Persistent behavioural phenotypes in the deer mouse and its response to serotonergic and dopaminergic intervention

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Preface

Yesterday is history, tomorrow is a mystery and today is a gift, that's why they call it present.

- Master Oogway

* * *

Hierdie referaat word opgedra aan my ouers, Johan en Elsa Fick

Spreuke 1, verse 8-9:

My dogter, luister
na wat jou pa jou leer.
Moenie wat jou ma vir jou sê
in die wind slaan nie.
Wat jy by hulle leer,
sal jou lewe
met goedheid kroon
en 'n versiering wees
om jou nek.

* * *

IN MEMORIAM

Arina Fick sadly passed away on 3 February 2019. This M.Sc. dissertation has been awarded to her posthumously.

* * *

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Alle eer aan God wat vir my die krag en moed gegee het om my Meestersgraad klaar te maak binne twee jaar. U het my gedra in die swaar tye, saam gedeel in die blydskap, leiding gegee in moeilike situasies en my geseën met wonderlike vriende.

''The Lord will guide you always'' – Isaiah 58:11

* * *

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- 'n Spesiale dankie aan Joné wat altyd seker gemaak het daar is tyd vir 'n vinnige grappie en *Happy Me* smoothies.

- *“A true friend accepts who you are but also helps you to become who you should be”*. Aan al my hartsvriendinne, Michelle Joubert, Isma, Natasia, Michelle Volkwyn, Bridgit en Lourené. Dankie dat julle altyd by my staan. Daar is nie woorde wat kan beskryf hoe baie ek julle waardeer nie.

- Dankie aan die vivarium-personeel, Cor, Antoinette, Kobus en Jaco vir die teling en versorging van die deermuise.

Congress proceedings and publications

Congress proceedings

The results of the current investigation was presented at the First Conference of Biomedical and Natural Sciences and Therapeutics (CobNeST), Spier Estate, Stellenbosch, October, 2018. The presenting author is underlined:

a) *ARINA FICK, BRIAN H HARVEY, DE WET WOLMARANS (2018): Different behavioural phenotypes and their relation to dopaminergic potentiation in the deer mouse model of obsessive-compulsive disorder: a balancing act for dopamine. Podium.*

Die resultate van die hierdie studie was ook aangebied by die Jaarlikse Studentesimposium in die Natuurwetenskappe, aangebied deur die Suid-Afrikaanse Akademie vir Wetenskap en Kuns, Arcadia, Oktober 2018.

b) *ARINA FICK, BRIAN H HARVEY, DE WET WOLMARANS (2018): Unieke gedragsfenotipes in die hertmuismodel van obsessiewe-kompulsiewe siekte (OKS) en die reaksie daarvan op serotonergiese en dopamienergiese geneesmiddelbehandeling. Podium.*

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Publications

Additional work by the candidate that contributed to the conceptualization of this dissertation (Addendum C):

GEOFFREY DE BROUWER[1], ARINA FICK[1], BRIAN H HARVEY[1,2], DE WET WOLMARANS[1] (2018) A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive–compulsive disorder: Mapping the way forward. Published online in Cognitive, Affective and Behavioral Neuroscience, 2018. DOI: 10.3758/s13415-018-00653-4.

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Abstract

Obsessive compulsive disorder (OCD)[1] is a debilitating psychiatric disorder that presents with intrusive thoughts (obsessions) and repetitive ritualistic behaviour (compulsions) as main symptoms. Selective reuptake inhibitors (SSRIs)[2] is regarded as first line intervention for OCD patients; however, only 40 - 60% of patients respond favourably to treatment. Moreover second-line interventions, which include switching to another SSRI or augmenting SSRI therapy with a low-dose antipsychotic, also demonstrates suboptimal response.

Recent psychological investigations have revealed different phenotypes of OCD, i.e. contamination/ washing; C/W)[3] and safety/checking; S/C[4], to be associated with unique dopaminergic constructs. Briefly, contrary to what was reported of individuals with S/C OCD, patients with C/W OCD seem to demonstrate more impulsive behaviour as well marked *reductions in dopaminergic reward anticipation* during tests of incentive-related action-outcome control. In this regard, adequate phasic striatal dopaminergic release prior and during presentation of a reward, is necessary for effective reward consolidation, while the opposite, i.e. inhibition of dopamine release, is needed to consolidate feedback learning from negative outcomes. However, irrespective of rewarding or punishing processing, simultaneous stimulation of the serotonergic pathways of the cortico-striatal-thalamic-cortical (CSTC)[5] circuitry is also needed to code an adequate feedback memory. Therefore, that C/W and S/C OCD seem to present with different neurological responses during reward processing, may provide valuable insight into what may possibly be unique neurobiological processes underlying its respective phenotypic presentations. It follows that while most SSRI-refractory cases of OCD are treated with anti-dopaminergic interventions, it should be considered that some individuals with treatment-refractory OCD may indeed benefit from interventions aimed at restoring the deficits in dopaminergic signalling we mention here. This idea forms the foundation of the current study.

Deer mice of both sexes that are housed in captivity naturally develop three forms of phenotypically heterogeneous but equally compulsive-like behaviours, i.e. high motor stereotypy, large nest building (LNB)[6], and high marble burying behaviour (HMB)[1]. These traits generally develop in different subjects

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[1] obsessive-compulsive disorder
[2] selective serotonin reuptake inhibitors
[3] contamination / washing
[4] safety / checking / symmetry
[5] cortico-striatal-thalamic-cortical
[6] large nest building

and are variably expressed across the deer mouse population. Of more importance for the present work is that high motor stereotypy and LNB is sensitive to chronic high dose SSRI intervention, while HMB remains refractory. As such, and taking into account that HMB is just as persistent and repetitive as LNB, we hypothesized that whereas LNB may be founded in a neurobiological construct closely related to the classic picture of hyposerotonergic, but overly normal dopaminergic functioning in OCD[2], *viz.* potentially resembling S/C[3] OCD, HMB may be a treatment resistant OC[4] phenotype of which the underlying mechanisms should be further studied in terms of its dopaminergic construct. More specifically, we hypothesized that HMB, but not LNB will respond to a combination of a SSRI (escitalopram) and a dopaminergic potentiator, i.e. the monoamine oxidase type B inhibitor, rasagiline. In fact, this concept has not yet been studied in either preclinical or clinical investigations.

As HMB is expressed in 11% of the deer mice only, a total number of 160 animals of both sexes (10 weeks of age at the onset of experimentation) first underwent screening for marble burying activity (3 x 30min trials over 3 consecutive days). Those individuals not identified as HMB were then further analysed for nesting building behaviour (7 consecutive days x 24h trials). Importantly, only animals presenting with HMB *or* LNB were selected for further treatment studies. The remainder of the subjects were either included in studies not related to this investigation, or euthanized. Treatment groups consisted of 1) water (control), 2) escitalopram (50 mg/kg/day), 3) rasagiline (5 mg/kg/day), or 4) a combination of escitalopram and rasagiline (n = 6 for all groups). All drugs were administered in the drinking water for 28 days. After treatment, the behavioural analyses were repeated as described above.

Following statistical analyses of the data generated, our results revealed 1) that HMB and LNB respond uniquely to dopaminergic potentiation, where 2) marble-directed behaviour (MDB)[5] as observed in the marble burying test is ameliorated by escitalopram and a combination of escitalopram and rasagiline, while only demonstrating modest response to rasagiline alone, and 3) LNB is sensitive to escitalopram alone only, while being exacerbated over time, irrespective of the administration of rasagiline alone or in combination with escitalopram. We also demonstrate the importance of appraising marble burying behaviour with *marble-directed behaviour* in mind, instead of focusing on the number of marbles buried, only.

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[1] high marble burying
[2] obsessive-compulsive disorder
[3] safety/checking
[4] obsessive-compulsive
[5] marble-directed behaviour

Our data seems therefore supportive of the hypothesis that LNB and HMB[1] are founded in unique neurobiological constructs as described by the inherent dopaminergic dysfunction underlying such behaviour. That HMB, but not LNB responded to dopaminergic intervention, putatively indicate that an improvement in reward feedback processing is associated with an improvement in compulsive-like burying behaviour. Although HMB also responded to escitalopram monotherapy, this response was suboptimal in the absence of rasagiline.

Taken together, the data presented in this dissertation provide a valuable and potentially important window on the neurobiological processes underlying symptom heterogeneous OCD. That HMB may be associated with deficits in reward related feedback, albeit being just as persistent and repetitive as SSRI-sensitive LNB, provides the necessary and much needed proof-of-concept to extend this work in clinical samples. Indeed, while OCD[2] may be appraised and diagnosed as a single condition, its treatment will possibly require an understanding of the unique perturbations in neurocognitive processes that promulgates the different symptomological clusters.

Keywords: Obsessive compulsive disorder (OCD), large nest building, high marble burying, escitalopram, rasagiline, dopamine, safety / checking / symmetry, contamination / washing

Solemn Declaration: I, Arina Fick (23371064) herewith declare that this dissertation is my own work and that no part thereof has been copied from other sources.

[1] high marble burying
[2] obsessive-compulsive disorder

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1 Introduction

1.1 Dissertation approach and layout

This dissertation has been prepared in article format according to the requirements of North-West University. This implies that the main body of the work is presented in the form of a journal article that will be submitted for publication following input from the examiners. The journal for which the work is intended is *Behavioural Pharmacology.*

The complete dissertation will however consist of four chapters. Chapter 1 provides a brief literature background, the problem statement, working hypothesis and experimental layout. Chapter 2 comprises a review of applicable literature, while Chapter 3 contains the journal article. Chapter 4 concludes the dissertation with a brief overall summary of the literature, the methods followed and the main findings of the current investigation. The manuscript was prepared in accordance with the 'Instructions to Authors' provided by *Behavioural Pharmacology* (link provided at the beginning of Chapter 3), while the referencing style used throughout this investigation is uniform in this regard. The different addenda contain letters of permission from co-authors to submit Chapter 3 for examination purposes, supplementary data tables and previous work by the candidate that was used in the conceptualization of the presented work.

1.2 Problem statement

Obsessive-compulsive disorder (OCD)[1] affects 2-3% of the global population (Sasson *et al.*, 1997; Angst *et al.*, 2004; De Bruijn *et al.*, 2010; Ruscio *et al.*, 2010). The condition is severe and debilitating to such an extent that it interferes significantly in the functional, occupational and social routines of patients (Bobes *et al.*, 2001; Angst *et al.*, 2005; De Bruijn *et al.*, 2010; Macy *et al.*, 2013; Schwartzman *et al.*, 2017). Even more worrisome is that only 40 – 60% of OCD patients respond favourably to first-line intervention, i.e. chronic, high dose selective serotonin reuptake inhibitors (SSRIs)[2] (Pallanti *et al.*, 2002; Pallanti & Quercioli, 2006). Apart from pharmacotherapeutic intervention, psychotherapeutic approaches, e.g. cognitive behavioural therapy (CBT)[3], can also be considered. Briefly, CBT comprises the cognitive reconstruction of behaviour by means of exposure and response prevention (ERP)[4] (Albert *et al.*, 2013). Currently, most treatment protocols specify that in cases of treatment refractory OCD, two strategies can be followed, i.e. 1) combining pharmacotherapy and psychotherapeutic intervention or 2) bolstering the effect of first-line drug therapy by either increasing the dose of the same drug, switching to another serotonin reuptake inhibitor (SRI)[5] / SSRI or augmenting SSRI treatment with a low-dose antipsychotic (Anand *et al.*, 2011; Albert *et al.*, 2013). Whereas up to 50% of treatment refractory cases respond to CBT-drug augmentation (Anand *et al.*, 2011), data pertaining to SSRI/anti-dopaminergic augmentation strategies is also promising (Bloch *et al.*, 2006; Murray *et al.*, 2017). In this case, an additional 40 – 60% of the refractory cases demonstrate adequate response (Maina *et al.*, 2003). However, discontinuation of antipsychotic augmentation treatment causes relapse within 2-4 weeks in up to 83% of patients after 2 months (Maina *et al.*, 2003), while patients that only achieve partial remission during treatment are more likely to relapse (Eisen *et al.*, 2013; Cherian *et al.*, 2014).

Recent findings indicate that differences in dopaminergic functioning may be central to the neurocognitive architecture underlying OCD and OC subtypes (Figee *et al.*, 2011; Figee *et al.*, 2014; Murray *et al.*, 2017). Indeed, research in the field of behavioural psychology demonstrated that patients with different phenotypes of OCD, most notably those with contamination/washing (C/W)[6] vs. safety/checking (S/C)[7] OCD[1], differ in terms of striatal activation when presented with a potential

[1] obsessive-compulsive disorder
[2] selective serotonin reuptake inhibitor
[3] cognitive behavioural therapy
[4] exposure and response prevention
[5] serotonin reuptake inhibitor
[6] contamination/washing
[7] safety/checking

reward (Figee *et al.,* 2011). Briefly, collective evidence from these studies demonstrate that patients with C/W OCD present with a marked reduction in striatal anticipatory response prior to receiving an expected reward. Further, these individuals generally respond impulsively in measures of action-outcome control, i.e. being unable to delay behavioural responses in return for greater rewards (Figee *et al.*, 2011), implicating deficits in reward-orientated processing. No such disturbances were found in patients presenting with S/C OCD (Figee *et al.*, 2011).

Considering the above, a closer look at the neurobiological mechanisms underlying reward and punishment learning is necessary to place the current investigation within context. Briefly, two theories have been proposed that attempt to explain how individuals process and learn from rewarding and punishing outcomes, i.e. the theory of phasic dopaminergic changes (Schultz *et al.*, 1993; Schultz *et al.*, 1997; Schultz, 2002; 2007) and the theory of dopaminergic and serotonergic opponency (Daw *et al.*, 2002). While the former explains reward and punishment learning on the basis of phasic increases and decreases in dopaminergic signalling respectively (Schultz *et al.*, 1993; Schultz *et al.*, 1997; Schultz, 2002; 2007), the latter suggests that while dopamine (DA)[2] is responsible for the coding of reward, serotonin (5HT)[3] could act as an opponent system by facilitating punishment learning (Daw *et al.*, 2002). That said, while these two concepts are essentially congruent with respect to suggesting a dichotomous role for DA in reward and punishment learning, it has also been found that neither of these learning processes can optimally transpire in the absence of sufficient serotonergic input (Palminteri *et al.*, 2012). Thus, while 5HT may be regarded as the functional opponent of DA, it can only fulfil this role in the absence of dopaminergic signalling. Therefore, the fact that monotherapeutic SSRIs[4] are often effective in the treatment of OCD, can possibly be ascribed to an already reduced dopaminergic tone in OCD patients diagnosed with generally responsive phenotypes OCD. On the other hand, DA can only adequately facilitate reward learning properly in combination with simultaneous 5HT release. It is in this principle that the current study is founded. In fact, it is likely that patients presenting with C/W[5] OCD and deficits in dopaminergic functioning (Figee *et al.*, 2011) will respond better to pharmacotherapeutic interventions that combine SSRIs with drugs that are known to bolster reward-related dopaminergic signalling, e.g. dopaminergic potentiators (Pilla *et al.*, 1999). On the other

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[1] obsessive-compulsive disorder
[2] dopamine
[3] serotonin
[4] selective serotonin reuptake inhibitor
[5] contamination/washing

hand, patients who present with no such deficit in striatal DA[1] release, may respond better to SSRI[2] monotherapy, and possibly even more so if this is augmented with compounds that inhibit dopaminergic responses, e.g. DA antagonists. Indeed, this concept has been illustrated before, albeit not in pre-clinical or clinical OCD[3] studies. For instance, Cools and colleagues (2009) demonstrated that DA enhancers, e.g. levodopa, will improve reward learning in patients with a low baseline dopaminergic tone, whereas DA blocking agents (antipsychotics) will cause further impairment of reward consolidation.

These concepts are of major importance for the current study, as we will apply two distinct persistent and repetitive behaviours expressed by deer mice, a prior validated model of OCD (Korff *et al.*, 2008; 2009; Wolmarans *et al.*, 2013), i.e. high marble-burying (HMB)[4] and large nest-building (LNB)[5], as frameworks in which to investigate the neurobiological differences underlying different OC[6] phenotypes. This study builds on previous findings from our laboratory that identified both HMB and LNB as aberrant, persistent, recurrent and seemingly purposeless behaviours in 11 – 15% and 30% of the deer mouse population, respectively (Wolmarans *et al.*, 2016b; Wolmarans *et al.*, 2016a). Further, we have shown that only LNB, but not HMB, responds to chronic high dose escitalopram intervention (50 mg/kg/day for 28 days), indicating that these two behaviours, although both resembling the compulsive phenotype, are founded in distinctly different neural correlates. Interestingly, the prevalence rates of HMB and LNB within the breeding colony seem to be congruent with clinical findings pertaining to the prevalence of treatment resistance in OCD patients (Wolmarans *et al.*, 2016b; Wolmarans *et al.*, 2016a).

Taken the literature summarized above into account, we therefore propose that as increased 5HT[7] release would be broadly effective in patients who demonstrate a low dopaminergic tone and that LNB is attenuated by bolstering serotonergic signalling (Wolmarans *et al.*, 2016a), such behaviour may potentially resemble the S/C[8] OC phenotype. On the other hand, given that HMB is completely non-

[1] dopamine
[2] selective serotonin reuptake inhibitor
[3] obsessive-compulsive disorder
[4] high marble burying
[5] large nest building
[6] obsessive-compulsive
[7] serotonin
[8] safety/checking

responsive to serotonergic interference (Wolmarans *et al.*, 2016b), we hypothesize that this may be due to inadequate simultaneous increases in DA[1] *and* 5HT[2] release.

[1] dopamine
[2] serotonin

1.3 Study hypothesis and objectives

1.3.1 Hypothesis

We hypothesize that two distinctly different but aberrant, persistent and repetitive behaviours expressed by deer mice, *viz.* HMB[1] and LNB[2], are representative of two different OC[3] phenotypes, being characterized by unique dysfunctions in dopaminergic activity. More specifically, we hypothesize that HMB, previously shown to be resistant to SSRI[4] monotherapy, will be attenuated by combination therapy comprising chronic high dose oral escitalopram (50 mg/kg/day) and rasagiline (5 mg/kg/day), a monoamine oxidase type-B inhibitor and dopaminergic potentiator; however, HMB will not respond to either drug alone. Further, we propose that while LNB will, as shown previously, be responsive to escitalopram alone, it will not respond to either rasagiline alone or in combination with escitalopram.

* * *

*A note on the context in which this dissertation is presented: Importantly, to achieve the outcomes of the larger investigation, which also includes treatment groups that comprises the use of a DA[5] antagonist, i.e. flupentixol either alone or in combination, this project has been divided into two separate phases that have been conducted in parallel, the findings of which will be disseminated in two separate dissertations by two separate candidates. For examination, the objectives of the full investigation will be provided here. However, for the perusal of the examiners, indications of the specific objectives addressed in each of the phases will be provided. * * **

[1] high marble burying
[2] large nest building
[3] obsessive compulsive
[4] selective serotonin reuptake inhibitor
[5] dopamine

1.3.2 Study Objectives

Based on the aforementioned summary of applicable literature and considering that deer mouse behaviour in general may provide a novel and necessary perspective on OC[1] symptom heterogeneity, the current project will attempt to elucidate the cognitive and neurobiological mechanisms underlying different OC phenotypes. More specifically, we will:

1. Characterize the behaviour of deer mice with respect to its resemblance of symptom heterogeneous OCD[2], with special emphasis on identifying either HMB[3] or LNB[4] expressing subjects within the normal deer mouse population housed in the Vivarium of North-West University; and

2. Employ distinct chronic pharmacological interventions via the drinking water to determine whether such behaviours may indeed be associated with unique DA[5] dysfunctions as shown in patients with different phenotypes of OCD. In the larger study, these interventions will aim to either bolster or inhibit dopaminergic responses alone or in combination with high dose SSRI[6] intervention in both behavioural cohorts, and will be structured as follows (n = 6 for all treatment groups in both behavioural cohorts):

    i. Escitalopram alone (50 mg/kg/day x 28 days) (Wolmarans *et al.*, 2013); **Findings reported in both the dissertations of **A Fick (2018)** and **A Lombaard (2019)***

    ii. Rasagiline alone (5 mg/kg/day x 28 days) (Eigeldinger-Berthou *et al.*, 2012); **Findings reported in the dissertation of **A Fick (2018)***

    iii. Combined escitalopram (50 mg/kg/day) (Wolmarans *et al.*, 2013) and rasagiline (5 mg/kg/day) (Eigeldinger-Berthou *et al.*, 2012) for 28 days; **Findings reported in the dissertation of **A Fick (2018)***

[1] obsessive compulsive
[2] obsessive-compulsive disorder
[3] high marble burying
[4] large nest building
[5] dopamine
[6] selective serotonin reuptake inhibitor

iv. Flupentixol alone (0.9 mg/kg/day x 28 days) (Engster *et al.*, 2015); **Findings reported in the dissertation of **A Lombaard (2019)***

v. Combined escitalopram (50 mg/kg/day) (Wolmarans *et al.*, 2013) and flupentixol (0.9 mg/kg/day) (Engster *et al.*, 2015) for 28 days; **Findings reported in the dissertation of **A Lombaard (2019)***

vi. Normal water – control in all cohorts
*Findings reported in both the dissertations of **A Fick (2018)** and **A Lombaard (2019)**.*

* * *

A note regarding the choice to exclude animals expressing normal behaviour as an additional control group from the current study: The main focus of the current investigation was to assess whether aberrant compulsive-like behaviours, purportedly representing different OC[1]-phenotypes, respond differentially to interventions that either bolster or inhibit dopaminergic signalling compared to its response to the relevant control treatments. As such, we did not include a normal behavioural control, as the only reason to do so would be to validate HMB[2] and LNB[3] as accurate frameworks in which to study OC-like behaviours. As this has been concluded before (Wolmarans et al., 2016a; Wolmarans et al., 2016b), it was decided not to include normal subjects in the current study design.

[1] obsessive compulsive
[2] high marble burying
[3] large nest building

1.4 Project layout

From this point forward, only those aspects of the study that are relevant for this dissertation, will be explained.

Please refer to *Infogram 1* for a detailed summary of the study layout and procedures followed. Taking into account that only 11 – 15% of the deer mouse colony housed at the NWU[1] express HMB[2], all animals (160 in the initially screened group; 10 weeks of age at onset of experiments; both sexes) were screened for HMB behaviour (Wolmarans *et al.*, 2016b; Wolmarans *et al.*, 2016a; Wolmarans *et al.*, 2017; de Brouwer & Wolmarans, 2018). Those that did not express HMB were subsequently screened for LNB[3]. HMB and LNB expressing animals were then divided into the four treatment groups (*n* = 6 per group) and treated for 28 days where after the relevant behavioural analyses were repeated.

Detailed schematic representation of study layout continues on next page .../..

[1] North-West University
[2] high marble burying
[3] large nest building

1.4.1 Detailed Study Layout

(A) First animal selection:

- HMB[1] is expressed in approximately 11-15% and LNB[2] in 30% of the population.
- Male and female mice (160) have randomly been sourced from the current breeding colony maintained at the vivarium of the NWU
- Age: 10 – 12 weeks.
- Housing: single animal per cage. from the onset of investigation.
- Caging: individual ventilated cages, standard approved bedding (corncob), food and water ad lib.
- Twelve-hour light/dark (6h/18h).
- Cleaning: once a week.

(B) Screening for HMB:

- 3 MB[3] assessments per animal (see methodology section, Manuscript A; Chapter 3).
- Light phase: dark.
- Behavioural caging: home cages.
- Bedding: river sand.
- Cleaning: daily cleaning and autoclaving of burying substrate that have been reused.
- Screening protocol: 30 min, 1 day apart.

(C) Screening for LNB:

- 7 NB[4] trials per animal (see methodology section, Manuscript A; Chapter 3).
- Light phase: 24 hrs.
- Behavioural cages: home cages.
- Bedding: standard laboratory bedding.
- Nesting material: standard non-odorized cotton wool.
- Cleaning: weekly, nests removed, and remaining cotton wool weighed daily
- Screening protocol: daily for 7 days.

(D) Treatment phase:

- Only the 24 animals expressing HMB and LNB respectively from the initial pool of 165 are used. The rest are either used in other investigations as per ethically approved protocol, or euthanized.
- Both the HMB and LNB cohorts will undergo the following 28-day treatments (*n* = 6 per treatment group per behavioural cohort).
- Water
- Escitalopram 50 mg/kg/day
- Rasagiline 5 mg/kg/day
- Escitalopram 50 mg/kg/day + rasagiline 5 mg/kg/day

(E) Post-treatment behavioural testing:

- Repetition of (B) and (C) in treated animals

Table 1-1 - Detailed Project Layout and Summary of Methods

[1] high marble burying
[2] large nest building
[3] marble burying
[4] nest building

1.5 Expected outcomes

We expect both phases of the current project to contribute to elucidating the underlying neurocognitive constructs of phenotypically heterogeneous compulsive-like behaviour. Specifically, with respect to the aspects of work that are disseminated in the current dissertation, we expect that:

- Deer mice can be separated into cohorts expressing aberrant HMB[1] and LNB[2] behaviours, respectively;

- HMB will be sensitive to chronic (28-day) intervention with a combination of the SSRI[3], escitalopram (50 mg/kg/day) and the MAO-B[4] inhibitor and dopaminergic potentiator, rasagiline (5 mg/kg/day), but not to escitalopram alone or rasagiline alone, thereby linking HMB with deficits in dopaminergic signalling as has also been observed in patients expressing C/W[5] OCD; and that

- LNB will be sensitive to escitalopram (50 mg/kg/day) alone, but not to rasagiline (5 mg/kg/day) either alone or in combination with escitalopram, thereby differentiating the underlying neurocognitive construct in LNB from that of HMB.

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[1] high marble burying
[2] large nest building
[3] selective serotonin reuptake inhibitor
[4] monoamine oxidase B
[5] contamination/washing

1.6 Ethical approval

The current investigation has been approved by the AnimCare Research Ethics Committee (NHREC reg. number AREC-130913-015) of North-West University (approval number NWU-00262-16-A5) and has been completed by the researcher, Miss A Fick, under constant supervision of the project supervisor, Dr PD Wolmarans. We have further aimed to follow the ARRIVE[1]-guidelines for animal experimentation as closely as possible by continuously refining the experimental protocol and reducing the sample sizes to the lowest number of animals per treatment group that would be sufficient to address the research theme.

