Dopaminergic and serotonergic modulation of reward appraisal in the zebrafish *(Danio rerio)*

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I dedicate this dissertation to my mom; without you it would not have been possible. Thank you for teaching me to persevere and never give up. Your love and motivation encouraged me. I will always look up to you.

“I imagined it. I wrote it. But I guess I never thought I'd see it.”

— Ken Follett, The Pillars of the Earth

Abstract

Obsessive-compulsive disorder (OCD)[1] is a chronic and debilitating neuropsychiatric condition affecting 1 – 3% of the world population. The condition is characterized by two main symptom cohorts namely recurrent, distressing and intrusive thoughts (obsessions) and seemingly purposeless rigid and repetitive behaviors (compulsions). Furthermore, OCD patients present with notable cognitive rigidity and behavioral inflexibility. This is characterized by deficits in reward and punishment feedback processing which are regulated by dopaminergic and serotonergic neurotransmission. In addition, OCD causes remarkable distress and severely impairs almost every aspect of an individual's life. Although chronic high-dose selective serotonin reuptake inhibitor (SSRI)[2] intervention is regarded as the first-line pharmacological treatment for OCD, 40 – 60% of patients remain symptomatic and full remission is usually not achieved.

Over the preceding decades, rodent models of OCD have helped us to further our understanding of the disorder and its treatment. However, these are characterized by noteworthy limitations for example, they are exceptionally time-consuming and expensive and have a low-throughput capacity. In this regard, zebrafish (*Danio rerio*) have emerged as an alternative framework for the pre-clinical study of neurobehavioral disorders. The most important advantages of applying zebrafish include 1) their broad homology to rodent and human neurobiology, presenting with almost fully conserved dopaminergic and serotonergic systems, 2) their highly social nature which enables them to shoal and seek out conspecifics (same species fish), 3) their ability to clearly distinguish between colors, which facilitates color-dependent learning, and 4) their sophisticated sensory, motor and motivational systems that are well-suited for experiments into associative learning.

As such, the present investigation was conceptualized to provide a foundation for establishing a novel, high-throughput screening test for anti-compulsive drug action using zebrafish as a model organism by exploiting their natural reward-seeking behavior such as elevated motivational drive to engage in social interaction in a cue-reward contingency learning paradigm. Considering the current theories describing the roles of dopamine and serotonin in OCD, we aimed to induce compulsive-like persistence with the dopaminergic agonist, apomorphine, and further investigated if such persistence, if present, would be reversed by chronic escitalopram, an SSRI.

Seven groups of zebrafish (n = 6 per group) were exposed for 24 days (1 hour per day) to either control (normal tank water), apomorphine (50 or 100 μg/L), escitalopram (500 or 1000 μg/L) or a combination

[1] obsessive-compulsive disorder
[2] selective serotonin reuptake inhibitor

(A100/E500 or A100/E1000 μg/L). Cue-reward learning was assessed over three phases i.e. *Phase 1* (contingency learning), *Phase 2* (dissociative testing), and *Phase 3* (re-associative testing).

We demonstrate that 1) sight of social conspecifics is an inadequate reinforcer of contingency learning under circumstances of motivational conflict, 2) dopaminergic and serotonergic intervention lessens the importance of an aversive stimulus, increasing the motivational valence of social reward, 3) while serotonergic intervention maintains reward-directed behavior, high-dose dopaminergic intervention bolsters cue-directed responses and 4) high-dose escitalopram reverses apomorphine-induced behavioral inflexibility. The results reported here are supportive of current dopamine-serotonin opponency theories and confirm that zebrafish may be a potentially useful species in which to emulate compulsive-like behaviors.

Although compulsive-like persistence toward habitual, cue-directed behavior was not induced by either dose of apomorphine, fish exposed to high-dose apomorphine present with behavior more akin to behavioral inflexibility compared to their counterparts in all other exposure groups. This was reversed by chronic high, but not lower dose escitalopram, a finding that is supportive of current dopamine-serotonin opponency theory. The apparent aversion demonstrated by drug-naive subjects to the color red was unexpected and has complicated the interpretation of our results. Indeed, it is likely that the use of a more-preferred color in this population may have yielded a more robust result, a possibility that we will investigate in future.

In conclusion, typical theories of neurotransmitter involvement in OCD[1] for example imbalanced crosstalk between dopamine and serotonin amongst others, provides a useful background for investigating compulsive-like behaviors in animals. Not only do the findings presented here confirm the viability of zebrafish as a model species in which to study the neurobiological and cognitive processes underlying dopamine-serotonin interactions under circumstances of motivational conflict, it also provides valuable direction for future endeavors toward the development of a novel screening framework that is sensitive for anti-compulsive drug action.

Keywords: dopamine; inflexibility; learning; obsessive-compulsive disorder; opponency; serotonin; zebrafish

[1] obsessive-compulsive disorder

Congress Proceedings

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Table of Contents

1 Introduction

1.1 Thesis Layout

The current dissertation is compiled in article format, as specified and approved by the North-West University (NWU)[1], Potchefstroom, South Africa. As such, the main body of the dissertation is presented as a single manuscript (Chapter 3) with the experimental work, results and main findings presented in the form of a journal article that has been accepted for publication in an accredited international, peer-reviewed neuroscience journal, i.e. *Behavioural Brain Research.*

Chapter 1 presents a concise description of the project problem statement, study questions, aims, project layout, hypothesis and expected outcomes. Chapter 2 comprises the applicable literature background to support the current project, while Chapter 3 will report the key findings of the investigation in the form of a scientific manuscript. Chapter 4 encapsulates the complete project. Addendum A contains the letters of permission of co-authors for subjecting the manuscript for assessment purposes. Addendum B contains the confirmation of acceptance to *Behavioural Brain Research* as well as the rebuttal to the reviewer comments from the first and second rounds of review. Addendum C contains the supplementary tables to Chapter 3. Addendum D contains additional detail on the methods followed during the execution of the project and Addendum E contains the published article in *Behavioural Brain Research*.

As there were no requirements from *Behavioural Brain Research* regarding a specific referencing style, we applied the style prescribed by the *European Journal of Neuroscience* throughout the dissertation as it is concise and easy to work with.

The dissertation is presented in US[2] English.

[1] North-West University
[2] United States

1.2 Problem Statement

Animal models are invaluable tools that can be applied to elucidate the etiopathological mechanisms underlying clinical illness (Pittenger *et al.*, 2019). Indeed, these models are also pivotal instruments for studying complex human neuropsychiatric disorders, e.g. obsessive-compulsive disorder (OCD)[1], performing pre-clinical drug evaluations and for simulating signs and symptoms of the applicable disorder with the intention of clarifying the underlying mechanisms (Szechtman *et al.*, 2017; Pittenger *et al.*, 2019). Historically, most animal models have been confined to the realms of rodent or other mammals that, despite their remarkable resemblance of human anatomy and physiology, are characterized by noteworthy disadvantages (D'Amico *et al.*, 2015; Fontana *et al.*, 2018). These include that they are often time-consuming, expensive and, if not performed in large and elaborate facilities, have a low-throughput capacity (Champagne *et al.*, 2010). This prompted recent research to expand its horizons to other vertebrates, i.e. bony fish (class *Osteichthyes*) to broaden the field of discovery (Maximino *et al.*, 2015). Over the preceding decade, zebrafish (*Danio rerio*) have become invaluable as a complementary model in behavioral pharmacology (Bailey *et al.*, 2015). As such, when considering the history of and our experience with the deer mouse model of OCD (Scheepers *et al.*, 2018), the current investigation aims to expand on the body of literature pertaining to persistent behavioral phenotypes by exploring a novel pharmacological model of compulsive-like behavior in zebrafish.

Briefly, OCD is a distressing and disabling neuropsychiatric condition that presents various challenges for neuroscience (Hoffman & Cano-Ramirez, 2018; Robbins *et al.*, 2019). Most individuals experience invasive thoughts or irresistible behavioral impulses. However, when these tendencies meet certain criteria, e.g. becoming excessive to the point of functional impairment, patients are diagnosed with OCD (Seli *et al.*, 2016; Seli *et al.*, 2017; Robbins *et al.*, 2019). According to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM[2]-5), obsessions are persistent, recurring thoughts, images or urges that are intrusive and unwanted, while compulsions are mental acts or repetitive behaviors that an individual feels compelled to perform, often but not always in response to an obsession (American Psychiatric Association, 2013; Hirschtritt *et al.*, 2017; Hoffman & Cano-Ramirez, 2018). Additionally, compulsions are often ostensibly executed to alleviate the anxiety arising from the obsessions (Hoffman & Cano-Ramirez, 2018; Winter *et al.*, 2018).

OCD is classified among the top ten worldwide causes of disability (Kessler *et al.*, 2005). The condition interferes with most aspects of the everyday life of an individual, impairing normal mental and social

[1] obsessive-compulsive disorder
[2] Diagnostic and Statistical Manual of Mental Disorders

functioning and disrupting family life (Coluccia *et al.*, 2016; Hirschtritt *et al.*, 2017). While it is estimated that 1 in 40 people is diagnosed with OCD[1] (Ruscio *et al.*, 2010; Hirschtritt *et al.*, 2017; Abramovitch *et al.*, 2019), existing OCD treatments are only moderately effective (Ahmari, 2016; Atmaca, 2016; Hirschtritt *et al.*, 2017; Robbins *et al.*, 2019). The mainstay of treatment comprises chronic treatment with the serotonin reuptake inhibitors (SRIs)[2], e.g. clomipramine, selective serotonin reuptake inhibitors (SSRIs)[3], e.g. escitalopram, and cognitive behavioral therapy (CBT)[4], including exposure response prevention (ERP)[5] (Atmaca, 2016; Hirschtritt *et al.*, 2017; Robbins *et al.*, 2019). Although SSRIs are regarded as the first-line pharmacological treatment for OCD, 40 – 60% of patients continue to experience symptoms, with full remission usually not achieved (Atmaca, 2016; Hirschtritt *et al.*, 2017; Robbins *et al.*, 2019). In such cases, increasing the dose of the SRI/SSRI, switching to a different SRI/SSRI or augmentation with additional medications may be useful (Fineberg & Craig, 2007; Atmaca, 2016; Hirschtritt *et al.*, 2017). Indeed, it has been reported that an estimated 11 – 33% of patients that do not respond to the first SRI, display clinically meaningful response to a second monotherapeutic drug choice, with a decreasing probability of therapeutic success for subsequent changes (Fineberg & Craig, 2007). One of the most commonly used augmentation strategies is to combine low-dose anti-dopaminergic intervention with an SSRI (McDougle *et al.*, 2000; Hollander *et al.*, 2003; Erzegovesi *et al.*, 2005; Denys *et al.*, 2013; Hirschtritt *et al.*, 2017; Murray *et al.*, 2019). In fact, a meta-analysis of randomized placebo-controlled trials reported significant efficacy of low-dose anti-dopaminergic augmentation of SSRIs in some patients with treatment-refractory OCD (Dold *et al.*, 2015). Treatment alternatives, including neurosurgical interventions, e.g. deep brain stimulation (DBS)[6], may also be considered in some refractory cases of OCD (Huff *et al.*, 2010; Zike *et al.*, 2017).

In line with these treatment strategies, it has been suggested that the serotonergic and dopaminergic neurotransmitter systems are involved in the pathogenesis of OCD (Abramowitz *et al.*, 2009; Markarian *et al.*, 2010). Moreover, as OCD is hypothesized to result from dysfunctional reward processing (Figee *et al.*, 2011), i.e. the lack of adequate closure following the reward of task completion, Daw *et al.* (2002) suggested serotonin to act as a motivational opponent to dopamine. Specifically, dopamine facilitates and promotes reward-seeking behavior, whereas serotonin is believed to be involved in the processing of negative affect and behavioral inhibition (Boureau &

[1] obsessive-compulsive disorder
[2] serotonin reuptake inhibitors
[3] selective serotonin reuptake inhibitors
[4] cognitive behavioral therapy
[5] exposure and response prevention
[6] deep brain stimulation

Dayan, 2011). Briefly, during the experience of reward, dopaminergic signaling is evoked (Arias-Carrión *et al.*, 2010). However, during the experience of unpleasant events, dopamine release is inhibited (Husted *et al.*, 2006). Further, persistent reward-seeking behaviors have been correlated with an increased cortico-striatal dopaminergic tone, pointing to deficits in reward-feedback processing following repeated experience of the same reward; this is believed to promulgate excessive engagement in reward-seeking persistence (Wise, 2004; Schultz, 2013). Considering that OCD[1] has previously been conceptualized as a condition akin to behavioral addiction, and that patients often fail to realize and appreciate the reward of task completion (Hinds *et al.*, 2012; Morein-Zamir *et al.*, 2013), it can be hypothesized that bolstered dopaminergic signaling will induce compulsive reward-seeking behavior that would be reduced or even prevented by the co-administration of serotonergic agents as per the opponency theory describing the functional interactions between dopamine and serotonin (Daw *et al.*, 2002). This theory is the basis of some current mammalian models of compulsion, i.e. compulsive checking in rats (Szechtman *et al.*, 2001; Tucci *et al.*, 2013) and will also constitute a primary foundation of the present work in which we will utilize apomorphine, a dopamine D_1/D_2-like receptor agonist (Sukhanov *et al.*, 2014) in an attempt to induce compulsive-like reward-seeking persistence and behavioral inflexibility, akin to that observed in OCD (Gruner & Pittenger, 2017).

As alluded to above, an almost exclusive focus on mammalian model species for the investigation of neuropsychiatric conditions is problematic (Ablain & Zon, 2013; Maximino *et al.*, 2015; Fontana *et al.*, 2018). As such, zebrafish have emerged as an attractive alternative avenue for investigation (Champagne *et al.*, 2010; Saif *et al.*, 2013; Stewart *et al.*, 2014). Zebrafish possess many inherent advantages, including their rapid development and prolific nature (Goldsmith, 2004; Champagne *et al.*, 2010), prolonged lifespan (Kalueff *et al.*, 2014), ease of drug administration (McGrath & Li, 2008; Stewart *et al.*, 2011; Kalueff *et al.*, 2014), and the facilitation of cost-effective high-throughput results-driven screening (Levin, 2011). Importantly, with regards to the focus on the current investigation, zebrafish also demonstrate remarkable homology to mammals with respect to the involvement of the major neurotransmitter systems and the degree to which its physiology overlaps with that of mammals (Kalueff *et al.*, 2014). Further, their sophisticated sensory, motor and motivational systems are well-suited for experiments into associative learning (Blaser & Vira, 2014), an aspect that will be exploited in this investigation. Indeed, it has previously been demonstrated that zebrafish are capable of associative and non-associative tasks, including appetitive choice discrimination and reversal learning (Al-Imari & Gerlai, 2008; Daggett *et al.*, 2019). Furthermore, zebrafish are shoaling animals

[1] obsessive-compulsive disorder

that seek out their conspecifics (same species fish), an aspect of their behavior that can be applied as a reinforcer for associative learning (Al-Imari & Gerlai, 2008; Saif *et al.*, 2013; Daggett *et al.*, 2019). Last, adult zebrafish are capable of being conditioned to color-dependent learning, as they clearly distinguish between different colors (Ahmad & Richardson, 2013). As such, the current study will leverage all of the aforementioned characteristics of zebrafish in a modified version of a T-maze used in rodent studies (Yadin *et al.*, 1991) in an attempt to induce repetitive and persistent one-arm choice. Importantly, in translating the concept of spontaneous alternation—which reflects the natural tendency of all animals to freely explore a novel environment and thereby being representative of behavioral flexibility—to zebrafish, reductions in spontaneous alternation and compulsive choice of a single arm can be measured (D'Amico *et al.*, 2015). This concept forms the major methodological focus of the current investigation.

Taken together, no reliable high-throughput screening test for anti-compulsive drug action exists as all currently applied animal models of compulsivity are restricted to mammalian research. Therefore, the current study will attempt to employ the robust sensory and cognitive abilities of zebrafish to investigate whether this model organism may represent a novel avenue for future *in vivo* behavioral and neurobiological investigations into persistent behaviors reminiscent of OCD[1]. Indeed, to further our understanding of the persistent behavioral phenotypes observed in OCD and to accelerate the discovery and development of novel pharmacotherapeutic agents, the addition of a cost-effective pre-clinical research paradigm to the current arsenal of animal models may be particularly useful.

[1] obsessive-compulsive disorder

1.3 Study Questions

1) Will zebrafish, following habituation to a T-maze, seek out a social reward (an unreachable shoal of conspecific fish) presented in one arm of the T-maze? Furthermore, by employing a cue-conditioned learning platform, will zebrafish be able to associate the presentation of the reward (sight of social conspecifics) with a visual cue (red cue card), i.e. demonstrating associative learning ability?
2) Following repeated exposure to the co-presented cue and reward as in (1), will zebrafish, in the absence of reward presentation, display spontaneous exploratory behavior in the T-maze by not overly seeking out the reward where it was conditioned to be presented (proximally to the red cue card), thereby demonstrating dissociative ability that is representative of cognitive flexibility?
3) Will zebrafish continue to display reward-orientated, as opposed to cue-directed behavior upon the reintroduction of the reward in the previously non-cued arm, thereby demonstrating re-associative ability?
4) Will chronic exposure to a dopaminergic agonist, i.e. apomorphine, a non-selective dopamine D_1/D_2 receptor agonist (Sukhanov *et al.*, 2014), bolster the acquisition of the cue-reward contingency in (1)? Further, will apomorphine induce a behavioral trait reminiscent of behavioral inflexibility as emulated by persistent cue-directed behaviors throughout (2) – (3)?
5) What will the effect of chronic exposure to the SSRI[1], escitalopram (Braestrup & Sanchez, 2004) alone, be on the behavior of zebrafish as per the conditions listed in (1) – (3).
6) How will escitalopram influence the observed behaviors under the conditions referred to in (4)?
7) Does the pharmacological manipulation of the natural reward-seeking behaviors of zebrafish hold promise as a potential model of compulsive-like persistent behaviors?

