre	10	/	(Egashira, Okuno, Abe, et al., 2008)
Ascorbic acid	Dietary supplement	250 mg/kg p.o. 30 min pre 250 mg/kg/day p.o. 14 days	250 250	#	(De Almeida et al., 2014) (De Almeida et al., 2014)
Atropine	Anticholinergic	0.46, 1, 2.2, 4.6, 10 mg/kg s.c. 30 min pre	1–4.6	#	(Broekkamp et al., 1986)
Carbamazepine	Anti-Epileptic	1, 3, 10 mg/kg i.p. 30 min pre 10, 100, 300 mg/kg p.o. 60 min pre	10 200, 300	/	(Nicolas et al., 2006) (Egashira et al., 2013)
Chlorpromazine	Antihistamine	0.32, 1, 3.2 mg/kg s.c. 30 min pre 1.25, 2.5, 5, 10 mg/kg p.o. 60 min pre 1–10 mg/kg i.p. 30 min pre	1, 3.2 10 10	#	(Broekkamp et al., 1986) (Abe et al., 1998) (Li et al., 2006)
Chlorpyrifos (Organophosphate)	Acetylcholine esterase inhibitor	0.3, 1, 3 mg/kg i.p. 30 min pre 1 mg/kg/day i.p. 5 days	3 1	– /	(Nicolas et al., 2006) (Savy et al., 2015)
Cilnidipine	Calcium channel blocker	3, 10 mg/kg i.p. 30 min pre	10	/	(Egashira, Okuno, Abe, et al., 2008)
Cyproheptadine	Antihistamine	1, 2, 5 mg/kg i.p. 30 min pre	5	–	(Njung'e & Handley, 1991a)
Diazinon	Acetylcholine esterase inhibitor	1 mg/kg/day i.p. 5 days	1	/	(Savy et al., 2015)
Diphenhydramine	Acetylcholine esterase inhibitor	2.2, 6.8, 22 mg/kg s.c. 30 min pre	22	#	(Broekkamp et al., 1986)
<i>Dolichandrone falcata</i> ethyl acetate extract	Novel plant extract	100, 200, 400 mg/kg p.o. 60 min pre	200, 400	#	(Badgujar & Surana, 2010)
<i>Dolichandrone falcata</i> TLC isolated compounds	Novel plant extract	50, 100, 200 mg/kg p.o. 60 min pre	50, 100, 200	#	(Badgujar & Surana, 2010)
Flunarizine	Calcium channel blocker	3, 10, 30 mg/kg i.p. 30 min pre	30	/	(Egashira, Okuno, Abe, et al., 2008)
GR205,171	Neurokinin NK ₁ receptor antagonist	0.63, 2.5, 10, 40 mg/kg i.p. 30 min pre	10, 40	–	(Millan et al., 2002)
HECE (<i>C. esculenta</i> extract)	Novel plant extract	25, 50 mg/kg i.p. 30 min pre	25, 50	#	(Kalariya et al., 2015)
<i>Hypericum perforatum</i> extract	Novel plant extract	150, 300 mg/kg p.o. 60 min pre	150, 300	/	(Skalisz et al., 2004)
Lamotrigine (ddY mice)	Anti-epileptic; sodium channel blocker	3, 10, 30 mg/kg i.p. 30 min pre	30	/	(Egashira et al., 2013)
<i>Lagenaria siceraria</i> extract	Novel plant extract	25, 50 mg/kg i.p. 30 min pre	25, 50	#	(Prajapati et al., 2011)
L-NAME	Neuronal nitric oxide synthase inhibitor	15, 30, 50 mg/kg 30 min pre	30, 50	/	(Gawali et al., 2016)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
L-NAME + Agmatine	Neuronal nitric oxide synthase inhibitor	15 mg/kg + 10 mg/kg (sub-effective doses)	15 + 10	/	(Gawali et al., 2016)
Leuprolide	Gonadotropin releasing hormone analogue	50, 100, 300, 600 µg/kg s.c. 30 min pre (acute)	100–600	/	(Uday et al., 2007)
		50, 100, 300, 600 µg/kg s.c. 30 min pre (acute)	100–600	/	(Umathe et al., 2008)
		25, 50 µg/kg s.c. twice daily 7 days (chronic)	50	/	(Umathe et al., 2008)
		100, 200, 300 µg/kg s.c. 30 min pre	200, 300	/	(Gaikwad et al., 2010)
Leuprolide + Fluoxetine	Gonadotropin releasing hormone analogue + SSRI	50 µg/kg s.c. & 5 mg/kg i.p. 30 min pre (sub-effective doses)	50 + 5	/	(Uday et al., 2007)
MCH (Melanin concentrating hormone)	Diverse hormone	i.c.v. 2 min pre	-	/	(Sanathara et al., 2018)
Metergoline	Dopamine agonist	0.1, 0.25, 1	1	–	(Njung'e & Handley, 1991a)
Morphine	Opioid analgesic	0.3, 1, 3, 10 mg/kg i.p. 30 min pre	10	+	(Nicolas et al., 2006)
Moxonidine	Imidazoline I ₂ receptor agonist	0.25, 0.5, 1 mg/kg i.p. 30 min pre	0.5, 1	#	(Dixit et al., 2014)
N-acetyl-L-cysteine (NAC)	Anti-oxidant; mucolytic	50, 100, 150 mg/kg i.p. 30 min pre	150	/	(Egashira, Shirakawa, et al., 2012)
Nilvadipine	Calcium channel blocker	1, 3, 10 mg/kg i.p. 30 min pre	3, 10	/	(Egashira, Okuno, Abe, et al., 2008)
Oxytocin	Hormone	i.c.v. 2 min pre	-	/	(Sanathara et al., 2018)
Phenelzine	Monoamine oxidase inhibitor	3, 10, 30, 100 mg/kg i.p. 30 min pre	100	/	(Nicolas et al., 2006)
Progesterone	Sex hormone	1 mg/kg s.c. 4h pre	1	–	(Schneider & Popik, 2007)
		4 mg/kg s.c. 3h pre	4	+	(Llaneza & Frye, 2009)
		5, 10, 20 mg/kg s.c. 60 min pre	20	/	(Umathe et al., 2009)
Progesterone + 17β-Estradiol	Sex hormone	4 mg/kg & 10 µg/rat	4 + 10	+	(Llanca & Frye, 2009)
PRE-084	Sigma σ ₁ agonist	10, 30, 60 mg/kg i.p. 30 min pre	60	/	(Egashira, Harada, et al., 2007)
Ro64-6198	Nociceptin opioid peptide receptor agonist	0.1, 0.3, 1, 3 mg/kg i.p. 30 min pre	1, 3	–	(Nicolas et al., 2006)
RP67,580	Neurokinin NK ₁ receptor antagonist	0.63, 2.5, 10, 40 mg/kg s.c. 30 min pre	10, 40	–	(Millan et al., 2002)
Scopolamine	Anticholinergic	0.046, 0.1, 0.22, 0.46, 1 mg/kg s.c. 30 min pre	0.046–1	#	(Broekkamp et al., 1986)
Tipecidine	GIRK inhibitor	5, 10, 20 mg/kg s.c. 30 min pre	5, 10, 20	+	(Honda et al., 2011)
TRIM	Nitric oxide synthase inhibitor	25, 50 mg/kg i.p. 30 min pre	25, 50	/	(Krass et al., 2010)
Valproate	Anti-epileptic; GABA potentiator	10, 30, 100 mg/kg i.p. 30 min pre	10, 30, 100	/	(Egashira et al., 2013)
<i>Withania somnifera</i> extract	Novel plant extract	10, 20, 40 mg/kg p.o. 12 days	20, 40	#	(Dey et al., 2016)

ED: effective dose; LMA: locomotor activity (/ no effect; # not measured/reported; + increased; – decreased)

NP-Y, TPH2, VDR, and V1a receptors (Angoa-Pérez et al., 2013; Egashira, Tanoue, et al., 2007; Gavioli et al., 2007; Lähdesmäki et al., 2002; Tasan et al., 2009). Taking this into account, it therefore must be considered that if marble-burying activity is triggered and driven by a possible anxiogenic and/or OC-like construct, the test itself as applied in the preclinical literature has shown poor predictive validity as a screening test for either behavior. Indeed, neither clinical anxiety nor OCD has demonstrated a response to many of the pharmacological agents listed in Table 1. Furthermore, even if it can be argued that preclinical experimental investigations may suffice only to contribute to our understanding of disease mechanisms, and that the answers obtained from such investigations do not have to

contribute to clinical treatment strategies, the findings reported in Tables 1, 2, and 3 are, in retrospect, also of little translational value. In this section, we will address this dilemma from a translational perspective.

Serotonergic drugs in studies of marble-burying behavior

Serotonin reuptake inhibitors Drugs targeting the serotonergic systems have, given their widespread application in OCD, anxiety, and other stress-related disorders (Bandelow et al., 2012; Bandelow et al., 2008; Dougherty et al., 2004), been

Table 2 Drugs exacerbating MBA/Blocking otherwise effective agents

Drug	Mechanism of action	Dose range	ED	LMA	Reference
Drugs targeting the serotonergic system					
ICI 169,369 PT to DOI	5-HT ₂ antagonist	10 mg/kg i.p. 40 min pre	10	/	(Njung'e & Handley, 1991a)
Ketanserin PT to trifluoperazine	5-HT ₂ antagonist	0.3 mg/kg i.p.	0.3	/	(Egashira et al., 2018)
mCPP	Non-specific 5-HT agonist	0.1, 0.3, 1 mg/kg i.p. 30 min pre	0.1, 0.3	/	(Nardo et al., 2014)
NAN-190 PT to fluvoxamine	5-HT _{1A} antagonist	0.3 mg/kg s.c. 30 min pre	0.3	#	(Ichimaru et al., 1995)
PCPA PT to leuprolide; fluoxetine	5-HT synthesis inhibitor	300 µg/kg i.p. 3 days	300	/	(Uday et al., 2007)
Ritanserin PT to leuprolide	5-HT _{2A/C} antagonist	20 mg/kg i.p. 30 min pre	20	/	(Gaikwad et al., 2010)
Ritanserin PT to DOI		0.2, 0.5, 1 mg/kg i.p. 40 min pre	0.5, 1	/	(Njung'e & Handley, 1991a)
SB-242084 PT to DOI	5-HT _{2C} antagonist	3 mg/kg i.p. 30 min pre	3	/	(Egashira, Okuno, et al., 2012)
SB-242084 PT to fluvoxamine; paroxetine		1 mg/kg i.p. 30 min pre	1	+	(Egashira, Okuno, et al., 2012)
SB-242084 PT to WAY-161503		3 mg/kg i.p. 30 min pre	3	+	(Egashira, Okuno, et al., 2012)
WAY-100135 PT to 8-OH-DPAT; perospirone	5-HT _{1A} antagonist	10 mg/kg i.p. 30 min	10	#	(Matsushita et al., 2005)
WAY-100635 PT to fluvoxamine	5-HT _{1A} antagonist	0.1, 1 mg/kg i.p. 60 min pre	1	/	(Ikarasawa et al., 2006)
WAY-100635 PT to 8-OH-DPAT		3 mg/kg i.p. 30 min pre	3	/	(Egashira, Okuno, Matsushita, et al., 2008)
WAY-100635 PT to paroxetine		3 mg/kg i.p. 30 min pre	3	#	(Casarotto et al., 2010)
WAY-100635 PT to tipecidine		3 mg/kg s.c. 60 min pre	3	+	(Honda et al., 2011)
Drugs targeting the dopaminergic system					
Raclopride PT to tipecidine	D ₂ antagonist	0.4 mg/kg s.c. 60 min pre	0.4	/	(Honda et al., 2011)
Quinpirole PT to trifluoperazine	D ₂ agonist	0.03 mg/kg i.p.	0.03	/	(Egashira et al., 2018)
Drugs targeting the adrenergic system					
Efaroxan PT to agmatine	α ₂ antagonist; imidazoline receptor antagonist	2 mg/kg i.p. 30 min pre	2	#	(Dixit et al., 2014)
Idazoxan PT to agmatine	α ₂ antagonist; imidazoline receptor antagonist	1 mg/kg i.p. 30 min pre	1	#	(Dixit et al., 2014)
Drugs targeting the GABAergic system					
Bicuculline PT to muscimol, valproate	GABA _A receptor antagonist	3 mg/kg 30 min pre	3	/	(Egashira et al., 2013)
Flumazenil PT to (+)-limonene epoxide; diazepam	Benzodiazepine receptor antagonist	5 mg/kg p.o. 60 min pre	5	#	(De Almeida et al., 2014)
Flumazenil PT to (+)-limonene epoxide; diazepam		5 mg/kg/day p.o. 14 days	5	#	(De Almeida et al., 2014)
Drugs targeting the glutamatergic system					
LY354740 PT to MGS0039	mGluR _{2/3} agonist	0.3, 1, 3 mg/kg i.p. 30 min pre	3	#	(Shimazaki et al., 2004)
Drugs targeting the endocannabinoid system					
AM-251 PT to cannabidiol	CB ₁ antagonist	1 mg/kg i.p. 30 min pre	1	#	(Casarotto et al., 2010)
AM-251 PT to WIN 55,212-2; URB597		1 mg/kg i.p. 60 min pre	1	/	(Gomes et al., 2011)
AM-251 PT to AN, AM404; URB597		1 µg/mouse i.c.v. 2 min pre	1	/	(Umathe et al., 2012)
AM404	Endocannabinoid reuptake inhibitor	0.05, 0.1, 1, 5, 10, 20 µg/mouse i.c.v. 2 min pre	20	/	(Umathe et al., 2012)
Anandamide	Endocannabinoid CB ₁ agonist	0.1, 0.5, 1, 5, 10, 20, 40 µg/mouse i.c.v. 2 min pre	40	/	(Umathe et al., 2012)
Capsaicin	TRPV1 agonist	0.1, 1, 10, 100 µg/mouse i.c.v. 2 min pre	100	/	(Umathe et al., 2012)
Rimonabant PT to PF-3845; JZL184	CB ₁ antagonist	0.3 mg/kg i.p. 10 min pre drug	0.3	/	(Kinsey et al., 2011)
URB597	FAAH inhibitor; cannabinoid potentiator	0.01, 0.05, 0.1, 1, 5, 10, 20 µg/mouse i.c.v. 2 min pre	20	/	(Umathe et al., 2012)
Drugs targeting miscellaneous systems					
BD 1047 PT to fluvoxamine	Sigma σ _{1/2} antagonist	1, 3, 10 mg/kg i.p. 30 min pre	3	/	(Egashira, Harada, et al., 2007)
BD 1063 PT to fluvoxamine	Selective sigma σ ₁ antagonist	0.3, 1 mg/kg 30 min pre	1	/	(Egashira, Harada, et al., 2007)
Cyproheptadine PT to DOI	Antihistamine	2 mg/kg i.p. 40 min pre	2	/	(Njung'e & Handley, 1991a)
DHAS (dehydroisoandrosterone 3-sulphate)	Neurosteroid	1, 2.5, 5 mg/kg i.p. 30 min pre	5	/	(Umathe et al., 2009)
GW803430 PT to oxytocin and MCH	Melanin concentrating hormone antagonist	3 mg/kg i.p. 30 min pre	3	/	(Sanathara et al., 2018)
L-arginine	Dietary supplement; nitric oxide precursor	200, 400, 800 mg/kg i.p. 30 min pre	800	/	(Gawali et al., 2016)
L-arginine 500 mg/kg PT to paroxetine; citalopram	Dietary supplement; nitric oxide precursor	500 mg/kg i.p. 10 min pre	500	#	(Krass et al., 2010)
LHRH antagonist, PT to leuprolide; fluoxetine	Sex hormone modulator	2.5 µg/mouse	2.5	/	(Uday et al., 2007)

ED: effective dose; LMA: locomotor activity; PT: pretreatment (/ no effect; # not measured/reported; + increased; - decreased)

Table 3 Drugs ineffective in the MBT

Drug	Mechanism of action	Dose range	LMA	Reference
Drugs targeting the serotonergic system				
Buspirone	5-HT _{1A} agonist	0.3–10 mg/kg i.p. 30 min pre 0.01–2.5 mg/kg s.c. 60 min pre	# #	(Li et al., 2006) (Bruins Slot et al., 2008)
Desipramine	Noradrenalin reuptake inhibitor	15, 30, 60 mg/kg p.o. 60 min pre 1, 3, 10, 30 mg/kg i.p. 30 min pre	# /	(Ichimaru et al., 1995) (Nicolas et al., 2006)
Escitalopram	Selective serotonin reuptake inhibitor	50 mg/kg/day p.o. 28 days (chronic)	—	(Wolmarans et al., 2016)
ICI 169,369	5-HT ₂ antagonist	1, 5, 10 mg/kg i.p. 30 min pre	/	(Njung'e & Handley, 1991a)
ICS 205-930	5-HT ₃ antagonist	0.1, 1, 10 mg/kg i.p. 30 min pre	#	(Njung'e & Handley, 1991a)
Ipsapirone	5-HT _{1A} partial agonist	5, 10, 20 mg/kg i.p. 30 min pre	/	(Njung'e & Handley, 1991a)
Ketanserin	5-HT ₂ antagonist	3 mg/kg i.p. 30 min pre 0.3 mg/kg i.p. 60 min pre	/ /	(Egashira, Okuno, Matsushita, et al., 2008) (Egashira et al., 2018)
LY266097	5-HT _{2B} antagonist	0.16–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
MDL100907	5-HT _{2A} receptor antagonist	—	#	(Bruins Slot et al., 2008)
MDMA	5-HT reuptake inhibitor	25 mg/kg 3 doses 3 hourly i.p. (neurotoxic dose)	#	(Saadat et al., 2006)
Metergoline	5-HT _{1/2} antagonist	0.25 mg/kg i.p. 30 min pre	/	(Njung'e & Handley, 1991a)
Ondansetron	5-HT ₃ antagonist	0.01, 0.1, 1 mg/kg i.p. 30 min pre	#	(Njung'e & Handley, 1991a)
Ritanserin	5-HT _{2A/C} antagonist	1, 2, 20 mg/kg i.p. 30 min pre	/	(Gaikwad et al., 2010)
RS127445	5-HT _{2B} antagonist	0.0025–10 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
SB-215505	5-HT _{2B} antagonist	0.16–10 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
SB242084	5-HT _{2C} receptor antagonist	0.01–2.5 mg/kg i.p. 60 min pre 1, 3 mg/kg i.p. 30 min pre	# /	(Bruins Slot et al., 2008) (Egashira, Okuno, et al., 2012)
S15535	5-HT _{1A} partial agonist	0.04–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
WAY-100635	5-HT _{1A} antagonist	1 mg/kg i.p. 60 min pre 0.04–10 mg/kg i.p. 60 min pre 3 mg/kg s.c. 60 min pre	/ # /	(Harasawa et al., 2006) (Bruins Slot et al., 2008) (Honda et al., 2011)
Drugs targeting the dopaminergic system				
ABT-724	D ₄ agonist	0.63–40 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
BP897	D ₃ partial agonist	0.04–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
F-15063	D ₂ /D ₃ antagonist, D ₄ agonist; 5-HT _{1A} partial agonist	0.01–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
L745870	D ₄ antagonist	0.01–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
Quinpirole	D _{2/3} agonist	0.03 mg/kg i.p. 60 min pre	/	(Egashira et al., 2018)
Raclopride	D ₂ antagonist	0.4 mg/kg s.c. 60 min pre	/	(Honda et al., 2011)
Ro10-5824	D ₄ partial agonist	2.5–40 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
Sarizotan	5-HT _{1A} agonist; D ₂ antagonist	0.16, 0.63, 2.5, 10 mg/kg s.c. 60 min pre	#	(Bruins Slot et al., 2008)
SB-277,011-A	D ₃ antagonist	0.01–10 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
SCH23390	D ₁ antagonist	7.5 µg/mouse s.c. 60 min pre	/	(Honda et al., 2011)
SCH 23390 PT to tipepidine	D ₁ antagonist	7.5 µg/mouse s.c. 60 min pre	/	(Honda et al., 2011)
SSR181507	D ₂ antagonist/partial agonist; 5-HT _{1A} agonist	0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	#	(Bruins Slot et al., 2008)
S33084	D ₃ antagonist	0.04–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
Drugs targeting the adrenergic system				
Atipamezole	α ₂ antagonist	0.04–2.5 mg/kg s.c. 60 min pre	#	(Bruins Slot et al., 2008)
Pindolol	Beta blocker; 5-HT _{1A} antagonist	5, 10 mg/kg i.p. 30 min pre	#	(Njung'e & Handley, 1991a)

Table 3 (continued)

Drug	Mechanism of action	Dose range	LMA	Reference
Drugs targeting the GABAergic system				
β CCE (β -carboline)	Benzodiazepine receptor inverse agonist	1, 5 mg/kg i.p. 30 min pre	/	(Njung'e & Handley, 1991b)
Bicuculline	GABA _A antagonist	3 mg/kg i.p. 30 min pre	/	(Egashira et al., 2013)
Bretazenil	GABA _A receptor modulator	1–100 mg/kg i.p. 30 min pre	#	(Li et al., 2006)
FG-7142 (β -carboline)	Benzodiazepine receptor inverse agonist	1, 3, 10, 30 mg/kg i.p. 30 min pre	+	(Nicolas et al., 2006)
Flumazenil	Benzodiazepine receptor antagonist	5 mg/kg p.o. 30 min pre	#	(De Almeida et al., 2014)
Pentobarbital	Barbiturate; GABA _A potentiator	3–17 mg/kg i.p. 15 min pre	#	(Li et al., 2006)
Drugs targeting the glutamatergic system				
LY354740	mGluR _{2/3} agonist	0.3, 1, 3 mg/kg i.p. 30 min pre	#	(Shimazaki et al., 2004)
NBQX	AMPA antagonist	0.1, 0.3, 1 mg/kg i.p. 30 min pre	–	(Egashira, Okuno, Harada, et al., 2008)
Riluzole	Glutamate release inhibitor	0.3, 1, 10 mg/kg i.p. 30 min pre	–	(Egashira, Okuno, Harada, et al., 2008)
Drugs targeting miscellaneous systems				
17 β -Estradiol	Sex hormone	10 μ g/rat 44–48 h pre	+	(Llancza & Fryc, 2009)
α -tocopherol	Dietary supplement	10, 30, 100 mg/kg p.o. 60 min pre	/	(Egashira, Shirakawa, et al., 2012)
Chlorpromazine	Antihistamine	2 mg/kg i.p. 1h pre	/	(Schneider & Popik, 2007)
Efaroxan	Imidazoline I ₁ /I ₂ antagonist	0.5, 1, 2 mg/kg i.p. 30 min pre	#	(Dixit et al., 2014)
Finasteride	5 α -reductase inhibitor	50 mg/kg i.p. 90 min pre	/	(Umathe et al., 2009)
GR226206	Neurokinin NK ₁ antagonist	0.63, 2.5, 10, 40 mg/kg i.p. 30 min pre	/	(Millan et al., 2002)
Hypericum perforatum extract	Novel plant extract	150, 300 mg/kg/day p.o. 21 days	/	(Skalisz et al., 2004)
Idazoxan	Imidazoline I ₁ /I ₂ antagonist	0.25, 0.5, 1 mg/kg i.p. 30 min pre	#	(Dixit et al., 2014)
L-arginine	Dietary supplement; Nitric oxide precursor	500 mg/kg i.p. 10 min pre	?	(Krass et al., 2010)
Lamotrigine (ICR mice)	Anti-epileptic	3, 10, 30 mg/kg i.p. 30 min pre	/	(Egashira et al., 2013)
Lithium (ICR mice)	Mood stabilizer	10, 30, 100 mg/kg i.p. 30 min pre	/	(Egashira et al., 2013)
Pregabalin	Anti-epileptic; calcium channel inhibitor	3, 10, 30, 100 mg/kg i.p. 30 min pre	–	(Nicolas et al., 2006)
Pyrimilamine	Antihistamine	0.04–2.5 mg/kg s.c. 60 min pre	#	(Bruins Slot et al., 2008)
SM-21	Selective sigma σ_2 antagonist	0.3, 1, 10 mg/kg i.p. 30 min pre	+	(Egashira, Harada, et al., 2007)

LMA: locomotor activity (/ no effect; # not measured/reported; + increased; – decreased)

well represented in the MBT. SSRI treatment, acting via increased synaptic 5-HT concentrations (Goddard et al., 2008), is the first line choice for the treatment of both anxiety (Bandelow et al., 2012; Bandelow et al., 2008) and OCD (Abramowitz et al., 2009; Fineberg, 2004), and has been well studied with the MBT (Table 1). These agents include fluoxetine (Kalariya, Prajapati, Parmar, & Sheth, 2015; Prajapati, Kalaria, Karkarc, Parmar, & Sheth, 2011; Uday et al., 2007), fluvoxamine (Harasawa et al., 2006; Matsushima et al., 2009), citalopram (Krass et al., 2010; Li et al., 2006), and paroxetine (Casarotto et al., 2010; Saadat et al., 2006). Furthermore, like the 5-HT releaser fenfluramine (Njung'e & Handley, 1991a),

SSRIs have generally been demonstrated to attenuate burying activity without affecting LMA. Drugs that deplete stores of 5-HT, such as para-chlorophenylalanine (PCPA), also negate the efficacy of fluoxetine (Uday et al., 2007). Further investigations into the function of SSRIs in this context have revealed that blockade of 5-HT_{1A} receptors reduces the efficacy of SSRIs (Casarotto et al., 2010; Harasawa et al., 2006; Ichimaru et al., 1995), whereas, *in most cases*, blockade of the 5-HT_{2A/C} receptors does not (Ichimaru et al., 1995; however, see Egashira et al., 2013). Nevertheless, given that 5-HT_{2A/C} blockers reversed the attenuating effects of the GnRH analogue leuprolide (Gaikwad et al., 2010) and of 1-

2,5-dimethoxy-4-iodophenyl-2-amino-propane (DOI; a potent 5-HT_{2A/C} agonist; Njung'e & Handley, 1991a) on marble-burying, etiological involvement of the 5-HT_{2A/C} receptors in MBA cannot be excluded. Sigma receptors, believed to be involved in neuroplasticity and cognitive function, have also been proposed to play a role in several affective disorders, although research is currently limited (Ishikawa & Hashimoto, 2010). In this regard, Egashira, Harada, et al. (2007) demonstrated that sigma receptor interactions may also play a role in the therapeutic response caused by SSRIs. Indeed, both the selective sigma-1 receptor antagonist BD 1063 and the non-selective sigma receptor antagonist BD 1047 reversed the attenuating effects of fluvoxamine on MBA, but not that of paroxetine. These findings suggest that unique within-class receptor-dependant mechanisms underlie the actions of fluvoxamine specifically, since activation of the sigma-1 receptor with (+)-SKF 10047 also reduced marble-burying behavior, whereas blockade of the sigma-2 receptor had no effect (Egashira, Harada, et al., 2007).

In line with the above findings, several other drug classes that also affect 5-HT reuptake have also been shown to reduce MBA. Indeed, the predominantly serotonergic tricyclic antidepressant (TCA) clomipramine (Tatsumi, Groshan, Blakely, & Richelson, 1997) reduces burying activity. However, the facts that the dominantly noradrenergic TCAs imipramine and desipramine attenuate MBA (Li et al., 2006; Schneider & Popik, 2007; although see also Ichimaru et al., 1995; Nicolas et al., 2006) and that these agents also target histamine, alpha-adrenergic, and muscarinic receptors (Owens, Morgan, Plott, & Nemeroff, 1997) undermine the predictive validity of these findings within the MBT as a screening paradigm, for both anxiety- and OC-like behavior. Indeed, a possible role for noradrenergic and dopaminergic signaling underlying MBA cannot be excluded, because a number of SNRIs—including duloxetine (Nicolas et al., 2006), milnacipran (Honda et al., 2011; Sugimoto et al., 2007), and venlafaxine (Li et al., 2006)—as well as 5-HT, NA, and DA releasers—including methamphetamine (Saadat et al., 2006), d-amphetamine (Jimenez-Gomez et al., 2011; Li et al., 2006; Nicolas et al., 2006), and methylphenidate (Saadat et al., 2006)—have also produced ameliorative effects in the MBT.

The role of specific serotonin receptors In attempting to elucidate the exact mechanisms whereby global increases in 5-HT concentrations modulate MBA, several receptor-specific approaches have been followed. In this regard, the role of the 5-HT₁- and 5-HT₂-receptor classes has been of major interest (Abe et al., 1998; Egashira, Okuno, et al., 2012; Ichimaru et al., 1995; Matsushita et al., 2005; Njung'e & Handley, 1991a).

The nonspecific serotonergic agonist meta-chlorophenylpiperazine (mCPP) has been found to reduce MBA in at least two investigations (Nicolas et al., 2006;

Njung'e & Handley, 1991a), whereas a recent report confirmed marble-burying to respond in a biphasic manner to this agent, with lower doses seemingly inducing burying behavior and higher doses resulting in attenuation (Nardo, Casarotto, Gomes, & Guimarães, 2014). Importantly, the attenuating effect of mCPP does not seem to be reversed by the co-administration of 5-HT-receptor antagonists as has been demonstrated for other 5-HT agonists—for example, DOI (Njung'e & Handley, 1991a). However, mCPP has been found to produce anxiogenic reactions in some individuals (Cornélio & Nunes-de-Souza, 2007; Fox, Hammack, & Falls, 2008) while exacerbating OC episodes in others (Khanna, John, & Reddy, 2001; Zohar, Mueller, Insel, Zohar-Kadouch, & Murphy, 1987). Taking into account the dichotomous effect of mCPP in the MBT, at least some clinical investigations have reported analogous findings with respect to its therapeutic applications in human samples (Pigott et al., 1993). In two other preclinical animal models of OCD—that is, quinpirole-induced compulsive checking and spontaneous stereotypy in the deer mouse—mCPP was also reported to modify compulsive-like behavior routines. Indeed, it reverses compulsive behaviors induced by the dopaminergic agonist quinpirole (Tucci et al., 2013), a response that seems unrelated to 5-HT_{2A/C} receptor functioning (Tucci, Dvorkin-Gheva, Johnson, Wong, & Szechtman, 2015). However, in the case of noninduced compulsive-like behaviors, both mCPP and quinpirole have been shown to attenuate compulsive-like behaviors (Korff et al., 2008). Although these findings may seem to contradict those reported by Tucci et al. (2013), it is important to note that the latter authors employed quinpirole-induced compulsive behaviors in an attempt to create an *etiologically homologous* population of test subjects, a concept that will be discussed later on.

Substantial evidence for the involvement of the 5-HT_{1A} receptor in marble-burying has been presented. Generally, findings are inconclusive, demonstrating that activation of the 5-HT_{1A} receptor either inhibits burying behavior (Abe et al., 1998; Egashira, Okuno, Matsushita, et al., 2008; Ichimaru et al., 1995; Matsushita et al., 2005; Nicolas et al., 2006; Njung'e & Handley, 1991a; Young et al., 2006), although in certain cases doing so only at doses that also reduced locomotor capability (Abe et al., 1998; Ichimaru et al., 1995; Njung'e & Handley, 1991a) or had no effect at all (Bruins Slot et al., 2008; Li et al., 2006). Possibly of more value for our understanding of the serotonergic mechanisms underlying purportedly aberrant burying activity is the finding that the selective 5-HT_{1A} antagonists WAY 100635 and WAY 100135 block 5-HT_{1A}-induced attenuation of burying behavior (Egashira, Okuno, Matsushita, et al., 2008; Matsushita et al., 2005). Interestingly, as we alluded to earlier, WAY 100635 also seems to reverse the attenuating actions of fluvoxamine (Harasawa et al., 2006) and paroxetine (Casarotto et al., 2010), implicating a specific role for the 5-

HT_{1A} receptor in the actions of the SSRIs in the MBT. That being said, a number of investigations have failed to replicate findings related to the effect of 5-HT_{1A} receptor agonists in the MBT (see, e.g., Li et al., 2006), whereas findings relating to the attenuation of burying behavior have often varied according to dose (Abe et al., 1998; Bruins Slot et al., 2008; Nicolas et al., 2006; Njung'e & Handley, 1991a; Young et al., 2006). These findings therefore cloud the overall conclusions that can be drawn from the collective literature. Moreover, the fact that 5-HT_{1A} agonists, such as 8-OH-DPAT, are used to *induce* compulsive-like behaviors in animals (Alkhatib, Dvorkin-Gheva, & Szechtman, 2013; Yadin et al., 1991), while they also result in anxiogenic effects in the EPM and SIT (Chceta, Kenny, & File, 2000), supports a conclusion that the marble-burying investigations are not in line with current theories regarding the neurobiology of anxiety and OCD. For example, in contrast to what has been shown in marble-burying investigations, although buspirone appears to be ineffective in treating OCD as a monotherapeutic intervention (Fineberg, 2004; Pigott et al., 1992), it may be of some benefit in combination with an SSRI (Pigott et al., 1992). On the other hand, buspirone has been shown to be very effective in the management of clinical anxiety (Bandelow et al., 2008; Goldberg & Finnerty, 1979; Rickels, 1990).

With respect to the 5-HT₂ receptor subclass, the 5-HT_{2A/C} agonist DOI (Egashira, Okuno, et al., 2012; Njung'e & Handley, 1991a) and the selective 5-HT_{2C} agonist WAY 161503 (Egashira, Okuno, et al., 2012) have been shown to reduce MBA. In addition, co-administration of the 5-HT_{2C} antagonist SB242084 seems also to reverse the attenuating effects of paroxetine, fluvoxamine, and WAY 161503 on MBA (Egashira, Okuno, et al., 2012). However, although the effects of the agonists DOI and WAY 161503 are antagonized by SB242084, ritanserin, and ICI 169,369 (all antagonists of 5-HT₂ receptors; Egashira, Okuno, et al., 2012; Njung'e & Handley, 1991a), the latter two drugs have also been shown to paradoxically *potentiate* the attenuating effect of the SSRIs zimeldine and fluvoxamine on marble-burying behavior (Njung'e & Handley, 1991a). Although the latter effect of ritanserin was not replicated in a later study (Ichimaru et al., 1995), both ritanserin and ICI 169,369 also inhibit marble-burying when administered as monotherapy (Bruins Slot et al., 2008; Njung'e & Handley, 1991a). In addition, Gaikwad et al. (2010) reported no effect of ritanserin administered in the same dose as that used by Njung'e and Handley (1991a; 20 mg/kg). Considering these data, and that ritanserin also reversed the seemingly beneficial effect of leuprolide (a gonadotropin-releasing hormone analogue) on burying behavior (Gaikwad et al., 2010; Uday et al., 2007; Umathe et al., 2008), the relevance of the findings relating to 5-HT₂ receptor function in the burying test is unclear.

Taking the main findings of serotonergic involvement in marble-burying together, the literature is mostly congruent in

showing that 5-HT seems to play a significant role in the neurobiology of marble-burying behavior. Indeed, SSRIs show demonstrable clinical efficacy in the treatment of both anxiety and OCD, as well as generally attenuating marble-burying behavior. However, whereas 5-HT_{2A/C} receptors have been mechanistically implicated in SSRI-induced attenuation of burying behavior in some studies (Egashira, Okuno, et al., 2012), this has not always been the case (Ichimaru et al., 1995). The collective translational value of these findings remains uncertain. Nevertheless, although the MBT as a means to understanding the role of 5-HT in such behavior has yielded inconsistent results, the test may be useful in studies of elicited behavior in which the involvement of specific serotonergic mechanisms may be investigated (Kedia & Chattarji, 2014). Furthermore, the translational relevance of the test to explain anxiolytic and anticomulsive responses following acute increases in serotonergic signaling remains poorly defined.

Dopaminergic system involvement in marble-burying

Considering the role of DA antagonists in treatment augmentation strategies for OCD (Fineberg, 2004), several compounds in this class, including amisulpiride (Bruins Slot et al., 2008), haloperidol (Broekkamp et al., 1986; Bruins Slot et al., 2008; Matsushita et al., 2005; Nicolas et al., 2006), L-741626 (Egashira, Okuno, Matsushita, et al., 2008), olanzapine (Bruins Slot et al., 2008; Egashira, Okuno, Matsushita, et al., 2008), perospirone (Matsushita et al., 2005), perphenazine (Nicolas et al., 2006), quetiapine (Egashira, Okuno, Matsushita, et al., 2008), remoxipride (Bruins Slot et al., 2008), risperidone (Bruins Slot et al., 2008; Li et al., 2006; Matsushita et al., 2005; Torres-Lista et al., 2015), aripiprazole (Gaikwad & Parle, 2011), and thioridazine (Broekkamp et al., 1986) have been investigated in the MBT, generally resulting in attenuation of burying behavior, with few exceptions (Honda et al., 2011). All of the said compounds have been administered as monotherapy in animals demonstrating inherent, nonprovoked burying behavior. These findings must therefore be viewed in light of DA's relevance within the context of OCD and anxiety. Although DA antagonists may be of value in treatment augmentation strategies with SSRIs in cases of treatment-refractory symptoms (Abramowitz et al., 2009; Dougherty et al., 2004; Fineberg, 2004), antidopaminergic treatment on its own is of negligible value in the treatment of anxiety disorders and OCD; DA antagonists are, however, often added to anxiolytic regimens to treat co-morbid symptoms of psychosis or in treatment resistant instances (Bandelow et al., 2008). Moreover, DA potentiators—that is, receptor agonists, including quinpirole (Egashira, Okuno, Abe, et al., 2008), pramipexole (Jimenez-Gomez et al., 2011), and PD168077 (Bruins Slot et al., 2008); DA reuptake inhibitors, including GBR 12909 (Saadat et al., 2006), bupropion (Honda et al.,

2011), and amantadine (Egashira, Okuno, Harada, et al., 2008); as well as DA precursors, including carbidopa (Njung'e & Handley, 1991b)—administered as monotherapy have also been demonstrated to attenuate burying activity. These findings are especially interesting, given that quinpirole have been used to *induce* OC-like behaviors in animal models of OCD (Albelda & Joel, 2012a; Szechtman et al., 1998). However, the effect of dopaminergic manipulation and its modulation of neuropsychiatric symptomology may be highly related to the context of investigation. In fact, while quinpirole induces compulsive-like checking behavior in rats (Szechtman et al., 1998), it also reduces naturally occurring OC-like stereotypy in the deer mouse (Korff et al., 2008).

Taken together, both dopaminergic and antidopaminergic compounds attenuate burying behavior, albeit variably so (Tables 1, 2, and 3), with few exceptions (Honda et al., 2011). These findings have invariably been reported following the application of tests of unprovoked marble-burying. Therefore, any conclusions drawn with respect to the neurobiological involvement of DA in “so-called” anxiety- or OC-like behaviors are speculative at best.

Glutamatergic involvement in marble-burying behavior

A number of findings support a role for altered glutamatergic signaling in both anxiety (Bergink, van Megen, & Westenberg, 2004; Cortese & Phan, 2005) and OCD (Abramowitz et al., 2009), constituting proof of principle for investigating the effects of drugs targeting glutamate receptors in the MBT. Apart from the attenuating effects of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor potentiators on burying behavior (Iijima et al., 2010; however, see also Egashira, Okuno, Harada, et al., 2008), the findings largely demonstrate that *N*-methyl-D-aspartate (NMDA) and metabotropic glutamate receptor (mGluR) antagonism attenuate burying behavior (Dixit et al., 2014; Egashira, Okuno, Harada, et al., 2008; Iijima et al., 2010; Nicolas et al., 2006; Shimazaki et al., 2004). Furthermore, fenobam, an mGluR 5 allosteric modulator, has also been shown to reduce burying activity (Nicolas et al., 2006). Interestingly, the response of antiglutamatergic compounds seem specific to the modulation of baseline glutamate signaling, since monotherapy with glutamate agonists does not seem to exacerbate burying behavior (Shimazaki et al., 2004). Furthermore, since animal studies have indicated NMDA receptor involvement in both the acquisition and extinction of fear responses, the modulation of MBA and inhibition of related fear responses by NMDA receptor antagonists seemingly supports a role for neophobic triggers in marble-burying behavior (Cortese & Phan, 2005). Indeed, mGluR modulation has also been found to elicit anxiolytic action in several other animal behavior paradigms (Cortese & Phan, 2005). Given

the evidence that agmatine blocks NMDA receptors (Yang & Reis, 1999), the administration of agmatine may also interact with this neurotransmission system in this manner (Dixit et al., 2014; Gawali et al., 2016; Krass et al., 2010; although see also “Miscellaneous pathways involved in marble-burying behavior” section).

Marble-burying and GABAergic signaling

The benzodiazepine class of drugs forms a core component in the short-term management of multiple anxiety disorders (Bandelow et al., 2012; Bandelow et al., 2008). Although they have no demonstrable effect in the treatment of OCD (Abramowitz et al., 2009; Fineberg, 2004), the benzodiazepines, which modulate the effects of the inhibitory neurotransmitter GABA via binding to GABA_A receptor complexes (Pym, Cook, Rosahl, McKernan, & Atack, 2005), have been studied extensively in the MBT. However, this work involved MBA as a screening test for anxiolytic drug action rather than applying the test itself as a framework in which to study the mechanisms underlying anxiety. Indeed, clonazepam (Broekkamp et al., 1986), alprazolam (Nicolas et al., 2006), and diazepam (Abe et al., 1998; Broekkamp et al., 1986; Kinsey et al., 2011) have all been studied in the MBT, mostly yielding results that supported the test's validity as a screening tool for anxiolytic drug action. However, many of these findings were often paired with significant locomotor suppression across most doses tested (Abe et al., 1998; Nicolas et al., 2006; Schneider & Popik, 2007; Young et al., 2006). Hence, follow-up investigations attempted to distinguish between actual anxiolytic responses and the coincidental effects of locomotor suppression by means of selectively targeting GABA receptors. These studies reported inconsistent results: The GABA_A agonists muscimol (which binds to the GABA-binding site of the GABA_A receptor complex) and meprobamate (binding to the barbiturate-binding site of the GABA_A receptor complex), as well as the GABA_B agonist baclofen, have all been demonstrated to reduce marble-burying (Broekkamp et al., 1986; Egashira et al., 2013). However, the same is true for the *anxiogenic* β -carboline, methyl beta-carboline-3-carboxylate (β -CCM), an inverse GABA_A benzodiazepine receptor agonist (Jimenez-Gomez et al., 2011). Furthermore, although the effects of β -CCM have also been shown to be associated with significant locomotor suppression (Jimenez-Gomez et al., 2011), other studies have failed to demonstrate changes in either MBA or LMA using the β -carboline derivative, ethyl-beta-carboline-3-carboxylate (β -CCE; Njung'e & Handley, 1991b). That said, the attenuating effect of GABA_A receptor modulation on burying behavior may be subject to activation-specific GABA_A receptor complexes, since the partial GABA_A agonist bretazenil, which binds to GABA_A complexes irrespective of the alpha subunit expressed (Pym et al., 2005), does not seem to affect the burying response

(Li et al., 2006); however, further investigation is warranted. Moreover, whereas the administration of the competitive GABA_A antagonist bicuculline alone fails to influence burying behavior, it prevents valproate- and muscimol-induced reductions in MBA (Egashira et al., 2013). In congruence with this finding, flumazenil, another GABA_A benzodiazepine receptor antagonist, also prevented the attenuating effects of benzodiazepines on MBA (De Almeida et al., 2014). Not surprising, though, is that given baclofen's use as a skeletal muscle relaxant, the GABA_B-mediated reductions of MBA induced by this compound are associated with notable impairments in locomotor capability (Egashira et al., 2013). However, quite remarkable is that the barbiturate phenobarbital failed to reduce marble-burying behavior (Li et al., 2006).

That compounds targeting GABAergic neurotransmission are effective against anxiety but not OCD somewhat clouds the interpretation of data relating to anxiolytic drug action generated in the MBT. It is common practice to manipulate a certain behavioral test pharmacologically, to allow it to constitute a valid framework that resembles a specific neurocognitive construct. This allows the behavioral test to be deployed as a screening tool for drug action following receptor-targeted interference. With respect to anxiety-related investigations, it would therefore be valuable to administer known anxiogenic substances and to quantify baseline MBA; subsequently, purported anxiolytic compounds may be administered in order to establish whether such pharmacological manipulations are successful, directly relating the behavioral outcome to a specific mechanism. However, given that the majority of investigations have employed unprovoked burying behavior, it is not possible to make any concrete deductions with respect to either anxiety or OCD.

Noradrenergic responses in the MBT

Although the noradrenergic system is typically not implicated in the neurobiology of OCD, it may have some role to play in the manifestation of anxiety (Hoehn-Saric & McLeod, 1988; Tanaka, Yoshida, Emoto, & Ishii, 2000). In this regard, a handful of studies have investigated the possible role of noradrenergic manipulation in the MBT. Indeed, the noradrenergic tricyclic antidepressant imipramine (Broekkamp et al., 1986; Li et al., 2006), the SNRI niooxetine (Li et al., 2006), and the nonselective NA, 5-HT, and DA releasing agents (Green et al., 2003; Rowley, Pinder, Kulkarni, Chettham, & Heal, 2015) methylphenidate (Saadat et al., 2006), d-amphetamine (Jimenez-Gomez et al., 2011; Li et al., 2006; Nicolas et al., 2006), and methamphetamine (Saadat et al., 2006) all reduce the expression of MBA.

NA has been demonstrated to influence anxiety-like behaviors in preclinical studies; levels are typically increased in a number of brain regions, including the hippocampus and

amygdala, in response to stressful events (Brunello et al., 2003; Tanaka et al., 2000). The neurobehavioral effects of such increases can typically be prevented by the administration of compounds not necessarily related to noradrenergic functioning—for instance, the benzodiazepines and opioids (Tanaka et al., 2000). Furthermore, anxious behaviors during these tests can be further exacerbated by drugs inducing NA release (Tanaka et al., 2000). Therefore, and also considering that potentiating noradrenergic signaling will also generally exacerbate OCD symptoms (Coşkun, 2011; Scrby, 2003; Shakeri et al., 2016), it is somewhat confounding that noradrenergic-bolstering agents are effective at *reducing* MBA (Jimenez-Gomez et al., 2011; Li et al., 2006; Saadat et al., 2006). This is especially problematic given that they have been administered acutely prior to execution of the MBT. Indeed, these effects may possibly be attributed to concomitant increases in the activity of other monoamines—that is, DA, in the case of amphetamine-like drugs (Green et al., 2003).

Mood stabilizers and anticonvulsants

Although anticonvulsants are not typically indicated for the treatment of either OCD (Abramowitz et al., 2009; Finberg, 2004) or anxiety (Bandelow et al., 2008), carbamazepine, lamotrigine, and valproate have indeed been shown to attenuate MBA. Interestingly, lamotrigine displayed strain-dependent treatment effects, in that it was effective in ICR but not ddY mice, supporting the findings previously discussed pertaining to strain-to-strain variation in nonmanipulated MBA (Egashira et al., 2013; Nicolas et al., 2006; Thomas et al., 2009). In fact, should such findings be reproducible, they will add weight to the argument for the standardization of strain selection in the MBT (see also “Final summary and recommendations for using the MBT” section). The aforementioned anticonvulsant drugs modulate a number of physiological processes, including the facilitation of GABAergic neurotransmission, blockade of sodium channels, and glutamate receptors, in addition to stimulating dopaminergic and serotonergic neurotransmission (Ambrósio, Soares-da-Silva, Carvalho, & Carvalho, 2002; Egashira et al., 2013; Perucca, 2002), complicating deductions regarding their specific therapeutic mechanisms. However, a role for GABAergic neurotransmission in their mechanism has been demonstrated by the ability of the GABA_A antagonist bicuculline to antagonize the attenuating effects of valproate in the MBT (Egashira et al., 2013). Oddly enough, the mood stabilizer lithium does not have a demonstrable effect on MBA, despite lowering LMA significantly (Egashira et al., 2013). This lack of effect may be due to lithium's known slow onset of action, which ranges from between 6 and 10 days, for the reduction of manic symptoms in clinical bipolar disorder, to more than 6 weeks, for the attenuation of depressive

symptoms (Malhi, Adams, & Berk, 2009); therefore, a single acute administration 30 min prior to testing (Egashira et al., 2013) is arguably insufficient to elicit observable change (Jope, 1999). Changes to LMA may be explained by transient changes to excitatory and inhibitory neurotransmission processes (Jope, 1999).

Cannabinoid receptor involvement in marble-burying

The endocannabinoid system represents a relatively novel array of therapeutic targets for drug discovery. The cannabinoid receptors (CB₁ and CB₂), the principal effectors of the system (Howlett et al., 2002), are typically activated by the endogenous cannabinoid anandamide, whereas a number of agonists and antagonists for these receptors have been characterized to date (Ameri, 1999; Pertwee, 1997). Although both receptors are widely distributed, the CB₁ receptor has been extensively studied for its CNS effects, whereas the CB₂ receptor is largely expressed on immune cells in the periphery (Ameri, 1999; Howlett et al., 2002). Presynaptically located CB₁ heteroreceptors function to reduce the release of a range of neurotransmitters, including glutamate and GABA (Schlicker & Kathmann, 2001), whereas acetylcholine and dynorphin release are also affected. Furthermore, neurotransmitter release may be manipulated by CB₁ agonists at multiple levels of control, such as the *bolstered* release of DA in certain brain regions, by means of removal of the inhibitory effects of tonically active GABAergic neurons following activation of presynaptic CB₁ receptors (Howlett et al., 2002; Schlicker & Kathmann, 2001).

A number of significant findings considering the role of cannabinoid-like effects in the MBT have been made, with agonists of cannabinoid receptors exerting dose-dependent actions on MBA. Anandamide (Umathe et al., 2012) and WIN55,212-2 (Gomes et al., 2011), both of which are cannabinoid receptor 1 (CB₁) agonists, attenuate MBA at lower doses, while exacerbating such behavior at higher doses. This seems to also be true for AM404 and URB597, which act to facilitate anandamide neurotransmission by inhibiting its reuptake or preventing its metabolism (Umathe et al., 2012), respectively. It is hypothesized that such a bimodal effect of anandamide can be attributed to the activation of transient receptor potential vanilloid type 1 (TRPV1) channels, which occurs when anandamide concentrations increase above those required for cannabinoid receptor stimulation (Umathe et al., 2012). Furthermore, these findings have been corroborated by the demonstration that capsazepine, a TRPV1 antagonist, reduces marble-burying, whereas capsaicin, a TRPV1 agonist, elicits pro-compulsive effects in the test (Umathe et al., 2012). Also, antagonism of the TRPV1 receptor by capsazepine blocks the pro-compulsive effects of high dose anandamide, AM404 and URB597, but has no effect

when these compounds are administered in lower doses, indicating a modulatory role for TRPV1 receptor activation underlying pro-compulsive responses. Considering receptor-specific effects, THC (Δ^9 -tetrahydrocannabinol), a nonselective cannabinoid receptor agonist, and cannabidiol (CBD), a nonselective indirect *antagonist*, also appear to also reduce MBA (Kinsey et al., 2011). Importantly, irrespective of receptor selectivity, the attenuating effects of CBD (Casarotto et al., 2010), anandamide (Umathe et al., 2012), AM404 (Gomes et al., 2011; Umathe et al., 2012), WIN55,212-2 (Gomes et al., 2011), and URB597 (Gomes et al., 2011; Umathe et al., 2012) are blocked by the CB₁ receptor antagonist AM251 (Casarotto et al., 2010; Gomes et al., 2011; Umathe et al., 2012). Since CBD itself also functions as an antagonist of the cannabinoid CB₁ and CB₂ receptors (Pertwee, 2008), the fact that it attenuates MBA on its own, and that such attenuation can be blocked by another antagonist, in the form of AM251, seems confounding. However, the attenuating effect of CBD on marble-burying behavior can possibly be ascribed to the decreased hydrolysis and reuptake of anandamide, and subsequent stimulation of CB₁ receptors, following the administration of CBD (Bisogno et al., 2001; Casarotto et al., 2010). Importantly, crosstalk between cannabinoid and serotonergic neurotransmission in the marble-burying response has been suggested by findings indicating that the co-administration of subeffective doses of CBD and fluoxetine reduces burying activity (Nardo et al., 2014). Furthermore, CBD was also able to reverse mCPP-induced MBA (Nardo et al., 2014). Finally, endogenous ligands of the endocannabinoid receptors also appear to modulate MBA, since inhibition of their catabolism also reduces MBA; this effect can also be blocked by CB₁ antagonist rimonabant (Kinsey et al., 2011).