All animals were bred and housed at the Vivarium (SAVC[2] reg. number FR15/13458; SANAS[3] GLP[4] compliance number G0019) of North-West University. All procedures performed were done so in accordance with the code of ethics and complied with national legislation (Ethics approval number NWU-00262-16-A5).

[1] Animal Research: Reporting of *In Vivo* Experiments
[2] South African Veterinary Council
[3] South African National Accreditation System
[4] good laboratory practice

1.7 References

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2 Literature Review

2.1 Obsessive-compulsive disorder in clinical practice

2.1.1 Epidemiology

The worldwide prevalence rate of obsessive-compulsive disorder (OCD)[1] has been reported at 2.5% (Ruscio *et al.*, 2010) and the condition is listed as the fourth most common psychiatric disorder (Basile & Mancini, 2014). It has consistently been suggested that OCD can broadly be clustered into five obsessive-compulsive symptom subtypes, *viz.* 1) obsessions about symmetry and ordering compulsions, 2) fear of contamination and cleaning or washing rituals, 3) concerns about harm to oneself or others associated with checking routines, 4) repugnant obsessions concerning sex, violence and religion, and 5) hoarding compulsions (see paragraph 2.1.2.1; McKay *et al.*, 2004; Mataix-Cols *et al.*, 2005; Rosario-Campos *et al.*, 2006; Abramowitz *et al.*, 2009). Irrespective of the symptom cluster(s) diagnosed, its presentation is time-consuming, while often incapacitating the social and familial relations of the patient, while significantly interfering with the general quality of life of patients (Steketee, 1997; Bobes *et al.*, 2001; Schwartzman *et al.*, 2017). Taylor and colleagues (2011) proposed two age groups i.e. early onset (EO)[2] OCD that manifests at a mean age of 11 years and late onset (LO)[3] OCD, manifesting by 23 years. It is notable that the EO subtype is associated with more severe symptoms whereas LO OCD is characterized by greater neuropsychological impairment, i.e. deficits in executive planning, organizational strategy and visual memory (Greisberg & McKay, 2003; Huyser *et al.*, 2010) compared to EO OCD. The distribution ratio of OCD between gender is roughly equal (Lochner & Stein, 2001; Van Oort *et al.*, 2009; Torresan *et al.*, 2013). However, men are often diagnosed with OCD symptoms at a younger age (18-24) compared to women (35-44) (Zohar *et al.*, 1997; Torresan *et al.*, 2013; Huang *et al.*, 2014). Interestingly, female OCD patients are generally married or cohabitating whereas men are more likely to be single (Bogetto *et al.*, 1999; Torresan *et al.*, 2013; Cherian *et al.*, 2014). Concerning symptom presentation, women more often present with obsessions and compulsions of the contamination/cleaning, hoarding and aggression clusters, while religious or sexual OCD symptoms are often observed in males (Labad *et al.*, 2008; Torresan *et al.*, 2013). Genetic involvement in the etiology

[1] obsessive-compulsive disorder;
[2] early onset
[3] late onset

of OCD is indicated by family studies demonstrating that relatives of OCD[1] patients present with an up to 10-23 % increased risk to develop OCD compared to the risk in the general population of 2-3% (Gottesman & Gould, 2003; Hanna *et al.*, 2005). Further, it is estimated that genetic predisposition accounts, at least to some extent, for approximately 40% of OCD cases (Pauls *et al.*, 2014), while the remainder of cases arise from other environmental factors, e.g. trauma and inflammatory processes (Murphy *et al.*, 2010), psychosocial stressors (Lafleur *et al.*, 2011), and adverse perinatal events (Geller *et al.*, 2008).

While some debate exists regarding the recent Diagnostic and Statistical Manual of Mental disorders DSM-5[2] classification of OCD not as an anxiety disorder, but rather as the archetype disorder in a new diagnostic category, i.e. obsessive-compulsive and related disorders (OCRDs)[3] (Abramowitz & Jacoby, 2015), the condition demonstrates co-diagnosis with anxiety in up to 70% of patients (Carter *et al.*, 2004). Indeed, high levels of anxiety or distress are associated with OCD, while patients report temporary relief of anxiety following engagement in compulsive and repetitive rituals in attempts to suppress feelings of distress caused by obsessions (Stein *et al.*, 2016). However, it has been shown that voluntarily attempts to *suppress* obsessional thoughts could contribute to symptom exacerbation (Tolin *et al.*, 2002). That said, considering its new classification status, OCD also demonstrates a high degree of comorbidity with other conditions in the class, i.e. trichotillomania (Lovato *et al.*, 2012; Brakoulias *et al.*, 2017), body dysmorphic disorder (Torres *et al.*, 2016; Brakoulias *et al.*, 2017), anorexia nervosa (Swinbourne *et al.*, 2012; Cederlöf *et al.*, 2015) and excoriation disorder (Torres *et al.*, 2016).

* * *

2.1.2 The diagnosis of OCD

As alluded to earlier, OCD can be described as a phenotypically heterogeneous psychiatric illness of which obsessions and compulsions are the main characteristics (Shapse, 2008). Obsessions are regarded as unpleasant images, doubts or feelings that vary in content between patients. Compulsions on the other hand, can be defined as repetitive and persistent behaviours that are often driven by an underlying obsession (Veale *et al.*, 2014). For instance, a personal experience that may have nothing to

[1] obsessive-compulsive disorder;
[2] Diagnostic and Statistical Manual of Mental Disorders
[3] obsessive-compulsive and related disorders

do with filth or contamination or even a distasteful place, can result in a person developing contamination related fears or feelings of disgust (Pauls *et al.*, 2014). These feelings will, in some individuals, translate into obsessive compulsive (OC)[1] psychopathology, e.g. obsessions about being afraid to acquire an undesirable trait, e.g. anti-social behaviour, from another person. Subsequently, this will translate into repetitive avoidance behaviours (Pauls et al., 2014). These obsession-compulsion associations manifest within several contexts that are related to the five main symptom clusters discussed above. Importantly, the relation between obsessions and compulsions are of such a nature that obsessions within one symptom domain, e.g. symmetry related thoughts, will only result in ordering compulsions, and not trigger compulsions related to another symptom cohort. However, patients often present with symptoms from more than one symptom cluster (Mataix-Cols *et al.*, 2005; Hasler *et al.*, 2007), an aspect of OCD[2] that will receive attention in the current study.

Briefly, the DSM-5[3] diagnostic criteria for OCD can be summarized as follows (APA, 2013); aspects relevant to the current investigation underlined):

A. The occurrence of compulsions, obsessions *or* both;

The two descriptions for obsessions are:

1. Persistent recurrent thoughts, ideas or images that are intrusive and unwanted and that causes noticeable anxiety or distress in individuals;
2. Attempts to ignore or neutralize these urges, recurrent thoughts, images or ideas with other actions or thoughts.

On the other hand, compulsions are described by:

1. Specific covert intellectual (i.e. mental counting or word repetition) or overt repetitive behaviours (e.g. checking or ordering) in response to strict rules or experienced obsessions;
2. Although such behaviours are performed in an attempt to reduce the level of anxiety or distress experienced, they are regarded as unrealistic.

[1] obsessive compulsive
[2] obsessive-compulsive disorder
[3] Diagnostic and Statistical Manual of Mental Disorders 5th Edition

B. Obsessive and/or repetitive rituals are time consuming, cause feelings of distress and impair the social and occupational routines of patients, while also interfering in normal daily life;

C. The presence of OCD[1] symptoms must not be attributable to another medical condition or the use of any substance; and

D. The obsessions and compulsions cannot be more appropriately explained by another possible DSM-5[2] psychiatric illness;

Furthermore, it is important to determine the level of insight a patient has into his/her symptoms (Phillips *et al.*, 2012). Indeed, data indicate that patients with poor insight may present with more severe symptoms, comorbid illness and poor treatment response (Kishore *et al.*, 2004; Catapano *et al.*, 2010; Jakubovski *et al.*, 2011).

* * *

2.1.3 Obsessive-compulsive phenotypes

As highlighted earlier, OCD can broadly be divided into 5 major symptom clusters i.e. symmetry/ordering, contamination/washing (C/W)[3], safety/checking (S/C)[4], repugnant intrusive thoughts, and collecting compulsions (Mataix-Cols *et al.*, 2005; Rosario-Campos *et al.*, 2006), the latter which under certain circumstances is diagnosed as a unique condition, i.e. hoarding disorder (APA, 2013). Of these phenotypes, C/W OCD has been extensively studied and described according to two broad ideas, *viz.* fears of being harmed or harming others – in this regard overlapping with the S/C phenotype, and feelings of discomfort when confronted with specific objects (Feinstein *et al.*, 2003; Calamari *et al.*, 2004). Considering the S/C phenotype, various stimuli (e.g. beliefs of theft or natural disasters) could provoke checking rituals while patients engage in such behaviour to curb the distress or uncertainty borne from an inflated fear of possible harm (Rachman & Rachman, 2003). These individuals incidentally reinforce their own beliefs that harmful events are more likely to happen by constantly imagining the worst possible outcomes of certain scenarios, e.g. strangers knocking on the front door or an oven that has been switched on with the intention to bake a cake (Shafran *et al.*, 1996). With respect to patients presenting with obsessive and often repugnant intrusive thoughts, themes commonly revolve around sexual images, religious guilt, or violence. While these obsessions

[1] obsessive-compulsive disorders
[2] Diagnostic and Statistical Manual of Mental Disorders fifth edition
[3] contamination/washing
[4] safety/checking

rarely result in the expression of overt behavioural symptomology that can be observed by others, they do significantly interfere with the normal functioning of the affected individual. For example, patients diagnosed with this OC[1] phenotype will for instance constantly seek reassurance from loved ones regarding spiritual beliefs and will commonly engage in excessive praying routines (Abramowitz *et al.*, 2002). Fears of losing objects and collecting compulsions is regarded to be one of the most disabling OC phenotypes; in fact, so much so that hoarding disorder has been introduced as a unique condition within the OCRD[2] category in the most recent version of the DSM[3] (Mataix-Cols *et al.*, 2010; APA, 2013). Patients with the hoarding phenotype commonly form emotional attachments towards worthless possessions (Frost *et al.*, 2004; Frost *et al.*, 2011). Further, these individuals also present with significantly more psychiatric comorbidities and a higher rate of treatment resistance compared to those diagnosed with other OC phenotypes (Wheaton *et al.*, 2008). Previously it has been suggested that different OC phenotypes may be founded in unique underlying neurological constructs, with at least some findings indicating that different symptomologies respond uniquely to pharmacotherapeutic intervention. That OCD[4] can be clustered into various symptomologies and that each may respond differently to treatment (see section 2.4) constitute the core focus of the current investigation.

* * *

2.2 The brain in OCD

2.2.1 The neuroanatomy of obsessive-compulsive disorder

It has been hypothesised that OCD results in part from or is associated with perturbations in specific loops within the cortico-striatal-thalamic-cortical circuitry (CSTC)[5] (Huyser *et al.*, 2009; Welter *et al.*, 2011). Briefly, the CSTC circuitry comprises the cortex, certain areas in the striatum which include the nucleus accumbens, putamen, and caudate, and the thalamus (Alexander *et al.*, 1986; Maia *et al.*, 2008; Kalra & Swedo, 2009; Bernstein *et al.*, 2016). Each of these brain structures, that together is responsible for the execution of complex motor behaviours as well as reward-based learning (Stocco *et al.*, 2010), plays a fundamental though distinct role in the functioning of the CSTC-circuitry. This will briefly be discussed below from the perspective of obsessive-compulsive symptomology.

[1] obsessive compulsive
[2] obsessive-compulsive and related disorders
[3] Diagnostic and Statistical Manual of Mental Disorders
[4] obsessive-compulsive disorder
[5] cortico-striatal-thalamic-cortical

The orbito-frontal cortex (OFC)[1] is mainly responsible for the normal processing of motivational and reward-related cues (i.e. perceiving an open door that needs to be locked) and emotions (Rolls, 2004; Oldham *et al.*, 2018). In fact, lesions in the OFC of animals and humans are associated with deficits in said reward-related processing (McEnaney & Butter, 1969; Rolls *et al.*, 1994). While hyperactivity of the OFC in patients with OCD[2] has been suggested and often demonstrated (Rauch *et al.*, 2007; Menzies *et al.*, 2008; Zurowski *et al.*, 2012), a clear and well-elucidated role for its involvement in the condition has not yet been established. Indeed, opposing findings prevail while different regions of the frontal cortex (FC)[3], including the anterior cingulate (AC)[4], have been implicated (Perani *et al.*, 1995; Busatto *et al.*, 2000; Menzies *et al.*, 2008), which somewhat clouds our understanding of the initial triggers for signal propagation in OCD.

The FC and the basal ganglia (BG)[5] are linked via the frontal-striatal loops (Alexander *et al.*, 1986; Busatto *et al.*, 2000; Nambu *et al.*, 2000; Nambu *et al.*, 2002; Nambu, 2008) which originate with projections from the OFC that can be traced to the caudate and the ventral striatum. From here they reach the dorsal thalamus from where the loop closes by once again entering relevant areas of the FC where outcome valuation is processed again (Alexander *et al.*, 1986; Maia *et al.*, 2008; Abramowitz *et al.*, 2009; Kalra & Swedo, 2009). Dysfunction within the BG has been implicated in numerous psychiatric conditions, including OCD (Welter *et al.*, 2011; Leisman & Melillo, 2013). The BG have a distinct role in cognitive control and the facilitation of executive processes, i.e. response inhibition, set-shifting and memory consolidation (Rubia *et al.*, 2001; Garavan *et al.*, 2006; Monchi *et al.*, 2006; Calabresi *et al.*, 2016). Further, the BG manifest the execution of reward related action plans propagated by the FC. Neuropsychological constructs of reward-related performance for which the BG are responsible include coding both the extent to which rewards are anticipated as well as the difference between the predicted and the actual reward, i.e. reward prediction errors and regulating incentive salience, i.e. promoting or inhibiting approach behaviour (Knutson & Cooper, 2005; Delgado, 2007).

The thalamus, located between the cortex and the midbrain, processes stimuli from different subcortical brain areas, including the BG[6] before reaching the cortex (Haber & Calzavara, 2009). The

[1] orbito-frontal cortex
[2] obsessive-compulsive disorder
[3] frontal cortex
[4] anterior cingulate
[5] basal ganglia
[6] basal ganglia

thalamus is structured into various lamellae that divide it into different functional regions (Jones *et al.*, 1991). This anatomical organization enables the thalamus to regulate a number of brain functions, including some that are especially significant in the case of OCD[1], e.g. the delivery of motor tasks to the cortex as conveyed to the thalamus by the BG (Steriade & Llinás, 1988). Thus, the thalamus ultimately functions as the final trigger for the execution of the motor plan as originally designed by the cortex.

Considering the anatomical organization of the CSTC[2]-circuitry as summarized above, its functional involvement in the pathogenesis of OCD will now be discussed. Briefly, the relay between the cortex and the thalamus via the BG involves both a direct (excitatory) and an indirect (inhibitory) pathway (Figure 2-1). These pathways oppose one another functionally, with the direct pathway facilitating signal propagation and subsequent motor execution, and the indirect pathway doing the opposite (Saxena & Rauch, 2000). Collectively, findings from animal and human studies have revealed a bias in favour of the direct over the indirect pathway in OCD (Gerfen, 2000; Saxena & Rauch, 2000; Mataix-Cols & van den Heuvel, 2006; DeLong & Wichmann, 2007; Perani *et al.*, 2008; Kravitz *et al.*, 2012; Ahmari *et al.*, 2013). For instance, hyper activation of the direct pathway will result in compulsive washing rituals in response to exaggerated concerns of danger or hygiene that are perceived as persistent threats (Saxena & Rauch, 2000; Ahmari *et al.*, 2013). Importantly, it has been suggested that the different symptom dimensions of OCD can be associated with unique demarcations in the CSTC circuits (Mataix-Cols *et al.*, 2004), an idea that is supported by findings that different phenotypes of OCD, e.g. hoarding, are associated with a higher degree of treatment resistance. Currently, studies are underway that attempt to gain more insight into this possibility.

[1] obsessive-compulsive disorder
[2] cortico-striatal-thalamic-cortical

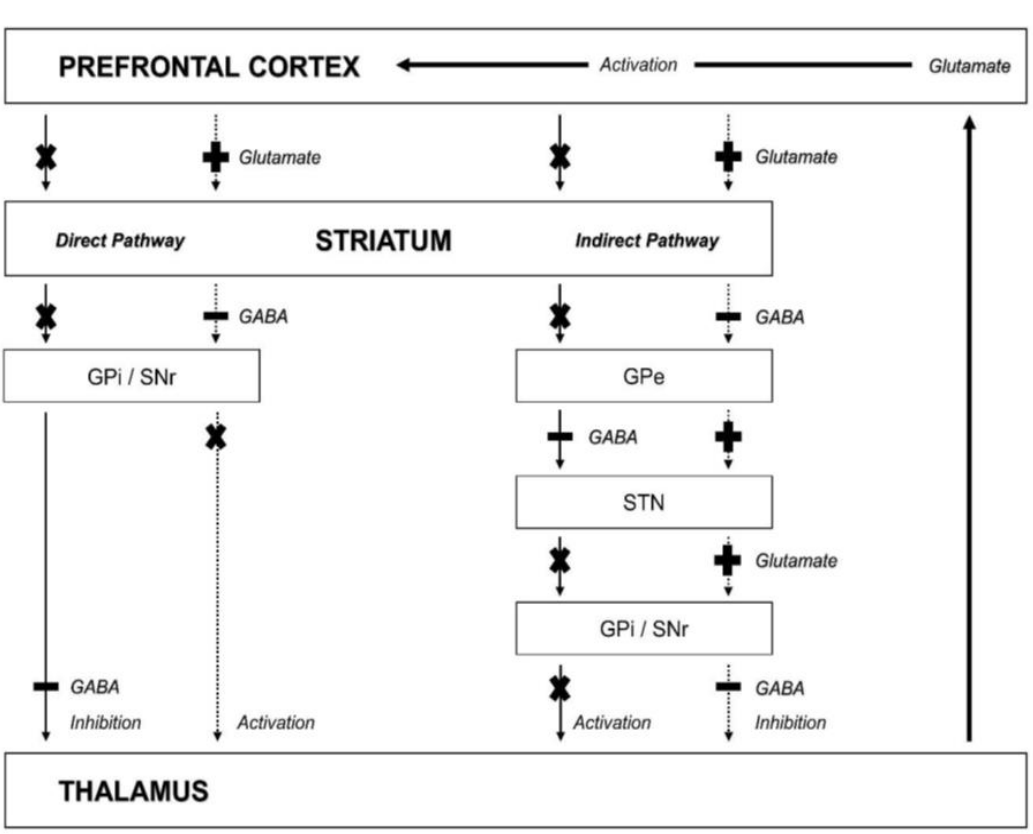

Figure 2-1 - The cortico-striatal-thalamic-cortical (CSTC) circuit

Solid lines, no cortical activation of pathways; dotted lines, cortically activated pathways; crosses, no considerable neurotransmitter release; minus signs, GABAergic inhibition; plus signs, disinhibition of target / glutamatergic activation; GPi / SNr, globus pallidus interna/substantia nigra pars reticulata; GPe, globus pallidus externa; STN, subthalamic nucleus; GABA, gamma-amino butyric acid

2.2.2 The neurotransmission of OCD

A substantial body of evidence indicates involvement of dopaminergic and serotonergic signalling in the neuropathology underlying OCD[1] (Denys *et al.*, 2004; Moresco *et al.*, 2007; Perani *et al.*, 2008; Hoffman & Rueda Morales, 2012; Ducasse *et al.*, 2014; Beaulieu *et al.*, 2015; Sinopoli *et al.*, 2017; Winter *et al.*, 2018a). However, given that the actual inhibitory and excitatory signals within the CSTC[2] circuitry are facilitated by gamma-aminobutyric acid (GABA)[3] and glutamate respectively, it is not surprising that both these neurotransmitters have also been implicated in the manifestation of OCD (Wu *et al.*, 2012; Mas *et al.*, 2014). In the following paragraphs, a synopsis of the neurotransmitter involvement in OCD will be provided.

2.2.2.1 Serotonin

The vast majority of serotonergic projections in the brain originates from the midbrain raphe nuclei from where it modulates various functions and processes regulated by the central nervous system, including eating and sleeping patterns, mood, reproduction, cognitive processing and motor functions (Murphy *et al.*, 2004; Murphy *et al.*, 2008; Murphy & Lesch, 2008). Serotonin (5HT)[4] has originally been implicated in the pathology of OCD following observations that treating patients with serotonin reuptake inhibitors (SRIs)[5] often, but not always, result in a positive treatment outcome (Vythilingum *et al.*, 2000; Grados & Riddle, 2001; Stein *et al.*, 2002; Fineberg *et al.*, 2007). Although a causal relationship between 5HT and OCD cannot be made based on the response to serotonin reuptake inhibitors (SRIs), such drugs act by blocking the reuptake of 5HT from the synaptic cleft (Blier & El Mansari, 2007) and hence the classical picture of OCD is that of a condition of hyposerotonergic signalling. However, the fact that only 40 – 60% of patients respond optimally to treatment (Pigott & Seay, 1998; Pallanti *et al.*, 2002; Maina *et al.*, 2003; Pallanti & Quercioli, 2006) and only following 8 – 12 weeks of uninterrupted drug administration (Hood *et al.*, 2001) somewhat clouds such a simplistic view. Further, neuroimaging studies in OCD patients have revealed confounding data regarding the availability of serotonin transporters (SERT)[6] and $5HT_{2A}$ receptors in the brain regions implied in OCD[7] (Pogarell *et al.*, 2003;

[1] obsessive-compulsive disorder
[2] cortico-striatal-thalamic-cortical
[3] gamma-aminobutyric acid
[4] serotonin
[5] serotonin reuptake inhibitors
[6] serotonin transporters
[7] obsessive-compulsive disorder

Stengler-Wenzke *et al.*, 2004; Adams *et al.*, 2005; Hesse *et al.*, 2005; Hasselbalch *et al.*, 2007; Reimold *et al.*, 2007; Perani *et al.*, 2008; Zitterl *et al.*, 2008; Matsumoto *et al.*, 2010).

The main theory regarding the involvement of 5HT[1] in OCD is founded on its functional opponency of dopamine (DA)[2] (Deakin & Graeff, 1991; Fletcher & Korth, 1999; Fletcher *et al.*, 1999; Daw *et al.*, 2002; Cools *et al.*, 2008a). Since behavioural inhibition has been associated with serotonergic neurotransmission (Daw *et al.*, 2002; Cools *et al.*, 2008a), it may provide a possible explanation for the positive treatment outcomes observed with long-term SRI treatment. A number of authors have proposed a role for the serotonergic system as a functional opponent of DA (Deakin & Graeff, 1991; Fletcher & Korth, 1999; Fletcher *et al.*, 1999; Daw *et al.*, 2002; Cools *et al.*, 2008a). Indeed, while the dopaminergic system propagates reward seeking behaviour, 5HT is mostly activated during aversive experiences (Fletcher, 1995; Kapur & Remington, 1996; Fletcher & Korth, 1999; Fletcher *et al.*, 1999; Daw *et al.*, 2002). Thus, it has been hypothesized that if DA is responsible for approach behaviour, motor excitement and reward processing, 5HT will be associated with avoidance behaviour, motor suppression and the processing of aversive or punishing stimuli. Several animal studies support this theory, showing that by enhancing serotonergic transmission, reward seeking behaviour can be attenuated (Parsons *et al.*, 1998; Harrison *et al.*, 1999; Harrison & Markou, 2001; Higgins & Fletcher, 2003). Recently, 5HT has also been linked to changes in risk-perceiving sensitivity (Balasubramani *et al.*, 2015; Cohen *et al.*, 2015), task disengagement and motor inhibition (Abrams *et al.*, 2004; Cools *et al.*, 2008a). Indeed, it has been shown that subjects with low baseline levels of 5HT present with impulsive behaviour and would rather opt for an immediate short-term reward rather than a larger, delayed, and longer-lasting reward (Tanaka *et al.*, 2007). This work has since been replicated and also in patients with deficits in striatal DA release (Figee *et al.,* 2011). Further, these findings were only applicable to patients diagnosed with the C/W[3] but not the S/C[4] phenotype, implicating and supporting theories that different phenotypes of OCD may be founded in unique underlying neurobiological involvement. This forms an important foundation for the current investigation.

5HT[5] elicits its effect via 14 different receptor subtypes (Nichols & Nichols, 2008). Those that are mostly relevant to OCD are the $5HT_1$ (Adams *et al.*, 2005) and $5HT_2$ receptor classes (Papakosta *et al.*,

[1] serotonin
[2] dopamine
[3] contamination/washing
[4] safety/checking
[5] serotonin

2013), which will briefly be highlighted here. Predominantly SRIs[1] and the highly selective reuptake inhibitors (SSRIs)[2] exert mostly an indirect influence on the $5HT_{1A/1B/2A/2C}$ receptors (Barnes & Sharp, 1999; Bergqvist *et al.*, 1999). 5-HT_1 receptors can be categorized into 5 different receptor subtypes *viz.*, $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, $5HT_{1E}$ and $5HT_{1F}$ (the $5HT_{1C}$ receptor was renamed as the $5HT_{2C}$ receptor; Hoyer & Martin, 1997). Serotonin $5HT_{1A}$ receptors are widely localized throughout the limbic regions of the brain and high densities have been demonstrated in the anterior cingulate cortex, the hippocampus, the dorsal and median raphe nuclei and the lateral septum (Barnes & Sharp, 1999).