[1] selective serotonin reuptake inhibitor

1.4 Project Aims

Following from the introduction and problem statement, the broad aim of this study will be to develop a novel screening test for anti-compulsive drug action that presents with good face and predictive validity. We aim to do so by pharmacologically inducing inflexible behavior in zebrafish that will be reminiscent of clinical compulsivity and determining whether such behavior is attenuated by a known anti-compulsive drug. We will do so by:

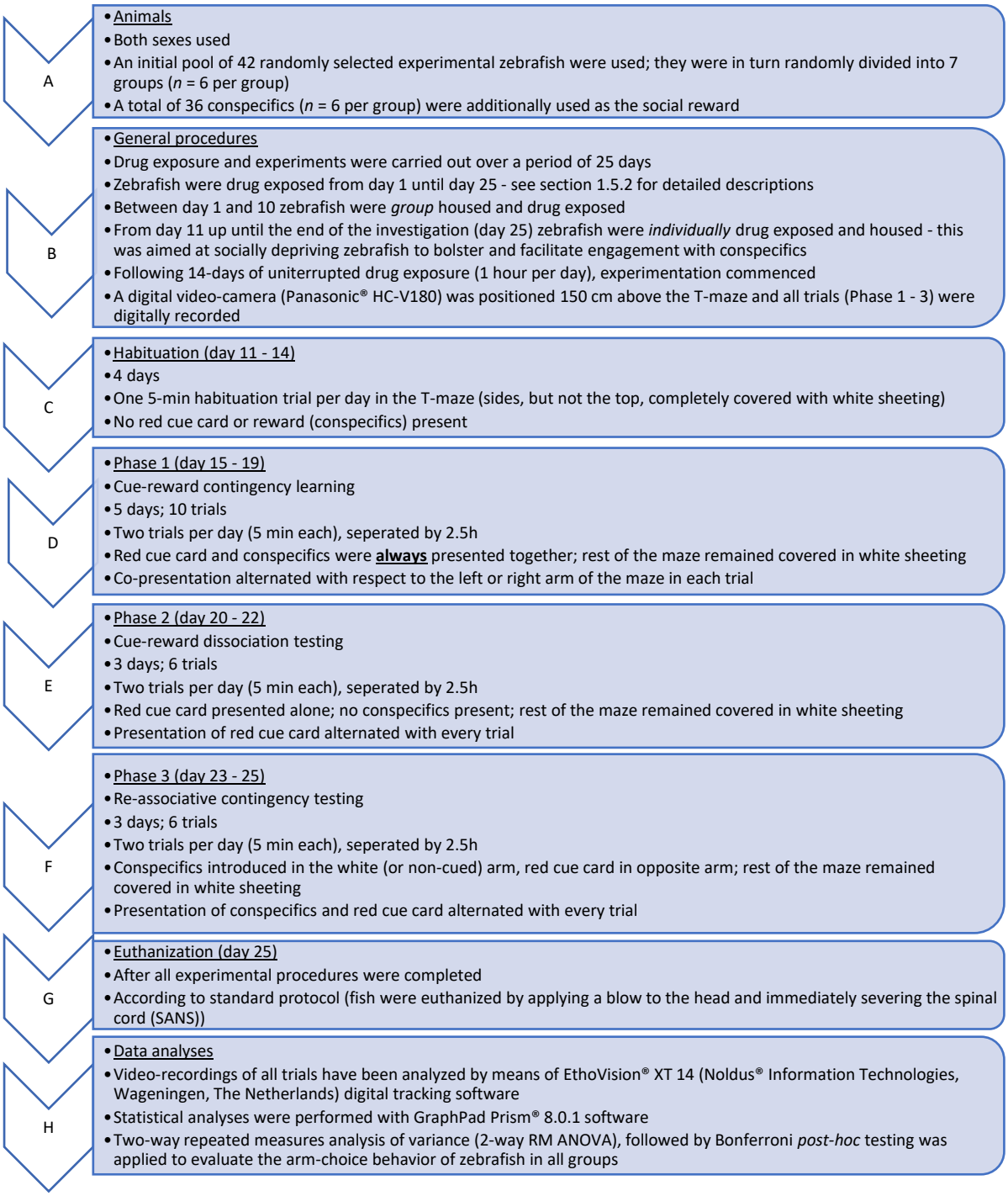
1) Observing the natural behavior of zebrafish (group 1; *n* = 6) in a T-maze under three distinct conditions. These will in future be referred to as Phases 1 - 3, and are described as follows:
    - Phase 1 (cue-reward contingency *learning* or associative learning): This phase consists of 10 individual 5-minute trials spaced over 5 days (2 trails per day, separated by 2.5 hours; Al-Imari & Gerlai, 2008). During this phase a social reward (shoal of unreachable conspecifics) is visually presented adjacent to one arm of the T-maze together with a red cue card (zebrafish can clearly distinguish between different colors; Ahmad & Richardson, 2013). The arm in which this reward and cue combination is presented, alternates with each successive trial (i.e. left, right, left, right).
    - Phase 2 (cue-reward dissociative *testing*): This phase consists of 6 individual 5-minute trials spaced over 3 days (2 trails per day, separated by 2.5 hours). During this phase, no reward is presented. Thus, only the red cue card is presented in alternating arms of the T-maze during each trial.
    - Phase 3 (re-associative contingency *testing*): This phase consists of 6 individual 5-minute trials spaced over 3 days (2 trails per day, separated by 2.5 hours). During this phase, the social reward is again presented, but in the previously non-cued arm, i.e. the white (or non)-colored arm.
2) Assessing the effect of chronic drug exposure (beginning 14 days before and continuing for 11 days during experimentation) to a lower and higher (50 and 100 μg/L; groups 2 and 3; *n* = 6 per group; see paragraph 1.5.2) concentration of apomorphine on the behavior of zebrafish in the experimental conditions specified for Phases 1 - 3. Only the concentration which best induces persistent cue-directed behaviors in Phase 3 will be selected for combined intervention with escitalopram.
3) Assessing the effect of chronic drug exposure (beginning 14 days before, and continuing for 11 days during experimentation) to a lower and higher (500 and 1000 μg/L; groups 4 and 5; *n* = 6 per group; see paragraph 1.5.2) concentration of escitalopram on the behavior of zebrafish in the experimental conditions specified for Phases 1 – 3.

4) Assessing the effect of chronic drug exposure (beginning 14 days before, and continuing for 11 days during experimentation) to a combination of apomorphine at a concentration selected in (2) and escitalopram at both concentrations stated in (3) (see paragraph 1.5.2) in the experimental conditions specified for Phases 1 – 3.

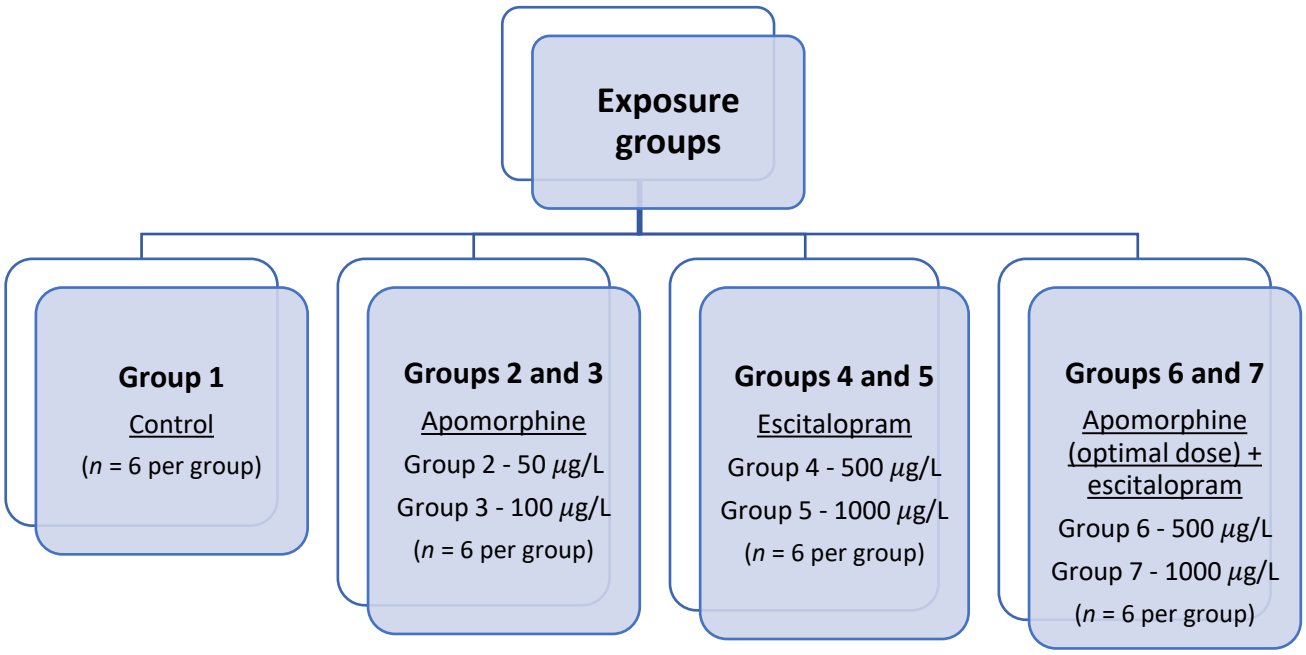
1.5 Project Layout

To address the study questions and aims, the current investigation was designed as follows:

1.5.1 General Procedures for all Zebrafish:



1.5.2 Detailed Description of Exposure Groups



1.6 Study Hypothesis and Expected Results

We hypothesize that cue-conditioned associative learning can successfully be established and quantified in zebrafish for exploitation as a potential framework in which to study the behavioral manifestations of dysfunctional reward-based learning and to characterize changes in goal-directed behavior. Further, we hypothesize that by bolstering dopaminergic neurotransmission, persistent choice for the cue only will be induced following dissociation of the cue-reward contingency, thereby representing behavioral inflexibility akin to compulsive-like persistence. Moreover, we hypothesize that upon representation of the reward in a non-cued context, fish subjected to dopaminergic potentiation during the initial cue-reward contingency learning phase, will persist in cue-directed behaviors, which will furthermore be affirmative of such inflexible responses. As such, the following study outcomes are expected:

1) That control-treated zebrafish will not only successfully acquire knowledge of the cue-reward contingency (Phase 1), but that they will successfully value and respond to a dissociation of the cue and reward during Phase 2, as well as the re-association between the cue and a previously non-cued context (Phase 3), thereby responding in a flexible manner to the absence or presence of the reward itself;
2) That apomorphine exposure alone will not only bolster cue-directed arm choice during Phase 1, but also during Phases 2 and 3, thereby inducing persistent and inflexible behavior;
3) That escitalopram exposure alone will, given its effect to induce an increased serotonergic tone and thereby counteract normal dopaminergic processes, induce behavior akin to that of control treated zebrafish under circumstances of associative learning (Phase 1), dissociative testing (Phase 2) and re-associative testing (Phase 3);
4) That, according to the theory of behavioral opponency (Daw *et al.*, 2002), co-exposure to escitalopram and apomorphine will prevent the effects of apomorphine exposure as observed in outcome (2), while eliciting behavioral responses analogous to that observed in control-treated animals; and
5) Given the similar application of comparable experimental procedures as those applied in rodent studies, we expect zebrafish to serve as a viable extension of mammalian work in investigations of pharmacologically induced compulsive-like behaviors related to the neurobiological mechanisms that may contribute to such behaviors.

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2 Literature Review

2.1 Clinical Overview of Obsessive-Compulsive Disorder

2.1.1 Description, Diagnosis, Classification and Comorbidity

Obsessive-compulsive disorder (OCD)[1] is a severe and disabling neuropsychiatric condition that is characterized by recurrent and persistent thoughts, mental images or impulses that are intrusive and unwanted, collectively known as obsessions, which result in noticeable anxiety or distress (American Psychiatric Association, 2013; Abramovitch & Cooperman, 2015). Subsequently, in an attempt to alleviate the level of anxiety experienced, rigid and repetitive overt (visible compulsive behaviors) or covert (repetitive compulsive cognitive patterns) symptoms are often experienced (American Psychiatric Association, 2013; Stewart, 2016; Robbins *et al.*, 2019). However, relief from the anxiety is only temporary, resulting in such compulsions being negatively reinforced (Pauls *et al.*, 2014; Abramowitz & Jacoby, 2015).

Although obsessions and compulsions are the hallmark symptoms of OCD, their content are marked by substantial heterogeneity and therefore considerable thematic diversity (Abramowitz *et al.*, 2009; Pauls *et al.*, 2014; Olatunji *et al.*, 2019). For example, obsessions may include chronic doubting, fear of contamination, thoughts of harm occurring to a loved one, preoccupation with symmetry or aggressive impulses. Conversely, compulsions include constant checking, excessive hand washing, arranging items symmetrically or ritualistic avoidance behaviors (Chamberlain *et al.*, 2005; Abramowitz *et al.*, 2010; Stewart, 2016). Research supports the notion that particular kinds of obsessions co-manifest with specific compulsions to represent five main subtypes of OCD *viz*. 1) fears of contamination associated with persistent washing rituals, 2) harm prevention obsessions associated with checking and reassurance-seeking, 3) symmetry obsessions and ordering and counting rituals, 4) pure obsessions (of a sexual, religious or aggressive nature) in the absence of overt compulsions, and 5) a hoarding phenotype characterized by excessive collecting behavior (Abramowitz *et al.*, 2009; Abramowitz *et al.*, 2010; Markarian *et al.*, 2010; Abramovitch *et al.*, 2015; Rowsell & Francis, 2015; Schwartzman *et al.*, 2017).

Like most psychiatric disorders, a diagnosis of OCD is based on a clinical assessment (Hirschtritt *et al.*, 2017). As alluded to above, the defining features of OCD include the presence of obsessions and compulsions, of which either symptom class or a combination of both is considered a requirement for diagnosis (American Psychiatric Association, 2013). A second criterion is that obsessions and compulsions must be time-consuming (occupying more than one hour per day), and therefore cause

[1] obsessive-compulsive disorder

remarkable distress and/or significantly interfere with daily functioning. Third, the presence of symptoms must not be attributable to a direct result of a substance use or medical condition, and last, the symptoms must not be related to another medical condition (American Psychiatric Association, 2013; Stewart, 2016; Hirschtritt *et al.*, 2017). The presentation of OCD[1] is often complicated by comorbidity with a number of other psychiatric disorders, i.e. major depressive disorder, bipolar disorder, and anxiety disorders (Adam *et al.*, 2012; Brakoulias *et al.*, 2017). Several well-validated screening tools and assessment tools have been developed to aid in the assessment and classification of OCD symptoms (Grabill *et al.*, 2008; Overduin & Furnham, 2012). Among these, the gold-standard is the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS)[2], given its proven reliability, extensive use in clinical research and robust internal construct validity (Goodman *et al.*, 1989; Grabill *et al.*, 2008; Hirschtritt *et al.*, 2017).

OCD has since the fifth publication of the Diagnostic and Statistical Manual of Mental Disorders (DSM[3]-5) been classified as the archetype disorder in a new category of obsessive-compulsive and related disorders (OCRDs[4]; American Psychiatric Association, 2013; Abramovitch *et al.*, 2015; Rowsell & Francis, 2015; Hirschtritt *et al.*, 2017); this as opposed to its previous classification as an anxiety disorder (American Psychiatric Association, 2000). This new category includes other conditions related to persistent and repetitive symptomologies, i.e. excessive hair-pulling (trichotillomania), skin picking disorder (excoriation), body dysmorphic disorder, and hoarding disorder (American Psychiatric Association, 2013; Abramowitz & Jacoby, 2015; Gillan *et al.*, 2017). The fact that OCD is no longer regarded as an anxiety disorder is noteworthy, as the current body of research suggests OCD is more consistently comorbid with anxiety disorders (up to 5 to 10 times more often) than the other OCRDs (Abramowitz & Jacoby, 2015). As such, many authors question the exclusion of OCD from the other anxiety disorders and deliberation about its clinical conceptualization continues (Bartz & Hollander, 2006). That said, on a phenotypical level, OCD is in fact more akin to the symptomology and cognitive psychology characteristic of other OCRDs, while comorbidity on its own is not the only factor for consideration in terms of disease classification (du Toit *et al.*, 2001; Stein *et al.*, 2016).

2.1.2 Epidemiology and Impact

OCD symptoms are highly ubiquitous (Stewart, 2016). In the United States the 12-month prevalence of OCD is 1.2% (Mathes *et al.*, 2019) with a similar international prevalence of 1.1 – 1.8% (Coluccia *et al.*, 2015). OCD affects 2 – 4% of children and adolescents; however, the disorder remains undetected

[1] obsessive-compulsive disorder
[2] Yale-Brown Obsessive-Compulsive Scale
[3] Diagnostic and Statistical Manual of Mental Disorders
[4] obsessive-compulsive and related disorders

and untreated in as many as 90% of suspected cases in this age group (Barzilay *et al.*, 2019), likely due to the shame that patients experience when disclosing their symptoms to family members, clinicians or caregivers (Neal-Barnett & Mendelson, 2003; Ahmed *et al.*, 2015; Stewart, 2016; Wheaton *et al.*, 2016; Barzilay *et al.*, 2019). The mean age of onset of OCD[1] is 19.5 years (Ruscio *et al.*, 2010; American Psychiatric Association, 2013; Abramowitz & Jacoby, 2015). However, up to 25% of cases manifest by the age of 14 years (American Psychiatric Association, 2013), while a first episode of OCD is rarely documented in men and women over the age of 35 years (Ruscio *et al.*, 2010; American Psychiatric Association, 2013). Prevalence rates decrease in the population over the age of 65 years (Fireman *et al.*, 2001; Wang *et al.*, 2009). Males tend to present an earlier age of onset, with the majority of OCD cases in women diagnosed during adolescence (Ruscio *et al.*, 2010; American Psychiatric Association, 2013; Barzilay *et al.*, 2019; Mathes *et al.*, 2019). Indeed, 25% of first-episode OCD cases in males are recorded before the age of 10 years (Ruscio *et al.*, 2010; American Psychiatric Association, 2013; Stewart, 2016). Furthermore, while men are more likely to present with symptoms in the symmetry/ordering and intrusive thoughts dimensions (Lochner & Stein, 2001; Mathes *et al.*, 2019), women are more frequently diagnosed with symptoms related to the contamination/washing symptom dimension (Lochner & Stein, 2001; Barzilay *et al.*, 2019; Mathes *et al.*, 2019). Data also suggest a correlation between OCD symptom fluctuations and changes in the female menstrual cycle, i.e. menstrual phases, pregnancy and menopause (Lochner *et al.*, 2004; Guglielmi *et al.*, 2014), where symptoms have been shown to aggravate during these significant events in the female hormonal cycle.

Although OCD is regarded as the tenth most disabling illness worldwide (Kessler *et al.*, 2007; Ahmed *et al.*, 2015), patients often endure their symptoms for up to 10 years before receiving a conclusive OCD diagnosis (Wheaton *et al.*, 2016). As briefly referred to above, this is likely due to the fact that patients are ashamed of their symptoms which prevent them from disclosing their concerns to clinicians (Neal-Barnett & Mendelson, 2003; Ahmed *et al.*, 2015; Stewart, 2016; Wheaton *et al.*, 2016; Barzilay *et al.*, 2019). Due to the nature of its symptom presentation, OCD ultimately results in a diminished quality of life across several domains, including social relationships, academic and work performance, financial status, and personal wellbeing (American Psychiatric Association, 2013; Macy *et al.*, 2013; Jacoby *et al.*, 2014; Coluccia *et al.*, 2015; Coluccia *et al.*, 2016; Schwartzman *et al.*, 2017). Patients with OCD often present with reduced quality of life compared to individuals diagnosed with other psychiatric conditions (Bobes *et al.*, 2001; Huppert *et al.*, 2009; Schwartzman *et al.*, 2017). For example, obsessions about symmetry can derail the timely completion of work or school projects resulting in job loss or academic failure. Obsessions about harm may lead to avoidance of

[1] obsessive-compulsive disorder

relationships as these relationships may feel hazardous (Abramowitz *et al.*, 2009; American Psychiatric Association, 2013). Furthermore, individuals with OCD[1] will often try to impose certain prohibitions and rules on family members, causing significant strain on family relationships (Lee *et al.*, 2015; Lebowitz *et al.*, 2016). Additionally, suicidal thoughts occur at some point in as many as half of those suffering from OCD, while up to a quarter of patients attempt suicide (American Psychiatric Association, 2013; Stewart, 2016). Considering these facts, it is clear that OCD is a devastating disorder impacting the everyday life of patients.

2.1.3 Treatment of OCD

Treatment algorithms for OCD are well-established yet still somewhat inadequate, comprising three major classes of intervention (Atmaca, 2016; Hirschtritt *et al.*, 2017), i.e. pharmacotherapy and psychological and neurosurgical intervention. Briefly, psychological interventions such as cognitive behavioral therapy (CBT)[2] includes strategies such as exposure and response prevention (ERP)[3] which are aimed at assisting patients to realize the unrealistic nature of their obsessions and to help them appreciate the futility of engaging in compulsive neutralizing rituals (McLean *et al.*, 2015). Neurosurgical techniques, including deep brain stimulation (DBS)[4], are reserved for highly treatment refractory patients, and involves the electrical stimulation of brain regions implicated in the manifestation of obsessive-compulsive symptoms (Kohl *et al.*, 2014). However, since the present investigation describes the conceptualization of a pharmacological model of compulsive-like persistence, closer attention will be afforded to the pharmacological treatment of the condition. For an in-depth review of the different cognitive and surgical therapeutic strategies, please refer to Jónsson *et al.* (2015) and Kohl *et al.* (2014).

The mainstay of the pharmacological treatment of OCD revolves around drugs that increase intrasynaptic serotonin concentrations, i.e. the tricyclic antidepressant (TCA)[5] drug clomipramine which exerts it's anti-obsessional effect as a serotonin reuptake inhibitor (SRI)[6] and the selective serotonin reuptake inhibitors (SSRIs)[7], such as escitalopram, fluoxetine and sertraline (Fineberg & Craig, 2007; Fineberg *et al.*, 2013; Atmaca, 2016; Stewart, 2016; Hirschtritt *et al.*, 2017). These can be administered either as monotherapy, or in combination with other interventions, e.g. low-dose anti-dopaminergic agents or psychological techniques in the case of refractory symptoms (Albert *et al.*,

[1] obsessive-compulsive disorder
[2] cognitive behavioral therapy
[3] exposure and response prevention
[4] deep brain stimulation
[5] tricyclic antidepressant
[6] serotonin reuptake inhibitor
[7] selective serotonin reuptake inhibitors

2018). Although the use of SSRIs[1] is preferred due to their favorable side-effect profile (Fineberg & Craig, 2007; Pittenger & Bloch, 2014; Seibell & Hollander, 2014; Marazziti *et al.*, 2016; Hirschtritt *et al.*, 2017), both clomipramine and SSRIs potently inhibit the action of the serotonin reuptake transporter (SERT)[2] expressed on the terminal ends of presynaptic serotonergic neurons (Goddard *et al.*, 2008). Further, as opposed to clomipramine, SSRIs are indicated for the treatment of OCD[3] in both children under the age of 12 years and adults (Fineberg & Craig, 2007). Considering the availability of numerous SSRIs, meta-analyses of placebo-controlled clinical trials revealed similar efficacy among the different compounds (Ackerman & Greenland, 2002; Geller *et al.*, 2003; Soomro *et al.*, 2008; Abudy *et al.*, 2011; Pittenger & Bloch, 2014; Fineberg *et al.*, 2015; Atmaca, 2016; Skapinakis *et al.*, 2016; Hirschtritt *et al.*, 2017). However, it is likely that individuals will still respond better to some SSRIs than others and as such, individualized approaches are important (Abudy *et al.*, 2011; Skapinakis *et al.*, 2016). Irrespective of the drug employed, treatment should be applied at the highest tolerated dose for a minimum of 8 – 12 weeks without interruption (Atmaca, 2016; Hirschtritt *et al.*, 2017). In the case of satisfactory symptom attenuation, treatment periods exceeding a year or even longer are typically advised (Hirschtritt *et al.*, 2017). In terms of onset of action, OCD symptoms display a delayed response to SRI[4]/SSRI treatment when compared to other psychiatric disorders, requiring at least 8 or more weeks before treatment response is noted (Fineberg & Craig, 2007; Goddard *et al.*, 2008; Markarian *et al.*, 2010; Atmaca, 2016). It is therefore not advised that treatment be interrupted or modified within the first three months following initiation (Fineberg & Craig, 2007; Pittenger & Bloch, 2014; Atmaca, 2016; Skapinakis *et al.*, 2016; Hirschtritt *et al.*, 2017).