The above findings are presented within the context of OC-like behavior; however, they must be viewed in light of the fact that endocannabinoid-like compounds do indeed exhibit anxiolytic action, particularly via CB₁ receptor activation (Haller, Varga, Ledent, & Freund, 2004; Rey, Purrio, Viveros, & Lutz, 2012). Anxiogenic effects can be precipitated by CB₁ blockade (Haller et al., 2004; Navarro et al., 1997), but similar manipulations can *also* reduce anxiety-like manifestations (Casarotto et al., 2010; Haller, Bakos, Szirmay, Ledent, & Freund, 2002). Additionally, this CB₁-mediated biphasic anxiety response is well known, and has in fact been replicated in other animal anxiety tests (Haller et al., 2004; Moreira & Wotjak, 2009; Rey et al., 2012). In this respect, the MBT appears to accurately mimic the behavioral manifestations of direct and indirect endocannabinoid receptor manipulation reported in other tests, so this test may be of particular interest for further study of drugs modulating this system. That said, because only some proof of concept exists that warrants further investigation of the effects of cannabinoid receptor modulation in OCD (Casarotto et al., 2010), the translational

relevance of these results with respect to OCD remains to be established.

Miscellaneous pathways involved in marble-burying behavior

A number of miscellaneous systems have been investigated in the MBT. Since the core focus of the present article is on anxiety and OCD, and considering that to date none of these have shown any clear and demonstrable role in current approaches to the treatment of either condition, they will only be briefly summarized.

Nitric oxide (NO) involvement in the MBA has been implicated in a few studies. It has been shown that administration of the NO precursor L-arginine, increases MBA (Gawali et al., 2016), while it also reverses the attenuating effect of SSRI administration on burying behavior (Krass et al., 2010). Furthermore, many inhibitors of NO synthesis—such as 7-nitroindazole (7-NI; Gawali et al., 2016; Krass et al., 2010), N^G-nitro-L-arginine methyl ester (L-NAME; Gawali et al., 2016), and 1-(2-trifluoromethylphenyl) imidazole (TRIM; Krass et al., 2010)—have demonstrated potential for reducing burying activity, implying a possible role for NO in the manifestation of MBA. In line with these findings, agmatine, a modulator of glutamatergic NMDA receptors that also decreases central NO activity, reduces MBA in a manner that can be reversed by L-arginine (Gawali et al., 2016). These findings are especially interesting with respect to OCD, in that previous reports had indicated that elevated NO levels in the rat brain suppress 5-HT activity in those same regions, whereas NO inhibitors overturn this effect (Gawali et al., 2016; Segieth, Pearce, Fowler, & Whitton, 2001; Wegener, Volke, & Rosenberg, 2000). Moreover, that agmatine has also demonstrated anxiolytic action in the EPM and SIT supports the abovementioned findings, in so far as the MBT is purported to represent both an anxiety-like and OC-like constructs (Gong et al., 2006). In physiological terms, elevated NO concentrations increase the activity of guanylyl cyclase, which increases cyclic guanosine 3′5′-monophosphate (cGMP) levels, an important second messenger known to interact with a number of neurotransmission processes (Feil & Kleppisch, 2008), most notably, those that also have established roles in OCD—for instance, GABA, glutamate, DA, and 5-HT. Indeed, NO may modulate 5-HT, GABA, and glutamate release (Kachler et al., 1999; Sandor, Brassai, Pliskas, & Lendvai, 1995; Spiacci, Kanamaru, Guimaraes, & Oliveira, 2008), and NO modulation has been demonstrated to elicit anxiolytic and antidepressant effects (Spiacci et al., 2008). As such, proof of concept exists for further investigations of NO manipulation in preclinical and clinical studies of anxiety and OCD.

Melanin-concentrating hormone (MCH) appears to modulate MBA, since administration of the hormone itself reduces this behavior. Oxytocin administration also reduces the

behavior, since many MCH neurons express oxytocin receptors that influence their activity levels. Finally, blockade of the MCH receptor with antagonist GW803430 prevents the attenuating actions of both the aforementioned hormones (Sanathara et al., 2018). Oxytocin has been linked to anxious behavior (Neumann & Landgraf, 2012).

Although antagonism of neurokinin receptor 1 (NK₁) has shown promise as a potential anxiolytic in both preclinical (Santarelli et al., 2001) and clinical (Tillisch et al., 2012) studies, the inhibition of MBA by NK₁ antagonists—for example, GR205,171 and RP67,580—is associated with significant locomotor impairment (Millan et al., 2002). It is therefore likely that the MBT is not sensitive enough to reveal possible anxiolytic responses following antineurokinin compounds.

Findings related to the effects of first-generation antihistamines—for instance, chlorpromazine (Abe et al., 1998; Broekkamp et al., 1986; Li et al., 2006; Nicolas et al., 2006), cyproheptadine (Njung'e & Handley, 1991a), and diphenhydramine (Broekkamp et al., 1986)—in the MBT are mixed. Although they have demonstrated positive effects in some investigations (Abe et al., 1998; Broekkamp et al., 1986; Li et al., 2006; Nicolas et al., 2006; Njung'e & Handley, 1991a), they have failed to do so in others (Schneider & Popik, 2007). Furthermore, since most of these compounds function as multipotent competitive antagonists at a number of receptors, including the noradrenergic alpha, dopaminergic D₁ and D₂, and cholinergic M-receptors (Owens et al., 1997), the translational relevance of these findings is difficult to interpret within the context of anxiety and OCD. Furthermore, since first-generation antihistamines cause significant motor impairment and sedation, their effects in the MBT are often confounded by parallel reductions in locomotor competence (Abe et al., 1998; Njung'e & Handley, 1991a; Nicolas et al., 2006).

What may be more important within the context of the present article is that, in addition to the major receptor systems discussed, a variety of compounds have been put forward as valid alternatives for reducing MBA, and thus for further study within the context of anxiety and OCD. These include N-acetyl-L-cysteine (NAC; Egashira, Shirakawa, et al., 2012) and ascorbic acid (De Almeida et al., 2014), which connects with evidence for redox disturbances in human OCD and animal models (Guldenpfennig, Wolmarans, du Preez, Stein, & Harvey, 2011; Szechtman et al., 2017); calcium channel blockers (Egashira, Okuno, Abe, et al., 2008), which connects with evidence of the efficacy of mood stabilizers in OCD (Egashira et al., 2013); anticholinergic drugs, such as atropine (Broekkamp et al., 1986; Nicolas et al., 2006) and scopolamine (Broekkamp et al., 1986); cholinergic compounds, such as organophosphates (Savy et al., 2015); novel plant extracts (Dey et al., 2016; Kalariya et al., 2015; Skalisz, Bejjamini, & Andreatini, 2004); and morphine (Nicolas et al., 2006). However, the relevance, usefulness, and translational contribution of these findings must be questioned, in light of the

concerns raised with respect to the methodologies followed in the execution of the MBT and the uncertain neuropsychological construct resembled by the MBT that we noted earlier. Suffice to say, with the exception of NAC, which has demonstrated a possible augmentation role in SSRI refractory OCD (Lafleur et al., 2006) and as a possible treatment for OC-spectrum disorders such as trichotillomania (de L. T. Vieira, Lossie, Lay, Radcliffe, & Garner, 2017; Özcan & Seckin, 2016), few of these findings have resulted in any clinical application. Although they may possibly contribute to our understanding of the mechanisms underlying MBA, this remains unlikely, given that these agents have shown little demonstrable effect with any other behavioral screening tools for either anxiety- or compulsive-like behaviors.

The role of pharmacologically induced burying behavior in the MBT

As we alluded to earlier, animal behavioral tests that are not only responsive to ameliorative therapeutic interventions, but additionally show detectable behavioral changes in response to exacerbating pharmacological manipulation (File & Seth, 2003; Tanaka et al., 2000), are useful for elucidating the specific neurobiological mechanisms underlying proposed anxiety- and compulsive-like behaviors. Considering that the MBT is indeed sensitive to anxiogenic (Kedia & Chattarji, 2014; Umathe et al., 2009) and pro-compulsive (Tucci et al., 2013) intervention and that it is applied as a measure of anxiety- and compulsive-like behavior, several investigations have attempted to bolster burying responses by administering drugs with known anxiogenic or pro-compulsive properties. However, the results have been inconsistent. For example, in terms of classic anxiogenic compounds, several have been shown to *reduce* marble-burying behavior. These include yohimbine (Nicolas et al., 2006; Njung'e & Handley, 1991b), β -carbolines (Jimenez-Gomez et al., 2011), and amphetamine (which are often paired with significant increases in LMA; Jimenez-Gomez et al., 2011; Li et al., 2006; Nicolas et al., 2006; Saadat et al., 2006). Furthermore, even this response is inconsistent, with some anxiogenic agents, such as FG-7142 (Nicolas et al., 2006) and β -carbolines (Njung'e & Handley, 1991b), not modulating the burying response at all. Furthermore, as we alluded to earlier, drugs known to induce compulsive-like behavioral persistence in animal models (which incidentally is what also characterizes MBA), such as quinpirole (Egashira, Okuno, Harada, et al., 2008; Szechtman et al., 1998; Tucci et al., 2013) and 8-OH-DPAT (Bruins Slot et al., 2008; Ichimaru et al., 1995; Yadin et al., 1991), also elicited ameliorating responses. Also, the DA agonist pramipexole has demonstrated pro-compulsive effects in human studies, yet it reduces MBA in rodents (Jimenez-

Gomez et al., 2011). Although demonstrating *sensitivity* to such interventions per se may potentially contribute to our understanding of the underlying neurobiology of MBA—that is, confirming a role for altered dopaminergic and serotonergic signaling—the indiscriminate and variable between-laboratory responses to the same compounds or drug classes negate the translational value of such findings. Indeed, reliability and reproducibility of experimental paradigms according to similar methodologies will be crucial before it will be possible to draw collective translational conclusions (Varga, Hansen, Sandøe, & Olsson, 2010). As such, given the data summarized above, marble-burying cannot be regarded as a reliable screening test for any *specific* neuropsychological construct.

Timing and duration of treatment

An important consideration in anxiety and OCD studies, and one that applies to all forms of treatment, concerns the timing and duration of a pharmacotherapeutic intervention. Apart from demonstrating the involvement of several neurotransmitter systems in MBA, few studies have addressed the fact that, with the exception of such drug classes as the benzodiazepines in the treatment of anxiety-like behavior, acute interference usually exacerbates rather than attenuates anxiety-like behavior (Bandelow et al., 2008; Fineberg, 2004). Indeed, with only a few exceptions (De Almeida et al., 2014; Ichimaru et al., 1995; Njung'e & Handley, 1991a; Savy et al., 2015; Taylor et al., 2017; Torres-Lista et al., 2015; Umathe et al., 2012; Wolmarans et al., 2016), most investigations into MBA have employed acute treatment 30 min prior to the test (Tables 1, 2, and 3). For instance, although a role for 5-HT has been implicated in the expression of MBA, the translational relevance of anxiolytic and anticomulsive responses subsequent to acute manipulations of neurotransmission has not been explained. Indeed, clinical responses to SSRIs typically require up to eight weeks for both OCD (Goddard et al., 2008) and anxiety (Bandelow et al., 2008). As such, studies testing MBA in response to chronic treatment with such agents will be vital for it to be considered a reliable measure of anxiolytic and/or anticomulsive drug action. Nevertheless, the effects of SSRIs on MBA have in some cases been shown to taper off during repeated administration (Njung'e & Handley, 1991a), while being maintained (Ichimaru et al., 1995; Umathe et al., 2012), or even demonstrating diminution of effect over 28 days of treatment (Wolmarans et al., 2016), in others. Reports concerning chronic diazepam treatment have also reported both successful (Taylor et al., 2017) and unsuccessful (Ichimaru et al., 1995) outcomes following sustained therapy. Similarly, most agents acting on the endocannabinoid system lose their efficacy after two weeks, whereas drugs such as the TRPV1 antagonists maintain suppression of MBA over this

period (Umathe et al., 2012). These unexplained findings warrant further study.

Concluding remarks on treatment responses in the MBT

Given the body of evidence presented here, it is clear that the MBT, as reported up to this point, has “responded” to a broad range of pharmacological interventions, some of which are in line with other preclinical and clinical evidence. That said, various compounds have also demonstrated attenuating effects, for which no current explanation of its contextual mechanism of action have been proposed. Furthermore, the evidence is replete with contradictions, in terms of the effects of specific agents, effective dosages, paradoxical effects of anxiogenic drugs, and divergent findings regarding agonist/antagonist pairings, as we highlighted in earlier sections. Since the MBT is often regarded as a relatively straightforward procedure to apply as a test for anxiolytic and anticomulsive drug action, we propose that such divergent findings have been influenced by the methodological inconsistencies summarized in “Digging, burrowing, and burying as natural behaviors” section, as well as by the general lack of standardization regarding the MBT as a whole. However, on the one hand, *aberrant* naturally occurring marble-burying behavior appears to hold some value as a potential *model* for neophobia or compulsive behavior. On the other hand, we posit that anxiety- or compulsive-like behaviors that are induced by pharmacological manipulation and are accurately highlighted by changes in normal burying behavior, as measured according to the criteria suggested for each condition in “Final summary and recommendations for using the MBT” section, could be of value as high-throughput *screening tests*, whereby any drugs that test positive may then be pursued further and characterized in more robust, pathology-driven models. Also, considering the potential ease, low cost, and high throughput capacity of the test, the MBT may potentially be ideal for examining the behavioral effects of specific receptor manipulations. This approach could allow for the *identification* of novel drug–receptor interactions that might be of relevance for anxiety and OCD, as has been demonstrated with respect to sigma receptor involvement (Egashira, Harada, et al., 2007), the various endocannabinoid-targeting drugs (Casarotto et al., 2010; Gomes et al., 2011; Kinscy et al., 2011; Umathe et al., 2012), antioxidants (Egashira, Shirakawa, et al., 2012), and drugs regulating NO synthesis (Gawali et al., 2016; Krass et al., 2010).

Final summary and recommendations for using the MBT

The body of literature reviewed in the present article has highlighted a number of between-laboratory inconsistencies.

To further the collective translational value of findings reported from investigations into marble-burying activity, and because methodological differences may have fundamentally contributed to the discrepancies in the findings reported, we suggest the alignment of methods, but at the same time consider that context-specific experimental design will be crucial. Briefly, we make the following recommendations with respect to execution of the MBT:

- Researchers should adopt a fixed size (e.g., 15 mm for mice, 20 mm for rats), number, and manner of placement of marbles in specific circumstances that are standardized for different species, cage sizes, and testing paradigms—that is, one-zone versus two-zone configurations and anxiety- versus compulsivity-related investigations.
- Burying substrates of a denser nature (i.e., river sand) should be used, since this prevents incidental settling of marbles during typical behavioral routines. Furthermore, irrespective of the neuropsychological construct investigated, the test subjects should be exposed to the burying substrate in home cages for at least 24 h prior to testing, to avoid novelty-induced burying activity—this will ensure that marbles are the only novel stimuli introduced in the testing paradigm. Furthermore, regardless of the substrate used, the average density and particle diameter thereof must be reported, to ensure adequate between-laboratory repeatability.
- Video tracking of the test procedure should form a core component of marble-burying investigations. Visual scrutiny of video should accurately confirm marble-directed behavior, as opposed to normal cage exploratory activity that results in coincidental covering of marbles. Furthermore, time spent on either side of a two-zone test paradigm should be applied as an indicator of avoidance or engagement behavior.
- Locomotor activity should always be accounted for. We propose that a separate group of animals be used for this objective, since simultaneous scoring of locomotor ability, typically quantified as the total distance traveled, may be confounded by marble-directed preoccupation during the test.
- The arena size should be standardized per species, whereas the spacing of marbles in arenas should allow sufficient room for avoidance, and therefore be sensitive to measuring behaviors related to *voluntary interaction*. The standard housing cages often used for mice and rats represent an ideal point of departure. Dimensions of $35 \times 20 \times 13$ cm and $40 \times 35 \times 21$ cm and for mice and rats, respectively, may be suitable. Naturally, interlaboratory differences in equipment will be apparent, but efforts should be made to minimize such differences.

- Since scoring of the test is arguably subjective, we recommend that two blind scorers be involved in the testing procedures. Applying score averages or any other viable method of normalizing scores will contribute to the validity and value of the assigned score, especially when combined with analogous use of burying substrates and analyses of video recordings. To ensure accurate between-laboratory methodological repetition, investigators should also describe in detail how their scoring procedures are carried out.
- Since different levels of inherent digging and MBA for different species and strains have been demonstrated, we recommend selecting specific strains for investigations of specific neurocognitive constructs, except in scenarios in which existing animal models of OCD or anxiety are scrutinized in the MBT for the presence of *additional* behavioral phenotypes—for example, deer mice (Wolmarans et al., 2016). The selected strains should demonstrate consistent burying behavior that is subject to modification by either known exacerbating or attenuating interventions. In this regard, it seems that C57BL mice may represent an ideal strain for such testing, since it has demonstrated sensitivity to mild anxiogenic behavioral interventions such as restraint, together with an apparent propensity to both avoid marbles and bury them when close by, indicating a likely neophobic behavioral phenotype (Kedia & Chattarji, 2014; Nicolas et al., 2006).
- Since the majority of reported anxiety studies have employed natural, noninduced burying behavior, the only possible anxiety-related trigger for burying activity has been the introduction of marbles as a novel stimulus. In this regard, a two-zone paradigm, in which the animal has the ability to avoid exposure to the marbles completely or can intentionally engage in active burying behaviors, is advised. Furthermore, it would be valuable to demonstrate that neophobic responses abate over time; hence, repeated testing over consecutive days is also advised.
- Since OC symptoms are inflexible, persistent, and directed at specific actions, these traits should ideally be emulated in the MBT, either with respect to natural behaviors or where such activity is artificially induced. In this regard, the use of a two-zone paradigm is crucial, in that (1) this allows the animal the choice of engagement, thereby emulating goal-directed responses, and (2) the test will be discriminative with regard to anxiety-related constructs—for example, avoidance. Furthermore, assessing burying behaviors over repeated trials both before (for baseline scoring) and after treatment will also indicate whether the burying construct is subject to habituation over time or whether it persists, thereby more accurately mimicking compulsive-like repetition.

Conclusion

The MBT is often applied as a model of anxiety- and compulsive-like behavior, as well as as a preclinical screening test to recognize potential anxiolytic or anticomulsive drug action. However, the findings from marble-burying investigations as they have been reported up to now have been inconsistent and often contradictory, and therefore have not contributed much in terms of translational usefulness. Although the present review has sought to highlight the various methodologies followed, we have also explained why the test in itself has not been used to its full potential. Furthermore, on the basis of anxiety- and OC-related theory, we have argued that certain methodological specifics are important for aligning the MBT as closely as possible to the illness under study. In this regard, we propose that the testing of anxiety or OCD should be assessed *after* anxiogenic or pro-compulsive interventions to facilitate marble-burying, be they behavioral (Kedia & Chattarji, 2014) or pharmacological (Nardo et al., 2014; Sanathara et al., 2018; Tucci et al., 2013; Umathe et al., 2012), so as to examine the effect of treatment in a more robust simulation of anxiety and compulsivity. With regard to using the MBT as a *model* of neophobia and OCD, emphasis should *ideally* rest on the demonstration of three key observations: namely, (1) that interactions with marbles are goal-directed and voluntary; (2) that MBA abates over time, with respect to anxiety, or persists over repeated trials in control groups, with respect to OCD; and (3) that the effects of anxiolytic and anticomulsive drugs are present only after the course of a chronic administration schedule, except in the case of drugs that could be of value in the acute treatment of anxiety. Furthermore, we have elaborated on the numerous pharmacological interventions that have been tested, described how these studies have extended (or not extended) understanding, and highlighted possible reasons why the results obtained have been counterproductive. That said, we reiterate the *potential* utility of appraising burying behavior, as either a possible model or a test for the identification of anxiety- or compulsive-like constructs and novel drug–receptor interactions, and as an instrument to screen for drugs that target novel physiological sites that have already demonstrated, or have been designed for, possible anxiolytic or anticomulsive activity, provided that a sound and contextually relevant experimental design is followed. Suggestions and recommendations for such methodologies have also been provided.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflicts of interest.

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5. Manuscript C

Article accepted for publication (rebuttal and revision accepted) in *Behavioural Pharmacology* titled:

“Naturalistic operant responses in deer mice (*Peromyscus maniculatus bairdii*) and its response to outcome manipulation and serotonergic intervention”

Author Contributions:

- *Geoffrey de Brouwer* assisted in the conceptualization of this study together with *De Wet Wolmarans* and *Brian Harvey*, performed all laboratory work, performed statistical processing and interpretation and conceptualized and wrote the manuscript.
- *De Wet Wolmarans* as the study leader, assisted in the conceptualization of this study together with *Geoffrey de Brouwer* and *Brian Harvey*, performed statistical processing and interpretation, and assisted the candidate with the writing of the manuscript.
- *Brian Harvey* provided professional insight and guidance with respect to the conceptualization of the study together with *Geoffrey de Brouwer* and *De Wet Wolmarans*. Further he proofread the manuscript and made suggestions for improvement.

Important Information:

- The manuscript is prepared according to the instructions to authors for *Behavioural Pharmacology*
- Instructions to the author may be accessed here:
<http://edmgr.ovid.com/bpharm/accounts/ifaauth.htm>
- As per the instructions, Figures are provided at the end of the manuscript.
- The co-authors have provided consent for this article to be assessed as part of the PhD thesis of *Geoffrey de Brouwer* (Addendum A).
- The article has been accepted for publication in *Behavioural Pharmacology*. The letter of acceptance is included in Addendum B.
- The responses to reviewer comments (rebuttal letter) are included in addendum F.
- Addendum F also contains a detailed description of the novel apparatus applied, to aid understanding.

“Naturalistic operant responses in deer mice (*Peromyscus maniculatus bairdii*) and its response to outcome manipulation and serotonergic intervention”

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Abstract

Investigating the motivational triggers underlying naturalistic compulsive-like behavior is generally regarded as challenging. To this extent, the current investigation aimed to establish a proof-of-concept for future investigation by probing unconditioned and naturalistic operant responses aimed at obtaining nesting material normal (NNB) and large nest building (LNB) deer mice (*Peromyscus maniculatus bairdii*). LNB mice and NNB controls were individually placed in cages equipped with a lever-operated nesting material (cotton rope) dispenser and allowed to become accustomed to the response (lever press)-outcome (obtaining cotton rope) contingency over 7 nights. Subsequently, the contingency was manipulated by withdrawing the nesting material (Experiment 1) or punishing the lever-press response with a mild electrical foot shock (Experiment 2). Mice were then treated for 28 days with escitalopram (50 mg/kg/day) and retested. Our results indicate that 1) LNB mice generally made more operant responses compared to NNB controls, 2) withdrawal of nesting material and institution of punishment bolstered responding in LNB but not NNB mice, and 3) escitalopram treatment tended to reduce increased responding in LNB mice following experimental manipulation, while normalizing the total number of lever-press counts in the LNB cohort. Therefore, LNB seems to diverge from NNB, not only as a spontaneous phenotype, but also in terms of the motivation to obtain nesting material, despite demotivating feedback. That such differences were abrogated by chronic escitalopram intervention, indicates that the uniquely motivated operant interactions displayed by LNB mice, may be founded upon serotonergic mechanisms, a finding in line with the neurobiological theory of OCD.

Key words: lever-press; obsessive-compulsive disorder; deer mouse model; large nest building; cognition; escitalopram; naturalistic behavior

Introduction

Obsessive compulsive disorder (OCD) is a chronic and disabling psychiatric condition with a lifetime prevalence of 2 – 3 % (Ruscio *et al.*, 2010). OCD is characterized by obsessions, which are recurrent, persistent and unwanted thoughts that is often anxiogenic or distressful to the patient (Chamberlain, *et al.*, 2005; American Psychiatric Association, 2013; Veale and Roberts, 2014). Obsessions are usually, but not exclusively, related to compulsions, i.e. repetitive thought or motor patterns that are often aimed at reducing the distress associated with obsessional thoughts (American Psychiatric Association, 2013; Veale and Roberts, 2014; Abramowitz and Jacoby, 2015). Further, obsessions and compulsions (OC) are performed relentlessly in spite of patients having insight into the irrationality of such actions (Marazziti *et al.*, 2002; Hinds *et al.*, 2012), while the patient is often compromised by the negative outcomes of engaging in OC routines, i.e. poor work performance, dysfunctional social relationships and large amounts of wasted time (Huppert *et al.*, 2009; Ruscio *et al.*, 2010; American Psychiatric Association, 2013). OCD is clinically heterogeneous, as symptoms manifest with respect to several specific themes, e.g. symmetry, contamination or safety (Markarian *et al.*, 2010; Abramowitz and Jacoby, 2015; Rowsell and Francis, 2015).

OCD demonstrates moderate response to chronic high dose treatment with selective serotonin reuptake inhibitors (SSRIs; El Mansari and Blier, 2006; Goddard *et al.*, 2008; Pittenger and Bloch, 2014). Such therapy is often augmented with low-dose dopamine antagonists in refractory cases (Dold *et al.*, 2013; Fineberg *et al.*, 2013; Veale *et al.*, 2014). That said, pharmacological treatment response remains suboptimal, and it is increasingly clear that interactions between neurobiological and cognitive deficits—of which a number of theories have been proposed—form a complex foundation for OC symptomology and the aforementioned poor response to treatment (Denys *et al.*, 2004; Chamberlain *et al.*, 2005; Fineberg *et al.*, 2010; Koo *et al.*, 2010; Markarian *et al.*, 2010; Gruner and Pittenger, 2017). In terms of the latter, a number of cognitive domains of interest have been identified. For example, research indicates that patients suffering from OCD commonly over-estimate the relevance of potential, but highly unlikely threats (Szechtman and Woody, 2004; Moritz and Jelinek, 2009; Cisler and Koster, 2010; Thomas *et al.*, 2013), and that such threat-overestimation may be a promulgating factor in the manifestation of safety and assurance seeking compulsions (Markarian *et al.*, 2010; Veale and Roberts, 2014). Further, taking into consideration that patients often only experience temporary relief from engaging in compulsive routines and that OC symptoms are thus subject to negative reinforcement (Gillan *et al.*, 2011; Fineberg, *et al.*, 2013; Gillan *et al.*, 2016), behavioral actions that may have been goal-directed initially may readily evolve to become more habitual in nature (Jager, 2003; de Wit and Dickinson, 2009; Kalanthroff *et al.*, 2016). OCD patients also often, although to varying

degrees, demonstrate impairments in a number of neurocognitive domains involved in the planning of and control over executive behavior (Lawrence *et al.*, 2006; Penades *et al.*, 2007; Gillan *et al.*, 2016; Gruner and Pittenger, 2017). More specifically, it has been shown that some OCD patients present with a diminished ability to update—or set-shift—their behavioral responses following negative outcomes leading to pronounced cognitive inflexibility (Nielen *et al.*, 2009; Morein-Zamir *et al.*, 2012; Haber and Heilbronner, 2013; Voon *et al.* 2015).

In this regard, Robbins *et al* (2012) succinctly conceptualize compulsion as “*the aberrant dysregulation of stimulus-response habit learning*”, providing the foundational concept for instrumental learning (IL). In simple terms, IL implies associating a specific action with its outcome (Colwill and Rescorla, 1990; Seymour *et al.*, 2007; Schultz, 2013), a process driven by two key learning components. First, optimal IL requires an organism to successfully acquire knowledge of the instrumental contingency between the response and outcome (R/O), while secondly *also* ascribing a motivational value to such an action dependent on the degree of salience held by any particular reinforcer (Boureau and Dayan, 2011; Schultz, 2013). In fact, the motivational salience of a reward appears to influence the development of OCD specific habitual responses (Voon *et al.*, 2015). This latter aspect of IL is driven by reward or punishment feedback processing, which appears to be founded on unique motivational systems and biological mediators (Schultz, 2013; Faulkner and Deakin, 2014; Meyniel *et al.*, 2016; Iigaya *et al.*, 2018). Punishment learning, known to be mediated primarily by serotonergic mechanisms (Seymour *et al.*, 2007; Cools *et al.*, 2008; Faulkner and Deakin, 2014; Iigaya *et al.*, 2018), is an important component of behavioral motivation (de Wit and Dickinson, 2009; Wise, 2009; Palminteri *et al.*, 2015). Indeed, serotonin is classically suggested to play an important role in the appraisal and processing of negative emotional affect, behavioral inhibition and effort cost assessments (Daw *et al.*, 2002; Faulkner and Deakin, 2014; Meyniel *et al.*, 2016). This theoretically contrasts with the purported role of dopamine, which is classically regarded to be closely involved with reward feedback processing, behavioral activation and invigoration (Wise, 2004; Schultz, 2007; Wise, 2008; Cools *et al.*, 2009; Schultz, 2013). That said, literature concerning serotonin/dopamine opponency theories (Daw *et al.*, 2002; Boureau and Dayan, 2011; Cools *et al.*, 2011) propose highly complex computational interactions between serotonergic and dopaminergic neurotransmission under punishing and rewarding circumstances (as encoders of positive and negative affect) respectively. However, this may be an oversimplification, since recent findings demonstrate that dopamine also governs defensive behaviors (Lloyd and Dayan, 2016)—a role classically ascribed to serotonin (Harvey *et al.*, 1975; Faulkner and Deakin, 2014)—while serotonin has been shown to influence rewarded responses (Iigaya *et al.*, 2018). Nevertheless, the outcomes of these computations ultimately inform the organism of the net

motivational value of certain actions, providing the necessary information for IL processes (Pessiglione *et al.*, 2006; de Wit and Dickinson, 2009; Palmiter *et al.*, 2015). Such integration of information is vital for action motivation and response selection, i.e. the ability to adapt behavior based on the likelihood of being rewarded or punished, and how much effort to expend to achieve desired outcomes (O'Doherty *et al.*, 2003; Seymour *et al.*, 2007; Cools *et al.*, 2011; Meyniel *et al.*, 2016).

Clinical investigations into the cognitive processes and affective states associated with OCD are typically performed using a number of clinical assessment scales to ascertain the effect of intervention i.e. improvement in rating scale scores (Overduin and Furnham, 2012). Naturally, such interrogations are impossible to execute in animals (Eilam *et al.*, 2012) and mostly rely on the study of learned, conditioned or pharmacologically induced behaviors (Albelda and Joel, 2012a, 2012b; Alonso *et al.*, 2015). To this extent, several models have been applied (Fineberg *et al.*, 2010; Ting and Feng, 2011; Albelda and Joel, 2012b; Monteiro and Feng, 2016). Despite this, the modelling of the elusive affective components of OCD, i.e. the obsessions which motivate compulsion, remains challenging (Fineberg *et al.*, 2011; Eilam *et al.*, 2012). The present investigation builds upon previous work into the deer mouse (*Peromyscus maniculatus bairdii*) model of compulsive-like persistence (Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2016a, 2016b, Wolmarans *et al.*, 2017). Deer mice of both sexes present to varying degrees with naturalistic and non-induced repetitive behaviors that are reminiscent of OC symptomology, as described in detail elsewhere (Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2016a, 2016b; Wolmarans *et al.*, 2017; De Brouwer and Wolmarans, 2018). These include spontaneous motor stereotypy (Wolmarans *et al.*, 2013), high marble burying (Wolmarans *et al.*, 2016b; De Brouwer and Wolmarans, 2018) and aberrant large nest building (LNB), the latter of which is observed in 30% of the population (Wolmarans *et al.*, 2016a). Further, while high stereotypy and LNB respond to chronic high-dose oral treatment with the SSRI, escitalopram (Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2016a), HMB seems less sensitive to such intervention (Wolmarans *et al.*, 2016b), potentially constituting a unique underlying neurobiological mechanism. Considering that said behaviors differ with respect to functional content, but that they are equally persistent, repetitive and compulsive-like in their expression, the study of naturally occurring aberrant behaviors in the deer mouse might provide a useful avenue in which to investigate the motivational constructs which may underlie compulsive-like behavior.

As such, the present work was conceptualized to investigate whether LNB expressing deer mice would present with a unique manner of engagement in operant responses, i.e. voluntary lever-pressing, aimed at acquiring nesting material compared to normal nest building (NNB) animals. We further wanted to establish how such behavior would respond to outcome

depreciation, i.e. withdrawal of the delivery of nesting material upon lever-pressing, and to aversive feedback, i.e. punishment via voluntary, self-administered electric foot-shock. Further, we also aimed to investigate how all of the said behaviors would respond to chronic high-dose oral escitalopram intervention. Importantly, as we specifically attempted to investigate how deer mice would perform under *natural* circumstances, the current work did *not* involve a training component as has been described in earlier investigations employing operant-outcome contingencies (Joel, 2006; Flaisher-Grinberg *et al.*, 2008). Indeed, unconditioned voluntary operant actions directed towards a highly specific contextual goal—in this case obtaining nesting material—may be better suited to study the phenotypic cognitive content motivating OC-like behavior.

Materials and methods

Animals

80 outbred deer mice (*Peromyscus maniculatus bairdii*; male and female), aged 12 weeks at the onset of experimentation were obtained from the in-house colony maintained at the North-West University (NWU) vivarium, Potchefstroom, South Africa. This study was approved by the local animal ethics committee AnimCare (ethical approval number: NWU-00261-16-A5; AREC-130913-015) and was performed in accordance with South African National Standard (SANS) for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008). We have previously demonstrated that LNB behavior is expressed without sex bias in deer mice (Wolmarans *et al.*, 2016a) and thus animals were selected only on the basis of their behavior. Food and water were provided *ad libitum* throughout all procedures. Animals were housed at 23°C ± 1°C on a twelve-hour light/dark cycle (06h00 / 18h00). Mice were housed in groups of 4 – 6 same-sex animals per individually ventilated cage until the onset of experimentation, where after they were single-housed. Housing cages [350 (l) x 200 (w) x 130 (h) mm; Techniplast®, Varese, Italy], prepared with a 3 cm thick layer of corncob bedding material, were cleaned and changed weekly. Animals remained housed in these cages throughout the investigation, except for the periods during which they were assessed for operant (lever-press) activity.

Baseline classification of NNB and LNB animals

A graphical representation of the study layout is provided in **Figure 1**. Before the onset of lever-press investigations, all mice were screened for nest building behavior over 7 consecutive nights by being provided with an excess of pre-weighed, unscented, hospital-grade cotton wool in the ceiling grids of each of the housing cages (Wolmarans *et al.*, 2016a). As mice are known to engage in nesting behavior immediately prior to dawn (Jirkof, 2014),

built nests were removed and weighed and new cotton wool supplied daily at 9h00 (Wolmarans *et al.*, 2016a). The daily and total weekly nesting scores were calculated based on the quantity of cotton wool utilized during each 24-hour period. After 7 days, animals were assigned to either of the two cohorts (NNB or LNB) based on 1) the total weekly nesting scores and 2) the degree of variance in the daily nesting scores (**Figure 2A**). As such, only animals that generated total nesting scores that clustered within (or as close as possible to) the upper quartile of the normal nesting score distribution, as well as presenting with the lowest between-day variance, were included in the LNB groups (**Figure 2A**; $n = 17$ in total). Conversely, animals that generated total nesting scores that clustered within (or as close as possible to) the lower quartile of nesting score distribution and presenting with the lowest between-day variance, were allocated to the NNB groups (**Figure 2A**; $n = 18$ in total) (Wolmarans *et al.*, 2016a). Animals were blindly selected with respect to sex by these two criteria alone. As only 25 – 30% of deer mice naturally engage in LNB behavior (Wolmarans *et al.*, 2016a), an original number of 80 deer mice were initially screened for nesting behavior. As such, the 45 remaining animals were either euthanized or allocated to other investigations not relevant to the current study. The latter was possible due to the non-anxiogenic nature of the baseline nest building screening procedure.

Operant assessment

General considerations and procedures for assessment of operant behavior

Each of the individually ventilated cages [380 (*l*) x 380 (*w*) x 230 (*h*) mm] in which goal-directed operant behavior was assessed were fitted with a cotton-rope dispensing operant cartridge (North-West University Instrument Making and Electronic Services, Potchefstroom, South Africa). Operant cartridges, measuring (315 (*l*) x 80 (*w*) x 155 (*h*) mm), were calibrated to deliver 10 mm of pliable cotton rope ($\varnothing = 4$ mm) each time the subject pressed a pedal-style lever situated within the wall of the cartridge approximately 5 mm from the floor of the cage. Levers were constructed with an incorporated foot-shock grid that could be switched on or off depending on the phase of the investigation. However, operant cartridges were programmed not to dispense more than a single 10 mm length of rope within 1 second, irrespective of the number of lever-presses executed within a single second. This was done to prevent the continued delivery of rope following potential rapid execution of a number of successive lever-presses, thereby maintaining clear separation between response and outcome. To attract attention to the operant cartridges, these were prepared every day with 10 mm of rope already dispensed and available for use at the start of each trial, regardless of the time point of investigation. The associated control software (Response Outcome Contingency Software [ROCS] v1.0.2; North-West University Electronic Services, Potchefstroom, South Africa)

continuously tracked both the number of lever presses executed and the length of rope dispensed during each of the assessment days respectively. During periods of lever-press assessment, bedding material was not provided as it interfered with the mechanical function of the operant cartridges. Cages were cleaned, nests removed, and operant cartridges serviced every day during these investigations. On each of the successive days during which mice were assessed for operant behavior, mice had access to the operant dispensers for at least 22-hours, providing a 2-hour window for daily cage maintenance routines.

Experiment 1: Natural goal-directed operant behavior and its response to reward devaluation before and after chronic escitalopram intervention

Figure 1A provides a schematic layout of the study progression for experiment 1. For this experiment, 12 NNB (6 males and 6 females) and 11 LNB (6 males and 5 females) animals were employed, selected only on the basis of behavioral expression during screening tests. Following the 7-day baseline nest-building screening (according to paragraph 2.2), each mouse was individually housed for 10 days prior to (**Figure 1A, pre-treatment testing**) and following (**Figure 1A, post-treatment testing**) 28-day treatment with high-dose (50 mg/kg/day) oral escitalopram (Wolmarans *et al.*, 2016a, 2016b) in the larger operant cartridge-containing cages. Following completion of Trials 1 – 10, animals were reintroduced to their home cages where they received treatment for 18 days before being returned to the operant cages, where treatment continued for another 10 days while being re-assessed in the operant cages (**Figure 1A, post-treatment testing**). Both before and after treatment, mice were allowed 7 days to freely interact with and become accustomed to the operant cartridges (**Figure 1A, lever press CP (conditioning phase)**). Following the 7-day CP, mice were left in the same cages for an additional three nights. However, during this time, cotton rope was *not* dispensed in response to the execution of lever presses (**Figure 1A, lever press WP (withdrawal phase)**). However, the number of presses was counted during both the CP and WP.

Experiment 2: Natural goal-directed operant behavior and its response to punishment before and after chronic escitalopram intervention

Figure 1B provides a schematic layout of the study progression for experiment 2. For this experiment, an additional 6 NNB and LNB (3 males and 3 females per cohort, again on the basis of behavioral expression only) were selected. Up until Trial 7 before treatment and Trial 17 after treatment, the experimental procedure was the same as explained in paragraph 2.4 (**Figure 1B**). Once again, the number of presses and length of cotton rope was counted, while mice were, as in Experiment 1, returned to their home cages for treatment following the completion of Trial 10. They were reintroduced to the operant cages after 18 days of

uninterrupted treatment and continued to receive treatment in the operant cages for a further 10 days while being reassessed.

Following completion of the CP, both before and after treatment (**Figure 1B**, *Pre-treatment and post-treatment testing*), mice were left in the operant cages for another three nights for assessment under circumstances of self-inflicted punishment when lever-pressing to obtain nesting material (**Figure 1B**, *lever press punishment phase (PP)*). During this time, operant cartridges were configured and inserted in the cages as explained previously. However, in this instance, the ROCS software was set to deliver a mild electric shock (0.05 mA) after the completion of two presses. Therefore, the electric shock pulse was delivered from the third press onwards. The choice of 0.05 mA (the lowest current output) was based on data from a prior pilot study (data not shown) in which shock was administered in 0.05 mA increments from 0.05 – 0.3 mA. Mice failed to engage in any lever-pressing activity from 0.1 mA.

Drugs and treatment

Following the pre-treatment assessment of operant behavior (Trials 1 – 10 in both experiments; **Figures 1A, 1B**) animals were returned to their standard home cages where treatment was administered for 18 days. The last 10 days of treatment was received in the operant cages during the post-treatment testing phase (Trials 11 – 20 in both experiments; **Figures 1A, 1B**). Escitalopram oxalate (Lundbeck A/S, Copenhagen, Denmark) was administered via the drinking water as previously reported (Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2016a). The drug concentration in the drinking water, *viz.* 20 mg/100 ml, was calculated based on the average fluid intake of deer adult mice, previously established to be 0.25 ml/g/day (Aschhoff *et al.*, 2000) and confirmed in our laboratory (data available on request; Wolmarans *et al.*, 2013). This concentration is equivalent to the administration of 50 mg/kg/day (Wolmarans *et al.*, 2013). Solutions were constituted and replaced every other day for the duration of the investigation. As this study builds on previous work, alternative routes of administration were not considered due to the significant risk of inducing injury or inducing anxiety (Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2016a, 2016b).

Statistics

Statistical analyses were performed with GraphPad Prism[®] version 8.0.1 (GraphPad[®] Software, San Diego, California, USA). Data sets were tested for normality using Shapiro-Wilk method. To compare the average pre- and post-treatment expression of operant responses, two-way repeated measures analysis of variance (2-way RM ANOVA) was applied. The average number of operant responses (lever-presses) was set as between-subject (dependent) factor, while nesting cohort and time or treatment were set as within-

subject (independent) factors. Statistical significance for all analyses was only inferred when $p < 0.05$. Differences between the operant responses of NNB and LNB animals between- and within-cohort, before and after treatment and with respect to the CP, WP and PP, were analyzed by means of linear regression. Slopes were regarded to be significantly different if $p < 0.05$. Due to the highly variable nature of operant responses observed in this investigation, all comparisons were followed up with calculations of Cohen's d effect size to establish the practical significance of the observations made. Effect sizes of > 0.8 were considered large and effect sizes of > 1.3 were considered very large.

Results

Baseline classification of NNB and LNB

In line with our previous findings regarding the percentage of animals that engage in LNB behavior (Wolmarans *et al.*, 2016a), 18 NNB and 17 LNB animals were identified from the initial pool of 80. The average total nesting sizes of the NNB group were significantly different from that of the LNB group as shown by an unpaired t-test (**Figure 2B**; 8.3 ± 0.5 g vs. 22.5 ± 0.8 g; $t(33) = 15.34$; $p < 0.0001$). Animals were subsequently randomly allocated to Experiment 1 (12 NNB and 11 LNB animals) and Experiment 2 (6 NNB and 6 LNB animals).

Experiment 1

The lever pressing data of both behavioral cohorts was tested for normality using the Shapiro-Wilk normality test. Neither of the cohorts expressed pressing responses that passed the test for normality (LNB $p < 0.0001$; NNB $p < 0.0001$). However, to analyze the pre- and post-treatment differences in pressing behavior we still employed 2-way RM-ANOVA as it is considered robust enough to account for non-normally distributed data (Maxwell and Delaney, 2004; Laerd Statistics, 2017). Thus, the analyses are based on the mean number of lever-presses executed, since there are no applicable non-parametric analyses which could be used to investigate 2-way interactions.

Pre-treatment: Animals of both the NNB and LNB cohorts acquired the necessary knowledge and competency to engage in goal-directed operant responses during the first trial of the CP, both before and after treatment (**Figure 3A**, trial 1). Two-way RM-ANOVA revealed a significant interaction between nesting cohort and trial with respect to the average daily number of operant responses executed (**Figure 3A**; $F(19, 399) = 2.15$, $p = 0.0036$). As such, Bonferroni post-hoc analyses were performed which revealed significant differences between the number of operant responses made by LNB animals on the first trial and all other trials except for one (**Figure 3A**; trial 3; descriptive statistics provided in **Table 1**) as well as between

the number of operant responses made during trial 1 by NNB and LNB animals (13.18 ± 15.21 , median 7 vs. 3.42 ± 5.58 , median 2, $p = 0.0003$). Although failing to reach statistical significance, the operant responses made by LNB animals remained higher compared to that made by NNB animals in most trials of the CP, except for trials 6 and 9 (**Figure 3A**; Cohen's d values provided in **Table 1**). In terms of behavioral adaptation over time, the operant responses of NNB animals remained relatively stable over the progression of the pre-treatment CP (**Figure 3A**; linear regression slope [trials 1 – 7]; -0.098 ± 0.23 , $r^2 = 0.002$). In contrast, LNB animals adapted their behavior over the course of the CP, engaging in more operant responses during the beginning of the pre-treatment CP compared to the end (**Figure 3A**; linear regression slope [trials 1 – 7]; -1.37 ± 0.44 , $r^2 = 0.11$). Furthermore, the average operant responses made by NNB animals throughout the pre-treatment phase remained more consistent in terms of between-animal deviation, compared to the more erratic response pattern observed in the LNB cohort (**Figure 3A**; average number of responses $\pm$ SD, LNB: 4.78 ± 7.75 , median 2 vs. NNB 2.34 ± 4.89 , median 1). The difference between these slopes is significant ($p = 0.009$). During the WP, NNB and LNB animals responded in a distinctly opposite manner to the response-outcome dissociation. On the first, but not subsequent WP trial(s) (**Figure 3A**; trial 8), LNB animals executed more operant responses compared to those expressed prior to withdrawal (**Figure 3A**; trial 8 vs. trial 7: $d = 0.43$). In contrast, the behavior of NNB animals remained the same as it was prior to the WP (**Figure 3A**; trial 8, NNB vs. LNB, $d = 0.8$).

Four weeks post-treatment: Although both groups of animals demonstrated initial interest to engage in operant responses during the first post-treatment trial (**Figure 3A**; trial 12 vs 11: NNB $d = 0.82$; LNB $d = 0.9$), chronic escitalopram treatment had a significant effect on the operant behavior of LNB, but not NNB animals (**Figure 3B**; $F(1, 21) = 6.782$, $p = 0.017$). Apart from LNB behavior returning to the stable operant response presentation of that observed in the NNB cohort during the CP (**Figure 3A**; linear regression slopes [trials 11 – 17]; **LNB** -0.32 ± 0.23 , $r^2 = 0.02$ vs. **NNB** -0.25 ± 0.08 , $r^2 = 0.099$), escitalopram had an overall attenuating effect on the average number of operant responses made by LNB animals during the post-treatment compared to the pre-treatment testing phase (**Figure 3B**; 1.92 ± 4.67 vs. 4.78 ± 7.75 , $p = 0.015$). Further, escitalopram stabilized the behavior of LNB animals during the WP, as evinced by the analogous operant behavior during trial 17 and trial 18 (**Figure 3A**).

While a scatter dot plot of the average operant responses over all of the trials (**Figure 3B**) confirm the stabilizing effect of escitalopram on the operant behavior of LNB animals, three subjects, one in the NNB cohort and two in the LNB cohort, were identified that engaged in an outlying number of operant responses compared to the average behavior of the respective cohorts (**Figure 3B**: NNB: blue dot; LNB: red and green dots). Further, although the behavior

of the NNB animal seemed to normalize, the two LNB animals persisted in making more operant responses after treatment, compared to the rest of the LNB animals.

Experiment 2

Pre-treatment: Operant behavior of the 6 NNB and LNB animals included in Experiment 2 generally followed the same trends as those observed in Experiment 1. We applied the Shapiro-Wilk test for normality and found that the data was not normally distributed (LNB $p < 0.0001$; NNB $p < 0.0001$). Again, we applied 2-way RM ANOVA according to the rationale explained above for experiment 1. However, in this case 2-way RM ANOVA failed to demonstrate a significant interaction between nesting cohort and trial ($F(19, 190) = 0.78, p = 0.73$). Analyses of the main effects of nesting cohort ($F(1, 10) = 1.26, p = 0.28$) and trial ($F(19, 190) = 1.38, p = 0.14$) also failed to yield significance. That said, animals again acquired the knowledge and competency to engage in operant responses during the first trial (**Figure 4A**; trial 1). Further, the number of operant responses made by LNB animals remained higher than those made by NNB animals throughout the CP (Cohen's d values provided in **Table 2**), except during trial 2, when a single NNB animal made 41 operant responses, hence resulting in the result observed as reported in **Figure 4A** (the apparatus was checked, tested and found to be in appropriate working order). As the operant responses of neither NNB nor LNB animals were normally distributed, this response could not be identified as an outlier. As such, linear regression failed to demonstrate a significant difference in the overall operant responses of NNB and LNB animals during the CP. That said, after electric foot-shock-punishment has been introduced, LNB, as opposed to NNB animals engaged in bolstered operant behavior from the second trial of the PP (**Figure 4A**; trial 9 and trial 10). Although the PP only consisted of three trials and linear regression therefore failed to yield significant slope differences between the PP behavior of NNB and LNB animals, the apparent differences in slopes are indicative of the orthogonal responses of NNB and LNB animals observed during the PP (**Figure 4A**; NNB $-0.5 \pm 0.39, r^2 = 0.09$ vs. LNB $1.92 \pm 1.73, r^2 = 0.07$).

Four weeks post-treatment: Escitalopram failed to elicit a statistically significant response in the average operant behavior of NNB and LNB animals during the CP (**Figure 4A and 4B**; $F(1, 10) = 0.065, p = 0.8$). However, during the PP, the behavior of LNB animals adjusted to a pattern akin to that expressed by NNB animals (linear regression slopes [trials 18 – 20]: NNB: $-0.42 \pm 0.41, r^2 = 0.06$ vs LNB: $-0.08 \pm 0.59, r^2 = 0.001$).

Discussion

The major findings of the current work are that 1) naturalistic operant behavior can likely be separated based on an inherent underlying motivational construct as reflected by the overall higher number of operant responses made by LNB compared to NNB mice, 2) LNB, previously validated as a repetitive, persistent and SSRI-sensitive phenotype (Wolmarans *et al.*, 2016a), is naturalistically associated with persistent outcome-seeking, despite the motivation for such behavior being devaluated and punished, and 3) chronic high dose oral escitalopram demonstrates promise in the attenuation of abnormal LNB-associated operant behavior and its response to reward devaluation and punishment.

This investigation attempted to constitute a proof-of-concept for continued exploration of the motivational triggers underlying aspects of *naturally* occurring persistent behavioral phenotypes. To this extent, we interrogated unconditioned operant responses in NNB and LNB expressing deer mice, a rodent model that presents with promising naturalistic compulsive-like phenotypes (Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2016a, 2016b, Wolmarans *et al.*, 2017; De Brouwer and Wolmarans, 2018). The model, seemingly representative of symptom heterogeneous OCD, presents with robust face, construct and predictive validity. On each of these levels, a significant body of work has already been performed and summarizing these is beyond the scope of this paper. For a complete review of the model and its relevance for the study of OCD, please refer to Scheepers *et al.*, 2018). To allow for deeper insights into the abnormal cognitive processes underlying LNB in these animals, we investigated the association between *intent* and the degree of operant engagement towards fulfilment of such intent, i.e. lever-pressing to obtain nesting material. Although conceptually based on the signal attenuation method that involves training protocols and conditioning (Joel and Doljansky, 2003; Joel *et al.*, 2005; Joel, 2006; Albelda and Joel, 2012b), the current approach relies on the inherent, natural differences in nesting behavior as a potential motivator for operant action. Indeed, nesting is a natural goal-directed behavior that is expressed by all rodents, aimed at protection from harsh environmental conditions and predation (Jirkof, 2014; Stewart and McAdam, 2017), thus inspiring the idea that naturalistic LNB may be representative of underlying threat bias, as is often characteristic of OCD (Cisler and Koster, 2010; Wang and Zhanjiang, 2017). As such, given that LNB is spontaneously expressed in approximately 30% of the laboratory housed deer mouse population (Wolmarans *et al.*, 2016a), it could be of value to study such naturalistic aberrant repetitive behavior to elucidate the underlying cognitive processes in OCD. This is especially true as obsessions and compulsions typically relate to one or more prevailing themes, such as excessive cleaning and compulsive checking (Rowell and Francis, 2015; Stewart, 2016). Moreover, while patients often demonstrate cognitive impairment in contexts related to such themes, i.e. threat-

overestimation in safety/checking OCD, they broadly function normally otherwise (Exner *et al.*, 2014). Our hypothesis that operant responses would hold a unique motivational value in the LNB compared to the NNB cohort due to a potential threat bias underlying the former, could thus potentially be tested by manipulating the *outcome* of successful operant engagement, allowing us to evaluate the ‘importance’ ascribed to such responses, thereby allowing an indirect evaluation of the cognitive processes underlying aberrant and repetitive animal behavior.