The behavioural effects on reward of the $5HT_{1A}$ receptor are well known (Hoyer *et al.*, 2002; Hannon & Hoyer, 2008; Hayes & Greenshaw, 2011). For example, low dose treatment with $5HT_{1A}$ agonists such as 8-OH-DPAT[3] increases behavioural responses to reward, while high doses exert the opposite effect (Papp & Willner, 1991; Harrison & Markou, 2001). The facilitation of reward-related behaviour at low dosages is postulated to be a consequence of decreasing serotonergic outflow from the raphe nuclei, while the opposite effect observed with high dose treatment purportedly result from the stimulation of post-synaptic $5HT_{1A}$ receptors, a dichotomous effect also observed with respect to the $5HT_{1B}$ receptor (Sharp *et al.*, 1989; Bari *et al.*, 2010). Another important set of behaviours associated with the stimulation of post-synaptic $5HT_{1A}$ receptors of relevance for OCD[4] is hyperphagia and an exacerbated tail flick response upon minor stimulation (Lucki, 1992; Millan *et al.*, 1994).

As is true for the $5HT_{1A}$ receptor, the $5HT_{1B}$ receptor is found both pre- and post-synaptically but only in the BG[5], FC[6], hippocampus and hypothalamus where it decreases the release of neurotransmitters from nerve terminals and cell bodies in these areas (Hoyer *et al.*, 2002; El Mansari & Blier, 2006). With respect to OCD, previous studies have provided evidence implicating $5HT_{1B}$ receptor activation in perseverative locomotor paths and that such behaviour is only attenuated after chronic but not sub-chronic treatment with SSRIs (Shanahan *et al.*, 2009). These findings could possibly relate to those of Blier and colleagues (1996), who demonstrated that terminal $5HT_{1B}$ autoreceptors desensitize only after 8 weeks of SSRI-treatment. Evidence exists that the $5HT_{1B}$ receptor functions as a heteroreceptor in the regulation of DA[7] release, amongst others (Pauwels *et al.*, 1997). Indeed, it has been shown that

[1] serotonin reuptake inhibitors
[2] selective serotonin reuptake inhibitors
[3] 8-hydroxy-2-(di-n-propylamino)-tetralin
[4] obsessive-compulsive disorder
[5] basal ganglia
[6] frontal cortex
[7] dopamine

$5HT^{1}_{1B}$ agonists indirectly stimulate frontal cortical and nigral DA release (Johnson *et al.*, 1992), especially within the indirect pathway.

The $5HT_2$ receptor class can be divided into 3 subtypes *viz.* $5HT_{2A}$, $5HT_{2B}$ and $5HT_{2C}$, of which the $5HT_{2A/2C}$ receptors are implicated in OCD (Pytliak *et al.*, 2011). $5HT_{2A}$ receptor activation is associated with the modulation of executive functioning, i.e. decision-making (Rogers, 2011; Seymour *et al.*, 2012), cognitive processes i.e. learning (Williams *et al.*, 2002; Harvey, 2003; Zhang & Stackman Jr, 2015), response inhibition and impulse control, and attention (Nichols & Nichols, 2008; Berger *et al.*, 2009). Direct evidence for the involvement of $5HT_{2A}$ receptors in OCD was presented by findings demonstrating increased receptor expression in the caudate nuclei of untreated OCD patients. In line with the theory of hypo serotonergic functioning, Adams and colleagues (2005) suggest that a compensatory up-regulation of the $5HT_{2A}$ receptor could follow in response to a decreased level of 5HT in CSTC[2] loops. $5HT_{2C}$ receptors, being highly expressed throughout hippocampus, nucleus accumbens, amygdala and frontal and parietal cortex (Sharma *et al.*, 1997; Clemett *et al.*, 2000; Barbon *et al.*, 2011; Finnema *et al.*, 2014), have been demonstrated to attenuate and blunt dopaminergic responses (Di Giovanni *et al.*, 2000). Further, this response seems reversible. In a study by Alex and colleagues (2005), the authors demonstrated that an increase in neuronal DA[3] activity and increased levels of striatal DA follows the administration of a selective $5HT_{2C}$ receptor antagonist. It can therefore be expected that $5HT_{2C}$ receptor agonists will result in abrogation of OC[4] symptomology, while blocking such receptors would exacerbate the illness (Di Giovanni *et al.*, 2000). However, this simplified hypothesis has been met with controversy (Zohar *et al.*, 1987; Tsaltas *et al.*, 2005; Nardo *et al.*, 2014; Reimer *et al.*, 2018).

2.2.2.2 Dopamine

As alluded to earlier, DA[5] plays an important, if not crucial, role in the manifestation of OCD[6]. This neurotransmitter elicits its effects via D_1[7] – D_5 receptors and has been implicated in a range of processes and functions, including but not limited to reward-related approach behaviour, attention and sleep regulation (Seeman, 2006; Harvey *et al.*, 2011; Nishi *et al.*, 2011; Jackson *et al.*, 2012;

[1] serotonin
[2] cortico-striatal-thalamic-cortical
[3] dopamine
[4] obsessive compulsive
[5] dopamine
[6] obsessive-compulsive disorder
[7] dopamine receptor abbreviation

Korshunov *et al.*, 2017). Dopaminergic neurons are located primarily in the BG[1] and hippocampus (Hall *et al.*, 1994; Khan *et al.*, 1998), the former being an integral brain area involved in the manifestation of OC[2] symptoms (Kalra & Swedo, 2009; Welter *et al.*, 2011; Leisman & Melillo, 2013).

DA, most notably so via dopamine-1 (D_1) and dopamine-2 (D_2) receptor signalling, is central to the functioning of the CSTC[3]-circuitry (Carmin *et al.*, 2002). As explained earlier, a bias in favour of the behaviourally activating D_1 receptor expressing direct pathway over that of the behaviourally inactivating D_2 receptor expressing indirect pathway in the BG, has been demonstrated in OCD (Perani *et al.*, 2008; Kravitz *et al.*, 2012). D_1 receptor stimulation, via their activation of glutamatergic neurons, activates the direct striatal pathway, while activation of D_2 receptors prevents excitation in said glutamatergic neurons, thereby inhibiting the propagation of motor behaviour via the indirect striatal pathway (Cepeda *et al.*, 1993). Also, as opposed to the reported reductions in central SERT[4] expression often observed in OCD patients (Reimold *et al.*, 2007; Zitterl *et al.*, 2008; Hesse *et al.*, 2011), some studies have found an increased expression of dopamine transporters (DAT)[5] in the prefrontal cortex (Minzer *et al.*, 2004; Yoon *et al.*, 2007) and the CSTC circuitry (Müller-Vahl *et al.*, 2009) of patients with Tourette syndrome (TS)[6], a motor disorder characterized by repetitive stereotyped movements and vocalizations in the absence of significant cognition (Krause *et al.*, 2002). Although TS and OCD differ with respect to the neuropsychological constructs driving the expression of repetitive behaviour (Coffrey, 1995; Miguel *et al.*, 1997; Ferrão *et al.*, 2009), persistent and often rigid motor repetition is a core symptom of both conditions; TS and OCD may therefore share common ground with respect to the striatal pathways involved (Figure 2-1). Importantly, these findings do not implicate dopaminergic hyperactivity in OCD[7]. Rather, they highlight an *imbalance* in dopaminergic signalling (Pitchot *et al.*, 1996; Brambilla, 2000). In fact, while both clinical (Borcherding *et al.*, 1990; Lemus *et al.*, 1991; Ahlskog, 2011) and preclinical (Szechtman *et al.*, 1998; Taylor *et al.*, 2010) evidence implicates DA[8] in the manifestation and exacerbation of OC[9] symptoms, selective mono-therapeutic interference with dopaminergic modulators is not successful in attenuating OC behaviour (Borcherding *et al.*, 1990; Lemus *et al.*, 1991; Szechtman *et al.*, 1998; Taylor *et al.*, 2010; Ahlskog, 2011). Also, amphetamine-like drugs may either exacerbate

[1] basal ganglia
[2] obsessive compulsive
[3] cortico-striatal-thalamic-cortical
[4] serotonin transporter
[5] dopamine transporter
[6] Tourette's syndrome
[7] obsessive-compulsive disorder
[8] dopamine
[9] obsessive compulsive

or improve OC-symptoms, while DA receptor agonists may result in symptom attenuation in some OCD patients (Denys *et al.*, 2004). Further, whereas motor stereotypy in both humans (Borcherding *et al.*, 1990; Lemus *et al.*, 1991; Ahlskog, 2011) and animals (Szechtman *et al.*, 1998; Taylor *et al.*, 2010) can be induced with DA agonists, it is often, but not always (Ghaleiha *et al.*, 2013; Veale *et al.*, 2014; McLean *et al.*, 2015) alleviated by the administration of DA antagonists (Connor *et al.*, 2005). Nevertheless, SSRI[1]-refractory patients often respond to augmentation therapy where an SSRI and a low-dose antipsychotic, e.g. risperidone, is combined (Bedingfield *et al.*, 1997; Muscatello *et al.*, 2011; Dold *et al.*, 2015). Considering these contradictions, recent clinical evidence proposes unique dopaminergic constructs underlying different OC-phenotypes. In fact, it is possible that either bolstering or suppressing dopaminergic signalling in patients diagnosed with different OC symptoms, may be key to understanding the role of DA in OCD (see paragraph 2.3). This idea forms a core component of the current study.

* * *

2.2.3 The cognitive neuropsychology of OCD: the role of feedback processing and its relation to modulation by serotonin and dopamine

Essentially, OCD can be regarded as a condition of dysfunctional reward- and punishment feedback processing. Fundamentally rewards can be described as environmental incentives to approach a specific sensation (Schneirla, 1959). Continuous seeking of such sensations, e.g. task completion from the perspective of OCD, could constitute the foundation of any habit or addiction (Wise, 2002). Therefore, abnormal reward valuation and seeking can alter the ways in which goal-directed behaviours are regulated (Schultz, 2006). Normally, reward and punishment processing is controlled and gated by reward prediction errors. Briefly, when an actual outcome is more rewarding than that which has been predicted, a positive dopaminergic reward prediction error will be coded, driving future behaviours to seek out the same reward. If the outcome is negative or associated with any form of punishment, the reward prediction error will be negative, i.e. a decrease in dopaminergic tone, preventing likewise future incidents. However, upon continued exposure to rewards of the same magnitude, i.e. the outcome repeatedly matches the expectation, reward seeking behaviour will reset to its default goal-directed function, e.g. only locking a door when it is unlocked (Kamin, 1969; Schultz, 2006). It is well

[1] selective serotonin reuptake inhibitors

established that DA[1] plays an important role in reward, motivation and choices (Robinson & Berridge, 1993; Schultz *et al.*, 1997; Berridge & Robinson, 1998; Bayer & Glimcher, 2005; Cools *et al.*, 2008b). In fact, repeated and rapid firing of ventral tegmental dopaminergic neurons has been proposed as a probable mechanism underlying the processing of prediction errors (Montague *et al.*, 1996; Schultz *et al.*, 1997). Nonetheless, findings that selective interference in the dopaminergic system is not sufficient to modify and improve reward-related deficits, implicates more than one role player in the coding and processing of prediction learning. Indeed, as has been highlighted earlier, 5HT[2] has been suggested as the functional opponent of DA (Schultz *et al.*, 1997; Daw *et al.*, 2002; Bayer & Glimcher, 2005). More specifically, it is hypothesized that 5HT, like DA, is released in a phasic manner during the valuation of outcome with a negative valence (Cools *et al.*, 2008b), thereby coding prediction errors related to adverse or punishing outcomes. That said, 5HT has been shown to modulate reward and punishment processing in various ways (Palminteri *et al.,* 2012). For instance, while 5HT is important to prevent punishment-related behaviours, it has also been implicated to act in concert with DA in normal reward processing (Seymour *et al.*, 2012).

Considering the above, reward could be conceptualized as task completion. For instance, when an OCD[3] patient concerned about contamination engages in washing rituals, the possible absence of adequate dopaminergic release following satisfactory task completion could drive persistent reward-seeking behaviour manifesting as constant hand-washing rituals. However, it is also possible that compulsive rituals can be conceptualized as behavioural addictions, i.e. expressed due to hypersecretion of DA during compulsive behaviours, continuously driving reward seeking behavioural rituals. That said, taking recent findings into consideration which demonstrated that patients diagnosed with the S/C[4] phenotype do not share the deficits in reward processing shown in C/W[5] OCD[6], it is possible that this subgroup of individuals are either unable to process signals related to dopaminergic suppression or punishment-related serotonergic release (Figee *et al.*, 2011; Pinto *et al.*, 2014). This hypothesis may provide some insight into treatment resistance and the differential response of patients with different phenotypes of OCD to pharmacotherapeutic intervention. This concept is of major importance in the current investigation.

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[1] dopamine
[2] serotonin
[3] obsessive-compulsive disorder
[4] safety/checking
[5] contamination-washing
[6] obsessive-compulsive disorder

2.3 The pharmacological treatment of OCD and treatment-resistance

2.3.1 First line treatment

As explained earlier, the gold standard of pharmacological treatment for OCD include agents that act mainly by bolstering serotonergic neurotransmission, i.e. SSRIs[1], e.g. escitalopram, fluoxetine and fluvoxamine and SRIs[2], e.g. clomipramine (Murphy *et al.*, 2004; Millan *et al.*, 2015). Other treatment approaches for OCD include cognitive behavioural therapy (CBT)[3] and deep brain stimulation (DBS)[4] (Pallanti *et al.*, 2004; Denys *et al.*, 2010). Irrespective of the SRI/SSRI used, OCD responds only following chronic (> 8 weeks), high dose treatment. In fact, daily doses of up to 3 – 4 times more than that usually used for the treatment of depression may be used (Stein *et al.*, 2007). SRIs/SSRIs are generally well tolerated; however, there are certain adverse effects, e.g. weight gain, nausea, impotence and anorgasmia that may adversely affect patient compliance (Marazziti & Consoli, 2010). Interestingly, one SSRI, escitalopram, is associated with improved remission rates and less adverse effects compared to other agents in this class (Stein *et al.*, 2007). Concerning the success rate of SSRI treatment in OCD patients, only 40-60% respond effectively to SSRIs (Pigott & Seay, 1998; Pallanti *et al.*, 2002). Nonetheless, it is still important to carefully contemplate the possible reasons for unsuccessful treatment, e.g. suboptimal doses, short treatment duration and patient compliance, before embarking on augmentative treatment (Hood *et al.*, 2001).

* * *

2.3.2 Treatment resistance

Refractory or treatment-resistant OCD[5] is clinically defined as those symptoms that fail to respond to the maximum doses of various SSRI[6] treatments after 3 months of treatment with each (Pallanti *et al.*, 2002). Clinically, treatment-resistant OCD is evinced by reductions of less than 25 – 35% in the total pre-treatment OC[7] score on the Yale-Brown Compulsive Scale (Jenike & Rauch, 1994). Importantly, four

[1] selective serotonin reuptake inhibitors
[2] serotonin reuptake inhibitors
[3] cognitive behavioural therapy
[4] deep brain stimulation
[5] obsessive-compulsive disorder
[6] selective serotonin reuptake inhibitor
[7] obsessive-compulsive

aspects should be considered before a patient can be classified as being treatment-resistant (Albert *et al.*, 2013):

- The diagnosis of OCD must be accurate while the symptoms should not be the result of a co-morbid psychiatric disorder, i.e. anxiety, depression, personality disorder, or others (Albert *et al.*, 2013);

- First-line treatment response should be evaluated only after 12 weeks; attenuation of OC symptoms happens slowly over time (Albert *et al.*, 2013);

- If the diagnosis of OCD is in fact co-morbid with other psychiatric illnesses, assessment of treatment response must take this into account, i.e. OCD diagnosed with bipolar disorder could worsen with high doses of SSRI treatment (Ghaemi *et al.*, 2008);

- Further, should a patient demonstrate a partial response to treatment after three months, it may be suggestive that more time is needed to achieve a full response. In this case, treatment must not be discontinued (McDonough & Kennedy, 2002).

Strategies to manage treatment-resistant OCD include augmentation therapies or switching (Abudy *et al.*, 2011) to a different SRI[1]/SSRI[2] (Marazziti *et al.*, 2008). Augmentation strategies for treatment-resistant OCD usually consists of a combination of a high dose of SSRI and a low-dose antipsychotic, e.g. risperidone (Hollander *et al.*, 2003). Normally, it is recommended that initial augmentative strategies are introduced at the lowest possible dose of the DA[3] antagonist and that treatment response is only evaluated after 12 weeks (Abudy *et al.*, 2011). Further, it is important to note that antipsychotics can also result in adverse reactions such as an increase in body mass index, an increase in total cholesterol, triglycerides and fasting blood glucose following long-term administration. Therefore, nutritional and metabolic evaluation is essential before and during such approaches are being taken (Abudy *et al.*, 2011).

Another approach in the management of refractory OCD[4] that often proves to be successful is a combination of CBT[5] and pharmacological treatment (Foa *et al.*, 2005; Abudy *et al.*, 2011). At least

[1] serotonin reuptake inhibitor
[2] selective serotonin reuptake inhibitors
[3] dopamine
[4] obsessive-compulsive disorder
[5] cognitive behavioural therapy

75% of patients diagnosed with OCD respond to this treatment. However, as alluded to earlier, patients expressing hoarding compulsions often remain refractory to even the most rigorous treatment schedules (Calamari *et al.*, 2004). The greatest advantage of a CBT-drug combination over drug-only interventions is that it is successful not only in treating the OC[1] symptoms, but also in moderating the comorbid symptoms such as a depressed mood, lack of self-care, as well as poor school performance and social functioning (Greenberg *et al.*, 2006). Indeed, OCD is commonly associated with other comorbid psychiatric illnesses such as anxiety, mood disorders, substance abuse and impulsive behaviour (Ruscio *et al.*, 2010; Adam *et al.*, 2012). Interestingly, hoarding present with the highest degree of comorbidity, especially with major depression and impulsive behaviours (Frost *et al.*, 2011) which could explain the poor treatment outcome of this phenotype.

Considering the background provided in section 2.2.3, it is important to note that there is currently no evidence that could indicate a possible role for dopaminergic potentiation in the treatment of OCD, either as a monotherapeutic intervention or in combination with an SSRI[2]. However, it may be possible that patients consistently engaging in compulsive rituals do so due to the lack of sufficient dopaminergic signalling during task completion. Therefore, to shed more light on the possible differential roles of DA[3] in the pathogenesis of various OC phenotypes, the current phase of the larger investigation (please refer to Chapter 1) will assess the effects of the SSRI, escitalopram and the dopaminergic potentiator, i.e. rasagiline, both alone and in combination with one another on the expression of large nest building (LNB)[4] and high marble burying (HMB)[5].

* * *

2.4 Animal models of OCD as preclinical frameworks for investigating complex mechanisms

2.4.1 An introduction to animal models of OCD

Suitable animal models of psychiatric conditions are necessary to understand the complex neurobiological mechanisms underlying these illnesses to develop essential treatment approaches

[1] obsessive compulsive
[2] selective serotonin reuptake inhibitors
[3] dopamine
[4] large nest building
[5] high marble burying

(Angoa-Pérez *et al.*, 2013). It is extremely difficult to model obsessions in animals. However, animals can express natural induced behaviours that may resemble human compulsions (Angoa-Pérez *et al.*, 2013). In general, animal models can be considered as tools to investigate certain aspects of the human condition but that is problematic to investigate in patients (Wang *et al.*, 2009). However, animal models need to be validated based on certain criteria before they can be considered useful. These criteria describe a model's face, construct, and predictive validity (Geyer & Markou, 1995). In the case of face validity, the animal model should display abnormal behaviour that is similar to the symptomology of the human disorder (Willner, 1991; Geyer & Markou, 1995) *viz.,* repetitive, persistent, time-consuming and seemingly purposeless compulsive-like behavioural routines (Veale *et al.*, 2014). Construct validity refers to the extent in which the underlying neurobiology of the animal phenotype and the psychiatric illness agrees. With respect to OCD[1], this refers to CSTC[2] circuit involvement parallel with perturbations in dopaminergic and serotonergic signalling. Predictive validity broadly refers to analogous treatment response and non-response in the human and animal. In the case of OCD, the foundation of predictively valid models can be found in a robust response to high dose, chronic serotonergic interference. Due to the large body of confounding results with respect to dopaminergic involvement, response to dopaminergic interferences in animal models of OCD are, as of yet, not regarded as a mandatory prerequisite (Wolmarans *et al.*, 2017b). However, this confound will be addressed in the current investigation. To date, several pharmacological, genetic and natural models of OCD have been described. For the purpose of the current investigation, we will only focus on the natural models, most notably the deer mouse model of OCD, LNB[3] and HMB[4], both of which are generally, albeit controversially so, not regarded as measures of compulsive-like behaviour, but as animal models in itself.

* * *

2.4.2 The deer mouse model

The deer mouse model has been the focus of validation studies in our laboratory since 2006. It is a natural animal model that may be representative of symptom heterogeneous human OCD (Güldenpfennig *et al.*, 2011b; Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2016b; Wolmarans *et al.*,

[1] obsessive-compulsive disorder
[2] cortico-striatal-thalamic-cortical
[3] large nest building
[4] high marble burying

2016a; Wolmarans *et al.*, 2017a). Under normal laboratory housing conditions, roughly 45% of deer mice *(Peromyscus maniculatus bairdii)* will develop spontaneous stereotypy (Powell *et al.*, 1999). However, 30% and 11% of animals will demonstrate persistent and repetitive LNB and HMB behaviour, respectively (Wolmarans *et al.*, 2016b; Wolmarans *et al.*, 2016a), which we have put forward as evidence for symptom heterogeneous OCD (Szechtman et al., 2017). Further, in line with epidemiological data, all three phenotypes manifest without sex bias (Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2016b; Wolmarans *et al.*, 2016a), and generally do not present in the same animal.

Briefly, stereotypy involves rigid routines of pattern running, backward somersaulting and repetitive jumping that are of a time-consuming nature (Wolmarans *et al.*, 2013). Further, the extent to which these behaviours manifest demonstrates a waxing and waning nature that resembles the behaviour of patients expressing compulsive routines in order to gain short-term relieve from the underlying obsession-related anxiety. However, these behaviours generally represent compulsions, while the fact that obsessions play a prominent role in the symptomology of OCD complicates the development of animal models. Cognitive abnormalities such as recurrent thoughts and obsessions are to say the least, nearly impossible to demonstrate in animals (d'Angelo *et al.*, 2014). However, by associating compulsive-like repetition of certain motor actions in animals with the fundamental constructs of OCD, certain conclusions can be made that may have direct relevance to the human disorder (Langen *et al.*, 2011). Since motor stereotypy was the founding focus of the model, considerable work has been put into elucidating its neurobiological construct. As such, it has been demonstrated that deer mouse stereotypy is characterized by perturbation in the same neural systems as those implicated in human OCD[1], i.e. the CSTC[2] circuitry (Presti *et al.*, 2003; Presti & Lewis, 2005; Korff *et al.*, 2009; Güldenpfennig *et al.*, 2011a; d'Angelo *et al.*, 2014). More specifically, not only has it been shown that an imbalance between the direct and indirect pathways modulates stereotypy (Presti & Lewis, 2005; Bechard *et al.*, 2016), but also that such modulation is associated with reduced striatal SERT[3] expression, indicating a role for hyposerotonergic signalling (Wolmarans *et al.*, 2013). The latter results are in line with that demonstrated in at least some patients with OCD (Hesse *et al.*, 2005; Reimold *et al.*, 2007; Zitterl *et al.*, 2008). Taken together with the findings of Korff and colleagues (2009) that showed $5HT_{2A/C}$[4] receptor involvement in stereotypy, these contributions support the construct validity

[1] obsessive-compulsive disorder
[2] cortico-striatal-thalamic-cortical
[3] serotonin transporter
[4] serotonin

of the model. Although the neurobiological underpinnings of LNB[1] and HMB[2] in the deer mouse are not yet established, studies are currently underway to elucidate more on these phenomena.

Considering the predictive validity of the model, Korff and colleagues (2008) demonstrated that chronic (21-day) intraperitoneal treatment with 10 and 20 mg/kg/day of the SSRI[3], fluoxetine, significantly decreased the expression of stereotypical behaviours. Furthermore, such a response was not achieved with desipramine, a noradrenergic tricyclic antidepressant, thus supportive of the specific response of OCD to serotonergic agents (Korff *et al.*, 2008). In a follow-up study, Wolmarans and colleagues (2013) demonstrated that the response of deer mouse stereotypy is highly sensitive to chronic, *but not sub-chronic* treatment with high dose SSRIs. Concerning nest building behaviour, while LNB in deer mice also respond to the same treatment regimen as stereotypy (Wolmarans *et al.*, 2016a), HMB (see paragraph 2.4.3) remains unresponsive, possibly resembling a treatment refractory behavioural phenotype (Wolmarans *et al.*, 2016b). Together, these results form the basis of the robust predictive validity of the model, i.e. resembling the response of OCD to pharmacological manipulation.

The deer mouse model of OCD presents with several advantages of over other animal models for exploitation in this work. First, as the heterogeneous behavioural phenotypes in the model develop naturally and only in some individuals, OCD can be studied from a developmental perspective, taking into consideration the natural variation in genetic predisposition (Shorter *et al.*, 2012). Further, the behaviours observed in deer mice are to various extents modifiable by both pharmacological and environmental interference, thereby constituting a framework in which to study both drug and behavioural intervention (Lewis *et al.*, 2007; Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2017b).

* * *

2.4.3 Marble burying as a purported animal model of OCD

In this section, we will not focus on marble burying (MB)[4] as unique behavioural phenotype within the deer mouse model, but rather as a separate model of OCD[5] that, although highly disputed (De Boer & Koolhaas, 2003; de Brouwer & Wolmarans, 2018), is widely applied in various laboratories.

[1] large nest building
[2] high marble burying
[3] selective serotonin reuptake inhibitor
[4] marble burying
[5] obsessive compulsive disorders

MB, without any clear guideline describing the requirements for its severity or persistence, was initially considered as a screening test for anxiety (Broekkamp *et al.*, 1986); however, it has later been utilised as a measure of compulsive-like behaviour. The premise on which application of the marble burying test (MBT)[1] for psychiatric illness is founded is twofold. First, in analyses of anxiety it is proposed that a more anxious cohort of rodents will engage in burying behaviour following a neophobic response elicited by exposure to harmless but foreign objects in a home cage environment (Pinel & Treit, 1978; de Brouwer & Wolmarans, 2018). Second, with respect to analyses of OC[2]-like behaviour, it has been hypothesized that animals engaging in MB behaviour do so without a clear goal in mind as marbles are regarded as non-anxiogenic and non-reactive (Thomas, 2006).