Even after applying the most intensive SRI/SSRI regimens, a considerable number of patients remain refractory to treatment. Indeed, 40 – 60% of patients still present with lingering symptoms after chronically using SSRIs (Bloch *et al.*, 2006; Abudy *et al.*, 2011; Veale *et al.*, 2014; Atmaca, 2016; Hirschtritt *et al.*, 2017; Robbins *et al.*, 2019). In such cases several approaches are followed to improve therapeutic outcomes, including increasing the dose of the current SRI/SSRI used, switching to a different SRI/SSRI or augmenting such therapy with low-dose anti-dopaminergic agents (Fineberg & Craig, 2007; Atmaca, 2016; Hirschtritt *et al.*, 2017) the latter of which, although lacking efficacy if used as monotherapy, have been proven useful in some cases of SRI/SSRI treatment resistance (McDougle *et al.*, 2000; Abudy *et al.*, 2011; Hirschtritt *et al.*, 2017; Murray *et al.*, 2019). Although both typical and atypical anti-dopaminergic agents have been shown to be effective, meta-analyses support the use of especially risperidone for this purpose (Dold *et al.*, 2015; Dougherty *et al.*, 2018). That said, in up to

[1] selective serotonin reuptake inhibitors
[2] serotonin reuptake transporter
[3] obsessive-compulsive disorder
[4] serotonin reuptake inhibitor

15% of patients, OCD[1] persists as a chronic condition that exacerbates over time (Simpson *et al.*, 2004; Van Oudheusden *et al.*, 2018). Another significant obstacle which undermines efforts to ensure optimal treatment response is patient adherence. It is believed that up to 40% of patients interrupt treatment within the first three months of therapy, likely due to the nature of the side-effects combined with a lack of noticeable symptom attenuation during these first few weeks (Simpson *et al.*, 2004; Bandelow *et al.*, 2017).

Considering the challenges remaining in our efforts to better understand and treat OCD, novel pre-clinical frameworks in which to investigate the neurobiological process underlying compulsive-like behavior and how such behaviors respond to novel treatment interventions, are invaluable. Therefore, delivering such a novel framework is the primary focus of the current work.

2.2 The Neurobiology of OCD

2.2.1 Neuroanatomy

OCD is regarded as a multifaceted neurobehavioral illness that is likely rooted in multiple etiologies. However, the two core features of OCD, i.e. intrusive thoughts and excessive repetition of executive routines, suggest that the orbitofrontal cortex (OFC)[2], the anterior cingulate cortex (ACC)[3], several structures of the basal ganglia, and the thalamus are the primary sites of neuroanatomical dysfunction (Maia *et al.*, 2008; Abramowitz *et al.*, 2009; Markarian *et al.*, 2010; Stocco *et al.*, 2010). This was demonstrated by neuroimaging studies which indicated that both the ACC and OFC are hyperactive in OCD patients, particularly during periods of OCD symptom expression (Saxena & Rauch, 2000; Maltby *et al.*, 2005; van den Heuvel *et al.*, 2005; Husted *et al.*, 2006; Maia *et al.*, 2008). More specifically, an overactive ACC results in the manifestation of repetitive behaviors while a hyperactive OFC signifies dysfunctional reward processing as will be explained in the following paragraphs (Maltby *et al.*, 2005; Husted *et al.*, 2006; Haber & Knutson, 2010). Collectively, all of the aforementioned brain regions are organized as a network of discrete circuits, known as the cortico-striatal-thalamic-cortical (CSTC)[4] circuit (Stocco *et al.*, 2010; Burguière *et al.*, 2015). These circuits are essential for the planning, execution and termination of intricate motor behaviors and reward-based learning (Graybiel & Rauch, 2000; Graybiel, 2008; Stocco *et al.*, 2010) and have therefore become central in theories attempting to describe OCD neurobiology. Importantly, the CSTC circuit is also pivotal for assessing the rewarding or punishing valence of external stimuli and the enactment of suitable approach or avoidance routines (Graybiel & Rauch, 2000; Pauls *et al.*, 2014).

[1] obsessive-compulsive disorder
[2] orbitofrontal cortex
[3] anterior cingulate cortex
[4] cortico-striatal-thalamic-cortical

Briefly, the CSTC[1] circuitry is arranged in such a way that the ACC[2], via innervation of the striatum, exerts feedback through the thalamus to the OFC[3] via two distinct relays, i.e. the direct and indirect pathways (Haber & Calzavara, 2009; Haber & Knutson, 2010). Whereas the direct (behaviorally activating) pathway expresses excitatory dopamine D_1 receptors, the indirect (behaviorally inactivating) pathway expresses thalamo-inhibitory D_2 receptors (Nambu *et al.*, 2002; Huey *et al.*, 2008; Nambu, 2008; Stocco *et al.*, 2010; Hong & Hikosaka, 2011). These dopamine receptor subtypes are of particular importance in OCD[4] (McDougle *et al.*, 1994; Korff *et al.*, 2008; Hoffman & Morales, 2012) as it is commonly accepted that a bias in favor of the direct pathway over that of the indirect pathway underlies obsessive-compulsive symptomology (Saxena & Rauch, 2000; Maia *et al.*, 2008; Abramowitz *et al.*, 2009). Briefly, for an executive 'go' signal to be propagated via the CSTC circuit, simultaneous activation of the direct pathway and inactivation of the indirect pathway is necessary (Nambu, 2008; Di Filippo *et al.*, 2009). As the cortex activates both the direct and the indirect pathway simultaneously, thereby propagating conflicting behavioral signals, another gating mechanism, i.e. dopaminergic signaling, is needed to ensure that the executive balance shifts in favor of the direct, activating pathway (Nambu *et al.*, 2002). Indeed, once the basal ganglia are activated by cortical input, the simultaneous release of dopamine in the striatal synapses results in the stimulation of both D_1 receptors (which maintain activation of the direct pathway), and D_2 receptors (which now *inhibits* the indirect pathway; Nambu, 2008). As such, for the brief period of simultaneous cortico-striatal activation and dopamine release, the executive balance shifts in favor of behavioral activation (Nambu *et al.*, 2002; Markarian *et al.*, 2010). Although causality has not yet been established, OCD patients invariably present with disturbances in CSTC circuit function, while successful SRI[5]/SSRI[6] treatment generally restores some balance to these processes (Maia *et al.*, 2008).

2.2.2 Serotonin and Dopamine in OCD: A Case of Reward and Punishment Processing

Although a number of neurotransmitters are variably implicated in the etiopathology of OCD, serotonin and dopamine are undoubtedly of major importance (Abramowitz *et al.*, 2009; Markarian *et al.*, 2010; Wong *et al.*, 2015). Considering the function of dopamine in the CSTC circuitry alluded to above, a clear role for serotonin is also implicated by the general anti-obsessional efficacy of SRIs/SSRIs (Denys *et al.*, 2004b; Zike *et al.*, 2017; Dougherty *et al.*, 2018). Briefly, serotonin can be regarded as the behavioral opponent of dopamine, whereby serotonergic afferents that project from the raphe

[1] cortico-striatal-thalamic-cortical
[2] anterior cingulate cortex
[3] orbitofrontal cortex
[4] obsessive-compulsive disorder
[5] serotonin reuptake inhibitor
[6] selective serotonin reuptake inhibitor

nuclei to the cortex and basal ganglia, counteract the effects of dopamine in the CSTC[1] circuits (Daw *et al.*, 2002; Boureau & Dayan, 2011). As such, whereas dopaminergic signaling is largely responsible for behavioral activation and approach behavior, serotonin will act to inhibit the execution of behavioral patterns (Daw *et al.*, 2002; Schultz, 2006; Boureau & Dayan, 2011).

Considering the above, OCD[2] is often hypothesized to be a condition of dysfunctional reward appraisal as patients often fail to consolidate the positive feedback—rewarding sensation on task completion—following the successful execution of a planned behavior such as, locking a door (Figee *et al.*, 2011). Against this background, it should be noted that dopaminergic signaling is responsible for the propagation and facilitation of reward-directed behavior (Higgins & Fletcher, 2003). Briefly, during the experience of reward, dopaminergic signaling is elevated whereas it is suppressed during the experience of aversive and negative consequences (Schultz, 2002; Husted *et al.*, 2006; Arias-Carrión *et al.*, 2010; Schultz, 2013; Fischer & Ullsperger, 2017; Murray *et al.*, 2019). While phasic increases in dopamine signaling would prime an individual for continued reward-directed behavior, the negative motivational valence coded by *suppressions in dopamine and increases in serotonergic signaling* during adverse experiences, typically serve to demotivate continued engagement during similar future circumstances (Boureau & Dayan, 2011; Cools *et al.*, 2011). However, following repetitive exposure to the same rewarding scenario, the extent of the phasic dopamine response should abate over time, resulting not in continuous reward-seeking responses, but rather in the maintenance of pure goal-directed responses (Schultz, 2002; Wise, 2004; Schultz, 2013). Therefore, if it is hypothesized that patients with OCD continuously engage in reward-seeking behavior, it is likely that they may present with excessive and persistent dopaminergic signaling (Denys *et al.*, 2004b). This idea is supported by both clinical (Denys *et al.*, 2004b) and pre-clinical (Bedingfield *et al.*, 1997; Szechtman *et al.*, 1998; Kontis *et al.*, 2008; Güldenpfennig *et al.*, 2011) findings demonstrating positive correlations between compulsivity and dopaminergic signaling. Further, decreased central D_2 receptor expression has been shown in OCD patients (Denys *et al.*, 2004a; Denys *et al.*, 2013) which is in line with proposed theories pertaining to the role of this receptor subtype in normal CSTC circuit functioning. Considering the functional interplay between dopamine and serotonin, it is not surprising that both conditioned (e.g. lever-pressing for food) and unconditioned (e.g. typical feeding) behaviors, which are normally associated with dopaminergic signaling, are suppressed by enhancing serotonergic signaling (Fletcher *et al.*, 1993; Fletcher, 1995; Fletcher *et al.*, 1999). However, research pertaining to the behavioral functions of serotonin is far from conclusive (see Faulkner and Deakin (2014) for review). For example,

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

the administration of serotonin agonists, e.g. meta-chlorophenylpiperazine (mCPP)[1], has been shown to exacerbate compulsivity in patients (Markarian *et al.*, 2010). Such findings stand in contrast to the effects of SRIs[2]/SSRIs[3] which in essence are indirect acting pro-serotonergic agents. Further, definitive evidence for the direct involvement of serotonergic receptor aberrations in OCD[4] has not yet been presented (Pogarell *et al.*, 2003; Reimold *et al.*, 2007; Zitterl *et al.*, 2008; Hesse *et al.*, 2011), likely pointing to complex interactions between serotonin and other neurotransmitter systems, rather than global dysfunctions in serotonergic signaling per se, underlying obsessive-compulsive symptom presentation.

That said, it is sufficient to say that the balance between reward-seeking and punishment-avoiding behavior is closely related to the balance between dopaminergic and serotonergic signaling (Deakin & Graeff, 1991; Seymour *et al.*, 2012) and how these two neurotransmitters interact in a number of complex, opposing ways (Daw *et al.*, 2002; Boureau & Dayan, 2011; Cools *et al.*, 2011). This concept is of major importance to the current investigation as we will attempt to induce compulsive-like persistence in zebrafish *(Danio rerio)* by means of dopaminergic drug intervention.

2.3 Cognitive Theories

2.3.1 Conditioning, Prediction and Flexibility

Following from the above, colors begin to emerge that can assist us in sketching a picture of how processes of learning, as influenced by dopamine and serotonin, may ultimately be involved in the pathogenesis of compulsive-like persistence. Considering the potential role that inadequate consolidation of goal-outcome feedback may play in the propagation of compulsive behavior, it is important to note that the CSTC[5] circuit is also a pivotal role player in processes of learning and decision-making, as well as being involved in flexible adaptations in response to changing external and internal conditions and demands (Endrass *et al.*, 2011). Since CSTC circuit dysfunction typically underlies OCD (see section 2.2), it can be presumed that the condition would also be characterized by deficits in learning and set-shifting, i.e. the ability to change one's responses based on outcome feedback; this has indeed been demonstrated in OCD patients (Endrass *et al.*, 2011; Endrass *et al.*, 2013; Hezel & McNally, 2016).

Briefly, humans and animals often, though not exclusively, guide their actions based on what is *expected* of key future events (Schultz *et al.*, 1997; Schultz, 2006). Against the background of

[1] meta-chlorophenylpiperazine
[2] serotonin reuptake inhibitors
[3] selective serotonin reuptake inhibitors
[4] obsessive-compulsive disorder
[5] cortico-striatal-thalamic-cortical

paragraph 2.2.2, such predictions, which are also coded by changes in dopamine (positive predictions) and serotonin (negative predictions), are applied to adapt future responses to attain a reward or to plan a suitable behavioral response to avoid adverse outcomes (Wise, 2004; Boureau & Dayan, 2011; Schultz, 2013). Therefore, reward and punishment are operational concepts that inform the positive or negative value assigned to a specific outcome, e.g. food pellets, a behavioral act, e.g. mating, an internal physical state, e.g. maintaining optimal body temperature, or risk of predation (Schultz *et al.*, 1997; Wise, 2004). However, following continuous exposure to the same outcome, rewarding and punishing feedback—if not consolidated properly—may act as positive or negative reinforcers of set behavioral routines, instead of being informative with respect to goal-directed action-outcome selection (Wise, 2004; Schultz, 2007); this may contribute to behavioral inflexibility.

An experiment often carried out in animals to study feedback processing revolves around the principle of classic Pavlovian conditioning (Gluck & Bower, 1988; Gould, 2002; Aguado, 2003). Usually, a conditioned stimulus (CS)[1], i.e. a previously neutral stimulus such as a bell sound (sensory cue), becomes associated with the unconditioned stimulus (US)[2], i.e. a biologically significant stimulus such as electrical foot shock (punishment) or food (reward) (Gluck & Bower, 1988; Blaser & Vira, 2014; Pezzulo *et al.*, 2015). Standard conditioning models postulate that for an association to develop, the sensory cue or CS, must consistently precede the presentation of the US, whether it be punishing or rewarding (Schultz *et al.*, 1997; Schultz, 2002; Schott *et al.*, 2008). Cue-directed or cue-avoidance behavior after such conditioning would indicate that the CS on its own generates an adequate prediction about the probable time and magnitude of the *expected* reward or punishment (Schultz *et al.*, 1997). As such, presentation of the CS alone becomes effective enough to direct future behavioral responses (i.e. Pavlovian conditioning has occurred). However, if the outcome changes, i.e. no reward or punishment is delivered, the contingency between the CS and the US should change. This is known as reversal learning, an important component of cognitive flexibility (Cools *et al.*, 2002; Jocham *et al.*, 2009; Ahmari, 2016; Zabegalov *et al.*, 2019). In this instance, subjects first acquire knowledge of a CS-US contingency. Subsequently, they are tested under conditions of CS devaluation, i.e. reward withdrawal upon CS presentation, or the delivery of the US after a novel CS (Remijnse *et al.*, 2006; Pittenger *et al.*, 2019). If animals (or humans) fail to recruit reversal learning mechanisms under circumstances of outcome change, they act in an inflexible manner, which is indicative of cognitive rigidity (Ahmari, 2016; Zabegalov *et al.*, 2019). It is hypothesized that in humans with OCD[3] and animals expressing compulsive-like behavior, both groups are insensitive to a gradually changing

[1] conditioned stimulus
[2] unconditioned stimulus
[3] obsessive-compulsive disorder

dopaminergic and serotonergic tone after continuous presentations of the same outcome, hence persisting in reward-seeking or punishment-avoiding behaviors (Palminteri *et al.*, 2012). Importantly, the rewards and punishments referred to here are all related to aspects of task completion, e.g. the actions of hand washing or door locking. Indeed, the rigid presentation of compulsive behavior can be considered as an inability to effectively switch between behaviors according to outcome valuation (Remijnse *et al.*, 2006). This concept is of major importance for this investigation.

2.3.2 Goal-Directed versus Habitual Responses in OCD

In line with, but expanding on the aforementioned concepts, it has been hypothesized that an imbalance between the goal-directed and habitual behavioral systems may also be informative for our understanding of compulsive behaviors (Vaghi *et al.*, 2019). Indeed, compulsivity is often seen as a form of behavioral addiction in association with diminished feedback processing (Gillan et al., 2016; Vaghi et al., 2019). However, as opposed to the general idea of conditioned reward-seeking or punishment-avoiding behaviors, which in essence are also related to goal-directed actions, habitual responses are behaviors which are often repeated in day-to-day life and therefore require little to no cognitive processing power; rather, they become automated after being repeated frequently to save valuable cognitive effort (de Wit & Dickinson, 2009; Gillan *et al.*, 2016; Vaghi *et al.*, 2019).

According to the dual-system theory of behavioral motivation, behaviors can be guided by either goal-directed or habitual systems (de Wit & Dickinson, 2009; Gillan *et al.*, 2011; de Wit *et al.*, 2012; Vaghi *et al.*, 2019). Whereas the goal-directed system allows individuals to flexibly direct their actions towards presently desirable outcomes (de Wit & Dickinson, 2009; de Wit *et al.*, 2012; Gottwald *et al.*, 2018), the habitual system begins to exert dominant control over such behaviors after multiple cycles of repetition (Gillan *et al.*, 2011; Gillan *et al.*, 2016). This is important, as it assists both humans and animals to function optimally without the need to recruit energy-intensive and time-consuming cognitive mechanisms. However, if habitual control dominates irrespective of context, loss of behavioral flexibility is evident which can result in so-called “slip of action” with respect to outcomes that are, at a specific time, not desirable (de Wit & Dickinson, 2009; Gillan *et al.*, 2011; de Wit *et al.*, 2012; Gottwald *et al.*, 2018), e.g. thoughtlessly washing one’s hands after entering the kitchen when the intention was simply to retrieve a set of keys. Here, the habitual response of washing was directly triggered by the contextual environment and not by the goal-directed intention to engage in hand washing (Gillan *et al.*, 2011; Gillan *et al.*, 2016). Considering the trait similarity between compulsivity and habitual responses, it has been hypothesized that overreliance on the habitual system may be

closely associated with compulsive responding in patients with OCD[1] (Gillan *et al.*, 2011; Gillan *et al.*, 2016).

2.4 The Zebrafish as a Novel Model Organism for Compulsive-Like Behaviors

For decades, mammalian species (especially rodents) have been pre-eminent in modeling complex human neuropsychiatric (and other) diseases due to their close resemblance of human anatomy and physiology (Lieschke & Currie, 2007; Champagne *et al.*, 2010; Ablain & Zon, 2013; D'Amico *et al.*, 2015; Fontana *et al.*, 2018). Despite these similarities, rodent models present research with several experimental limitations that ultimately restrict their utility for disease modeling. For example, rodent models are often time-consuming and expensive, they have a low-throughput capacity in terms of relatively slow maturation, labor intensive maintenance and behavioral assessment, making large-scale studies based on chemical and genetic screens technically challenging to perform (Lieschke & Currie, 2007; Champagne *et al.*, 2010; Ablain & Zon, 2013). As such, research has increasingly begun to turn to other model organisms for foundational investigations (as in the *in vitro* stage) that can inform the subsequent extension of studies in rodents (Maximino *et al.*, 2015).