Our finding that both NNB and LNB deer mice engaged in operant action on the first night after being introduced to the operant cages (**Figures 3A and 4A**), is important as it demonstrates the natural ability of laboratory-housed deer mice to engage in voluntary exploration of unknown, and what could potentially be anxiogenic, scenarios. Further, as animals cannot directly be assessed in terms of the cognitive processes that may underlie the manifestation of unconditioned behavior, the ability to experimentally analyze the *intent* of spontaneous, unforced and untrained behaviors may potentially be valuable for our understanding of behavioral processes. With respect to the current investigation, in which animals selected for NNB and LNB were tested in a contextual response-outcome scenario, the question could be asked whether LNB animals will continue to make a unique effort to obtain nesting material under circumstances of contextual manipulation. Indeed, we found that apart from differing in their *natural* expression of NB behavior in a typical home cage environment (*paragraph 2.2*; (Wolmarans *et al.*, 2016a), LNB animals executed a significantly higher number of operant responses compared to NNB animals during the first night of having access to the nest-building material (**Figure 3A; Figure 4A**). Further, although the separation in the operant behavior of LNB and NNB animals did not remain as clear on subsequent nights, potentially pointing to a bolstered initial interest that habituated over time, the behavior of LNB animals mostly remained more persistent throughout the pre-treatment testing phases of both experiments (**Figures 3A and 4A; Tables 1 and 2**). Therefore, it can be concluded that although both cohorts of animals have the ability to learn and adapt to a novel environment, operant engagement as it is expressed in this model, presents with natural differences in the drive to engage in nest-building. This provides us with a proof-of-concept for investigating the cognitive processes that may uniquely underlie the expression of compulsive-like large nest-building behavior under different circumstances of contextual manipulation, *viz.* response-outcome dissociation (reward devaluation) and penalizing the *intent* to obtain a reward by means of administering punishment.

With respect to dissociating operant actions from its expected outcome, i.e. the dispensing of nesting material, LNB animals seemed to respond distinctly from the NNB group (**Figure 3A**; trials 8 – 10), trending towards increased levels of operant responding ($d = 0.8$). Although the

behavior of NNB and LNB animals during trials 9 and 10 seemed to present in an opposite manner, this could neither be confirmed statistically, nor by means of effect size. That said, a single NNB animal made 38 operant responses during trial 9, explaining the increased average operant actions executed by the NNB cohort during trial 9. If the responding of this subject during trial 9 presented at a level in line with its responding over all other trials, it is likely that the NNB group as a whole would have presented with a reduced level of operant engagement throughout trials 8 – 10. As such, future investigation is needed to establish why such highly discrepant behavior is shown by certain animals. Nevertheless, that the average standard deviation on the number of operant responses made by NNB compared to LNB animals remained lower across all pre-treatment trials, clearly indicate a unique action-outcome trait exhibited by LNB animals.

We further hypothesized that animals naturally engaging in aberrant nest building behavior may present with an inflated motivational drive to engage in such behavior irrespective of such action being paired with a negative outcome (Moritz and Jelinek, 2009; Cisler and Koster, 2010; Exner *et al.*, 2014). Indeed, recent evidence indicates maternal nesting behavior of *Peromyscus* species is sensitive to temperature change (Stewart and McAdam, 2017), thus representing not only a natural goal-directed behavior, but one which is sensitive to external feedback. To test whether this extends to artificially administered negative stimuli, we introduced voluntary foot-shock as a means of penalizing the motivational intent and ultimate execution of goal-directed operant responses. Remarkably, the results reported here seem to support such theory in that NNB and LNB animals responded in an opposing manner immediately after the infliction of punishment (**Figure 4A**; trials 8 – 10). Although both groups of animals executed on average an equal number of operant responses during trial 8, hence demonstrating appreciation of the outcome, LNB animals bolstered their operant actions on the subsequent two trials, while the response in NNB animals further reduced. This behavior is well in line with clinical observations in patients with OCD, who are often noted to prevail in engaging in compulsive rituals, despite punishing outcomes resulting from among others, cognitive inflexibility, poor set-shifting ability and dysfunctional reversal learning paired with the condition (Hinds *et al.*, 2012; Morein-Zamir *et al.*, 2012; Gruner and Pittenger, 2017). In humans, such inability to prevent or reduce the level of behavioral engagement is explained by the overwhelming nature of intrusive thought processes (American Psychiatric Association, 2013; Hinds *et al.*, 2012; Julien *et al.*, 2007). Hence, the data presented here showing that LNB animals are more prone to endure unpleasant circumstances in order to satisfy an underlying drive to engage in contextual operant responses, provide putative evidence that the very act of nesting may be of importance and uniquely significant to LNB subjects. Further, the change in behavior of LNB animals further indicate that they are differentially responding

to a change in the response and the negative stimulus feedback after willingly engaging in punished lever presses. It would be of value to examine how chronic punishment would affect this behavior, thereby providing insight into processes of behavioral adaptation. By overcoming punishment in order to acquire nesting material, more complex cognitive processes than pure motor-stereotypical abnormalities underlie the expression of naturalistic compulsive-like large nest building behavior. Moreover, the findings presented here are in agreement with observations that penalizing contextual responses may result in behavioral disinhibition, as has been shown in clinical OCD (Nielen *et al.*, 2009; Morein-Zamir *et al.*, 2012).

As alluded to earlier, OCD generally responds to a varying extent to chronic high-dose SSRI intervention. We have previously demonstrated that LNB but not NNB is attenuated by 28-day high-dose (50 mg/kg/day) oral escitalopram (Wolmarans *et al.*, 2016a). However, as such forms of intervention also reduce the expression of stereotypical behavior (Wolmarans *et al.*, 2013), it is uncertain whether an increase in cortico-striatal serotonergic tone only influences the motor component underlying the expression of compulsive-like behavior or whether it also addresses broader neurocognitive processes. Considering this question, our findings relating to the effect of said intervention on the different phases of operant responding, is informative. First, while both cohorts once again demonstrated an initial interest to engage in operant responding in both experiments (**Figure 3A** and **4A**, trial 11), the behavior of LNB animals quickly returned to a more uniform and stable presentation, while the behavior of NNB animals remained unchanged (**Figure 3A** and **3B**). Although the same trend was not observed in the LNB group in experiment 2 (**Figure 4B**), again the influence of the behavior of a specific animal during the first post-treatment trial—executing 44 operant responses—as well as the fact that we only included 6 subjects in this phase of the experiment based on the robust differences observed between the behavior of NNB and LNB animals during experiment 1, could possibly provide an explanation. We also considered the possibility that the average lower level of operant engagement of LNB animals after treatment may have been an artefact of habituation. However, this is unlikely since preliminary data from our laboratory indicate naturalistic LNB in deer mice to exacerbate over time (supplementary data). Therefore, we can conclude with a certain degree of certainty that the overall change in the behavioral responses of LNB animals after treatment, has indeed resulted from the serotonergic intervention. The finding that two LNB animals generally remained refractory to escitalopram intervention (**Figure 3B**) is in line with previous findings in our model with respect to high stereotypy (Wolmarans *et al.*, 2013) and high marble burying (Wolmarans *et al.*, 2016b) and highlights the importance of an individualized, rather than a grouped appraisal of deer mouse behavior. Indeed, it may be possible that these two subjects may present with a behavioral trait that is conceptually

different from the overall LNB trait characterized here. That said, it is the effect of escitalopram on operant responses during the respective phases of devaluation (**Figure 3A**, trials 18 – 20) and penalization (**Figure 4A**, trials 18 – 20), that are noteworthy. Indeed, in contrast to the pre-treatment behavioral expression of LNB deer mice, escitalopram resulted in responding patterns analogous to what has been observed in the NNB cohort, providing putative evidence in an animal model that bolstered serotonergic signaling may also modify the potential cognitive processes that naturally motivate voluntary and goal-directed operant engagement in spite of such behavior being devalued and penalized. Importantly, we believe the behavioral responses observed during trials 18 – 20 to be especially founded upon the serotonergic influence of escitalopram, since the operant responses of NNB and LNB animals over trials 11 – 17 remaining largely similar. The data presented here is encouraging as it aligns with the clinical picture of OCD, where patients are found to be inhibited with respect to learning from negative affect (Nielen *et al.*, 2009; Exner *et al.*, 2014), and that such deficits are often counteracted by the administration of SSRIs (Palminteri *et al.*, 2012). Although previous non-OCD-related findings also showed that the processing of reward and punishment may be inhibited by the administration of SSRIs treatment (McCabe *et al.*, 2010), our data is not fully concordant. However, behavioral adaptation to rewarding and punishing feedback is a complex process that relies on the interplay between dopamine and serotonin (Boureau and Dayan, 2011; Cools *et al.*, 2011; Dalley *et al.*, 2011). Further, it is widely accepted that although no causal relationship has been established, OCD is generally associated with large-scale serotonergic dysfunction (Westenberg *et al.*, 2007; Goddard *et al.*, 2008; Markarian *et al.*, 2010). Hence, the apparent ability of escitalopram to improve abnormal reward and punishment valuation in LNB animals suggests a dysfunctional serotonergic system underlying the unique behavior in question here. Serotonin is indeed implicated in calculating effort cost, while SSRIs typically bolster effort output towards goals (Meyniel *et al.*, 2016). Conversely, serotonin depletion, leads to impulse control deficits (Crockett *et al.*, 2009; Cools *et al.*, 2011), in line with literature showing that serotonin depletion promotes behavioral perseveration in previously-rewarded tasks both in animal models (Clarke *et al.*, 2004; Clarke *et al.*, 2006) and in humans (Worbe *et al.*, 2015).

Despite the novel nature of the work presented here, there are some shortcomings of note that prompt further study and refinement. First, since the overall average operant engagement of LNB animals reduced over all trials during the post-treatment testing phase, it remains to be established whether LNB animals specifically, habituate to the action of lever pressing. Since we built on our own previous work and employed repeated measures analysis, we did not employ a control-treated group, which would arguably have provided some insight into this matter. That said, the aim of this investigation was not to use lever-pressing as a *measure of*

nest building activity per se, but rather to establish whether LNB animals would exhibit a unique behavioral profile in a contextual goal-outcome paradigm. Second, considering the relatively low-level of operant engagement across both behavioral phenotypes and experimental designs, it is possible that the cotton rope used as nesting material may not have been malleable enough to serve as an ideal nesting material for the mice. Indeed, we concede that the cotton rope could arguably be more difficult to fashion into a nest than the non-rope cotton wool used in the home cage environment (see *paragraph 2.2*). Although the use of cotton rope facilitated the well-calibrated and measurable dispensing of nesting material by the operant cartridges, it is entirely possible that a softer and more malleable material could have served as a more suitable motivator for operant action. That said, as the fundamental purpose here was to assess how animals selected for different spontaneous nest-building traits would respond under changing contextual circumstances relating to their motivational drive to engage in such behavior, we believe having used cotton rope would not undermine the ultimate value of the conclusions drawn. Third, our data cannot exclude the possibility that operant actions as assessed in this investigation, may be associated with and be representative of a different behavioral trait that may be conceptually distinct from LNB, i.e. impulsivity. This may be especially true for some animals, considering our finding that two LNB subjects remained refractory to escitalopram intervention, similar to that observed in related marble-burying investigations (Wolmarans *et al.*, 2016b). This question needs further clarification, especially considering the erratic responses noted in the behavior of some animals.

In conclusion, this work has presented proof-of-concept support for investigating the role of cognitive processes in a naturalistic animal model of OCD. More specifically, we have shown that mice presenting with an inherent drive to express LNB are more prone to engage in operant actions to obtain nesting material than their NNB counterparts. Further, treatment-naïve LNB animals persist in such operant engagement in spite of reward devaluation and punishment, pointing to potential dysfunction with respect to outcome feedback processing, as is also typical of clinical OCD. This observation strengthens both the face and construct validity of the model, since it is reminiscent of dysfunctional or inflated underlying motivational processes (Willner, 1986; Geyer and Markou, 1995; Markou *et al.*, 2009). Lastly, the response of operant action to reward devaluation and punishment seems to be normalized by chronic escitalopram treatment to a behavioral expression akin to that observed in the NNB cohort. This suggests that serotonergic mechanisms are involved in both the neurocognitive and motor processes that underlie compulsive-like behavior in the deer mouse model of OCD and contributes to the predictive validity of the model (Willner, 1986; Geyer and Markou, 1995; Markou *et al.*, 2009). That said, we believe the most valuable contribution of this work to be

the fact that it constitutes the foundational steps for investigating the cognitive processes and motivational triggers that may underlie seemingly abnormal animal behavior in a pre-clinical setting. Limitations notwithstanding, the current work adds to the current body of literature pertaining to compulsive-like behavior in animals and provides a valuable point-of-departure for future investigations.

Conflict of Interest

The authors declare no conflict of interest. The escitalopram used in this investigation was a kind sponsorship from Lundbeck A/S, Copenhagen, Denmark.

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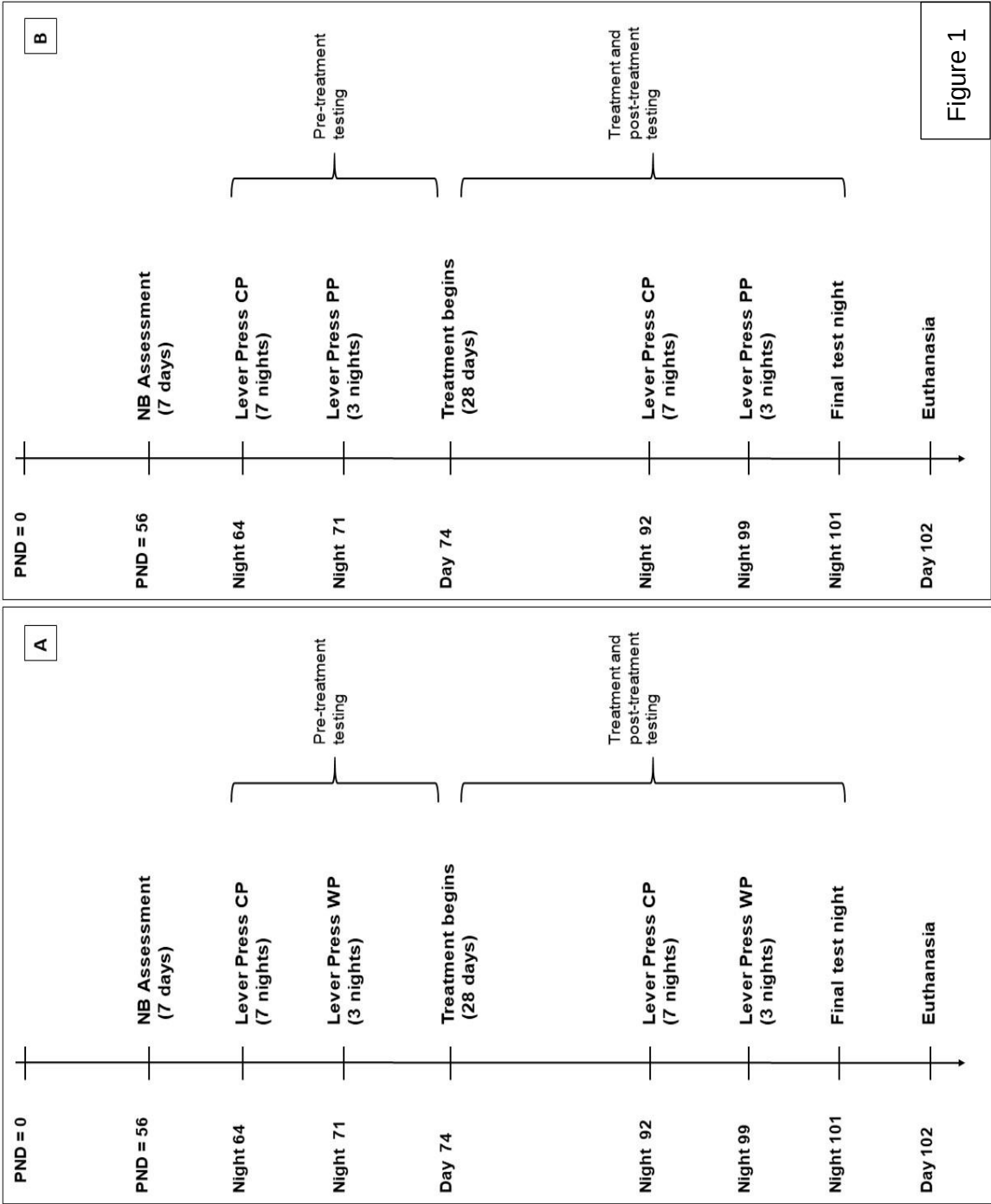
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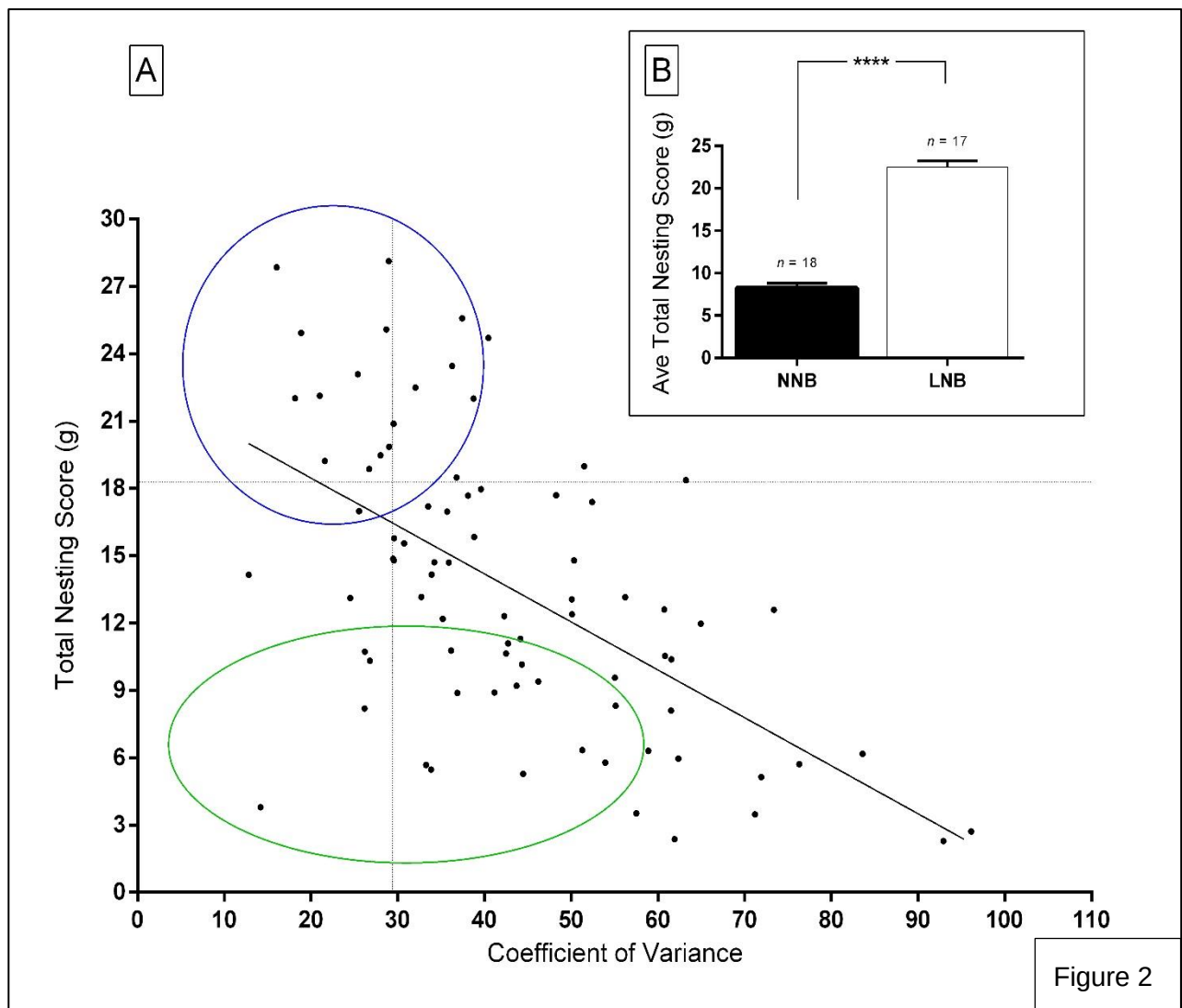
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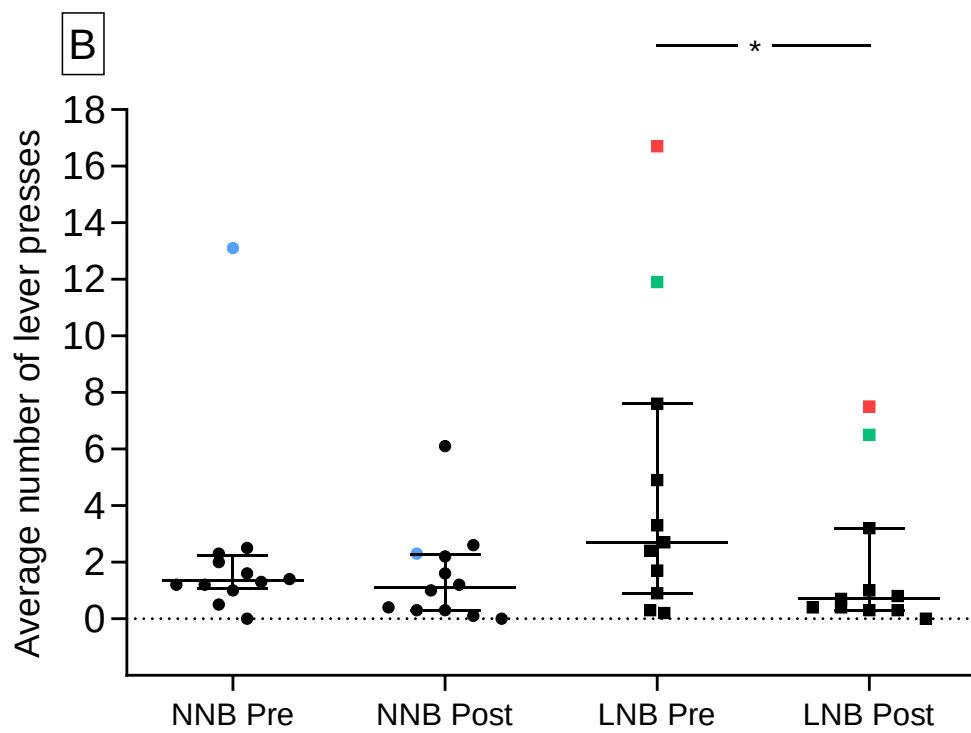
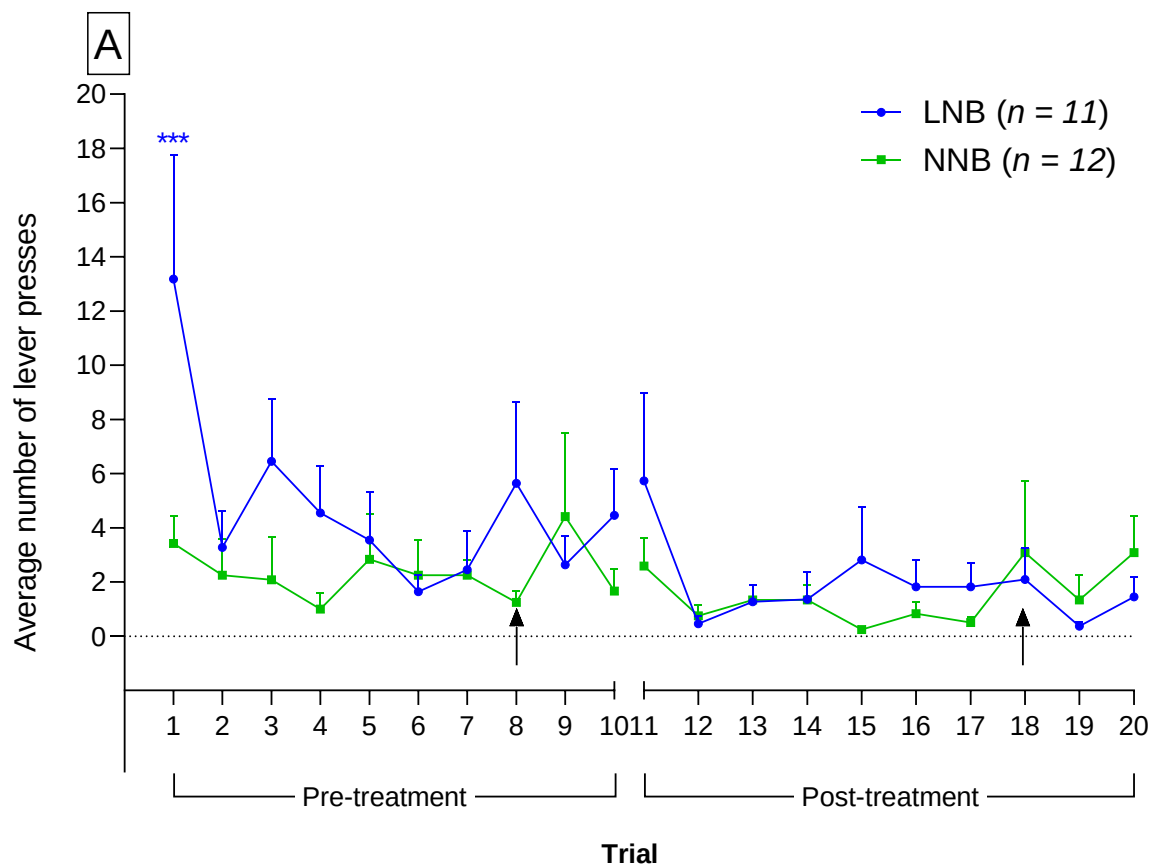


Figure 3

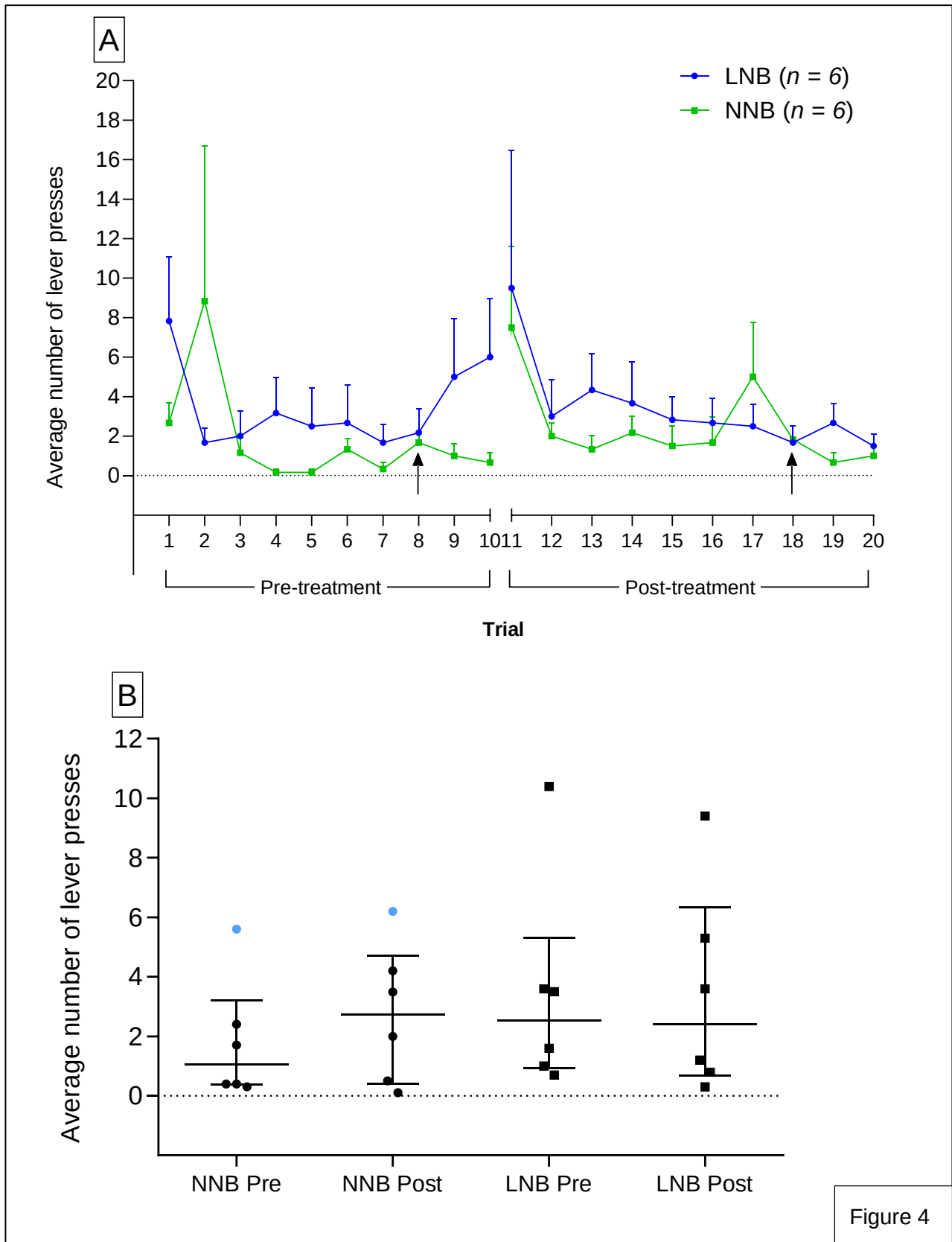


Figure 4

Table 1: Descriptive statistics of Experiment 1

Within cohort comparisons			Between-cohort comparisons		
Comparison of trial (LNB)	<i>p</i> value (2-way RM ANOVA with Bonferroni's multiple comparisons)		Trial (LNB vs. NNB)	Mean number of lever presses±SD (LNB vs. NNB)	Cohen's <i>d</i>
1 vs 2	****	<0.0001	1	13.18±15.21 vs. 3.42±3.58	1.04
1 vs 3	<i>ns</i>	0.0894	2	3.27±4.41 vs. 2.25±4.58	0.23
1 vs 4	**	0.0015	3	6.45±7.58 vs. 2.08±5.42	0.67
1 vs 5	***	0.0001	4	4.55±5.77 vs. 1.00±2.09	0.90
1 vs 6	****	<0.0001	5	3.55±5.94 vs. 2.83±5.87	0.12
1 vs 7	****	<0.0001	6	1.64±2.11 vs. 2.25±4.43	-0.19
1 vs 8	*	0.0172	7	2.45±4.78 vs. 2.25±2.01	0.06
1 vs 9	****	<0.0001	8	5.64±9.90 vs. 1.33±1.44	0.76
1 vs 10	**	0.0012	9	2.64±3.47 vs. 4.33±10.71	-0.24
1 vs 11	*	0.0208	10	4.45±5.65 vs. 1.67±2.81	0.66
1 vs 12	****	<0.0001	11	5.73±10.82 vs. 2.67±3.50	0.43
1 vs 13	****	<0.0001	12	0.45±0.93 vs. 0.67±1.37	-0.18
1 vs 14	****	<0.0001	13	1.27±2.05 vs. 1.33±1.87	-0.03
1 vs 15	****	<0.0001	14	1.36±3.32 vs. 1.33±1.97	0.01
1 vs 16	****	<0.0001	15	2.82±6.48 vs. 0.33±0.49	0.71
1 vs 17	****	<0.0001	16	1.82±3.28 vs. 0.75±1.48	0.45
1 vs 18	****	<0.0001	17	1.82±2.89 vs. 0.58±0.67	0.69
1 vs 19	****	<0.0001	18	2.09±3.83 vs. 3.08±9.15	-0.15
1 vs 20	****	<0.0001	19	0.36±0.50 vs. 1.42±3.15	-0.58
-	-	-	20	1.45±2.38 vs. 3.08±4.74	-0.46

Table 1 – Descriptive statistics of the within-cohort comparisons of the LNB (large nest building) cohort (no significant between-trial differences were demonstrated for the normal NNB cohort) and the between-cohort (LNB vs NNB) comparisons of operant responses made during experiment 1. SD - standard deviation; $p < 0.05$.

Table 2: Descriptive statistics of experiment 2

<i>Trial (LNB vs NNB)</i>	<i>Mean number of operant responses$\pm$SD (LNB vs. NNB)</i>	<i>Cohen's d</i>
1	7.83 $\pm$ 7.99 vs. 2.67 $\pm$ 2.50	0.99
2	1.67 $\pm$ 1.75 vs. 8.83 $\pm$ 19.29	-0.68
3	2.00 $\pm$ 3.10 vs. 1.17 $\pm$ 1.94	0.33
4	3.18 $\pm$ 4.45 vs. 0.17 $\pm$ 0.41	1.24
5	2.50 $\pm$ 4.72 vs. 0.17 $\pm$ 0.41	0.91
6	2.67 $\pm$ 4.68 vs. 1.33 $\pm$ 1.37	0.44
7	1.67 $\pm$ 2.25 vs. 0.33 $\pm$ 0.82	0.87
8	2.17 $\pm$ 2.99 vs. 1.67 $\pm$ 1.37	0.23
9	5.00 $\pm$ 7.27 vs. 1.00 $\pm$ 1.55	0.91
10	6.00 $\pm$ 7.24 vs. 0.67 $\pm$ 1.21	1.26
11	9.50 $\pm$ 17.09 vs. 7.50 $\pm$ 10.07	0.15
12	3.00 $\pm$ 4.52 vs. 2.00 $\pm$ 1.67	0.32
13	4.33 $\pm$ 4.55 vs. 1.33 $\pm$ 1.75	0.95
14	3.67 $\pm$ 5.09 vs. 2.17 $\pm$ 2.04	0.42
15	2.83 $\pm$ 2.86 vs. 1.50 $\pm$ 2.51	0.50
16	2.67 $\pm$ 3.01 vs. 1.67 $\pm$ 3.14	0.33
17	2.50 $\pm$ 2.74 vs. 5.00 $\pm$ 6.78	-0.53
18	1.67 $\pm$ 2.07 vs. 1.83 $\pm$ 1.72	-0.09
19	2.67 $\pm$ 2.42 vs. 0.67 $\pm$ 1.21	1.10
20	1.50 $\pm$ 1.52 vs. 1.00 $\pm$ 1.26	0.36

Table 2 – Descriptive statistics of between-cohort (LNB vs NNB) comparisons of operant responses made during experiment 2. SD - standard deviation.

Figure 1. Study layout of experiment 1 (A) and experiment 2 (B).

Figure 2. Expression of nesting activity during pre-selection. (A) Correlation between the total nesting score (g) and coefficients of variance for each individual animal. LNB (large nest building) animals were selected from the blue circle (highest score/lowest individual variance) while NNB (normal nest building) animals selected were from the green circle (lowest use/lowest variance). (B) Comparison of the average expression of nest building in the selected LNB ($n = 17$) and NNB ($n = 18$) cohorts. Unpaired student t-test; **** $p < 0.0001$; data represented as mean $\pm$ SEM.

Figure 3. (A) Devaluation and response to escitalopram (Exp. 1): Average number of operant responses made by the LNB (large nest building; $n = 11$) and NNB (normal nest building; $n = 12$) cohorts across 20 trials (Trial 1 - 20). Trials 1 - 10 represent the pre-treatment testing scores, while trials 11 - 20 represent post-treatment test scores. Arrows indicate the beginning of the withdrawal phase (WP; trials 8 - 10 and 18 - 20), from which time operant cartridges did not release nesting material upon a successful operant response. 2-Way Repeated Measures ANOVA, Trial 1 LNB vs. NNB, *** $p = 0.0003$. Data represented as mean $\pm$ SEM. (B) Comparison of the average number of lever-presses per subject per cohort across 10 pre-treatment and 10 post-treatment trials. 2-Way Repeated Measures ANOVA, * $p = 0.0218$. Data represented as mean $\pm$ SEM.

Figure 4. (A) Punishment learning and response to escitalopram (Exp. 2): Average number of operant responses made by the LNB (large nest building; $n = 6$) and NNB (normal nest building; $n = 6$) cohorts across 20 trials (Trial 1 - 20). Trials 1 - 10 represent the pre-treatment testing scores, while trials 11 - 20 represent post-treatment test scores. Arrows indicate the beginning of the punishment phase (PP; trials 8 - 10 and 18 - 20), from which time voluntary foot shocks had been delivered. Data represented as mean $\pm$ SEM. (B) Comparison of the average number of lever-presses per subject per cohort across 10 pre-treatment and 10 post-treatment trials. Data represented as mean $\pm$ SEM.

6. Conclusion

Abbreviations used in Chapter 6 (in order of appearance)

<i>O/C(s)</i>	–	<i>obsessive-compulsive/obsessions and compulsions</i>
<i>OCD</i>	–	<i>obsessive-compulsive disorder</i>
<i>MBT</i>	–	<i>marble-burying test</i>
<i>MB</i>	–	<i>marble-burying</i>
<i>LNB</i>	–	<i>large nest building</i>
<i>NNB</i>	–	<i>normal nest building</i>
<i>HMB</i>	–	<i>high marble-burying</i>

6.1. Summary of key findings

The current work contributed to the body of literature pertaining to the pre-clinical modeling and assessment of obsessive-compulsive (O/C)-like behaviors in rodents by applying the deer mouse model of obsessive-compulsive disorder (OCD; Guldenpfennig et al., 2011; Wolmarans et al., 2013; Wolmarans et al., 2016a, 2016b, 2017). Firstly, the marble-burying test (MBT), commonly applied as a model of or a screening test for compulsive-like behavior, was dissected in terms of methodology. The results of this investigation, which were presented in Chapter 3, broadly highlighted the significant influence of a number of methodological manipulations on both the burying behavior of deer mice and the quantification of burying outcomes. Secondly, we performed a critical review of marble-burying (MB) investigations conducted prior to 2017 (Chapter 4) and concluded that given the drastic variation in its application, research has gained very little in terms of our understanding of OCD. Lastly, we aimed to appraise another compulsive-like phenotype expressed by deer mice, i.e. large nest building (LNB), against the background of an inflated motivational drive to nest. This investigation (Chapter 5) sought to establish whether the seemingly goal-directed expression of LNB will persist in the wake of changing outcome feedback and was designed in an attempt to interrogate the potential cognitive processes underlying the expression of highly planned, but excessive behaviors. Such approaches are crucial, since studies of animal behavior can provide useful insights into psychiatric disorders, provided that every care is taken to emulate the given disorder as closely as is reasonably possible. Indeed, it is important that the methodologies employed in such investigations be continually refined so that the modelling of ‘abnormal’ animal behaviors and the testing thereof be contextually relevant.

* * *

Our investigation into the influence of methodological manipulation in the MBT (Manuscript A; Chapter 3) was prompted by observations from our own laboratory (Wolmarans et al., 2016b) and others (Slot et al., 2008; Umathe et al., 2012; Egashira et al., 2013) that while being widely used, the assay as a whole is characterized by inherent conceptual flaws which arguably undermine any deductions made from its application, particularly with respect to modeling of or screening for compulsive-like behaviors of a purportedly thoughtful, highly goal-directed origin (Alonso et al., 2015). Chief amongst these was the prevailing assertion that the simple quantification of a number of marbles 'buried' in a single test session is sufficient to infer a dedicated preoccupation with the act of burying marbles; this without any attempt to control for other possible confounds, e.g. the incidental settling of marbles during cage exploration (Nicolas et al., 2006; Egashira et al., 2007; Egashira et al., 2013). Further, since the test is *subjectively* scored by investigators, we aimed to demonstrate how scoring data can vary between observers. To this extent, we manipulated two aspects of the MBT that vary significantly between different laboratories, i.e. burying substrate and zone configuration, while employing two observers to quantify the burying responses. As such, we not only demonstrated that observers score identical test cages differently, but also that the nature of the burying substrate used has a significant effect on the type of burying response elicited. Irrespective, the MBT is applied in various laboratories according to vastly different methodologies; however, deductions are made pertaining to the same supposed neuropsychological constructs, i.e. O/C- and anxiety-like responses. Considering the MBT being entirely subject to subjective interpretation, methodological manipulation and observer bias, it is remarkable how the collective of MB research is proceeding without consideration of various methodological constraints. In this regard, the findings presented in Chapter 3 contributes to the broad base of MB literature by illustrating the importance of sound experimental design, that will contribute to the execution of studies which may better elaborate on our understanding of the modeled conditions.

* * *

Following from the completion of the work presented in (Chapter 3; Manuscript A), we embarked on a critical review of all available MB research conducted prior to 2017. This review, published in *Cognitive, Affective and Behavioral Neuroscience*, is presented in (Chapter 4; Manuscript B). The major conclusions drawn from this review was that 1) the MBT is applied equally often as a test for compulsive- and anxiety-like behavior, 2) the assessment of MB is characterized by drastic within- and between-laboratory variation with respect to a number of experimental parameters, 3) little to no modification is made when the test is applied to investigate OCD or anxiety, both of which reflect key applications of the assay despite being two rather unique constructs, and 4) taking into account its response to a range of

pharmacological interventions, its translational utility for the broader neuropsychiatric research community is questionable. One aspect that this work highlighted which is of particular importance for the testing and modeling of OCD, is the ubiquitous application of a single testing session following acute drug treatment for the quantification and characterization of so-called ‘compulsive-like behaviors’ and anti-compulsive drug effects, which stands in obvious contention of modelling compulsive-like persistence.

In addition to the arguments made throughout this review, and taking into account the experimental findings that arose from the work presented in Manuscript A, the review also concluded with a number of recommendations that should guide future applications of the MBT as a measure of both compulsive- and anxiety-like behaviors, thereby strengthening the practical utility of the test as a whole. These included calls to standardize the pre-exposure procedures, test cage sizes, marble size and number, zone configuration, scoring procedures, burying substrate, behavioral scoring.

* * *

Moving away from investigations into the methodological validity of currently employed techniques, we also aimed to assess a repetitive and persistent behavioral phenotype, i.e. LNB, commonly argued to be reminiscent of compulsive-like behavior (Greene-Schloesser et al., 2011; Mitra et al., 2016; Wolmarans et al., 2016a; Mitra et al., 2017), in terms of its psychobiological construct. We have previously shown that 30% of the deer mouse population (with no specific or selective breeding intervention) express LNB and that such behavior, but *not* normal nest building (NNB) is responsive to chronic high-dose oral escitalopram treatment (Wolmarans et al., 2016a). The current work (Chapter 5; Manuscript C) further confirmed LNB as a distinctive behavioral aberrancy in the laboratory housed deer mouse population (see also Addendum G). That said, considering the highly planned and goal-directed (albeit purposeless) expression of LNB, we believe this phenotype can present research with a useful avenue for investigating the psychobiology, i.e. processes more akin to cognitive mechanisms, that may underlie its expression. Indeed, we asked the question: Why do some animals, despite being housed under identical circumstances to their conspecifics, engage in such energy-taxing and needless behavior? To explore this question, we designed a novel test paradigm in which animals had to execute operant actions in order to obtain nesting material on their own and without prior training. We did so because it allowed us to manipulate the ultimate goal as well as the feedback associated with the execution of nesting material-seeking behavior. Briefly, the manuscript describes the implementation of a lever-press paradigm that allowed mice to obtain nesting material. We further demonstrated that although both NNB and LNB expressing mice could acquire the contingent-lever-pressing response, LNB subjects

displayed a greater overall *drive* to engage in lever-pressing compared to their normal nest building counterparts. This was noted even when the outcome contingency was modified by removing the reward or when the contingent action was *punished* by means of a weak self-administered electric footstock. Moreover, our finding that such persistent and pronounced engagement in lever-pressing is normalized by chronic high-dose escitalopram, is congruent with clinical literature (Palminteri et al., 2012). Not only do we demonstrate that LNB expressing mice potentially present with an inflated motivational drive to engage in nesting behavior despite the negative consequences of such engagement, we also show that the currently employed first-line treatments for OCD, e.g. selective serotonin reuptake inhibitors like escitalopram, potentially also acts to modify such cognitive constructs. Furthermore, the construct of HMB and LNB behavior is currently being investigated (Addendum C). Regardless, the investigation reported in Chapter 5 arguably adds value to ethological studies, as it goes to show that by examining *naturally aberrant behaviors* in the face of changing outcome contingencies, we are able to gain insight into the cognitive processes which may potentially underlie behaviors which deviate from the typical.

* * *

In conclusion, the current study set out to critically examine and interrogate the way in which pre-clinical literature seeks to answer crucial questions relating to translational aspects of human neuropsychiatric disease. We did so by investigating behaviors naturally expressed by deer mice. First, this work brought valuable progression to the scientific literature base describing the MBT, which still offers substantial value as an assay for anti-obsessional drug effects despite its arguably flawed implementation in the past. Apart from and critically dissecting and arguing against the application of burying behavior as a mirror for human compulsion and anxiety, we provide valuable directives for future research. Indeed, with minor modification, much more can be gained from this simple, cost-effective and relatively uncomplicated behavioral test. Last, we explored how naturally developing, goal-directed behavior that clearly deviates from the norm and which is borne from within an evolutionary background, can be explored on a psychobiological level. In fact, the analysis of nesting behavior by means of the novel lever-pressing infrastructure developed here, represents a new direction for basic research. What makes this approach especially unique, is that it was designed to target *naturally occurring* behavioral deviations, rather than initiating such behavior by means of training or another intervention, which is arguably preferable for studies that ask fundamental ethological questions.

Summarized Study Question	Final Outcome(s)
1.1) How does the marble-burying performance of deer mice and the quantification thereof respond when tested repeatedly under circumstances of methodological manipulation?	The outcomes of the MBT are significantly affected by the type of burying substrate used, zone configuration and repeated exposure. Marbles spaced on sparser substrates like sawdust, as opposed to heavier substrates like course river sand, settle to the bottom of the arena quicker. Further, sparse substrates facilitate naturalistic burrowing behavior which ultimately causes coincidental coverage of marbles. As these behaviors are not related to the goal-directed preoccupation with marbles, they should not be considered as being indicative of compulsive burying.
1.2) How is MBT scoring influenced by observer bias?	Applying the scoring criteria most often employed, i.e. marbles being considered as buried if only two thirds of their size is covered in burying substrate, observers scores were remarkably different. This is especially true for burying experiments conducted in sparse substrates, as these allow for the settling of marbles without any significant and purposeful burying behavior. In fact, the scores of observers differed less in heavier substrates, e.g. course river sand. As such, we conclude the MBT to be highly manipulatable and subject to observer bias but underscored by methodological inconsistencies.
1.3) What constitutes <i>abnormal</i> behaviors beyond a <i>normal</i> behavioral repertoire?	With respect to OCD, abnormal behaviors viewed against the background of the otherwise 'normal' behaviors expressed by the larger population of animals should be appraised based on voluntary goal-directed engagement with marbles that persist over the course of several trials. This is especially important as <i>all</i> rodents express digging, burrowing and burying behavior.

Following from the literature review (study question 2.1), is the MBT as reported adequate, relevant and accurate for studies into O/C- or anxiety-like behavior? If not, what are the major aspects that undermine its usefulness (study question 2.2) and how should studies that apply the MBT be applied and interpreted in future in order to better elaborate on our understanding of OCD?

The reported literature concerning the MBT varies widely in terms of a number of experimental variables, leading to a test which is poorly standardized. Furthermore, treatment protocols and test parameters often used to test for anti-compulsive or anxiolytic effects are typically inappropriate. The review further concludes with a valuable directive for future research into studies related to both compulsivity and anxiety.

3.1) Will differences in lever-pressing behavior directed at the acquisition of nesting material exist between NNB and LNB animals?

Indeed, although animals of both cohorts engaged in lever-press behavior, LNB animals did so to a greater extent and therefore obtained more nesting material compared to NNB controls.

3.2) Will the lever-pressing behavior of NNB and LNB expressing animals differ under circumstances of reward (nesting material) withdrawal and contextual punishment?

Data demonstrated a remarkable difference in the lever-press responses of LNB, compared to NNB animals. Although these differences were not as striking following the withdrawal of nesting material upon lever-presses, LNB animals endured more punishment in order to obtain nesting material, while the lever-press behavior of NNB animals demonstrated immediate reductions. The behavior of LNB animals is indicative of an inflated motivational drive to engage in compulsive-like rituals despite the negative outcomes resulting from such behavior.

3.3) Will chronic high dose oral escitalopram modify the behavioral responses of LNB, but not NNB animals as potentially highlighted in 3.1 and 3.2?

Escitalopram reversed the excessive drive to engage in lever-pressing behavior despite the punishing feedback introduced by electric foot shock. Apart from attenuating LNB behavior, serotonergic intervention also results in the improvement of punishment feedback processing. This is in line with clinical literature and adds to our current understanding of the motivational triggers of LNB.

6.2. Shortcomings and future studies

While the study questions answered above added knowledge to the field of ethological investigations into psychiatric disorders, there are a number of areas where the various investigations could be refined and improved or further investigated. As Manuscript B is a critical review in itself, this section will briefly focus on the work presented in Manuscripts A and C, as well as the preliminary results reported in Addendum C.

* * *

With respect to Manuscript A (Chapter 3), marble-burying was investigated in an environment which introduced as few as possible instigators for digging, so as to measure specific marble-directed digging. To this end, we pre-exposed the subjects to the burying substrates before testing, so as to prevent novelty induced digging. This was indeed a step in the right direction. However, one of the reviewers of this paper pointed out the importance of odor to rodents (Addendum D). Therefore, the methodology could be refined by pre-exposing each mouse to its own test cage some time before testing so that their own burying substrate is familiar to them at the time of testing, further eliminating novelty induced digging. In general, the reviewers also had a particularly strong stance regarding examining marble-burying data with sex as factor (see Addendum D). Therefore, it will be of value to replicate the experiment, perhaps with larger group sizes (as was also suggested) and analyze the data with sex as a main factor. That said, for this investigation we opted not to go this route, as we position the deer mouse model as one that is reminiscent of sex-non-specific OCD. Indeed, HMB occurs without sex bias in a small minority of deer mice.

* * *

The data presented in Manuscript C (Chapter 5) are undoubtedly of a preliminary nature. Further, given that this was the first application of this methodology, many avenues for improvement exist, alongside many potential follow-up investigations. One of the primary problems we had to overcome was to find a material which was robust enough to be fed through the dispensers while also being desirable to the mice. The cotton rope which was ultimately employed was possibly too tough to be used to easily fashion a nest, which potentially deterred nesting engagement relative to the easy-to-use cotton wool used during the pre-screening phase. Therefore, a more suitable material may foreseeably improve outcomes. Further, the data generated during the study indicated that certain individuals, regardless of cohort, tended to exhibit many more lever-presses than their peers. It is therefore possible that excessive lever-pressing is a distinct compulsive-like phenotype. This reveals two primary possibilities for future investigation. Animals could potentially be pre-

selected on the basis of their engagement in lever-pressing during a short screening exposure to the lever-press apparatus, or lever-pressing could be investigated by delivering some other type of incentive through the dispensers. This is especially true since we did not employ a non-rewarded phase at the beginning of the experiments, which excludes a definitive conclusion in terms of whether mice in actual fact learned to press levers *to obtain a reward*. To this extent, it would also be of value to perform a study where two dispensers are provided to each test subject, one of which would be inactive (not deliver any material upon a lever-press) so as to measure whether nesting material is indeed a motivator for increased engagement with the lever versus an ‘unrewarded’ lever. Furthermore, while the current work was indeed aimed at furthering the development of the deer mouse model of OCD, it must be kept in mind that different species of *Peromyscus* present with notably different average nesting activity levels (Lewarch and Hoekstra, 2018), and therefore that a different species, perhaps even outside the genus, may represent a superior model organism for such investigations.

* * *

The preliminary results reported in Addendum C remain relevant, considering that HMB and LNB have been well validated in terms of face and predictive validity, but that construct validity is still lacking. Therefore, it will be useful to quantify the expression of receptors which are likely effector sites of treatment, such as 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, D₁, and D₂ (Goddard et al., 2008). To this end, continued studies concerning 5-HT_{1A} expression are underway. The successful completion of this work is especially important since LNB, and not HMB, responds to chronic high dose oral escitalopram treatment.