However, the face validity of MB as an animal model and screening test for either psychiatric construct must be evaluated after considering the ethological value of burying behaviour. Indeed, digging, burrowing and burying serve analogous purposes across both natural and laboratory settings and are central to rodent survival and social structure (Ebensperger & Blumstein, 2006; Deacon, 2012). Fundamentally, burying is motivationally driven by the need to store food (Fleming & Brown, 1975; Jenkins & Breck, 1998), to control temperature (Ellison, 1995; Tracy & Walsberg, 2000), to facilitate social interaction, to nurture and protect young (Denenberg *et al.*, 1969) and to avoid predation (Ruffer, 1965; Tracy & Walsberg, 2002; Ebensperger & Blumstein, 2006). Further, such behaviours can also be regarded as a *mandatory behavioural need* as such behaviours persists in cages that already contain extensive burrow networks (Sherwin *et al.*, 2004). 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 behaviour is modifiable by a number of factors including pre-exposure to the burying substrate that results in decreased, albeit persistent burrowing activity (Schultz, 1972). Further, the burying substrate itself can influence the number of burying episodes, and the overall measurable digging activity (Layne & Ehrhart, 1970; Webster *et al.*, 1981). Overall digging behaviour can also be influenced by genetics, even in closely-related species which can exhibit notably different burrow architecture and digging activity (Layne & Ehrhart, 1970; Webster *et al.*, 1981; Dudek *et al.*, 1983; Weber & Hoekstra, 2009). Considering that common standard housing conditions in most rodent housing facilities constitute only a thin layer of any given form of bedding material, e.g. corn cob (Jimenez-Gomez *et al.*, 2011), wood chips (Deacon, 2012), or paper (Burn *et al.*, 2006), digging and burrowing cannot be readily expressed. It can therefore be expected that such behaviours will be

[1] marble burying test
[2] obsessive compulsive

expressed to a greater extent following the provision of ample bedding or burrowing substrate (Adams & Boice, 1981; Webster *et al.*, 1981).

As opposed to the natural tendency of rodents to burrow, dig and bury as described above, defensive burying is founded upon a clear goal of self-defence. However, to elucidate the neurobiological mechanisms underlying defensive burying, Pinel and Treit (1978) introduced a shock prod paradigm and observed that a rat will present with a strong tendency to bury a shock prod or *any* novel object if confronted in its familiar surroundings. Various objects have been identified as aversive stimuli e.g., electrified shock prods (Pinel & Treit, 1978), mouse traps, flash bulbs (Terlecki *et al.*, 1979; Gray *et al.*, 1981), noxious smells (Silverman, 1978), pepper sauce and other unpleasant liquids (Wilkie *et al.*, 1979; Poling *et al.*, 1981). However, studies also found that rodents bury harmless and non-fear-provoking objects, i.e. glass marbles (Broekkamp *et al.*, 1986) and rat chow pellets (Poling *et al.*, 1981; Londei *et al.*, 1998), thus undermining the value of introducing marbles in home cages as a neophobic stimulus. Rather, burying behaviour may be dependent on the emotional state of a subject rather than be propagated by a clear outcome in mind (Londei *et al.*, 1998). In fact, it has been shown that different levels of aggression (Koolhaas *et al.*, 1999) or stress could influence burying behaviour (Londei *et al.*, 1998). This is evinced by findings that compared defensive burying responses in rats and hamsters during exposure to a shock prod (Whillans & Shettleworth, 1981), demonstrating that even though hamsters associate shock with the presence of a prod, they do not engage in burying behaviour similar to that expressed by rats (Whillans & Shettleworth, 1981).

Thus, taking aforementioned into account, the face validity of the MBT[1] can only be evaluated knowing that such behaviour is natural and that it occurs in most rodent species both in the absence and presence of anxiogenic stimuli. Therefore, if such behaviour is natural, which criteria must be met if MB[2] is to be applied as a screening tool for anxiety- and/compulsive like behaviour? First, anxiety (or neophobia as in the case of the MBT) and compulsions differ in meaningful ways, foremost of which is that neophobic responses should abate over time, while compulsive behaviour should persist (de Brouwer & Wolmarans, 2018). Indeed, with respect to anxiety-like behaviours related to novelty in other behavioural paradigms, such behaviours 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 *et al.*, 2001). On the other hand, considering that compulsions are mostly *directed* towards reducing the distress caused by *persistent* intrusive thoughts related to specific scenarios, MB behaviour should be

[1] marble burying test
[2] marble burying

appreciated based on an animal's persistent, repetitive and behaviourally inflexible preoccupation and direct interaction with marbles (Njung'e & Handley, 1991; Wolmarans *et al.*, 2016b; Wolmarans *et al.*, 2017b). However, as MB is often only applied during a single session, neither of these criteria is met, undermining the face validity of the model with respect to both conditions.

As the MBT is mostly employed as a rapid screening tool to establish whether an animal may be anxious or engaging in compulsive behaviour, no study has yet investigated the construct validity of the model for either condition per se. However, some insight into the neurobiological basis of MB behaviour can be found in pharmacological treatment data, of which numerous results have been published. Nevertheless, a brief overview of these findings indicate that MB respond to several interventions that include agents which target the noradrenergic, dopaminergic, serotonergic, cholinergic, glutamatergic, and GABA[1]ergic systems (for a complete review of these and other pharmacological treatment findings, refer to (de Brouwer *et al.*, 2018, in press, Addendum C). Further, several miscellaneous receptors have also been targeted, including neurokinin, imidazoline, calcium, and endocannabinoid receptors, while genetic studies have also considered a number of putative anxiety-linked receptor targets (Lähdesmäki *et al.*, 2002; Egashira *et al.*, 2007; Gavioli *et al.*, 2007; Tasan *et al.*, 2009; Angoa-Pérez *et al.*, 2013). Taking this into account, it therefore must be considered that if MB[2] activity is triggered and driven by a possible anxiogenic and/or obsessive-compulsive-like construct, the test itself as applied in preclinical literature presents with poor construct and predictive validity as a screening test for either behaviour. Indeed, neither clinical anxiety nor OCD[3] demonstrates response to many of these.

2.4.3.1 *A perspective on the current investigation: methodological aspects of the marble burying setup*

2.4.3.1.1 *Zone configuration*

The MBT[4] can be performed in a one zone (marbles spaced evenly on the burying substrate throughout the test cage) or a two zone (marbles spaced evenly on one side of the testing arena only) paradigm (Handley, 1991; Gyertyan, 1995; Takeuchi *et al.*, 2002; Li *et al.*, 2006; Nicolas *et al.*, 2006; Slot *et al.*, 2008; Thomas *et al.*, 2009; Badgujar & Surana, 2010; Kinsey *et al.*, 2011; Prajapati *et al.*, 2011; Nardo *et al.*, 2014). However, as a one-zone paradigm does not allow the animal to avoid exposure to marbles

[1] gamma-aminobutyric acid
[2] marble-burying
[3] obsessive-compulsive disorder
[4] marble burying test

and therefore is insensitive to behaviours driven by *voluntary* engagement, the current investigation will employ a two-zone paradigm only. Following placement of the marbles, the mouse is introduced to the experimental cage and left to interact with the marbles for 30 minutes. Thereafter, the number of marbles buried is counted by observers that are unfamiliar with the cohort to which the tested individuals belong (Harasawa *et al.*, 2006; Angoa-Pérez *et al.*, 2013; de Almeida *et al.*, 2014; Wolmarans *et al.*, 2016b). Importantly, different investigators apply different criteria to determine if a marble is buried (or covered). Whereas some studies refer to buried marbles as those covered up to two-thirds of its size in bedding material (as applied in this investigation; Shimazaki *et al.*, 2004; Slot *et al.*, 2008; Badgujar & Surana, 2010; Shimada *et al.*, 2011) others apply a 50% (Kinsey *et al.*, 2011) or fully (Torres-Lista *et al.*, 2015) covered criterion.

2.4.3.1.2 Burying Substrates

Substrates commonly used include corn cob (Thomas *et al.*, 2009; Jimenez-Gomez *et al.*, 2011; Angoa-Pérez *et al.*, 2013), sawdust (Harasawa *et al.*, 2006; Slot *et al.*, 2008; Krass *et al.*, 2010; Dixit *et al.*, 2014), wood chips (Londei *et al.*, 1998; Saadat *et al.*, 2006; Llaneza & Frye, 2009; Thomas *et al.*, 2009), wood shavings (Poling *et al.*, 1981), river sand (de Brouwer & Wolmarans, 2018) and Sani-chips© (Young *et al.*, 2006; Thomas *et al.*, 2009; Kinsey *et al.*, 2011). However, the choice of an appropriate burying substrate is important for several reasons. Due to the sparse and light nature of burying substrates such as sawdust (pine, weighed and calculated at 0.17 g/cm^3 (de Brouwer & Wolmarans, 2018) and wood shavings (pine, weighed and calculated at 0.07 g/cm^3 (de Brouwer & Wolmarans, 2018), marbles simply placed gently on the surface of these substrates may appear from outset to be covered to a depth of at least two-thirds of its size when compared to more dense substrates (de Brouwer & Wolmarans, 2018). Further, as most investigations do not report the use of video tracking, 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, e.g. corncob (weighed and calculated at 0.38 g/cm^3 de Brouwer & Wolmarans, 2018), or river sand (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[1]. In fact, in substrates such as sawdust and wood shavings, marbles of a greater mass would settle to the bottom of the testing arena quicker compared to marbles of a lower mass; these substrates are therefore subject to disturbance by any routine movement of the animals during the test

[1] marble burying test

session (de Brouwer & Wolmarans, 2018). For this reason, the current investigation made use of river sand as the burying substrate of choice.

2.4.3.1.3 Habituation before exposure

Since MB[1] behaviour 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 (Gyertyan, 1995; Thomas *et al.*, 2009). Therefore, to exclude the possible effects of novel cage exploration on burying outcomes, it is important to consider adequate habituation in burying substrates before the onset of behavioural analysis. This may be more applicable for anxiety than compulsivity-related studies. This is because in compulsivity studies, animals should be exposed over the course of repetitive trials, instead of a single trial (Njung'e & Handley, 1991; Gyertyan, 1995; Thomas *et al.*, 2009; Wolmarans *et al.*, 2016b; Taylor *et al.*, 2017; de Brouwer & Wolmarans, 2018); hence, habituation is introduced coincidentally in the experimental design. In fact, it has been shown previously that when the test is repeated on up to five consecutive days with the same subjects, no significant differences in burying activity ensue (Poling *et al.*, 1981; Njung'e & Handley, 1991; Gyertyan, 1995; Thomas *et al.*, 2009; Wolmarans *et al.*, 2016b). However, with investigations into anxiety-like behaviour, it is important therefore to exclude the possible effects of other novelty factors, e.g. burying substrate on burying performance (Casarotto *et al.*, 2010; Umathe *et al.*, 2012; Gawali *et al.*, 2016; Taylor *et al.*, 2017). With respect to the current investigation, we attempted to exclude the influence of a possible neophobic trigger underlying the burying response, and therefore habituated all individuals in the burying substrate for at least 24 hours prior to the first test.

* * *

2.4.4 Large nest building as an animal model of OCD

In the wild, mice build nests to protect themselves against predators or harsh elements as well as to conserve heat, to breed and as nurseries to raise young (Latham & Mason, 2004; Hess *et al.*, 2008). Various aspects, i.e. genetics (Lynch, 1980), environmental configuration (Porter & Busch, 1978) and endocrinological status (Voci & Carlson, 1973) may influence nest building (NB)[2] behaviour. However, non-breeding mice will spontaneously build complex nests without prior experience with nesting material (Sherwin, 1997) and will repeatedly engage in such behaviour (Lynch, 1977). Although being a

[1] marble burying
[2] nest-building

normal behavioural manifestation in rodents, previous investigation into the nesting behaviour of house mice *(Mus musculus)* (Greene-Schloesser *et al.*, 2011) and deer mice (Wolmarans *et al.*, 2016a) demonstrated *aberrant* NB that is persistent, repetitive and seemingly purposeless compared to normal nest building behaviour in some individuals only within a normal laboratory colony. These findings provide the basis for the face validity of LNB[1] behaviour as an animal model of OCD[2], in this study hypothesized to be resembling of the S/C[3] phenotype. Further, LNB is responsive to SRI[4] (clomipramine) and SSRI[5] (fluoxetine and escitalopram; Greene-Schloesser *et al.*, 2011; Wolmarans *et al.*, 2016a) treatment, but not to the norepinephrine reuptake inhibitor (NRI)[6], desipramine (Greene-Schloesser *et al.*, 2011). Thus, the model presents with good predictive validity. Further, recent results also demonstrated reduced 5HT[7] levels in the brain regions implicated in OCD (Winter *et al.*, 2018b; Mitra *et al.*, 2016; Mitra *et al.*, 2017), thereby strengthening the construct validity of the model. From a different OC[8] perspective that addresses the construct of perfectionism, a recent series of studies have investigated nest building behaviour in rabbits as a model of OCD (Hoffman & Rueda Morales, 2009; 2012). This model is proposed to be relevant to understanding compulsions related to feelings of incompleteness, "just right" sensations, and the perception of task completion and has provided some interesting evidence in support of its face validity for OCD.

2.4.4.1 Perspectives on the current investigation: the assessment of nest-building behaviour

To characterize persistence and repetition, nest building analyses are conducted over 7 consecutive days (Wolmarans *et al.*, 2016a). Briefly, an excess of pre-weighed cotton wool is placed on top of the metal grid of the animal's home cage and left for 24 hours. On every following day, the remaining cotton wool is weighed, and an excess of cotton wool again provided (Wolmarans *et al.*, 2016a). Following completion of the 7-day trial period, the total nesting score (in grams) is calculated and applied to characterize a compulsive-like large nest-building phenotype within the normal deer mouse

[1] large nest building
[2] obsessive-compulsive disorder
[3] safety/checking
[4] serotonin reuptake inhibitor
[5] selective serotonin reuptake inhibitor
[6] norepinephrine reuptake inhibitor
[7] serotonin
[8] obsessive compulsive

population. Indeed, we have shown previously that 20 – 30% of deer mice, irrespective of stereotypical cohort or sex, express aberrant large LNB[1] behaviour (Wolmarans *et al.*, 2016a).

* * *

2.4.5 The relevance of high marble burying and large nest building as different obsessive-compulsive phenotypes in the current investigation

Considering the literature background we have presented here, little progress has been made in addressing the poor treatment response of patients with OCD. Recent findings relating to the neurocognitive constructs underlying different OC[2] phenotypes (Figee *et al.,* 2011) provide some indication that a novel understanding of OCD may be necessary. The fact that HMB[3] by an 11% minority of deer mice seems just as persistent and purposeless as LNB or high stereotypy, but that it does not respond to serotonergic intervention, may indicate that aberrant burying behaviour may be a unique OC-like phenotype within the deer mouse model of OCD, especially one that is treatment resistant. Therefore, based on the matter of persistence, repetition and seemingly purposelessness, the current investigation sets out from the perspective that HMB and LNB are two different phenotypes of the same condition but that may provide a valuable framework in which to investigate unique treatment interventions. Further, we propose HMB to resemble treatment-resistant OC-like behaviour related to deficits in dopaminergic signalling. Moreover, we hypothesize that based on its response to SSRI[4] intervention, LNB will resemble an OC-like phenotype akin to the classic picure of OCD, i.e. a condition of hyposerotonergic signalling. Moreover, we propose that HMB and LNB will diverge on the basis of their unique response to dopaminergic intervention administered either alone or in combination with escitalopram, as alluded to earlier.

[1] large nest building
[2] obsessive compulsive
[3] high marble burying
[4] selective serotonin reuptake inhibitor

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* * *

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- *Arina Fick* designed the investigation in consultation with *De Wet Wolmarans and Brian Harvey*, performed all behavioural and pharmacological experiments and assisted with statistical analyses. She also wrote the first version of the manuscript, and edited the manuscript following input from the co-authors.

- *Brian H Harvey* was study co-supervisor, funded the project and assisted in the interpretation of results.

- *Dan J Stein* provided significant and valuable input into the interpretation of the results and highlighted key issues of the data reported here and how to address them.

- *De Wet Wolmarans* acted as supervisor of this study. He has conceptualized and designed this work and were instrumental in every phase of this investigation. He also revised the first version of this dissertation, including this article.

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Important Information:

- As per the instructions to the author, figures and legends are provided at the end of the manuscript.
- All co-authors provided consent for the paper to be assessed as part of the MSc. thesis of *Arina Fick* (Addendum A).

Two compulsive-like behavioural phenotypes in the deer mouse (*Peromyscus maniculatus bairdii*) and their response to serotonergic, dopaminergic and combination intervention

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ABSTRACT

Obsessive-compulsive disorder (OCD) is treated with selective serotonin reuptake inhibitors (SSRIs), while in treatment-resistant cases, augmentation of SSRIs with anti-dopaminergic agents is typical. There is increasing evidence that different OCD phenotypes have somewhat different neurobiological underpinnings in terms of dopaminergic involvement. Although a number of experimental models of OCD have been proposed, further work is needed to determine whether these are useful in shedding light on differences in neurobiology across OCD phenotypes. Therefore, we aimed to investigate the neurobiological mechanisms underlying two phenotypes of persistent repetitive behaviour in the deer mouse, i.e. SSRI-responsive large nest-building (LNB) and SSRI-resistant high marble-burying (HMB). 160 deer mice of both sexes underwent screening for marble burying and nest building behaviour. Animals presenting with HMB or LNB were selected for treatment with one of four interventions: water control, the SSRI escitalopram (50 mg/kg/day), the MAO-B inhibitor and dopamine potentiator rasagiline (5 mg/kg/day), or a combination of rasagiline and escitalopram at the aforementioned doses (28 days; n = 6 for all groups). Behavioural analyses were repeated following treatment. HMB responded to escitalopram and even most robustly to the combination of escitalopram and rasagiline ($p < 0.0001$). In contrast, LNB responded to escitalopram, but was worsened by rasagiline and the combination of escitalopram and rasagiline. Our findings indicate that 1) HMB and LNB are founded in different neurobiology and 2) whereas LNB resembles an OCD phenotype responding to pharmacological interventions used in clinical samples, HMB may be a behavioural paradigm in which to investigate compulsive behaviour associated with unique dopaminergic involvement.

KEYWORDS

Obsessive-compulsive disorder; marble burying; nest building; animal model; escitalopram; deer mouse model

INTRODUCTION

Obsessive compulsive disorder (OCD) is a debilitating psychiatric illness (Basile & Mancini, 2014) that affects up to 2.5% of the global population (Ruscio *et al.*, 2010). The two main diagnostic traits of OCD are obsessions (invasive thoughts or ideas) and / or compulsions (repetitive persistent behaviours) (Veale *et al.*, 2014) which impair normal functioning, including in the social and occupational domains (Bobes *et al.*, 2001; De Bruijn *et al.*, 2010; Schwartzman *et al.*, 2017). OCD demonstrates symptom heterogeneity within 5 typical main symptom domains i.e. 1) contamination/washing (C/W), 2) safety/checking (S/C), 3) symmetry/ordering, 4) repugnant intrusive thoughts related to religion, violence and sex, and 5) collecting compulsions (McKay *et al.*, 2004; Mataix-Cols *et al.*, 2005; Rosario-Campos *et al.*, 2006; Abramowitz *et al.*, 2009), the last of which has recently been classified as a unique disorder, i.e. hoarding, within the obsessive-compulsive and related disorders category of the DSM-5 (APA, 2013).

OCD is treated with selective serotonin reuptake inhibitors (SSRIs) (Murphy *et al.*, 2004; Millan *et al.*, 2015), while in treatment-resistant cases, augmentation of SSRIs with anti-dopaminergic agents is used (Marazziti *et al.*, 2008; Dold *et al.*, 2015). Still, up to 15% of patients remain refractory to these interventions (Pallanti *et al.*, 2002; Pallanti & Quercioli, 2006). Further, there is increasing evidence that different OCD phenotypes have somewhat different neurobiological underpinnings. For example, individuals with contamination/washing (C/W) OCD have been shown to present with marked dysfunctional striatal activation compared to patients with safety/checking (S/C) OCD, prior to receiving an expected reward (Figee *et al.*, 2011). This point to unique dopaminergic involvement (Schultz, 2007) possibly underlying different phenotypes of OCD.

With respect to the current investigation, few pre-clinical investigations have aimed to divulge the neurobiological underpinnings of different OC phenotypes. Further, investigations into the effects of dopamine-modulating drugs in animal models of OCD, delivered inconsistent results (Hatalova et al., 2017 Mitra et al., 2017; Dorfman et al., 2018; Egashira et al., 2018). Considering that OCD has previously been associated with a hyperactive D_1-expressing cortico-striatal-thalamo-cortical (CSTC) pathway in some patients (Rauch *et al.,* 2007) and that bolstered serotonergic signalling in the CSTC-circuitry is believed to underlie the efficacy of SSRIs (Goddard *et al.*, 2008), the anti-compulsive role of serotonin could possibly be ascribed to it acting as a behavioural opponent to the actions of dopamine (Daw *et al.,* 2002). Thus, based on the aforementioned potential differences in dopaminergic involvement in different phenotypes of OCD, the question arises whether individuals expressing differences in SSRI-related treatment response, will respond uniquely following dopaminergic manipulation.

To investigate this point, the deer mouse *(Peromyscus maniculatus bairdii)* model of OCD has been employed. The model is especially suited for the current investigation as we have previously identified three naturally occurring compulsive-like behaviours in both male and female deer mice that are variably expressed across the population, *viz.* high stereotypical behaviour (HSB; Wolmarans *et al.*, 2013), large nest-building behaviour (LNB; Wolmarans *et al.*, 2016a), and high marble burying behaviour (HMB; Wolmarans *et al.*, 2016b). Importantly, these phenotypes resemble the persistent, ritualistic and seemingly purposeless symptomology of clinical OCD. Further, while both HSB (Wolmarans *et al.*, 2016b) and LNB (Wolmarans *et al.*, 2016a) respond favourably to chronic high-dose oral escitalopram (50 mg/kg/day), HMB seems more resistant to this agent (Wolmarans *et al.*, 2016b). Therefore, we hypothesize that these phenotypes are founded on distinct neurobiological correlates. Specifically, we propose that since HMB is non-responsive to serotonergic intervention, it may rather involve unique perturbations in the dopaminergic system and respond to SSRIs only if co-administered with drugs that manipulate dopaminergic signalling. On the other hand, as LNB demonstrates response to SSRI intervention, it may demonstrate a different response to dopaminergic potentiation compared to HMB, thereby resembling a distinct neurobiology.

MATERIALS AND METHODS

Animals

As only 11 - 15% of deer mice demonstrate HMB (Wolmarans *et al.*, 2016b), 160 deer mice of both sexes were initially obtained from the deer mouse colony of the North-West University (NWU), Potchefstroom, South Africa (ethical approval number: NWU-00262-16-A5; AnimCare Research Ethics Committee, NHREC Registration Number: AREC-130913-015). The original breeding pairs were established using breeding pairs obtained from the *Peromyscus* Genetic Stock Centre at the University of South Carolina, USA. All animals were aged 10 weeks at the onset of experimentation and were randomly selected without litter or weight bias. One week prior to the first behavioural assessment, animals were separately housed in individually ventilated and climate-controlled mouse cages (35cm (l) x 20cm (w) x 13cm (h); Techniplast® S.P.A., Varese, Italy). All experiments were conducted in the good laboratory practice (GLP) area of the vivarium of the NWU. Throughout the investigation, animals were maintained at 23°C on a 12-hour light/dark cycle (06h00/18h00). Food and water were provided ad lib for the duration of the study. Cages were cleaned, and new bedding material (ground corncob) added weekly, except when stated otherwise. Animals that were not identified as either HMB or LNB were euthanized immediately following the last NB assessment (see below).

Drugs

Both drugs, namely the SSRI, escitalopram oxalate (50 mg/kg/day; Wolmarans *et al.*, 2013) and the monoamine oxidase type B (MAO-B) inhibitor, rasagiline mesylate (5 mg/kg/day; Eigeldinger-Berthou *et al.*, 2012) were dosed orally by dissolving them in the drinking water of each individual animal (Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2016b; Wolmarans *et al.*, 2016a). Fresh drug solutions were prepared daily using Milli-Q® water for dissolution. Animals of both behavioural cohorts, i.e. HMB and LNB were divided into four treatment groups that received chronic (28-day) treatment with either 1) normal drinking water (control), 2) escitalopram alone, (3) rasagiline alone, or 4) a combination of escitalopram and rasagiline. To prevent any possible anxiogenic effect that may arise from excessive handling, neither parenteral administration, nor oral gavage was considered, while the handling of animals was kept to a minimum. As deer mice consume liquid at a rate of 0.25 ml/g/day (Aschhoff *et al.*, 2000; Wolmarans *et al.*, 2016b) and that the addition of escitalopram and rasagiline does not modify this, the dose received by each animal over 24 hours could be calculated and confirmed within close margins.

The marble burying test

The marble burying test (MBT) has been applied as reported previously (Broekkamp *et al.*, 1986), albeit with some modification based on previous findings from our laboratory (de Brouwer *et al.*, 2018; de Brouwer & Wolmarans, 2018). Considering that rodents are nocturnal animals, all marble burying (MB) experiments were conducted under dim red light (40 lux) during the dark phase of the 24-hour cycle. Briefly, the experimental cage setup involved 9 glass marbles (ø=15 mm) being evenly placed in one half of the cage only (two-zone paradigm) on a 5 cm-thick layer of course river sand (de Brouwer *et al.*, 2018). As opposed to employing sawdust (Krass *et al.*, 2010; Dixit *et al.*, 2014), wood shavings (Poling *et al.*, 1981) and husk (Kedia & Chattarji, 2014), course river sand was used as a burying substrate as it is largely resistant to the co-incidental covering of marbles when animals, especially highly motor-active deer mice, engage in their normal behavioural repertoire (de Brouwer *et al.*, 2018). Further, we adopted a two-zone paradigm as this allows the animal to completely avoid exposure to the marbles in the event of experiencing neophobic anxiety. In studies of relevance for OCD, directed and intentional engagement with the marbles is important as compulsions are preoccupied with and aimed at specific outcomes (Londei *et al.*, 1998; Thomas *et al.*, 2009).