Over the preceding decade, teleost (a fish of a large group that comprises all ray-finned fish) zebrafish have emerged as one such a novel model organism (Bailey *et al.*, 2015; Maximino *et al.*, 2015; Fontana *et al.*, 2018). Experimental applications of zebrafish, both as adult animals and during the larval stages of development, are gaining momentum in neuroscience, pharmacogenetics and neuropharmacology (Champagne *et al.*, 2010; Kalueff *et al.*, 2014a; Kalueff *et al.*, 2014b). Additionally, zebrafish are particularly useful for modeling complex psychiatric and neurological disorders (Fontana *et al.*, 2018). Moreover, zebrafish have both a high genetic (80 – 85%) and physiological homology to humans (Kalueff *et al.*, 2014a; D'Amico *et al.*, 2015; Naderi *et al.*, 2016) and are remarkably similar to mammals with respect to the involvement of the classic neurotransmission and other physiological systems (Kalueff *et al.*, 2014a; Kalueff *et al.*, 2014b; Maximino *et al.*, 2015). Furthermore, a growing body of evidence suggests that zebrafish can be used to model various aspects of almost every brain disorder which are currently modelled in rodents, typically in a more cost-effective manner (Kalueff *et al.*, 2014a; Kalueff *et al.*, 2014b; D'Amico *et al.*, 2015). In the following paragraphs, a brief overview of zebrafish and how they will be applied as a model organism in this investigation, will be provided.

[1] obsessive-compulsive disorder

2.4.1 The Normal Developmental Stages of Zebrafish

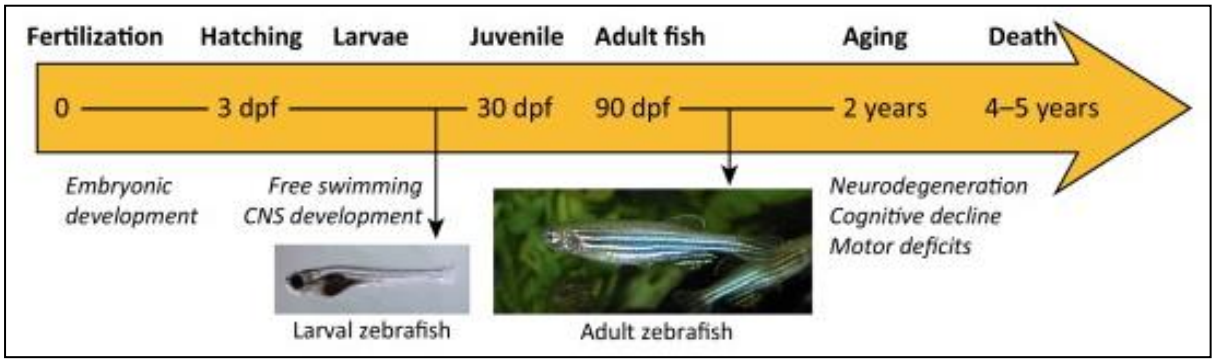

Figure 1: The normal developmental stages of zebrafish taken from (Stewart et al., 2014); dpf - days post-fertilization.

The life cycle of zebrafish is divided into several distinct stages, spanning from the embryonic pre-hatching stage (0 – 3 dpf[1]) to the different post-hatching stages, *viz.* larval (3 – 29 dpf), juvenile (30 – 89 dpf), adult (90 dpf – 2 years) and finally aging/aged zebrafish (from 2 years onwards) (Kalueff *et al.*, 2014b; Stewart *et al.*, 2014). After spawning, zebrafish develop externally in eggs which become translucent following fertilization, allowing for continuous visualization and monitoring of the anatomical changes in these developing embryos (Kari *et al.*, 2007; Kalueff *et al.*, 2014b; Bailey *et al.*, 2015). This also enables the manipulation of these processes, be it injecting drugs or altering genes, *in vivo* (Kalueff *et al.*, 2014b). Zebrafish models are therefore ideal for neurodevelopmental studies (Kalueff *et al.*, 2014a). Additionally, embryonic development is extraordinarily rapid since all major organs form within 1 dpf. Hatching followed by feeding and free swimming starts within 3 dpf at the onset of the larval stage (Kari *et al.*, 2007; Kalueff *et al.*, 2014a; Kalueff *et al.*, 2014b). A few days after hatching, larval zebrafish possess the major central nervous structures found in adult animals which continue to develop over the proceeding days (Bögli & Huang, 2017). Indeed, since the capability for complex behavior is established at the onset of the larval stage, zebrafish models are ideal for measuring changes to the developing nervous system (Bailey *et al.*, 2015). Furthermore, the expanded lifespan of zebrafish (4 – 5 years versus 3 years in mice) promotes translational studies of aging related behavioral deficits (Kalueff *et al.*, 2014b). Adult zebrafish have been shown to excel in multiple memory-related experiments (Bögli & Huang, 2017) including spatial alternation (Cognato *et al.*, 2012), olfactory conditioning (Braubach *et al.*, 2009) as well as associative (Valente *et al.*, 2012) and non-associative learning (Tran & Gerlai, 2014). Conversely, studies using larval zebrafish pertaining to memory have only commenced recently (Roberts *et al.,* 2013). Best and Alderton (2008) demonstrated successful non-associative forms of learning in larval zebrafish including habituation and sensitization to various drugs. However, associative learning capabilities have only been shown by a limited amount of studies at later developmental stages (Bögli & Huang, 2017). Adult zebrafish

[1] days post-fertilization

are therefore better suited toward associative learning tasks than their larval counterparts - and as such, adult fish (90 dpf[1]) were employed in the current investigation.

2.4.2 Key Advantages of Using Zebrafish as a Pre-Clinical Model Species

The utility of zebrafish in research display numerous advantages that can be summarized as follows:

1. *Time efficiency*: Transgenic lines of zebrafish can be generated in roughly a third of the time required to develop stable transgenic mouse lines (D'Amico *et al.*, 2015; Meshalkina *et al.*, 2017). Rapid external fertilization and embryonic development yields developed basic physiological systems within 24 hours after fertilization (Goldsmith, 2004; Kari *et al.*, 2007; Champagne *et al.*, 2010). Furthermore, zebrafish have a robust reproductive capacity in standardized laboratories (Champagne *et al.*, 2010; Blaser & Vira, 2014) with a single pair of adults capable of producing up to 100 – 200 offspring per week (Goldsmith, 2004; Kari *et al.*, 2007; Ablain & Zon, 2013; Kalueff *et al.*, 2014a; Kalueff *et al.*, 2014b; Meshalkina *et al.*, 2017). Rapid maturation is also an advantage, since larval zebrafish are suitable for certain types of experimentation and require no parental support, they can be used as early as 3 dpf in contrast to rodents which require maternal support for at least (though typically more than) three weeks following birth (Möller *et al*., 2018).
2. *Lengthy lifespan*: Compared to rodents, zebrafish have a relatively long lifespan (Kalueff *et al.*, 2014b), enabling research of a developmental nature over a chronic period of time.
3. *Cost-efficiency*: Zebrafish is a more cost-effective model organism to breed, house and treat compared to rodents (Goldsmith, 2004; Champagne *et al.*, 2010; Maximino *et al.*, 2015; Meshalkina *et al.*, 2017; Fontana *et al.*, 2018). In fact, zebrafish larvae can be accommodated in volumes of as little as 50 μl, hence requiring microgram quantities of the experimental compounds used for pharmacological or chemical intervention (Goldsmith, 2004; Kari *et al.*, 2007). With respect to the adult fish, numerous fish can simultaneously be exposed to specific interventions by means of simple aqueous immersion (Ablain & Zon, 2013; Fontana *et al.*, 2018). This is in contrast to drug administration in rodent studies, which often rely on oral, subcutaneous or intraperitoneal administration routes which present risk of injury and substantial anxiogenesis in addition to being labor intensive and time-consuming (Möller *et al.*, 2018; Steyn *et al.*, 2018; Zhu *et al.*, 2018; Harvey *et al.*, 2019).
4. *High-throughput results-driven organism*: Zebrafish models can deliver a relatively quick indication of the putative biological activity of novel compounds (Levin, 2011). Therefore, the organism may fill a much-needed gap between cell-based and other *in vitro* systems and

[1] days post-fertilization

classic mammalian models for drug screening. Further, zebrafish models are also valid translational frameworks in which to study reward and drug abuse, complementing the use of rodent models (Kalueff *et al.*, 2014b)—a feature that is both relevant and advantageous for the current study. High sensitivity to various drugs of abuse (with respect to tolerance and a clear preference for these agents) is evident in both larval and adult zebrafish (Kalueff *et al.*, 2014b). It is therefore not surprising that the use of zebrafish is becoming a valuable tool for pre-clinical research (Champagne *et al.*, 2010; Ablain & Zon, 2013), and accordingly the species has been rapidly propelled to the forefront of developing modern technology for high-throughput drug screening and discovery (Champagne *et al.*, 2010).

Another advantage of using zebrafish is their similar neurobiology to humans and mammals, which will be discussed in the following paragraphs.

2.4.3 Neurobiology of Zebrafish and Their Homology to Humans and Mammals

Zebrafish demonstrate remarkable similarity to humans with respect to their neurochemical systems and basic neuronal organization (D'Amico *et al.*, 2015). Although relatively small, the overall organizational features of the adult zebrafish brain are similar to its mammalian counterparts (Best & Alderton, 2008; Stewart *et al.*, 2014). Areas such as the hypothalamus and olfactory bulb (including structures of the lateral pallium) seem to be homologous to the mammalian correlates (Best & Alderton, 2008). Moreover, zebrafish possess all of the major nervous systems components, i.e. receptors, transmitters, transporters and metabolic enzymes, in a manner that closely parallels that of mammals (Best & Alderton, 2008; Kalueff *et al.*, 2014b; D'Amico *et al.*, 2015; Maximino *et al.*, 2015). The zebrafish central nervous system (CNS)[1], like that of mammals, possesses a functional blood-brain barrier (Best & Alderton, 2008; D'Amico *et al.*, 2015).

In the following paragraphs, a brief overview of the serotonergic and dopaminergic systems of zebrafish will be provided. However, for a detailed review of the neurotransmitter systems in zebrafish, please see Schweitzer and Driever (2009), Maximino and Herculano (2010), Panula *et al.* (2010), Schweitzer *et al.* (2012) and Maximino *et al.* (2013).

2.4.3.1 The Zebrafish Serotonergic System

Serotonin, like in humans, is an important modulator of zebrafish behavior (Stewart *et al.*, 2013). Zebrafish possess a well-developed serotonergic system that is functionally similar to that of mammals (Stewart *et al.*, 2011b; Maximino *et al.*, 2013; Stewart *et al.*, 2013). The expression patterns, signaling properties and binding of serotonin to SERT[2] and other receptors also resembles those seen in

[1] central nervous system
[2] serotonin reuptake transporter

mammals (Stewart *et al.*, 2011b; Maximino *et al.*, 2013; Stewart *et al.*, 2013). Interestingly, a genome duplication event in the phylogenetic development of teleosts like zebrafish generated numerous genes that code for functional proteins, such as the tryptophan hydroxylase enzyme (THP)[1], SERT[2] and the serotonin 5-HT_{1A} receptor, which are all relevant to central serotonergic processes (Maximino *et al.*, 2013; Stewart *et al.*, 2013). For example, zebrafish possess three copies of the *tph* gene (*tph1a*, *tph1b* and *tph2*), all of which encode for THP enzyme, the rate limiting enzyme in serotonin synthesis (Maximino *et al.*, 2013; Stewart *et al.*, 2013). The *tph1a/b* genes are expressed in the hypothalamus, pineal gland, spinal cord and retina, whereas *tph2* is expressed in the raphe nuclei, pretectal area and the reticular formation. Similarly, the duplicated *SERT* gene is expressed in a corresponding fashion, displaying similar pharmacological profiles with both its isoforms (SERTa and SERTb; Maximino *et al.*, 2013; Stewart *et al.*, 2013). The SERTa protein is widely distributed throughout the brain, binds SRIs[3]/SSRIs[4] with high affinity and is more homologous to mammalian SERTa, than SERTb, which seems to be limited to the retina and medulla (Stewart *et al.*, 2013). Therefore, SERTb is less likely to be implicated in behavioral responses after SRI/SSRI administration. Furthermore, both the 5-HT_{1A} and 5-HT_2 receptor subclasses that are also important in clinical OCD[5], are expressed in zebrafish (Stewart *et al.*, 2013).

2.4.3.2 The Zebrafish Dopaminergic System

Dopaminergic pathways in zebrafish display remarkable similarities to the corresponding mammalian systems; in fact, all mammalian dopamine receptor subtypes, apart from the D_5 receptor, are also found in zebrafish (Naderi *et al.*, 2016). However, there are some noteworthy differences between zebrafish and mammals with respect to the localization pattern of dopaminergic neurons. Whereas the midbrain in mammals constitutes the main dopaminergic domain, specifically the ventral tegmental area and the substantia nigra, most zebrafish dopaminergic neurons are located across the diencephalon, with ascending projections to the telencephalon (Naderi *et al.*, 2016). However, it has been suggested that dopaminergic neurons in zebrafish located in the posterior tuberculum represent a functional equivalent of the primary dopaminergic pathways in the mammalian brain (Naderi *et al.*, 2016). Moreover, since all types of mammalian dopamine receptors, excluding the D_5 receptor, have been identified in zebrafish, the similarity is even more striking (Naderi *et al.*, 2016). Dopaminergic neurotransmission in the vertebrate brain is mediated by two distinct categories of G protein-coupled receptors (Naderi *et al.*, 2016). Five types of dopamine receptors, grouped as D_1-like (D_1 and D_5) and

[1] tryptophan hydroxylase enzyme
[2] serotonin reuptake transporter
[3] serotonin reuptake inhibitors
[4] selective serotonin reuptake inhibitors
[5] obsessive-compulsive disorder

D_2-like (D_2, D_3 and D_4), have been identified in mammalian neurons and astrocytes (Boehmler *et al.*, 2004; Li *et al.*, 2007; Maximino & Herculano, 2010; Naderi *et al.*, 2016). Receptors from the D_1 subfamily are coupled to G_s, thereby stimulating intracellular 3'-5'-cyclic adenosine monophosphate (cAMP)[1] production by the induction of adenylyl cyclase (AC)[2] enzymatic activity (Boehmler *et al.*, 2004; Li *et al.*, 2007; Naderi *et al.*, 2016). Conversely, receptors from the D_2 subfamily are coupled to $G_{i/o}$, thereby inhibiting AC function and reducing cAMP synthesis (Boehmler *et al.*, 2004; Li *et al.*, 2007; Naderi *et al.*, 2016). Moreover, D_1- and D_2-like dopamine receptors are mainly located extra-synaptically, although D_2-like receptors are so expressed to a lesser extent (Maximino & Herculano, 2010). Furthermore, evidence from the same study revealed two members of the D_2 subfamily (D_2 and D_4 receptors) have undergone evolutionary multiplication in the zebrafish. As classic neuroleptics (anti-dopaminergic drugs) target this receptor specifically, there is mounting interest in the fact that zebrafish express more than one type of D_2 receptor (Maximino & Herculano, 2010). Although further investigation is needed, the multiplication event that generated different D_2 subtype receptors is thus far not evident in D_1-like receptors. That said, while zebrafish do express D_1 receptors, these also display a high degree of homology to mammalian analogue (Maximino & Herculano, 2010).

In summary, zebrafish may represent an ideal model organism in which to study the neurobiology underlying potential compulsive-like behaviors. This is due to the species' marked homology to mammals with respect to the two neurotransmitter systems that play fundamental roles in the etiopathology of obsessive-compulsive symptomology. As such and toward a novel pre-clinical model of compulsivity, the present investigation will be based on a pharmacological exploration of these systems in zebrafish.

2.4.4 Learning and Conditioning in Zebrafish

Considering that the current investigation will be based on an interrogation of cue-reward learning under the influence of dopaminergic and serotonergic drug intervention, a brief background of what is known about processes of learning in zebrafish, is necessary.

Zebrafish have previously demonstrated similar performance compared to mammals in tests of choice and reversal learning, behavioral reinforcement, response timing and behavioral extinction (Blaser & Vira, 2014). Collectively, research indicates that although zebrafish might recruit different neurobiological processes than mammals during the various phases of learning, sufficient parallels can be drawn between fish and mammals with respect to both conditioned and unconditioned learning (Blaser & Vira, 2014). That said, although it is speculated that fish may provide a useful and simplified

[1] 3'-5'-cyclic adenosine monophosphate
[2] adenylyl cyclase

framework to examine the neurobiology of basic learning phenomena, caution must be applied when translating the findings from piscine experiments to mammalian paradigms (Blaser & Vira, 2014). As the current study is aimed at an initial investigation of zebrafish learning and how it may be influenced by dopaminergic and serotonergic processes, this is especially important.

Nevertheless, zebrafish are capable of performing both associative and non-associative tasks including habituating to novel environments, reversal learning, appetitive reinforcement-based learning and avoidance learning (Al-Imari & Gerlai, 2008; Daggett *et al.*, 2019). Within the context of the current investigation, associative learning, a form of Pavlovian conditioning, can be defined as the learning of an association between a cue and an outcome that over time results in certain goal-directed behavioral manifestations upon presentation of the cue alone (Roy *et al.*, 2019). In zebrafish, associative learning tasks have been carried out using a variety of motivating stimuli/reinforcers (outcomes) including food. That said, not all investigations using food report successful findings in terms of cue-reward conditioning, most likely since fish quickly develop tolerance to the rewarding value of food. This is due to the fact that zebrafish experience rapid satiation and can remain healthy over several days of food deprivation (Al-Imari & Gerlai, 2008; Blaser & Vira, 2014; Daggett *et al.*, 2019). This is somewhat counterproductive for investigations that are aimed at establishing successful learning of cue-reward contingencies, which require the reward to maintain a high level of motivational salience (Daggett *et al.*, 2019). Also, due to zebrafish typically being housed in large groups, it is difficult to accurately control food deprivation and food intake across individuals (Blaser & Vira, 2014). Zebrafish are not equally motivated to feed in novel experimental tanks, and the timing and rationing of food proves to be a major obstacle (Blaser & Vira, 2014). That said, procedures using appetitive stimuli have involved pairing either olfactory or visual cues with food, drugs of abuse (e.g. opioids) or social contact, and then measuring approach to the conditioned stimulus (Blaser & Vira, 2014). Therefore, zebrafish offer tremendous promise as a model for learning-related research. The current work will attempt to manipulate processes of learning in such a way that the outcomes thereof represent compulsive-like traits, e.g. behavioral inflexibility, persistence and repetition, and an inability to appropriately appraise changes in learned outcomes.

To this extent, the aforementioned constraints regarding the use of food reward as behavioral reinforcer have to be considered against suitable alternatives. Here, the highly social nature of zebrafish, which happens to be more robust than that of other classical laboratory vertebrates (Saverino & Gerlai, 2008; Buske & Gerlai, 2011; Mueller & Neuhauss, 2012; Saif *et al.*, 2013), will be exploited. Zebrafish are known to spontaneously exhibit behaviors such as aggregation or forming groups, i.e. shoaling (Al-Imari & Gerlai, 2008; Saverino & Gerlai, 2008; Saif *et al.*, 2013; Daggett *et al.*, 2019), both in nature as well as in the laboratory (Al-Imari & Gerlai, 2008; Saif *et al.*, 2013). Other

traditional laboratory animal species, such as mice and rats, do not exhibit the same extent of group preference and social cohesion (Saverino & Gerlai, 2008). Moreover, as a shoaling species, zebrafish express a powerful motivational drive to interact with conspecifics, i.e. same species fish (Al-Imari & Gerlai, 2008; Saverino & Gerlai, 2008; Saif *et al.*, 2013; Meshalkina *et al.*, 2018; Daggett *et al.*, 2019). Social behaviors displayed by zebrafish are already evident 6 dpf[1] and a rapid increase in shoal cohesion is observed within the first month of development which gradually continues to increase until the third month (Meshalkina *et al.*, 2018). In fact, such social responses are so profound that the mere sight of a conspecific in the absence of physical contact shows rewarding properties in instrumental conditioning paradigms (Al-Imari & Gerlai, 2008). This has been corroborated in other fish species such as the paradise fish *(Macropodus opercularis)* as well (Al-Imari & Gerlai, 2008). Indeed, it has been shown that sight of conspecifics is not only rewarding, but that it facilitates associative and other types of learning (Al-Imari & Gerlai, 2008). Interestingly, computer-simulated 'fish' (a shoal displayed on a liquid crystal display (LCD)[2] screen) serve as an effective alternative reinforcer to live conspecifics, reducing the number of fish required for such studies (Saif *et al.*, 2013; Daggett *et al.*, 2019). In a study by Saif *et al.* (2013), the authors confirmed that conspecific images induce strong behavioral responses in experimental zebrafish that are partially mediated by the dopaminergic system. In fact, they found that dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC[3]; a metabolic product of dopamine) levels significantly increased in response to the presentation of conspecific images; however, serotonin and 5-hydroxyindoleacetic acid (5-HIAA[4]; a serotonin metabolite) levels did not change. These findings, which are in congruence with others (Al-Imari & Gerlai, 2008; Buske & Gerlai, 2012), are important in demonstrating the role of dopaminergic signaling in the reward processing of zebrafish. Indeed, the present investigation will seek to manipulate such dopaminergic responses to induce compulsive-like reward-seeking behavior. Another advantage of using a social reward construct as a behavioral reinforcer which is of critical importance for the current study is that zebrafish do not seem to habituate to the rewarding properties of social interaction (Daggett *et al.*, 2019), as opposed to their reduced response toward food reward over time.