*** * * THE END * * ***

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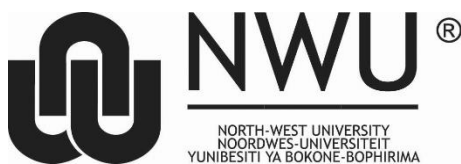
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Addendum A - Co-authors Letters of Consent



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13 September 2019

Dear examiner,

As study leader and senior corresponding co-author on three manuscripts first authored by Mr. Geoffrey de Brouwer, I hereby approve that the manuscripts listed below be included as part of the requirements of his PhD degree, and that these manuscripts may be submitted for examination of Mr. de Brouwer's thesis.

The articles are as follows:

Manuscript A

"Back to basics: A methodological perspective on marble-burying behavior as a screening test for psychiatric illness"

Published in *Behavioural Processes* (2018)

Manuscript B

"A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: Mapping the way forward"

Published in *Cognitive, Affective, & Behavioral Neuroscience* (2019)

Manuscript C

*"Naturalistic operant responses in deer mice (*Peromyscus maniculatus bairdii*) and its response to outcome manipulation and serotonergic intervention"*

Accepted for publication in *Behavioural Pharmacology*

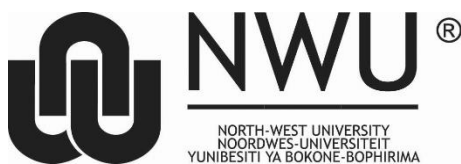
A letter of confirmation for each respective submission is included in addendum B.

I trust that all is in order.

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13 September 2019

Dear examiner,

As co-author on two manuscripts first authored by Mr. Geoffrey de Brouwer, I hereby approve that the manuscripts listed below be included as part of the requirements of his PhD degree, and that these manuscripts may be submitted for examination of Mr. de Brouwer's thesis.

The articles are as follows:

Manuscript B

"A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: Mapping the way forward"

Published in *Cognitive, Affective, & Behavioral Neuroscience* (2019)

Manuscript C

*"Naturalistic operant responses in deer mice (*Peromyscus maniculatus bairdii*) and its response to outcome manipulation and serotonergic intervention"*

Accepted for publication in *Behavioural Pharmacology*

A letter of confirmation for each respective submission (chapters 4-5) is included in addendum B.

I trust that all is in order.

Prof. Brian H. Harvey, PhD

Co-author

North-West University, Potchefstroom, South Africa

Addendum B - Confirmations of Submission/Publication

Manuscript A - *Behavioural Processes*

*This communication was formatted to better fit the style of this thesis without content changes.
Original correspondence available upon request.*

Date: Mon, 16 Apr 2018 10:11:58

Subject: Your manuscript BEPROC_2017_304_R2 has been accepted

Ref: **BEPROC_2017_304_R2**

Title: Back to Basics: A Methodological Perspective on Marble-Burying Behavior as a Screening Test for Psychiatric Illness

Journal: Behavioural Processes

Dear Dr. Wolmarans,

I am pleased to inform you that your paper has been accepted for publication. I appreciated your careful response to the reviewers' concerns and I look forward to seeing your work in print.

Your accepted manuscript will now be transferred to our production department and work will begin on creation of the proof. If we need any additional information to create the proof, we will let you know. If not, you will be contacted again in the next few days with a request to approve the proof and to complete a number of online forms that are required for publication.

Thank you for submitting your work to Behavioural Processes. We hope you consider us again for future submissions.

Kind regards,

Olga Lazareva

Editor-in-Chief

Behavioural Processes

Manuscript B - Cognitive, Affective, and Behavioral Neuroscience

*This communication was formatted to better fit the style of this thesis without content changes.
Original correspondence available upon request.*

27-Sep-2018

Dear Dr Wolmarans,

It is my pleasure to accept your manuscript **CABN-TR-18-207.R1** entitled "*A critical inquiry into marble burying as a preclinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: Mapping the way forward*" in its current form for publication in Cognitive, Affective, and Behavioral Neuroscience. Your manuscript has been sent to the Journal's Production Department and you can expect to receive proofs within approximately three weeks. You will have the opportunity to proofread your article and make minor corrections at that time if necessary.

If you think your article may be of interest to a broader scientific audience, please email Dr. Stephan Lewandowsky, stephan.lewandowsky@bristol.ac.uk, the Digital Content Editor of the Psychonomic Society. Dr. Lewandowsky may decide to do a featured blog post on your article for the society website. Furthermore, if your article might appeal to a general lay-person audience, Dr. Lewandowsky will forward it to the press team at Springer for a possible press release.

Thank you for your fine contribution. On behalf of the Editors of Cognitive, Affective, and Behavioral Neuroscience, we look forward to your continued contributions to the Journal.

Cognitive, Affective, and Behavioral Neuroscience is a journal of the Psychonomic Society. If you are not already a member of the society, we encourage you to join. For more details about the benefits (and low cost) of membership, please visit <http://www.psychonomic.org/>

Sincerely,

Stan Floresco

Action Editor, Cognitive, Affective, and Behavioral Neuroscience

Editor:

Marie T. Banich

This communication was formatted to better fit the style of this thesis. Extraneous information was removed. Original correspondence available upon request.

Nov 07, 2019

RE: BP-19-153R1, entitled "Naturalistic operant responses in deer mice (*Peromyscus maniculatus bairdii*) and its response to outcome manipulation and serotonergic intervention"

Dear Dr. Wolmarans,

I have received the reports of the two initial reviewers on your revised manuscript that you can find below. Since the opinions of these reviewers widely diverged (one being satisfied with your response; the other being not convinced and having major remaining concerns), I have consulted a third reviewer. As you can see, the third reviewer agreed with the more positive review. Therefore, I am pleased to let you know that your manuscript should be acceptable for publication in Behavioural Pharmacology after appropriate revision.

Please address both concerns of Reviewer #2 in your revision. For this, you can follow the recommendations made by Reviewer #3, by addressing these points in the Discussion of your manuscript. Point 1: It would be helpful if you could include the data you show in the rebuttal from your submitted study in the present paper in some form, but this should at the very least be mentioned in the Discussion. Point 2: Please also address in the Discussion. In fact, are you sure that 'p-values of between-group differences before escitalopram treatment are all >0.9999' - meaning that they all approached $p=1$?

Please include with your revised submission an itemized, point-by-point response to the comments of the reviewers. The revisions should be completed by Feb 05, 2020 to avoid being considered as a new submission.

Finally, if you haven't already done this, please add your twitter handle to your address on your manuscript's title page. This will help us promote your article!

With kind regards,

Prof Louk J.M.J. Vanderschuren

Editor

Behavioural Pharmacology

Addendum C - Preliminary results:
Prefrontal-cortical 5-HT_{1A} receptor-
binding

Introduction

In an attempt to divulge the potential neurobiological differences which may potentially underlie the different behavioral phenotypes explored in this thesis, i.e. large nest-building (LNB) and high marble-burying (HMB), it will be of value to characterize the prefrontal-cortical 5-HT_{1A} receptor expression density in both of these cohorts, as well as in normal control subjects, viz. animals that do not express either of the mentioned behaviors. Contained in this addendum is a brief summary of literature which prompted and informed the design of the work presented in this addendum, a detailed description of the experimental methods which were applied, and the preliminary results. Lastly, we will consider the possible factors that potentially contributed to the data reported here and make recommendations for future improvement and elaboration. This work is not intended for assessment.

* * *

Obsessive-compulsive disorder (OCD) is a disabling chronic condition affecting up to 2-3% of the population (Murray and Lopez, 1996; Kessler et al., 2009; Ruscio et al., 2010). The disorder is characterized by two distinct symptoms i.e. obsessions, which are intrusive mental images that are typically associated with affective outcomes such as anxiety and rumination (American Psychiatric Association, 2013; Stewart, 2016), and compulsions which can be regarded as seemingly purposeless, repetitive and persistent behavioral routines (American Psychiatric Association, 2013; Veale and Roberts, 2014).

Much work has been done to elucidate the possible neurobiological mechanisms underlying the condition (Wood and Ahmari, 2015). At a causal level, observations of the efficacy of drugs modulating serotonin (5-HT), such as selective serotonin reuptake inhibitors (SSRIs; Stewart, 2016; Hirschtritt et al., 2017), have convincingly implicated serotonergic hypofunction, in the development of symptoms (Goddard et al., 2008). Closely related to this is dysregulated dopaminergic (DA) functioning (Denys et al., 2004; Westenberg et al., 2007; Markarian et al., 2010). Typically, these two neurotransmitters interact at both physiological (Westenberg et al., 2007; Markarian et al., 2010; Cools et al., 2011) and neurocognitive levels (Boureau and Dayan, 2011; Cools et al., 2011), both of which have important implications for the genesis and maintenance of OCD-like behavioral expression.

From an anatomical perspective, attention has been focused on the cortico-striatal-thalamic-cortical (CSTC) circuitry (Di Filippo et al., 2009; Milad and Rauch, 2012; Harrison et al., 2013; Wood and Ahmari, 2015; Ahmari, 2016; Manning, 2016). Indeed, the CSTC circuitry has been shown to regulate a number of the maladaptive cognitive processes implicated in OCD (Milad and Rauch, 2012; Wood and Ahmari, 2015; Gruner and Pittenger, 2017). With respect to the

interactions between the serotonergic system and the CSTC circuitry, serotonergic neurons originate primarily from a large population of fibers named the raphe nuclei (RN), projecting globally to forebrain regions, including the anatomical components of the CSTC circuits (Hornung, 2003; Fink and Göthert, 2007). Specifically, and with regards to the modulation of DA release, serotonergic fibers project to the substantia nigra and ventral tegmental area as well as their subsequent projection areas, including the striatum, nucleus accumbens and prefrontal cortex (Fink and Göthert, 2007). Here, the DA outflow is modulated to varying extent by the action of specific 5-HT receptors. Serotonin receptors include a large number of subtypes belonging to seven families, i.e. 5-HT₁₋₇ (see Barnes and Sharp (1999) for review). OCD is rather unique in terms of its response to SSRIs, since therapeutic changes are typically only observed after periods of ten to twelve weeks of treatment (Nutt et al., 1999; Goddard et al., 2008; Hirschtritt et al., 2017) at doses that are significantly higher than those normally employed in the treatment of other psychiatric conditions, including major depression (Goddard et al., 2008; Stewart, 2016). Such response led to mechanistic theories focused on the role of long-term changes in synaptic expression of specific receptors, both in serotonergic and other systems, to explain the therapeutic response of OCD to SSRI intervention (Benmansour et al., 1999; Kugaya et al., 2003; Blier and El Mansari, 2007; Koo et al., 2010). In addition to changes in serotonin transporter (SERT) expression, chronic SSRI treatment also influences the expression of 5-HT_{1A} receptors (Descarries and Riad, 2012), believed to be a major effector of SSRI treatment efficacy (Goddard et al., 2008). The 5-HT_{1A} receptor is a G_i-coupled receptor expressed both somatodendritically on pre-synaptic neurons as well as post-synaptically (Barnes and Sharp, 1999; Goddard et al., 2008; Descarries and Riad, 2012). Activation of the receptor results in cellular hyperpolarization (Barnes and Sharp, 1999). When located pre-synaptically, inhibitory processes initiated by the 5-HT_{1A} receptor generally prevent the outflow of 5-HT and other neurotransmitters, e.g. DA either directly or indirectly (Fink and Göthert, 2007; see also chapter 2, section 2.2). The receptor has been rather extensively mapped and found to be highly expressed in dorsal and medial RN, cortical and hippocampal regions (Barnes and Sharp, 1999).

That said, the fact that not all phenotypes of OCD respond equally well to treatment (Thorsen et al., 2018), was proof-of-concept for this planned trajectory of the work presented in this thesis. Indeed, we aimed to establish whether HMB, a SSRI-resistant phenotype (Wolmarans et al., 2016a), would present with unique 5-HT_{1A} receptor expression patterns compared to animals expressing LNB, which is indeed treatment-sensitive (Wolmarans et al., 2016b), thereby gaining more insight into the role of different baseline neurobiological footprints in the treatment response of compulsive-like behavior. Indeed, the deer mouse model is a robust,

well validated, naturalistic animal model of symptom heterogeneous OCD, which lends itself toward research relating to the nature of different OC-like symptom phenotypes.

Therefore, to investigate whether different densities of 5-HT_{1A} receptors in the prefrontal cortex associate with LNB and HMB behavior respectively, a radioactive ligand-receptor binding assay was applied (De Jong et al., 2005; Oikonen, 2013) where the competitive binding of a specific analyte (cold ligand; which binds all receptors in a family) is compared to that of a radiolabeled, receptor specific ligand (hot ligand). The binding of the hot ligand can be quantified by counting the latent radioactivity of the ligand bound to receptors in a tissue sample. The radiolabeled ligand (RAL) that we chose for this purpose was tritiated 8-hydroxy-DPAT, while the cold ligand used was serotonin.

Methods

Animals

27 adult deer mice (*Peromyscus maniculatus bairdii*), aged at least 10-12 weeks were obtained from the colony maintained at the North-West University vivarium. These were behaviorally screened according to the methods below. Selected mice were divided into one of three behavioral cohorts (each $n = 9$) normal behavior (control cohort; CC), LNB cohort and HMB cohort, from an initial pool of 60 mice were used in the work reported here. Mice were housed in cages measuring 350 (*l*) x 200 (*w*) x 130 (*h*) mm (Techniplast® S.P.A., Varese, Italy) on corncob bedding material with unrestricted access to food and water unless otherwise stated. The facility was maintained at 23±1°C on a twelve-hour light/dark cycle, with lights switched on and off at 06h00/18h00. Mice (10 weeks old at the onset of behavioral screening tests), were housed in same sex litters of 5 – 6 mice per cage, until the onset of the nest building (NB) and marble-burying (MB) screening tests. At this point in time, mice were individually housed as necessitated by the methods discussed below. All procedures were ethically approved by the AnimCare Animal Research Ethics Committee of the NWU (registration number: AREC-130913-015, project approval number: NWU-00261-16-A5).

Nest Building

As LNB is only expressed by 30% of the deer mouse population, 30 mice were initially screened for nest building (NB) behavior by providing them with an excess of pre-weighed, unscented cotton wool in the ceiling grids of each of the individual mouse housing cages at approximately 09h00 each day for 7 consecutive days. On each subsequent morning, built nests were removed and the cotton wool remaining in the ceiling grid weighed and supplemented with additional material. This process was carried out over eight days, allowing

for seven consecutive nights of nest building analyses. Animals were then assigned to the large nest building (LNB), based on the total cotton wool used and the consistency of building activity throughout these seven nights (for a detailed review of this methodology, please refer to Chapter 5). To ensure adequate separation between the three groups, all mice, including the 9 LNB expressing subjects, plus another 30 animals underwent marble burying screening (see below).

Marble-Burying Screening

As only 11 – 15% of deer mice present with HMB, we screened 60 mice in total (including the 30 animals screened for nest building above). The marble burying test (MBT) was carried out according to a previously published method with minor modifications (Wolmarans et al., 2016a; de Brouwer and Wolmarans, 2018). Briefly, marble-burying cages (exact replicas of the home cages) were prepared with a 5 cm thick layer of coarse river sand on which nine glass marbles ($\varnothing = 15$ mm) were arranged in a 3 x 3 grid equidistant to one another in *one half* of the cage only, thereby constituting a two-zone MBT setup. This allowed mice to willingly and voluntarily engage with marbles while also ensuring that novelty-induced avoidance behavior could be identified. The use of coarse river sand, as opposed to sawdust or husk as often reported (Krass et al., 2010; Egashira et al., 2018), prevents marbles being unintentionally covered due to routine cage locomotion (de Brouwer and Wolmarans, 2018). Since deer mice are nocturnal animals, all marble-burying assessments were carried out from 1 hour after onset of the dark cycle under dim red light (40 lux).

On the morning of the day prior to the first marble-burying assessment, i.e. 36 hours before the first marble-burying assessment, mice were habituated to the burying substrate by replacing the corncob home cage bedding with coarse river sand; this was necessary to prevent novelty induced digging during the first MB trial (Handley, 1991; Gyertyan, 1995). Subsequently, each animal was assessed for MB activity over three nights in three separate 30-minute trials (one per night), separated by 24 hours. At the time of assessment, animals were removed from their home cages and introduced into pre-prepared marble-burying cages. Due to the highly active nature of deer mice, cages were covered with clear Plexiglass® covers to prevent animals from leaving the cages, whilst still allowing them to be videotaped. Mice were subsequently left to explore and interact with marbles for 30 minutes in the absence of human observers, while being videotaped for later analysis for specific marble-directed interactions. After each 30-minute session, animals were returned to their respective home cages which still contained the same burying substrate and left for 24 hours. This procedure was repeated twice over the next two days, until all animals underwent three separate screens.

To identify the 9 HMB expressing animals, the number of marbles covered in burying substrate to a depth at least two-thirds of the marble size (Wolmarans et al., 2016a) were quantified after the completion of each 30-minute screening session. This was done by two experimentally blind observers of whom the scores were averaged. Importantly, after the first three pre-treatment MB assessments had been completed, the 9 HMB-expressing animals were selected based on 1) the average number of marbles covered, 2) the persistence of burying behavior over three trials as evinced by lower coefficients of variance with respect to the between the daily burying scores *and*, 3) evidence of marble-directed activity (Wolmarans et al., 2016b; de Brouwer and Wolmarans, 2018).

Tissue Collection, Pooling & Preparation

Using the screening procedures described above, three cohorts of animals were selected. The control cohort (CC; $n = 9$) were those animals who expressed neither of the phenotypical behaviors. Two additional cohorts were selected for HMB behavior (HMB; $n = 9$) and LNB behavior (LNB; $n = 9$) scores respectively. None of the HMB and LNB expressing animals, also presented with the other behavioral phenotype.

Following the last behavioral screen, mice were euthanized by means quick decapitation. Subsequently, the brain was removed, the prefrontal cortex (PFC) removed on ice and flash frozen in liquid nitrogen. Samples were kept frozen at -80°C until the day of tissue preparation.

Due to the low mass of the brain tissues harvested from each animal, a single radioactive ligand binding assay required the pooling of PFC tissue of three animals (**Figure 1**), yielding approximately 60 mg of tissue per sample. Hence, the 9 animals included in each cohort, enabled three separate replications of the binding assay. Tissue homogenates were prepared by means of rapid homogenization (Brinkman Polytron® PT10/35in) in 10 ml of ice-cold, freshly prepared 50 mM TRIS buffer containing 4 mM CaCl_2 and buffered to a pH of 7.7 (two rounds of 5 seconds each). Next, the samples were transferred to ultra-centrifuge tubes and centrifuged at 49 000g for 10 minutes at 4°C . All subsequent instances of centrifuging employed these same parameters.

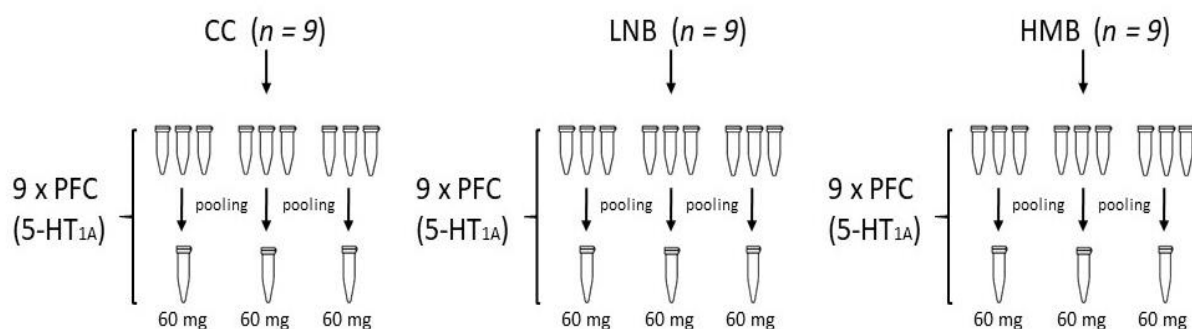


Figure 1. Tissue pooling procedure

Following the first round of ultra-centrifugation, the resultant supernatants were discarded and the remaining tissue pellets carefully dislodged. The specified volume of ice-cold buffer, now in a ratio of 10 ml / 10 mg tissue, was added to each tube once again. Then, the pellet with the buffer was transferred to a glass tube, for two slow bouts of homogenization via Teflon® bit rotation in a cold room maintained at 4°C. The resultant homogenate was then again transferred to the ultra-centrifugation tubes and recentrifuged as per the methodology mentioned above. This process was repeated once more. After the last round of ultra-centrifugation, the Teflon®-homogenates were incubated in a water bath maintained at 37°C for 10 minutes and then transferred to an Erlenmeyer flask for a final round of homogenization via Polytron®, resulting in the final homogenates diluted to 100 volumes.

Saturation Binding Assays

Buffer and Cold (non-radioactive) Ligand Solution Preparation

To prepare the 50mM/4mM TRIS/CaCl₂ buffer, which was used in all preceding steps as well as in the constitution the of final assays as described below, 39.4 g TRIS-hydrochloride and 2.2 g CaCl₂ was dissolved in a 5 L volumetric flask. The pH of the solution was raised to 7.7 by the step-wise addition of a concentrated NaOH solution.

To prepare the 20 µM serotonin solution, 850 µg of serotonin hydrochloride salt (Sigma-Aldrich; Johannesburg, South Africa) was dissolved in 10 ml of Milli-Q® water and mixed via Vortex® mixer for 2 minutes. This solution was used for all the assays.

Radioactive Standard Preparation

A series of 8 points containing increasing amounts of RAL ([³H] OH-DPAT; PerkinElmer; Massachusetts, USA); with specific activity 135.2 Ci/mmol) were prepared according to (**Table 1**). Note that 2 series of standards were prepared. The CC group was tested using one set of standards (**Figure 2A**) whereas the LNB and HMB cohorts were tested using the second set of standards (**Figure 2B**).

Table 1: Constitution of radioactive standards

Number	Point	RA Ligand (µl)	TRIS Buffer (µL)
1	0.2	2	198
2	0.7	7	193
3	1.2	12	188
4	2.0	20	180
5	5.0	50	150
6	10.0	100	100
7	15.0	150	50
8	20.0	200	0
	<i>Total</i>	<i>441</i>	

Total Binding Series

This comprised a series of 8 points (see **Table 2**) which were prepared by pipetting 20 µl TRIS buffer into a sealable vial. Next, 20 µl of the RAL ([³H] OH-DPAT) from the respective range of eight standard points (0.2 - 20 nM) in **Table 1** was added to the respective vials. Lastly, 360 µl of cortical homogenate was transferred by pipette, resulting in a final assay volume of 400 µl.

Non-Specific Binding Series

This comprised a series of 8 points (see **Table 2**) which were prepared by pipetting 20 µl of cold ligand (serotonin solution) into a sealable vial. Next, 20 µl of the RAL ([³H] OH-DPAT) from the respective range of eight standard points (0.2 - 20 nM) in **Table 1** was added to the respective vials. Lastly, 360 µl of cortical homogenate was transferred by pipette, resulting in a final assay volume of 400 µl.

Standard Series

This comprised a series of 8 points (see **Table 2**), each containing 4 ml of Filter-Count® scintillation fluid (PerkinElmer) scintillation fluid to which 20 µl of the radioactive ligand ([³H] OH-DPAT) from the range of eight respective standard concentration points (0.2 - 20 nM) in **Table 1** was added by pipette. Two standard curves were used, which are depicted in **Figure 2A** and **2B**.

Table 2: Constitution of the various series for the assay

Total binding series				
<i>Numbers</i>	<i>Points</i>	<i>RA-Ligand (µL)</i>	<i>TRIS Buffer (µL)</i>	<i>Homogenate</i>
1 - 8	0.2 - 20	20	20	360
Non-specific binding series				
<i>Numbers</i>	<i>Points</i>	<i>RA-Ligand (µL)</i>	<i>Serotonin sol (µL)</i>	<i>Homogenate</i>
1 - 8	0.2 - 20	20	20	360
Standard series				
<i>Numbers</i>	<i>Points</i>	<i>RA-Ligand(µL)</i>	<i>Scint. Liquid (mL)</i>	-
1 - 8	0.2 - 20	20	4	-

After preparation of these final test solutions, samples were incubated for sixty minutes at room temperature after which binding was terminated by means of vacuum filtration through Whatman GF/B® glass microfiber filters. Filters were rinsed twice with 4 ml of ice-cold buffer solution, removed and carefully placed into scintillation-counter tubes which were filled with 4 ml scintillation fluid (Filter-Count®). Samples rested in this state for three hours at room temperature before latent radioactivity trapped in the filters was counted using a liquid scintillation counter (PerkinElmer Tri-Carb 2810 TR®).

Statistics

Linear regressions of counts per minute (CPM) of the standard solutions were drawn (**Figure 2**) by plotting the respective concentration points (see **Table 1**) against the CPM.

The baseline corrected CPM values for the respective cohort homogenates (**Figure 3**) were calculated by subtracting the CPM of the respective total binding (TB) series from the corresponding non-specific (NS) binding series.

Results

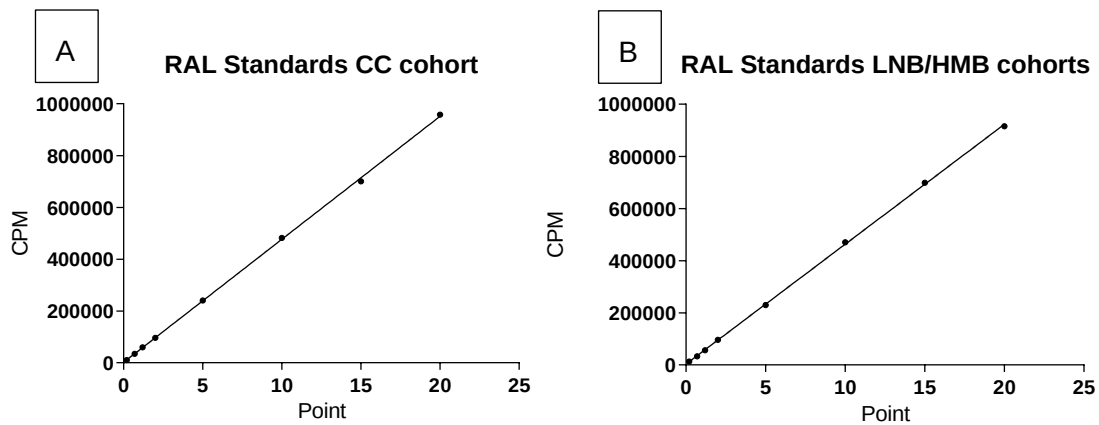


Figure 2. Standards used for the control cohort (CC; A) and the large nest building (LNB) and high marble-burying (HMB) cohorts (B). Data represented as linear regressions of the counts per minute (CPM) of the respective selected concentration points.

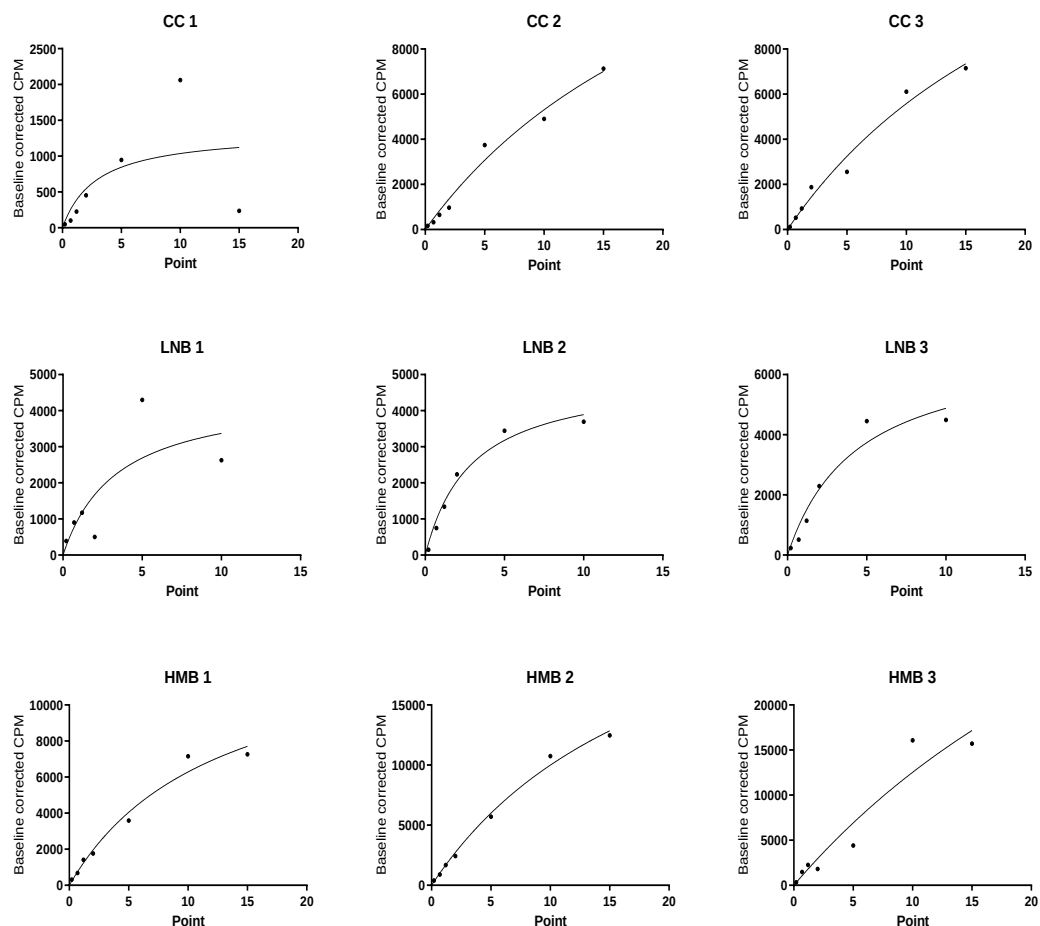


Figure 3. Non-linear fit of the difference in counts per minute (CPM) between the respective total binding and non-specific binding series for the three respective cohorts; CC - control cohort, HMB - high marble-burying cohort, LNB - large nest building cohort.

Due to the fact that the binding results in 4 of the 9 binding studies delivered ambiguous results, and that receptor saturation did not successfully occur, the dissociation constant of the RAL (K_d) could not be calculated and reported and therefore receptor quantification equations (B_{max}) could not be carried out.

Conclusion and Suggestions for Improvement

The two standard curves depicted in **Figure 2** showed accurate linear regressions (r^2 : **2A** = 0.0097; **2B** = 0.0098), indicating that the calculations for the amounts of RAL to be added were accurate, that pipette function and pipetting technique were up to standard and that the scintillation counter was functioning correctly. Therefore, we must conclude that the error must have been introduced at some other stage of the procedure. This could have happened at a variety of steps:

- *RAL ligand* ($[^3H]$ OH-DPAT) - Concentrations applied may have been insufficient to saturate targeted receptors. This is possible because even though the RAL used here is highly selective for 5-HT_{1A}, it does also bind to other serotonin receptors such as 5-HT₇ (Sprouse et al., 2004).
- *Low tissue sample mass* - Due to the small size of deer mice, tissue homogenate binding studies are problematic. It may well have been that the assay volume was too low to detect reliable results.
- *Two-site binding* - We found preliminary evidence for two-site binding. To this extent, our concentration series was insufficient and spaced too far apart to come to any conclusion.
- *Vacuum filtration* - It is possible that there may have been malfunctions in the vacuum filtration system, resulting in radioactive noise on the counted filters.
- *Pipetting* - It is possible that errors were made with pipetting, but this is highly unlikely as it was all performed by a highly trained laboratory technician and all amounts were carefully double-checked during the constitution of the various series.

As such, the current work is *still underway*, and the procedures are being repeated with several improvements, including a wider RAL dose range and larger tissue pools.

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Addendum D - Rebuttal letter for Manuscript A

Important!

This rebuttal letter comprises comments and responses from both the first and second round of reviewing. All writing by the authors is depicted in italics, whilst all commentary by reviewers is depicted in standard text. The text was edited without content changes to better fit the formatting of this dissertation.

Ms. Ref. No: BEPROC_2017_304

“Back to Basics: A Methodological Perspective on Marble-Burying Behavior as a Screening Test for Psychiatric Illness”

De Brouwer, G. et al., submitted to Behavioral Processes.

Response to review comments:

The Editor and referee comments have been copied across below, beneath which we have responded to each comment (in italics), describing how we have addressed the raised concerns and where these amendments can be found in the revised manuscript. As laid out in the Decision letter from the editor, all amendments in the manuscript and elsewhere are indicated in track changes highlighted text.

13-Feb-2018

Ref: BEPROC_2017_304

Title: Back to Basics: A Methodological Perspective on Marble-Burying Behavior as a Screening Test for Psychiatric Illness

Journal: Behavioural Processes

Dear Dr Wolmarans,

Thank you for submitting your manuscript to Behavioural Processes. First of all, please accept my apologies for the delays in the review process. Fortunately, I have received reviews from the experts in the area, and I am now able to make an editorial decision on your manuscript.

As you will see, both reviewers are highly positive about merits of your work and its potential impact on the field. However, both reviewers also raise several important concerns that need to be addressed before your manuscript can be considered for publication. Therefore, I would like to invite you to revise and resubmit your work carefully addressing the reviewers' concerns as well as a few concerns of my own outlined below.

Statistical analyses:

When reporting significant comparisons, please add an appropriate measure of effect size.

- *We now included the effect size of significant comparisons in Tables 1 – 6*

Please add 95% confidence interval to significant correlations.

- *We now have no included the 95% CI on **p. 17, line 342**.*

Figures:

Figure 1: This figure does not seem to be necessary as it can be easily described in the text.

- *Dear Editor, thank you for this. We initially thought of omitting this figure based on the description in the text. However, if reading the methodology section, it may be complicated to visualize the layout of the investigation, especially if linking this with the results disseminated from Figure 3, onwards. However, we removed it and amended all other remaining figure numbers accordingly.*

Tables: I am not convinced that the tables are necessary; figures convey the main message clearly and convincingly. I strongly recommend excluding them from the MS and/or reporting the means in the text if desired.

- *The only driver for the inclusion of these tables was the relative complicated structure of the data presented. As a RM-Three Way ANOVA was performed for some analyses, the results cannot be represented by any other means graphically. Thus, and also in response to the reviewer comment below, it would not be possible to perform more simplified statistics. As such, the within- and between-group comparisons are quite extensive, layering down from three-way interactions, through simple two way interactions, ending in simple-simple main effects. As such, one needs to provide the appropriate statistical descriptors for which the figures are not suitable, as it simply is too much to represent better. Also, the number of significant comparisons as requested to be provided in the text, will complicate the reading process significantly.*

Reviewer 1

This MS describes a very detailed study that was designed to evaluate a number of aspects in the marble burying test (MBT) and examine its validity as a model for anxiety, OCD and other disorders. The results demonstrate significant variability in results across conditions, across experimenters and across order of tests. The authors interpret the results to suggest that the validity of the test is limited and that the utilization of this test should be done with significant caution.

Studies dedicated to examining the validity and reproducibility of standard animal models and tests for neuropsychiatric disorders are very important and therefore the task taken by the authors is of value to the field. Moreover, the detailed approach and the examination of a number of sources of variability is to be commended. Yet, there are some issues that cast some doubts on the results and the conclusions.

Conceptual issues

The MS suggests that one problem with the test is that it may not be representing a pathology but the normal behaviour of animals. I suggest that this is the case for most tests used in the context of neuropsychopharmacology research. Animals perform ONLY within their “normal” repertoire of behaviours and intact animals are not model for disease. The tests by themselves are not representative of a pathology but a way to measure the effects of interventions that are related to a disease or a disorder. Accordingly, a test such as the MBT can assist in the research of anxiety (as an example) if the number of buried marbles will increase in animals after an anxiogenic manipulation (be it environmental, pharmacological or genetic) and will bury less marbles after an anxiolytic manipulation (again, be it environmental, pharmacologic or genetic). Measuring marble burying in vacuum indeed may not be representative of anything but this is also correct for other tests such as the elevated plus maze (in the context of anxiety), sucrose preference (in the context of depression) and so on. Accordingly, the fact that the test represents a “normal” behaviour does not invalidate it to evaluate changes in anxiety levels. This needs to be clarified in the MS.

- *We thank the reviewer for this valuable perspective. In fact, we agree and have thus indicated this in the text **(p 4, lines 65 - 71)**. This is true, as indicated by a number of reports that applied marble burying post-external intervention, e.g. (Kedia and Chattarji, 2014; Schneider and Popik, 2007). That said, we are concerned in as far as that most investigations employ marble burying with no pre-test manipulation, i.e. applying marble burying as a natural behavioural response, while subsequently drawing conclusions on the efficacy of anti-compulsive and anxiolytic interference.*

*This in itself is not undermining translational research per se; however, it is such application of the test without methodological congruence that confounds between-laboratory interpretation of reported findings. Thus, our line of thought concerns investigations where marble burying is applied without consistent methodology within the conceptual domains of anxiety and/or compulsivity and where it is used as a natural behaviour to make translational deduction. This is also addressed on **p 5, lines 85 - 92.***

The MS suggests that the lack of specificity in the response of the MBT might represent a problem with its validity. However, one may want to consider that the disorders themselves are not totally unique and that the same drugs are used to treat different disorders. For example, SSRIs are used in depression, anxiety disorders and OCD, atypical antipsychotics are used in schizophrenia, bipolar disorder, autism and more. It is more than possible that there is an overlap in the underlying biological mechanisms of different neuropsychiatric diseases resulting in the fact that some drugs may be efficacious in treating more than one disorder. This may also be the case for models.

- *Thank you for this consideration. We agree completely that certain drugs may be efficacious in reducing a number of aberrant behavioural manifestations due to overlapping effects as mentioned in the examples above. However, the problematic case of predictive validity (and by implication also the construct) discussed in our paper, refers largely to the manner of drug administration, for which we lay the foundation on **p5, lines 88 – 92.** Although demonstrating neurochemical involvement in any specific behavioural model highlights a role for a specific transmitter or transmission system in the observed behaviour, it does not necessarily contribute to our understanding of disease mechanisms. Indeed the mechanisms of action of SSRIs in OCD, anxiety and depression diverge based on treatment duration, dose and underlying neuropsychology and it is for such differences that the marble burying test does not seem to be sensitive. Also, that the (singular) behaviour responds to a diverse number of agents, often with opposing neurobiological and behavioural actions, complicates the translational interpretation of data. The aim of the present investigation is thus simply to divulge whether inconsistent application of methodology may contribute to such data being reported. This we refer to on **p5, lines 93 – 96.***

Standardization of tests had been repeatedly proposed as an important factor that should be addressed. I think that the current study can contribute by emphasizing the main factors that should be standardized (maybe the type of bedding or the version of the test). At the same

time, it would be appropriate previous efforts that were done in the area of standardization of other relevant tests.

- *Thank you for this valuable comment. We have now included this summarizing detail in the Conclusion on **p 29-30, lines 605 – 634**. We have now also included references on the importance of this and to previous attempts to standardize behavioural experiments.*

I suggest that the significant outcomes of the study are two: (1) that there is no advantage to the two-zone configuration over the one zone configuration. (2) There is an advantage to using sand over sawdust or shavings as the burying material for the test. I think these are two important outcomes with very practical meaning and should be emphasized.

- *Thank you for this. We disagree on the first (and apologize for not making the reason for this clearer in the text), and agree on the second, and have modified and included said detail in the conclusion on **p. 22, lines 447 – 452, p 24, lines 493 – 500 and lines 508 – 510, p 25, lines 513 – 520, and p. 29-30, lines 608 - 617**.*

Other comments

The groups in this study include both females and males. The inclusion of both sexes in animal studies is certainly a positive thing and is encouraged by many scientists as well as funding agencies. However, it is a question whether females and males can be pooled together into one group or that sex should be a main factor in analysis. The MS justifies pooling based on previous studies that show lack of difference between sexes in deer mice in some behavioural tests. However, there is so much work showing behavioural differences between sexes in rodents that I am wondering if sex should not be included as a factor in the analysis. But that brings up the next issue which is number of animals per group.

- *We fully appreciate the reviewer's concerns about possible sex differences. Although oestrous cycling female animals are known to respond differently in various behavioural assessments, we impress upon the preceding work done in our own, as well as other laboratories which specifically ruled out significant sex differences in the marble burying behaviour of the currently utilized deer mice and all other measures of psychiatry related assessments of psychiatry related behaviours (Güldenpfennig, Wolmarans, du Preez, Stein, & Harvey, 2011; J. R. Powell, Bennett, Waters, Skinner, & Reed, 2013; S. B. Powell, Newman, Pendergast, & Lewis, 1999; Presti, Gibney, & Lewis, 2004; Presti & Lewis, 2005; Presti, Mikes, & Lewis, 2003; Presti, Powell, & Lewis, 2002; Presti, Watson, Kennedy, Yang, & Lewis, 2004; Wolmarans de, Stein, & Harvey, 2016; De Wet Wolmarans, Brand, Stein, & Harvey, 2013; D. W. Wolmarans,*

Stein, & Harvey, 2016, 2017). Due to ethical constraints, and that the influence of sex on measures of anxiety- or compulsive-like behaviours in the current investigation. This is in fact planned from the perspective on OCD for current and future investigation.

The experiments start with n=12/group but groups are then divided again to result in an n = 6/group. With this small n, animals are repeatedly tested under different conditions. The authors took all precautions to reduce the chance of type one statistical error but with the small number of animals per group I am not sure regarding type 2 errors. I suggest to solve the question by computing the power of the experiments.

- *The current investigation is based on extensive previous studies using small numbers of deer mice. Indeed, the behavioural manifestations in deer mice is consistent, repetitive and highly persistent over time, allowing for smaller numbers to be used. Further, the fact that we use a highly quantifiable parameter in the current investigation, excludes large variance in findings normally reported with behavioural investigations e.g. the forced swim test. In order to better support the data presented, we have also now included effect sizes with respect to all pairwise comparisons of burying activity.*

Experimental procedure – to reduce novelty the study introduced mice to testing environment that is very similar to their home cage. However, a critical factor for rodents is odour of the cage and as they are introduced to a cage with fresh bedding it can be suggested that the novelty effect is quite significant.

- *We thank the reviewer for this valuable consideration. Habituation to the relevant burying substrate (to avoid novelty seeking behaviour during test sessions) is discussed more clearly on **p8-9, lines 173 – 186**. Furthermore, a sentence has been added to explain how crossover effects of odours have been excluded between trials. Briefly, animals were introduced each to his or her own experimental cage to avoid the effect of between trial novelty. Cages were only cleaned and autoclaved following the completion of all six nights of experiments. We now disseminate this information on **p 8-9, lines 173 - 186**.*

Regarding the differences between scorers, I would suggest to examine if there is a correlation between the scores; that is whether an animal that was scored “high” by one is also scored “high” by the other scorer. If there is a correlation it will suggest that both scorings are valid except that there is a shift of “sensitivity” from one person to the other, if there is no correlation it would suggest a significant problem with the scores.

- *Based on the significant differences reported in Figure 2 with respect to observer scores, no correlation exist between the phenotype scores assigned by both observers. In fact, scoring seemed to be highly biased in terms of what is regarded as a marble being two-thirds buried. We refer especially to Figure 2(i), bars 1c and 2c where observer 1 scored 'high' and observer 2 scored 'low'. Again, the only consistent pattern between observers was found in fine sand, strengthening our conclusion that finer and denser substrates are more appropriate to exclude the possible effects of observer bias.*

The MS suggest that the MBT is conducted with 6-15 marbles. In fact I know a version with 25 marbles (see for example Ene et al., 2015, *Acta Neuropsychiatrica* 28(2): 85-91).

- *Addressed in text. This is true, marble numbers reach up to 25 in many investigations, particularly in one-zone conditions; see review by (Çalışkan et al., 2017)). We have amended the discussion on **p. 21, line 435** to refer directly to the present investigation and avoid the interpretation regarding the MBT as a whole using 6-15 marbles.*

All tables as well as all the pictures should be moved to supplementary materials. I would also consider reducing the number of figures.

- *This recommendation was under serious consideration during the initial draft of the MS. In fact, that some pictures did not pull through as supplementary files, is a mistake. All photos and videos are intended as supporting, supplemental data. We struggled with the issue of figures and tables, however. The only driver for the inclusion of these tables was the relative complicated structure of the data presented. As a RM-Three Way ANOVA was performed for some analyses, the results cannot be represented by any other means graphically. Thus, and also in response to comment of the editor, it would not be possible to perform more simplified statistics. As such, the within- and between-group comparisons are quite extensive, layering down from three-way interactions, through simple two way interactions, ending in simple-simple main effects. As such, one needs to provide the appropriate statistical descriptors for which the figures are not suitable.*

Reviewer 2

Excellent topic for study. Understanding how the 3 methodological variables (the substrate, consideration of avoidance, and quantification of behaviour) influence the test results in the marble burying test is critical for understanding the advantages and limitations of this test, and how results should be interpreted. This assessment of the marble burying test is a much-needed addition to the literature. In my view, the most important results of the study have to do with the substrate variable: that substrates like sawdust are more sensitive to coincidental burying (as evidenced by sensitivity to trial repetition), while burying in sand seems to reflect burying behaviour directed specifically at the marbles. However, the analysis of the data and presentation of the results are quite complicated/confusing, making it difficult for the reader to extract a “take home” message.

- *Thank you for the positive commentary! Indeed, we enjoyed investigating this topic as it has been food for thought in our laboratory for the last few years. We have also now addressed the take home messages clearer in the conclusion and refer the reviewer to p. 29-31, lines 603 - 639.*

Page 6, Line 113: Please change “have” to “has”

- *Agreed and amended, please see p. 6, line 120.*

Page 6, line 121: I would suggest changing “ability” to “opportunity”

- *Agreed and amended here (p. 6, line 128) as well as on page 29, line 613.*

Page 12, line 243: “resemble” seems to be the wrong word here

- *Agreed. Amended in the text on p. 13, line 254 - Changed to ‘represents’*

Page 12, line 247: Please change to: “...collated according to the zone-configuration that was applied at the first trial, and a three-way...”

- *Addressed in text on page 13, lines 258 – 259.*

Methodology, page 8, lines 171-172: The way this is written, it sounds like the animal was kept in cages containing the substrate for the 24 hours immediately preceding the test: is this correct? Also, was the habituation cage containing the substrate the exact same form (dimensions, material) as the test cage? Was the habituation carried out in the same place (e.g., testing room) as the experimental tests? Please provide a little more detail.

- *We can answer positively on all of these questions and have redrafted this section to more accurately reflect this information. Please see p 8-9, lines 173 – 186.*

Results:

Table 1 and Figure 2: These data show that there is a significant main effect of observer on scores, and there are significant differences between observer scores specifically for sand, sawdust and corncob. This seems to be the main result of this part of the experiment; for me the pairwise comparisons within an observer (for example, Table 1: Figure 2i, Observer 1, 1ac/2b) are not helpful here, and they make reading the graphs and tables more confusing than they need to be.

- *Thank you for this view. If possible, we would like to request keeping the within-scorer data as well; this indicate the differences in between-trial scores that can be resultant from unique scoring measures applied by each observer and that therefore may influence findings relating to repeated exposure.*

Figure 3a and 3b: The nomenclature for the bar labels “1ai, 1bi, 1ci, 1aii, 1bii, 1cii” is unnecessarily confusing. The graph axis legend already clarifies that the first three sets of bars are first exposure 1 zone, and the second three sets of bars are first exposure 2 zone, so the “i” and “ii” are not necessary. It could be stated as a graph heading or in the figure legend that graph 3a deals with a 1 zone test and 3b deals with a 2 zone test, and therefore the “1” and “2” in the bar labels would be unnecessary. Finally, there are 3 successive trials for each first exposure type, these could be labelled as Trial 1, Trial 2, and Trial 3 (instead of 1ai, 1bi, 1ci, and 1aii, 1bii, and 1cii, for example).

- *Concurred. The only reason why we opted for this approach is to clarify the between trial comparisons of data. If the same labels are applied for those exposed first to either paradigm, pairwise comparisons will be impossible to make. We would like to request keeping Figures 3A and 3B as is, for practical reasons, if possible.*

I am not sure that it will be clear for all what a “simple simple main effect” is... Could this be explained in a clear concise way in the methods section?

- *A simple-simple main effect is a statistical indicator for the main significant effects of any one or more of three factors that could have interacted significantly to bring about the reported results. However, to divulge where the main source of such interaction lies, one needs to run simple-simple main effects as data is stratified according to interaction factors. As such, a two-way ANOVA will be followed by analyses of simple main effects, while a three-ANOVA will be followed by analyses of simple simple main effects, as any two of the three factors could also have interacted to result in the data presented. We now include a reference to an appropriate source (Gamst, Meyers, & Guarino, 2008). Please see p. 10, line 219.*

The description and results of the statistical analysis of the data shown in Figs 3a and 3b is very difficult to follow. What is the main “take home” message, or outcome, of this analysis? It is understood that the objective was to examine whether or not the zone configuration of the first trial affected the performance in each of the three trials, and whether this depends on the specific substrate, but I am left confused by the end of the section. If one just looks at the graphs, one can see clear differences between substrates that are generally consistent across First Exposure Zone Type and Trial Zone Type (course dust and fine dust generally greater than sand and corncob). Beyond that, it is difficult to extract the important results here, and to me it is questionable whether or not the First Zone, Test Zone, and Trial number effects are relevant in practice (even though they may be statistically significant). ***But see comments on section 4.3, below***. Maybe a Generalized Linear Model analysis would give a clearer idea about how large each of these effects are?

- *Thank you for this. The statistical design of the current study indeed posed some difficulty, especially in reporting the data as streamlined and focussed as possible. Following several rewrites, we opted for the presented layout, as it seems to carry the message clearest. However, one can always improve on this and we thank the reviewer for these valuable perspectives. Indeed, the main take home message is that –*
1. *Sand and fine dust are more reflective of marble-directed action, as measurable differences were observed in the behaviour of animals exposed in sand, most notably in the two-zone configuration, i.e. animals voluntary engaged in burying activity and this can be seen in Figure 3B. The interpretation of this is difficult, however. We included the supplementary video files for the specific reason of indicating the phenotypic differences between behaviours in sand and fine dust. Although both of these substrates seem to reflect burying behaviour better, animals are able to burrow dust, which is simply not possible in sand. Thus, the data pertaining to burying performance in sand, although not seeming as profound as in fine dust, is the result of marble-directed burying behaviour, while that of fine dust may be confounded by burrowing activity. As it is impossible to note this simply by looking at data, we included the supplementary files as an indication. One cannot therefore assume that fine dust is a more appropriate substrate in terms of burying ability. It may well be that animals simply indulged more in burrowing activity within said substrate. Burying behaviour in sand is possibly thus more reflective of directed goal-orientated engaging as demonstrated in the three trials. In the one zone paradigm, this is less obvious and probably due to animals having been forced to interact with the marbles. The same is true for corncob, the other substrate that demonstrated a simple-simple main effect.*

We have now amended the discussion of the findings on **p. 22, lines 448 – 450, p. 24, lines 493 – 500 and 508 – 510, p. 25-26, lines 534 – 549, and p. 30, lines 618 – 621.**

2. *first exposure have a significant influence on burying activity, as animals first exposed in a two-zone paradigm, actually buried more marbles during the subsequent one-zone, and two-zone trials (also in fine dust), compared to those exposed first in a one-zone paradigm. An explanation for this is difficult aside from speculation, but it may well be that the opportunity to avoid marbles in the first exposure, could have resulted in animals learning in the absence of forced interaction, that marbles are actually non-reactive, harmless and worth investigating. We have now amended the discussion of findings on **p. 25, lines 513 – 520 and p. 29-30, lines 608 – 617.***

Also, it seems that Figs 4 and 5 give a clearer idea of the relevant result: that on the first trial, there is an interaction between zone configuration and substrate (Fig 4), but that this difference disappears across repeated testing (Fig 5), and that the type of zone configuration on the first trial does not affect performance across subsequent trials (Fig 5).