After introducing each animal to its own experimental cage, they were allowed to explore the area for 30 minutes, after which they were returned to their home cages. The same procedure was followed in all animals for three consecutive nights before and after treatment, respectively, with each animal introduced to the same, but newly prepared experimental cage for each trial. Thus, three pre- and post-treatment MB scores were generated for each animal. Importantly, all animals were habituated to the burying substrate for 24 hours preceding the first MB trial both before, and after treatment. To quantify

the number of marbles buried and identify the HMB expressing animals, a marble was regarded 'buried' if two-thirds of its size has been covered in burying substrate. However, although being adequate to provide a robust baseline separation in burying cohorts, this criterion falls short to identify the effects of treatment on the burying response, for which the additional criterion of 'marble-directed behaviour' (MDB) has been introduced *(see 'Results' as well as Addendum B)*. To score MDB, which included 1) rolling, 2) sniffing and licking, 3) touching with forepaws and 4) standing on or over marbles, all experiments were video recorded and analysed retrospectively by an investigator blind to treatment condition. As burying behaviour is a naturally occurring behaviour that is expressed by all rodents, albeit demonstrating within- and between-species variance, HMB was regarded as those burying scores that clustered within the upper 25th percentile of the individual average 3-night burying scores, while also being associated with the lowest coefficients of variance with respect to the daily expression of burying behaviour (**Figure 1**). Locomotor behaviour was assessed simultaneously with MB activity, and has been scored using Ethovision XT® 14 software (Noldus Information Technologies, Wageningen, The Netherlands).

Nest building analysis

Following the initial pre-treatment analysis of MB activity, all animals that did not present with HMB were screened for nest building (NB) over the course of seven consecutive nights. On each of the respective days, an excess of pre-weighed cosmetic cotton wool was provided above the steel grid roof of the home cages (Wolmarans *et al.*, 2016a). On subsequent days, the remaining cotton wool, i.e. that which had not been used for NB, was weighed and the built nests removed from the cages. Animals were therefore allowed 24 hours to interact with the nesting material. A seven-day analysis period was necessary, as deer mice demonstrate within-individual variance in NB behaviour (Wolmarans *et al.*, 2016a). As with MB, the daily nesting scores were recorded and totalled after seven days to provide a total one-week nesting score (Wolmarans *et al.*, 2016a). These experiments were conducted in the home cages of individually housed animals with food and water being supplied ad lib. During this time, animals did not have access to other forms of laboratory nesting material. Like MB, NB is also a naturally occurring behaviour and as such, LNB was identified based on the same criteria as those used for MB, albeit applying total nesting score, instead of the number of marbles buried. Therefore, if an animal expressed LNB behaviour during a single or two of the seven nights only, it was excluded from the current investigation (**Figure 2**; Wolmarans *et al.*, 2016a).

Statistical analysis

Statistical analysis was performed with GraphPad Prism® 6 under guidance of the Statistical Consultation Service of NWU, Potchefstoom. Linear regression and column statistics (25th and 75th

quartiles) were applied to identify HMB and LNB expressing subjects. Pre-and post-treatment expression of HMB and LNB, as well as locomotor behaviour (LMB; in the case of the HMB groups) were analysed and compared by applying of two-way repeated measures analysis of variance (2-Way RM-ANOVA) followed by Bonferroni post-hoc tests (Wolmarans *et al.*, 2016b; Wolmarans *et al.*, 2016a). Behavioural expression (MB and NB scores respectively) was set as between subject factor and time and treatment as within subject factors. Statistical significance was determined at $p < 0.05$ for all analyses. Cohen's *d* was used to determine the effect sizes of interventions, where a large effect is indicated by a $d > 0.8$, and a very large effect by $d > 1.3$.

RESULTS

Figure 3: Marble burying – number of marbles buried

A total of 24 HMB expressing animals were identified from the initial pool of 160 (**Figure 1**). Although no statistically significant two-way interaction was observed between time and treatment (F [3,20] = 1.46, $p = 0.25$), time had a significant main effect on the burying scores observed in the post-treatment phase (**Figure 3**; F [1,20] = 45,73; p = < 0,0001). As such, post-hoc pairwise comparisons revealed significant reductions in the number of marbles buried within the control (1.17 vs 3.72; $p = 0.004$; CI: 0.7—4.4; Cohen's $d = 2.48$) and the rasagiline (0.44 vs 3.67; $p = 0.0004$; CI: 1.4—5.0; Cohen's d = 3.88) treated groups compared to the respective pre-treatment values. Neither escitalopram, nor the combination had any significant effect on the number of marbles buried, although the main effect of time also tended to decrease behavioural expression in these groups (Cohen's $d = 1.15$ and 1.6 respectively).

Figure 4: Marble burying – marble-directed behaviour (MDB)

A statistically significant two-way interaction between time and treatment was shown (**Figure 4**; F [3,20) = 5.29, $p = 0.008$). Further, both time and treatment were shown to be significant sources of the observed interaction (time: F [1,20] = 41.8, $p < 0.0001$; treatment: F [3,20] = 7.84, $p = 0.001$). Post-hoc analysis revealed a significant difference in the pre-treatment MDB of animals within the escitalopram and rasagiline groups (20.7 vs 30.4; $p = 0.008$; CI: 1.9—17.4; Cohen's $d = 1.79$) which can only be explained as naturally occurring behavioural variation, since no behaviour-modifying interventions had taken place at this time. Considering the effect of treatment over time, both escitalopram (19.0 vs 30.4; $p = 0.001$; Cohen's $d = 1.98$) and the combination of escitalopram and rasagiline (8.4 vs 23.7; $p < 0.0001$; Cohen's $d = 3.06$) elicited significant and large reductions in the number of MDB episodes compared to the respective pre-treatment scores. As opposed to the number of marbles buried (**Figure 3**), neither the control, nor rasagiline alone had any statistical or practical effect. This data is supported by comparisons of the number of MDB episodes within the post-

treatment groups. Indeed, significant differences with large effect sizes have been demonstrated between the number of MDB expressed by the combination group compared to all other treatment groups (control: $p < 0.0001$, CI: 6.3—21.7, Cohen's $d = 3.08$; escitalopram: $p = 0.003$, CI: 2.9—18.4, Cohen's $d = 2.16$; rasagiline: $p = 0.013$; CI: 1.4—16.9, Cohen's $d = 2.19$).

Figure 5: Marble burying – locomotor activity

A two-way RM ANOVA was run to determine whether time or treatment had any significant effect on motor ability. In a random sample of three animals in each treatment group, no significant interaction between time and treatment was found (**Figure 5**; F [3,8] = 0.16, $p = 0.922$). However, although time did not have any significant main effect on the results obtained (F [1,8] = 0.28, $p = 0.612$), the effect of treatment was significant (F [3,8] = 5.63, $p = 0.023$). However, this effect is negligible as no significant differences between either of the groups, both within and between treatment groups was demonstrated.

Figure 6: Nest building

A total of 24 LNB expressing subjects were identified from the remaining 136 animals, i.e. those that remained following the identification of 24 HMB expressing mice (**Figure 2**). A statistically significant two-way interaction between time and treatment was evident with respect to the pre- and post-treatment nesting scores of LNB animals (**Figure 6**: F [3,20] = 9.56, $p = 0.0004$). Further, time had a significant effect on the way in which NB responded to treatment (F [1,20] = 9.37, $p = 0.0062$). Subsequent pairwise analyses revealed that although no differences in the pre-treatment nesting behaviour of the different treatment groups were observed, the average expression of LNB increased significantly over time in the control group (34.1g vs 19.8g; $p = 0.0014$; CI: 5.2g—23.3g; Cohen's $d = 1.8$), which may indicate that LNB is a type of self-reinforcing behaviour. However, although not demonstrating statistical significance, LNB behaviour expressed by rasagiline and combination-treated animals also trended towards an increment in nest building score following treatment (Cohen's $d = 1.63$ and 1.05, respectively). On the other hand, administration of escitalopram alone significantly reduced the expression of LNB (14.9g vs 24.5g; $^p = 0.0356$; CI: 0.5g—18.7g, Cohen's $d = 1.2$). The marked reduction in NB scores elicited by escitalopram was also evinced by the significant differences with large effect sizes in post-treatment NB scores between this and the other treatment groups (control: $p = 0.0002$, CI: 7.7g—30.5g, Cohen's $d = 2.04$; rasagiline: $p = 0.017$; CI: 1.6g—24.4g, Cohen's $d = 1.87$; combination: $p = 0.016$; CI: 1.8g—24.6g, Cohen's $d = 1.52$).

DISCUSSION

The major findings of the present work, which investigated the response of two compulsive-like phenotypes in the deer mouse model of OCD, i.e. HMB (Wolmarans *et al.*, 2016b) and LNB (Wolmarans *et al.*, 2016a) to serotonergic and dopaminergic intervention are 1) MDB is ameliorated by escitalopram and a combination of escitalopram and rasagiline while only demonstrating modest response to rasagiline alone and 2) LNB is sensitive to escitalopram alone only, while being worsened by rasagiline alone or in combination with escitalopram. These findings indicate that LNB and HMB is associated with unique underlying neurobiological constructs, which may be founded on differences in dopaminergic involvement.

Despite the fact that OCD affects 2-3% of the global population (Sasson *et al.*, 1997; Angst *et al.*, 2004; De Bruijn *et al.*, 2010; Ruscio *et al.*, 2010), only 40 – 60% of OCD patients respond favourably to first-line pharmacotherapeutic intervention, i.e. chronic, high SSRIs (Issari *et al.*, 2016; Locher *et al.*, 2017). Although psychotherapeutic approaches, e.g. cognitive behavioural therapy (CBT), can also be considered, treatment response to these is also suboptimal (Abramowitz *et al.*, 2002; Ost *et al.*, 2015; Ost *et al.*, 2016). Current guidelines for treatment refractory OCD suggest several strategies, e.g. increasing the dose of current SSRI used (Liebowitz *et al.*, 2002; Bloch *et al.*, 2010), switching to another SSRI (Marazziti *et al.*, 2008), combining pharmacotherapy and psychotherapeutic interventions (Abudy *et al.*, 2011), or bolstering the effect of first-line drug therapy by augmenting SSRI treatment with a low-dose antipsychotic (Dold *et al.*, 2015; Murray *et al.*, 2017). In this regard, data are promising (Bloch *et al.*, 2006; McLean *et al.*, 2015; Murray *et al.*, 2017). Nevertheless, a better understanding of the neurobiology of OCD would be valuable to further current attempts to improve treatment outcomes.

Considering that OCD has previously been described as a condition closely related to perturbations in cortico-striatal reward-feedback processes (Welter *et al.*, 2011), findings from different investigations collectively point to the unique involvement of striatal dopaminergic signalling in different phenotypes of OCD (Rauch *et al.*, 2007; Figee *et al.*, 2011). Such differences in dopaminergic signalling may potentially be related to the way in which humans process and learn from rewarding and punishing outcomes as explained by the theories of phasic dopaminergic signalling (Schultz *et al.*, 1993; Schultz *et al.*, 1997; Schultz, 2002; Schultz, 2007) and serotonin-dopamine opponency (Daw *et al.*, 2002). While the former explains reward and punishment learning on the basis of phasic increases and decreases in dopaminergic signalling respectively (Schultz *et al.*, 1993; Schultz *et al.*, 1997; Schultz, 2002; Schultz, 2007), the latter suggests that while dopamine is responsible for the coding of reward, serotonin could act as an opponent system by facilitating punishment learning (Daw *et al.*, 2002). That said, while these two concepts are essentially congruent with respect to suggesting a dichotomous role for dopamine in reward and punishment learning, it has also been found that neither can optimally

transpire in the absence of sufficient serotonergic input (Palminteri *et al.*, 2012). Considering current treatment approaches, it may therefore be possible that unique involvement of serotonin and dopamine in different phenotypes of OCD, may be associated with varying treatment response.

As alluded to earlier, deer mice of both sexes naturally develop different phenotypes of compulsive-like behaviour that manifest in some individuals only. These behaviours, i.e. spontaneous motor stereotypy, HMB, and LNB, are generally expressed by different animals; however, all three phenotypes resemble compulsions in that they are persistent, recurrent and seemingly purposeless (for a more detailed overview, please see Wolmarans *et al.*, 2013; Wolmarans *et al.*, 2016b; Wolmarans *et al.*, 2016a). The deer mouse model can thus be regarded as a potentially useful framework in which to study the etiology and neurobiological foundations of phenotypically heterogeneous OCD. Of more importance for the current study is that while spontaneous stereotypy (Wolmarans *et al.*, 2013) and LNB (Wolmarans *et al.*, 2016a) respond to chronic high dose escitalopram intervention (50 mg/kg/day for 28 days), HMB—at the time of its initial characterization quantified only based on the number of marbles buried—remains overly insensitive to such intervention (Wolmarans *et al.*, 2016b). Hence, it seems that these behaviours diverge from one another on a neurobiological level.

Indeed, our findings indicate that MDB demonstrated a significant reduction following chronic administration of the combination treatment only, as neither escitalopram nor rasagiline alone groups demonstrated any significant behavioural changes after treatment, while the behaviour of the control group did remain consistent after the treatment period (**Figure 4**). It must be noted however that the impact of drug treatment may have been blunted by the significantly increased MDB measured in the escitalopram group during the pre-treatment testing. Further, these results were obtained in the absence of any significant changes in the general locomotor activity of the animals (**Figure 5**). Importantly, this finding was not supported by the data generated by counting the number of marbles buried (**Figure 3**). This is noteworthy as the majority of investigations that apply the MBT as a measure of compulsive-like behaviour, only quantify the number of marbles 'buried' as an indicator of behavioural severity. Such an approach is inaccurate for a number of reasons which have been reviewed elsewhere (de Brouwer *et al.*, 2018). Suffice to say that appraisals of MB activity from an OCD perspective should be based on observations of persistent, recurrent and goal-directed preoccupation with the objects. In fact, marbles coincidentally become covered due to the normal cage exploration activity of rodents, *viz.* digging and burrowing (Thomas *et al.*, 2009). Only focusing on this parameter may yield a false picture of the actual underlying burying phenotype. For example, here we show that over time, all interventions, including the control, were associated with large, although not always significant, reductions in the number of marbles buried (**Figure 3**). Rather than being a true reflection of a reduction in burying activity, this result is likely an artefact of diminished novelty-induced exploratory activity which would have played a significant role in the burying scores generated before the onset of

treatment (de Brouwer & Wolmarans, 2018). The findings related to escitalopram intervention reported here, differ from what we have shown before (Wolmarans *et al.*, 2016b), i.e. that HMB is refractory to monotherapeutic SSRI intervention. However, this discrepancy can be explained by methodological differences pertaining to zone setup (one-zone vs two-zone), burying substrate (ground corncob vs course river sand), and scoring criteria (number of marbles buried vs MDB). In fact, we show here that an appraisal of actual marble-directed activity highlights key characteristics of compulsive-like behaviours that would otherwise have been overseen. Taken together, it seems that aberrant marble burying activity is associated with deficits in dopaminergic signalling which may possibly play a role in the promulgation of this compulsive-like phenotype, and which is subject to modification with dopaminergic potentiators. Therefore, it can be posited that HMB resembles an OC phenotype in which unique dopaminergic mechanisms underlying compulsive-like behaviour, may be investigated.

On the other hand, we demonstrate that LNB exacerbates over time as evinced by the significant increase in post-treatment nest-building activity of control-treated LNB animals (**Figure 6**). Although not statistically significant, LNB animals treated with rasagiline alone or in combination with escitalopram, also trended towards increased building activity, indicating that although not worsening LNB more than what was simply a consequence of time, it also did not prevent or reverse this phenomenon. In fact, in line with our previous observation (Wolmarans *et al.*, 2016a), only escitalopram when administered alone resulted in a significant reduction in the expression of LNB, thereby overcoming the effect of time on building activity. It can therefore be considered that as opposed to HMB, LNB may be representative of an OC phenotype that is associated with a neurobiological construct in which a reduced dopaminergic tone is not disadvantageous, but rather instrumental to the effect of serotonergic intervention. In this regard, LNB may be more representative of the 'classic' neurobiological picture of OCD that describes the condition as a manifestation of hyposerotonergic signalling (Goddard *et al.*, 2008).

CONCLUSION

Here we provide pharmacological evidence that two compulsive-like phenotypes as expressed by deer mice of both sexes, i.e. HMB and LNB, are founded in different neurobiological constructs. More specifically, the two behaviours can be separated based on the unique involvement of serotonin and dopamine in its expression, where simultaneous serotonergic and dopaminergic potentiation attenuates HMB, while having no significant effect in LNB. In fact, LNB is responsive to escitalopram treatment alone. Therefore, we propose that HMB and LNB are representative of two OC-phenotypes which together, may be of value to investigated different neurobiological underpinnings of compulsive-like behaviour.

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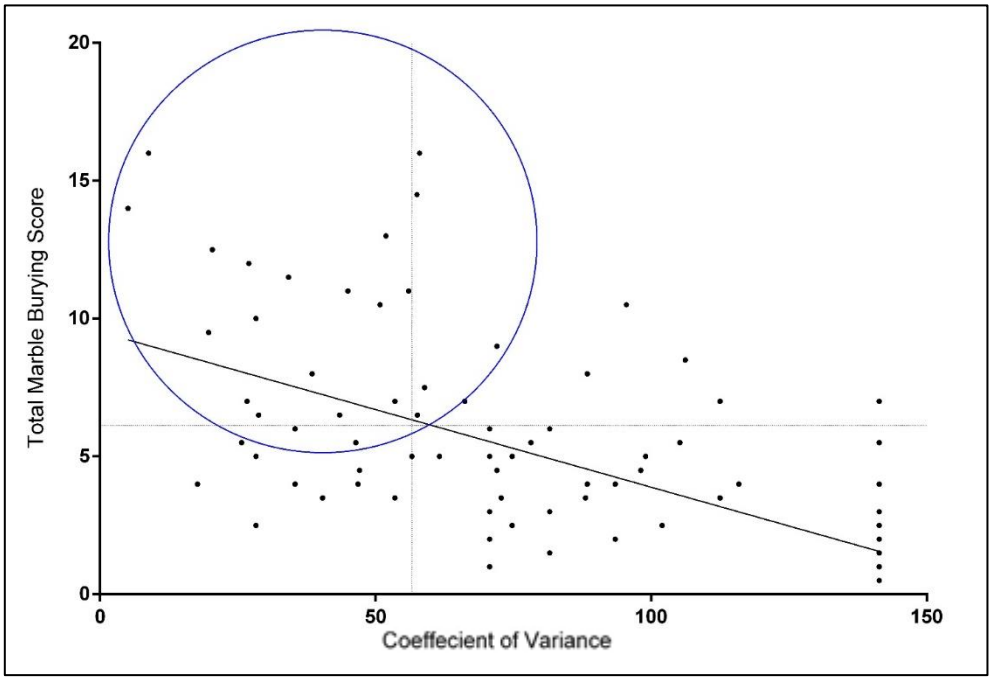

Figure 1

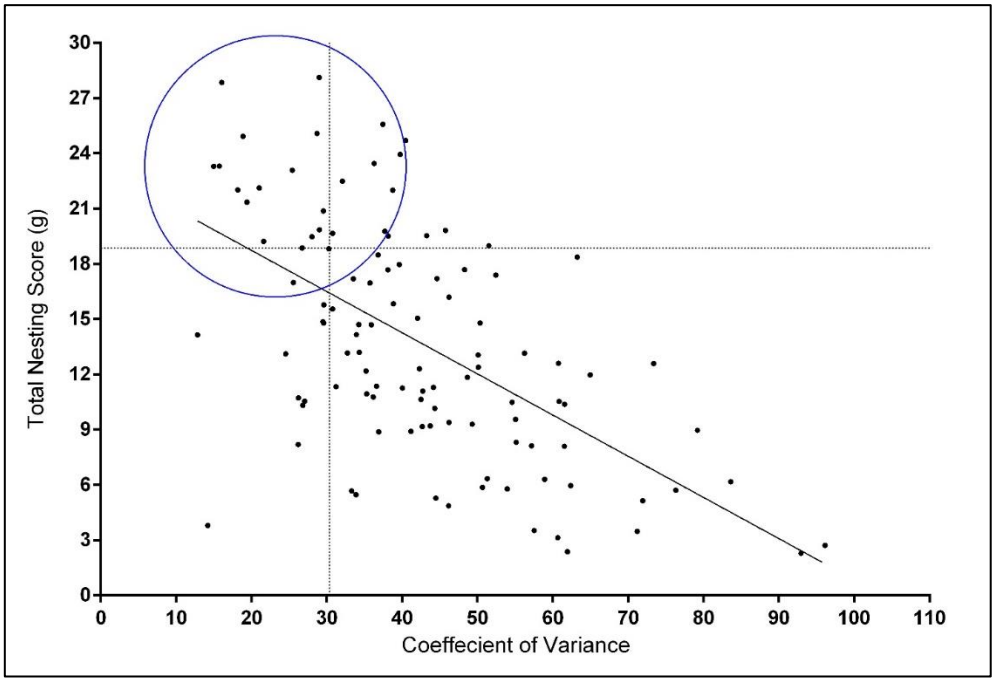

Figure 2

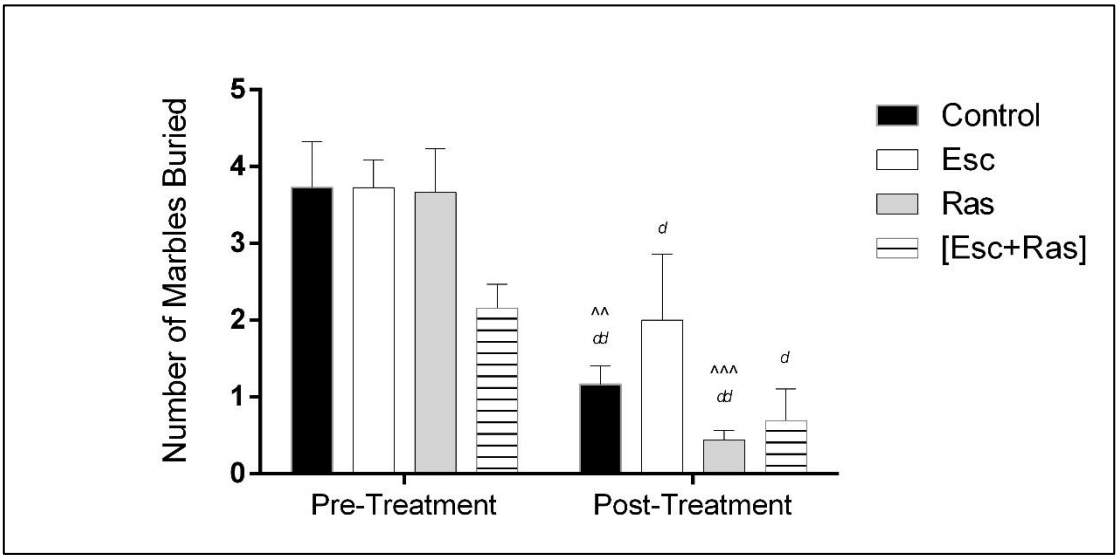

Figure 3

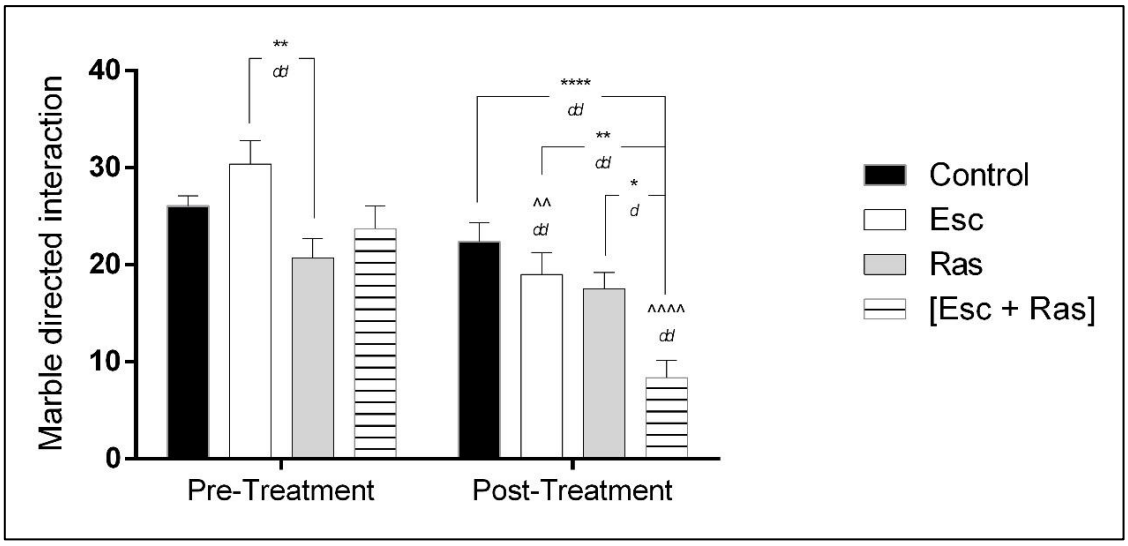

Figure 4

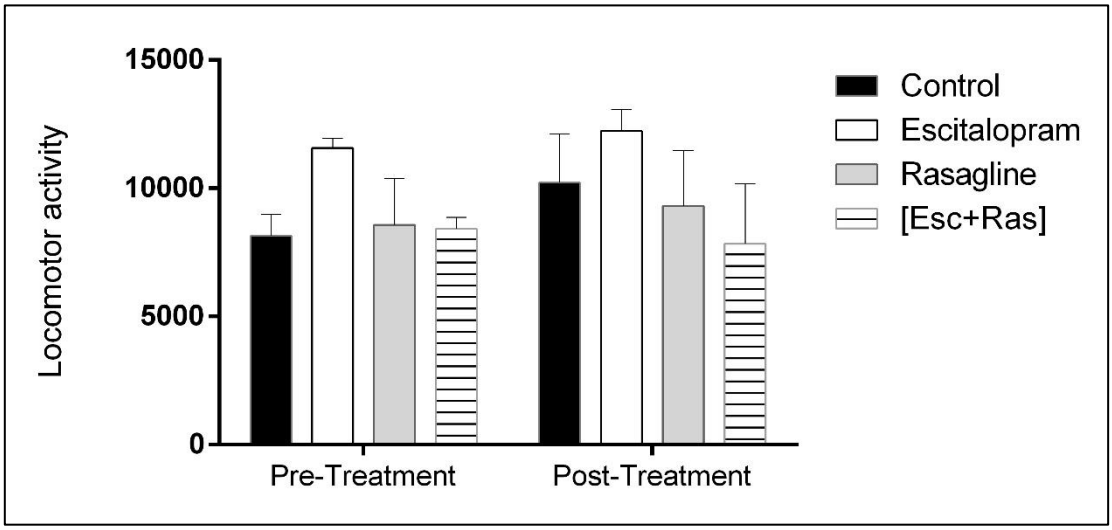

Figure 5

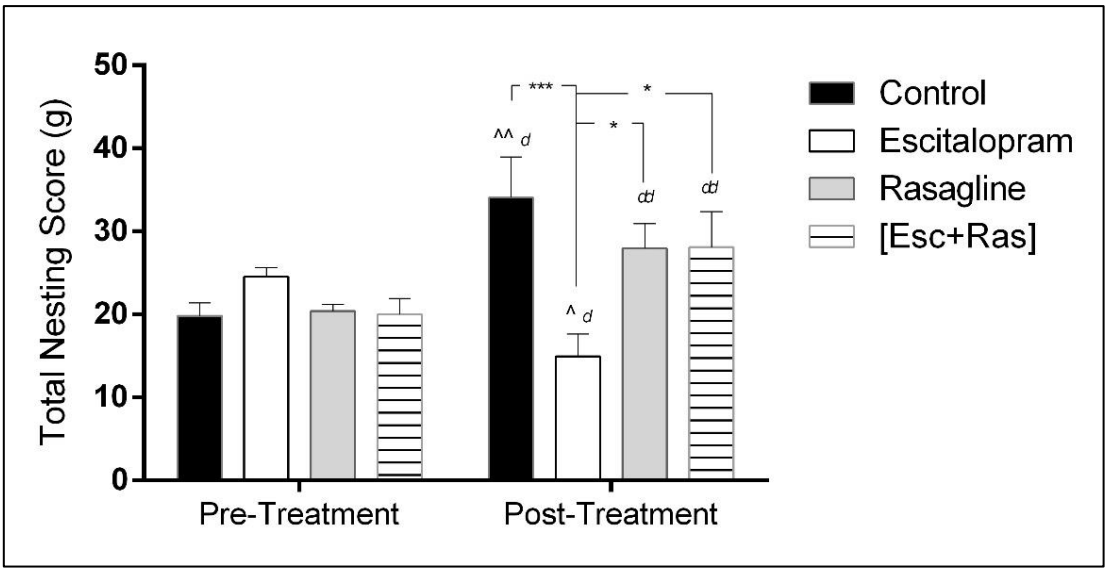

Figure 6

FIGURE CAPTIONS

Figure 1

Plot of total marble burying scores after three nights and the coefficients of variance with respect to the daily burying activity. Data reflects all animals that were initially screened. Blue circle: animals selected for treatment.