In terms of successfully establishing cue-reward contingency learning, adult zebrafish can clearly distinguish between different colors and are therefore capable of color-dependent conditioning (Ahmad & Richardson, 2013). In addition, color perception in zebrafish influences learning, decision-making and memory formation (Oliveira *et al.*, 2015). Although it has been demonstrated that zebrafish seemingly prefer red (as used in this investigation) and green equally over no color, yellow

[1] days post-fertilization
[2] liquid crystal display
[3] 3,4-dihydroxyphenylacetic acid
[4] 5-hydroxyindoleacetic acid

and blue (Avdesh *et al.*, 2012), findings with respect to color perception still remain largely inconclusive (Oliveira *et al.*, 2015). For example, Roy *et al.* (2019) suggested zebrafish may exhibit preference for colors that match the resources in their housing environments. That said, they also found that fish from different source populations differ in their color preferences and that the ability to learn associations as well as reverse these associations upon modification of a color-reward contingency may be impacted by these preferences (Roy *et al.*, 2019).

2.4.5 Administration of Drugs to Zebrafish

Drug administration to zebrafish is a relatively simple procedure, since small water-soluble molecules (including oxygen) can be dissolved in the surrounding water. These in turn are readily absorbed through the skin and gills (McGrath & Li, 2008; Meshalkina *et al.*, 2017; Fontana *et al.*, 2018). Due to scales not developing until later developmental stages, whole body surface diffusion represents a major route for the absorption of small molecules (Goldsmith, 2004), especially during the early stages of development. Further, it is easy to manage drug dose and concentrations as the volume of exposure solutions can be easily controlled. Apart from diffusion, drugs are also delivered orally (Meshalkina *et al.*, 2017) as zebrafish begin to swallow at 3 dpf[1] (McGrath & Li, 2008), while absorption also occurs readily across the gills in older fish (Goldsmith, 2004). That said, lipid-soluble drugs, which comprise the majority of CNS[2] active drugs, can pose some difficulty with regard to aqueous immersion; however, a number of techniques are available to ensure adequate delivery of these lipophilic molecules using this route of administration (Stewart *et al.*, 2011a; Kalueff *et al.*, 2014a). In this investigation, both of the pharmacological compounds chosen for the different interventions, i.e. apomorphine (R-(−)-apomorphine hydrochloride hemihydrate) and escitalopram oxalate, are readily water-soluble (Managò *et al.*, 2012; Wolmarans *et al.*, 2013; Ang *et al.*, 2016).

Since zebrafish are a relatively novel model organism, exact dosing algorithms that accurately translate the effective concentration of various psychotropic compounds used in rodent studies to zebrafish, are not readily available (Stewart *et al.*, 2011b). As explained above, the primary route of drug exposure in zebrafish is aqueous immersion, which differs in notable ways from the administration routes used in rodent models. Translating an injected drug dose in rodents to an aqueously immersion-administered drug dose in zebrafish may also create potential difficulties (Stewart *et al.*, 2011b). Although immersion is for a number of reasons the preferred route of drug administration in zebrafish (Stewart *et al.*, 2011b; Meshalkina *et al.*, 2017), certain compounds may affect water oxygen content, water pH, gaseous exchange in the swim bladder or gill physiology, all of

[1] days post-fertilization
[2] central nervous system

which may confound the interpretation of behavioral data (Stewart *et al.*, 2011b). Also, in terms of bench to bedside translation, it is likely that it might not be possible to parallel clinical drug administration dosages in zebrafish (Bailey *et al.*, 2015). Compared to humans and rodents, some drugs may display different pharmacokinetic profiles in zebrafish (Bailey *et al.*, 2015) and as such, careful consideration of these aspects in the selection of compounds and dose ranges is required (Bailey *et al.*, 2015). Species-specific differences in animal biology (i.e. physiological and biochemical differences) also play a significant role in the choice of appropriate interventions that will speak to the content of the research question asked. For example, as alluded to earlier, zebrafish, as opposed to humans and rodents, express two forms of SERT[1], i.e. SERTa and SERTb. Although it is believed that only SERTa will play a role in the behavioral responses following SRI[2]/SSRI[3] administration, this remains to be established (Stewart *et al.*, 2011a).

2.4.6 Zebrafish Behaviors that may be of Relevance for Emulating Clinical OCD

Although zebrafish models of psychiatric conditions are gaining importance (Zabegalov *et al.*, 2019), behavioral tests that can describe and quantify behaviors that resemble compulsive-like symptomology have not yet been described. As this shortcoming in the pre-clinical literature is the focus of this work, the conceptualization and validation of a robust zebrafish model of OCD[4] (or indeed compulsive-like behavior) has yet to be attempted or accomplished (D'Amico *et al.*, 2015). Before the application of costly and time-consuming rodent models, research employing zebrafish could help validate or discard candidate OCD therapeutic targets identified in human whole-genome sequencing studies (D'Amico *et al.*, 2015). However, the lack of a prefrontal cortex and expanded telencephalon, the regions involved in executive functions commonly disrupted in psychiatric disorders, could be a possible drawback for using zebrafish in OCD research (D'Amico *et al.*, 2015; Meshalkina *et al.*, 2017); this warrants consideration and further investigation. Regardless, it has been shown that zebrafish are capable of complex cognitive processing despite these anatomical discrepancies (D'Amico *et al.*, 2015; Meshalkina *et al.*, 2017). Also, most behavioral endophenotypes associated with clinical OCD, including anxiety-like behavior, impulsivity, stereotypy, decision-making, and attention deficit have already been demonstrated in adult zebrafish, demonstrating their relevance as a translational model organism, irrespective of potential differences in neurocognitive pathways (D'Amico *et al.*, 2015). The phenotypical presentation of these behaviors and how they resemble analogous rodent behaviors and the symptomology observed in clinical OCD, are summarized in Table 1 below.

[1] serotonin reuptake transporter
[2] serotonin reuptake inhibitor
[3] selective serotonin reuptake inhibitor
[4] obsessive-compulsive disorder

Table 1 - OCD phenotypical modeling in animals. Comparison of OCD diagnostic criteria and phenotypical features shown in rodents and zebrafish (adapted from D'Amico et al. (2015)). OCD: obsessive-compulsive disorder; DSM-5: Diagnostic and Statistical Manual of Mental Disorders, 5th ed.

Phenotypical modeling validity in animal models			
OCD Diagnostic criteria (DSM-5)		***Rodents OCD-like behaviors***	***Zebrafish OCD-like behaviors***
Obsessions	Recurrent and persistent thoughts, urges, or impulses causing anxiety or distress. Individuals attempt to ignore or suppress such thoughts, urges or images, or to neutralize them with some other thought or action.	Anxiety-like behavior, deficiency in set-shifting, decreased natural alternation.	Anxiety-like behavior, stereotypy, impulsivity, deficits in decision-making, attention deficit.
Compulsions	Repetitive behaviors (e.g. hand washing, ordering, checking) or mental acts (e.g. praying, counting, repeating words silently). The behaviors or mental acts are aimed at preventing or reducing anxiety or distress, although it is not connected in a realistic way with what it is attempting to neutralize or prevent.	Excessive grooming, hair removal, altered pattern of "neat" chewing, wheel-running activity, marble-burying, compulsive checking, impaired goal-directed response inhibition.	Stereotypic movements (dashing, freezing or repetitive rotational turns), spontaneous alternation deficits.

From Table 1 (D'Amico *et al.*, 2015), it is evident that by assessing stereotypical movements and compulsive-like persistent choices, some phenotypic traits that are characteristic of clinical OCD[1], may provide an indirect means of measuring compulsions in zebrafish (Zabegalov *et al.*, 2019). In earlier studies, the novel tank test—a paradigm similar in purpose to the open field test used for rodents—was utilized to better understand stereotypical behavior by focusing on drug-induced locomotor effects (Egan *et al.*, 2009). Indeed, stereotypy is often proposed to be a pre-clinical correlate of compulsions (Wolmarans *et al.*, 2013). In this regard, stereotypical behaviors, e.g. repetitive rotations or "circling behavior" have been demonstrated in adult zebrafish (Riehl *et al.*, 2011; Zabegalov *et al.*, 2019). However, great caution is to be exercised if behavioral stereotypy forms the primary construct of animal models of OCD, since stereotypical behavior is a core characteristic of multiple psychiatric and neurological disorders, including Tourette's syndrome (Mercadante *et al.*, 2004), and autism

[1] obsessive-compulsive disorder

(Delmonte *et al.*, 2012). Moreover, although the same brain regions are involved in the pathogenesis of most forms of stereotypy (Garner and Mason, 2002; Haber and Calzavara, 2009; Joel and Weiner, 2000; Langen *et al.*, 2011a; Langen *et al.*, 2011b; Maia *et al.*, 2008), the various conditions respond differently to the available treatment options. If any success is to be gained during the development of an animal model of OCD[1], the differences between the neurobiological and behavioral presentation of the stereotypies observed in conditions associated with motor repetition, need to be understood. To this extent, the following paragraphs will briefly discuss the stereotypies associated with Tourette's syndrome, autism and OCD respectively.

Tourette's syndrome is characterized by *non-goal-directed* verbal and motor tics (American Psychiatric Association, 2013). An inverse relationship has been demonstrated between clinical symptom severity and the volume of the caudate nucleus and, although the condition is postulated to be of a hyper-dopaminergic nature due to its *favorable* response to dopaminergic antagonists, it can also be treated with the SRIs[2]/SSRIs[3] (Langen *et al.*, 2011a; Makki *et al.*, 2008; Rasmussen *et al.*, 1994). Caudate involvement distinguishes the stereotypy observed in Tourette's from pure motor repetition.

Patients with *autism* present with three main symptoms namely stereotypy, non-stereotypical repetitive behavior, and restricted interests (American Psychiatric Association, 2013); *these are all also not directed at realizing any specific goal*. A number of studies have associated autism with abnormalities of the ACC[4] and posterior parietal cortices (Shafritz *et al.*, 2008). Furthermore, patients with autism make more mistakes in experimental tasks and fail to distinguish between correct and incorrect responses compared to healthy control subjects (Thakkar *et al.*, 2008). Although the repetitive behavior expressed in autistic patients responds to some degree to SRIs/SSRIs (Kolevzon *et al.*, 2006), dopamine antagonists are still the mainstay of therapy (Barnard *et al.*, 2002).

Stereotypical behavior in most patients with OCD involves motor and cognitive repetition (Abramowitz *et al.*, 2003; Bartz and Hollander, 2006; Rasmussen *et al.*, 1994; Stein, 2002; Tynes *et al.*, 1990) that are not sensitive to monotherapeutic interventions with anti-dopaminergic agents. As patients most often engage in compulsive rituals in order to reduce the level of distress caused by intrusive thought processes, their behavior has a clear outcome in mind, *which therefore makes it goal orientated*. As referred to earlier, OCD is also associated with increased activity in the ACC and OFC[5], as well as in the caudate nucleus (Maia *et al.*, 2008; Maina *et al.*, 2001; Maltby *et al.*, 2005; Markarian

[1] obsessive-compulsive disorder
[2] serotonin reuptake inhibitors
[3] selective serotonin reuptake inhibitors
[4] anterior cingulate cortex
[5] orbitofrontal cortex

et al., 2010; Saxena and Rauch, 2000). Further, it seems that the repetitive behavior observed in OCD[1] is unique in that it involves motor *and* cognitive repetition and responds mostly to monotherapy with SSRIs[2] (Markarian *et al.*, 2010). Subsequently, these two parameters should be the principle targets for consideration in an animal model of OCD. In this work, we will first attempt to induce persistent behavioral cue-directed arm choice in zebrafish by means of chronic exposure to a dopaminergic drug intervention. We will then aim to establish whether fish exposed to such dopaminergic intervention engage in persistent goal-directed reward-seeking behavior compared to control-exposed zebrafish, thereby being provided with a window into both the motor and cognitive aspects of compulsive-like persistence.

2.4.7 Concluding Remarks with Respect to Zebrafish as a Model Organism

Zebrafish are increasingly sought after as a complementary model to rodents in the field of behavioral neuroscience (Bailey *et al.*, 2015; Maximino *et al.*, 2015). The species fills a significant void in the transitional spaces that exist between traditional mammalian models and *in vitro* studies. Zebrafish present numerous advantages, e.g. being time- and cost-efficient, results-driven, and in their demonstration of sufficient neurochemical and behavioral overlap with mammalian models (Bailey *et al.*, 2015). The administration of drugs to zebrafish is also relatively simple and cost-effective, and by employing aqueous immersion as route of administration, physical stress to the fish is restricted to a minimum, while also ensuring uniform doses are administered to each subject (McGrath & Li, 2008; Stewart *et al.*, 2011b; Meshalkina *et al.*, 2017). Moreover, zebrafish possess sophisticated sensory, motor and motivational systems that are well-suited to experiments based on associative learning tasks (Blaser & Vira, 2014). With respect to the current investigation, in which persistent goal-dissociated behavior is the focus of investigation, we will employ color and conspecifics as cue and reward respectively in an associative learning task to establish how such learning is modified by dopaminergic and serotonergic drug intervention.

2.5 Translating Rodent Models of OCD to Zebrafish

2.5.1 General Considerations in the Design of Animal Models of OCD

Animal models can be defined as experimental constructs that permit phenomena of interest in one species (typically humans), to be studied in another distinct species in a controlled environment (McKinney, 1984; Joel, 2006a; Wang *et al.*, 2009; Albelda & Joel, 2012a), allowing deductions to be made about the former. Over the past three decades, significant effort has been dedicated to the establishment of reliable animal models of human OCD (Albelda & Joel, 2012a; Albelda & Joel, 2012b;

[1] obsessive-compulsive disorder
[2] selective serotonin reuptake inhibitors

Alonso *et al.*, 2015; D'Amico *et al.*, 2015). The broad collective aim is to apply these models to help advance our understanding of the genetic, neurochemical and neuroanatomical underpinnings of the human disorder, to help characterize the underlying neural mechanisms by which current treatments exert their beneficial effects, and to screen for novel therapeutic approaches (Albelda & Joel, 2012a; Albelda & Joel, 2012b; d'Angelo *et al.*, 2014; Alonso *et al.*, 2015; D'Amico *et al.*, 2015; Szechtman *et al.*, 2017; Pittenger *et al.*, 2019). Despite these efforts, some authors have questioned whether it is possible to develop a valid animal model of OCD[1] (Alonso *et al.*, 2015). This argument is based on the difficulty in assessing obsessions, since to obsess is an abstract, internal experience that can only be measured by means of self-reporting, therefore depending on verbal or written communication for its assessment (Hoffman, 2011; Szechtman *et al.*, 2017; Zike *et al.*, 2017; Pittenger *et al.*, 2019). For obvious reasons, animal models can never be interrogated for constructs of such a nature (Alonso *et al.*, 2015). However, animals are well suited for studying other aspects of OCD phenomenology that manifest visibly and that can therefore be quantified as measures of perseveration, compulsivity or stereotypy (Hoffman, 2011; Alonso *et al.*, 2015; Monteiro & Feng, 2016; Szechtman *et al.*, 2017).

It is unrealistic to expect any animal model to fully resemble all aspects of the symptomology and physiology of complex human neuropsychiatric conditions (Joel, 2006a; Fineberg *et al.*, 2011; d'Angelo *et al.*, 2014; Alonso *et al.*, 2015; Szechtman *et al.*, 2017; Zike *et al.*, 2017; Pittenger *et al.*, 2019). Therefore, rather than focusing on emulating the entire syndrome, certain significant condition-like trait features can be selectively investigated (Wang *et al.*, 2009; Hoffman, 2011). Furthermore, although animal models must meet several validation criteria before they can be regarded as reliable frameworks for investigation, the intended purpose of the model will ultimately define the specific criteria it will be measured against (Joel, 2006a; Fineberg *et al.*, 2011; d'Angelo *et al.*, 2014; Alonso *et al.*, 2015). In general, the validation criteria that animal models of OCD should meet can be summarized according to three criteria as initially defined by Willner (1984; 1986), later refined by Geyer and Markou (1995) and Geyer (2002) and reconceptualized for the purposes of OCD (Wolmarans *et al.*, 2017):

1) *Face* validity refers to significant phenomenological resemblance between the specific symptoms of the human disorder and the behavior observed in the animal model. It is typically based on the induction of behaviors that are similar to compulsions; however, some animal models can be representative of other aspects of OCD such as perseveration. In this regard, and as briefly alluded to in paragraph 2.4.6, demonstrations of stereotypical motor repetition form the corner stone of most currently employed animal models of OCD.

[1] obsessive-compulsive disorder

2) *Predictive* validity refers to analogous treatment-induced symptom reduction (therapeutic effects) observed in both the clinical condition and the animal model. From a pharmacological perspective, this is typically based on the attenuation of specific stereotypical behaviors by the administration of SRIs[1]/SSRIs[2] and/or a lack of response to drug interventions that are typically ineffective in the treatment of clinical OCD[3]. Further, considering the chronology of the anti-compulsive treatment response in the human condition, the predictive validity of animal models of OCD will be strengthened if it could be demonstrated that chronic, *but not acute or sub-chronic*, and high, but not nominal dose treatment with SRIs/SSRIs is successful in alleviating said behaviors. In addition, it would be of interest to assess whether certain subjects that remain refractory to SRI/SSRI treatment, will respond to augmentation strategies with low-dose neuroleptic agents.
3) Construct validity refers to the degree of neurobiological overlap between the human condition and the animal model. With respect to OCD, this typically implies that the anatomical regions that constitute the CSTC[4] circuitry, or their analogous regions in fish, are implicated in the animal model. Further, considering the treatment responses of clinical OCD, a clear involvement of serotonin and dopamine should be demonstrated. Although a number of other factors, such as an altered oxidative status (Güldenpfennig *et al*., 2011), increased glutamatergic signaling (Pittenger *et al*., 2006), increased nitric oxide levels (Atmaca *et al*., 2005), the circadian effects of the female hormonal cycle (Abramowitz *et al*., 2003), and increased growth hormone levels (Kluge *et al*., 2006) to name but a few, may contribute to the symptomology of OCD, these are likely to be secondary to changes in serotonin and/or dopamine.

Due to the fact that the present investigation is aimed at establishing a novel model pharmacological framework in which to study the nature and presentation of compulsive-like behavior in zebrafish, the work presented in Chapter 3 considers all three of the abovementioned criteria of validity.

2.5.2 A Review of Current Animal Models

To place the current work within the appropriate context, these paragraphs will briefly review a handful of currently employed rodent models of OCD of different etiopathological classes that collectively constituted the conceptual foundation of this investigation.

[1] serotonin reuptake inhibitors
[2] selective serotonin reuptake inhibitors
[3] obsessive-compulsive disorder
[4] cortico-striatal-thalamic-cortical

2.5.2.1 8-OH-DPAT-Induced Decrease in Spontaneous Alternation: A Pharmaco-Behavioral Model

Pharmacological models of OCD[1] employ drug interventions to induce behavioral alterations that are reminiscent of the symptomology of human OCD. Such behaviors include compulsive checking (Szechtman *et al.*, 1998) or motor perseveration and indecision (Joel, 2006a; Albelda & Joel, 2012b; d'Angelo *et al.*, 2014; Alonso *et al.*, 2015; Zike *et al.*, 2017). In addition to the overlap in behavioral presentation, such behaviors are induced by the manipulation of neurotransmitter systems that are also implicated in the human condition, *viz.* the dopaminergic and serotonergic systems (Joel, 2006a; d'Angelo *et al.*, 2014; Alonso *et al.*, 2015; Zike *et al.*, 2017).