- **In fact, what Figures 4 and 5 shows us, when combining with analyses of Figures 3A and 3B, is indeed not that performance on subsequent trials isn't affected by zone configuration. Rather, it demonstrates that if one looks at averages of burying behaviour only, one runs the risk of missing detailed interactions with marbles. Interestingly, varying patterns of burying activity were only observed in sand, fine dust and corncob (two-zone data), but this seems to disappear when looking at averages. In fact, time had a significant effect on burying activity the two zone configurations (Figure 3B), something that is missed in generalized two-way ANOVA. We have now included the significance of the data presented in Figures 4 and 5 to a greater extent in the discussion and apologize for this not being clearer from the outset. Please refer to **p 24, lines 493 - 500.***

For Figure 6, consider using an open circle to represent the sand points, in order to see more easily the points that correspond to the significant correlation. With all points represented by filled symbols, it is difficult quickly see the ones that correspond to sand.

- *Concurred. Figures 6 and 7 was amended as per request.*

Discussion

- *We have significantly reorganized the discussion to address the concerns raised below. Please see references to exact page numbers as indicated.*

Section 4.1, Lines 408-410: It is not clear to where “see next paragraph” refers.

- *We now refer to the exact paragraph number. Please see p. 21, line 425.*

A more straightforward interpretation/conclusion of this result is that the lack of significant between-observer differences in scoring in the fine sawdust substrate is because it is easier to score consistently in this substrate compared to the other substrates. And given that inter-observer differences for fine sawdust are the lowest, wouldn't it make sense to limit the analysis of the Zone and Trial effects (Fig 3) to just the fine sawdust substrate?; i.e., eliminate the substrate factor in the Zone and Trial analysis (**But see comments on section 4.3, below**). This would simplify the presentation and interpretation of the analysis of the Zone and Trial effects. In fact, by looking at the graphs, one can see that the fine sawdust is the only substrate that varies across trials in a potentially meaningful way (generally increasing across trials in the 2Z conditions (3b), and showing a depressed effect only in Trial one of first exposure 1Z (3a)).

- *Thank you for this suggestion. We did think of approaching the data in this manner during the initial drafts of the paper. However (and please also refer to our response to your comment above*), that fine dust seemed to be a more accurate burying substrate, is from a burying phenotype point of view, not the conclusion we came to. That course dust seemed not be sensitive to changes in any of the variables, and that sand were associated with marked inter-observer differences, are just as important to indicate. In fine dust, marbles simply settle quicker below the surface of the burying substrate. This can be due to any number of reasons, e.g. burrowing or locomotor rituals. However, course dust on the other hand is susceptible to these effects to such an extent, that the total effect over time becomes masked, hence being the worst possible substrate in which to perform marble burying procedures. Fine sand on the other hand are resistant to these effects to such an extent, that animals simply have to directly engage in covering them. The fact that the two observer scores differ significantly, is most possibly due to individual perspectives on what constitutes a two-thirds buried criterion. We now elaborate on this to a greater extent in the text and thank the reviewer for this valuable perspective that was lost in the final draft of the MS. Please see p. 20-21, lines 415-427.*

Lines 429-431: Please say explicitly here what the principal effects are. Although the description of the statistical analysis is difficult to follow, with respect to Trial/Zone effects, it seems that the bottom line is that 1Z tests (Fig 3a) are modified by repetition (when first

exposure is 1Z, but not if it is 2Z), and 2Z tests (Fig 3b) are modified by repetition, regardless of the nature of the first test exposure. Is this correct?

- *Indeed. In brief, animals first exposed in a two-zone paradigm demonstrates bolstered activity in the subsequent first one-zone trial, while such activity does not seem to change subsequently. However, those first exposed in a one-zone paradigm demonstrates lower burying activity in the first one-zone trial, and then only habituate to testing environment; **p 22, lines 447-448 and 450-452 and p. 22-23, lines 464 - 467.** However, it seems that burying activity in two-zone paradigms are much more sensitive to changes in burying phenotypes, as if choice to engage in burying activity, modifies the resultant action of doing so. Given the data presented, no other conclusions apart from speculation can be made. Please also see **p. 25, lines 513 – 520** and **p. 29-30, lines 608-617.***

Again, I suggest carrying out this analysis and present the Trial/Zone effect results considering only the fine sawdust substrate, since the effects of substrate are clearly shown in Figs 2, 4 and 5 (**But see comments on section 4.3, below**). The discussion in this section (4.2) should clearly address possible reasons why burying for 2Z tests generally increased across trials regardless of the nature of the first exposure, while a first exposure to a 2Z test eliminated this effect for the 1Z trials.

- *Please refer to our answer in this regard, above. Please also see the revised section in paragraph 4.2 on **p. 25, lines 513 – 520.***

Line 443: Is it not possible that a particular “experimental interference” can reveal an aberrant cognitive construct? For example, the elevated plus maze is an experimental manipulation that is proposed to distinguish between more anxiously reactive and less anxiously reactive individuals. “Anxiety” behaviour in the elevated plus maze also is reduced across repeated trials. Or maybe I am not clear on what is meant here by “experimental interference”.

- *By ‘experimental interference’ we do not imply the nature of the test used, as with the example of the elevated plus maze, used above. We mean this within the context of the current investigation, i.e. that any change to any of the parameters investigated, can result in unique results. Further, different laboratories approach the marble burying test differently; however, the same deductions with respect to anxiety or compulsivity are being made.*

Lines 448-451: It is not clear how “investigative behaviour” is distinguished from compulsive- or anxiety-like behaviour here. Is it assumed that compulsive- and anxiety-like behaviour should be stable across repeated trials?

- *Indeed not. We move to propose that while anxiety as measured by the marble burying test, should demonstrate habituation over time, compulsive-like activity should not. Investigative behaviour can be distinguished from compulsive- or anxiety-like behaviour on the basis of direct, focussed burying interaction. Investigative covering of marbles is not characterized by the physical activity of burying. If we want to apply ‘marble burying’ in its essence as a behaviour that reflects an aberrant cognitive process, it therefore needs to reflect preoccupation with the objects itself.*

With respect to “anxiety”, it is unclear how an “anxious” mouse would be predicted to respond to a marble: proactive individuals might actively bury it, while passive individuals might avoid it. This makes arguments like that given in lines 463-467 dubious.

- *We now omitted this whole section and simply stated that, based on our data, anxiety-like behaviour is unlikely to be reflected in MB if positive anxiogenic interference is not also introduced. We therefore agree with the reviewer on this. We now modified this part of the discussion to better illustrate the meaning. Please see p. 23 – 24, lines 473 – 493.*

Section 4.3. The authors provide an important and interesting interpretation of the results of the sand vs. other substrates: that the nature of the sand substrate makes it more difficult to accidentally cover the marbles while doing other general activities that are not directed at the marble itself. Now, the relevance of the substrate effects described in sections Figure 3a and Figure 3b of the results, and section 4.2 of the discussion becomes clearer. Given this result, I think the discussion of the Zone/Trial results (section 4.2) needs to be revised in order to incorporate this interpretation. Note that (if I am interpreting the results accurately) fine sawdust was sensitive to Trial effects, but sand was not.

- *Yes and no. Please see our response relating to fine dust vs sand on pages 8 and 9 of this rebuttal letter. Further, see also the amended section 4.2 on p. 22, lines 448 – 450, as well as p. 24, lines 508 – 510, p. 26, lines 535 – 550, and p. 30, lines 618 – 621 requested.*

Given the discussion in 4.3, the significant Zone/Trial effects on burying shown in Fig 3 (e.g., for fine sawdust substrate) could be attributed to alterations in general exploration/digging (behaviours that might be predicted to change with repeated testing), while the absence of an

effect of Trial repetition on burying in the sand substrate is consistent with the conclusion that burying in sand is stable across trials, and not sensitive to habituation or changes in exploratory behaviour, and therefore more consistent with a compulsive-like behaviour.

- *Concurred.*

Lines 577-578: By “most investigations” do you mean those investigations that use substrates such as sawdust (sensitive to coincidental burying) as opposed to sand (argued here to be insensitive to coincidental burying)?

- *Indeed. We also now amended this section. Please refer to **p. 29, lines 605 – 607.***

Line 580-581: Here and throughout: exactly what is meant by “aberrant cognition”, and how is it distinguishable from normal exploratory behavior? Is it not possible that differences between individuals with respect to exploration of a novel spatial environment (i.e., activity, habituation across trials) could reflect “aberrant cognition”?

- *Indeed, this is the case. See also the amendment to the text in this regard. However, such behaviours should abate over time as exploration is a functional outcome of introduction to novel environments. In as far as marble burying is concerned, however, deductions are made based on ‘the number of marbles’ buried, which apart from being highly modifiable by the experimental design, is applied as a measure of aberrant cognition, i.e. anxiety or compulsivity, as opposed to its resemblance of ‘normal’ behaviours, such as exploration.*

Lines 582-584: Isn’t this conclusion consistent with the proposal that marble burying (at least in sand) might reflect a behavioural construct similar to compulsion?

- *Yes, indeed.*

Line 589: By “often inadequate” do you mean inadequate in circumstances in which substrates other than sand are used?

- *Addressed in text. Yes, this has been amended in line with the findings presented in the current investigation. Please refer to the amended wording on **p. 30-31, lines 627-639.***

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“Back to Basics: A Methodological Perspective on Marble-Burying Behavior as a Screening Test for Psychiatric Illness”

De Brouwer G et al, submitted to Behavioural Processes.

Round 2 – Response to review comments:

The Editor and referee comments have been copied across below, beneath which we have responded to each comment (in green italics), describing how we have addressed the raised concerns and where these amendments can be found in the revised manuscript. As laid out in the Decision letter from the editor, all amendments in the manuscript and elsewhere are indicated in track changes highlighted text.

Editor

Dear Dr Wolmarans,

Thank you for submitting your revision to Behavioural Processes. The same two reviewers who read the original manuscript have commented on your revision. As you will see, both reviewers found that your manuscript has improved; however, both reviewers have also suggested additional changes before it can be considered for publication. Therefore, I would like to invite you again to revise and resubmit your manuscript addressing these remaining concerns.

- Thank you for the valuable input. We carefully considered each of the issues raised and responded accordingly. We appreciate the meticulous attention of both reviewers that will result in a much improved paper and its usefulness for future investigations relating to translational neuroscience.*
- We further restructured and ordered the objectives to clearly link with the outcomes reported.*

With respect to the number of figures and tables (Reviewer 1): much of information presented in the tables could be conveyed in the text by placing statistical information in parentheses instead of table reference. I recommend doing this to eliminate the tables. Please check all figures for typos and for correspondence with the text (also noted by the Reviewer 2).

- We have now moved all applicable tables (where possible) to supplementary data and highlighted relevant statistics in text. We further reduced the number of figures to only four now and checked the captions as well.*

When preparing your revision, please include the detailed, point-by-point response to the reviewers' concerns in addition to the cover letter. Your revision is due in 60 days; if you do not submit your revised paper by this deadline, please note that the Editors reserve the right

to withdraw the paper. If the paper is withdrawn, you can submit your revision as a new submission.

Again, thank you for submitting your work to Behavioural Processes. I look forward to receiving your revised manuscript as soon as possible.

Sincerely,

Dr Lazareva
Editor-in-Chief
Behavioural Processes

Reviewer 1

The authors have answered many of my concerns, but some issues are still open.

Regarding my first comment in the previous version related to “normal” behavior. The MS now attempts to address the issue (pg. 4 lines 65-71). Yet, I think that the authors are still too demanding, as anxiolytic drug treatment may also be a relevant intervention. There is vast literature on the effects of such drugs in human healthy volunteers, including effects on emotional behavior and emotional processing. Hence, it is only reasonable to expect that potential anxiolytics will also influence the behavior of model animals in anxiety-like related tests. I strongly suggest addressing this issue.

- *Again, we agree completely. In fact, as with our response to the initial comments regarding this dilemma, we also feel this topic needs to be addressed somehow. With respect to the current paper that primarily investigates the possible role of between-laboratory methodological differences on outcomes of marble burying assessments, a detailed discussion of the topic falls outside the current scope. However, as a fundamental foundation for the current paper was in fact that the marble burying test is applied without methodological congruence and standardization with respect to specific neuropsychiatric constructs, this issue indeed needs some thought. We have now rewritten this part of the Introduction once more and refer the reviewer in this regard to Pages 4 – 5, lines 65 – 100.*
- *On this note, what we struggle with in this paper is not the fact that behavioural tests can only be regarded as accurate only if aberrant or abnormal behaviour is modified. As also highlighted by your earlier comment, this is indeed not true. What is the focus though, is the fact that using a singular, monodimensional behavioural phenotype and its equally monodimensional quantification criterion, i.e. the ‘counting of marbles that have seemingly been buried’, that is applied under various circumstances and under numerous conditions, often from the perspective of the same neuropsychiatric construct, yields inconsistent results that does not seem to be repeatable between laboratories. That said, the conclusions drawn from a number of investigations as cited in the manuscript are mostly directly translated to anxiety and OCD, i.e. by treating, exposing and observing animal behaviour in the marble burying test, novel hypotheses relating to anxiety and OCD are proposed as targets for future investigations. What the current MS therefore address, is whether such an approach is useful, given that a few methodological manipulations can yield significantly different results, this in the absence of any pharmacological interference whatsoever.*

- *We hope that this response, with the changes made in the manuscript, better summarize and carries what we attempt to demonstrate here.*

The authors added some emphasis regarding the issues of standardization, but I also suggested that the MS should include some references to previous work that examined effects of standardization.

- *We have now included reference to the importance of standardization, as well as to specific studies that addressed issues of standardization. Please refer to pages 5 – 6, lines 105 – 117 of the manuscript.*

Regarding the issue of sex as a factor, I am still not completely convinced. I suggest that the best approach would be to perform even just one of the analyses with sex as a main factor and if there is no sex effect then pool data across sexes for the entire study. Alternatively, if there are previous studies from the authors' laboratory that specifically tested sex differences in one of the measures presented in the study I suggest to include the data of that previous study.

- *Again, thank you for highlighting the subject of sex in behavioural measurements. We now include reference to the fact that, as is true for high stereotypical behaviour and large nest building behaviour, deer mice display high marble-burying behaviour irrespective of sex. A figure that graphically represents this is now included as supplementary data (supplementary Figure 1 – we also refer to this on page 8, line 166, and page 21, line 432). Importantly, as per the ARRIVE criteria and based on our previous findings disseminated during the past decade, we included only six animals per group per exposure paradigm (one- or two-zone), equally distributed between males and females. It will therefore not be possible to perform sex-specific statistics on the data presented here.*

Please re-consider the issue of figures and tables. There are too many in the manuscript. See what you can move to supplemental data.

- *We have now omitted where possible and where no statistical significance needed to be conveyed. We have now removed all tables as well as two more figures from the MS (moved to supplementary data). Only figures that indicate the statistical significance of findings are now included.*

Reviewer 2

Line 234: change “reflect” to “represent”

- *Done. Please see page 13, line 263.*

Line 236 change “resemble” to “represent”

- *Done. Please see page 13, line 265.*

Line 78: Do you mean to say Figure 2B?

- *Concurred. Changed as well as double-checked all other reference to Figures.*

Line 306: the representation “T1, T2, T3” is not found in figure 1; I assume T refers to “Trial”, but it is not completely clear, and as it is written the reader assumes that these abbreviations will be used within Figure 1. This might be unnecessarily confusing because in Figure 1 the trials are not designated by T1, T2, etc.

- *Concurred. This unfortunate mistake was introduced upon the reorganization of figures during the first review round. We changed all references to figures now accordingly.*

Line 194: T1-6 are referred to in the context of Figure 1, but in that figure the trials are not designated in that manner.

- *Concurred. This unfortunate mistake was introduced upon the reorganization of figures during the first review round. We changed all references to figures now accordingly.*

When re-reading the article, it occurred to me that there may be a confounding factor that has been overlooked: the possible effect of Trial (regardless of first zone configuration). I think this may be important to consider for the following reason: Considering the data shown in Figure 2a, for example, the first trial in a First Exposure 1Z, one-zone series (1ai) is also necessarily the first exposure of the animal to the marbles (Trial 1, immediately after the 24 h habituation; at this trial the marbles are novel). However, the first trial of First Exposure 2Z in the one-zone series (1aai) would correspond to Trial 2, or the second exposure to the marbles (presumably, the previous 24 hours were spent in its home cage, and also at that trial the marbles are no longer a novelty).

- *This analysis and summary of the reported results is accurate!*

Therefore, within a single zone configuration analysis (Figure 2a, lines 251-274 of Results, for example), trial and first zone configuration are not independent of each other. A repetition effect that might be seen among trials 1ai, 1bi, 1ci would not be comparable to a repetition effect seen among trials 1aii, 1bii, 1cii, because 1ai and 1aii are not equivalent; that is, 1aii was necessarily the second trial of a 6-trial series, whereas 1ai was the first; in one case (1ai) the marbles were novel and in the other case (1aii) they were not (in addition to the differences in handling during the previous 24 hours). This might, for example, complicate the interpretation of the following observation (lines 453-455) "...results from one-zone trials only demonstrate that animals having been first exposed in a two-zone configuration, covered significantly more marbles during their first one-zone trial compared to those first exposed in a one-zone configuration...." In this case, the first one-zone trial of the animals that began with a two-zone configuration corresponds to Trial 2 (marbles are no longer novel), while the first one-zone trial of animals that began with a one-zone configuration corresponds to Trial 1 (marbles are novel). Playing the devil's advocate, one could suggest that, in the former case, the mouse covered more marbles because of reduced neophobia and therefore interacted with them more.

- *We also considered this. In fact, we did investigate this possibility. That animals having first been exposed in a two-zone paradigm bury more marbles in the subsequent 1Z paradigm, could possibly have resulted from reduced novelty-induced anxiety. However, from the current data, this possibility was excluded based on the collective appraisal of both 1Z and 2Z data, demonstrating that animals having first been exposed in a 1Z paradigm, also do not engage in burying behaviour during subsequent 2Z trials. In fact, both groups of animals that have been first exposed in the 1Z and 2Z paradigms respectively, buried equal numbers of marbles during their first 2Z trials. Further, all animals, irrespective of first exposure zone configuration, buried more marbles during the first 2Z trial, compared to the first 1Z trial (Figure 3). A reduced level of neophobia and increased investigative / engaging behaviour could therefore, in our minds, reasonably be excluded.*

Can the authors comment on the general possibility that repeated exposure to marbles (i.e., across Trials 1-6, regardless of first zone configuration) might also be a relevant factor in determining the behavioral outcome? Is there a main effect of trial (across T1, T2, T3, for example), independent of zone configuration?

- *Indeed, we can. A main effect of Trial yielded insignificant p-values across all burying substrates and zone configurations in a three-way RM ANOVA. Trial, although interacting with zone configuration and burying substrate, therefore had no initial*

significant main effect on the results displayed, either dependent or independent of zone configuration, and hence data was collated according to zone configuration.

- *See also page 11, lines 231 – 232.*
- *An important consideration in the present article was the representation of data. Given the number of possible interactions, we began with the broadest approach and then narrowed it down. As such, where data could be collapsed and collated according to the statistical results generated by SPSS, we only represent the essence of findings in various figures. In this specific example, both Figures 2 and 3 are important to allow for and exclude the possible reasons for the results as highlighted above. It was indeed a difficult task to contemplate and decide which data to show, and how best to represent it. We hope that in answering this query, as well as referring to Figures 2 and 3 holistically, we addressed this concern of the reviewer satisfactorily.*

Addendum E - Rebuttal letter for Manuscript B

Important!

This rebuttal letter comprises comments and responses from the first round of reviewing. All writing by the authors is depicted in italics, whilst all commentary by reviewers is depicted in standard text. The text was edited without content changes to better fit the formatting of this dissertation.

29 August 2018

Ms. Ref. No.: CABN-TR-18-207

“Back to Basics: A Methodological Perspective on Marble-Burying Behavior as a Screening Test for Psychiatric Illness”

De Brouwer, G., Fick, A., Harvey, B.H., Wolmarans, D.

Response to review comments:

The Editor and referee comments have been copied across below, beneath which we have responded to each comment (in italics), describing how we have addressed the concerns and where these amendments can be found in the revised manuscript (referring to page and line number etc.). As laid out in the decision letter from the editor, all amendments in the manuscript and elsewhere are indicated in blue font together with an appropriate commentary box explaining our response in brief that compliments the more detailed explanation provided in this response.

First, we would like to express our sincerest gratitude to the editors and reviewers for considering and scrutinizing this work. The idea to draft this manuscript was born from our own experience with, and doubts concerning the MBT, but also from observations that some laboratory animals indeed exhibit unique burying behaviours compared to the larger populations that cannot simply be discarded as insignificant. Hence began the journey to evaluate what has been done, how it has been done and how one can distinguish between truly valuable findings and those findings that are in simple terms, meaningless. That said, the work proved to be a greater challenge than we originally thought. We soon came to know that a critical review of 4 decades of published findings would, although we would have liked to nullify most of it in one sweep, have to be sensitive to a lot of hard work and good intention that may have been applied before. As such, we decided to systematically dissect step-by-step the intentions, methods, pharmacological interventions and conclusions reported in previous work to put forward an argument that in the end, would use these elements to speak for itself. It is truly remarkable that most of the concerns raised below, also plagued us as authors which not only confirms to us the value of your comments, but also let us realize once

again the potential meaning and novel contribution of this work to the current literature base. We hope that in addressing the concerns of the reviewers, that this paper will be better off for it, providing food for thought as well as be an objective, holistic and comprehensive roadmap for future researchers that intend to apply the MBT in their investigations.

Reviewer 1

Comments to the Author

In general, this is a truly comprehensive and thus potentially invaluable review of the marble burying test as a model for anxiolytic and anti-compulsive drug action. The review is very clearly structured and covers conceptual, methodological and pharmacological issues. There is a comprehensive bibliography and this literature is nicely summarised in a series of well laid-out and readily accessible tables.

- *Thank you!*

However, that said:

There is some very awkward and cumbersome phraseology which may lead to misunderstandings, e.g. 'pharmacological or behavioural interferences'? Such problems will need to be tidied up in revision or at the copy-editing stage.

- *Noted. We have searched the document and replaced such phrases with the more appropriate ones such as 'pharmacological or behavioural manipulations' or 'pharmacological or behavioural interventions'. Where necessary, we also rephrased the applicable sentences or sections to better carry the meaning and intention of the respective ideas. Due to the review-wide attention to this point of critique, we do not indicate page and line numbers here. However, all such changes are highlighted in blue text throughout.*

Given that page 7 refers to burying as a robust, naturally-occurring behaviour, I am a loss to understand repeated reference to 'aberrant marble burying' – if rodents naturally bury marbles (most probably as a defensive response), how can this be consistently referred to as 'aberrant' behaviour?

- *The question of 'naturally occurring/normal' vs 'aberrant' behaviour is indeed difficult. We agree with the reviewer in terms of using the word to describe burying behaviour observed in the MBT when it was performed in randomly selected animals (which is the case in most of the investigations). In this regard, we have removed possible confusing references to 'aberrant' marble burying activity where it has been applied*

within the context of such investigations. Please see lines 50, 79, 240, 247-249, 393-395, 451-454, 458-459, 605-606, 960.

However, in certain rare cases, researchers did indeed preselect animals, since they show a unique abnormal burying phenotype compared to the larger population.

- *In these instances, we continue to use the term ‘aberrant’ to describe a behaviour that is unique, different and abnormal – indeed the behaviours and its applications we wanted to highlight against the background of work performed in randomly selected subjects.*
- *Based on the literature reviewed in Section 2, we also conclude that rodents will bury marbles based on an investigative drive, and not as a defensive response. We have now made several subtle changes to convey this message in Section 2 and elsewhere. Please see lines 159-167,*
- *We hope we have addressed the concern satisfactorily.*

Although some reference to DSM-5 is made in later sections, Section 3 does not seem to recognise the changes to the traditional classification of anxiety disorders in DSM5? Section 3 (page 9 onwards) really needs to take more care in using (perhaps by defining) the phrases animal ‘tests of anxiety’ and animal ‘models of anxiety’ – the former referring to naturally-occurring defensive behaviours, and the latter to (environmentally- or genetically-induced) alterations to such patterns.

- *Thank you. We have now rewritten sections of Paragraph 3.1 to more closely align our views with the diagnostic categories put forward in the DSM-V. Please see, lines 172-182.*
- *We agree with respect to the need to carefully distinguish between ‘models’ and ‘tests’ and have now rewritten all paragraphs, sentences and phrases to carry the context of the respective arguments with more care. Please refer to lines 14-15, 50-53, 81-83, 224-237, 239, 253-258, 266-267, 285-286, 289, 597, 789-790, 1007-1009, 1466-1467, 1474.*

It is very clear indeed that the existing literature on the MBT is highly inconsistent, arising from major methodological variations in the test itself as well as in the pharmacological interventions employed. As such, although the MBT is ‘a relatively straightforward test to apply’ (p54), one has to ask – to what end? The authors (p55) go on to argue that ‘the test does appear to have some value in terms of early-stage preclinical screening’ – how so? The well-described inconsistency in results from this test would surely argue against any such proposal as it could/would lead to large numbers of false positives and false negatives – and considerable

waste of time and money – nevermind the ethical dimension. Indeed, the final paragraph of section 6.11, to me, does not logically follow from the preceding demolition of the predictive validity of the MBT. Thus, while the authors' final recommendations are well-intentioned, they avoid the fairly obvious conclusion that – as the MBT literature stands at present - this test falls way short on reliability and validity – and is simply NOT useful as a means either to understand the underlying mechanisms of anxiety and/or OCD or as a preclinical screening tool.

- *Thank you for this perspective. By and large, we agree completely. In this regard, some context (with modification in the manuscript to provide support) need to be provided. It was important for us to carry the message that the collective of investigations that used the marble burying test (MBT) up to this point in time, mostly did so without applying valid methodologies in terms of really separating normal from abnormal behaviour. Further, the majority of investigations also lack a clear theoretical foundation for why the MBT has been put forward as a valid measure of anxiety- and compulsive-like behaviour, while simply using the, might we add highly manipulatable, test as a yes-no confirmation of anxiety- and compulsive-like behaviour. It is in this that we disagree and want to nullify the meaning of the test. However, it was also important for us to highlight the fact that indeed, something like aberrant, persistent and not-normal burying phenotypes do exist and that this was in fact investigated and reported accurately in some literature. Therefore, instead of only breaking and entering, we aimed to give attention to this aspect as well. Indeed, on the one hand, if one looks back over 40 years, very little can be learned from marble burying data as a whole; this is important and needs to be conveyed. On the other, data from a few studies do indeed support the identification of a naturally occurring abnormal burying phenotype in some animals that will support a role for marble burying, not as a test, but as a potential model of neophobia (in some scenarios) and compulsive behaviour in others. However, it is also evident that aberrant behaviour can be induced in animals (or naturally occurring; identified and selected for via a pre-screening procedure) otherwise expressing normal inherent burying behaviours, which could be of benefit for screen testing potential anxiolytics and anti-compulsive drugs. Thus, what we intended to do was to provide a overview of what has transpired thus far, why its meaning is overly insignificant and how we can map a road forward. It is with respect to the latter idea, where Sections 6.11 and 7 are of utmost importance.*
- *We have now rewritten several phrases in these and other sections to convey this message better. Please refer to lines 1369-1370, 1376, 1378, 1380-1387, and 1468 in the manuscript.*

- *We also provide a foundation for this approach now in the Section 1, lines 89-94.*

In sum, my major criticism of the review is that the conclusions do not seem to follow logically from the authors' comprehensive analysis of the literature.

- *We sincerely hope that we addressed most, if not all, of these concerns.*

Reviewer 2

Comments to the Author

This review article on the translational significance or insignificance of the marble burying test (MBT) is certainly well-written, comprehensive and thought provoking. The authors have provided an exhaustive coverage of the entire literature and the review article is encyclopaedic with regard to the inclusion of pharmacological agents and how they modify behaviour on the test. The paper can serve as a valuable resource for laboratories that are already using the MBT and as an important instructional guide for investigators interested in using these behavioral tests. The review is timely and could help insert more consistency into this particular test and thereby enhance the translational value of the MBT. A few minor questions and points arise when reading the review and these are presented below for consideration by the authors.

The review is well balanced, but the title is quite negative in its stance- “translational insignificance” seems overly harsh and somewhat out of line with parts of the review. The authors catalogue probably every pharmacological agent ever used in the MBT and some of these drugs seem to have some clinical validity. However, as pointed out by the authors, many of the drugs that reduce MB have not made it into clinical testing. Therefore, the MBT seems of have some translational significance and a mild toning down of the title by using a term other than “translational insignificance” would be more consistent with the text.

- *We agree and have now retitled the manuscript to provide a better idea of what the paper will entail and how it should be interpreted. We hope this addresses the concern of the reviewer. We also focus more throughout the paper on a clear separation between a collective summary of past papers up until now, and the way forward, which should still carry the message, albeit in a softer tone.*

The review is so comprehensive, it would probably be impossible to expand it, but one wonders if the major drugs used treat humans with OCD and anxiety disorders are really effective. This is reminiscent of the situation with regard to the treatment of depression with SSRIs and Irving Kirsch’s multiple meta-analyses showing that they are no more effective than placebo. Perhaps a sentence or two could be added to make the point that SSRIs should be given the same scrutiny with regard to treatment outcome in OCD as has been applied to depression. If an SSRI is no better in treating human OCD, how can its effectiveness in the MBT be validated. Just because SSRIs are used frequently in treating OCD and anxiety (and every other psychiatric condition), it does not mean that they are effective.

- *Again, we agree! In fact, it was something we also struggled but knew that to begin touching on this topic, could be a review of its own. To address this, but still keep the paper focused on marble burying, we now introduced subtle changes to the text to be reminiscent of this perspective. Please see lines 221-223 and 357-358.*

On page 44 and 45, the authors discuss noradrenaline releasing agents such as methylphenidate, d-amphetamine and methamphetamine and mention how these drugs alter MBT outcomes. However, these drugs are probably more powerful as releasers of dopamine and serotonin. This fact should be acknowledged as noradrenaline release is minor by comparison to dopamine and serotonin release resulting from these particular drugs.

- *We have now introduced a subtle change to this phrasing in line 1128-1129 on page 46 as well in the discussion of serotonergic agents in lines 918-919 and 922-924. The mechanism of these drugs involving other monoamines has been stated.*

Addendum F - Rebuttal letter for Manuscript C

i) Rebuttal Letter to *Behavioural Pharmacology*

Important!

This rebuttal letter comprises comments and responses from the first round of reviewing. All writing by the authors is depicted in italics, whilst all commentary by reviewers is depicted in standard text. The text was edited without content changes to better fit the formatting of this dissertation.

Rebuttal to the reviewer comments: Manuscript (BP-19-153) submitted to *Behavioural Pharmacology* titled:

“Naturalistic operant responses in deer mice (*Peromyscus maniculatus bairdii*) and its response to outcome manipulation and serotonergic intervention”

1 October 2019

Dear Editor,

Following discussion and deliberation between co-authors, please find herewith a detailed rebuttal to the comments raised by the reviewers. We sincerely hope and believe that our approach and responses to these issues are addressed to the satisfaction of all the reviewers.

Reviewing comments have, beginning on the next page, been copied over to this rebuttal in black text, each time followed by our detailed response in blue. To facilitate revision crosscheck, we indicate where changes have been made with exact referral to page and line numbers in the manuscript.

Thank you for the effort and your valuable time. It is appreciated!

Warm regards,

GdB; BHH; DWW

Reviewer 1

The manuscript by de Brouwer and colleagues reports on naturalistic operant responses in deer mice and its response to outcome manipulation and serotonergic intervention. This is an interesting study that assessed the changes in operant responses to nest building material by deer mice selected as a possible phenotype of obsessive-compulsive disorder. The study is well designed and conducted, exploring different conditions such as devaluation and punishment on the persistent response. The methods, results and discussion are clear, well written and relevant to the data obtained.

- *Thank you for the lovely feedback and for the time you took to read through our work.*

There are, however, some concerns:

In the Material and Methods section. Please, if it is the case, specify the country ethical guidelines or law on animal procedures where the study has been conducted.

- *This has been addressed. Please see page 7, lines 148-150.*

Do the authors try to analyse the data within sex? In this case, it's true that split the n by sex, will have a reduced number of animals for the analyses. However, it's well known that OCD is more prevalent in the male population, and most of the literature on animal models are also relative to males. I wonder if nesting behavior could also be influenced by female animals by the ovarian cycle.

- *While we agree that it may be valuable to analyze the influence of sex in a model of OCD, we have a few other studies in the pipeline specifically designed to address this need. Indeed, ultimately both men and women present with OCD to the same extent. What does differ it seems, is the age of onset and phenotype expressed – in this, we have a great interest. Historically therefore, we have positioned the deer mouse model as sex-non-specific model with all previous investigations indicating that all of the phenotypes observed, are expressed in a roughly 50/50 sex-based split. Interestingly, we have previously asked ourselves the question, especially since nesting may be seen as a form of nursing-driven trait, whether the sexes would differ. Given the very brief estrous cycle of female deer mice (4 days), we repeat nesting over 7 days. From these data it was obvious that the willingness to engage in the act of nesting, is not driven by a biological rhythm, but rather by another ever-present construct, whichever form this may take in future. The benefit is that one does not have to screen twice the number of animals to obtain a yield of 3/10 LNB animals, as this would effectively double the number of subjects needed. Rather, we are looking toward retrospective*

data analyses in future to determine age of onset of LNB in the two different sexes. Lastly, we would like to reiterate the highly foundational nature of the current work in terms of not only establishing a novel way of interrogating potential compulsion, but also in drawing possible associations between a measurable behavior and the drive to engage in such behaviour of which neither requires a sex-based split explicitly, even though this would have been informative.

The concentration of the drug is administered in the drinking water, but is it possible to know the final dose that the animals have intake? Did the authors find any neophobic effect on the taste of the water at the beginning of the administration? Did the authors find variations in the intake between the different days? In my opinion, these questions should be explained as it could affect the purposed effect of the dose.

- *This is a question we often get and agree that it is indeed fair. Escitalopram administration in the drinking water is used because other routes of administration used in rodents is not practically viable. In fact, when employing deer mice, which are extremely small, belligerent and difficult to handle, repeated injections over the course of a month would prove an enormous stressor as well as risk to their wellbeing. We have added a line to reflect this. As we have validated the water and drug-solution intake before, we do not include this data in our newer work. However, we have now, in this rebuttal (see response to reviewer 2), added water intake data of mice receiving only water compared to those that receive escitalopram solutions – the intake maintains a stable volume of 0.25 ml/g/24 hrs. Please also refer to lines 247, 250-252 in the manuscript.*

In the discussion section, the authors should emphasize the characteristic of their model in terms of face, construct and predictive validity for OCD according to the criteria for assessing animal models by Geyer and Markou 1995, 2002; Markou et al. 2009; Willner 1984.

- *We have now included this. This has been briefly addressed in the concluding remarks as it best fits with the text already there. Please see lines 367-369; 526-529 & 531-533.*
- Moreover, the interesting explanation about stimulus-response habit learning behaviour proposed in the introduction should be could be retaken in the discussion to better explain the results.
- *We have now made subtle reference to this in lines 449-451.*

Reviewer 2

The manuscript investigates the effects of altered outcome contingency, punishment, and chronic citalopram treatment on operant responses aimed at obtaining nesting material in deer mice. This research attempts to provide much-needed understanding of the motivational background for unconditioned nest-building behavior as a compulsive-like behavior. The contribution of this work is especially important as nest building is a natural, goal-directed behavior in mice and methods for assessing when this behavior becomes irrational/pathological are lacking. However, there are some problems with interpretation of results, data analysis, and missing information in the manuscript that requires attention.

Major points:

The authors conclude that chronic citalopram treatment normalized behavior of LNB mice because of the smaller number of lever presses post-treatment in this group, compared to pre-treatment (Figure 3B). In figure 3A, in the pre-treatment phase, it however appears that LNB animals exhibit very high lever press engagement during the first session, with this behavior dropping drastically on the following sessions (Table 1). The authors mention in the discussion that there is habituation to lever presses over time. It does not appear that a control-group with LNB animals receiving vehicle was included in this study as a control. How did the authors account for that the proposed anti-compulsive-like effect of citalopram is simply not an effect of time/habituation?

- *This is a very valuable and necessary question which needs a clear answer. We will begin by placing the current work squarely against the background of previous work. Since 2013, we have performed work in deer mice by means of repeated measures analysis. In other words, each animal was screened for the same behaviors twice—once before, and once after treatment—thereby constituting its own control. As such, in order to prevent doubling numbers for both the normal- and compulsive-like controls, we did not include a non-treated control before. That said, recently, a change in study design (in a study different to this one) was necessary to realize the answers of the study question. Here, we now include an excerpt from that data (please see images below; paper is submitted for publication and we can therefore not include all the data). Interestingly from this, we found that LNB exacerbates over time. Indeed, in untreated animals, the behavior of LNB-expressing animals become more inflated over time, a pattern that is completely prevented by escitalopram. We can therefore, with relative certainty, conclude that what is observed in this study is not habituation to the lever-presses in the four weeks between testing, but rather the effect of escitalopram on*

overall motivation to nest and engage in lever responses (Wolmarans, Stein, and Harvey 2016a). Indeed, that all animals still engaged in more presses on the first night following treatment, is indicative of the fact that the interest to engage in pressing is still there, but that the drive to do so continually has been diminished by escitalopram. Conversely, we believe what we see before treatment is habituation to the action itself, not to the willingness to engage in nesting, hence the continued generally higher presses made by LNB animals prior to treatment.

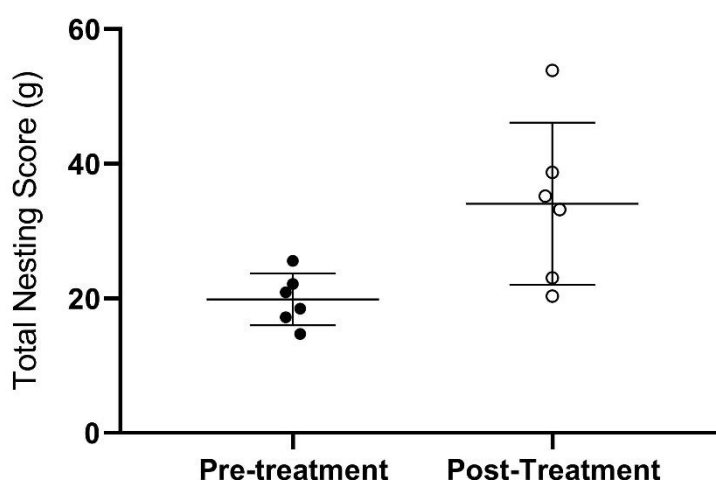


Figure 1 - Pre- vs. Post-treatment average total nest-building scores of LNB mice prior to and after 28-day normal water treatment. Student's paired t-test, $p = 0.0105$

The authors state that the animals received 50 mg/kg/day chronic citalopram treatment in the drinking water. They state the concentration used as well as an estimated water intake from a previous study. The water intake can however vary in the same animals over time as well as between different cohorts of animals. The authors should state the actual amount citalopram consumed by the animals with a confidence interval.

- *Thank you for the valuable comment. The average water-intake (and thus self-dosing) of deer mice has been well validated. As this has been performed before, we do not include this data in recent work due to space. However, here we include a graph of the average daily water intake of mice with and without escitalopram added. Also included are results of the 2-way RM ANOVA followed by Bonferroni's post hoc comparisons and the respective 95% CI values, indicating that no significant differences between the water consumption of the two groups as a function of treatment are evident. Further, since repeated injections over the course of a month*

would prove an enormous stressor as well as risk to the wellbeing of deer mice, no alternate routes of administration were considered. We have added a line to reflect this in the MS. Please see lines 247; 250-252. Lastly, this method of dosing has been employed several times previously (Wolmarans et al. 2013; Wolmarans, Stein, and Harvey 2016a, 2016b); these references are added in the lines 250-252 also. We hope these changes are satisfactory to address the concerns of the reviewer.

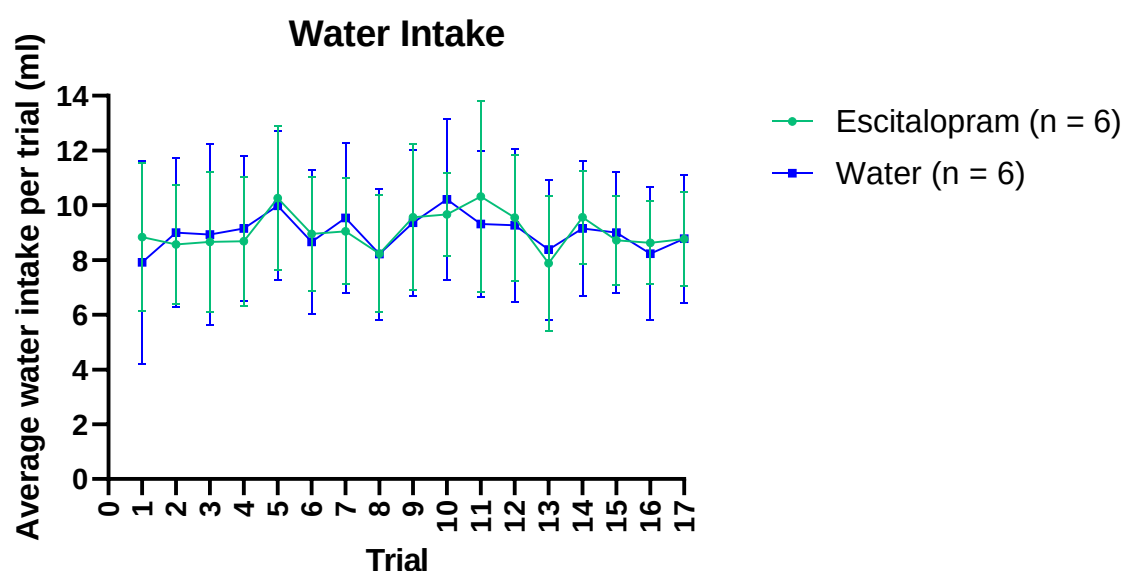


Figure 2: Average daily water intake of deer mice during pilot study with and without escitalopram. Data represented as mean $\pm$ 95% CI

Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	1.073	0.4147	ns	No	
Time	6.469	<0.0001	****	Yes	
Treatment	0.01075	0.9719	ns	No	
Subject	82.16	<0.0001	****	Yes	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	11.28	16	0.7051	F (16, 160) = 1.043	P=0.4147
Time	68.00	16	4.250	F (16, 160) = 6.287	P<0.0001
Treatment	0.1129	1	0.1129	F (1, 10) = 0.001308	P=0.9719
Subject	863.5	10	86.35	F (10, 160) = 127.7	P<0.0001
Residual	108.2	160	0.6760		

Figure 3: 2-way RM ANOVA data of water ingestion (see tables below also).

Bonferroni's multiple	Mean Diff.	95.00% CI of diff.	Adjusted P Value
Escitalopram - Water			
Row 1	0.9167	-3.248 to 5.082	>0.9999
Row 2	-0.4333	-4.598 to 3.732	>0.9999
Row 3	-0.2667	-4.432 to 3.898	>0.9999
Row 4	-0.4667	-4.632 to 3.698	>0.9999
Row 5	0.2833	-3.882 to 4.448	>0.9999
Row 6	0.2833	-3.882 to 4.448	>0.9999
Row 7	-0.4833	-4.648 to 3.682	>0.9999
Row 8	0.01667	-4.148 to 4.182	>0.9999
Row 9	0.2000	-3.965 to 4.365	>0.9999
Row 10	-0.5500	-4.715 to 3.615	>0.9999
Row 11	1.000	-3.165 to 5.165	>0.9999
Row 12	0.2833	-3.882 to 4.448	>0.9999
Row 13	-0.5000	-4.665 to 3.665	>0.9999
Row 14	0.4167	-3.748 to 4.582	>0.9999
Row 15	-0.2833	-4.448 to 3.882	>0.9999
Row 16	0.4000	-3.765 to 4.565	>0.9999
Row 17	-0.01667	-4.182 to 4.148	>0.9999

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	t	DF
Escitalopram - Water								
Row 1	8.833	7.917	0.9167	1.380	6	6	0.6641	170.0
Row 2	8.567	9.000	-0.4333	1.380	6	6	0.3139	170.0
Row 3	8.667	8.933	-0.2667	1.380	6	6	0.1932	170.0
Row 4	8.683	9.150	-0.4667	1.380	6	6	0.3381	170.0
Row 5	10.27	9.983	0.2833	1.380	6	6	0.2053	170.0
Row 6	8.950	8.667	0.2833	1.380	6	6	0.2053	170.0
Row 7	9.050	9.533	-0.4833	1.380	6	6	0.3502	170.0
Row 8	8.233	8.217	0.01667	1.380	6	6	0.01207	170.0
Row 9	9.567	9.367	0.2000	1.380	6	6	0.1449	170.0
Row 10	9.667	10.22	-0.5500	1.380	6	6	0.3985	170.0
Row 11	10.32	9.317	1.000	1.380	6	6	0.7245	170.0
Row 12	9.550	9.267	0.2833	1.380	6	6	0.2053	170.0
Row 13	7.883	8.383	-0.5000	1.380	6	6	0.3622	170.0
Row 14	9.567	9.150	0.4167	1.380	6	6	0.3019	170.0
Row 15	8.717	9.000	-0.2833	1.380	6	6	0.2053	170.0
Row 16	8.633	8.233	0.4000	1.380	6	6	0.2898	170.0
Row 17	8.767	8.783	-0.01667	1.380	6	6	0.01207	170.0

The authors should state the age of the animals included in this study.

- *Fixed. See lines 145-146.*

The authors do not state how statistical model assumptions were investigated (diagnostic plots) or ensured (transformation) in the statistical section. The authors state that "operant responses of neither NNB nor LNB animals were normally distributed". How did the authors ensure that their data met the model assumptions before statistical analysis? Furthermore, the authors use mean values to describe the data. Means are used to describe data that is normally distributed and using means to describe skewed data gives a misrepresentation of what one would expect for an "average animal". Median values would be more informative to describe data that is not normally distributed.

- *The data in question was analyzed by means of 2-way repeated measures ANOVA, a test that can account for data which is not normally distributed (Maxwell and Delaney 2004; Laerd Statistics 2017). We have added lines in the Results section to clarify this. Please see lines (255; 276-282; 327-330). Furthermore, when referring to specific mean values (in keeping with the parametric test used—no suitable non-parametric test exist which specifically perform repeated measures analysis of two factors), we have now also added the median values. See lines 291; 302.*

The authors report interesting differential responses to change in outcome contingency and punishment in LNB compared to NNB mice that support the use of nest building behavior in LNB as a compulsivity measure. It does not appear that this effect reached statistical significance, due to the small power of the study. The authors used Cohen's d to describe effects sizes, but it would be more informative to also add results of between-group RM ANOVA.

- *Indeed, this is important. The work reported here was characterized by substantial between-animal variation. This was unexpected but also understandable given that animal behavior is governed by several processes of which we aimed to analyze the association between a phenotype like nest building and a singular executive correlate. As such, remarkable different responses were observed, while we attempted to highlight the broad pattern differences between the NNB and LNB cohorts. As such, the between group comparisons (which we presume are referred to and of which the data is provided in Table 1), the p values all of which were > 0.9999, and were thus not reported in the narrative or the Tables. We do however provide the ANOVA statistics in the narrative for a better view of the lack of between group significance.*

Minor points:

The authors used a paired t-test to analyze baseline nest-building behavior in figure 2. What is the rationale for using a paired t-test?

- *Thank you for picking up on this. We checked again, and did indeed use an unpaired student t-test, but incorrectly reported this in the MS. Please see line 801 (fixed in caption).*

Please avoid using the word "gender" to describe the sex of animals.

- *Fixed. Please see line 175.*

The study includes baseline measurements of nest building behavior used to allocate animals into LNB and NNB groups. It would be interesting to see whether within-group lever pressing corresponds to baseline nesting scores and strengthen the rationale for allocating animals by this method.

- *Agreed! However, this is difficult as the nature of lever-pressing in our view and experience cannot be compared directly with the act of naturalistic nesting under laboratory conditions. As such, more validation work is needed in order to establish the most accurate comparisons between lever-pressing and inherent baseline nesting. The purpose here was to apply previous methodology and measure whether animals will actually engage in lever pressing to obtain nesting material, and not to provide a direct comparison between the actual magnitude of nesting between the two methods. LNB animals can indeed be separated from normal controls based on serotonergic response and behavioral persistence (Wolmarans et al. 2013; Wolmarans, Stein, and Harvey 2016a, 2016b; Scheepers, Stein, and Harvey 2018), a separation which formed the basis of this work.*

Furthermore, these data can be used to investigate how stable the phenotype is over time. Did the authors perform the same baseline screening test at the end of the experiments?

- *No, there was no manual nest building scoring following the post-treatment phase. However, although previous studies shown the stability of nesting behavior in normal NNB animals before, we have now included preliminary data to support the fact that LNB behavior exacerbates over time (see figures introduced above in response to a different comment raised by the same reviewer). In other words, the LNB phenotype fortifies over time, in that regard being stable.*

Do the authors have locomotor data for these animals to ensure that lever engagement and nesting behavior is not due to non-specific differences in activity of the two phenotypes?

- *No, unfortunately there is not locomotor data for these specific animals, as we also previously have shown the locomotor behavior of animals to stay unaltered as a function of treatment with the same regimen (in this regard, please refer to the figure below and the work we have done before in different studies; Wolmarans, Stein, and Harvey 2016b). However, as nesting is a behavior related to higher cognitive planning and execution, we have previously published work on nesting without concurrent locomotor behavior. This is opposed to work in marble burying, of the quantification can be potentially affected by hyper and hypoactivity (Nicolas, Kolb, and Prinssen 2006; Wolmarans, Stein, and Harvey 2016b; De Brouwer and Wolmarans 2018).*

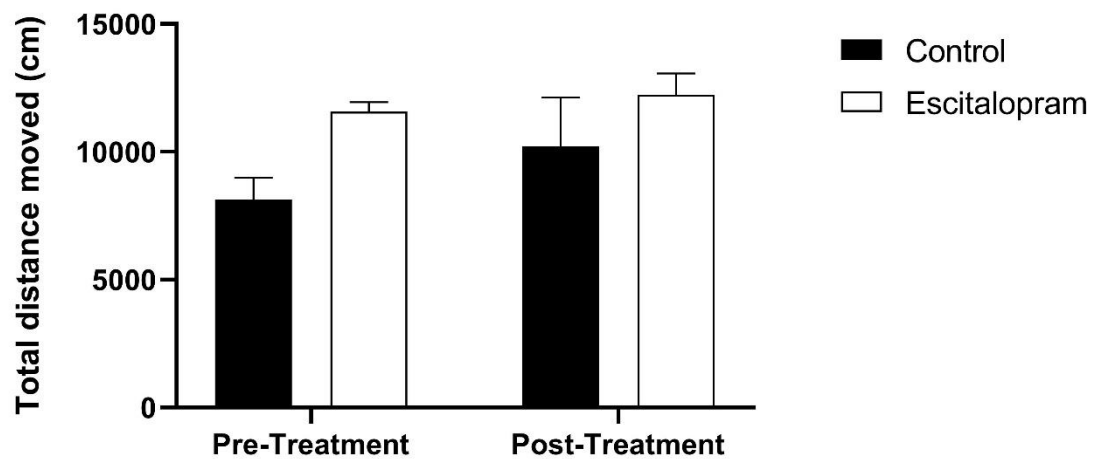


Figure 4 - Pre and post-treatment locomotor activity of deer mice treated with either control or escitalopram

References

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- Wolmarans, D.W., Stein, D.J., Harvey, B.H. 2016a. Excessive nest building is a unique behavioural phenotype in the deer mouse model of obsessive-compulsive disorder. *Journal of Psychopharmacology*. 30, 867-874.
- Wolmarans, D.W., Stein, D.J., Harvey, B.H. 2016b. Of mice and marbles: Novel perspectives on burying behavior as a screening test for psychiatric illness. *Cognitive, Affective, and Behavioral Neuroscience*. 16, 551-560.

ii) Apparatus Used for the Lever-Press Investigation

Design and Construction

The study described in manuscript C made use of a novel apparatus designed and constructed following a collaborative effort between the Instrument Making and Electrical and Electronic Services departments of the North-West University, Potchefstroom. The respective representatives from each department were Mr. Thys Taljaard and Mr. Albert Pienaar. All aspects of the design and construction of the apparatus were carried out by these individuals and their teams.