Figure 2

Plot of total nest building scores after seven 24h periods and the coefficients of variance with respect to the daily nesting activity. Data reflect all animals that were initially screened. Blue circle: animals that were selected for treatment.

Figure 3

Average pre- vs. post-treatment number of marbles buried over three trials by high marble burying (HMB) animals. ^Pre- vs. post-treatment; within treatment groups. ^^$p < 0.01$; ^^^$p < 0.001$ (Two-way RM-ANOVA, Bonferroni post-hoc). Cohen's effect size: $0.8 > d < 1.3 > dd$. Data is mean ± SEM.

Figure 4

Average pre- vs. post-treatment number of marble-directed interactions over three trials by high marble burying (HMB) animals. ^Pre- vs. post-treatment; within treatment groups. *Pre- and post-treatment comparison between treatment groups. *$p < 0.05$; ^^/**$p < 0.01$; ^^^^/****$p < 0.0001$ (Two-way RM-ANOVA, Bonferroni post-hoc). Cohen's effect size: $0.8 > d < 1.3 > dd$. Data is mean ± SEM.

Figure 5

Average pre- vs. post-treatment expression of motor activity in three animals of each treatment group over three trials. Two-way RM ANOVA (no significant interaction or main effects reported).

Figure 6

Average pre- vs. post-treatment total nesting scores after seven trials in large nest building (LNB) animals. ^Pre- vs. post-treatment; within treatment groups. *Pre- and post-treatment comparison between treatment groups. ^/*$p < 0.05$; ^^$p < 0.01$; ***$p < 0.001$ (Two-way RM-ANOVA, Bonferroni post-hoc). Cohen's effect size: $0.8 > d < 1.3 > dd$. Data is mean ± SEM.

4 Conclusion

The major findings of the present work, which investigated the response of two obsessive-compulsive (OC)[1]-like phenotypes in the deer mouse model of obsessive compulsive disorder (OCD)[2], i.e. HMB[3] (Wolmarans *et al.*, 2016b) and LNB[4] (Wolmarans *et al.*, 2016a) to serotonergic, dopaminergic and combination intervention are 1) that HMB and LNB respond uniquely to dopaminergic potentiation, where 2) marble directed behaviour (MDB)[5] as observed in the marble burying test (MBT)[6] is ameliorated by escitalopram and a combination of escitalopram and rasagiline while only demonstrating modest response to rasagiline alone, and 3) LNB is sensitive to escitalopram alone only, while being exacerbated over time, irrespective of the administration of rasagiline alone or in combination with escitalopram. We also demonstrate the importance of appraising marble burying behaviour with *marble-directed behaviour* (MDB) in mind, instead of focusing on the number of marbles buried, only.

While OCD affects 2-3% of the global population (De Bruijn *et al.*, 2010; Ruscio *et al.*, 2010), only 40 – 60% of OCD patients respond favourably to first-line pharmacotherapeutic intervention, i.e. chronic, high selective serotonin reuptake inhibitors (SSRIs)[7] (Albert *et al.*, 2017). Guidelines for treatment refractory OCD suggest several strategies, foremost of which is to augment SSRI treatment with a low-dose antipsychotic (Albert *et al.*, 2017). In this case, it has been shown that up to 60% of SSRI-refractory patients demonstrate at least some response (Albert *et al.*, 2017), leaving roughly 15 – 20% of patients suffering from OCD with no alternative.

In this regard, findings from psychological investigations pointed to unique associations between dopaminergic involvement in different OC subtypes (Rauch *et al.*, 2007; Figee *et al.*, 2011). For instance, whereas patients with contamination/washing (C/W)[8] OCD are often more impulsive and present with reduced neural reward anticipatory activity, such deficits are not seen in patients with safety/checking (S/C)[9] OCD (Figee *et al.*, 2011). The clinical dilemma is founded in the fact that

[1] obsessive-compulsive
[2] obsessive-compulsive disorder
[3] high marble burying
[4] large nest building
[5] marble-directed behaviour
[6] marble burying test
[7] selective serotonin reuptake inhibitor
[8] contamination/washing
[9] safety/checking

reductions in dopamine (DA)[1] code learning responses to aversive stimuli, while dopaminergic increases are responsible for coding rewarding stimuli (Schultz *et al.*, 1993; Schultz *et al.*, 1997; Schultz, 2002; Schultz, 2007; 2016). Moreover, adequate serotonergic neurotransmission is also needed for both of these processes to be facilitated (Fischer & Ullsperger, 2017). It can therefore be hypothesized that patients responding to SSRI monotherapy, present with an underlying neurobiological construct already akin to low dopaminergic tone, but in which hyposerotonergic signalling is driving the symptomology. On the other hand, SSRI refractory OCD, may be founded on the basis of a reduced dopaminergic tone and it may be likely that augmentation of SSRI[2] therapy with dopaminergic potentiators, may prove to be beneficial.

In this investigation we attempted to apply HMB[3] and LNB[4] as two phenotypes of OC[5]-like behaviour that resemble a refractory and a SSRI sensitive behaviour, respectively. We have previously shown that while LNB is completely reversible by SSRI treatment, HMB remains largely unresponsive (Wolmarans *et al.*, 2016b; Wolmarans *et al.*, 2016a). Briefly, we hypothesized that as LNB is already attenuated by bolstering serotonergic signalling alone (Wolmarans *et al.*, 2016a), such behaviour should demonstrate no response to the co-administration of a dopaminergic potentiator. On the other hand, given that HMB is overly refractory to serotonergic interference (Wolmarans *et al.*, 2016b), we hypothesized that this may be due to inadequate simultaneous increases in serotonin (5HT)[6] *and* DA release. Considering the data presented here, this hypothesis is largely supported.

With respect to the compulsive-like directed action aimed at the marbles itself and how such behaviour responded to the different interventions, it seems that aberrant marble burying activity is associated with deficits in dopaminergic signalling which may possibly play a role in the promulgation of this compulsive-like phenotype, and which is subject to modification with dopaminergic potentiators. This is reflected by the marked and large reduction in MDB[7] following treatment with a combination of escitalopram and rasagiline. Although escitalopram elicited some response, it could only maximally do

[1] dopamine
[2] selective serotonin reuptake inhibitors
[3] high marble burying
[4] large nest building
[5] obsessive-compulsive
[6] serotonin
[7] marble directed behaviour

so when administered in combination with rasagiline. Therefore, it can be posited that HMB[1] resembles an OC[2] phenotype that may be related to inadequate dopaminergic signalling (Figee *et al.*, 2011).

On the other hand, our findings relating to LNB[3] also supports the working hypothesis of the investigation that was performed. As opposed to HMB, LNB may indeed be representative of the 'classic' neurobiological picture of OCD[4] that describes the condition as a manifestation of hyposerotonergic signalling (Goddard *et al.*, 2008).

To conclude, we provide pharmacological evidence that two compulsive-like phenotypes as expressed by deer mice of both sexes, i.e. HMB and LNB, are founded in different neurobiological constructs. More specifically, the two behaviours can be separated based on the unique involvement of 5HT[5] and DA[6] in its expression, where simultaneous serotonergic and dopaminergic potentiation attenuates HMB, while having no significant effect in LNB. In fact, LNB is responsive to escitalopram treatment alone (Table 4-1). Therefore, considering theories explaining how reward feedback is processed, we propose HMB and LNB may be representative of two OC-phenotypes which may be associated with unique dopaminergic involvement. Further, it could be of value to assess the response of OCD patients presenting with perturbations in reward-feedback processing, to simultaneous serotonergic and dopaminergic intervention.

[1] high marble burying
[2] obsessive-compulsive
[3] large nest building
[4] obsessive compulsive disorder
[5] serotonin
[6] dopamine

4.1 References

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Addendum A

Letters of permission to submit Chapter 3 for examination purposes

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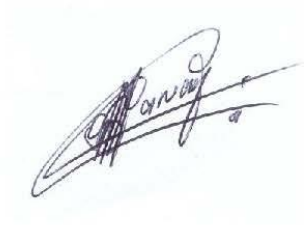
15 November 2018

Dear examiner

MSc Dissertation – A FICK
PERMISSION TO INCLUDE MANUSCRIPT FOR EXAMINATION PURPOSES

As co-study leader and contributing author on the manuscript first authored by Miss Arina Fick, entitled "Two compulsive-like behavioural phenotypes in the deer mouse (*Peromyscus maniculatus bairdii*) and their response to serotonergic, dopaminergic and combination intervention", I hereby approve that this concept manuscript be included as part of the requirements for fulfilment of the MSc. degree, and that this manuscript may be submitted for examination purposes by the candidate.

Sincerely,



Brian H Harvey, PhD

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Entrance at the maternity car park (2nd exit from the circle, turn right at the next stop), 2nd Floor, Human Resources and Development Centre (aka Drs' Bungalows)
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13 November 2018

Re: **Permission to submit dissertation for examination _ Arina Fick**

This serves to confirm my approval of the above submission

Yours Sincerely,

Dan J Stein

Dan J. Stein, FRCPC, PhD
Professor and Chair
Dept. of Psychiatry & Mental Health
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Addendum B

Addendum B includes notes and comments regarding the burying behaviour of each animal (pre-treatment and post-treatment) that presented with high marble burying (HMB)[1] during this investigation. As previously mentioned in paragraph 2.4.3, rodents display burrowing, burying and digging behaviour in natural and laboratory settings that is essential for optimal social structures and survival (Ebensperger & Blumstein, 2006; Deacon, 2012). For preclinical studies, burying behaviour is regarded as a measuring tool for anxiety (Broekkamp *et al.*, 1986) and/or compulsive-like behaviour (De Boer & Koolhaas, 2003; de Brouwer & Wolmarans, 2018). Due to the natural tendency of animals to bury both noxious and non-noxious objects, we included an extra parameter to exclude the effects of natural burying behaviour on the results obtained. Indeed, from a compulsive perspective, burying behaviour must persist, must be directed at the objects of interest and must be seemingly purposeless. As such, the marble burying test (MBT)[2] was video recorded over three consecutive nights and the number of marble directed interactions counted. The following pages contain the bulk of these observations. Marble directed interactions included the following behaviours: 1) rolling the marble, 2) standing over the marble, 3) touching and sniffing the marble, and 4) rolling the marble and finally covering the marble with sand.

[1] high marble burying
[2] marble burying test

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Escitalopram and rasagiline drug combination group

Pre-treatment

MOUSE NUMBER	12	13	19	20	21	44
TRIAL 1	DB observed in corner nearest to marbles. Investigative and sniffing behaviour towards marbles. Displays DB near marble. Displays DB in non-marble zone. Mouse keeps on returning to marble side. Mouse digs a hole and marble is displaced. Displayed less stereotypical behaviour.	Stereotypical jumping in corner near the marbles. Mouse did display BB towards marbles. DB in NMZ was observed. Mouse also displayed investigative and digging behaviour.	Rearing behaviour. Stereotypical jumping in MZ corner. DB in MZ corner spraying sand backwards. DB in NMZ area. DB in MZ corner displaces nearest marble and covers another marble with sand. DB in NMZ corner spraying sand backwards. Mouse dug a hole in the corner. Investigative behaviour viz., touching and sniffing marble. Mouse rolls marble out of original setting. Stereotypical jumping in NMZ corner. Mouse spent a lot of time displaying stereotypical jumping.	DB in NMZ area. Investigative behaviour viz., sniffing, rolling and touching marbles. Rearing behaviour. Mouse runs over marbles. DB in MZ corner spraying sand backwards covering the nearest marble with sand. DB between marbles. Stereotypical jumping in NMZ corner.	Rearing behaviour. Investigative behaviour. Mouse rolls marbles. DB in NMZ area and corner. DB between marbles. DB in MZ corner spraying sand backwards covering nearest marbles. Mouse buries marble. Stereotypical jumping in MZ corner. Somersaulting on marbles causes the marble to move from original setting.	Mouse displayed neophobic behaviour. Touching marble and running away. DB in corner of the NMZ. Rearing, sniffing and investigative behaviour. Mouse roles marble and displaced marble.
TRIAL 2	Displayed same behaviour as in trial 1. Digging and investigative behaviour was observed. Mouse sat by marble and covered it.	Mouse displayed digging behaviour near the marbles. DB, investigative behaviour and sniffing behaviour were observed. Mouse buried marble. Mouse engaged a great deal of time in DB in both sides of the cage. DB greatly observed in corners of cage.	DB in MZ corner spraying sand backwards covering nearest marble. Rearing behaviour. Stereotypical jumping in corners of the cage. Investigative behaviour viz., sniffing and touching marble. DB in MZ corner and marble roles into the corner and mouse buries it partially. DB near marbles. Mouse buries another marble partially. Stereotypical jumping on marble	DB in MZ corner spraying sand backwards covering the nearest marbles with sand. Rearing behaviour. Stereotypical jumping in NMZ corners. DB in NMZ area. DB between marbles. DB displaces marble into corner. Mouse displays stereotypical jumping on marble in the corner. DB in NMZ corner. Mouse buries marble partially then completely. Buries	DB in MZ corner displaces the marble from original setting into the corner. Mouse present with stereotypical jumping on the marble in the corner. DB between marbles. DB in NMZ area. Rearing behaviour. Mouse stands over marble and tries to bury the marble. Investigative behaviour viz., touching and sniffing marble.	DB in NMZ area. Mouse roles marbles and then burying the marble. Mouse buries marble that was bumped into the corner of the MZ. Mouse buried the marbles. DB in the corner of the NMZ. When the mouse engage in DB in the corners of the cage the nearest marbles are displaced or covered with sand. Mouse displayed digging

			presses the marble deeper in the sand. Mouse buries the partially cover marble completely.	another marble.		and rearing behaviour. Mouse buried marbles.
TRIAL 3	Displayed sniffing and investigative behaviour. Spends an amount of time digging in the NMZ. Spends time with marble. Displayed digging behaviour	Stereotypical behaviour in NMZ but mouse buried a marble in the MZ. Sniffing behaviour observed and DB next to the marble.	Stereotypical jumping in NMZ and MZ corner. Rearing behaviour. DB in top MZ corner causes the marble to roll into the corner and the nearest marble to the corner is covered with sand. Mouse displays stereotypical jumping on the marble pressing it deeper in the sand. Mouse then buried this marble in the sand completely.	Rearing behaviour. Mouse rolls marble. Stereotypical jumping in NMZ and MZ corner. Mouse stands over marble and tries to bury it causing the marble to move from its original setting. DB in NMZ areas. Mouse buries a marble partially. Mouse stands over marble and tries to bury it. Mouse buried marbles. Mouse rolls marbles. Mouse also partially buries another marble	Stereotypical jumping in MZ corner. Rearing behaviour. DB in NMZ area. DB in MZ corner spraying sand backwards covering the nearest marble. Mouse rolls marble and buries it. Mouse then returns and uncovers buried marble. DB between marbles. Investigative behaviour viz., touching, sniffing and rolling marble.	DB in NMZ area. Rearing behaviour. DB between marbles and in corner of the MZ. Mouse roles marble and tries to bury the marble. Mouse displayed HMB towards the marbles and spent time burying the marbles.
NUMBER OF MARBLES BURIED OVER 3 CONSECUTIVE NIGHTS	Observer 1: 1.3 Observer 2: 1	Observer 1: 1.3 Observer 2: 3	Observer 1: 2 Observer 2: 1.67	Observer 1: 2 Observer 2: 2.33	Observer 1: 1.33 Observer 2: 3	Observer 1: 3.33 Observer 2: 3.67
PREOCCUPIED BEHAVIOUR OVER 3 CONSECUTIVE NIGHTS	29.67	29	17.33	25.67	16.33	24.33

Post-Treatment

MOUSE NUMBER	12	13	19	20	21	44
TRIAL 1	DB observed in NMZ. Less DB between marbles. Somersaulting on marbles. Displayed stereotypical behaviour. Rearing behaviour observed. Photo relates with less BB and more stereotypy.	Photo supports that the mouse displayed BB in MZ and NMZ. Stereotypical behaviour observed. DB near marbles and in corners of cage (NMZ and MZ). Rearing behaviour was also observed. Mouse rolled marble. When the mouse digs in the corners of the MZ the nearest marble is displaced and falls into the hole.	Displayed stereotypical behaviour in NMZ. DB was observed in corners of the cage. Mouse spent a great amount of time digging.	Stereotypical jumping was observed. Mouse presented with rearing behaviour and digging behaviour. Mouse displaced marble when digging gin the corner of the MZ. Digging behaviour in corners and throughout the cage.	DB in the corner of the NMZ. Stereotypical behaviour in corner of the MZ. Mouse displaced marble when digging in the corner of the MZ. Mouse buried marbles. Displayed rearing behaviour. Sometimes the mouse ran over the marbles covering the marbles with sand this phenomenon can also be seen when the mouse digs in the corner of the MZ. Stereotypical jumping in the corner of the MZ also covers the marble in some way. The mouse will also roll the marble and then engage in DB.	Investigative and rearing behaviour. Mouse rolls marble. Digging NMZ. Digging behaviour between marbles and in the corners of the MZ. Mouse bumped marble and marble rolled to the nearest corner of the MZ. The mouse ran over the marble in the corner covering it with sand. Mouse runs over marbles pressing the marbles deeper in the sand. Mouse buries marble that was already covered therefore, covering it completely. One marble was buried.
TRIAL 2	BB observed in corners of the cage. Rearing behaviour observed. Mouse displayed DB in corner of MZ and covered the nearest marble slightly. Repetitive somersaulting on marbles. When mouse jumps marbles are displaced.	A little stereotypical behaviour observed however rearing and DB was observed. DB in both the MZ and NMZ. Most of the time the mouse was engaged in BB however none of the marbles were buried.	Stereotypical behaviour on the marbles. Mouse also displayed rearing behaviour as well as digging behaviour. DB in NMZ.	Rearing behaviour and DB was observed. Mouse constantly moved from MZ to NMZ. Stereotypical jumping in the corner of the MZ.	Rearing behaviour and DB observed in NMZ. Mouse digs in corner of the MZ a marble falls into the hole and the mouse buries the marble. In the 3rd trial the mouse also rolled the marble. Overall the mouse presented greatly with digging behaviour.	Rearing behaviour. DB in the NMZ. Mouse displayed DB in the corner of the MZ, the DB covered the nearest marble with sand. Stereotypical jumping in NMZ corners. Mouse runs over marbles pressing marbles deeper in the sand.
TRIAL 3	Stereotypical behaviour (somersaulting) on marbles. Displayed rearing behaviour as well	Displayed rearing behaviour as well as stereotypy. DB in NMZ observed. Stereotypical	Repetitive somersaulting on marbles. Rearing and stereotypical behaviour in MZ and NMZ.	Rearing behaviour observed as well as DB in corners in the NMZ. Stereotypical jumping	Rearing behaviour. DB in NMZ as well as the corners of the NMZ. Mouse rolled the marble	Digging in the NMZ area. DB in the NMZ corner. Stereotypical jumping in the NMZ corner. Mouse

	as DB in NMZ.	jumping on the marble nearest to the corner of the MZ. No direct BB towards marbles. DB especially in the corners of the MZ and NMZ.	Somersaulting displaced marbles. DB in corners of MZ and NMZ. Mouse displayed a lot of DB. DB between and near marbles	observed. Mouse displayed DB in NMZ and MZ.	and showed DB near the marble. When the mouse engaged in DB near the marble the marble rolled into the hole and mouse buried it. Important to note, mouse did also accidentally covered marbles when DB is near marbles. Burrowing behaviour was also observed. Mouse buried marbles.	displayed rearing behaviour. DB between and next to marbles. Mouse spent most of the time in the NMZ
NUMBER OF MARBLES BURRIED OVER 3 CONSECUTIVE NIGHTS	Observer 1: 0.33 Observer 2: 0.33	Observer 1: 1 Observer 2: 0.67	Observer 1: 0 Observer 2: 0	Observer 1: 0 Observer 2: 0	Observer 1: 3 Observer 2: 2.33	Observer 1: 0.33 Observer 2: 0.33
PREOCCUPIED BEHAVIOUR OVER 3 CONSECUTIVE NIGHTS	7.33	13.33	4.67	5.33	14.33	5.33

Rasagiline alone group

Pre-treatment

MOUSE NUMBER	29	30	31	36	55	56
TRIAL 1	DB in MZ corner. Stereotypical jumping in MZ area. DB between marbles. Rearing behaviour. Mouse rolled marble. DB in corners of cage (NMZ and MZ). DB in MZ corner partially covered the nearest marbles.	DB in the NMZ corner and area. Rearing behaviour. DB between marbles. DB in MZ corner. Rolled the marble but did not bury marbles. Stereotypy in NMZ corner.	DB in NMZ corner and area. DB and BB near marble. DB in MZ corner. Rearing behaviour. Mouse buried marble. Mouse rolled marble.	Rearing behaviour. DB between marbles. Stereotypical behaviour observed in corners of cage. Mouse rolled marbles. Mouse buried marble. DB in NMZ area.	Rearing behaviour. DB in top corner of MZ displacing marble from original setting. Investigative behaviour viz., touching, sniffing and rolling marble. DB in MZ and NMZ corners. Mouse pushes sand backwards by using forepaws thereby covering nearby marbles. Mouse rolls marble and tries to bury it. DB caused the marble to fall into the corner itself and the mouse buried it. DB observed throughout the cage especially in NMZ area. Stereotypical jumping in cage. Mouse buries marbles.	Stereotypical jumping in corners of cage. Rolling marbles. DB in NMZ and MZ corners spraying sand backwards. DB between marbles. Mouse buries marbles. Mouse spent a lot of time digging in the sand throughout the cage. Mouse digs a hole in the corner of the MZ. The mouse investigated the marble and displaced it from its original setting and buried it.
TRIAL 2	DB in corners of cage (NMZ and MZ). Mouse displaced marble into the corner. Rearing behaviour. Stereotypical jumping on the marble that was displaced into the corner thereby covering it. Mouse buried marbles. Mouse displayed HMB. It seems the mouse returns to marbles to bury them.	DB in corner displaced the nearest marble. Stereotypical behaviour in NMZ corner. Burrowing and rearing behaviour. Mouse buried marble.	DB in NMZ corner. DB in MZ corner. Stereotypical behaviour was observed. Mouse buried marbles. Mouse dug a hole near the marble and the marble had fallen into the hole and then the mouse buried the marble. Burrowing and rearing behaviour observed.	Mouse rolled marble. Mouse buried marbles. DB in NMZ corners and area. Displayed stereotypical behaviour a lot. DB in corners of cage.	Mouse rolls marble out of original setting. Mouse digs between marbles and throughout the cage. Rearing behaviour. DB in NMZ area as well as corners. Mouse buried marbles. DB in MZ corner the marble falls into the corner and mouse buried the marble.	Investigative behaviour viz., sniffing and touching. DB in the corners of the MZ and NMZ. Rearing behaviour observed. DB next to the marbles. Mouse rolls marble. DB displaced the marble into the MZ bottom corner. Mouse displayed stereotypical jumping on the marble pressing it deeper into the sand. The mouse tried to bury the marble and this behaviour displaced the marble from

						its original setting. Mouse rolls marble. DB in top NMZ corner. Mouse buries marble which had fallen into the corner bottom corner MZ. Mouse buries marbles. DB between marbles. Stereotypy in the corners of the cage
TRIAL 3	DB in MZ corners covered the nearest marbles due to mouse pushing sand backwards with its hind legs. Mouse dug a hole near the marble and the marble had fallen into the hole and then the mouse buried the marble. Stereotypical behaviour on marbles that had fallen into a hole in the corner. Mouse rolled marble.	Rearing behaviour. DB in NMZ area and corner. Mouse tries to bury marble but merely displaced it. Mouse rolled marble. Mouse dug a hole near the marble and the marble had fallen into the hole and then the mouse buried the marble. Mouse buried marbles. Mouse displayed lesser stereotypy.	DB in MZ corner displaced the nearest marble and partially covered the marble with sand. Stereotypical and rearing behaviour were observed. Mouse buried marbles. Mouse rolled the marbles.	Rearing behaviour. DB in MZ corner. Mouse buried marbles. DB in MZ corner and area. DB between marbles. Mouse buried marbles that were displaced in the corners of the cage.	Mouse engages in DB in the MZ top corner and this behaviour caused the marble to fall into the corner and partially buried it. Mouse rolls marble. Mouse buries in bottom corner of MZ spraying sand backwards covering nearby marbles. Investigative behaviour touching, sniffing and rolling marble. Mouse buries marble. Stereotypical jumping in the corners of the cage. DB in NMZ corners. Mouse digs near or next to marbles.	Stereotypical jumping. DB in NMZ area. DB in MZ corner caused the marble to fall into the corner (top). Mouse present stereotypical jumping on top of this marble in the corner pressing it deeper into the sand. Investigative behaviour viz., touching, sniffing and rolling marble. DB in the NMZ area. DB in MZ corner displaced nearest marble from its original setting and the marble is partially covered with sand due to sand that is pushed backwards. Rearing behaviour observed. DB in corners of cage. Mouse stands over marble and tries to bury it.
NUMBER OF MARBLES BURIED OVER 3 CONSECUTIVE NIGHTS	Observer 1: 2.67 Observer 2: 3.33	Observer 1: 2.33 Observer 2: 2.33	Observer 1: 5.33 Observer 2: 5.33	Observer 1: 2.33 Observer 2: 2.33	Observer 1: 5 Observer 2: 5.67	Observer 1: 3 Observer 2: 4.33
PREOCUPIED BEHAVIOUR OVER 3 CONSECUTIVE NIGHTS	21	17	21.67	13.33	26.33	25