In one such model, 8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide (8-OH-DPAT)[2], a 5-HT_{1A} receptor agonist, is used to induce a reduction in the natural spontaneous alternation behavior of rats in a T-maze (Yadin *et al.*, 1991; Tucci *et al.*, 2013). Indeed, interrogations of how rodents explore novel spaces (i.e. a T-maze) in succession may provide useful insight into the processes that produce compulsive-like persistent arm choice in this scenario. Briefly, food deprived rats are observed in a T-maze where the two goal boxes (in the respective T-maze arms; one white and the other black) are always baited with an identical food reward (Yadin *et al.*, 1991). Each rat is placed in the start box located at the terminal end of the T-maze stem and allowed to choose one of the goal arms over several successive trials per day. The mean number of choices made until an alternation occurs is set as the critical measure (a score of 7 represents perseveration, whereas a score of 1 represents perfect alternation). Against this background, following the acute administration of 8-OH-DPAT, rats are shown to persist in their choice of a specific arm (Albelda & Joel, 2012b; Albelda & Joel, 2012a). However, since decreased alternation (or task-switching) is a common neurocognitive trait observed in a number of neurological and psychiatric conditions other than OCD, the face validity of this model has been questioned (Joel, 2006a; Albelda & Joel, 2012a; Alonso *et al.*, 2015). Furthermore, both sub-chronic and chronic administration of the SSRI[3] fluoxetine as well as sub-chronic administration of the SRI[4] clomipramine, but not desipramine (TCA)[5], prevented said reductions in spontaneous alternation (Joel, 2006a; Albelda & Joel, 2012a; Albelda & Joel, 2012b; d'Angelo *et al.*, 2014; Alonso *et al.*, 2015; Zike *et al.*, 2017). These findings, although implicating a role for serotonin in reduced alternation, are somewhat inconclusive toward the elucidation of the neurobiology of OCD, since patients generally respond to chronic, and not sub-chronic or acute SSRI treatment. Nevertheless, the concept of spontaneous alternation in a T-maze will be adapted for use in this investigation.

[1] obsessive-compulsive disorder
[2] 8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide
[3] selective serotonin reuptake inhibitor
[4] serotonin reuptake inhibitor
[5] tricyclic antidepressant

2.5.2.2 Quinpirole-Induced Compulsive Checking: A Pharmacological Model

Szechtman *et al.* (1998) introduced a rat model of OCD[1] based on compulsive-like checking behavior induced by chronic treatment with a $D_{2/3}$ receptor agonist, quinpirole (administered twice weekly at a dose of 0.5 mg/kg, over a 5-week period). Specifically, quinpirole-treated rats, but not their control treated counterparts, display preference for specific locations in an open field equipped with four objects fixed at different locations (Joel, 2006a; Fineberg *et al.*, 2011; Hoffman, 2011; Albelda & Joel, 2012a; Albelda & Joel, 2012b; d'Angelo *et al.*, 2014; Alonso *et al.*, 2015; Ahmari, 2016; Szechtman *et al.*, 2017; Zike *et al.*, 2017; Hoffman & Cano-Ramirez, 2018). Such checking behavior is proposed to be a pre-clinical correlate of human compulsive checking, as it is representative of i) a preoccupation with and exaggerated hesitancy to leave an item of interest, ii) ritual-like motor activity patterns, and iii) a dependence on environmental context (Szechtman *et al.*, 2001). These observations constitute the face validity of the model. Furthermore, quinpirole-induced checking is partially and transiently attenuated by chronic SRI[2] administration, suggesting pharmacological overlap with clinical OCD patients that display adequate treatment response to SRIs/SSRIs[3] (Joel, 2006a; Fineberg *et al.*, 2011; Hoffman, 2011; Albelda & Joel, 2012a; Albelda & Joel, 2012b; d'Angelo *et al.*, 2014; D'Amico *et al.*, 2015; Ahmari, 2016; Zike *et al.*, 2017). Moreover, the construct validity of the model is supported by the involvement of dopamine D_2 receptors. The current investigation will use the dopamine $D_{1/2}$ receptor agonist apomorphine, rather than quinpirole, as a potential inducer of compulsive-like persistence.

2.5.2.3 The Signal Attenuation Model: A Trained Behavioral Model

Behavioral models are suitable to study the natural etiology and pathogenesis of compulsive-like behaviors and can be divided into naturally occurring or trained repetitive behaviors, e.g. large nest-building (Hoffman & Rueda Morales, 2009; Wolmarans *et al.*, 2016), fur chewing (Galeano *et al.*, 2013), tail chasing (Moon-Fanelli, 2005), or lever-pressing (Joel, 2006b). As the current investigation will involve a training phase to establish adequate cue-reward contingency learning, we will briefly review another model founded on behavioral training, i.e. the signal attenuation model (Albelda & Joel, 2012b). Joel and Avisar (2001) were the first authors to describe a model inspired by the concept that compulsive behaviors may result from deficient feedback associated with the completion of normal goal-directed responses—this is a principle that will be exploited in the current work as well. As alluded to earlier, optimal processing of external feedback prevents the senseless repetition of what

[1] obsessive-compulsive disorder
[2] serotonin reuptake inhibitor
[3] selective serotonin reuptake inhibitors

should be goal-directed responses once such goals have been achieved or when actions are no longer rewarded (Fineberg *et al.*, 2011).

To describe the methodology of the signal-attenuation model, the approaches of several investigations were integrated for clarity (Joel, 2006a; Fineberg *et al.*, 2011; Hoffman, 2011; Albelda & Joel, 2012a; Albelda & Joel, 2012b; d'Angelo *et al.*, 2014; Alonso *et al.*, 2015; Hoffman & Cano-Ramirez, 2018). Briefly, rats are first conditioned to associate the delivery of a reward with the co-presentation of two cues, i.e. light and sound, in the absence of any lever-pressing action (classical conditioning). Rats are then trained to press a lever in order to receive the food reward (goal-directed behavior) while the feedback for a successful lever-press is co-presented by the light/sound cues that accompanies the delivery of the food reward. After the completion of the training period, 'signal attenuation' trials are implemented, whereby the 'feedback deficit', presumed to underlie the expression of compulsive behavior, is induced. Now, the signaling accuracy of the cues is attenuated by repeatedly presenting the cues without the delivery of the food reward during trials where operant lever-pressing is not possible. Finally, during the test phase, the effects of signal attenuation are evaluated under extinction conditions, whereby the pressing of the lever will only result in the presentation of the cues without the delivery of the food reward. As persistent lever-pressing in the absence of reward presentation is both senseless and excessive, thereby being reminiscent of compulsion, this model presents with good face validity (Joel, 2006b; Albelda & Joel, 2012b; Albelda & Joel, 2012a). Furthermore, acute treatment with the SSRIs[1], paroxetine and fluvoxamine, have been found to attenuate compulsive lever-pressing, whereas diazepam (anxiolytic benzodiazepine), desipramine (TCA)[2] and haloperidol (neuroleptic agent) did not (Joel, 2006b; Fineberg *et al.*, 2011; Hoffman, 2011; Albelda & Joel, 2012a; Albelda & Joel, 2012b; d'Angelo *et al.*, 2014; Alonso *et al.*, 2015). This supports the model's predictive validity to some extent; however, chronic administration of drugs has not been tested (Alonso *et al.*, 2015). Moreover, lesions to the OFC[3] and subthalamic nuclei increase compulsive responding and reduce the concentration of striatal dopamine and serotonin (Fineberg *et al.*, 2011; Hoffman, 2011; Albelda & Joel, 2012a; Albelda & Joel, 2012b; d'Angelo *et al.*, 2014; Alonso *et al.*, 2015). Such dysregulation of dopamine and serotonin in the striatum proposes a common pathway that may underlie the expression of compulsive-like behaviors (Albelda & Joel, 2012b). In this current work, we will attempt to first let zebrafish learn the contingency between a cue (red color) and reward (sight of social conspecifics). We will then test persistent cue-directed responses during a phase where the reward-predicting properties of the color

[1] selective serotonin reuptake inhibitors
[2] tricyclic antidepressant
[3] orbitofrontal cortex

red is deconstructed by presenting the color red in the absence of a reward. Ultimately, we aim to establish whether fish exposed to dopaminergic treatment would respond under circumstances of changing outcomes, ultimately being provided with a window into the neurobiological processes that may underlie senseless and repetitive behaviors in zebrafish.

2.6 Developing a Zebrafish Model of OCD

Referring to the previous discussion on the characteristics of zebrafish (section 2.4), the current study will borrow theoretical and methodological approaches from the three rodent models of OCD[1] summarized in section 2.5 towards developing a high-throughput screening test in zebrafish that can detect potential anti-compulsive drug action. The following aspects will be taken from each of the three models in our attempt to develop a new model in zebrafish:

We will adopt the T-maze approach of Yadin *et al.* (1991) to interrogate the concept of naturalistic spontaneous alternation and its modification by dopaminergic and serotonergic drug intervention. Specifically, reductions in spontaneous alternation as well as persistent arm choice will be measured. Thereby, we will be able to investigate two aspects of the Yadin *et al.* (1991) rodent-based model in zebrafish, i.e. perseveration and indecision. Further, we will incorporate certain aspects of the quinpirole model (Szechtman *et al.*, 1998) by attempting to induce persistent arm choice by means of dopaminergic drug intervention. Last, we will combine the aforementioned approaches with the conceptual construct of the signal attenuation model (Joel & Avisar, 2001), by investigating whether the potential compulsive-like behaviors displayed by zebrafish, will be reminiscent of deficient feedback processing, resulting in behavioral inflexibility that visibly manifests as a habitual response to certain environmental stimuli.

Based on this approach and expanding on previous work from different laboratories (Colwill *et al.*, 2005; Al-Imari & Gerlai, 2008), we aim to develop a zebrafish model of compulsive-like persistence behavior in a T-maze. We will first apply a cue-conditioning learning platform (classical conditioning) by which zebrafish will associate the presentation of a reward (social conspecifics) with a visual cue (red cue card). The reward will subsequently be dissociated from the cue in a shorter testing phase, i.e. the cue will be presented without the reward, to measure cue-directed behaviors in zebrafish. Finally, during the last testing phase, the reward will be reintroduced in the non-cued arm, during which phase persistent approaches to the cued arm will again be measured. These experiments will be carried out during chronic exposure to dopaminergic and serotonergic drug interventions in order to investigate the potential contribution of the two neurotransmitters in the observed behaviors.

[1] obsessive-compulsive disorder

Upon completion of this study, the work presented here will provide some useful insight into the etiopathology of compulsivity as well as fill a significant void in current pre-clinical literature.

2.7 Summary of Chapter 2

OCD[1] displays at best a modest response to the currently available treatment options, leaving many patients with residual symptoms. Despite the public health burden of OCD, relatively little is understood about the specific etiology, neurobiology and cognitive dysfunction of the condition compared to other neuropsychiatric conditions (Chamberlain *et al.*, 2005; Wang *et al.*, 2009). There have been many attempts throughout the last three decades to develop valid and useful animal models of OCD in order to further our understanding of the disorder and its treatment (Albelda & Joel, 2012b). Furthermore, as alluded to earlier, current animal (rodent) models have some significant limitations, i.e. they are exceptionally time-consuming and expensive, while not being overly robust. Zebrafish have emerged as an attractive alternative model species to rodents, boasting numerous advantages as highlighted in section 2.4. Currently, no reliable baseline screening tools exist for detecting and quantifying compulsive behaviors in fish prior to initiating similar studies in rodents. Therefore, the current study will attempt to develop such a framework for the study of compulsive-like behavior in zebrafish. If the validity of a zebrafish model of OCD can be demonstrated, it can potentially be used for drug screening and compliment (or even precede and limit) the testing of compounds in rodent models following *in vitro* investigations. This will ultimately broaden the number of experimental resources available to researchers to aid in dissecting the complexities of OCD and its response to treatment.

[1] obsessive-compulsive disorder

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Author Contributions:

- *Cailin van Staden* designed the investigation in consultation with *De Wet Wolmarans* and *Sarel Brand*, performed all behavioral 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.
- *De Wet Wolmarans* and *Sarel Brand* acted as supervisor and co-supervisor of this study respectively. They conceptualized and designed this work and were instrumental in every phase of this investigation. They also revised the first version of this dissertation, including this article.
- *De Wet Wolmarans* funded the project, assisted in the interpretation of results and was corresponding author in the submission of the manuscript to *Behavioural Brain Research*.
- *Geoffrey de Brouwer* assisted with data processing and interpretation and assisted with the writing of the manuscript.
- *Karin Finger-Baier* served as a professional consultant with regards to the use of zebrafish in psychiatric research and proof-read the manuscript.
- *Tarryn Botha* served as the professional consultant regarding the use of zebrafish.

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Important Information:

- All co-authors provided consent for the article to be assessed as part of the M.Sc. dissertation of Cailin van Staden (Addendum A).
- The title of the article changed during revision stages (See Addendum B), therefore the article title reflected on some of the co-authors letters of consent are inaccurate.
- As per the instructions to the authors, figures, tables and legends are provided at the end of the manuscript following the reference list.
- Supplementary tables not included in Chapter 3 are included in Addendum C.
- The submitted manuscript has been accepted for publication in *Behavioural Brain Research* and is currently undergoing proofing at the time of submission of this dissertation (Addendum B). A letter of confirmation of acceptance from *Behavioural Brain Research* and the responses to reviewer comments from the first round of the review process are included in Addendum B.
- **Note:** We received feedback during the second round of review (see Addendum B) at the last minute before the submission of this work. One of the reviewing parties took issue with the use of the word 'learning'. As such certain phrases which were used throughout this dissertation were slightly adjusted *only in this scientific manuscript* and not throughout the remaining content of this dissertation. Therefore, to avoid confusion the prior applied concept of 'learning' was changed to 'association' and 'cue-reward learning' was changed to 'contextual reward appraisal'. Since the reviewer was satisfied with these changes and the paper has since been accepted for publication, we would like to indicate that, for the purpose of this work, these terms are used interchangeably throughout the dissertation.

Title Page

Dopaminergic and serotonergic modulation of social reward appraisal in zebrafish (Danio rerio) under circumstances of motivational conflict: Towards a screening test for anti-compulsive drug action

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Abbreviations

1. Obsessive-compulsive disorder (OCD)
2. Selective serotonin reuptake inhibitors (SSRIs)
3. Serotonin; 5-hydroxytryptamine (5-HT)
4. Time spent in the proximity area of the non-cued arm (NCT)
5. Time spent in the proximity area of the red-colored cued arm (RT)
6. Number of entries made into the proximity area of the non-cued arm (NCE)
7. Number of entries made into the proximity area of the red-colored cued arm (RE)
8. Total time spent in the proximity areas of both arms (TPAT)
9. Total number of entries made into the proximity areas of both arms (TPAE)
10. Two-way repeated measures analysis of variance (2-way RM ANOVA)
11. 8-hydroxy-2-[di-n-propylamino] tetralin (8-OH-DPAT)

Abstract

Cognitive flexibility, shown to be impaired in patients presenting with compulsions, is dependent on balanced dopaminergic and serotonergic interaction. Towards the development of a zebrafish *(Danio rerio)* screening test for anti-compulsive drug action, we manipulated social reward appraisal under different contexts by means of dopaminergic (apomorphine) and serotonergic (escitalopram) intervention. Seven groups of zebrafish (*n* = 6 per group) were exposed for 24 days (1 h per day) to either control (normal tank water), apomorphine (50 or 100 μg/L), escitalopram (500 or 1000 μg/L) or a combination (A100/E500 or A100/E1000 μg/L). Contextual reward appraisal was assessed over three phases i.e. *Phase 1* (contingency association), *Phase 2* (dissociative testing), and *Phase 3* (re-associative testing). We demonstrate that 1) sight of social conspecifics is an inadequate motivational reinforcer under circumstances of motivational conflict, 2) dopaminergic and serotonergic intervention lessens the importance of an aversive stimulus, increasing the motivational valence of social reward, 3) while serotonergic intervention maintains reward directed behavior, high-dose dopaminergic intervention bolsters cue-directed responses and 4) high-dose escitalopram reversed apomorphine-induced behavioral inflexibility. The results reported here are supportive of current dopamine-serotonin opponency theories and confirm the zebrafish as a potentially useful species in which to investigate compulsive-like behaviors.

Keywords

obsessive-compulsive disorder; zebrafish; dopamine; serotonin; inflexibility; opponency

Introduction

Obsessive-compulsive disorder (OCD), like several other neuropsychiatric disorders, e.g. autism (Delmonte *et al.*, 2012), gambling disorder (Reuter *et al.*, 2005), eating disorders (Simon *et al.*, 2016), and schizophrenia (Whitton *et al.*, 2015), is often associated with behavioral inflexibility and dysfunctional reward-feedback processing (Koch *et al.*, 2018). OCD is primarily characterized by persistent and anxiety-provoking thoughts (obsessions) which are typically associated with rigid, repetitive behavioral routines (compulsions), initiated to alleviate the symptoms of anxiety (Figee *et al.*, 2011; American Psychiatric Association, 2013; Winter *et al.*, 2018). Sensations of relief generated by such obsession-compulsion loops are typically temporary (Abramowitz & Jacoby, 2015) leading to worsening cycles of repetitive behavior.

Although the exact etiology and pathophysiology of OCD remains unclear, the condition is often associated with cortico-striatal-thalamic dysregulation (van den Heuvel *et al.*, 2005; Maia *et al.*, 2008; Stocco *et al.*, 2010; Ahmari *et al.*, 2013; Winter *et al.*, 2018). The prefrontal cortex, striatum and thalamus are all involved in the planning, execution and termination of goal-rewarded behavior (Graybiel & Rauch, 2000; Stocco *et al.*, 2010; Vaghi *et al.*, 2017). Dysfunction within these regions is similarly associated with changes in the expression of goal-directed behavior and abnormal reward feedback processing (Stocco *et al.*, 2010; Vaghi *et al.*, 2017). From a neurobiological perspective, cortico-striatal interactions between dopaminergic and serotonergic signaling are pivotal in the regulation of these processes. Indeed, in line with the proposed role of dopamine in reward processing (Schultz, 2002; 2007; 2013) and the opponency theory, describing the motivational dopamine-serotonin interactions under circumstances of rewarding and negative feedback respectively (Daw *et al.*, 2002; Rogers, 2011; Balasubramani *et al.*, 2015), it is believed that a bias in favor of behaviorally activating dopaminergic signaling (Salamone *et al.*, 2015; Nieh *et al.*, 2016) exists (Maia & Cano-Colino, 2015; Wong *et al.*, 2015; Lissemore *et al.*, 2018). Although the neurobiology of OCD and other disorders of behavioral persistence is more complex (Fischer & Ullsperger, 2017), this theory provides a rationale for the use of selective serotonin reuptake inhibitors (SSRIs) as a first-line intervention for the treatment of OCD (Pittenger & Bloch, 2014; Zike *et al.*, 2017). Briefly, chronic administration of SSRIs, e.g. escitalopram, increases the concentration of synaptic serotonin (Lissemore *et al.*, 2018), thereby presumably attenuating excessive behavioral activation, a theory which is supported by data from a number of pre-clinical investigations (Szechtman *et al.*, 2001; Tsaltas *et al.*, 2005; Kontis *et al.*, 2008; Güldenpfennig *et al.*, 2011). However, not all patients respond to SSRI monotherapy, in which case treatment is often augmented with low-dose dopamine receptor antagonists (Bedingfield *et al.*, 1997; Denys *et al.*, 2013).

Observations of dopamine-serotonin interactions are critical when explaining animal behavior since it is often functionally directed at obtaining a reward or avoiding potential aversive outcomes. More specifically, a novel rewarding scenario would initially provoke mass phasic dopaminergic activation (Schultz, 1998; 2002; Pessiglione *et al.*, 2006). Although repeated presentation of the same rewarding scenario within a specific context is, over time, associated with a diminishing dopaminergic response (Schultz, 2002; 2013)—thereby preventing engagement in persistent reward-seeking behaviors—it also enables an organism to acquire knowledge of cue-outcome contingencies, whereby the presentation of specific contextual cues *predicts* potential rewarding outcomes (Schultz, 1998; 2002; Schott *et al.*, 2008). Conversely, serotonergic signaling is typically activated during the experience of aversive feedback and is integral in the learning of future avoidance behaviors (Faulkner & Deakin, 2014). As alluded to earlier, OCD patients often present with dysfunctions related to such constructs, including abnormal reward feedback processing (Figee *et al.*, 2011), altered neurobiological reward anticipatory responses (Figee *et al.*, 2011), overinflated threat estimation (Cisler & Koster, 2010; Exner *et al.*, 2014) and often also being relatively insensitive to punishment (Hinds *et al.*, 2012; Morein-Zamir *et al.*, 2013). It has also been proposed that individuals suffering from OCD may be indifferent to the external feedback cues associated with adequate task completion, which may ultimately manifest as repetitive behavioral engagement (Nielen *et al.*, 2009; Endrass *et al.*, 2013).