Inventory and Description

The lever pressing apparatus consists of:

- 1 x Computer running custom written control software;
- 1 x Integration unit;
- 1 x UPS capable of powering the entire system in the event of power failure;
- 24 x Nesting material dispensers containing integral levers;
- 24 x Connecting cables;
- 24 x Cable shields;
- 24 x Connection ports wired into a rat-housing rack;

**Photographs of all the described components follow this text.*

The apparatus consists of a computer (**Figure 1A**) which is connected to a custom-designed integration unit (**Figure 1A**), containing various micro-circuits (**Figures 1B and C**) which are in turn wired into one of the frames typically used to house the standard rat cages* (**Figures 4A and 4C**). Thus, a series of 24 ports are wired into the frame, to which the material dispensers connect directly (**Figure 4B**). The system further comprises 24 individual nesting material dispensers, each containing an integral lever press (**Figures 2A, 2B and 2C**). The cartridges are pre-loaded with nesting material (**Figure 3B**) and placed inside the rat cages (**Figures 3C and 3D**) after which they are placed into the frame (**Figure 4C**) and connected to the software system.

Once cages are prepared in this manner, the experiment can be programmed using the program that was custom written to control the apparatus (Response Outcome Contingency Software, ROCS; v1.0.2, Electronic Services, North-West University, Potchefstroom Campus). This program has several functionalities which can be used to calibrate the system, and to design (**Figure 6**) and monitor experiments (**Figure 7**).

**The use of rat cages was necessitated by the dimensions of the lever press dispensers. To ensure that the mice could comfortably drink, water bottles with extra-long necks were used.*

System Capabilities

The system can primarily be programmed to roll out a specified length of nesting material (or any other substance/material which can be fashioned into a lengthy, relatively flat ribbon shape no wider than 15 mm), in multiples of 10 mm following successful lever presses. The rollers which deliver nesting material lock when inactive, making the acquisition of material impossible without a successful press. Lever presses are counted per cage and can be timestamped. The levers can be independently programmed to deliver electric shocks of a range of amperages. Any specific combination of cages can be selectively punished by using the experimental design interface (**Figure 6**). Furthermore, the lever press dispensers and ports are designed in such a way that two dispensers can be inserted per cage should experimental design require this. Lastly, the tension on the levers is governed by simple springs which, at a relatively low effort, can be retrofitted with more robust springs to adapt the dispensers to function with heavier animals such as rats.

(Figures follow on the next page)

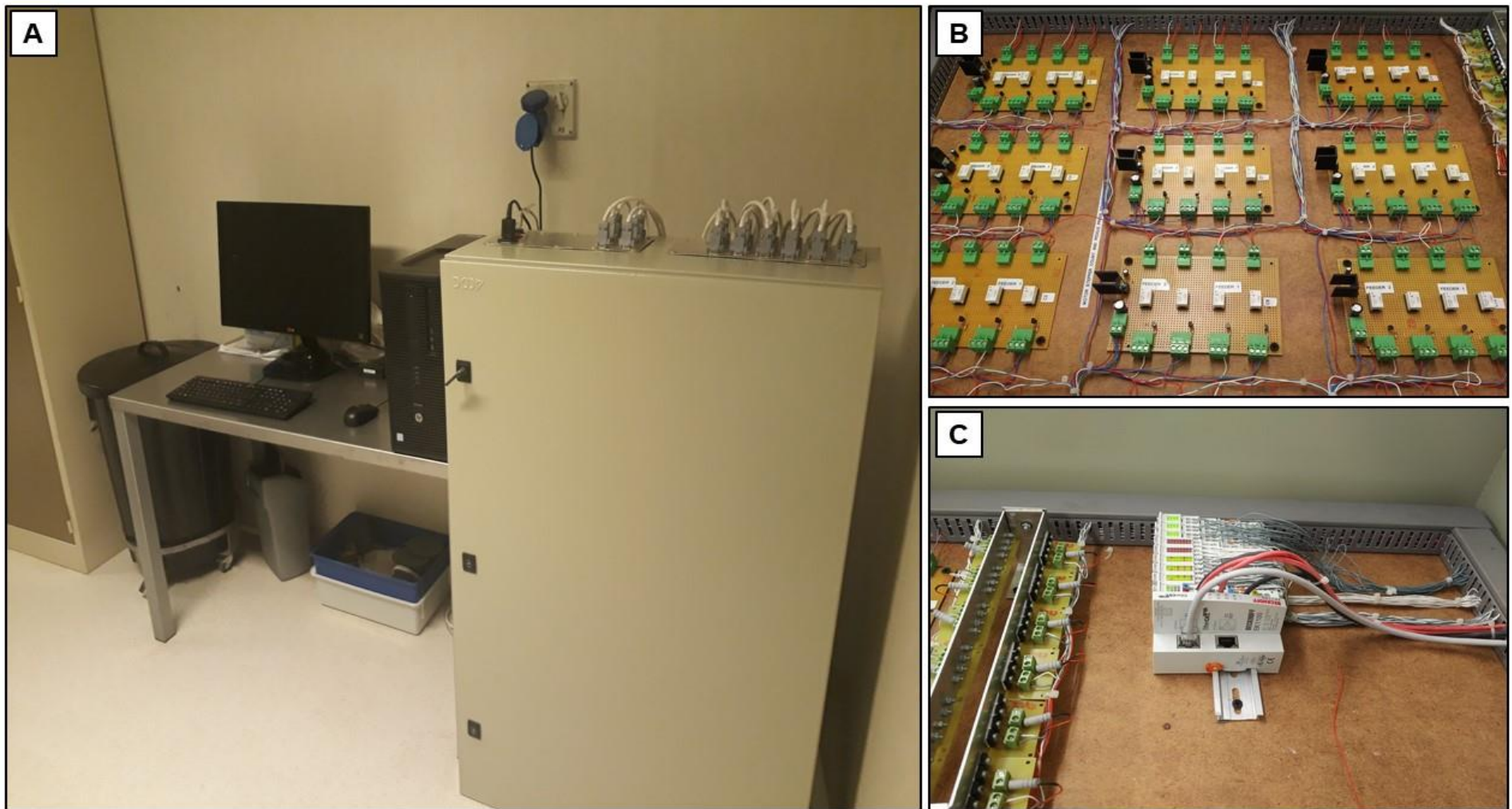


Figure 1 - The control system for the entire lever-press apparatus. A) The workstation computer which operates the system and processes data, alongside the integration unit (beige box to the right of workstation) which receives data from the various test cages; B) microcircuits inside the integration unit which control the shock amperage delivered to each respective dispenser if activated; and C) the programmable central control unit inside the integration unit which sends instructions to the dispenser units and relays data back to the computer.

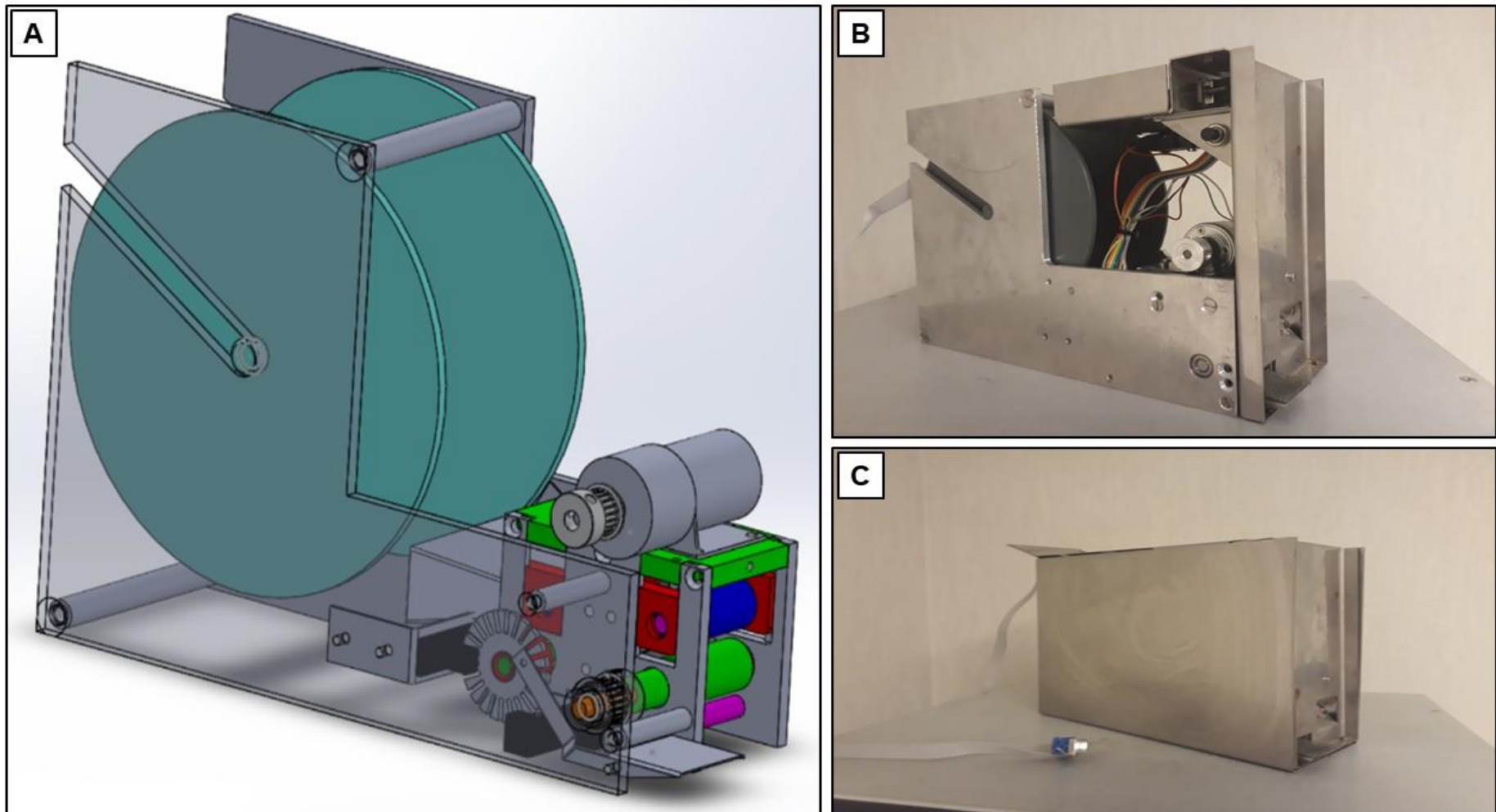


Figure 2 - The cage inserted lever-press nesting material dispensers. A) the design drawing of the internal construction of the dispensers; notably visible is the large removable spool which contains the nesting material, the motor which drives the rollers and the depressible lever; B) the constructed dispenser without protective cover and face plate in place; and C) the dispenser with the protective cover and cable guard attached.

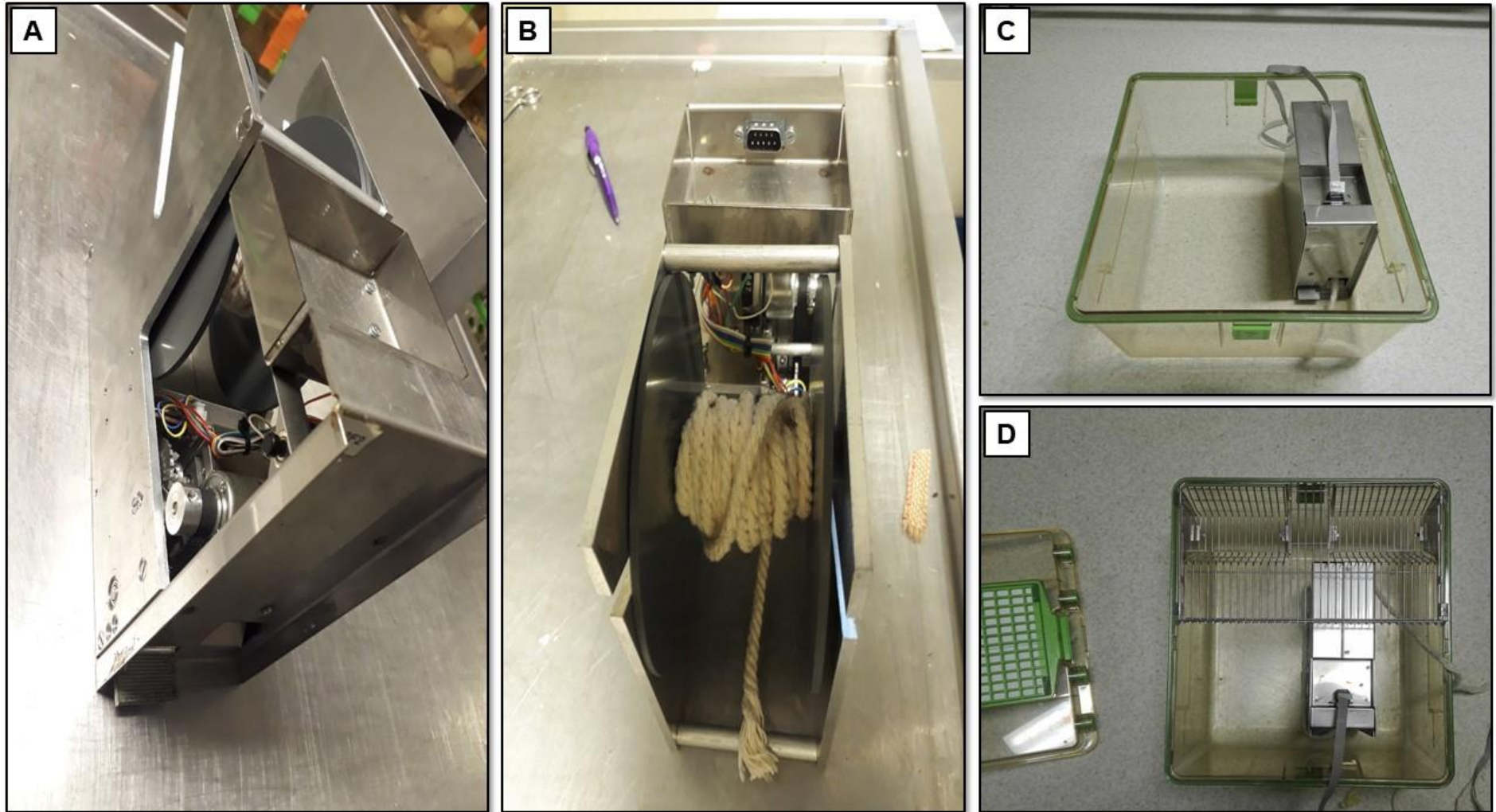


Figure 3 - The preparation of test cages. A) dispenser without any material loaded; B) the spool is removed and material is wound around it and then fed through the rollers, see also the connection port where the connector cable is plugged into the unit; C) the protective cover is replaced and the loaded dispenser is placed in the test cage; and D) the food grid (empty in this example) is placed. The cage is now ready to be placed in the housing system and connected to the integration unit.

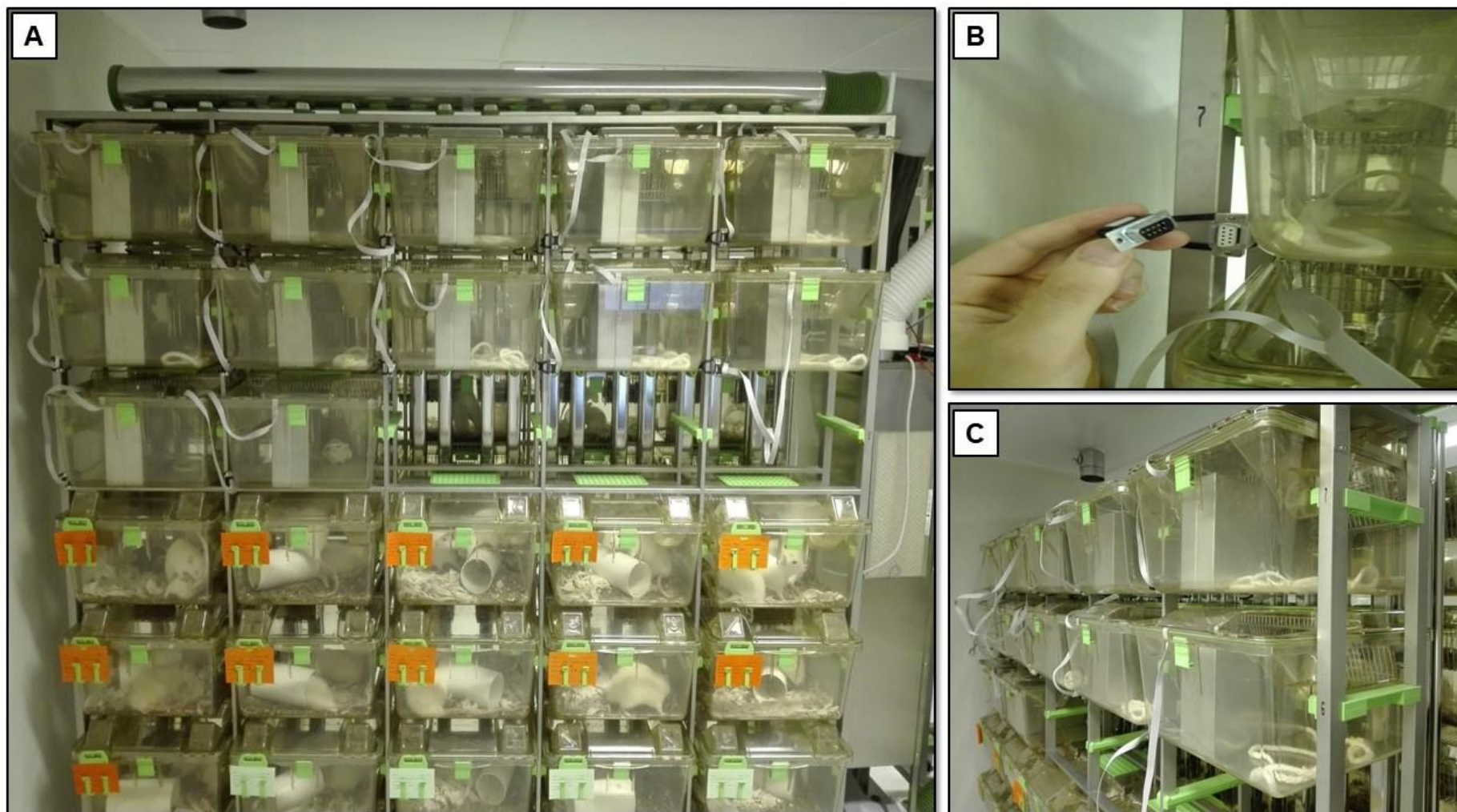


Figure 4 - The dispensers as used in the housing framework. A) 12 cages placed in the rodent housing rack and connected to the port available for each cage slot, only the top three rows are currently connected to the system; B) the connection port which relays information back and forth between the dispensers and the integration unit; and C) side view of the test cages showing the connected dispensers, with food grids and cage lids (which normally hold water bottles) in place.



Figure 5 - Test cages immediately after being removed from the rodent housing rack following a twenty-four-hour test session.

Setup Experiments

Filter: Status: Experiments:

Current Experiment Details

Name:

	Enable Cage	Cage Name:	Material Type 1:	Material Type 2:	Enable Feeder 1	Enable Feeder 2	Feeder 1 Feed Length (mm)	Feeder 2 Feed Length (mm)	Enable Pedal Current 1	Enable Pedal Current 2	Pedal Current 1	Pedal Current 2	Enable Pedal Count 1	Enable Pedal Count 2
Cage 1 =>	☒	Cage 1	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 2 =>	☒	Cage 2	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 3 =>	☒	Cage 3	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 4 =>	☒	Cage 4	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 5 =>	☒	Cage 5	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 6 =>	☒	Cage 6	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 7 =>	☒	Cage 7	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 8 =>	☒	Cage 8	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 9 =>	☒	Cage 9	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 10 =>	☒	Cage 10	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 11 =>	☒	Cage 11	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒
Cage 12 =>	☒	Cage 12	Sponge	Sponge	☒	☒	10	10	☒	☒	01 mA	01 mA	☒	☒

Feeder Detect Distance: mm

Status:

Add New Experiment

Edit Experiment

Cancel

Save

Figure 6 - Screenshot of the form used to program the experimental parameters for a test session.

Experiment GUI

Summary | Cage 1 | Cage 2 | Cage 3 | Cage 4 | Cage 5 | Cage 6 | Cage 7 | Cage 8 | Cage 9 | Cage 10 | Cage 11 | Cage 12

Summary

Cage No	Nest Material 1	Nest Status 1	Nest Feed 1 (mm)	Peddle Current 1 (mA)	Peddle Count 1	Nest Material 2	Nest Status 2	Nest Feed 2 (mm)	Peddle Current 2 (mA)	Peddle Count 2
1	Sponge	OK	10	0	1	Sponge	N/A	0	0	0
2	Sponge	OK	20	0	2	Sponge	N/A	0	0	0
3	Sponge	OK	40	0	4	Sponge	N/A	0	0	0
4	Sponge	OK	60	0	6	Sponge	N/A	0	0	0
5	Sponge	OK	10	0	1	Sponge	N/A	0	0	0
6	Sponge	OK	0	0	0	Sponge	N/A	0	0	0
7	Sponge	OK	0	0	0	Sponge	N/A	0	0	0
8	Sponge	OK	10	0	1	Sponge	N/A	0	0	0
9	Sponge	OK	10	0	1	Sponge	N/A	0	0	0
10	Sponge	OK	0	0	0	Sponge	N/A	0	0	0
11	Sponge	OK	30	0	3	Sponge	N/A	0	0	0
12	Sponge	OK	10	0	1	Sponge	N/A	0	0	0

Settings



Filter - Choose Experiment: _____
Status: Enabled
Experiments: Compulsive press

Experiments Name: Night 6

Stop Experiment

Start Timestamp: 6/14/2018 8:48:55 AM
End Timestamp: Not Set
Runtime: Hour:23 Min:57 Sec:8

Status: Running...

Addendum G - Additional Article I

Manuscript prepared for submission to *Journal of Psychopharmacology* titled:

“Large nest building and high marble-burying: two compulsive-like phenotypes expressed by deer mice (*Peromyscus maniculatus bairdii*) are distinctly regulated by serotonergic and dopaminergic intervention”

Author Contributions:

- *Geoffrey de Brouwer* assisted with the behavioral tests, data processing and interpretation and wrote this manuscript.
- *Arina Fick* assisted in the conceptualization of the investigation with *De Wet Wolmarans* and *Ané Lombaard*, performed the behavioral tests, captured and processed data and reported a portion of this data in her M.Sc. thesis*.
- *Ané Lombaard* assisted in the conceptualization of the investigation with *De Wet Wolmarans* and *Arina Fick*, performed the behavioral tests, captured and processed data and reported a portion of this data in her M.Sc. thesis*.
- *Dan Stein* and *Brian Harvey* provided expert insight regarding this investigation as a whole and assisted in the writing of this manuscript.
- *De Wet Wolmarans* as the study leader, assisted in the conceptualization of the study together with *Arina Fick* and *Ané Lombaard*, processed and interpreted the data, and co-wrote the manuscript.

Important Information:

- Instructions to the author can be accessed here:
<https://journals.sagepub.com/author-instructions/JOP>
- The article has been prepared for publication in *the Journal of Psychopharmacology* and will be submitted there.
- The data reported in this manuscript was generated throughout the course of the M.Sc. studies of two students of the NWU, namely *Mrs. Arina Fick* and *Ms. Ané Lombaard*. As such, certain portions of the data have been reported in the dissertations of these two students. Since the candidate of this thesis (*Mr. Geoffrey de Brouwer*) assisted with the research, he was given the opportunity to first author the manuscript presented here, by synthesizing the data generated by these two students. As such this work is presented as additional evidence of the candidate's experience and is therefore not intended for examination purposes, as it did not form part of the initial scope of this thesis as a whole and much of the experimentation was carried out by the two aforementioned students. None of the data presented here has been published as of yet.

“Large nest building and high marble-burying: two compulsive-like phenotypes expressed by deer mice (*Peromyscus maniculatus bairdii*) are distinctly regulated by serotonergic and dopaminergic intervention”

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Abstract

The current investigation aimed to further dissect the deer mouse (*Peromyscus maniculatus bairdii*) model of obsessive-compulsive disorder (OCD) with respect to two compulsive-like behavioral phenotypes viz. large nest building (LNB) and high marble-burying (HMB) as characterized in the species. Since LNB is sensitive to chronic, high dose escitalopram intervention while HMB seems less so, we aimed to determine whether the two behaviors could be further separated based on their response to 4 weeks of serotonergic (i.e. escitalopram 50 mg/kg/day) or dopaminergic modulation, i.e. the dopamine D_{1/2} receptor blocker, flupentixol (0.9 mg/kg/day) and the monoaminoxidase type B inhibitor, rasagiline (5 mg/kg/day), and their combinations with escitalopram. We found that LNB is responsive to chronic escitalopram with or without co-administration of flupentixol, while HMB remained insensitive to either of these interventions. However, HMB showed significant reduction following chronic combined escitalopram and rasagiline intervention, pointing to a unique involvement of the dopaminergic system in HMB compared to LNB. Additionally, we report for the first time that scoring preoccupied interaction with marbles over several trials is a more appropriate measure of compulsive-like behavioral persistence. In conclusion, the data presented here provide evidence that two unique, naturally occurring compulsive-like behaviors in deer mice have different neurobiological underpinnings. This has valuable implications for the pre-clinical modelling and our understanding of symptom heterogeneity and treatment resistant OCD.

Keywords:

Obsessive-compulsive disorder; marble-burying test; nest building; animal model; escitalopram; flupentixol; rasagiline; deer mouse model; treatment resistance

Introduction

Obsessive-compulsive disorder (OCD) is a neuropsychiatric disorder that affects 2-3% of the global population. The condition is characterized by obsessions, i.e. intrusive, unwanted thoughts, and/or compulsions, i.e. persistent, repetitive and time-consuming behavioral routines (Ruscio et al., 2010; American Psychiatric Association, 2013). Classically, five broad phenotypes of obsessive-compulsive (OC) symptom phenotypes have been identified, i.e. contamination obsessions with washing compulsions, safety obsessions with checking compulsions, obsessions concerning symmetry paired with ordering/organizing compulsions, covert intrusive thoughts with repugnant themes, i.e. violent or sexual acts that are associated with ritualistic, self-directed acts, e.g. praying, and fears of losing objects and hoarding compulsions (Mataix-Cols et al., 2005; Hirschtritt et al., 2017). Although excessive collecting symptoms are frequently noted in OCD (Boerema et al., 2019), fewer than 5% of OCD patients are diagnosed with hoarding as the primary obsessive compulsive-phenotype (Albert et al., 2015). In fact, hoarding had been afforded unique disorder status in the DSM-5 (American Psychiatric Association, 2013). A diagnosis of hoarding disorder differs in meaningful ways from OCD, with or without collecting compulsions. For example, hoarding-related thoughts are usually not experienced as intrusive or distressing, rather eliciting a sense of pleasure; therefore, as opposed to patients suffering from OCD, individuals with hoarding disorder do not feel overly compelled to engage in symptom-correcting behaviors (Albert et al., 2015). Further, in hoarding disorder, patients describe symptoms from an ego-syntonic perspective, rather than the ego-dystonic symptom experience of patients with OCD (Albert et al., 2012). Last, and important for the current investigation, hoarding disorder seems overly treatment refractory (Bloch et al., 2014) to the first line pharmacotherapeutic interventions for the treatment of OCD, i.e. selective serotonin reuptake inhibitors (SSRIs; Fineberg et al., 2013; Oregan ECHO Network, 2018).

That said, a considerable proportion of OCD patients also remain unresponsive to SSRI treatment (Atmaca, 2016; Hirschtritt et al., 2017). In such cases, SSRI therapy is often augmented with low-dose dopamine-2 (D_2) receptor antagonists, e.g. haloperidol, risperidone or quetiapine (Bloch et al., 2006; Fineberg et al., 2013; Veale et al., 2014). Importantly, these drugs, most notably the second generation neuroleptic compounds, e.g. risperidone, also inhibit serotonin receptors, including the 5-HT_{1A/D} and 5-HT_{2A/C} subtypes (Richelson and Souder, 2000), which may contribute to their efficacy as augmenting agents (Ramasubbu et al., 2000; Westenberg et al., 2007; Goddard et al., 2008).

Increasingly, evidence suggests that different subtypes of OCD vary in their underlying neurobiology (Mataix-Cols et al., 2004; Leckman et al., 2009; Thorsen et al., 2018), cognitive

neuropsychology (McKay et al., 2004; Hashimoto et al., 2011; Martoni et al., 2015), and treatment response (Rufer et al., 2006; Stein et al., 2007; Grados and Riddle, 2008). There is also growing interest in whether particular Research Domain Criteria (RDoC) constructs, such as reward processing, are more relevant to particular OCD subtypes (Figeet al., 2011; Hauser et al., 2017). Indeed, recent evidence highlighted potentially unique reward-feedback processes underlying different phenotypes of obsessive-compulsive (OC) symptomology, which, considering the role of serotonin and dopamine in reward feedback processing, could implicate unique serotonergic and dopaminergic involvement in the manifestation of different symptom dimensions of OCD (Cavedini et al., 2006; Figeet al., 2011; Wong et al., 2015)

Few pre-clinical studies have explored the neurobiological underpinnings of different compulsive-like phenotypes within a specific model framework. This is likely due to the lack of animal models that are representative of naturalistic, but heterogeneous OC-like behavior (Scheepers et al., 2018). In this regard, we have previously described three unique naturalistic compulsive-like phenotypes exhibited by deer mice (*Peromyscus maniculatus bairdii*), i.e. spontaneous stereotypy (Wolmarans et al., 2013), large nest-building (LNB; Wolmarans et al., 2016a) and high marble-burying (HMB; Wolmarans et al., 2016b). These three behaviors, which have been reviewed in depth before (Scheepers et al., 2018), are equally repetitive, persistent and seemingly purposeless. Whereas spontaneous stereotypy (Wolmarans et al., 2013) and LNB (Wolmarans et al., 2016a) demonstrate therapeutic response to chronic high dose (50 mg/kg/day) oral SSRI administration, HMB is less responsive to such intervention (Wolmarans et al., 2016b). As such and taking its phenotypic presentation in mind, we have previously proposed that HMB may provide a useful avenue for exploring hoarding-related mechanisms (Scheepers et al., 2018).

Considering the literature summarized above, it would be of interest to determine whether selective pharmacological manipulation of serotonergic and dopaminergic activity would modify the expression of LNB and HMB in unique ways. To this extent, we employed chronic, high-dose oral administration of the SSRI, escitalopram (50 mg/kg/day), alone or in combination with either a dopamine-selective $D_{1/2}$ receptor antagonist, flupentixol (0.9 mg/kg/day; Jenner and Marsden, 1987), or the dopaminergic monoamine oxidase B (MAO-B) inhibitor, rasagiline (5 mg/kg/day; Eigeldinger-Berthou et al., 2012). Specifically, we hypothesized that since LNB demonstrates robust response to SSRI intervention alone, such behavior will not show additional response to anti-dopaminergic modulation either alone or in combination with escitalopram. In contrast, since HMB is less responsive to SSRI intervention, we propose it to be possible that such behavior represents a compulsive-like phenotype more

founded within dopaminergic perturbations, thereby demonstrating unique response to interventions targeting dopamine signaling.

Materials and methods

Animals

Since only 11 – 15% and 30% of deer mice, regardless of sex, express HMB (Wolmarans et al., 2016b; de Brouwer and Wolmarans, 2018) and LNB respectively (Wolmarans et al., 2016a), a total of 280 deer mice were initially screened for marble-burying (MB) behavior, followed by nest building (NB) screening, allowing for the selection of 36 HMB and 36 LNB expressing subjects, respectively. Each of the respective groups were further divided into six drug groups ($n = 6$ per group; see below). All mice (outbred; both sexes; 10 – 12 weeks old at the onset of investigation) were obtained from the in-house deer mouse colony of the Vivarium of North-West University, Potchefstroom, South Africa. The study was approved by the relevant ethics committee prior to the onset of investigation (approval number: NWU-00262-16-A5; AnimCare Research Ethics Committee, registration number: AREC-130913-015). All steps of animal handling conformed to the South African National Standard (SANS) for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008). The original breeding pairs were acquired from the *Peromyscus* Genetic Stock Centre at the University of South Carolina, USA. Animals were group-housed in same-sex groups of four to six animals per individually ventilated and climate-controlled home cage (35 cm (l) × 20 cm (w) × 13 cm (h); Techniplast SPA, Varese, Italy) until one week prior to the first behavioral assessment. From this point onwards, animals were single housed; this is necessary in assessments of NB as experiments are carried out over the course of 24 hours (Wolmarans et al., 2016a). Environmental conditions were maintained at 23°C, 50% humidity, and a 12-hour light/dark cycle (06h00/18h00). Food and water (or drug solutions where applicable) were provided *ad libitum* for the duration of the study, except during the execution of the 30-minute marble-burying tests. Cages were changed and cleaned once a week. Prior to behavioral experimentation, bedding consisted of ground corncob (particle $\varnothing$ 3–4 mm) and nesting material was provided in the form of unscented, white paper towel. Animals not selected for inclusion into either cohort, i.e. HMB or LNB, were excluded from further investigation and used for studies unrelated to this work.

Behavioural tests – general background

The aim of the investigation was to study associations between different behavioral phenotypes and unique drug responses. Therefore, clear separation between animals that expressed either behavior, *but not both*, was imperative. As such, all 280 animals were first

screened for HMB activity (paragraph 2.3.1). Following the selection of the 36 HMB expressing animals they, along with an additional 144 mice from the original pool of 280, were screened for LNB (paragraph 2.3.2). Although none of the HMB-expressing individuals also engaged in LNB, animals that expressed neither of the behaviors were also excluded from further study. Further, as the present investigation sought to characterize the drug-response of compulsive-like cohorts, non-compulsive-like animals were excluded. The normal behavioral controls for LNB and HMB, as well as their responses to chronic high-dose oral escitalopram (50 mg/kg/day) have been described elsewhere (Wolmarans et al., 2016a; Wolmarans et al., 2016b; however, please refer to the supplementary data for a comparison between NNB and LNB animals).

The marble-burying test

The marble-burying test (MBT) was carried out as described previously (de Brouwer and Wolmarans, 2018). Briefly, marble-burying cages (exact replicas of the home cages) were prepared with a 5-cm thick layer of course river sand on top of which nine glass marbles ($\varnothing=15$ mm) were arranged in a 3 x 3 grid equidistant to one another on *one half* of the cage only, thereby constituting a two-zone MBT setup. This allowed mice to willingly and voluntarily engage with marbles while also ensuring that novelty-induced avoidance behavior could be expressed if present and so identified. The use of course river sand, as opposed to sawdust, husk or wood chips as often reported in this test (Poling et al., 1981; Young et al., 2006; Kedia and Chattarji, 2014; Taylor et al., 2017), prevents marbles being unintentionally covered due to routine cage locomotion (de Brouwer and Wolmarans, 2018; de Brouwer et al., 2019). Since deer mice are nocturnal animals, all marble-burying assessments were carried out from 1 hour after onset of the dark cycle under dim red light (40 lux).

Thirty-six hours prior to the first marble-burying assessment before (pre-intervention screen) and again after four weeks of drug administration (post-intervention screen), mice were habituated to the burying substrate by replacing the corncob home cage bedding with the same coarse river sand used in the MBT; this was to prevent novelty induced digging (Handley, 1991; Gyertyan, 1995). Subsequently, each animal was assessed for marble-burying activity over three nights in three separate 30-minute trials (one per night), separated by 24 hours. This process was repeated before and after chronic drug administration. At the time of testing, animals were removed from their home cages and introduced into pre-prepared marble-burying cages. Due to the highly active nature of deer mice, cages were covered with clear covers to prevent animals from leaving the cages. Mice were subsequently left to explore and interact with marbles for 30 minutes in the absence of human observers, while being videotaped for later analysis. After each 30-minute session, animals were returned to their

respective home cages which still contained sand and left under standard housing conditions for approximately 24 hours until the next trial. This procedure was repeated twice over the next two days, until all animals underwent three separate pre- and post-intervention screens.

To identify the 36 HMB expressing animals, and appraise the findings reported in this investigation against the background of previously reported results (Egashira et al., 2013; Egashira et al., 2018), the number of marbles covered in burying substrate to a depth at least two-thirds of the marble size were quantified after completion of each 30-minute screening session. This was performed by two experimentally blind observers of whom the scores were averaged. Importantly, after the first three pre-intervention marble-burying assessments had been completed, the 36 HMB-expressing animals were selected based on 1) the average number of marbles covered *and* 2) the persistence of burying behavior over three trials as evinced by lower coefficients of variation with respect to the between daily burying scores (Wolmarans et al., 2016b; **Figure 1A**). Only the 36 animals selected for HMB during the pre-intervention phase were retested in the MBT after 28 days of drug intervention.

When MB is used as a screening test for anti-compulsive drug action, quantification of the number of marbles ‘buried’ is most often reported (Egashira et al., 2013; Wolmarans et al., 2016b; Taylor et al., 2017; Egashira et al., 2018). However, this measure is inadequate for highlighting seemingly purposeless compulsive-like persistence (de Brouwer and Wolmarans, 2018). Thus, marble-directed behaviors (MDBs) were assessed to better establish behaviors that are indicative of *preoccupation* with the marbles (de Brouwer et al., 2019). MDBs were scored by visual inspection of video-taped trials and assigned a single count for any of the following: 1) sniffing and licking of marbles, 2) rolling or touching of marbles with forepaws, 3) standing on marbles, and 4) any movement directed in the immediate vicinity of a marble that was aimed at covering the marble. As for the number of marbles buried, scorers were blind to the experimental conditions.

Nest building analysis

Following the last pre-intervention MBT, home cages of the 36 HMB expressing subjects and an additional 144 mice were prepared with an excess of pre-weighed, non-scented hospital grade cosmetic cotton wool placed above the steel grid within the roof of the home cage. Every subsequent day between 13h00 and 14h00, built nests were removed, discarded and the unused cotton wool weighed (Wolmarans et al., 2016a). Mice were therefore left to utilize the cotton wool for approximately 23 hours of every day for 7 consecutive days. As NB analyses were conducted in the home cages, food and water were available *ad-libitum* for the duration of this screening. Animals did not have access to any additional form of nesting material during this time. As with marble-burying, classification of LNB (**Figure 1B**) was based

on 1) total nesting score generated over seven days, and 2) the behavioral variance over the course of seven days as reflected by the coefficients of variation with respect to the daily nesting scores. Only the 36 animals which consistently built the largest nests, were included in the LNB cohort (**Figure 1B**; Wolmarans et al., 2016a) and retested after 28-days of drug intervention.

Locomotor activity

As adequate locomotor ability (LMA) is an important prerequisite for behavioral expression as well as being an accurate identifier of drug-induced locomotor activation or inhibition, general locomotor behavior was assessed by tracking the total distance moved during the execution of each MBT by means of Ethovision XT[®] 14 software (Noldus[®] Information Technologies, Wageningen, The Netherlands). Importantly, although it is possible that marble-directed preoccupation could result in falsely lowered ambulatory scores, we have previously shown that no correlation exists between the number of MDB and the total distances moved by deer mice during a 30-minute session (de Brouwer and Wolmarans, 2018).

Drug interventions and post-intervention testing

36 HMB and 36 LNB animals were selected following the first round of MB and NB assessments. Each group was divided randomly into 6 intervention groups ($n = 6$), each receiving chronic (28-day) administration of either 1) normal drinking water, 2) high-dose escitalopram (50 mg/kg/day; Wolmarans et al., 2013; Wolmarans et al., 2016a; Wolmarans et al., 2016b), 3) rasagiline (5 mg/kg/day; Eigeldinger-Berthou et al., 2012), 4) flupentixol (0.9 mg/kg/day; Jenner and Marsden, 1987), 5) a combination of escitalopram and rasagiline, or 6) a combination of escitalopram and flupentixol at the aforementioned doses. Higher than normally used oral doses of rasagiline were administered (Huang et al., 1999) as 13 – 15% of orally administered rasagiline promptly redistributes to the skin (Meier-Davis et al., 2012) while another 36 – 40% is eliminated during the first pass effect (Mittal et al., 2016). To mimic its application in clinical OCD, a low dose of flupentixol (0.9 mg/kg/day) was chosen based on earlier work that employed oral drug administration via drinking water (Murugaiah et al., 1982; Murugaiah et al., 1983; Jenner and Marsden, 1987). All drugs (H. Lundbeck[®] A/S, Ottilavej, Denmark) were administered via the drinking water at concentrations calculated to deliver the stated doses according to the daily water intake of deer mice (0.25 mg/kg/day) as previously determined in our laboratory (Wolmarans et al., 2013) and confirmed by others (Aschhoff et al., 2000). Fresh drug solutions were prepared every second day (Wolmarans et al., 2013; Wolmarans et al., 2016a; Wolmarans et al., 2016b). As per the observations reported previously (Jenner and Marsden, 1987; Huang et al., 1999; Eigeldinger-Berthou et al., 2012; Wolmarans et al., 2013), the addition of the drugs to the drinking water did not alter the

average water consumption (data not shown). Oral drug administration by means of drinking water is the preferred route in our laboratory due to the potential influence of injection- or oral gavage-induced anxiety on the behavioral expression of deer mice, particularly when considering the lengthy 28-day drug administration period. Following 28 days of drug administration, mice were retested according to identical procedures as those explained above.

Statistical analysis

Statistical analyses were performed using GraphPad Prism® 8.01 (GraphPad® Software, San Diego, USA). As the behavioral data generated for the complete pool of 280 animals were not normally distributed (**Figure 1A**, total burying scores: $p < 0.0001$; **Figure 1B**, total NB scores: $p < 0.01$; Shapiro-Wilk test), Spearman's correlational analyses and column statistics (broad clustering within the upper 75th percentile for burying scores, MDBs and total nesting scores, and the lower 25th percentile for the respective coefficients of variation) were applied to identify HMB- and LNB-expressing subjects (Wolmarans et al., 2016a; Wolmarans et al., 2016b). Pre-and post-intervention expression of HMB and LNB, as well as LMA in the case of the HMB expressing animals were analyzed and compared by means of two-way repeated measures analysis of variance (2-way RM-ANOVA) followed by Bonferroni post-hoc comparisons (Wolmarans et al., 2016a; Wolmarans et al., 2016b). Behavioral scores (of MB, MDB and NB) were set as between subject factor and time and intervention as within subject factors. Statistical significance was set as $p < 0.05$ for all analyses. All statistical analyses were followed by pairwise calculations of Cohen's d effect size to determine effect size. Effect sizes were considered large when $d > 0.8$, and very large when $d > 1.3$.

Results

Marble-burying and nest building screening and cohort selection

Selection of the HMB cohort

36 HMB expressing animals were selected from the initial pool of 280 animals (**Figure 1A**: $r_s(239) = -0.68$, $p = < 0.0001$). As 39 animals failed to cover a single marble over the course of the three consecutive pre-intervention trials, they were excluded from further MB analysis. Hence, **Figure 1A** is representative of 241 data points only. Subjects presenting with HMB are enclosed within the oval.

Selection of the LNB cohort

None of the 36 HMB expressing animals engaged in LNB activity as well. Thus, a total of 36 individuals with LNB were selected (**Figure 1B**: $r_s(178) = -0.51$, $p < 0.0001$). Identified LNB expressing individuals are enclosed within the oval.

Pre- and post-intervention marble-burying comparisons

With respect to the number of marbles buried (**Figure 2A**), no significant interaction was demonstrated between time and intervention ($F[5, 30] = 2.24$; $p = 0.076$). However, time ($F[1, 30] = 46.78$; $p < 0.0001$), but not intervention ($F[5, 30] = 1.87$; $p = 0.13$), had an overall significant main effect on the burying scores; post-hoc comparisons: control group (1.17 ± 0.56 vs. 3.73 ± 1.47 ; $p = 0.003$; CI: $0.73 - 4.40$; $d = 2.49$), escitalopram alone group (2.0 ± 2.1 vs. 3.73 ± 0.89 ; $p = 0.077$; CI: $-0.11 - 3.57$; $d = 1.16$), rasagiline alone group (0.44 ± 0.29 vs. 3.67 ± 1.38 ; $p = 0.0002$; CI: $1.38 - 5.06$; $d = 3.85$), flupentixol/escitalopram group (1.25 ± 1.29 vs. 2.81 ± 0.48 ; $p = 0.14$; CI: $-0.28 - 3.40$; $d = 1.77$), and the rasagiline/escitalopram group (0.69 ± 1.02 vs. 2.16 ± 0.77 ; $p = 0.19$; CI: $-0.8 - 3.30$; $d = 1.65$) groups as well. No significant differences between the burying scores generated by mice of the different drug groups were observed (**Figure 2A**, post-intervention).

Concerning MDBs (**Figure 2B**), a significant interaction between time and intervention was demonstrated ($F[5, 30] = 3.20$; $p = 0.019$), while both time ($F[1, 30] = 47.94$; $p < 0.0001$) and intervention ($F[5, 30] = 4.22$; $p = 0.005$) had significant main effects on the results reported. Post-hoc analyses revealed statistically significant reductions in the post-intervention MDBs expressed by animals in the escitalopram alone group (19.0 ± 5.57 vs. 30.39 ± 5.92 ; $p = 0.0013$; CI: $3.74 - 19.04$; $d = 1.98$), the flupentixol alone group (21.95 ± 9.05 vs. 29.61 ± 8.54 ; $p = 0.049$; CI: $0.01 - 15.32$; $d = 0.87$) and the escitalopram/rasagiline group (8.39 ± 4.32 vs. 23.72 ± 5.71 ; $p < 0.0001$; CI: $7.68 - 22.99$; $d = 3.06$). Also, reductions in MDBs of large effect sizes were also observed at 4 weeks in the control (22.39 ± 4.77 vs. 26.06 ± 2.58 , $p = >0.99$; $d = 1.0$) and the escitalopram/flupentixol combination: 15.61 ± 5.69 vs. 20.33 ± 6.58 , $p = 0.549$; $d = 0.8$). The main effect of intervention on the number of MDBs observed is apparent from the post-intervention differences observed between the different drug groups (**Figure 2B**, post-intervention). The combination of escitalopram and rasagiline resulted in a significantly lower number of MDBs (8.38 ± 4.32) compared to the control group (22.39 ± 4.77 ; $p = 0.002$; CI: $3.61 - 24.40$; $d = 3.08$), the escitalopram alone group (19.0 ± 5.57 ; $p = 0.042$; CI: $0.22 - 21.01$; $d = 2.14$), and the flupentixol alone group (21.95 ± 9.05 ; $p = 0.003$; CI: $3.16 - 23.96$; $d = 2.03$). Further, while not reaching statistical significance, the combination of escitalopram and rasagiline was more effective than rasagiline alone (17.57 ± 4.03 ; $p = 0.136$; $d = 2.19$).

Pre- and post-intervention nest-building comparisons

A statistically significant two-way interaction was demonstrated between time and intervention with respect to the nesting scores of LNB animals ($F[5, 30] = 4.350$; $p = 0.0043$), while neither time ($F[1, 30] = 2.461$, $p = 0.127$), nor intervention ($F[5, 30] = 1.473$, $p = 0.2279$) had a main effect on the result (**Figure 3**). Subsequent pairwise analyses revealed the average expression of naturalistic LNB to increase over time (control: $34.06 \pm 12.05\text{g}$ vs. $19.83 \pm 3.86\text{g}$; $p = 0.0151$; CI: $-26.43\text{g} - -2.04\text{g}$; $d = 1.79$). Although not statistically significant, such exacerbation was reversed by the administration of escitalopram as evidenced by the large effect size noted ($14.92 \pm 6.67\text{g}$ vs. $24.51 \pm 2.80\text{g}$, $p = 0.20$; $d = 2.03$). While the combination of escitalopram and rasagiline ($28.08 \pm 10.6\text{g}$ vs. $20.0 \pm 4.7\text{g}$, $p = 0.426$; $d = 1.05$) somewhat reversed this result, the combination of flupentixol and escitalopram resulted in nest building scores akin to that observed in the escitalopram alone group at four weeks ($16.53 \pm 7.57\text{g}$ vs. $14.92 \pm 6.67\text{g}$, $p > 0.99$; $d = 0.23$). Rasagiline ($27.94 \pm 7.3\text{g}$ vs. $20.41 \pm 1.98\text{g}$, $p = 0.547$; $d = 1.62$) and flupentixol ($27.08 \pm 12.86\text{g}$ vs. $25.18 \pm 5.9\text{g}$, $p > 0.99$; $d = 0.2$) administered alone, failed to elicit any noteworthy response.

Only escitalopram alone ($14.92 \pm 6.67\text{g}$ vs. $34.06 \pm 12.05\text{g}$; $p = 0.003$; CI: $4.42\text{g} - 33.87\text{g}$; $d = 2.05$) and the escitalopram/flupentixol combination ($16.53 \pm 7.57\text{g}$ vs. $34.06 \pm 12.05\text{g}$; $p = 0.009$; CI: $2.78\text{g} - 32.24\text{g}$; $d = 1.78$) were able to significantly reduce LNB behavior compared to the control group at 4 weeks.

Locomotor activity

Two-way RM ANOVA failed to reveal a significant interaction between time and intervention with respect to the total distance moved (**Figure 4**; $F[5, 12] = 0.215$, $p = 0.95$). Furthermore, neither time ($F[1, 12] = 0.58$, $p = 0.461$), nor intervention ($F[5, 12] = 1.812$, $p = 0.185$) had a significant main effect on the result observed.

Discussion

The main findings of this investigation were that LNB and HMB show unique responses to serotonergic and dopaminergic intervention in that 1) HMB is more responsive to combined escitalopram and rasagiline, compared to escitalopram or flupentixol alone, or a combination of flupentixol and escitalopram, and 2) LNB is responsive to escitalopram alone or combined with flupentixol, but not to rasagiline alone, flupentixol alone, or combined escitalopram and rasagiline.

Considering the burden of treatment resistance in OCD (Husted et al., 2006; Brakoulias and Tsalamaniotis, 2017), there is a need to better understand the mechanisms underlying OC

symptomology. This is important, as the development of novel pharmacological alternatives ultimately depends on elucidating the underlying neurobiological processes which play a role in psychiatric disorders. While a significant number of patients do not demonstrate adequate response to the current first line interventions, e.g. SSRIs or cognitive behavioral therapy (CBT), second line interventions, e.g. augmenting SSRIs with antidopaminergic drugs, only provides relief in 40 – 60% of SSRI refractory patients (Atmaca, 2016). Further, the neurobiological mechanisms via which current pharmacotherapies elicit their effects are largely unknown. For example, anti-dopaminergic treatment is typically ineffective, and may even exacerbate OC symptomology if used as monotherapy (Lykouras et al., 2003; Westenberg et al., 2007; Kim et al., 2019), often only being effective in some patients when used in combination with SSRIs (Atmaca, 2016). This points to potential crosstalk between serotonergic and dopaminergic mechanisms in the treatment response (Lykouras et al., 2003; Westenberg et al., 2007; Kim et al., 2019). On a psychobiological level, unique involvement of goal-directed action motivation (Voon et al., 2015) and reward-feedback processing have been shown in patients with different phenotypes of OCD (Palminteri et al., 2012; Ferreira et al., 2017; Murray et al., 2019). These processes are all regulated by dopamine and serotonin (Boureau and Dayan, 2011; Schultz, 2013; Faulkner and Deakin, 2014; Gruner and Pittenger, 2017). Moreover, putative evidence points to unique dopaminergic processes in patients with hoarding disorder, i.e. resembling a state more akin to that observed in patients suffering from addiction (McLaughlin et al., 2018) and impulsivity (Suñol et al., 2019), while patients suffering from hoarding disorder broadly describe their symptoms from a pleasurable, ego-syntonic perspective; this as opposed to the ego-dystonic experiences of OCD patients. Also, hoarding disorder and hoarding symptoms in OCD demonstrate poor treatment response to SSRIs and its augmentation with low-dose anti-dopaminergic agents (Bloch et al., 2006; Fineberg et al., 2013; Veale et al., 2014), while some reports even indicate symptom attenuation after pro-dopaminergic intervention (McLaughlin et al., 2018).