Post-Treatment

MOUSE NUMBER	29	30	31	36	55	56
TRIAL 1	Rearing behaviour. DB in NMZ area and corner. Stereotypical jumping in NMZ corner. Roles marble and buries it a little. Stereotypy in MZ corner. Mouse buried marble. Mouse digs in the MZ corner and displaces sand on the nearest marble thereby covering it. Stereotypical behaviour also displaces sand. Mouse runs over marbles and digs between marbles.	Rearing and investigative behaviour. DB in NMZ corner. Stereotypical jumping in the MZ corner. Stereotypy displaced the marble nearest to the MZ corner. Mouse roles marbles and displayed DB. Mouse engaged in DB between marbles. Stereotypy displaced sand and covered marbles. Displayed DB throughout the cage.	Rearing and investigative behaviour. DB in corner of MZ and shoots sand backwards on the marbles. Stereotypical jumping in the same corner where mouse first displayed DB. Runs over marbles and roll the marbles as well. Mouse displaced marble by DB. Runs over marbles. Mouse returned to same corner and covered the sand and uncover the same marble again and covered it again. Roll marble. DB in corner and shoots sand backwards and covered marble with sand.	Rearing and sniffing behaviour. DB in the NMZ area and corners. DB in MZ corners thereby causing the marble to fall into the hole in the corner and then the mouse buried marble. The mouse then uncovered the buried marble then runs away. The mouse then goes to the same uncovered marble and buried it again. The mouse returns to the same marble recently covered and then uncovered it again. Stereotypical jumping on the partially uncovered marble forced the marble deeper in the sand thereby completely covering the marble.	DB in corner of MZ cage displaced marble away from corner. DB between marbles and in MZ corners. Rearing behaviour observed. Mouse rolls marble. DB in bottom corner of MZ pushing sand backwards thereby covering nearest marbles with sand. DB in corners of cage (MZ and NMZ). Mouse walks and digs throughout the cage. Mouse digs a hole in bottom corner in MZ. DB in NMZ area. Stereotypical behaviour observed viz., stereotypical jumping and pattern running. DB between marbles the mouse will also roll the marbles. Mouse buries the marble only partially. DB near marbles also displaces marble from original setting. Mouse buried marble. DB in top corner of MZ covers nearest marbles partially.	DB in bottom NMZ corner. Rearing and grooming behaviour. Stereotypical jumping in corners of cage. DB in corners of cage displaced sand backwards. Stereotypical jumping in bottom corner of MZ displaces sand. Mouse stands over marbles. DB in top MZ corner. Investigative behaviour viz., sniffing, touching and rolling. DB between marbles. DB in NMZ area. Rolling of marbles causes the displacement of marbles from its original place.

TRIAL 2	Stereotypical jumping most of the time in the NMZ corner. Rearing behaviour. DB in the NMZ area and corner. Mouse roles marble tries to bury it and only displaced the marbles.	Rearing and grooming behaviour. DB in the corners of the cage (NMZ and MZ). Stereotypical jumping in corners of the cage (MZ and NMZ). Investigative and sniffing behaviour. Stereotypy in the MZ corner displaced marbles from original place.	Rearing, sniffing and investigative behaviour. DB in corner of MZ and marble is displaced and covered with sand. Stereotypical jumping in corners of the MZ and NMZ. DB and BB near marble. Mouse runs over marbles. DB in MZ corners.	DB in the NMZ area and corner. Stereotypical jumping in the NMZ. Mouse rolled marble. Mouse runs over and between marbles. DB between marbles. Rearing behaviour. Stereotypical jumping in corner near marbles.	Investigative behaviour viz., sniffing and rolling, displaced marble. Grooming behaviour in NMZ corner. Rearing behaviour. DB in NMZ corners the mouse pushed the sand backwards using its forepaws. DB in top MZ corner. DB between marbles. Stereotypical jumping in the NMZ corner. Mouse spent most of the time in the NMZ corner. DB in top MZ corner, covers the nearest marbles due to spraying of sand backwards. DB in NMZ area. Rolling of marbles as well as rearing behaviour observed. Spent most of the time engaged in stereotypical jumping	Rearing behaviour. DB between marbles. Stereotypical jumping in NMZ corners. DB in NMZ area. Spent most of the time presenting with stereotypical jumping. Mouse touches marble.
TRIAL 3	Mouse digs in corner of MZ and displaced the marble. Stereotypy in NMZ and MZ corner. Mouse roles marble. Mouse buried marble. Displays investigative and digging behaviour. Mouse roles marble again and tries to bury the marble. The mouse displays DB and BB.	BB near marble thereby displaced marble. DB in MZ corner. Stereotypical jumping in NMZ and MZ corner. DB near marble and covered the marble partially with sand. Rearing behaviour. DB near between marbles. Running over marbles.	Rearing behaviour. DB in the MZ corner and the nearest marble is displaced. DB in NMZ corner. Stereotypical jumping in NMZ corner. DB near marble slightly covered the marble. DB in the corners of the MZ caused the marbles to be partially covered. DB between marbles	DB in NMZ and MZ corners. Rearing and investigative behaviour. Stereotypical jumping in MZ and NMZ corners. Mouse tried to bury marble and displayed DB between marbles.	DB in NMZ area. DB in MZ area spraying corner backwards covering nearest marbles. Rearing behaviour. Stereotypical jumping in NMZ corner. DB in MZ bottom corner displaces marble from original setting. Rearing behaviours against walls of cage. DB between marbles. Spent most time engaged in stereotypical jumping	Rearing and grooming behaviour. DB in NMZ area. Stereotypical jumping in NMZ area. Spent most of the time presenting with stereotypical jumping. DB between marbles. Rearing against wall of cage. Mouse didn't really display BB/DB
NUMBER OF MARBLES BURIED OVER 3 CONSECUTIVE NIGHTS	Observer 1: 0.67 Observer 2: 0.6	Observer 1: 0.33 Observer 2: 0.33	Observer 1: 0.67 Observer 2: 1	Observer 1: 0.67 Observer 2: 0.33	Observer 1: 0.33 Observer 2: 0.33	Observer 1: 0 Observer 2: 0
PREOCUPIED BEHAVIOUR OVER	15	13.67	14.67	17.33	24	20.67

3 CONSECUTIVE NIGHTS						

Control

Pre-treatment

MOUSE NUMBER	49	51	52	71	74	81
TRIAL 1	Rearing behaviour. DB in MZ corner displaces marble from its original setting. Marble rolled into the corner due DB. Investigative behaviour viz., sniffing, touching and rolling. DB in corners of cage spraying sand backwards. Mouse buries marble in the corner. Mouse buries marbles. DB between marbles.	DB in NMZ area and corner. Rearing behaviour. DB in MZ corner. Mouse displaces marble into top corner of MZ. Mouse buries marble in corner. DB throughout the cage. Stereotypy observed viz., somersaulting. Running over marbles. DB near marble. Mouse buries marble. DB in NMZ area. Mouse rolls marble and buries the marble. Stereotypical jumping in NMZ corner.	Rearing behaviour. DB throughout the cage. Investigative behaviour viz., touching and rolling of marble. DB near marble displaces the marble from the original setting. DB in NMZ and MZ corner. Stereotypical jumping in corners of cage. Mouse buries marbles. Mouse rolls marble and buries the marble. DB in MZ bottom corner displaces nearest marble from original setting.	Mouse buries 1st marble in 1st row in MZ corner. Rearing behaviour. DB in NMZ area and corner. Investigative behaviour viz., touching and sniffing marble. DB in bottom MZ corner displacing nearest marble from original setting. DB between marbles and buries marble. Mouse buries another marble. Mouse displays DB throughout the cage. DB in MZ corner pushing sand backwards covering nearest marbles partially then the mouse buries the marble completely. Stereotypical jumping in MZ corner. Mouse digs a hole in the sand.	Rearing behaviour. Investigative behaviour viz., touching marble. DB in NMZ area. DB in MZ corner spraying a sand backwards covering marbles with sand. DB between marbles. DB in NMZ area and corner. Rearing and grooming behaviour. Mouse stands over marble and tries to bury it. Mouse buries marble. Mouse spent a lot of time digging.	DB between marbles displaces marble from original setting. Rearing behaviour. Mouse buries marble. DB in NMZ area. DB in MZ corner pushing sand backwards covering nearest marble. Stereotypical jumping in NMZ and MZ corners. Stereotypical jumping on marble that rolled into the MZ corner. Mouse partially buries marble. Mouse stands over marble and tries to bury it.
TRIAL 2	Mouse rolls marble. DB in corner of NMZ. Mouse buries marbles. DB in MZ corner displaces nearest marble from original setting. Rearing behaviour. Mouse digs between marbles.	Mouse buries marble that rolled into the corner. Stereotypical jumping in MZ corner. Investigative behaviour viz., rolling, touching and sniffing marble. Rearing behaviour. Mouse buries another marble which rolled into the corner however, DB in corner	Rearing behaviour. Investigative behaviour viz., touching and rolling of marble. DB between marbles. DB throughout the cage. Mouse rolls the marble and tries to bury the marble. Mouse buries marble. DB in NMZ area and corners. Stereotypical jumping	DB in MZ displaces nearest marble into the corner. Mouse buries marbles partially. DB between marbles. Rearing behaviour. Mouse digs between marbles and covers nearest marbles with sand. DB in NMZ areas covers the nearest	DB in NMZ area. Rearing behaviour. DB in MZ corners spraying sand backwards covering nearest marbles. DB in NMZ corners and areas. Stereotypical jumping in MZ corners. DB between marbles. Investigative behaviour viz., touching, rolling and sniffing	DB in MZ corner displaces nearest marble from original setting. Rearing behaviour. DB in NMZ corner pushing sand backwards. Stereotypical jumping in NMZ corner. DB between marbles. DB in NMZ area and corner. Mouse spent a lot of time digging in bedding

		also covered nearest marbles with sand. Running over marbles displaces the marble from the original setting. Mouse buries marble. DB in NMZ area.	observed.	marbles with sand. Mouse displays stereotypical jumping on top of the marble in the corner pressing the marble deeper in the sand. Investigative behaviour viz., sniffing, touching and rolling marble.	marble. Mouse rolls and buries marble. Mouse stands over marble and tries to bury it.	material. DB in MZ corner displacing the marble into the corner the mouse then buries it. Investigative behaviour viz., sniffing, touching and rolling marble. Stereotypy covers marble partially
TRIAL 3	Rearing behaviour. DB in corners of MZ. DB in NMZ areas and corners. Mouse rolls marbles (investigative behaviour). DB in MZ corner causes the marble to roll into the corner. Mouse buries marbles. Mouse displaces marble from original setting when the mouse tries to bury it. DB in corners of MZ covers the nearest marbles with sand.	DB in corners of the cage (NMZ and MZ). Investigative behaviour viz., touching, sniffing and rolling marble. Stereotypical jumping in NMZ corners. DB throughout the cage. DB near the marbles.	Rearing behaviour. DB in MZ and NMZ corners. Mouse tries to bury marble. Stereotypical jumping observed. DB in MZ corner. DB near marble also causes the marble to move. Mouse stands over marble and tries to bury it. DB between marbles. Mouse buries marbles. Mouse rolls marble.	Mouse buries 2 marbles completely. DB in MZ corners spraying sand backwards covering nearest marbles with sand. DB in NMZ area. DB in MZ area and between marbles. Investigative behaviour viz., sniffing and touching marble. Mouse rolls and buries marble. Spent a lot of time burying.	DB in MZ corner pushing sand backwards covering nearest marble with sand. Rearing behaviour. DB between marbles. DB in NMZ area. DB in NMZ corner pushing sand backwards. Investigative behaviour viz., sniffing, touching and rolling marble. Mouse rolls marble and buries it. Mouse digs a hole near marble covering nearest marbles with sand. Mouse buries marble. Stereotypical jumping in NMZ corner.	Rearing behaviour. DB in MZ corner displaces the nearest marble from its original setting. DB in NMZ area and corner. Mouse buries marble. Stereotypical jumping in NMZ corners. DB in MZ corner pushing sand backwards covering the nearest marble. Spent a lot of time digging bedding material. Investigative behaviour viz., sniffing, touching and rolling marble.
NUMBER OF MARBLES BURIED OVER 3 CONSECUTIVE NIGHTS	Observer 1: 4.67 Observer 2: 5	Observer 1: 3.67 Observer 2: 3.67	Observer 1: 2.67 Observer 2: 3.67	Observer 1: 6.33 Observer 2: 5.67	Observer 1: 3 Observer 2: 2.33	Observer 1: 2.33 Observer 2: 1.67
PREOCCUPIED BEHAVIOUR	28	26	27	27.67	26.67	21

Post-Treatment

MOUSE NUMBER	49	51	52	71	74	81
TRIAL 1	DB in MZ corners sprays sand backwards covering nearest marble with sand.	DB in NMZ area and corner. Investigative behaviour viz., touching,	DB in corners of NMZ and MZ. Rearing behaviour. DB next to marble causes	Rearing behaviour. DB in MZ bottom corner. Investigative behaviour	Mouse rolls marble and displays investigative behaviour viz., sniffing	Rearing behaviour. DB in NMZ area. Stereotypical jumping in NMZ area.

	Rearing behaviour. DB in corner of NMZ. DB between marbles. Investigative behaviour viz., rolling, sniffing and touching of marbles. DB in NMZ area. DB near marbles displaces the marble from its original setting. Mouse stands over marble and partially buries it. Somersaulting over marbles. Stereotypical jumping covered one marble completely. Mouse displays high DB however, seldom shown interest in marbles.	sniffing and rolling marble. Rearing behaviour. DB in corner of MZ spraying sand backwards on top of nearest marble. DB between and next to marble. Grooming behaviour observed. A lot of time spent digging in NMZ area. Buries a marble only partially. Stereotypical jumping on top of marble that rolled into the corner pressing it deeper in the sand. Stereotypical jumping in NMZ corners. Mouse rolls marble into the bottom corner of the MZ.	the marble to roll into the top MZ corner. DB in NMZ area. Mouse stands over marble and tries to bury the marble. DB between and next to marble. Stereotypical jumping in NMZ corner. Stereotypical jumping in NMZ corner runs over marbles and returns to the same corner. Investigative behaviour viz., touching and sniffing marble.	viz., sniffing, touching and rolling marble. Mouse rolls marble out of original setting. DB in NMZ corner spraying sand backwards. Stereotypical jumping in NMZ corner DB between marbles. Mouse rolls another marble out of original setting. Mouse rolls a marble and partially buries it. DB in NMZ area. DB throughout the cage. A lot of stereotypical behaviour observed. Mouse buries the partially buried marble completely. Mouse displays less DB in corners.	touching and rolling. Rearing behaviour. DB in NMZ corner spraying sand backwards. DB in MZ corner displacing nearest marble from original setting. DB between marbles. Stereotypical jumping in NMZ corner. Pattern running. Most of the time stereotypical behaviour observed. DB in NMZ area. Mouse rolls and buries it. DB in MZ corner spraying sand backwards covering nearest marble.	Stereotypical behaviour displaces marble in MZ area. DB between marbles. Stereotypical jumping in MZ corner. Investigative behaviour viz., sniffing, touching and rolling marble. DB in MZ corner displacing marble from original place and covers it with sand. Mouse rolls marble. Mouse spent a lot of time displaying stereotypical jumping.
TRIAL 2	Grooming behaviour. DB between marbles. Mouse rolls marble. Somersaulting on top of marbles pressing them deeper into the sand. Investigative behaviour viz., touching, sniffing and rolling of marbles. Mouse stands over marble and partially buries it. DB between marbles covers the marble with sand. DB in NMZ area. Mouse present with DB throughout the cage.	Db between marbles. Grooming and rearing behaviour. Investigative behaviour viz., rolling, touching and sniffing marble. DB in NMZ area. DB between marbles. DB in MZ corners spraying sand backwards covering nearest marbles. Sitting in NMZ area. Stereotypical jumping in NMZ corners. Mouse rolls marble. Pattern running in NMZ corners. DB next and between marbles. Spent most of the time in NMZ area.	DB in MZ corner displaces marble from originals setting. Investigative behaviour viz., touching, rolling and sniffing marble. DB in NMZ area. Rearing and grooming behaviour. Mouse rolls marble into bottom corner of MZ corner. DB between marbles. DB in MZ corner spraying sand backwards on top of nearest marbles. Mouse stands over marble and partially buries it. Mouse rolls another marble and partially buries the marble. Mouse rolls the marble and tries to bury it only to displace the	Rearing behaviour. DB in NMZ corner. DB between marbles. Rearing behaviour displaces marble from original setting. DB between marbles sprays sand backwards covering other marbles. DB in NMZ corner spraying sand backwards. Mouse stands over marble and completely buries it. DB in MZ and NMZ corners. Most of the time stereotypical behaviour observed.	Rearing and grooming behaviour. DB in MZ corner. DB between marbles displaces it from original setting. Mouse stands over and partially buries it. Pattern running. DB next to marble covers nearest marble with sand. Mouse buries partially covered marble.	Rearing behaviour. Mouse rolls marble. DB in MZ corner displacing nearest marble from original setting. Stereotypical jumping in MZ corner. DB in MZ sprays sand backwards covering nearest marble. DB in NMZ area. DB between marbles. Mouse DB between marbles covers one marble partially. Mouse stands over marble and tries to bury it.

			marble from original setting. Stereotypical jumping in NMZ corner. Somersaulting over marble covering marbles with sand.			
TRIAL 3	DB in the NMZ corner. Somersaulting over marbles. DB in MZ corners spraying sand backwards on top of the marbles. Investigative behaviour viz., touching, rolling and sniffing of marbles. Rearing behaviour. DB between marbles. Standing over marble rolling it then burying it.	Rearing behaviour. Mouse digs in MZ corner this behaviour causes the marble to roll into the corner however, the mouse continuous to dig in the MZ corner shooting the marble out of the corner into the cage. DB between marbles. Rolls marble and tries to bury it. DB in NMZ area and corner. Mouse stands over marble and buries it completely. DB in MZ area. Mouse uncovers buried marble partially. Stereotypical jumping in NMZ and MZ corner. Rearing against walls of NMZ area. Grooming and rearing behaviour. Spent most of the time displaying stereotypical behaviour.	Mouse rolls marble and tries to bury the marble. Stereotypical jumping in NMZ corner. DB in NMZ area and corner. Rearing behaviour. DB in MZ corners mouse pushes sand backwards covering nearest marbles. DB in bottom MZ corner displaces nearest marble from original setting. Investigative behaviour viz., touching, rolling marble. Mouse rolls marble into MZ corner and partially buries it. DB in corners of MZ sprays sand backwards covering marbles. Mouse stands over marble and tries to bury the marble however, this only displaces the marble from original setting. Mouse buries one of the partially buried marbles. Mouse buries the marble in the corner. Mouse uncovers buried marble and later buried it again.	DB between marbles. DB in NMZ area. Rearing behaviour. Mouse rolls marble. DB in MZ corner displaces nearest marble from original setting. DB in NMZ corner spraying sand backwards. Mouse rolls marble and buries it. DB in NMZ area. Mouse buries marble completely. Stereotypical jumping in NMZ and MZ corner.	DB in MZ corner. Stereotypical jumping in MZ corner. Pattern running. DB in NMZ area. Rearing behaviour. DB in NMZ corner spraying sand backwards. Investigative behaviour viz., touching, sniffing and rolling marble. DB in MZ corner displaces nearest marble and covers it with sand. Mouse rolls marble. A lot of DB in NMZ area.	Rearing and grooming behaviour. Mouse rolls marble. DB between marbles. Stereotypical jumping in MZ corner. DB in NMZ corner spraying sand backwards. Stereotypical jumping in NMZ corner. Stereotypical jumping in MZ corner displaces a marble from original setting. Most of the time mouse displays stereotypical behaviour.
NUMBER OF MARBLES BURIED OVER 3 CONSECUTIVE NIGHTS	Observer 1: 1.33 Observer 2: 1.33	Observer 1: 0.33 Observer 2: 1	Observer 1: 2 Observer 2: 2	Observer 1: 1.33 Observer 2: 1.33	Observer 1: 1.33 Observer 2: 1.33	Observer 1: 0.33 Observer 2: 0.33
PREOCCUPIED	17.67	21.67	31.67	21	21.33	21

BEHAVIOUR						

Escitalopram alone

Pre-treatment

MOUSE NUMBER	100	103	109	105	104	106
TRIAL 1	Rearing as well as rearing behaviour against the walls. DB in NMZ spraying sand backwards. DB in MZ top corner spraying sand backwards covering nearest marbles with sand. Mouse digs a hole. DB in NMZ area and between marbles. DB in bottom MZ corner displaces the nearest marble causing it to roll into the corner the mouse partially buries it. Stereotypy in MZ bottom corner pressing the marble deeper in the sand. Mouse rolls marbles. Mouse buries marbles.	DB in NMZ area. Rearing behaviour observed. DB in MZ corner spraying sand backwards. Somersaulting in MZ corner displaces marbles from original setting. Somersaulting displaces marble into corner. Grooming behaviour observed. DB in NMZ mouse sprays sand backwards. DB near marbles or between marbles covers the marble with sand. DB throughout the cage. Mouse buries marble. Mouse partially buries marble.	Rearing behaviour observed. DB in NMZ area. DB near marble and covers marble partially. Stereotypical jumping in MZ corner. DB between marbles. Mouse buries marble partially. Mouse rolls marble. Investigative behaviour viz., sniffing, touching and rolling marble. DB in MZ corner spraying sand backwards. Mouse buries marble. DB in MZ corner covers the nearest marble with sand.	DB in NMZ corner. DB between marbles. Rearing behaviour observed. Stereotypical jumping in NMZ corner. DB in bottom corner displacing marble and covering marbles with sand. Stereotypical jumping in NMZ corner. DB in corner covers nearest marble. Mouse rolls marble. Mouse partially buries marble. DB between marbles. Stereotypy in corner on top of marble. Mouse buries marble. Mouse uncovers marble partially covered marble. Mouse rolls marble and partially covers the marble. Mouse buries marble.	Repeated rearing. Stereotypical vertical jumping in corners of cage. DB close to the marble. DB between marbles. DB in corners of cage. Possibly buried more once the session ended and in the period when the mice were returned to home cages.	Rearing behaviour observed. Mouse displayed pattern running for the 1st minute of the recording. Mouse stretches towards the marble and sniffs. Investigative behaviour viz., touching, sniffing. Spends a great deal of time in NMZ area. About 16:30 of the recording the mouse crossed the NMZ. Mouse preoccupied with marble however brief visits but behaviour directed towards marbles. DB between marble. Mouse buries marbles. DB in corners of cage. Mouse displaces marble from original setting (Top left corner of MZ).
TRIAL 2	DB in bottom corner covering nearest marbles completely. DB between marbles. Mouse rolls marble into hole which the mouse dug. DB in NMZ corner. Stereotypy in top MZ corner. Mouse stands over marble and tries to bury it but only displaces the marble from its original setting. DB	Rearing behaviour observed. DB between marbles. DB in NMZ area and corner spraying sand backwards. DB in MZ corner spraying sand backwards covering nearest marble. Rolling the marble into the corner. Mouse partially buries marble in the corner. Somersaulting	Stereotypy in NMZ corner. Rearing behaviour observed. DB in NMZ area. DB in MZ area and corner. DB between marbles. DB in MZ corner spraying sand backwards. DB near marble displaces marble from original setting. Mouse stands over marble. Investigative behaviour	Rearing behaviour. Stereotypical jumping in MZ corner. DB in NMZ area. Rearing behaviour against cage walls. DB in MZ area. DB between marbles. Mouse stands over marble and partially covers the marble with sand. Stereotypical jumping in NMZ corner. Mouse buries a marble	DB in corners of the cage. Rearing behaviour observed. Vertical jumping in corners of the cage. DB near marbles. Grooming behaviour observed. Mouse attempt to bury marbles. Prolonged DB throughout the cage. Mouse buried marble. Mouse rolls marble.	Rearing behaviour observed. Mouse initially in NMZ area of cage seems afraid of marbles. Cautious investigation of marbles with brief numerous interactions with marbles. Mouse moves quicker from NMZ to MZ when compared with 1st night. DB in top NMZ

	between marbles covers the nearest marbles with sand. Rearing and grooming behaviour observed. Mouse buried 2 marbles completely.	behaviour observed. Again buries marble in the corner. Mouse stands over marble and tries to bury it. Mouse somersaults on top of marbles pressing the marbles deeper in the sand. DB in MZ area. Mouse rolls marble and tries to bury it. Stereotypy displaces marble.	viz., touching, sniffing and rolling marble. DB between marbles covering the marbles with sand. Mouse stands over marble and tries to bury it.	partially.	Mouse uncovered a marble which was buried.	corner. Intentional burying behaviour observed. Mouse engages in grooming behaviour. Mouse intentionally buries marble. Rearing behaviour presses marbles deeper into the sand therefore covering the marble completely. Burying in proximity to marbles. Spends a great deal of time in lower left corner rearing or burying. Mouse uncovers a marble which was buried the mouse then buries the marble again and uncovers it again and DB displaces marble. Mouse rolls marble. DB in NMZ bottom corner.
TRIAL 3	Rearing behaviour observed. Stereotypy in NMZ corner. DB in MZ corner displaces marble from original setting. DB between marbles. Investigative behaviour displaces marble. DB in NMZ corner spraying sand backwards. DB in MZ corner covers nearest marble completely. Mouse sprays sand backwards using forepaws.	DB in top MZ corner displacing nearest marble and covers other marbles with sand by spraying sand backwards. Rearing behaviour observed. Somersaulting on top of marbles this stereotypy displaces marble from original setting. DB in NMZ and MZ area. DB between marbles. Grooming behaviour observed. Mouse rolls marbles. DB in NMZ corner spraying sand backwards. DB in bottom MZ corner displaces	Rearing behaviour observed. Stereotypy in MZ corner. DB next to marble. DB in bottom MZ corner spraying sand backwards. Investigative behaviour viz., rolling, touching and sniffing marble. Mouse stands over marble. DB in NMZ area as well as stereotypy. Stereotypy on top of marbles pressing marbles deeper in the sand. Mouse stands over marble and tries to bury it. Grooming behaviour observed. Mouse stand	Mouse tries to bury marble and displaces the marble from its original setting. Mouse rolls marble. DB in NMZ area. Rearing behaviour observed. Rearing displaces marble from original setting. Stereotypy in NMZ area and corner. DB between marbles. Mouse buries marble. Mouse rolls marble into the corner. Mouse stands over marble tries to bury it but displaces the marble from its original setting. DB	Vertical jumping in corners of cage. Mouse runs from marble to marble. DB next to marble. Seemingly intentional BB. DB between marbles. Intentional attempt at burying a marble. Mouse rolls marble and buries it. DB in top MZ and NMZ corner. Intentional burying behaviour observed.	Mouse appears no longer afraid of marbles. Rearing behaviour observed. Mouse interacts with marbles. DB in top NMZ corner. Burying / DB in close proximity to marbles. Mouse displaces marble from original setting in an attempt to bury it. Grooming behaviour observed. Mouse rolls marble. DB in corners of cage (MZ and NMZ). Mouse attempts to bury marble.