The present investigation attempts to investigate how natural reward-seeking behavior in the zebrafish (*Danio rerio*), i.e. the motivational drive to engage in social interaction (Al-Imari & Gerlai, 2008; Saif *et al.*, 2013; Daggett *et al.*, 2019), can be modified by the pharmacological manipulation of dopaminergic and serotonergic neurotransmission. Taken from the literature summarized here, it is possible that bolstered dopaminergic signaling would be associated with persistent reward-seeking behaviors and that such behavior would be subject to attenuation by serotonergic agents. Although this concept has been investigated in rodent studies (Flagel *et al.*, 2010), promising zebrafish models of neuropsychiatric conditions may present a unique avenue to complement rodent models (Fontana *et al.*, 2018). Indeed, the numerous advantages of zebrafish as a model species, including their prolific nature and rapid development (Goldsmith, 2004; Champagne *et al.*, 2010), lengthy lifespan (Kalueff *et al.*, 2014b), ease of drug administration through aqueous immersion (McGrath & Li, 2008; Stewart *et al.*, 2011; Kalueff *et al.*, 2014a) and that it enables cost-effective high-throughput results-driven screening (Levin, 2011), explain why zebrafish are rapidly emerging as a complementary pre-clinical framework for neurobiological research (Flinn *et al.*, 2008; Levin & Cerutti, 2009; Zabegalov *et al.*, 2019). With respect to the focus of this work, the species shows a strong homology to rodent and human neurobiology (Kalueff *et al.*, 2014a; Kalueff *et al.*, 2014b), presenting with almost fully conserved dopaminergic and serotonergic systems (Maximino *et al.*, 2013; Saif *et al.*, 2013; Stewart *et*

al., 2013; D'Amico *et al.*, 2015; Naderi *et al.*, 2016). In addition, zebrafish demonstrate both associative and non-associative cognitive ability, including adequate reversal learning, appetitive reinforcement-based learning and avoidance learning (Al-Imari & Gerlai, 2008; Daggett *et al.*, 2019). Moreover, zebrafish are highly social and are known to shoal and seek out conspecifics, constituting the basis of the present investigation (Al-Imari & Gerlai, 2008; Saverino & Gerlai, 2008; Saif *et al.*, 2013; Daggett *et al.*, 2019). Indeed, it has been shown that simple observation of conspecifics is highly rewarding to zebrafish (Al-Imari & Gerlai, 2008; Saif *et al.*, 2013). A further advantage of a social, as opposed to a food-based, reward construct is that zebrafish do not seem to develop tolerance to social interaction (Daggett *et al.*, 2019). This is of particular value for the present work, which attempts to employ social reinforcement as a suitable reward stimulus for socially deprived zebrafish. Further, in terms of ensuring robust acquisition of cue-reward contingencies, adult zebrafish can clearly distinguish between different colors and are therefore capable of conditioning-based color-dependent learning (Ahmad & Richardson, 2013). In addition, color perception in zebrafish influences learning, decision-making and memory formation (Oliveira *et al.*, 2015). Although it has been demonstrated that zebrafish seemingly prefer red (as used in this investigation) and green equally over no color, yellow and blue (Avdesh *et al.*, 2012), findings with respect to color perception still remain largely inconclusive (Oliveira *et al.*, 2015).

Currently, no rapid and cost-effective screening test exists that can detect potential anti-compulsive drug action. Hence, to this extent and considering the literature summarized here, the current investigation will elaborate on previous work (Colwill *et al.*, 2005; Al-Imari & Gerlai, 2008) by interrogating contextual reward appraisal in zebrafish exposed to either no drug, the dopaminergic $D_{1/2}$ receptor agonist, apomorphine, the SSRI, escitalopram, and combinations of both.

Experimental procedures

Animals and housing

A total of 42 randomly chosen adult wild-type long-fin zebrafish (*Danio rerio*) of both sexes (experimental fish; 3-5 months old; ± 30-40 mm in length; 6 fish per exposure group; refer to '*Exposure groups and drug administration'*) were assessed in this investigation. A further 36 fish were used as social conspecifics (see below). The progenitor stock was originally obtained from Aquaworld Tropical Fish (Singapore) via a national South African importing supplier (WCB Imports, Pretoria, South Africa). All fish used were subsequently bred and housed in the National Aquatic Bioassay Facility (NABF), North-West University, Potchefstroom, South Africa. All procedures and experimental methods were approved by the AnimCare Research Ethics Committee, North-West University; Registration Nr. AREC-130913-015. Subjects were housed according to standard laboratory conditions as prescribed for zebrafish (Reed & Jennings, 2011) and maintained on a 12-hour light/dark cycle (06h00/18h00) in a fully automated system (ZebTec® Zebrafish Housing System, Techniplast®, Varese, Italy) that regulated water quality (pH: 7; conductivity: 600 µS), temperature (26 $\pm$ 0.1 ˚C), and aeration (7.2 mg O_2/L). Experimental zebrafish were group-housed (n = 6 per group) in 3.5 L acrylic tanks for the first 10 days of the investigation and then individually allocated to replicas of the group tanks for the remainder of the study (15 days). Food (ZM-400 fry food, Zebrafish Management Ltd, Twyford, United Kingdom) was provided once daily after the first trial or at 10h00 on exposure days (days 1 - 10). Conspecifics were group-housed (*n* = 6 per tank) for the duration of the study.

Apparatus

Testing procedures involved the use of a transparent Plexiglas® T-maze, the stem of which measured 50 cm x 10 cm x 10 cm. To form a start box, an area (10 cm x 10 cm x 10 cm) was closed off at the foot of the stem. Each arm of the maze measured 20 cm x 10 cm x 10 cm. The cross-arm is defined as the entire arm perpendicular to the arm containing the start box. One separate 10 cm x 10 cm x 10 cm tank of clear Plexiglas® was placed adjacent to the extreme end of each arm of the T-maze (**Figure 1**). Depending on the specific phase of investigation, six (6) conspecific fish were placed into the tank adjacent to one arm of the T-maze. The tank located at the other arm contained only water. At no time were conspecifics introduced to both adjacent tanks simultaneously. The terminal 10-cm end of one of the arms (cued arm) was colored red by covering all relevant surface areas (including that of the adjacent tank, but excluding the adjoining surface between the T-maze and the adjacent tank) as well as those areas of the arm that were located directly opposite the adjacent tank, with a red-colored cue card ('cued arm'; **Figure 1**). The other adjacent arm was covered with the same white plastic sheets as the rest of the maze ('non-cued arm'; **Figure 1**). The resulting setup ensured that the

experimental fish could only see into the adjacent tanks by swimming to and entering the proximity area *directly* in front of the adjacent tank (**Figure 1**). The maze was filled with water from the home tank to a depth of 8 cm and maintained at 26˚C, with the water changed and the maze cleaned every day to prevent excessive growth of biofilm. A digital video-camera (Panasonic® HC-V180) was positioned 150 cm above the T-maze and all trials were digitally recorded. Recordings were subsequently analyzed at the same time by means of EthoVision® XT 14 (Noldus® Information Technologies, Wageningen, The Netherlands) digital tracking software.

Exposure groups and drug administration

Experimental zebrafish were randomly divided into 7 groups (*n* = 6 per group). The different groups were constituted by randomly allocating 6 fish, each from a different home tank to the respective groups. Each group was exposed for 1 h per day for 24 days to one of the respective drug exposures (**Figure 2A**). First, we aimed to establish what the effect of a lower (50 μg/L) and a higher (100 μg/L) dose of apomorphine (Sigma-Aldrich®, Johannesburg, South Africa) would be on contextual reward appraisal. These dosages were based on data from rodent studies (Sukhanov *et al.*, 2014) and adapted for translational application in zebrafish according to previously reported guidelines (Irons *et al.*, 2013; Pinho *et al.*, 2016). As such, the first three exposure groups consisted of 1) control (normal tank water), 2) apomorphine 50 μg/L, and 3) apomorphine 100 μg/L. Subsequently, we aimed to establish the effect of escitalopram (Lundbeck A/S®, Copenhagen, Denmark) alone at both a lower and a higher dose, *viz.* exposure groups 4) escitalopram 500 μg/L and 5) escitalopram 1000 μg/L. Based on earlier work in studies of neuropsychiatric states in rodents and zebrafish, escitalopram oxalate equivalent to 50 mg/kg/day (Wolmarans *et al.*, 2013) roughly equates to 500 μg/L for aqueous immersion studies (Egan *et al.*, 2009; Wong *et al.*, 2010; Stewart *et al.*, 2011; Pittman & Lott, 2014). Last we aimed to establish the effect on contextual reward appraisal following exposure to a combination of apomorphine (at the optimal dose established, i.e. 100 μg/L; refer to *'Results'*) and escitalopram at both doses, *viz.* apomorphine/escitalopram 100/500 μg/L (group 6) and 100/1000 μg/L (group 7). All exposures were administered by means of aqueous immersion throughout the study. During the first ten days of the study, zebrafish were group-housed and group-exposed (**Figure 2A**). For the remainder of the study (days 11 - 25), zebrafish were individually housed, drug exposed and tested, therefore being socially isolated throughout the behavioral testing phase (**Figure 2A**). Drug solutions were constituted by dissolving appropriate quantities of drug in a volume of 1.5 L (for group exposure) or 1.2 L (for individual exposure) tank water. Group-exposed fish were placed into tanks identical to the housing tanks and contained 1.5 L of the relevant drug solutions. During individual exposure, fish were placed into 600 mL beakers containing 250 mL of drug solution. During individual exposure and social

isolation, non-reflective white polystyrene separators were placed between exposure beakers and tanks to prevent zebrafish from seeing one another.

Procedures

Habituation

Prior to the start of Phase 1, zebrafish were habituated individually in the T-maze during a single daily 5-minute session repeated over four consecutive days (Figure 2A; days 11 – 14). During habituation, neither conspecifics nor a red cue card was presented, with all of the T-maze outside walls being covered with white plastic sheeting. Each habituation session on each of the four respective habituation days consisted of a 7-minute exposure session adapted from literature (Al-Imari & Gerlai, 2008). For each of the habituation sessions, a single fish was gently introduced to the start box in the stem of the T-maze for 2 minutes. Next, the start box door was manually raised and lowered again once the fish exited the start box. Each fish was then left to explore the T-maze without restriction for a duration of 5 minutes. Upon completion of the 5-minute session, fish were netted out of the T-maze and placed back into the home tanks. This procedure was repeated for all the fish in the group. Following the completion of all the habituation trials, experimental fish were tested individually in the T-maze according to the procedures explained below (**Figure 2B**).

Phase 1: Cue-reward association

To establish whether fish already chronically exposed to each of the respective interventions would value and appraise the presentation of social conspecifics in the red-colored arm, Phase 1 was executed over 5 days, with two 5-minute T-maze trials per fish per day, separated by 2.5 hours. As such, each fish was assessed across 10 trials during Phase 1 (Al-Imari & Gerlai, 2008). During all trials of Phase 1, one arm of the maze was colored with the red cue card as described under *'Apparatus'*, while a shoal of 6 conspecific fish (social reward), was always presented adjacent to the proximity area of this cued arm. That said, to prevent the potential influence of place preference on arm choice, the cue-reward presentation (red-colored cued arm with conspecific shoal) alternated, i.e. left or right, with each successive trial. Each trial was started by introducing a single zebrafish to the start box for 2 minutes. Subsequently, the start box door was raised and then lowered after the fish exited. Free exploration of the T-maze was allowed for a 5-minute, videotaped session. After the 5-minute trial, the fish was gently netted and placed back into its home tank. This procedure was repeated for each fish. Each trial began at roughly the same time each day, approximately 8h00 for the first trial, followed by the second trial after the inter trial period (2.5 hours), starting at approximately 10h30. This procedural flow was maintained throughout all phases.

Phase 2: Cue-reward dissociation testing

To establish whether zebrafish engaged in cue-directed responses, fish were assessed in Phase 2 over 3 days of testing, with two trials per fish per day, separated by 2.5 hours, beginning on the day following the last Phase 1 trial. Phase 2 proceeded identically to Phase 1 in that the red cue card was once again presented in alternate arms of the maze during each successive trial. However, during this phase, *no social reward (conspecifics) was presented* at any time, hence only filling the adjacent tanks with water.

Phase 3: Re-associative contingency testing

Phase 3 was, as Phase 2, conducted over another 3 days of testing with two daily 5-minute trials, separated by 2.5 hours, completed in a single day. During Phase 3, the experimental setup was applied exactly as in Phases 1 and 2. However, here the presentation of the conspecific-containing tank was paired with the non-cued arm.

The use of conspecifics in Phases 1 and 3

The 36 conspecific fish were divided into 6 groups (A – F) of 6 fish each and used as follows: Groups A and B were used for exposure groups 1 – 3. Groups C and D were used for exposure groups 4 and 5. Groups E and F were used for exposure groups 6 and 7. During each of the phases, the first group of conspecifics was used during the first trial of the day, and the second group of conspecifics, during the second trial of the day.

Statistical analyses

Statistical analyses were performed with GraphPad Prism® 8.0.1 software. Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni post-hoc testing was applied to evaluate the arm-choice behavior of fish in the different exposure groups over the three separate phases. As behaviors relating to arm-choice were primarily expressed as the indices of entry frequency and time spent in the red-colored cued vs. the non-cued arm (see below), these were set as between-subjects (dependent) factor, and phase and exposure as within-subjects (independent) factors, except if stated otherwise. Statistical significance was set at $p < 0.05$ for all analyses. All comparisons, irrespective of being statistically significant or not, were informed with calculations of Cohen's *d* effect size to establish the magnitude of the effects observed. Effect sizes are considered large at values between 0.8 and 1.29 and very large at values higher than 1.3 (Cohen, 1988).

The values expressed on the Y-axis of **Figures 3** and **4** are representative of an index describing the time spent in ([RT-NCT]/TPAT; **Figure 3**) and entries made into ([RE-NCE]/TPAE; **Figure 4**) the cued

compared to the non-cued arm of the maze. These indices were calculated by subtracting the time spent in (NCT) and the entries made into (NCE) the non-cued proximity area from the time spent in (RT) and entries made into (RE) the red-colored proximity area of the cued arm divided by the total time spent in (TPAT) and the number of entries made into (TPAE) the proximity areas.

Results

Comparisons of the effect of phase and drug exposure on the indices of time spent in and number of entries made into the arms

Two-way RM ANOVA did not reveal significant interactions between phase and exposure for either the index of time spent in [F (12,64) = 0.7910, p = 0.658] or the index of entries made into the cued vs. the non-cued arm [F (12,64) = 1.211, p = 0.296]. However, with respect to the index of time spent in the cued vs. the non-cued arm, significant main effects of phase [F (2,64) = 35.27, p < 0.0001] and exposure [F (6,32) = 2.421, p = 0.048] were demonstrated (**Figure 3**). In terms of the index of entry frequency into the cued- vs. the non-cued arm, a significant main effect of phase [F (2,64) = 43.18, p < 0.0001], but not exposure [F (6,32) = 2.176, p = 0.072] was shown (**Figure 4**).

Establishing the most appropriate dose of apomorphine

Based on the observation that apomorphine 100 μg/L, and not 50 μg/L induced *both* a higher degree of cued-arm persistence during Phase 2, *as well as* indiscriminate arm-choice behavior during Phase 3 compared to control exposure (**Figures 3** and **4**; descriptive statistics provided in **Tables 1 and 2**), this dose was chosen for combined exposure with escitalopram in exposure groups 6 and 7.

Within phase comparisons of the indices of time spent in and number of entries made into the arms

Phase 1: Although no statistically significant differences were demonstrated between any of the exposure groups during Phase 1 with respect to either the indices of time spent in (**Figure 3**), or entries made into (**Figure 4**) the cued- vs. the non-cued arm, differences with large effect sizes were observed between the control group and all other exposure groups (**Tables 1A** and **2A**). Further, the difference in the index of time spent in the cued- vs. the non-cued arm between the control-exposed fish and group exposed to 100 μg/L apomorphine only narrowly missed statistical significance (p = 0.076). Moreover, despite conspecific presentation in the cued arm, control-exposed fish avoided the color red, which was reversed by all the exposure groups, as indicated by the negative, as opposed to the positive index values calculated for the control-exposed and all other exposure groups, respectively. This trend was demonstrated with respect to both the indices of time spent in (**Figure 3**) and the number of entries made into (**Figure 4**) the cued- vs. the non-cued arm.

Phase 2: No statistically significant differences were found between exposure groups. In the absence of reward presentation in either arms, control-exposed fish maintained their preference for the non-cued arm, demonstrating continued cued arm avoidance (**Figures 3** and **4**; **Tables 1A** and **2A**). In contrast, all other exposure groups, except for fish exposed to escitalopram 500 μg/L, spent more time in the cued arm compared to the non-cued arm (**Figure 3**; **Table 1A**). However, considering the number of entries made into the arms, escitalopram-alone exposed groups trended toward showing a higher interest for exploring the non-cued arm, as evinced by the negative index of entry frequency into the cued- compared to the non-cued arm of the maze (**Figure 4**, **Table 2A**). Moreover, while fish exposed to apomorphine 50 μg/L and 100 μg/L spent an equal period of time in the cued arm (**Figure 3**, time index 0.33 ± 0.34 vs. 0.26 ± 0.43; **Table 1A**; supplementary data), the group exposed to 100 μg/L also trended towards increased exploration of the non-cued arm in the absence of reward presentation (**Figure 4**, entrance index 0.02 ± 0.49 (A0.1) vs. 0.25 ± 0.28 (A0.05); **Table 2A**; supplementary data).

Phase 3: Again, no statistically significant differences were observed with respect to any of the pairwise comparisons between the different exposure groups. However, in Phase 3, where social conspecifics were presented in the non-cued arm, all exposure groups, except for fish exposed to apomorphine 100 μg/L and a combination of apomorphine and low-dose escitalopram (100/500 μg/L) spent more time in (**Figure 3**) and made more entries into (**Figure 4B**) the non-cued arm, compared to the cued arm (**Tables 1A** and **2A**, respectively). However, only low-dose escitalopram administered alone, induced behavior akin to that observed in the control-exposed fish with respect to both arm choice parameters (**Figures 3** and **4**; **Tables 1A** and **2A**). Apart from the near-zero indices calculated with respect to both the time spent in (**Figure 3**; **Table 1A**) and the number of entries made into (**Figure 4**; **Table 2A**) the cued and non-cued arms respectively, the behavior of animals in the apomorphine 100 μg/L- and combination 100/500 μg/L groups differed to the greatest extent from that of control-exposed fish (**Tables 1A** and **2A**), demonstrating indiscriminate arm-choice behavior.

Between-phase comparisons of the indices of time spent in and number of entries made into the arms

Control group: Control-exposed fish demonstrated a persistent preference for the non-cued arm throughout all phases of the investigation, irrespective of reward presentation in the cued arm during Phase 1, as measured by both the time spent in (**Figure 3**; **Table 1B**) and the number of entries made into (**Figure 4**; **Table 2B**) the arms. Moreover, once sight of conspecifics was introduced in the non-cued arm, control-exposed fish made more entries into the non-cued arm compared to Phase 1 (-0.59 ± 0.27 vs. -0.26 ± 0.46, p = 0.032, d = -0.9) and Phase 2 (-0.59 ± 0.27 vs. -0.33 ± 0.27, p = 0.131, d = -

1.0). Further, although not statistically significant, a difference with a large effect size was observed between the time spent in the non-cued arm during Phase 3, compared to Phase 1 (-0.61 ± 0.29 vs. -0.31 ± 0.45, p = 0.272, d = -0.8).

Apomorphine-alone exposed groups: Considering the time spent in the respective arms, both 50 μg/L and 100 μg/L induced persistent cued arm choice in the absence of reward presentation during Phase 2, compared to Phase 1 (**Figure 3**; **Table 1B**). However, the behavior of 50 μg/L exposed fish was once again more orientated towards the reward as evinced by both the time spent in (**Figure 3**, **Table 1B**; Phase 3 vs. Phase 1: -0.36 ± 0.26 vs. 0.32 ± 0.61, p = 0.0009, d = -1.6; Phase 3 vs. Phase 2: -0.36 ± 0.26 vs. 0.33 ± 0.34, p = 0.0009, d = -2.3) and the number of entries made into (**Figure 4**, **Table 2B**; Phase 3 vs. Phase 1: -0.23 ± 0.22 vs. 0.19 ± 0.58, p = 0.005, d = -1.0; Phase 3 vs. Phase 2: -0.23 ± 0.22 vs. 0.25 ± 0.28, p = 0.001, d = -1.9) the non-cued, compared to the cued arm.