Animal models of OCD are usually representative of a single behavioral phenotype based on its face similarity to OCD routines (Yadin et al., 1991; Tsaltas et al., 2005; Joel, 2006; Hoffman and Morales, 2009; Egashira et al., 2018). This complicates investigations into symptom-heterogeneous OCD (McKay et al., 2004; Nedeljkovic et al., 2009). Indeed, to further our understanding of the mechanisms underlying OCD treatment resistance and how this relates to the neurobiological constructs, studies of heterogeneous, but spontaneous and naturalistic compulsive-like behaviors within a specific model species will be a valuable extension of current research. To this extent, the persistent behavioral phenotypes expressed by deer mice, i.e. LNB and HMB, may prove useful. Deer mice of both sexes present to a varying extent with LNB (Wolmarans et al., 2016a) and HMB (Wolmarans et al., 2016b). Whereas

roughly 30% of the laboratory-housed deer mouse colony develop LNB and 45% highly stereotypical behaviors, only 11 – 15% of animals express HMB—see Scheepers et al. (2018) for an extensive review. These behaviors are equally persistent and repetitive over several successive trials, and seemingly purposeless under normal laboratory conditions (Scheepers et al., 2018). Moreover, LNB is responsive to SSRI intervention (Wolmarans et al., 2016a); HMB is not (Wolmarans et al., 2016b). These phenotypes may therefore also be founded on different neurobiological underpinnings. Against this background, the clinical utility of low-dose anti-dopaminergic augmentation for the treatment of SSRI-refractory OCD, is well established. However, considering recent literature (Figuee et al., 2011; Pinto et al., 2014; Ferreira et al., 2017; McLaughlin et al., 2018; Suñol et al., 2019) and taking into account the unresponsiveness of HMB to SSRIs, we also aimed to investigate how LNB and HMB would respond to the actions of a pro-dopaminergic intervention, i.e. rasagiline, a strategy often employed in conditions characterized by impulsivity (Caye et al., 2019) and dysfunctional reward processing (Spijker and Nolen, 2010).

With respect to burying activity, our results demonstrate a time-dependent adaptation to the burying scores, without any significant main effect of intervention, nor an interaction reported between time and intervention. As such, we could not confirm an effect of drug within any of the groups after four weeks of drug administration, as all interventions, except for the flupentixol alone group, presented with substantial reductions in burying scores at 4 weeks of drug exposure. This is a noteworthy observation, especially since the locomotor behavior of all the groups remained unaltered after chronic drug administration (**Figure 4**). The *burying* scores reported here can likely be contributed to initial interest in the objects shown by most animals which abated over time. Further, we applied previously reported scoring methodology (Egashira et al., 2013; Egashira et al., 2018) and characterized a marble as ‘buried’ if two-thirds of its size was covered in burying substrate. However, this approach is arguably not sensitive enough to highlight actual ‘burying’ performance, rather being more indicative of investigative or exploratory behavior which ultimately results in the partial covering of objects (de Brouwer and Wolmarans, 2018). As such, we also scored MDBs. Here, several noteworthy observations were made. First, intervention and time interacted significantly on the findings reported, while both time and intervention had significant main effects on the MDB of HMB deer mice. Second, it is evident that the behavior of control-exposed animals remained at the same level of preoccupation with the objects as they did prior to intervention. Third, animals that were administered escitalopram alone, a combination of escitalopram and rasagiline, and flupentixol alone, demonstrated post-intervention reductions in MDB. Importantly, while no significant differences between any of the drug groups prior to administration has been noted, the number of MDB (**Figure 2B**) differed significantly between

the different groups after 4 weeks of drug administration. In fact, although escitalopram and flupentixol alone elicited significant reductions in the MDB of HMB mice, the combination of rasagiline with escitalopram proved to be the most effective intervention in this regard, with no differences demonstrated between the post-intervention control animals and any of the other groups. This indicates that the post-intervention behavior of animals receiving escitalopram and flupentixol can likely be ascribed to a time-dependent adaptation, which is not true for the combined escitalopram and rasagiline group. That no significant difference in the post-intervention MDB behavior of escitalopram exposed animals could be demonstrated compared to the control cohort, confirms our previous results (Wolmarans et al., 2016b). However, that such behavior responds to a combination of escitalopram and rasagiline, is interesting given that dopamine bolstering drugs typically result in exacerbation of compulsive-like behaviors (Aarons et al., 2012; Sevincok et al., 2014; Voon et al., 2015). This finding highlights a unique role of dopamine in this phenotype and is indicative of a specific neurobiological mechanism underlying HMB that may not necessarily be implicated in SSRI or combined SSRI and anti-dopaminergic drug sensitive phenotypes. In fact, it is likely that HMB resembles a bio-behavioral trait akin to clinical hoarding disorder. This possibility needs further investigation.

In contrast to the expression of burying activity, LNB exacerbated over time (**Figure 3**), which from an adaptational perspective, separates LNB on a phenotypical level from HMB. In fact, in contrast to the expression of burying behavior, LNB expressing animals are even more compelled to engage in excessive nesting behavior as time progresses. Although further study is required, such natural exacerbation in nesting behavior may point to an underlying anxiogenic construct. Considering the evolutionary function of nesting behavior (Jirkof, 2014; Stewart and McAdam, 2017; Lewarch and Hoekstra, 2018) and that LNB serves no functional purpose, such compulsive-like behavior may be borne from inflated fear-anxiety. This hypothesis is supported by the response of LNB to high-dose chronic escitalopram and the combination of escitalopram and flupentixol. Although pre- and post-intervention nesting scores of neither of these groups differed significantly (**Figure 3**), only escitalopram and the combination of escitalopram and flupentixol prevented the exacerbation of nesting behavior versus control animals; in fact, it reversed this trend. This is not only congruent with response in treatment-sensitive OCD but may also be supportive of anxiogenic involvement in the expression of LNB. Indeed, both chronic high dose SSRIs and low-dose anti-dopaminergic drugs are known anxiolytic treatments (Spijker and Nolen, 2010; Oregan ECHO Network, 2018), even though the clinical anti-obsessive-compulsive effect of these agents are not exclusively related to an anxiolytic response (Baldwin et al., 2014; Hirschtritt et al., 2017). However, until the potential influence of anxiety on the expression of LNB has been adequately

investigated, these findings could also be related to a neurocognitive construct other than anxiety, more in line with feedback processing theory. In fact, that both rasagiline and combined escitalopram plus rasagiline exacerbated LNB expression, suggests LNB to be representative of some inflated form of goal-directed outcome (Lim et al., 2008; Ondo and Lai, 2008), eliciting a sense of reward that if not gated adequately, becomes akin to behavioral addiction (Robbins and Clark, 2015). This has been proposed as a core neurocognitive construct of OCD (Voon et al., 2015; Gillan et al., 2016). In fact, since nesting behavior is inherently rewarding, it could likely be bolstered by either natural feedback interpretation or by increased dopaminergic activity geared towards achieving reward (Boureau and Dayan, 2011; du Hoffmann and Nicola, 2014; da Silva et al., 2018). Also, dopaminergic potentiation has been shown in pre-clinical (Presti et al., 2004; Sesia et al., 2013; Tucci et al., 2013) and clinical literature (Pessiglione et al., 2006; Voon et al., 2009; Garcia-Ruiz et al., 2014) to facilitate and fortify persistent and repetitive behaviors characterized by cognitive inflexibility.

A potential shortcoming of this work is the small sample sizes. Given the often-large effect sizes reported in the absence of statistical significance, the statistical power of the investigation could have been improved with larger group sizes. Nevertheless, taking into account the large number of animals needed to yield sufficient HMB and LNB expressing subjects, this work was aimed at a theoretical pharmacological dissection of different OC-like phenotypes; to this extent, we believe our findings are valid.

Conclusion

Our data broadly supports the notion that different behavioral OC-related phenotypes are supported by underlying differences in neurobiology. We have confirmed that LNB responds robustly to SSRI therapy, while further demonstrating that the addition of flupentixol to escitalopram parallels the response of escitalopram alone on nesting behavior. In contrast, HMB is less responsive to either escitalopram, flupentixol or their combination, only being attenuated by a combination of escitalopram and rasagiline. Therefore, HMB seems less representative of the classic hyposerotonergic, hyperdopaminergic picture of OCD (Westenberg et al., 2007), paving the way for its interrogation as a behavioral phenotype founded upon a unique neurocognitive construct. In conclusion, this work provides first-of-its-kind pharmacological evidence that LNB and HMB in deer mice, both previously proposed to be pre-clinical models of compulsivity, differ in response to pharmacotherapy. Further work is needed to determine whether these distinctions have parallels in humans.

Declarations of Interest

The authors declare no conflicts of interest.

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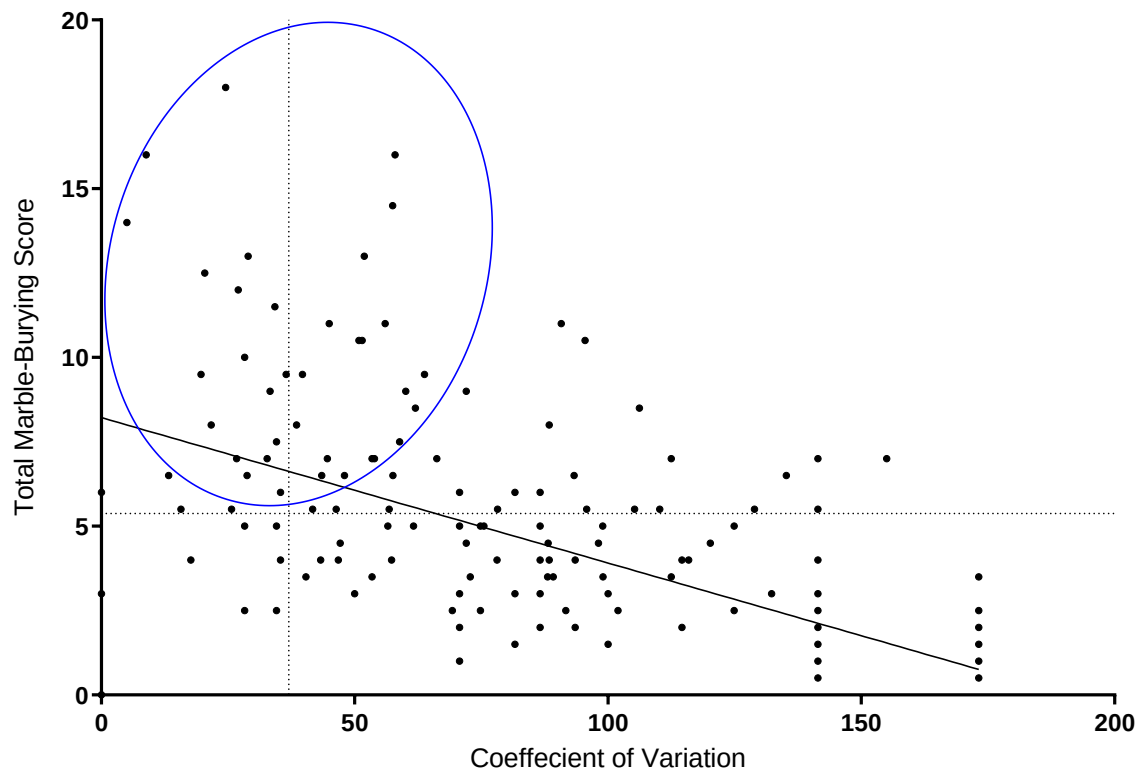
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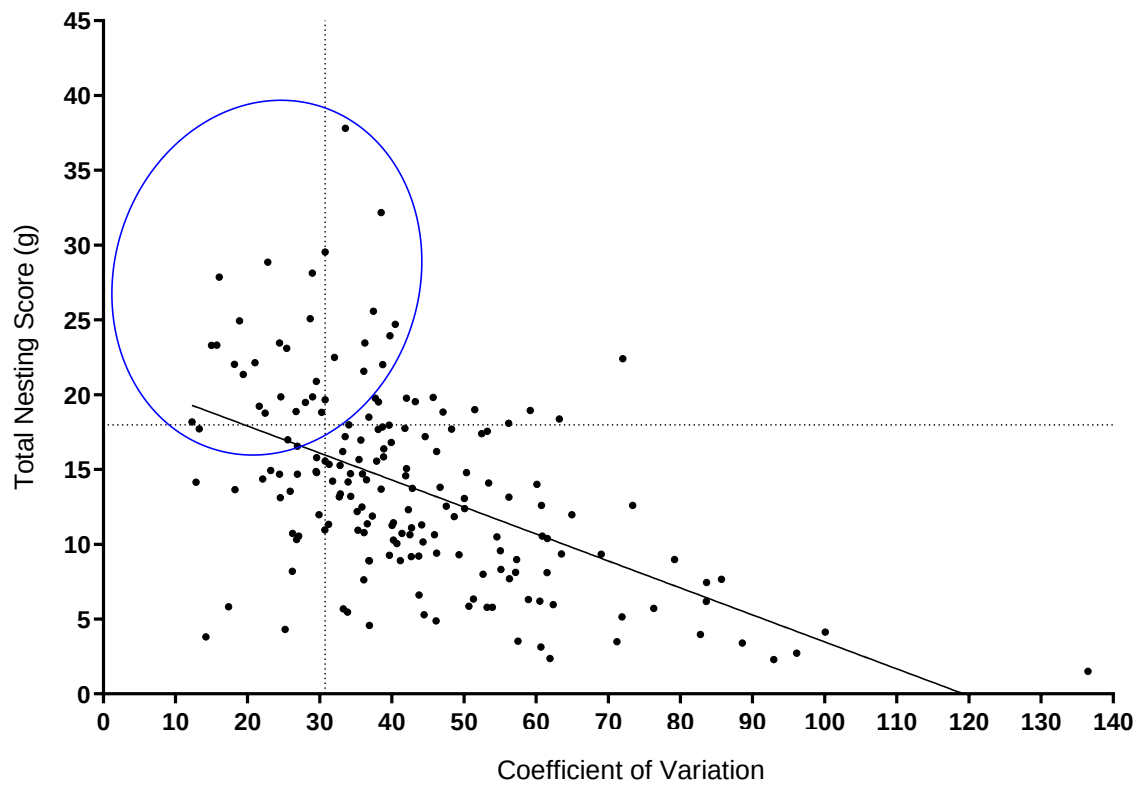
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A

Figure 1

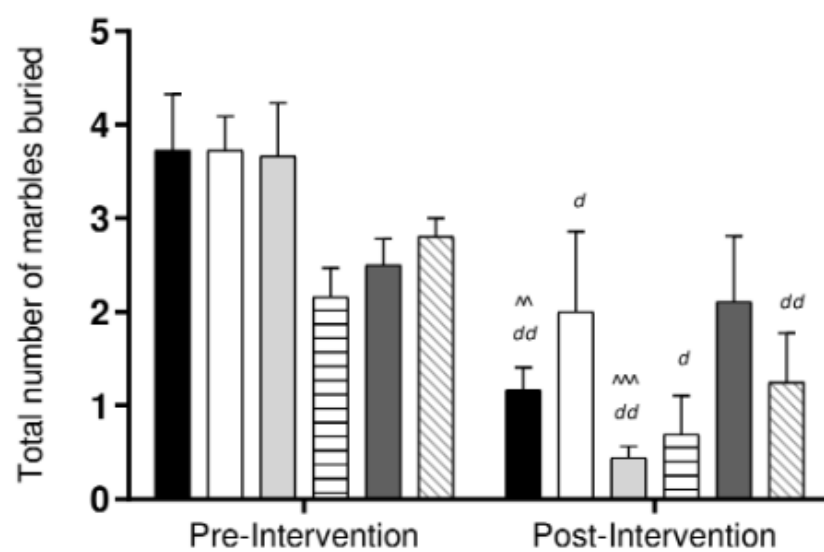


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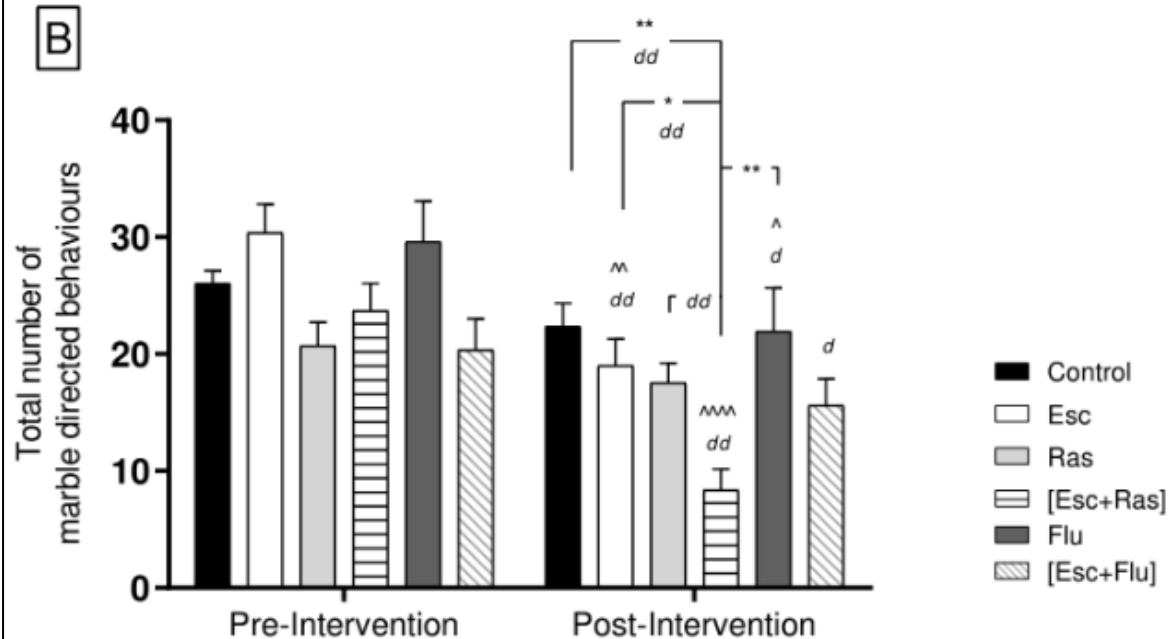


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Figure 2



B



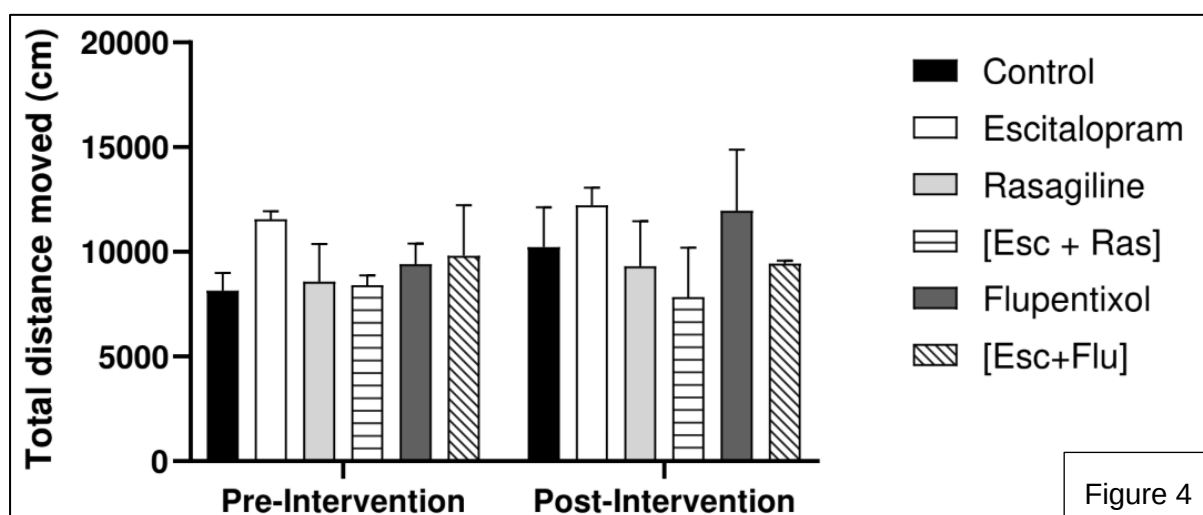
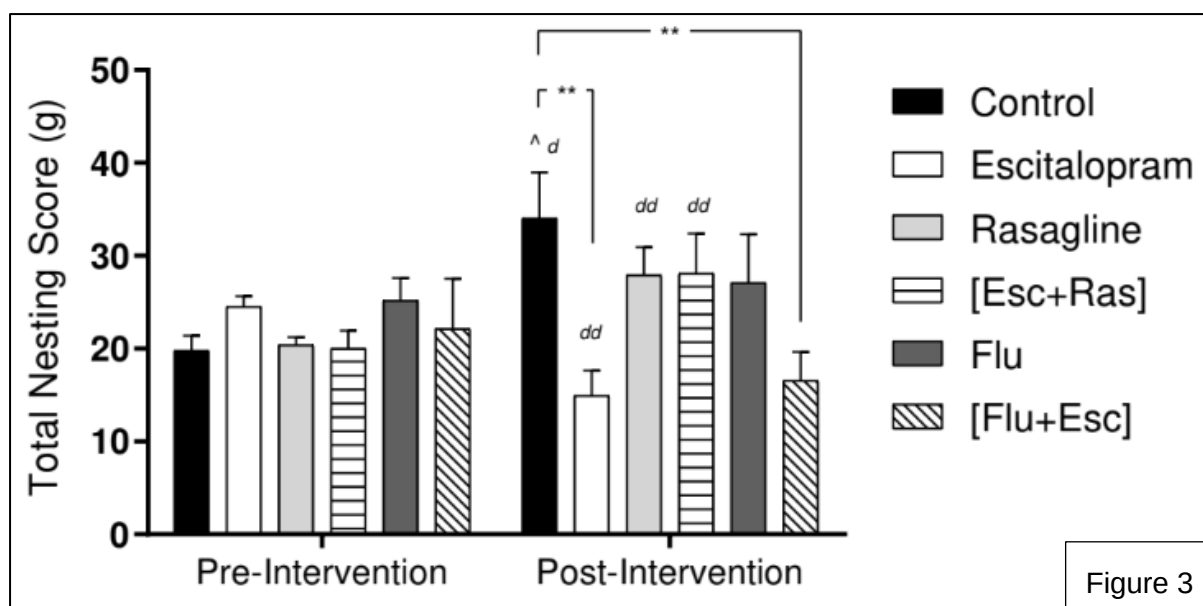


Figure Captions

Figure 1. Pre-selection screening data used for cohort selection. **A)** Marble-burying behavior during screening, expressed as Pearson's correlation ($r_s(239) = -0.68, p = < 0.0001$) between the total number of marbles buried and coefficients of variance for each individual animal over three test sessions. HMB (high marble-burying) animals were selected from the oval (highest marble-burying scores/lowest individual variance). **B)** Nest building activity during screening, expressed as Pearson's correlation ($r_s(178) = -0.51, p = < 0.0001$) between the total nesting score (g) and coefficients of variance for each individual animal over seven nights of screening. LNB (large nest building) animals were selected from the oval (highest total nesting score/lowest individual variance). All data represented as mean $\pm$ SEM.

Figure 2. Marble-burying data. **A)** Average pre- vs. post-intervention number of marbles buried over three pre- and three post-intervention trials by high marble-burying (HMB) animals. ^ Indicates significant pre- vs. post-intervention interactions within drug groups. ^^ $p = 0.0028$; ^^ $p = 0.0002$ (Two-way RM-ANOVA, Bonferroni post-hoc). Cohen's effect size: $d > 0.8$; $dd > 1.3$. **B)** Average pre- vs. post-intervention number of marble-directed behaviors (MDBs) over three pre- and three post-intervention trials by high HMB animals. ^ Indicates pre- vs. post- intervention interactions within drug groups. * Indicates post-intervention interactions between drug groups. ^ $p = 0.0494$; ^^ $p = 0.0013$; ^^ $p < 0.0001$; * $p = 0.0416$; control vs. escitalopram & rasagiline ** $p = 0.0018$; flupentixol vs. escitalopram & rasagiline ** $p = 0.0028$ (Two-way RM-ANOVA, Bonferroni post-hoc). Cohen's effect size: $d > 0.8$; $dd > 1.3$. All data represented as mean $\pm$ SEM.

Figure 3. Average total nesting scores after seven days of pre- and seven days of post-intervention nest building screening in large nest building (LNB) animals. ^ Indicates significant pre- vs. post-intervention interactions within drug groups. * indicates post-intervention comparisons between drug groups. ^ $p = 0.0151$; control vs. escitalopram ** $p = 0.0029$; control vs. flupentixol ** $p = 0.0087$ (Two-way RM-ANOVA, Bonferroni post-hoc). Cohen's effect size: $d > 0.8$; $dd > 1.3$. Data represented as mean $\pm$ SEM.

Figure 4. Average pre- vs. post-intervention expression of motor activity in three randomly selected animals per drug group over three pre- and post-intervention trials. Two-way RM ANOVA revealed no significant interactions or main effects.

Addendum H - Additional Article II

Article accepted for publication in *Behavioural Brain Research* titled:

“Dopaminergic and serotonergic modulation of contingency learning in zebrafish (*Danio rerio*) under circumstances of motivational conflict: towards a screening test for anti-compulsive drug action”

Author Contributions:

- *Cailin van Staden* assisted in the conceptualization of the study with *De Wet Wolmarans* and *Sarel Brand*, performed the behavioral analysis, scoring and statistical processing and wrote the manuscript.
- *Geoffrey de Brouwer* assisted with data processing and interpretation and assisted with the writing of the manuscript.
- *Tarryn Botha* served as the professional consultant regarding the use of zebrafish.
- *Karin Finger-Baier* served as a professional consultant with regard to the use of zebrafish in psychiatric research and proof-read the manuscript.
- *Sarel Brand* assisted with the conceptualization of the study and proof-read the manuscript.
- *De Wet Wolmarans* as the study leader, assisted in the conceptualization of the study together with *Cailin van Staden* and *Sarel Brand*, processed the data, interpreted the statistical analyses and assisted in the writing of the manuscript.

Important Information:

- Instructions to the author can be accessed here:
<https://www.elsevier.com/journals/behavioural-brain-research/0166-4328/guide-for-authors>
- The article is accepted for publication in *Behavioural Brain Research*.
- This work was primarily written by first author *Cailin van Staden*, however, the candidate *Geoffrey de Brouwer* assisted with the writing of the manuscript, and as such this work is presented as additional evidence of the candidate's experience and is therefore not intended for examination purposes.

“Dopaminergic and serotonergic modulation of contingency learning in zebrafish (*Danio rerio*) under circumstances of motivational conflict: towards a screening test for anti-compulsive drug action”

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Abbreviations

OCD - Obsessive-compulsive disorder

SSRIs - Selective serotonin reuptake inhibitors (SSRIs)

5-HT - Serotonin; 5-hydroxytryptamine (5-HT)

NCT - Time spent in the proximity area of the non-cued arm (NCT)

RT - Time spent in the proximity area of the red-colored cued arm

NCE - Number of entries made into the proximity area of the non-cued arm

RE - Number of entries made into the proximity area of the red-colored cued arm

TPAT - Total time spent in the proximity areas of both arms

TPAE - Total number of entries made into the proximity areas of both arms

2-way RM ANOVA Two-way repeated measures analysis of variance

8-OH-DPAT - 8-hydroxy-2-[di-n-propylamino] tetralin

Abstract

Cognitive flexibility, shown to be impaired in patients presenting with compulsions, is dependent on balanced dopaminergic and serotonergic interaction. Towards the development of a zebrafish (*Danio rerio*) screening test for anti-compulsive drug action, we manipulated socially driven cue-contingency learning by means of dopaminergic (apomorphine) and serotonergic (escitalopram) intervention. Seven groups of zebrafish (n = 6 per group) were exposed for 24 days (1 h per day) to either control (normal tank water), apomorphine (50 or 100 $\mu\text{g/L}$), escitalopram (500 or 1000 $\mu\text{g/L}$) or a combination (A100/E500 or A100/E1000 $\mu\text{g/L}$). Cue-reward learning was assessed over three phases i.e. Phase 1 (contingency learning), Phase 2 (dissociative testing), and Phase 3 (re-associative testing). We demonstrate that 1) sight of social conspecifics is an inadequate reinforcer of contingency learning under circumstances of motivational conflict, 2) dopaminergic and serotonergic intervention lessens the importance of an aversive stimulus, increasing the motivational valence of social reward, 3) while serotonergic intervention maintains reward directed behavior, high-dose dopaminergic intervention bolsters cue-directed responses and 4) high-dose escitalopram reversed apomorphine-induced behavioral inflexibility. The results reported here are supportive of current dopamine-serotonin opponency theories and confirm the zebrafish as a potentially useful species in which to mimic compulsive-like behaviors.

Keywords

obsessive-compulsive disorder; zebrafish; dopamine; serotonin; inflexibility; learning; opponency

Introduction

Obsessive-compulsive disorder (OCD), like several other neuropsychiatric disorders, e.g. autism (Delmonte et al., 2012), gambling disorder (Reuter et al., 2005), eating disorders (Simon et al., 2016), and schizophrenia (Whitton, Treadway, & Pizzagalli, 2015), is often associated with behavioral inflexibility and dysfunctional reward-feedback processing (Koch et al., 2018). OCD is primarily characterized by persistent and anxiety-provoking thoughts (obsessions) which are typically associated with rigid, repetitive behavioral routines (compulsions), initiated to alleviate the symptoms of anxiety (American Psychiatric Association, 2013; Figeet al., 2011; Winter et al., 2018). Sensations of relief generated by such obsession-compulsion loops are typically temporary (Jonathan S Abramowitz & Jacoby, 2015) leading to worsening cycles of repetitive behavior.

Although the exact etiology and pathophysiology of OCD remains unclear, the condition is often associated with cortico-striatal-thalamic dysregulation (Ahmari et al., 2013; Maia, Cooney, & Peterson, 2008; Stocco, Lebiere, & Anderson, 2010; van den Heuvel et al., 2005; Winter et al., 2018). The prefrontal cortex, striatum and thalamus are all involved in the planning, execution and termination of goal-rewarded behavior (Graybiel & Rauch, 2000; Stocco et al., 2010; Vaghi et al., 2017). Dysfunction within these regions is similarly associated with changes in the expression of goal-directed behavior and abnormal reward feedback processing (Stocco et al., 2010; Vaghi et al., 2017). From a neurobiological perspective, cortico-striatal interactions between dopaminergic and serotonergic signaling are pivotal in the regulation of these processes. Indeed, in line with the proposed role of dopamine in reward processing (Schultz, 2002, 2007, 2013) and the opponency theory, describing the motivational dopamine-serotonin interactions under circumstances of rewarding and negative feedback respectively (Balasubramani, Chakravarthy, Ravindran, & Moustafa, 2015; Daw, Kakade, & Dayan, 2002; Rogers, 2011), it is believed that a bias in favor of behaviorally activating dopaminergic signaling (Nieh et al., 2016; Salamone et al., 2016) exists (Lissemore et al., 2018; Maia & Cano-Colino, 2015; Wong et al., 2015). Although the neurobiology of OCD and other disorders of behavioral persistence is more complex (Fischer & Ullsperger, 2017), this theory provides a rationale for the use of selective serotonin reuptake inhibitors (SSRIs) as a first-line intervention for the treatment of OCD (Pittenger & Bloch, 2014; Zike, Xu, Hong, & Veenstra-VanderWeele, 2017). Briefly, chronic administration of SSRIs, e.g. escitalopram, increases the concentration of synaptic serotonin (Lissemore et al., 2018), thereby presumably attenuating excessive behavioral activation, a theory which is supported by data from a number of pre-clinical investigations (Güldenpfennig, Wolmarans, du Preez, Stein, & Harvey, 2011; Kontis et al., 2008; Szechtman et al., 2001; Tsaltas et al., 2005). However, not all patients respond to SSRI monotherapy, in which case treatment is often

augmented with low-dose dopamine receptor antagonists (Bedingfield, Calder, Thai, & Karler, 1997; Denys et al., 2013).

Observations of dopamine-serotonin interactions are critical when explaining animal learning since the behavior of an organism is often functionally directed at obtaining a reward or avoiding potential aversive outcomes. More specifically, a novel rewarding scenario would initially provoke mass phasic dopaminergic activation (Pessiglione, Seymour, Flandin, Dolan, & Frith, 2006; Schultz, 1998, 2002). Although repeated presentation of the same rewarding scenario within a specific context is, over time, associated with a diminishing dopaminergic response (Schultz, 2002, 2013)—thereby preventing engagement in persistent reward-seeking behaviors—it also enables an organism to acquire knowledge of cue-outcome contingencies, whereby the presentation of specific contextual cues *predicts* potential rewarding outcomes (Schott et al., 2008; Schultz, 1998, 2002); this is the basis of reinforcement learning. Conversely, serotonergic signaling is typically activated during the experience of aversive feedback and is integral in the learning of future avoidance behaviors (Faulkner & Deakin, 2014). As alluded to earlier, OCD patients often present with dysfunctions related to learning-related processes, including abnormal reward feedback processing (Figuee et al., 2011), altered neurobiological reward anticipatory responses (Figuee et al., 2011), overinflated threat estimation (Cisler & Koster, 2010; Exner, Zetsche, Lincoln, & Rief, 2014) and often also being relatively insensitive to punishment (Hinds, Woody, Van Ameringen, Schmidt, & Szechtman, 2012; Morein-Zamir et al., 2013). It has also been proposed that individuals suffering from OCD may be indifferent to the external feedback cues associated with adequate task completion, which may ultimately manifest as repetitive behavioral engagement (Endrass, Koehne, Riesel, & Kathmann, 2013; Nielen, Den Boer, & Smid, 2009).

The present investigation attempts to investigate how natural reward-seeking behavior in the zebrafish (*Danio rerio*), i.e. the motivational drive to engage in social interaction (Al-Imari & Gerlai, 2008; Daggett, Brown, & Brennan, 2019; Saif, Chatterjee, Buske, & Gerlai, 2013), can be modified by the pharmacological manipulation of dopaminergic and serotonergic neurotransmission. Taken from the literature summarized here, it is possible that bolstered dopaminergic signaling would be associated with persistent reward-seeking behaviors and that such behavior would be subject to attenuation by serotonergic agents. Although this concept has been investigated in rodent studies (Flagel et al., 2010), promising zebrafish models of neuropsychiatric conditions may present a unique avenue to complement rodent models (Fontana, Mezzomo, Kalueff, & Rosemberg, 2018). Indeed, the numerous advantages of zebrafish as a model species, including their prolific nature and rapid development (Champagne, Hoefnagels, de Kloet, & Richardson, 2010; Goldsmith, 2004), lengthy lifespan (Kalueff, Stewart, & Gerlai, 2014), ease of drug administration through

aqueous immersion (Kalueff, Echevarria, & Stewart, 2014; McGrath & Li, 2008; A. Stewart et al., 2011) and that it enables cost-effective high throughput results-driven screening (Levin, 2011), explain why zebrafish are rapidly emerging as a complementary pre-clinical framework for neurobiological research (Flinn, Bretaude, Lo, Ingham, & Bandmann, 2008; Levin & Cerutti, 2009; Zabegalov et al., 2019). With respect to the focus of this work, the species shows a strong homology to rodent and human neurobiology (Kalueff, Echevarria, et al., 2014; Kalueff, Stewart, et al., 2014), presenting with almost fully conserved dopaminergic and serotonergic systems (D'Amico, Estivill, & Terriente, 2015; Maximino et al., 2013; Naderi, Jamwal, Chivers, & Niyogi, 2016; Saif et al., 2013; A. M. Stewart et al., 2013). In addition, zebrafish demonstrate both associative and non-associative cognitive ability, including adequate reversal learning, appetitive reinforcement-based learning and avoidance learning (Al-Imari & Gerlai, 2008; Daggett et al., 2019). Moreover, zebrafish are highly social and are known to shoal and seek out conspecifics, constituting the basis of the present investigation (Al-Imari & Gerlai, 2008; Daggett et al., 2019; Saif et al., 2013; Saverino & Gerlai, 2008). Indeed, it has been shown that simple observation of conspecifics is highly rewarding to zebrafish (Al-Imari & Gerlai, 2008; Saif et al., 2013). A further advantage of a social, as opposed to a food-based, reward construct is that zebrafish do not seem to develop tolerance to social interaction (Daggett et al., 2019). This is of particular value for the present work, which attempts to employ social reinforcement as a suitable reward stimulus for socially deprived zebrafish. Further, in terms of ensuring robust acquisition of cue-reward contingencies, adult zebrafish can clearly distinguish between different colors and are therefore capable of conditioning-based color-dependent learning (Ahmad & Richardson, 2013). In addition, color perception in zebrafish influences learning, decision-making and memory formation (Oliveira, Silveira, Chacon, & Luchiari, 2015). Although it has been demonstrated that zebrafish seemingly prefer red (as used in this investigation) and green equally over no color, yellow and blue (Avdesh et al., 2012), findings with respect to color perception still remain largely inconclusive (Oliveira et al., 2015).

Currently, no truly cost-effective screening test exists that can detect potential anti-compulsive drug action. Hence, to this extent and considering the literature summarized here, the current investigation will elaborate on previous work (Al-Imari & Gerlai, 2008; Colwill, Raymond, Ferreira, & Escudero, 2005) into zebrafish learning by interrogating the acquisition and manipulation of cue-reward contingency learning in zebrafish exposed to either no drug, the dopaminergic $D_{1/2}$ receptor agonist, apomorphine, the SSRI, escitalopram, and combinations of both.

Experimental procedures

Animals and housing

A total of 42 randomly chosen adult wild-type long-fin zebrafish (*Danio rerio*) of both sexes (experimental fish; 3-5 months old; $\pm$ 30-40 mm in length; 6 fish per exposure group; refer to '*Exposure groups and drug administration*') were assessed in this investigation. A further 36 fish were used as social conspecifics (see below). The progenitor stock was originally obtained from Aquaworld Tropical Fish (Singapore) via a national South African importing supplier (WCB Imports, Pretoria, South Africa). All fish used were subsequently bred and housed in the National Aquatic Bioassay Facility (NABF), North-West University, Potchefstroom, South Africa. All procedures and experimental methods were approved by the AnimCare Research Ethics Committee, North-West University; Registration Nr. AREC-130913-015. Subjects were housed according to standard laboratory conditions as prescribed for zebrafish (Reed & Jennings, 2011) and maintained on a 12-hour light/dark cycle (06h00/18h00) in a fully automated system (ZebTec[®] Zebrafish Housing System, Techniplast[®], Varese, Italy) that regulated water quality (pH: 7; conductivity: 600 μ S), temperature (26 ± 0.1 °C), and aeration (7.2 mg O₂/L). Experimental zebrafish were group-housed ($n = 6$) in 3.5 L acrylic tanks for the first 10 days of the investigation and then individually allocated to replicas of the group tanks for the remainder of the study (15 days). Food (ZM-400 fry food, Zebrafish Management Ltd, Twyford, United Kingdom) was provided once daily after the first trial or at 10h00 on exposure days (days 1 - 10). Conspecifics were group-housed ($n = 6$ per tank) for the duration of the study.

Apparatus

Testing procedures involved the use of a transparent Plexiglas[®] T-maze, the stem of which measured 50 cm x 10 cm x 10 cm. To form a start box, an area (10 cm x 10 cm x 10 cm) was closed off at the foot of the stem. Each arm of the maze measured 20 cm x 10 cm x 10 cm. The cross-arm is defined as the entire arm perpendicular to the arm containing the start box. One separate 10 cm x 10 cm x 10 cm tank of clear Plexiglas[®] was placed adjacent to the extreme end of each arm of the T-maze (**Figure 1**). Depending on the specific phase of investigation, six (6) conspecific fish were placed into the tank adjacent to one arm of the T-maze. The tank located at the other arm contained only water. At no time were conspecifics introduced to both adjacent tanks simultaneously. The terminal 10-cm end of one of the arms (cued arm) was colored red by covering all relevant surface areas (including that of the adjacent tank, but excluding the adjoining surface between the T-maze and the adjacent tank) as well as those areas of the arm that were located directly opposite the adjacent tank, with a

red-colored cue card ('cued arm'; **Figure 1**). The other adjacent arm was covered with the same white plastic sheets as the rest of the maze ('non-cued arm'; **Figure 1**). The resulting setup ensured that the experimental fish could only see into the adjacent tanks by swimming to and entering the proximity area *directly* in front of the adjacent tank (**Figure 1**). The maze was filled with water from the home tank to a depth of 8 cm and maintained at 26°C, with the water changed and the maze cleaned every day to prevent excessive growth of biofilm. A digital video-camera (Panasonic® HC-V180) was positioned 150 cm above the T-maze and all trials were digitally recorded. Recordings were subsequently analyzed at the same time by means of EthoVision® XT 14 (Noldus® Information Technologies, Wageningen, The Netherlands) digital tracking software.

Exposure groups and drug administration

Experimental zebrafish were randomly divided into 7 groups ($n = 6$ per group). The different groups were constituted by randomly allocating 6 fish, each from a different home tank to the respective groups. Each group was exposed for 1 hour per day for 24 days to one of the respective drug exposures (**Figure 2A**). First, we aimed to establish what the effect of a lower (50 $\mu\text{g/L}$) and a higher (100 $\mu\text{g/L}$) dose of apomorphine (Sigma-Aldrich®, Johannesburg, South Africa) would be on cue-reward contingency learning. These dosages were based on data from rodent studies (Sukhanov et al., 2014) and adapted for translational application in zebrafish according to previously reported guidelines (Irons, Kelly, Hunter, MacPhail, & Padilla, 2013; Pinho et al., 2016). As such, the first three exposure groups consisted of 1) control (normal tank water), 2) apomorphine 50 $\mu\text{g/L}$, and 3) apomorphine 100 $\mu\text{g/L}$. Subsequently, we aimed to establish the effect of escitalopram (Lundbeck A/S®, Copenhagen, Denmark) alone at both a lower and a higher dose, *viz.* exposure groups 4) escitalopram 500 $\mu\text{g/L}$ and 5) escitalopram 1000 $\mu\text{g/L}$. Based on earlier work in studies of neuropsychiatric states in rodents and zebrafish, escitalopram oxalate equivalent to 50 mg/kg/day (Wolmarans, Brand, Stein, & Harvey, 2013) roughly equates to 500 $\mu\text{g/L}$ for aqueous immersion studies (Egan et al., 2009; Pittman & Lott, 2014; A. Stewart et al., 2011; K. Wong et al., 2010). Last we aimed to establish the effect on cue-reward contingency learning following exposure to a combination of apomorphine (at the optimal dose established, *i.e.* 100 $\mu\text{g/L}$; refer to '*Results*') and escitalopram at both doses, *viz.* apomorphine/escitalopram 100/500 $\mu\text{g/L}$ (group 6) and 100/1000 $\mu\text{g/L}$ (group 7). All exposures were administered by means of aqueous immersion throughout the study. During the first ten days of the study, zebrafish were group-housed and group-exposed (**Figure 2A**). For the remainder of the study (days 11 - 25), zebrafish were individually housed, drug exposed and tested, therefore being socially isolated throughout the behavioral testing phase (**Figure 2A**). Drug solutions were constituted by dissolving

appropriate quantities of drug in a volume of 1.5 L (for group exposure) or 1.2 L (for individual exposure) tank water. Group-exposed fish were placed into tanks identical to the housing tanks and contained 1.5 L of the relevant drug solutions. During individual exposure, fish were placed into 600 mL beakers containing 250 mL of drug solution. During individual exposure and social isolation, non-reflective white polystyrene separators were placed between exposure beakers and tanks to prevent zebrafish from seeing one another.

Procedures

Habituation

Prior to the start of Phase 1, zebrafish were habituated individually in the T-maze during a single daily 5-minute session repeated over four consecutive days (Figure 2A; days 11 – 14). During habituation, neither conspecifics nor a red cue card was presented, with all of the T-maze outside walls being covered with white plastic sheeting. Each habituation session on each of the four respective habituation days consisted of a 7-minute exposure session adapted from literature (Al-Imari & Gerlai, 2008). For each of the habituation sessions, a single fish was gently introduced to the start box in the stem of the T-maze for 2 minutes. Next, the start box door was manually raised and lowered again once the fish exited the start box. Each fish was then left to explore the T-maze without restriction for a duration of 5 minutes. Upon completion of the 5-minute session, fish were netted out of the T-maze and placed back into the home tanks. This procedure was repeated for all the fish in the group. Following the completion of all the habituation trials, experimental fish were tested individually in the T-maze according to the procedures explained below (**Figure 2B**).

Phase 1: Cue-reward contingency learning

For each respective fish, Phase 1 was executed over 5 days of testing, with two 5-minute T-maze trials per day, separated by 2.5 hours. As such, each fish was assessed across 10 trials during Phase 1 (**Al-Imari & Gerlai, 2008**). During all trials of Phase 1, one arm of the maze was colored with the red cue card as described under '*Apparatus*', while a shoal of 6 conspecific fish (social reward), was always presented adjacent to the proximity area of this cued arm. That said, to prevent the potential influence of place preference on arm choice, the cue-reward presentation (red-colored cued arm with conspecific shoal) alternated, i.e. left or right, with each successive trial. Each trial was started by introducing a single zebrafish to the start box for 2 minutes. Subsequently, the start box door was raised and then lowered after the fish exited. Free exploration of the T-maze was allowed for a 5-minute, videotaped session. After the 5-minute trial, the fish was gently netted and placed back into its home tank. This procedure was repeated for each fish. Each trial began at roughly the same time each

day, approximately 8h00 for the first trial, followed by the second trial after the inter trial period (2.5 hours), starting at approximately 10:30. This procedural flow was maintained throughout all phases.

Phase 2: Cue-reward dissociation testing

To establish whether zebrafish engaged in cue-directed responses, fish were assessed in Phase 2 over 3 days of testing, with two trials per fish per day, separated by 2.5 hours, beginning on the day following the last Phase 1 trial. Phase 2 proceeded identically to Phase 1 in that the red cue card was once again presented in alternate arms of the maze during each successive trial. However, during this phase, *no social reward (conspecifics) was presented* at any time, hence only filling the adjacent tanks with water.

Phase 3: Re-associative contingency testing

Phase 3 was, as Phase 2, conducted over another 3 days of testing with two daily 5-minute trials, separated by 2.5 hours, completed in a single day. During Phase 3, the experimental setup was applied exactly as in Phases 1 and 2. However, here the presentation of the conspecific-containing tank was paired with the non-cued arm.

The use of conspecifics in Phases 1 and 3

The 36 conspecific fish were divided into 6 groups (A – F) of 6 fish each and used as follows: Groups A and B were used for exposure groups 1 – 3. Groups C and D were used for exposure groups 4 and 5. Groups E and F were used for exposure groups 6 and 7. During each of the phases, the first group of conspecifics was used during the first trial of the day, and the second group of conspecifics, during the second trial of the day.

Statistical analyses

Statistical analyses were performed with GraphPad Prism® 8.0.1 software. Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni post-hoc testing was applied to evaluate the arm-choice behavior of fish in the different exposure groups over the three separate phases. As behaviors relating to arm-choice were primarily expressed as the indices of entry frequency and time spent in the red-colored cued vs. the non-cued arm (see below), these were set as between-subjects (dependent) factor, and phase and exposure as within-subjects (independent) factors, except if stated otherwise. Statistical significance was set at $p < 0.05$ for all analyses. All comparisons, irrespective of being statistically significant or not, were informed with calculations of Cohen's d effect size to establish the

magnitude of the effects observed. Effect sizes are considered large at values between 0.8 and 1.29 and very large at values higher than 1.3 (Cohen, 1988).

The values expressed on the Y-axis of **Figures 3** and **4** are representative of an index describing the time spent in ([RT-NCT]/TPAT; **Figure 3**) and entries made into ([RE-NCE]/TPAE; **Figure 4**) the cued compared to the non-cued arm of the maze. These indices were calculated by subtracting the time spent in (NCT) and the entries made into (NCE) the non-cued proximity area from the time spent in (RT) and entries made into (RE) the red-colored proximity area of the cued arm divided by the total time spent in (TPAT) and the number of entries made into (TPAE) the proximity areas.

Results

Comparisons of the effect of phase and drug exposure on the indices of time spent in and number of entries made into the arms

Two-way RM ANOVA did not reveal significant interactions between phase and exposure for either the index of time spent in [$F(12,64) = 0.7910, p = 0.658$] or the index of entries made into the cued vs. the non-cued arm [$F(12,64) = 1.211, p = 0.296$]. However, with respect to the index of time spent in the cued vs. the non-cued arm, significant main effects of phase [$F(2,64) = 35.27, p < 0.0001$] and exposure [$F(6,32) = 2.421, p = 0.048$] were demonstrated (**Figure 3**). In terms of the index of entry frequency into the cued- vs. the non-cued arm, a significant main effect of phase [$F(2,64) = 43.18, p < 0.0001$], but not exposure [$F(6,32) = 2.176, p = 0.072$] was shown (**Figure 4**).

Establishing the most appropriate dose of apomorphine

Based on the observation that apomorphine 100 $\mu\text{g/L}$, and not 50 $\mu\text{g/L}$ induced *both* a higher degree of cued-arm persistence during Phase 2, *as well as* indiscriminate arm-choice behavior during Phase 3 compared to control exposure (**Figures 3** and **4**; descriptive statistics provided in **Tables 1** and **2**), this dose was chosen for combined exposure with escitalopram in exposure groups 6 and 7.

Within phase comparisons of the indices of time spent in and number of entries made into the arms

Phase 1: Although no statistically significant differences were demonstrated between any of the exposure groups during Phase 1 with respect to either the indices of time spent in (**Figure 3**), or entries made into (**Figure 4**) the cued- vs. the non-cued arm, differences with large effect sizes were observed between the control group and all other exposure groups (**Tables 1A**

and **2A**). Further, the difference in the index of time spent in the cued- vs. the non-cued arm between the control-exposed fish and group exposed to 100 $\mu\text{g/L}$ apomorphine only narrowly missed statistical significance ($p = 0.076$). Moreover, despite conspecific presentation in the cued arm, control-exposed fish avoided the color red, which was reversed by all the exposure groups, as indicated by the negative, as opposed to the positive index values calculated for the control-exposed and all other exposure groups, respectively. This trend was demonstrated with respect to both the indices of time spent in (**Figure 3**) and the number of entries made into (**Figure 4**) the cued- vs. the non-cued arm.

Phase 2: No statistically significant differences were found between exposure groups. In the absence of reward presentation in either arms, control-exposed fish maintained their preference for the non-cued arm, demonstrating continued cued arm avoidance (**Figures 3** and **4**; **Tables 1A** and **2A**). In contrast, all other exposure groups, except for fish exposed to escitalopram 500 $\mu\text{g/L}$, spent more time in the cued arm compared to the non-cued arm (**Figure 3**; **Table 1A**). However, considering the number of entries made into the arms, escitalopram-alone exposed groups trended toward showing a higher interest for exploring the non-cued arm, as evinced by the negative index of entry frequency into the cued- compared to the non-cued arm of the maze (**Figure 4**, **Table 2A**). Moreover, while fish exposed to apomorphine 50 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$ spent an equal period of time in the cued arm (**Figure 3**, time index 0.33 ± 0.34 vs. 0.26 ± 0.43 ; **Table 1A**; supplementary data), the group exposed to 100 $\mu\text{g/L}$ also trended towards increased exploration of the non-cued arm in the absence of reward presentation (**Figure 4**, entrance index 0.02 ± 0.49 (A0.1) vs. 0.25 ± 0.28 (A0.05); **Table 2A**; supplementary data).

Phase 3: Again, no statistical differences were observed with respect to any of the pairwise comparisons between the different exposure groups. However, in Phase 3, where social conspecifics were presented in the non-cued arm, all exposure groups, except for fish exposed to apomorphine 100 $\mu\text{g/L}$ and a combination of apomorphine and low-dose escitalopram (100/500 $\mu\text{g/L}$) spent more time in (**Figure 3**) and made more entries into (**Figure 4B**) the non-cued arm, compared to the cued arm (**Tables 1A** and **2A**, respectively). However, only low-dose escitalopram administered alone, induced behavior akin to that observed in the control-exposed fish with respect to both arm choice parameters (**Figures 3** and **4**; **Tables 1A** and **2A**). Apart from the near-zero indices calculated with respect to both the time spent in (**Figure 3**; **Table 1A**) and the number of entries made into (**Figure 4**; **Table 2A**) the cued and non-cued arms respectively, the behavior of animals in the apomorphine 100 $\mu\text{g/L}$ - and combination 100/500 $\mu\text{g/L}$ groups differed to the greatest extent from that of control-exposed fish (**Tables 1A** and **2A**), demonstrating indiscriminate arm-choice behavior.