		marble into the corner and covers the marble completely. Mouse partially buries marbles.	over marble tries to bury it however the mouse only buries the marble partially. Mouse buries marble. Mouse partially buries marble in MZ corner.	throughout the cage. Mouse buries marble. Stereotypy on top of displaced marble in the MZ corner pressing the marble deeper in the sand. DB in MZ corner spraying sand backwards covering nearest marbles		
PREOCCUPIED BEHAVIOUR	21.67	25.67	37.33	31	31	35.67
NUMBER OF MARBLES BURIED OVER 3 CONSECUTIVE NIGHTS	Observer 1: 5 Observer 2: 4.33	Observer 1: 3.67 Observer 2: 3	Observer 1: 4.67 Observer 2: 4	Observer 1: 2.33 Observer 2: 2	Observer 1: 4.33 Observer 2: 3.67	Observer 1: 4 Observer 2: 3.67

Post-Treatment

MOUSE NUMBER	100	103	109	105	104	106
TRIAL 1	DB near marbles displaces marble from original setting. Rearing behaviour observed. DB rolls nearest marble into the corner and mouse stands on it. Mouse runs over marble in corner and completely covers it. Mouse stands over marble and tries to bury it. DB between marbles. DB in corners of cage. Investigative behaviour viz., rolling, sniffing and touching marble. DB in NMZ area. Mouse buries a partially covered marble. Grooming behaviour observed. DB between marbles. Rearing against cage walls. Stereotypical	DB in top MZ corner displacing marble from original setting. Rearing and behaviour observed. Stereotypical somersaulting. Investigative behaviour viz., touching, rolling and sniffing marble. DB in NMZ area. DB between marbles. DB in MZ corner spraying sand backwards covering the nearest marble partially. DB in NMZ area. Mouse displays a lot of stereotypy. Mouse covers marble by spraying sand backwards. Mouse stands over marble and tries to bury it. Walking over marbles. Spent a lot of time engaged in	Rearing behaviour. Running displaces marble from original setting. Investigative behaviour viz., sniffing, rolling and touching marble. DB in NMZ area. DB between marbles. Stereotypy in top MZ corner. DB partially covers marble. Stereotypy covers marble. Stereotypy in NMZ corners. Grooming behaviour observed. Mouse buries marble. Mouse partially buries marble.	Mouse displays rearing and grooming behaviour. DB in MZ corner pushing sand backwards and displacing marble from original setting. DB in corner of NMZ area. DB in MZ area. Investigative behaviour viz., sniffing, rolling and touching marble. Stereotypy in NMZ corner. DB between marbles. DB in top MZ corner partially covering nearest marbles. Mouse tries to bury marble however, displaces marble further. Mouse displays a lot of stereotypy. Mouse stands over marble.	DB between marbles. Rearing against cage walls. DB in corner of cage. Standing over marble. Investigative behaviour viz., touching, rolling and sniffing marble. Somersaulting on top of marbles. DB in NMZ. DB in bottom of MZ corner spraying sand backwards covering nearest marble. Mouse partially covers marble. DB between marbles. DB near marbles displaces marble. Mouse displays grooming behaviour. Mouse partially buries a marble and displaces another marble in the process. Stereotypy covers marbles and	Investigative behaviour viz., sniffing, rolling and touching marble. Mouse rolls marble and buries it. Mouse displays rearing and grooming behaviour. Rearing against cage walls. Mouse stands over marble. DB in bottom MZ corner displaces marble from original setting. DB in corner of cage and sprays sand backwards. Mouse partially buries marble. Running displaces marble. DB between marbles. Mouse buries marble and displaces another.

	jumping in NMZ corner. Mouse stands over marble.	stereotypical behaviour. DB in corners of cage.			presses marbles deeper in the sand. Mouse buries marble.	
TRIAL 2	Mouse displaces marble from original setting. DB in NMZ corner. Rearing behaviour. Stereotypy in NMZ area. Stereotypical jumping in MZ corner. Grooming behaviour observed. DB in MZ corner spraying sand backwards covering nearest marbles. DB near marbles causes nearest marble to roll into the corner. DB and stereotypy covers the marble in the top MZ corner partially. Investigative behaviour viz., touching and sniffing. Mouse spent most of the time displaying stereotypical behaviour. Mouse stands over marbles. DB between marbles. Stereotypy near marbles presses marbles deeper into the sand.	Mouse stands over marble and displaces marble from original setting. Somersaulting. Mouse touches and sniffs marble. Stereotypical behaviour displaces behaviour. DB in NMZ area. Mouse rolls and sniffs marble. Mouse displays DB near marble. DB between marble. Rearing behaviour observed. Spent most of the time displaying stereotypy	Investigative behaviour viz., touching, sniffing and rolling marble. Rearing behaviour. DB in NMZ area. Stereotypy displaces marble from original setting. Stereotypy in corners of cage. DB between marbles. Mouse displays a lot of stereotypy.	Investigative behaviour viz., rolling, sniffing and rolling marble. Displays rearing behaviour. DB in NMZ area. Mouse partially buries marble. Rearing against cage walls. Stereotypy in NMZ corner. DB between marbles. DB in corners of cage. Mouse buries marbles. Running over marbles. Mouse displays a lot of DB. DB displaces marble from original setting. DB covers marbles.	Stereotypy displaces marble. DB in corners of cage spraying sand backwards. Stereotypy covers marble. DB in NMZ area. Mouse displays grooming behaviour. Mouse spent most of the time in NMZ area digging. Mouse didn't display a real interest in marbles.	Mouse displays grooming and rearing behaviour. Investigative behaviour viz., sniffing, touching and rolling marble. Rearing against cage walls. DB in bottom MZ corner spraying sand backwards covering nearest marbles with sand. Mouse tries to bury marble but only displaces marble. Mouse partially buries marble. Stereotypy in MZ corner. DB between marbles. Mouse buries marble.
TRIAL 3	Rearing and DB observed. Mouse rolls marble. DB in top MZ corner. Mouse stands over marble and tries to bury it. DB between marbles displaces marble from original setting. Stereotypical behaviour in NMZ and MZ corner. Rearing against cage	Somersaulting on marbles. Rearing against cage wall. Investigative behaviour viz. touching, sniffing marble. Grooming behaviour observed. DB in top MZ corner displacing marble into the corner. DB in NMZ area. DB near and between marbles. DB	Investigative behaviour viz., sniffing and touching marble. Rearing behaviour observed. DB in NMZ area. Standing over marble. Stereotypy against NMZ and MZ corners. DB between marbles. Stereotypy displaces marble from original setting. Mouse	DB in NMZ area. DB displaces marble from original setting. DB in corner displaces marble from original setting. Rearing against cage walls. DB in MZ area. Mouse spent most of the time engaging in stereotypy. Investigative behaviour viz., sniffing	Rearing and grooming behaviour observed. Stereotypical somersaulting on top of marbles. DB in NMZ corners. DB between marbles. Investigative behaviour viz., sniffing and touching marble. Somersaulting covers one marble completely.	Rearing against cage walls. DB in corners of cage. Mouse displays grooming behaviour. DB in bottom MZ corner displaces marble. Investigative behaviour viz., sniffing, rolling and touching marble. Mouse buries 2 marbles. DB in corners of cage. Mouse

	walls. DB in NMZ area. Investigative behaviour viz., sniffing and touching. Stereotypical behaviour on top of marbles. Most of the time mouse displayed stereotypical behaviour. Mouse did not bury marbles. When mouse engages in DB in the corners of the cage the mouse sprays sand backwards using forepaws. DB in bottom MZ corner covers marble partially.	corners of cage. Spent most of the time displaying stereotypy. Investigative behaviour viz., sniffing. DB in top MZ corner covering marble partially. DB decreases. Rearing against cage walls. Standing over marble.	displays grooming behaviour. Mouse directs body towards marble as if the mouse wants, to bury it.	and touching marble. Displaying less DB than previous nights.	Rearing against cage walls. Mouse displays nearest marble in bottom MZ corner and buries it partially. DB in corners of cage. Mouse spent a lot of time digging in MZ area.	partially buries marble. Standing over marble. DB in NMZ area. Mouse rolls marble and tries to bury it but only pushes the marble backwards. Mouse displays a lot of DB. Stereotypy in top MZ corner pressing marble deeper into the sand.
PREOCCUPIED BEHAVIOUR	19.33	21.67	15.67	12.33	16.67	28.33
NUMBER OF MARBLES BURIED OVER 3 CONSECUTIVE NIGHTS	Observer 1: 1.33 Observer 2: 1.33	Observer 1: 0.33 Observer 2: 0.33	Observer 1: 1.33 Observer 2: 1	Observer 1: 2 Observer 2: 1.67	Observer 1: 1.33 Observer 2: 1	Observer 1: 5.67 Observer 2: 6.67

Addendum C

The review paper contained in Addendum C was co-written by the candidate. The work is a critical appraisal of the marble burying test as it is often applied, and supports the methodology followed in Chapter 3 of this investigation.

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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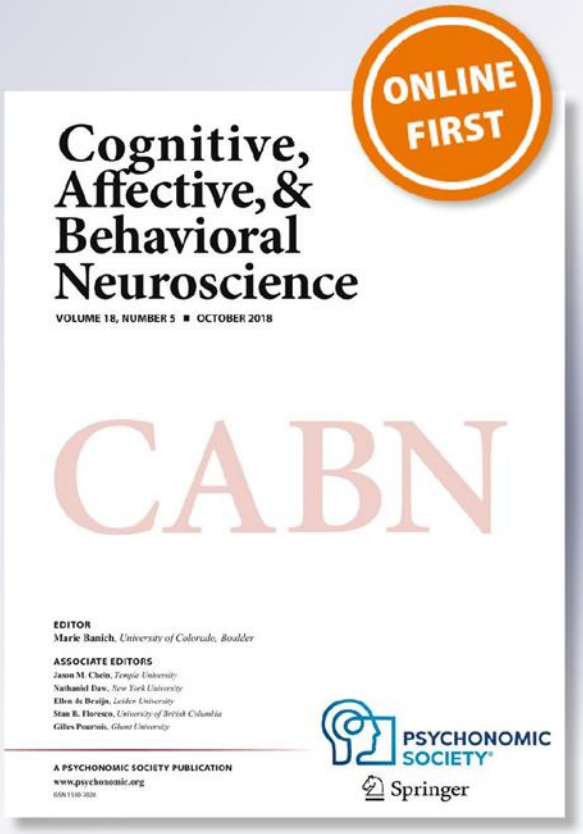

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THEORETICAL REVIEW

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 anticompulsive 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 anticompulsive (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, Mullot, 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 (Çalişkan 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 symptomology 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 anticompulsive 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), tha 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 Psychatric 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, & Iobst, 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 (Rowsell & 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, & McDougle, 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; Bechard & 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 anticompulsive 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 anticompulsive 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 anticompulsive 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, Shirota, 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; Llaneza & 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/cm^3; de Brouwer & Wolmarans, 2018) and wood shavings (pine, weighed and calculated at 0.07 g/cm^3; 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/cm^3; de Brouwer & Wolmarans, 2018) or fine river sand (Supplementary Fig. 2b; weighed and

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 anticompulsive 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 anticompulsive 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;

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 (Çalişkan 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 ø = 4 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 ø = 8 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 & Frye, 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; Kaehler, 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-

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 × 25 × 19 cm (Poling et al., 1981) to 45 × 24 × 21 cm (Llaneza & Frye, 2009) and 47 × 27 × 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 sizees range from 23 × 17 × 14 cm (25 marbles; Broekkamp et al., 1986) and 38 × 32 × 28 cm (25 marbles; Casarotto et al., 2010) to 45 × 60 × 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 cm^2/marble (Bruins Slot et al., 2008) or 23.8 cm^2/marble (Dixit et al., 2014) stand in stark contrast to lower densities of 112.5 cm^2/marble (Badgujar & Surana, 2010) or 75 cm^2/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 (Çalişkan 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; Çalişkan 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, Llaneza and Frye (2009) found that both MBA and vigilant/avoidant behaviors were minimized during the oestrous phase, in line with the observations of Schnieder 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, Schnieder 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), C57Black/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 (de Brouwer & Wolmarans, 2018; Wolmarans et al., 2016). Wistar (Schneider & Popik, 2007) and Long-Evans/hooded (Llaneza & Frye, 2009; Poling et al., 1981) rats have also been used, as well as a number of miscellaneous strains (Çalişkan 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) knockout (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 noradrenergic, 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)
Buspirone	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	#	(Badgujar & 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	#	(Broekkamp 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)
Psilocybe argentipes (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)
		0.1, 0.5, 4, 6 i.p. 30 min pre	0.5, 4, 6	/	(Gaikwad & Parle, 2011)
Aripiprazole + Ethanol	D_2 antagonist/partial agonist; 5-HT_{1A} partial agonist	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;5-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 5-HTP	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)
Olanzepine	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/D_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/D_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	1	–	(Matsushita et al., 2005)
		0.03–1 mg/kg i.p. 30 min pre	1	#	(Li et al., 2006)
		0.01, 0.04, 0.16, 0.63 mg/kg s.c. 60 min pre	0.16, 0.63	–	(Bruins Slot et al., 2008)
		1 mg/kg/day s.c. 21 days	1	#	(Torres-Lista et al., 2015)
SLV-313	D_2 antagonist/HT_{1A} agonist	0.01, 0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	2.5	#	(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	#	(Broekkamp et al., 1986)
Trifluperidol	D_2 antagonist	0.1, 0.32, 1 mg/kg s.c. 30 min pre	0.32, 1	#	(Broekkamp 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	0.03, 0.3	–	(Young et al., 2006)
		15, 30, 60 mg/kg i.p. 30 min pre	60	#	(Dixit et al., 2014)
d-Amphetamine	Monoamine releaser/reuptake inhibitor*	0.1–10 mg/kg i.p. 10 min pre	1–10	+	(Li et al., 2006)
		0.1, 0.3, 1, 3 mg/kg i.p. 30 min pre	1, 3	+	(Nicolas et al., 2006)
	Also affects DA, 5-HT	0.5, 1, 5 mg/kg i.p.	1,5	+	(Jimenez-Gomez et al., 2011)
Desipramine	Noradrenalin reuptake inhibitor	3–30 mg/kg i.p. 30 min pre	30	#	(Li et al., 2006)
		10 mg/kg i.p. 4 h pre	10	–	(Schneider & Popik, 2007)
Methamphetamine	Monoamine releaser/reuptake inhibitor*	0.5, 2.7, 4.8 µmol/kg i.p. 30 min pre	2.7, 4.8	/	(Saadat et al., 2006)
	Also affects DA, 5-HT				
Methylphenidate	Monoamine releaser/reuptake inhibitor*	9.3, 18.5, 37 µmol/kg i.p. 30 min pre	37	/	(Saadat et al., 2006)
	Also affects DA, 5-HT				
Mianserin	Noradrenalin reuptake inhibitor; anti-serotonergic	3.2, 10, 32 mg/kg s.c. 30 min pre	10, 32	#	(Broekkamp et al., 1986)
Milnacipran	Serotonin-noradrenalin reuptake inhibitor	5, 10, 20 mg/kg i.p.30 min pre	10, 20	/	(Sugimoto et al., 2007)
		20 mg/kg s.c. 30 min pre	20	/	(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)
		0.0025, 0.01, 0.04, 0.16, 0.63, 2.5 mg/kg	2.5	#	(Bruins Slot et al., 2008)
para--chloroamphetamine (PCA)	Monoamine releaser/reuptake inhibitor*	15 mg/kg 2 doses 6 hours apart (neurotoxic dose, day 28 post)	15	#	(Saadat et al., 2006)
	Also affects DA, 5-HT	2.4, 3.9, 4.8, 9.7 µmol/kg i.p. 30 min pre	4.8–9.7	/	(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	5, 10	–	(Njung'e & Handley, 1991b)
		0.3, 1, 3, 10 mg/kg i.p. 30 min pre	3, 10	–	(Nicolas et al., 2006)
Drugs targeting the GABAergic system					
(+)-limonene epoxide	GABA potnetiator	25, 50, 75 mg/kg p.o. 30 min pre	25, 50, 75	#	(De Almeida et al., 2014)
		25, 50, 75 mg/kg/day p.o. 14 days	25, 50, 75	#	(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	10, 22	#	(Broekkamp et al., 1986)
		3–30 mg/kg i.p. 30 min pre	30	#	(Li et al., 2006)
		10, 30, 100 mg/kg i.p. 30 min pre	30, 100	–	(Nicolas et al., 2006)
Clonazepam	Benzodiazepine receptor agonist	0.22, 0.46, 1 mg/kg s.c. 30 min pre	0.22–1	#	(Broekkamp 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. 30 min pre	2.2, 4.6, 10	#	(Broekkamp et al., 1986)
		0.05, 0.1, 0.25, 1, 5 mg/kg i.p. 30 min pre	1, 5	+	(Njung'e & Handley, 1991b)
		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. 30. min pre	3, 10, 30	–	(Young et al., 2006)
		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)
		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)
Ethanol	GABA potentiator; NMDA receptor antagonist	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)
		0.1, 1.2, 2.4, 4.8% w/v solution i.p. 30 min pre	1.2, 2.4, 4.8	/	(Gaikwad & Parle, 2011)
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. 30 min pre	150, 220	#	(Broekkamp et al., 1986)
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. 2 min pre	0.1, 1, 5	/	(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	1, 5, 10	/	(Umathe et al., 2012)
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 ic.v. 2 min pre	100	/	(Umathe et al., 2012)
JZL184	MAGL inhibitor; cannabinoid potentiator	4, 16, 40 mg/kg i.p. 2h 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)
Tetrahydrocannabidiol (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	0.1, 0.3, 1	/	(Gomes et al., 2011)
		0.01, 0.05, 0.1, 1, 5, 10, 20 μg/mouse i.c.v. 2 min pre	0.05–5	/	(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, 10mg/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	50	–	(Krass et al., 2010)
		10, 20, 40 mg/kg i.p 30 min pre	20, 40	/	(Gawali et al., 2016)
Agmatine	Imidazoline receptor agonist; Nitric oxide synthesis inhibitor	10, 50 mg/kg 30 i.p. min pre	10, 50	/	(Krass et al., 2010)
		10, 20, 40 mg/kg i.p. 30 min pre	20, 40	/	(Dixit et al., 2014)
		10, 20, 40 mg/kg i.p. 30 min pre	20, 40	/	(Gawali et al., 2016)
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	#	(De Almeida et al., 2014)
		250 mg/kg/day p.o. 14 days	250	#	(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)
		1, 3, 10 mg/kg i.p. 30 min pre	10	/	(Nicolas et al., 2006)
Carbamazepine	Anti-Epileptic	10, 100, 300 mg/kg p.o. 60 min pre	200, 300	–	(Egashira et al., 2013)
Chlorpromazine	Antihistamine	0.32, 1, 3.2 mg/kg s.c. 30 min pre	1, 3.2	#	(Broekkamp et al., 1986)
		1.25, 2.5, 5, 10 mg/kg p.o. 60 min pre	10	–	(Abe et al., 1998)
		1–10 mg/kg i.p. 30 min pre	10	#	(Li et al., 2006)
		0.3, 1, 3 mg/kg i.p. 30 min pre	3	–	(Nicolas et al., 2006)
Chlorpyrifos (Organophosphate)	Acetylcholine esterase inhibitor	1 mg/kg/day i.p. 5 days	1	/	(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)
Dolichandrone falcata ethyl acetate extract	Novel plant extract	100, 200, 400 mg/kg p.o. 60 min pre	200, 400	#	(Badgujar & Surana, 2010)
Dolichandrone falcata 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 (*C. esculenta* extract)	Novel plant extract	25, 50 mg/kg i.p. 30 min pre	25, 50	#	(Kalariya et al., 2015)
Hypericum perforatum 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)
Lagenaria siceraria 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	+	(Llaneza & 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)
Tipepidine	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)
Withania somnifera 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	/	(Harasawa 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 tipepidine		3 mg/kg s.c. 60 min pre	3	+	(Honda et al., 2011)
Drugs targeting the dopaminergic system					
Raclopride PT to tipepidine	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 $\sigma_{1/2}$ 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	#	(Li et al., 2006)
		0.01–2.5 mg/kg s.c. 60 min pre	#	(Bruins Slot et al., 2008)
Desipramine	Noradrenalin reuptake inhibitor	15, 30, 60 mg/kg p.o. 60 min pre	#	(Ichimaru et al., 1995)
		1, 3, 10, 30 mg/kg i.p. 30 min pre	/	(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	/	(Egashira, Okuno, Matsushita, et al., 2008)
		0.3 mg/kg i.p. 60 min pre	/	(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	#	(Bruins Slot et al., 2008)
		1, 3 mg/kg i.p. 30 min pre	/	(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	/	(Harasawa et al., 2006)
		0.04–10 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
		3 mg/kg s.c. 60 min pre	/	(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				
βCCE (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	Glutmate release inhibitor	0.3, 1, 10 mg/kg i.p. 30 min pre	–	(Egashira, Okuno, Harada, et al., 2008)
Drugs targeting miscellaneous systems				
17β-Estradiol	Sex hormone	10 μg/rat 44–48 h pre	+	(Llaneza & Frye, 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	5α-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)
Pyrilamine	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, Karkare, 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 $5\text{-}HT_{1A}$ 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 $5\text{-}HT_{2A/C}$ receptors does not (Ichimaru et al., 1995; however, see Egashira et al., 2013). Nevertheless, given that $5\text{-}HT_{2A/C}$ 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 nonselective 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-HT_1- and 5-HT_2-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 (Cheeta, 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 monotherapueutic intervention (Fineberg, 2004; Pigott et al., 1992), it may be of some benefit in combination with an SSRI (Piggot 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-HT_2 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-HT_2 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-HT_2 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 anticompulsive 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 nisoxetine (Li et al., 2006), and the nonselective NA, 5-HT, and DA releasing agents (Green et al., 2003; Rowley, Pinder, Kulkarni, Cheetham, & 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; Serby, 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; Fineberg, 2004) or anxiety (Bandelow et al., 2008), carbamazepine, lamotrigine, and valproate have indeed been shown to attenuate MBA. Interestingly, lamotrigine displayed strain-dependant 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 (CB_1 and CB_2), 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 CB_1 receptor has been extensively studied for its CNS effects, whereas the CB_2 receptor is largely expressed on immune cells in the periphery (Ameri, 1999; Howlett et al., 2002). Presynaptically located CB_1 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 CB_1 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 CB_1 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 (CB_1) 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 CB_1 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 CB_1 and CB_2 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 CB_1 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 CB_1 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 CB_1 receptor activation (Haller, Varga, Ledent, & Freund, 2004; Rey, Purrio, Viveros, & Lutz, 2012). Anxiogenic effects can be precipitated by CB_1 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 CB_1-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 (Kaehler 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 (NK_1) 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 NK_1 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 D_1 and D_2, 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; Njunge & 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, Beijamini, & 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 anticompulsive 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 anticompulsive 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 anticompulsive 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; Kinsey 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 × 20 × 13 cm and 40 × 35 × 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 anticompulsive 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 anticompulsive 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 anticompulsive 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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