Between-phase comparisons of the indices of time spent in and number of entries made into the arms

Control group: Control-exposed fish demonstrated a persistent preference for the non-cued arm throughout all phases of the investigation, irrespective of reward presentation in the cued arm during Phase 1, as measured by both the time spent in (**Figure 3; Table 1B**) and the number of entries made into (**Figure 4; Table 2B**) the arms. Moreover, once sight of conspecifics was introduced in the non-cued arm, control-exposed fish made more entries into the non-cued arm compared to Phase 1 (-0.59 ± 0.27 vs. -0.26 ± 0.46 , $p = 0.032$, $d = -0.9$) and Phase 2 (-0.59 ± 0.27 vs. -0.33 ± 0.27 , $p = 0.131$, $d = -1.0$). Further, although not statistically significant, a difference with a large effect size was observed between the time spent in the non-cued arm during Phase 3, compared to Phase 1 (-0.61 ± 0.29 vs. -0.31 ± 0.45 , $p = 0.272$, $d = -0.8$).

Apomorphine alone exposed groups: Considering the time spent in the respective arms, both 50 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$ induced persistent cued arm choice in the absence of reward presentation during Phase 2, compared to Phase 1 (**Figure 3; Table 1B**). However, the behavior of 50 $\mu\text{g/L}$ exposed fish was once again more orientated towards the reward as evinced by both the time spent in (**Figure 3, Table 1B**; Phase 3 vs. Phase 1: -0.36 ± 0.26 vs. 0.32 ± 0.61 , $p = 0.0009$, $d = -1.6$; Phase 3 vs. Phase 2: -0.36 ± 0.26 vs. 0.33 ± 0.34 , $p = 0.0009$, $d = -2.3$) and the number of entries made into (**Figure 4, Table 2B**; Phase 3 vs. Phase 1: -0.23 ± 0.22 vs. 0.19 ± 0.58 , $p = 0.005$, $d = -1.0$; Phase 3 vs. Phase 2: -0.23 ± 0.22 vs. 0.25 ± 0.28 , $p = 0.001$, $d = -1.9$) the non-cued, compared to the cued arm.

In contrast, although spending significantly less time in (**Figure 3, Table 1B**; 0.01 ± 0.48 vs. 0.47 ± 0.38 , $p = 0.035$, $d = -1.1$) and making fewer entries, albeit not significantly so, into the cued arm (**Figure 4, Table 2B**; -0.01 ± 0.32 vs. 0.27 ± 0.32 , $p = 0.106$, $d = -0.9$) during Phase 3, compared to Phase 1, fish exposed to apomorphine 100 $\mu\text{g/L}$ expressed indiscriminate arm choice behavior during Phase 3 as indicated by near-zero discrimination indices calculated for both time and entry frequency (**Tables 1B and 2B**).

Escitalopram alone exposed groups: In the absence of reward presentation (Phase 2 vs. Phase 1), chronic intervention with lower dose escitalopram tended to reduce the index of time spent (-0.02 ± 0.10 vs. 0.20 ± 0.45 , $d = -0.8$) and the number of entries made into the cued vs. the non-cued arm (-0.21 ± 0.20 vs. 0.07 ± 0.29 , $d = -1.1$). In contrast, compared to Phase 1, high-dose escitalopram exposed fish failed to modify their behavior in the absence of reward presentation (**Figures 3 and 4; Tables 1B and 2B**).

However, upon reintroducing sight of conspecifics in the non-cued arm, both groups of escitalopram-exposed fish directed their behavioral response toward the reward. Compared to both Phase 1 and Phase 2, both groups spent significantly more time in [Figure 3, Table 1B; Phase 3 vs. Phase 1: (500 $\mu\text{g/L}$: -0.60 ± 0.38 vs. 0.20 ± 0.45 , $p = 0.0004$, $d = -1.9$; 1000 $\mu\text{g/L}$: -0.31 ± 0.78 vs. 0.35 ± 0.48 , $p = 0.004$, $d = -1.1$); Phase 3 vs. Phase 2: (500 $\mu\text{g/L}$: -0.60 ± 0.38 vs. -0.02 ± 0.015 , $p = 0.015$, $d = -2.4$; 1000 $\mu\text{g/L}$: -0.31 ± 0.78 vs. 0.23 ± 0.47 , $p = 0.022$, $d = -0.9$)] and trended towards making more entries into the non-cued, compared to the cued arm [Figure 4, Table 2B; Phase 3 vs. Phase 1: (500 $\mu\text{g/L}$: -0.51 ± 0.34 vs. 0.07 ± 0.29 , $d = -1.9$; 1000 $\mu\text{g/L}$: -0.43 ± 0.46 vs. 0.11 ± 0.42 , $d = -1.2$); Phase 3 vs. Phase 2: (500 $\mu\text{g/L}$: -0.51 ± 0.34 vs. -0.21 ± 0.20 , $d = -1.1$; 1000 $\mu\text{g/L}$: -0.43 ± 0.46 vs. -0.07 ± 0.29 , $d = -1.0$)].

Combination exposed groups: Compared to the behavior observed in fish exposed to 100 $\mu\text{g/L}$ apomorphine only, the addition of the lower dose of escitalopram had no overall effect. In fact, the behavior of fish exposed to combined apomorphine and escitalopram (100/500 $\mu\text{g/L}$) remained analogous to the behavior of apomorphine-alone exposed fish (Figures 3 and 4, Tables 1 and 2). In contrast, the combination of apomorphine and high-dose escitalopram, although not affecting cue-directed reward-seeking behavior in the absence of reward presentation compared to apomorphine 100 $\mu\text{g/L}$ alone-exposed fish (Phase 2, A0.1E1.0 vs. A0.1: Figure 3, Table 1A, $d = 0.3$; Figure 4, Table 2A, $d = 0.4$), trended towards attenuating the indiscriminate arm choice during Phase 3 by modifying the behavior of apomorphine-alone exposed fish to a presentation more akin to escitalopram alone- and control-exposed fish (Phase 3, A0.1E1.0 vs. A0.1: Figure 3, Table 1A, -0.17 ± 0.49 vs. 0.01 ± 0.48 , $d = -0.4$; Figure 4, Table 2A, -0.36 ± 0.30 vs. -0.01 ± 0.32 , $d = -1.1$).

Total distance moved, total arm entries, and time per cross-arm visit

A statistically significant interaction was demonstrated between phase and exposure with respect to the average total distance moved, an indication of general locomotor activity ($[F(12.64) = 1,925, p = 0.048]$), with significant main effects of phase [$F(2, 64) = 5,327, p = 0.007$], as well as exposure [$F(6, 32) = 3,509, p = 0.009$]. Indeed, drug exposure induced significant within-phase differences between the distances swam by the control group and the apomorphine 100 $\mu\text{g/L}$ and escitalopram 500 $\mu\text{g/L}$ exposed groups (Phase 1 and 2) and the apomorphine 100 $\mu\text{g/L}$ and escitalopram 500 $\mu\text{g/L}$ exposed groups (Phase 3) (See **Figure 5**, and supplementary data). Further, with respect to between-phase behavior, pairwise comparisons revealed apomorphine 100 $\mu\text{g/L}$ -exposed fish to present with shorter distances swam in Phase 3 compared to Phase 1 ($p = 0.002, d = -0.9$), and Phase 3 compared to Phase 2 ($p = 0.041, d = -0.5$) (**Figure 5** and supplementary data). However, said reduction in the total distance swum, neither confounds nor undermines the results reported in the results, as fish exposed to apomorphine (100 $\mu\text{g/L}$) made an equal number of entries into the cross-arms throughout all phases of the investigation (supplementary data). Furthermore, although the average total distance moved by apomorphine 100 $\mu\text{g/L}$ -exposed fish did not differ significantly from any other exposure group, with the exception of control-exposed fish (**Figure 5**), apomorphine 100 $\mu\text{g/L}$ exposure was associated with an increased time spent per cross-arm visit during each of the phases, compared to all other exposure groups (supplementary data).

Discussion

In an effort towards developing a novel screening test for anti-compulsive drug action, the present work investigated cue-reward contingency learning in zebrafish and its modification with dopaminergic and serotonergic intervention. Our main findings were that 1) sight of social conspecifics, although previously reported to be rewarding for socially deprived zebrafish, was an insufficient reinforcer of cue-reward contingency learning under circumstances of motivational conflict, 2) apomorphine, escitalopram and combinations thereof lessened the negative valence of an aversive scenario, i.e. bolstering reward appraisal and facilitated cue-reward contingency learning, 3) high-concentration apomorphine exposure generally maintained cue-directed responses throughout all testing phases, 4) escitalopram facilitated and maintained reward-directed behavior, and 5) high-dose, but not low dose escitalopram intervention reversed apomorphine-induced indiscriminate arm choices during processes of re-associative testing.

Repetitive behavioral rituals such as hand washing, compulsive checking of locks or re-arranging of items, among others, are characteristic of OCD (Abramowitz, Taylor, & McKay, 2009). By themselves, these actions often demonstrate a clear goal-directed outcome; however, in OCD, they are performed beyond any functional value (American Psychiatric Association, 2013; Eisen et al., 2006). Chronic high-dose SSRI intervention is regarded as the first-line pharmacological treatment for OCD; however, a substantial number of OCD patients remain treatment refractory (Fineberg, Reghunandanan, Brown, & Pampaloni, 2013; Huff et al., 2010). In order to extend the number of currently available treatment options, accurate and robust screening tests for putative anti-compulsive drugs are of great importance.

Irrespective of the manner in which persistent repetitious behavior arises, organisms normally expend effort to perform actions that are often, but not always, aimed at achieving specific goal-directed outcomes (Banca et al., 2015; de Wit & Dickinson, 2009), e.g. seeking out a food reward (Joel, 2006), constructing a nest (Greene-Schloesser et al., 2011) or grooming (Greer & Capecchi, 2002). However, if such actions are frequently repeated, they become *habitual*, i.e. expressed in an automated fashion which reduces the need for intensive cognitive decision-making efforts (Banca et al., 2015). That said, over-reliance on habitual responding styles is viewed by contemporary literature as being conducive to compulsive routines (Fineberg et al., 2013; Gillan, Robbins, Sahakian, van den Heuvel, & van Wingen, 2016; Gottwald et al., 2018; Vaghi et al., 2017; Robbins, Vaghi, & Banca, 2019). Any action, be it goal-directed, habitual or otherwise, is preceded by learned associations between the action and its outcome (Gillan et al., 2011; Seymour, Singer, & Dolan, 2007). In this regard, behavior is governed by two primary learning pathways i.e. reward and punishment feedback processing (Cools, Roberts, & Robbins, 2008; S Palminteri, Khamassi, Joffily, & Coricelli, 2015; Wise, 2004) which are regulated by dopaminergic and serotonergic neurotransmission (Boureau & Dayan, 2011; Cools, Nakamura, & Daw, 2011; Faulkner & Deakin, 2014; Wise, 2009). Disruption of both systems within the neurobiological architecture that regulates learning, i.e. the cortico-striatal-thalamic circuitry (Gillan et al., 2011; Gillan et al., 2016; Morris, Nevet, Arkadir, Vaadia, & Bergman, 2006; Schott et al., 2008) is often characteristic of OCD (Koo, Kim, Roh, & Kim, 2010; Markarian et al., 2010).

Targeted manipulation of the serotonergic and dopaminergic neurotransmission systems has previously been shown to induce ritualistic behaviors reminiscent of compulsive-like rituals in rodents (Szechtman, Sulis, & Eilam, 1998; Yadin, Friedmand, & Bridger, 1991). Further, in line with the clinical response of OCD to SSRI intervention, SSRIs have proven useful to attenuate compulsive-like behaviors expressed in pre-clinical models (Albelda & Joel, 2012; Fernández-Guasti, Agrati, Reyes, & Ferreira, 2006; Joel, 2006; Wolmarans et al., 2016).

Considering the expensive, time-consuming and labor-intensive nature of rodent models, the work presented here extends this body of evidence in a putative zebrafish model of compulsive-like persistence. As alluded to earlier, zebrafish demonstrate significant potential as a model species for cost-effective, high-throughput drug screening tests.

Phase 1 - Cue-reward contingency learning

Our findings with respect to the influence of dopaminergic and serotonergic drug intervention on cue-reward contingency learning (Phase 1), must be regarded against the background of the behavioral response of control-exposed animals. Although sight of conspecifics has previously been shown to be highly rewarding to socially deprived zebrafish (Saif et al., 2013), control-exposed zebrafish generally avoided cued arm exposure, irrespective of the fact that the red color was cued with the presentation of conspecifics. As such, it is possible that as far as natural preference is concerned, the negative valence carried by the color red outweighed the potential rewarding value of visual interaction with conspecifics. Current research is inconclusive with respect to the color preference of zebrafish (Ahmad & Richardson, 2013; Avdesh et al., 2012; Oliveira et al., 2015; Park et al., 2016; Spence & Smith, 2008). For example, Avdesh et al. (2012) reported zebrafish to display a natural preference for reds and greens, while demonstrating strong aversion towards blue. However, in accordance with our findings, Oliveira et al. (2015) reported that zebrafish display an aversion towards red and yellow while preferring blue and green. That said, the collective of research seems to point to color preference in zebrafish being subject, rather than species-specific (Roy, Suriyampola, Flores, López, Hickey, Bhat, & Martins, 2019).

Irrespective, the results reported here show that chronically bolstered dopaminergic *and* serotonergic neurotransmission resulted in improved reward appraisal as applied in this investigation, outweighing the negative impact of the red color. Indeed, irrespective of being administered alone or in combination, both apomorphine and escitalopram increased the time spent in and the number of entries made into the cued arm compared to the non-cued arm during Phase 1. Thus, under the influence of both drugs, the red color adopted reward-predicting properties (Schultz, 2002, 2013; Wise, 2009) as was evident from the responses of drug exposed fish during Phase 2 (refer to '*Phase 2 – Cue-reward dissociation testing*') indicating that indeed, successful associative learning has taken place during Phase 1. Although our data pertaining to the influence of apomorphine on cue-reward learning is in line with the well-known role of dopamine in reward-orientated behavior (Adamantidis et al., 2011; Cools et al., 2009; Stefano Palminteri et al., 2009; Pessiglione et al., 2006; Rutledge et al., 2009; Schultz, 2002), it is the actions of serotonin that are noteworthy. Low-dose acute SSRI intervention has been shown to increase the effect of aversive feedback on behavioral

outcomes, i.e. resulting in avoidance behavior (Bari et al., 2010), a finding that is supported by the results reported here. Recent evidence also points to the long-term importance of serotonin in the appraisal of rewards and decision making with respect to learned cue-reward contingencies (Iigaya et al., 2018). Furthermore, SSRI-bolstered serotonergic tone also promotes increased effort cost towards achieving rewarding results (Meyniel et al., 2016), possibly explaining the currently observed behaviors.

Phase 2 – Cue-reward dissociation testing

In the absence of reward presentation, cued arm approach bias would confirm successful cue-reward contingency acquisition during Phase 1, manifesting as reward-seeking behavior. However, behavioral flexibility, if intact, should also quickly promote reversal learning and favor exploration of the alternate, non-cued arm. Indeed, considering the apparent aversive nature of the color red, the *absence* of social reward presentation when it was *expected*, should prompt dissociation between the cue and its conditioned outcome and facilitate an alternative behavioral choice, an ability which is impaired in OCD (Hinds et al., 2012; Gruner & Pittenger, 2017). Drug-naïve zebrafish continued to display aversion to the red color. However, the behavioral adaptation of apomorphine and escitalopram seemed to diverge, not only as a function of drug but also of dose. First, both doses of apomorphine resulted in continued dwelling in the cued arm, a finding that points to an overall lack of dissociative ability or dopamine-facilitated invigoration towards reward predicting cues (du Hoffmann & Nicola, 2014). This is in line with previously reported literature implicating bolstered dopaminergic signaling in persistent reward-seeking behavior (Beierholm et al., 2013; den Ouden et al., 2013). Further, our data seem support literature in that transiently depressed, not increased, dopaminergic signaling has been shown to act in concert with serotonin when learning from worse than expected outcomes (Schultz, 2013), a process that should assist with cognitive flexibility. This may have been prevented by the administration of apomorphine. Still, considering the fact that the two arms of the T-maze differed significantly in presentation, we cannot rule out the potential influence of possible persistent spatial memory, a behavioral effect also fortified by bolstered dopaminergic signaling (McNamara, Tejero-Cantero, Trouche, Campo-Urriza, & Dupret, 2014). However, this possibility seems less likely than a reward-seeking response per se, as zebrafish exposed to higher dose apomorphine demonstrated increased exploration of the non-cued arm as reflected by the equal number of entries made into both arms of the maze by this group. Irrespective, regarded against the background of the red color aversion shown by drug-naïve zebrafish, it can be concluded that the presentation of social conspecifics in the cued arm during Phase 1 resulted in an initial preference for this arm by exposed groups. As such, spatial memory, if playing a role in the

data presented here, was primarily founded in the co-presentation of the red color and the reward.

We hypothesized that subjects exposed to escitalopram would learn to disengage their cued arm directed behavior during Phase 2, a process governed chiefly by serotonin, (Boureau & Dayan, 2011; Faulkner & Deakin, 2014). Although our data is to some extent congruent with previous findings that demonstrated both lower and higher doses of escitalopram to facilitate reversal learning (Brown, Amodeo, Sweeney, & Ragozzino, 2012), the difference observed in the effect of lower and higher dose escitalopram on the time spent in the previously cued arm, warrants further investigation. Sadly, most investigations into serotonergic influences on behavioral flexibility and reversal learning, employ serotonin depletion as the primary intervention (Evers et al., 2005; Tanaka et al., 2009), with very little data existing that divulge the role of different serotonin concentrations in cognitive flexibility within analogous experimental paradigms as followed here (Bari et al., 2010).

Our findings related to combination intervention are interesting. Indeed, it seems that combined intervention with both doses of escitalopram maintained the cued arm-directed behavior observed in high-dose apomorphine-alone exposed groups. The addition of escitalopram, regardless of dose, seemed to increase the number of entries made into the cued arm, i.e. masking the effect of apomorphine and escitalopram exposure alone on exploratory activity. In this regard it is likely that, in the absence of reward, the combined effects of dopamine on reward-seeking behavior and that of chronic serotonin in as far as reducing the impact of aversive feedback on decision making (Bari et al., 2010), converges to facilitate continued exploration of a previously reward-cued context.

Phase 3 – Re-associative contingency testing

Considering that zebrafish demonstrated a natural aversion to the color red and that all drug interventions resulted in reward-directed behavior in spite of this, it could have been expected that once introducing the reward to the naturally preferred arm, bolstered reward-directed behavior should be facilitated during Phase 3. This was true for all exposure groups, except the two groups exposed to high-dose apomorphine and a combination of apomorphine and low-dose escitalopram. From an obsessive-compulsive perspective, the ideal response in the high-dose apomorphine exposed group of fish would have been persistent entries and dwelling time in the cued arm, an effect that would have been indicative of the effect of apomorphine previously shown in other species, e.g. pigeons (Keller, Delius, & Acerbo, 2002). It is likely that the natural aversive nature of the red color as reported in this investigation may have contributed to dampening the effect of high-dose apomorphine on persistent cue-directed responses; this needs further clarification. Nevertheless, our data would suggest that,

as opposed to low-dose apomorphine intervention, chronic high-dose apomorphine intervention resulted in a phenotype more akin to behavioral inflexibility. Further, that high-dose apomorphine exposed fish persisted in cue-directed behavior throughout this investigation points to the fact that once a cue-outcome contingency has been learned under the influence of said intervention, such behavior would be more resistant to adaptation over time and irrespective of changing contexts, albeit being reversible by high-dose SSRI intervention (Boureau & Dayan, 2011; Cools et al., 2011).

Importantly, it is unlikely that any of the results obtained and conclusions drawn here were confounded by the locomotor ability of the animals (**Figure 5**). Although the total distance moved was the highest in drug-naïve fish over the course of the investigation, all exposure groups displayed relatively similar locomotor activity during each phase, apart from the high-dose apomorphine exposed group, which demonstrated a slight reduction in locomotor activity over time. However, rather than being indicative of a general motor inability, this points to an increased interest in cued arm exploration. This is true as the data presented here are expressed as indices of the time spent in and number of entries made into the cued vs. the non-cued arm. Indeed, the number of entries made into the respective arms did not change in parallel with a reduction in the total distance swum (**Supplementary Figures i & ii**).

The present investigation was conceptualized to provide a foundation for establishing a novel, high-throughput screening test for anti-compulsive drug action using zebrafish as a model organism. Considering the current theories describing the roles of dopamine and serotonin in OCD, we aimed to induce compulsive-like persistence with the dopaminergic agonist, apomorphine, and further investigated if such persistence, if present, would be reversed by chronic escitalopram. To this extent, the results reported here provide sufficient grounds for further investigation. Although compulsive-like persistence toward habitual, cue-directed behavior was not induced by either dose of apomorphine, fish exposed to high-dose apomorphine did, in fact, present with behavior more akin to behavioral inflexibility compared to their counterparts in all other exposure groups; this was reversed by chronic high, but not lower dose escitalopram, a finding that is supportive of current dopamine-serotonin theory. The apparent aversion shown by drug-naïve subjects to the color red was unexpected and has complicated the interpretation of our results. Indeed, it is likely that the use of a more-preferred color in this population may yield a more robust result, a possibility that we will investigate in future.

In conclusion, typical theories of neurotransmitter involvement in OCD, i.e. imbalanced crosstalk between dopamine and serotonin (Markarian et al., 2010), provide a useful background for investigating compulsive-like behaviors in animals (Gillan et al., 2016). Not

only do the findings presented here confirm the viability of zebrafish as a model species in which to study the neurobiological and cognitive processes underlying dopamine-serotonin interactions under circumstances of motivational conflict, it also provide valuable direction for future endeavors toward the development of a novel screening framework that could be sensitive for anti-compulsive drug action.

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Declarations of interest:

The authors declare no conflicts of interest.

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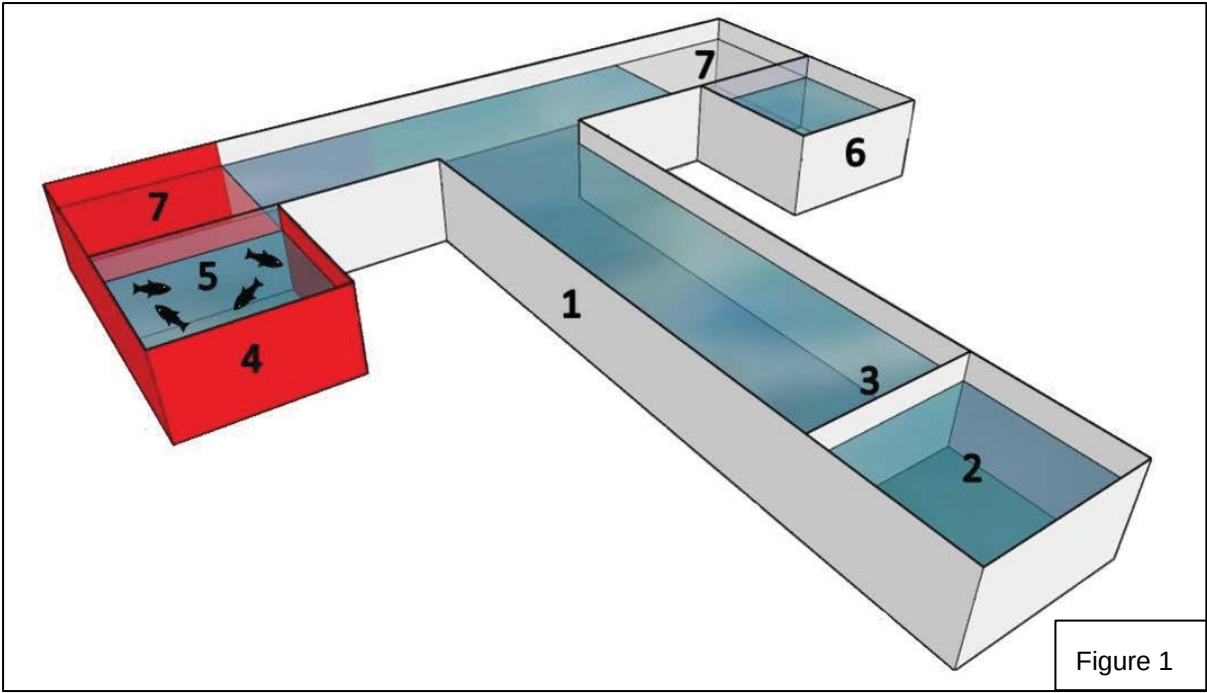
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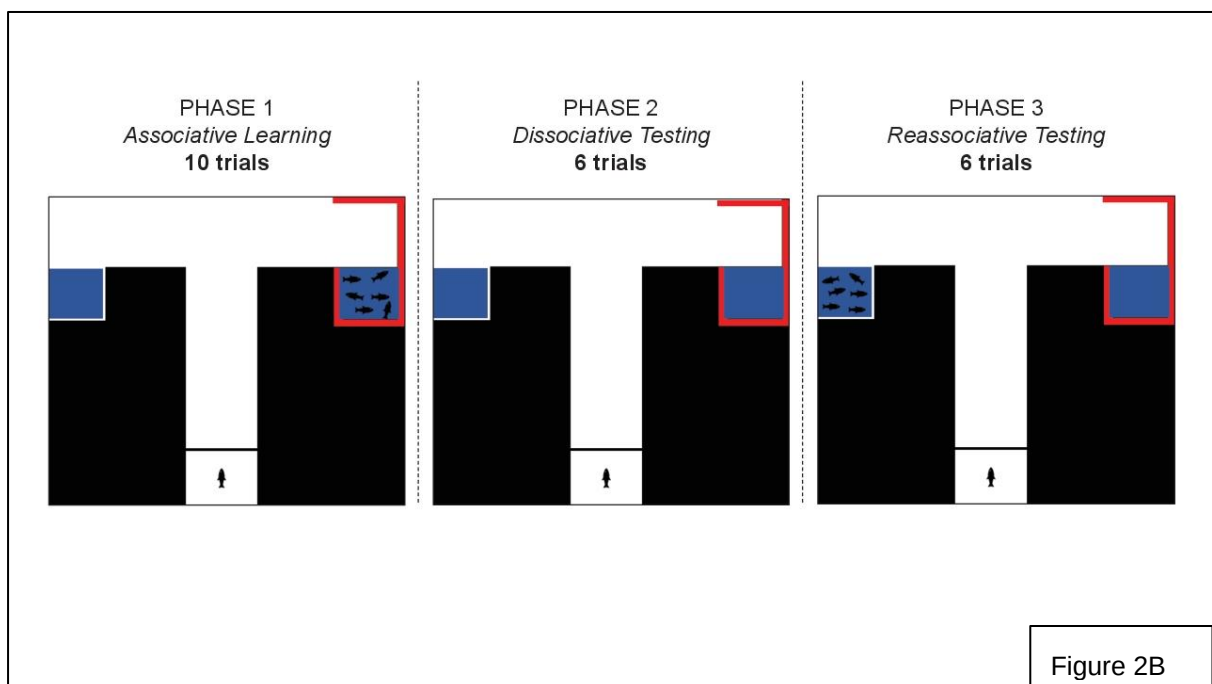
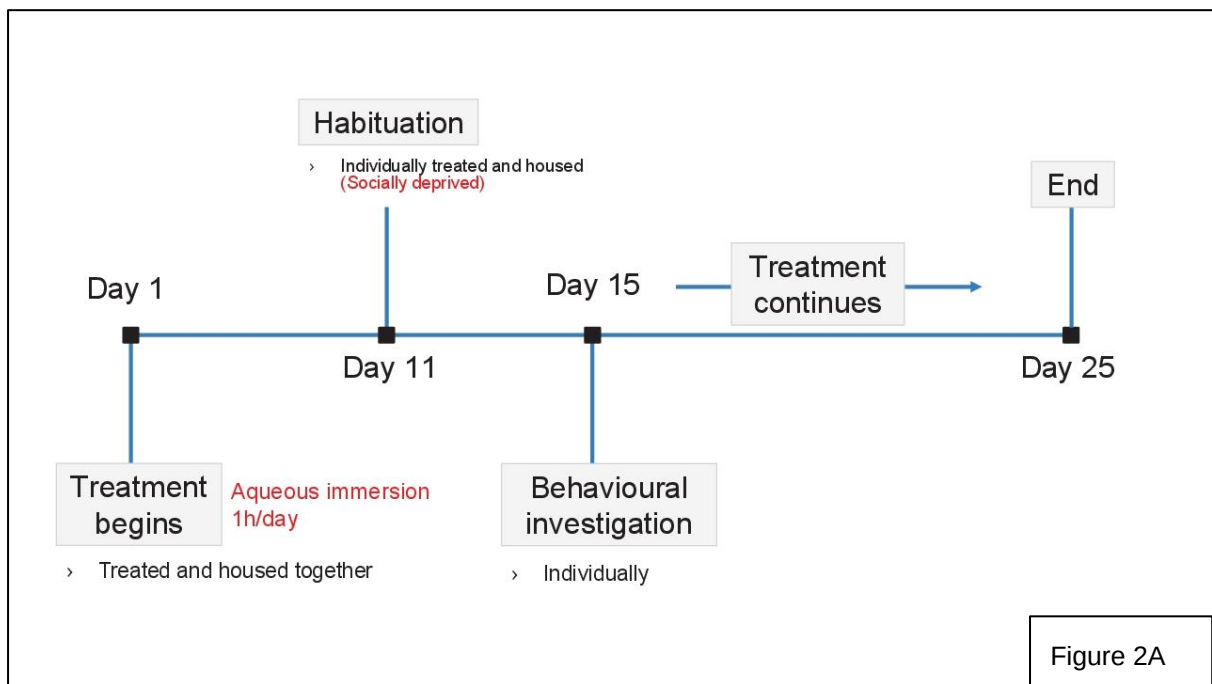
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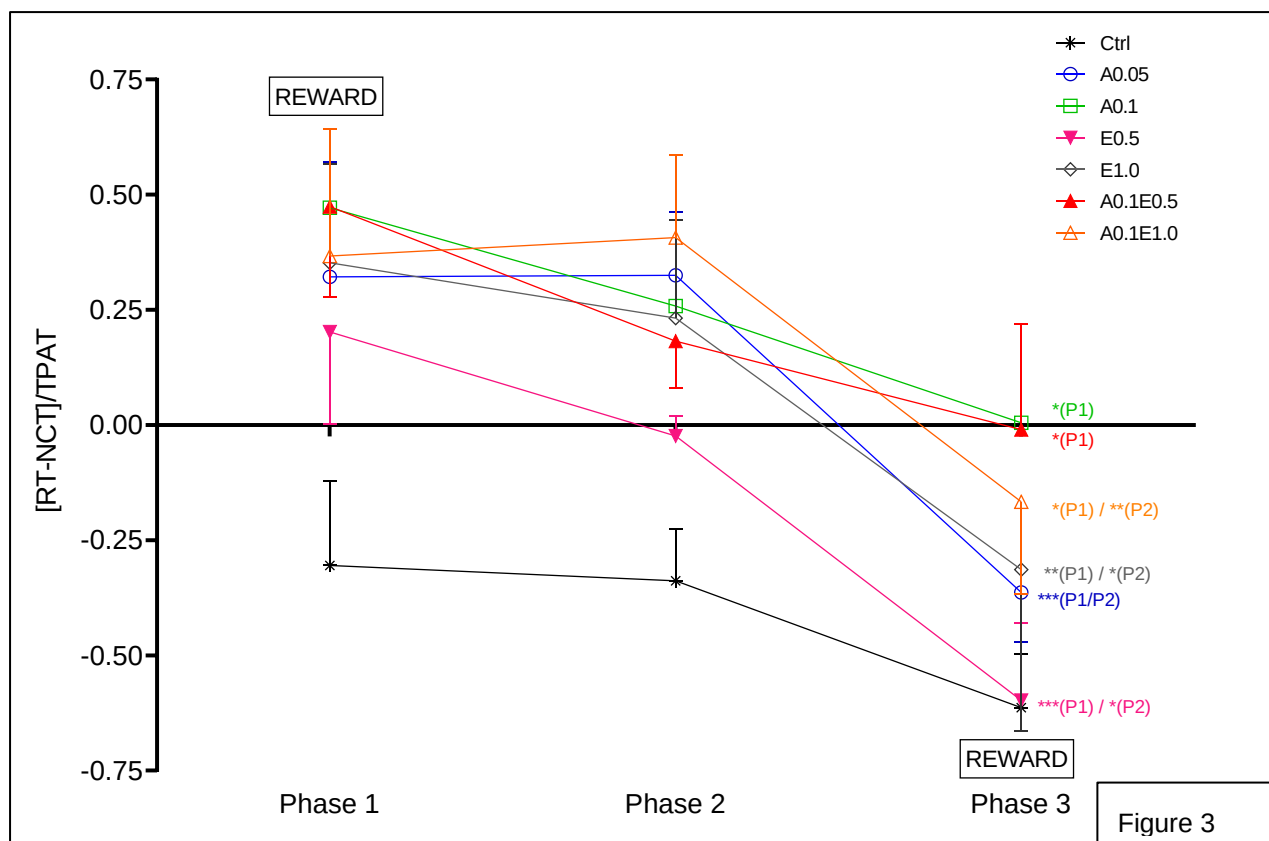
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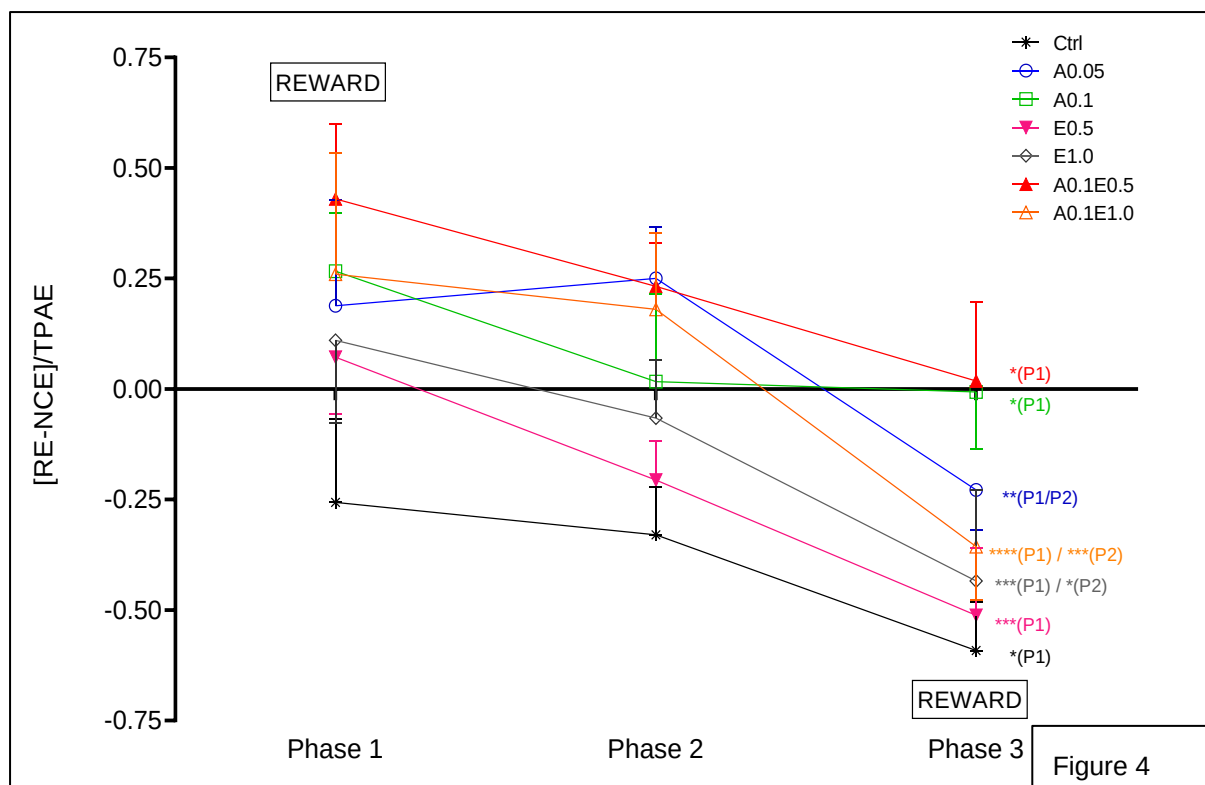
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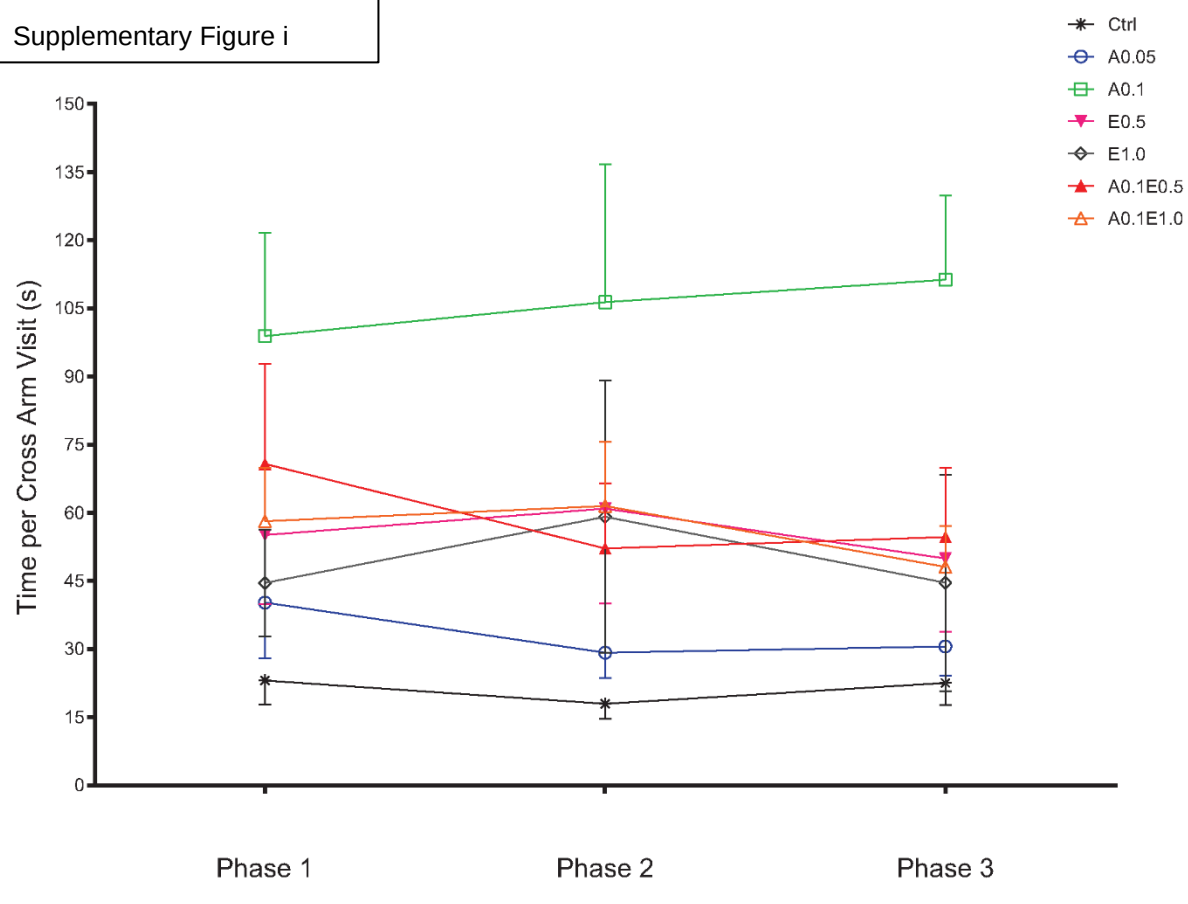


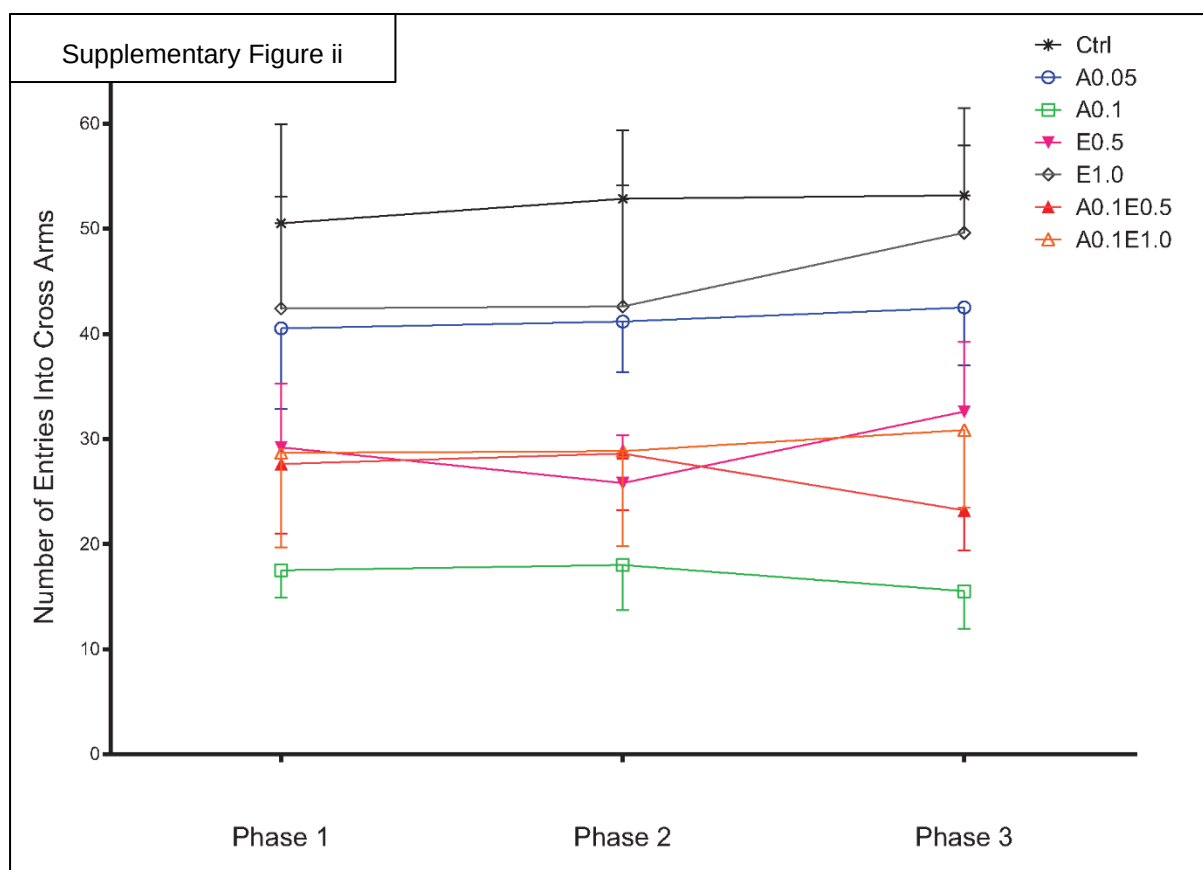






Supplementary Figure i





Exposure groups		Phase 1	Phase 2	Phase 3
		Cohen's <i>d</i> All <i>p</i> values > 0.05	Cohen's <i>d</i> All <i>p</i> values > 0.05	Cohen's <i>d</i> All <i>p</i> values > 0.05
Ctrl vs.	A0.05	1.2	2.2	0.9
	A0.1	1.9	1.7	1.6
	E0.5	1.1	1.7	0.1
	E1.0	1.4	1.5	0.6
	A0.1E0.5	1.8	2.1	1.5
	A0.1E1.0	1.2	2.1	1.2
A0.05 vs.	A0.1	0.3	0.2	1.0
	E0.5	0.2	1.6	0.7
	E1.0	0.1	0.2	0.1
	A0.1E0.5	0.3	0.5	0.9
	A0.1E1.0	0.1	0.2	0.5
A0.1 vs.	E0.5	0.7	1.1	1.4
	E1.0	0.3	0.1	0.5
	A0.1E0.5	0.002	0.2	0.03
	A0.1E1.0	0.2	0.3	0.4
E0.5 vs.	E1.0	0.3	0.9	0.5
	A0.1E0.5	0.6	1.3	1.3
	A0.1E1.0	0.3	1.6	1.0
E1.0 vs.	A0.1E0.5	0.3	0.1	0.5
	A0.1E1.0	0.02	0.4	0.2
A0.1E0.5 vs.	A0.1E1.0	0.2	0.7	0.3

Table 1 – (A) Cohen's *d* values of the within-phase comparisons of the indices of time spent in the cued vs. non-cued arms.

Exposure groups		Phase 1	Phase 2	Phase 3
		Cohen's <i>d</i> All <i>p</i> values > 0.05	Cohen's <i>d</i> All <i>p</i> values > 0.05	Cohen's <i>d</i> All <i>p</i> values > 0.05
Ctrl vs.	A0.05	0.9	2.1	1.5
	A0.1	1.3	0.9	2.0
	E0.5	0.9	0.5	0.3
	E1.0	0.8	0.9	0.4
	A0.1E0.5	1.6	2.3	1.8
	A0.1E1.0	0.9	1.5	0.8
A0.05 vs.	A0.1	0.2	0.6	0.8
	E0.5	0.3	1.9	1.0
	E1.0	0.2	1.1	0.6
	A0.1E0.5	0.5	0.1	0.8
	A0.1E1.0	0.1	0.2	0.5
A0.1 vs.	E0.5	0.6	0.6	1.6
	E1.0	0.4	0.2	1.1
	A0.1E0.5	0.5	0.6	0.1
	A0.1E1.0	0.01	0.4	1.1
E0.5 vs.	E1.0	0.1	0.6	0.2
	A0.1E0.5	1.1	2.1	1.4
	A0.1E1.0	0.4	1.2	0.5
E1.0 vs.	A0.1E0.5	0.8	1.2	1.0
	A0.1E1.0	0.3	0.7	0.2
A0.1E0.5 vs.	A0.1E1.0	0.3	0.2	1.1

Table 2 – (A) Cohen's *d* values of the within-phase comparisons of the indices of the number of entries made into the cued vs. non-cued arms.

Exposure group	Phase	Level of significance	
		<i>p</i> value	<i>d</i> value
Control	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	0.272	0.8
	Phase 2 vs. Phase 3	0.391	1.0
A0.05	Phase 1 vs. Phase 2	-	0.01
	Phase 1 vs. Phase 3	0.0009	1.6
	Phase 2 vs. Phase 3	0.0009	2.3
A0.1	Phase 1 vs. Phase 2	0.716	0.5
	Phase 1 vs. Phase 3	0.035	1.1
	Phase 2 vs. Phase 3	0.488	0.6
E0.5	Phase 1 vs. Phase 2	0.763	0.8
	Phase 1 vs. Phase 3	0.0004	1.9
	Phase 2 vs. Phase 3	0.015	2.4
E1.0	Phase 1 vs. Phase 2	-	0.3
	Phase 1 vs. Phase 3	0.004	1.1
	Phase 2 vs. Phase 3	0.022	0.9
A0.1E0.5	Phase 1 vs. Phase 2	0.427	0.9
	Phase 1 vs. Phase 3	0.0495	1.0
	Phase 2 vs. Phase 3	0.997	0.5
A0.1E1.0	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	0.013	0.9
	Phase 2 vs. Phase 3	0.007	1.2

Table 1 – (B) Pairwise between-phase comparisons of the indices of time spent in the cued vs. non-cued arms.

Exposure group	Phase	Level of significance	
		p value	d value
Control	Phase 1 vs. Phase 2	-	0.2
	Phase 1 vs. Phase 3	0.032	0.9
	Phase 2 vs. Phase 3	0.131	1.0
A0.05	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	0.005	1.0
	Phase 2 vs. Phase 3	0.001	1.9
A0.1	Phase 1 vs. Phase 2	0.160	0.6
	Phase 1 vs. Phase 3	0.106	0.9
	Phase 2 vs. Phase 3	-	0.1
E0.5	Phase 1 vs. Phase 2	0.150	1.1
	Phase 1 vs. Phase 3	0.0003	1.9
	Phase 2 vs. Phase 3	0.095	1.1
E1.0	Phase 1 vs. Phase 2	0.632	0.5
	Phase 1 vs. Phase 3	0.0007	1.2
	Phase 2 vs. Phase 3	0.031	1.0
A0.1E0.5	Phase 1 vs. Phase 2	0.479	0.7
	Phase 1 vs. Phase 3	0.013	1.1
	Phase 2 vs. Phase 3	0.387	0.7
A0.1E1.0	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	< 0.0001	1.3
	Phase 2 vs. Phase 3	0.0002	1.5

Table 2 – (B) Pairwise between-phase comparisons of the number of entries made into the cued vs. the non-cued arms.

Figure 1 – Illustration of basic experimental setup. 1) T-maze, 2) starting box, 3) start box door, 4) red cue card, 5) tank containing conspecifics (phase dependent), 6) tank without conspecifics and 7) proximity areas (opposite the two adjacent conspecific tanks).

Figure 2 – **(A)** Schematic layout of the timeline followed for all experimental fish; **(B)** Schematic layout of the phasic setup of the T-maze, indicating the presence of social conspecifics in the vicinity of the red cue card in Phase 1, no social conspecifics presented in Phase 2, and reintroduction of conspecifics in the white arm during Phase 3.

Figure 3 – Comparisons of the average index of time spent in the cued vs. non-cued proximity areas (RT-NCT/[TPAT]; index for time spent in respective arms; see *statistical analysis* for complete explanation) for the different exposure groups over three phases of testing. Positive values on the y-axis represent more time spent in the red proximity area, while negative values indicate more time spent in the white non-cued proximity area. *Phase 1* represents 10 trials (5 days), *Phase 2* represents 6 trials (3 days) and *Phase 3* represents 6 trials (3 days). ‘Reward’ indicates where the sight of social conspecifics was introduced, i.e. either the cued arm (Phase 1), neither of the arms (Phase 2), or the non-cued arm (Phase 3). Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni’s multiple comparisons; statistics are mean $\pm$ SEM. Significant between-phase differences within the respective exposure groups are indicated on the right side. ‘P’ indicates the specific phase compared to *Phase 3*. Descriptive statistics provided in **Tables 1A & B**, and **supplementary Table i a**.

Figure 4 – Comparisons of the average index of entries made into the cued vs. non-cued proximity areas (RE-NCE/[TPAE]; index for number of entries made into respective arms; see *statistical analysis* for complete explanation) for the different exposure groups over three phases of testing. Positive values on the y-axis represent more entries into the red proximity area, while negative values indicate more entries into the white non-cued proximity area. *Phase 1* represents 10 trials (5 days), *Phase 2* represents 6 trials (3 days) and *Phase 3* represents 6 trials (3 days). ‘Reward’ indicates where the sight of social conspecifics was introduced, i.e. either the cued arm (Phase 1), neither of the arms (Phase 2), or the non-cued arm (Phase 3). Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni’s multiple comparisons; statistics are mean $\pm$ SEM. Significant between-phase differences within the respective exposure groups are indicated on the right side. ‘P’ indicates the specific phase compared to *Phase 3*. Descriptive statistics provided in **Tables 2A & B**, and **supplementary Table i b**.

Figure 5 – Comparisons of the average total distance moved for the different exposure groups over the different phases. Two-way repeated measures analysis of variance (2-way RM

ANOVA), followed by Bonferroni's multiple comparisons; statistics are mean $\pm$ SEM. Significant between-phase differences within the respective exposure groups are indicated on the right side. 'P' indicates the nature of specific phase comparisons. Descriptive statistics provided in **supplementary Table ii a & b**.

Supplementary Figure i – Comparisons of the time spent per cross-arm visit (seconds) throughout all phases of the investigation for the different exposure groups. Statistics are mean $\pm$ SEM. Descriptive statistics provided in **supplementary Tables iii a & b**.

Supplementary Figure ii – Comparisons of the number of entries into the cross-arms throughout all phases of the investigation for the different exposure groups. Statistics are mean $\pm$ SEM. Descriptive statistics provided in **supplementary Table iv a & b**.

*** END OF SUPPLEMENTARY MATERIAL AND THESIS ***