In contrast, although spending significantly less time in (**Figure 3**, **Table 1B**; 0.01 ± 0.48 vs. 0.47 ± 0.38, p = 0.035, d = -1.1) and making fewer entries, albeit not significantly so, into the cued arm (**Figure 4**, **Table 2B**; -0.01 ± 0.32 vs. 0.27 ± 0.32, p = 0.106, d = -0.9) during Phase 3, compared to Phase 1, fish exposed to apomorphine 100 μg/L expressed indiscriminate arm choice behavior during Phase 3 as indicated by near-zero discrimination indices calculated for both time and entry frequency (**Tables 1B** and **2B**).

Escitalopram-alone exposed groups: In the absence of reward presentation (Phase 2 vs. Phase 1), chronic intervention with lower dose escitalopram tended to reduce the index of time spent (-0.02 ± 0.10 vs. 0.20 ± 0.45, d = -0.8) and the number of entries made into the cued vs. the non-cued arm (-0.21 ± 0.20 vs. 0.07 ± 0.29, d = -1.1). In contrast, compared to Phase 1, high-dose escitalopram exposed fish failed to modify their behavior in the absence of reward presentation (**Figures 3** and **4**; **Tables 1B** and **2B**).

However, upon reintroducing sight of conspecifics in the non-cued arm, both groups of escitalopram-exposed fish directed their behavioral response toward the reward. Compared to both Phase 1 and Phase 2, both groups spent significantly more time in [**Figure 3**, **Table 1B**; Phase 3 vs. Phase 1: (*500 μg/L*: -0.60 ± 0.38 vs. 0.20 ± 0.45, p = 0.0004, d = -1.9; *1000 μg/L*: -0.31 ± 0.78 vs. 0.35 ± 0.48, p = 0.004, d = -1.1); Phase 3 vs. Phase 2: (*500 μg/L*: -0.60 ± 0.38 vs. -0.02 ± 0.015, p = 0.015, d = -2.4; *1000 μg/L*: -0.31 ± 0.78 vs. 0.23 ± 0.47, p = 0.022, d = -0.9)] and trended towards making more entries into the non-cued, compared to the cued arm [**Figure 4**, **Table 2B**; Phase 3 vs. Phase 1: (*500 μg/L*: -0.51 ± 0.34 vs. 0.07 ± 0.29, d = -1.9; *1000 μg/L*: -0.43 ± 0.46 vs. 0.11 ± 0.42, d = -1.2); Phase 3 vs. Phase 2: (*500 μg/L*: -0.51 ± 0.34 vs. -0.21 ± 0.20, d = -1.1; *1000 μg/L*: -0.43 ± 0.46 vs. -0.07 ± 0.29, d = -1.0)].

Combination exposed groups: Compared to the behavior observed in fish exposed to 100 μg/L apomorphine only, the addition of the lower dose of escitalopram had no overall effect. In fact, the behavior of fish exposed to combined apomorphine and escitalopram (100/500 μg/L) remained analogous to the behavior of apomorphine-alone exposed fish (**Figures 3** and **4**, **Tables 1** and **2**). In contrast, the combination of apomorphine and high-dose escitalopram, although not affecting cue-directed reward-seeking behavior in the absence of reward presentation compared to apomorphine 100 μg/L alone-exposed fish (*Phase 2*, A0.1E1.0 vs. A0.1: **Figure 3**, **Table 1A**, d = 0.3; **Figure 4**, **Table 2A**, d = 0.4), trended towards attenuating the indiscriminate arm choice during Phase 3 by modifying the behavior of apomorphine-alone exposed fish to a presentation more akin to escitalopram-alone- and control-exposed fish (*Phase 3*, A0.1E1.0 vs. A0.1: **Figure 3**, **Table 1A**, -0.17 ± 0.49 vs. 0.01 ± 0.48, d = -0.4; **Figure 4**, **Table 2A**, -0.36 ± 0.30 vs. -0.01 ± 0.32, d = -1.1).

Total distance moved, total arm entries, and time per cross-arm visit

A statistically significant interaction was demonstrated between phase and exposure with respect to the average total distance moved, an indication of general locomotor activity ([F (12.64) = 1,925, p = 0.048]), with significant main effects of phase [F (2, 64) = 5,327, p = 0.007], as well as exposure [F (6, 32) = 3,509, p = 0.009]. Indeed, drug exposure induced significant within-phase differences between the distances swam by the control group and the apomorphine 100 μg/L and escitalopram 500 μg/L exposed groups (Phase 1 and 2) and the apomorphine 100 μg/L and escitalopram 500 μg/L exposed groups (Phase 3) (See **Figure 5**, and supplementary data). Further, with respect to between-phase behavior, pairwise comparisons revealed apomorphine 100 μg/L-exposed fish to present with shorter distances swam in Phase 3 compared to Phase 1 (p = 0.002, d = -0.9), and Phase 3 compared to Phase 2 (p = 0.041, d = -0.5) (**Figure 5** and supplementary data). However, said reduction in the total distance swum, neither confounds nor undermines the results reported in the results, as fish exposed to apomorphine (100 μg/L) made an equal number of entries into the cross-arms throughout all phases of the investigation (supplementary data). Furthermore, although the average total distance moved by apomorphine 100 μg/L-exposed fish did not differ significantly from any other exposure group, with the exception of control-exposed fish (**Figure 5**), apomorphine 100 μg/L exposure was associated with an increased time spent per cross-arm visit during each of the phases, compared to all other exposure groups (supplementary data).

Discussion

In an effort towards developing a novel screening test for anti-compulsive drug action, the present work investigated cue-reward contingency learning in zebrafish and its modification with dopaminergic and serotonergic intervention. Our main findings were that 1) sight of social conspecifics, although previously reported to be rewarding for socially deprived zebrafish, was an insufficient behavioral reinforcer under circumstances of motivational conflict, 2) apomorphine, escitalopram and combinations thereof lessened the negative valence of an aversive scenario, i.e. bolstering reward appraisal and facilitated reward valuation, 3) high-concentration apomorphine exposure generally maintained cue-directed responses throughout all testing phases, 4) escitalopram facilitated and maintained reward-directed behavior, and 5) high-dose, but not low-dose escitalopram intervention reversed apomorphine-induced indiscriminate arm choices during processes of re-associative testing.

Repetitive behavioral rituals such as hand washing, compulsive checking of locks or re-arranging of items, among others, are characteristic of OCD (Abramowitz *et al.*, 2009). By themselves, these actions often demonstrate a clear goal-directed outcome; however, in OCD, they are performed beyond any functional value (Eisen *et al.*, 2006; American Psychiatric Association, 2013). Chronic high-dose SSRI intervention is regarded as the first-line pharmacological treatment for OCD; however, a substantial number of OCD patients remain treatment refractory (Huff *et al.*, 2010; Fineberg *et al.*, 2013). In order to extend the number of currently available treatment options, accurate and robust screening tests for putative anti-compulsive drugs are of great importance.

Irrespective of the manner in which persistent repetitious behavior arises, organisms normally expend effort to perform actions that are often, but not always, aimed at achieving specific goal-directed outcomes (de Wit & Dickinson, 2009; Banca *et al.*, 2015), e.g. seeking out a food reward (Joel, 2006a), constructing a nest (Greene-Schloesser *et al.*, 2011) or grooming (Greer & Capecchi, 2002). However, if such actions are frequently repeated, they become *habitual*, i.e. expressed in an automated fashion which reduces the need for intensive cognitive decision-making efforts (Banca *et al.*, 2015). That said, over-reliance on habitual responding styles is viewed by contemporary literature as being conducive to compulsive routines (Fineberg *et al.*, 2013; Gillan *et al.*, 2016; Vaghi *et al.*, 2017; Gottwald *et al.*, 2018; Robbins *et al.*, 2019). Any action, be it goal-directed, habitual or otherwise, is preceded by associating certain actions with specific outcomes (Seymour *et al.*, 2007; Gillan *et al.*, 2011). In this regard, behavior is governed by reward and punishment feedback processing (Wise, 2004; Cools *et al.*, 2008; Palminteri *et al.*, 2015) which are regulated by dopaminergic and serotonergic neurotransmission (Wise, 2009; Boureau & Dayan, 2011; Cools *et al.*, 2011; Faulkner & Deakin, 2014).

Disruption of both systems within the neurobiological architecture that regulates voluntary and planned behavior, i.e. the cortico-striatal-thalamic circuitry (Morris *et al.*, 2006; Schott *et al.*, 2008; Gillan *et al.*, 2011; Gillan *et al.*, 2016) is often characteristic of OCD (Koo *et al.*, 2010; Markarian *et al.*, 2010).

Targeted manipulation of the serotonergic and dopaminergic neurotransmission systems has previously been shown to induce ritualistic behaviors reminiscent of compulsive-like rituals in rodents (Yadin *et al.*, 1991; Szechtman *et al.*, 1998). Further, in line with the clinical response of OCD to SSRI intervention, SSRIs have proven useful to attenuate compulsive-like behaviors expressed in pre-clinical models (Fernández-Guasti *et al.*, 2006; Joel, 2006b; Albelda & Joel, 2012; Wolmarans *et al.*, 2016). Considering the expensive, time-consuming and labor-intensive nature of rodent models, the work presented here extends this body of evidence in a putative zebrafish model of compulsive-like persistence. As alluded to earlier, zebrafish demonstrate significant potential as a model species for cost-effective, high-throughput drug screening tests.

Phase 1 - Cue-reward association

Our findings with respect to the influence of dopaminergic and serotonergic drug intervention on reward appraisal during Phase 1, must be regarded against the background of the behavioral response of control-exposed animals. Although sight of conspecifics has previously been shown to be highly rewarding to socially deprived zebrafish (Saif *et al.*, 2013), control-exposed zebrafish generally avoided cued arm exposure, irrespective of the fact that the red color was cued with the presentation of conspecifics. As such, it is possible that as far as natural preference is concerned, the negative valence carried by the color red outweighed the potential rewarding value of visual interaction with conspecifics. Current research is inconclusive with respect to the color preference of zebrafish (Spence & Smith, 2008; Avdesh *et al.*, 2012; Ahmad & Richardson, 2013; Oliveira *et al.*, 2015; Park *et al.*, 2016). For example, Avdesh *et al.* (2012) reported zebrafish to display a natural preference for reds and greens, while demonstrating strong aversion towards blue. However, in accordance with our findings, Oliveira *et al.* (2015) reported that zebrafish display an aversion towards red and yellow while preferring blue and green. That said, the collective of research seems to point to color preference in zebrafish being subject, rather than species-specific (Roy *et al.*, 2019).

Irrespective, the results reported here show that chronically bolstered dopaminergic *and* serotonergic neurotransmission resulted in improved reward appraisal as applied in this investigation, outweighing the negative impact of the red color. Indeed, irrespective of being administered alone or in combination, both apomorphine and escitalopram increased the time spent in and the number of entries made into the cued arm compared to the non-cued arm during Phase 1. Thus, under the

influence of both drugs, the motivation value of social conspecifics was highlighted over and above the potential negative valence carried by the color red. In fact, considering the data presented with respect to Phase 2 (refer to *'Phase 2 – Cue-reward dissociation testing'*) we can with some certainty conclude that zebrafish associated the presentation of conspecifics with the color red. Although our data pertaining to the influence of apomorphine on reward appraisal is in line with the well-known role of dopamine in reward-orientated behavior (Schultz, 2002; Pessiglione *et al.*, 2006; Cools *et al.*, 2009; Palminteri *et al.*, 2009; Rutledge *et al.*, 2009; Adamantidis *et al.*, 2011), it is the actions of serotonin that are noteworthy. Low-dose acute SSRI intervention has been shown to increase the effect of aversive feedback on behavioral outcomes, i.e. resulting in avoidance behavior (Bari *et al.*, 2010), a finding that is supported by the results reported here. Recent evidence also points to the long-term importance of serotonin in the appraisal of rewards and decision making with respect to learned cue-reward contingencies (Iigaya *et al.*, 2018). Furthermore, SSRI-bolstered serotonergic tone also promotes increased effort cost towards achieving rewarding results (Meyniel *et al.*, 2016), possibly explaining the currently observed behaviors.

Phase 2 – Cue-reward dissociation testing

In the absence of reward presentation, cued arm approach bias would confirm successful cue-reward association during Phase 1, manifesting as reward-seeking behavior. However, behavioral flexibility, if intact, should also quickly promote reversal learning and favor exploration of the alternate, non-cued arm. Indeed, considering the apparent aversive nature of the color red, the *absence* of social reward presentation when it was *expected*, should prompt rapid dissociation between the cue and its conditioned outcome and facilitate an alternative behavioral choice, an ability which is impaired in OCD (Hinds *et al.*, 2012; Gruner & Pittenger, 2017). Drug-naive zebrafish continued to display aversion to the red color. However, the behavioral adaptation of apomorphine and escitalopram seemed to diverge, not only as a function of drug but also of dose. First, both doses of apomorphine resulted in continued dwelling in the cued arm, a finding that points to an overall lack of dissociative ability or dopamine-facilitated invigoration towards reward predicting cues (du Hoffmann & Nicola, 2014). This is in line with previously reported literature implicating bolstered dopaminergic signaling in persistent reward-seeking behavior (Beierholm *et al.*, 2013; den Ouden *et al.*, 2013). Further, our data seem to support literature in that transiently depressed, not increased, dopaminergic signaling has been shown to act in concert with serotonin when learning from worse than expected outcomes (Schultz, 2013), a process that should assist with cognitive flexibility. This may have been prevented by the administration of apomorphine. Still, considering the fact that the two arms of the T-maze differed significantly in presentation, we cannot rule out the potential influence of possible persistent spatial memory, a behavioral effect also fortified by bolstered dopaminergic signaling (McNamara *et al.*,

2014). However, this possibility seems less likely than a reward-seeking response per se, as zebrafish exposed to higher dose apomorphine demonstrated increased exploration of the non-cued arm as reflected by the equal number of entries made into both arms of the maze by this group. Irrespective, regarded against the background of the red color aversion shown by drug-naive zebrafish, it can be concluded that the presentation of social conspecifics in the cued arm during Phase 1 resulted in an initial preference for this arm by exposed groups. As such, spatial memory, if playing a role in the data presented here, was primarily founded in the co-presentation of the red color and the reward.

We hypothesized that subjects exposed to escitalopram would learn to disengage their cued arm directed behavior during Phase 2, a process governed chiefly by serotonin, (Boureau & Dayan, 2011; Faulkner & Deakin, 2014). Although our data is to some extent congruent with previous findings that demonstrated both lower and higher doses of escitalopram to facilitate reversal learning (Brown *et al.*, 2012), the difference observed in the effect of lower and higher dose escitalopram on the time spent in the previously cued arm, warrants further investigation. Sadly, most investigations into serotonergic influences on behavioral flexibility and reversal learning, employ serotonin depletion as the primary intervention (Evers *et al.*, 2005; Tanaka *et al.*, 2009), with very little data existing that divulge the role of different serotonin concentrations in cognitive flexibility within analogous experimental paradigms as followed here (Bari *et al.*, 2010).

Our findings related to combination intervention are interesting. Indeed, it seems that combined intervention with both doses of escitalopram maintained the cued arm-directed behavior observed in high-dose apomorphine-alone exposed groups. The addition of escitalopram, regardless of dose, seemed to increase the number of entries made into the cued arm, i.e. masking the effect of apomorphine and escitalopram exposure alone on exploratory activity. In this regard it is likely that, in the absence of reward, the combined effects of dopamine on reward-seeking behavior and that of chronic serotonin in as far as reducing the impact of aversive feedback on decision making (Bari *et al.*, 2010), converges to facilitate continued exploration of a previously reward-cued context.

Phase 3 – Re-associative contingency testing

Considering that zebrafish demonstrated a natural aversion to the color red and that all drug interventions resulted in reward-directed behavior in spite of this, it could have been expected that once introducing the reward to the naturally preferred arm, bolstered reward-directed behavior should be facilitated during Phase 3. This was true for all exposure groups, except the two groups exposed to high-dose apomorphine and a combination of apomorphine and low-dose escitalopram. From an obsessive-compulsive perspective, the ideal response in the high-dose apomorphine exposed group of fish would have been persistent entries and dwelling time in the cued arm, an effect that

would have been indicative of the effect of apomorphine previously shown in other species, e.g. pigeons (Keller *et al.*, 2002). It is likely that the natural aversive nature of the red color as reported in this investigation may have contributed to dampening the effect of high-dose apomorphine on persistent cue-directed responses; this needs further clarification. Nevertheless, our data would suggest that, as opposed to low-dose apomorphine intervention, chronic high-dose apomorphine intervention resulted in a phenotype more akin to behavioral inflexibility. Further, that high-dose apomorphine exposed fish persisted in cue-directed behavior throughout this investigation points to the fact that once a cue-outcome contingency has been acquired under the influence of said intervention, such behavior would be more resistant to adaptation over time and irrespective of changing contexts, albeit being reversible by high-dose SSRI intervention (Boureau & Dayan, 2011; Cools *et al.*, 2011).

Importantly, it is unlikely that any of the results obtained and conclusions drawn here were confounded by the locomotor ability of the animals (**Figure 5**). Although the total distance moved was the highest in drug-naive fish over the course of the investigation, all exposure groups displayed relatively similar locomotor activity during each phase, apart from the high-dose apomorphine exposed group, which demonstrated a slight reduction in locomotor activity over time. However, rather than being indicative of a general motor inability, this points to an increased interest in cued arm exploration. This is true as the data presented here are expressed as indices of the time spent in and number of entries made into the cued vs. the non-cued arm. Indeed, the number of entries made into the respective arms did not change in parallel with a reduction in the total distance swum (**Supplementary Figures i & ii**).

The present investigation was conceptualized to provide a foundation for establishing a novel, high-throughput screening test for anti-compulsive drug action using zebrafish as a model organism. Considering the current theories describing the roles of dopamine and serotonin in OCD, we aimed to induce compulsive-like persistence with the dopaminergic agonist, apomorphine, and further investigated if such persistence, if present, would be reversed by chronic escitalopram. To this extent, the results reported here provide sufficient grounds for further investigation. Although compulsive-like persistence toward habitual, cue-directed behavior was not induced by either dose of apomorphine, fish exposed to high-dose apomorphine did, in fact, present with behavior more akin to behavioral inflexibility compared to their counterparts in all other exposure groups; this was reversed by chronic high, but not lower dose escitalopram, a finding that is supportive of current dopamine-serotonin theory. The apparent aversion shown by drug-naive subjects to the color red was unexpected and has complicated the interpretation of our results. Indeed, it is likely that the use

of a more-preferred color in this population may yield a more robust result, a possibility that we will investigate in future.

In conclusion, typical theories of neurotransmitter involvement in OCD, i.e. imbalanced crosstalk between dopamine and serotonin (Markarian *et al.*, 2010), provide a useful background for investigating compulsive-like behaviors in animals (Gillan *et al.*, 2016). Not only do the findings presented here confirm the viability of zebrafish as a model species in which to study the neurobiological and cognitive processes underlying dopamine-serotonin interactions under circumstances of motivational conflict, it also provide valuable direction for future endeavors toward the development of a novel screening framework that could be sensitive for anti-compulsive drug action.

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None.

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Exposure groups		Phase 1	Phase 2	Phase 3
		Cohen's *d* All *p* values > 0.05	Cohen's *d* All *p* values > 0.05	Cohen's *d* All *p* values > 0.05
Ctrl vs.	A0.05	**1.2**	**2.2**	**0.9**
	A0.1	**1.9**	**1.7**	**1.6**
	E0.5	**1.1**	**1.7**	0.1
	E1.0	**1.4**	**1.5**	0.6
	A0.1E0.5	**1.8**	**2.1**	**1.5**
	A0.1E1.0	**1.2**	**2.1**	**1.2**
A0.05 vs.	A0.1	0.3	0.2	**1.0**
	E0.5	0.2	**1.6**	0.7
	E1.0	0.1	0.2	0.1
	A0.1E0.5	0.3	0.5	**0.9**
	A0.1E1.0	0.1	0.2	0.5
A0.1 vs.	E0.5	0.7	**1.1**	**1.4**
	E1.0	0.3	0.1	0.5
	A0.1E0.5	0.002	0.2	0.03
	A0.1E1.0	0.2	0.3	0.4
E0.5 vs.	E1.0	0.3	**0.9**	0.5
	A0.1E0.5	0.6	**1.3**	**1.3**
	A0.1E1.0	0.3	**1.6**	**1.0**
E1.0 vs.	A0.1E0.5	0.3	0.1	0.5
	A0.1E1.0	0.02	0.4	0.2
A0.1E0.5 vs.	A0.1E1.0	0.2	0.7	0.3

Table 1 – (A) Cohen's *d* values of the within-phase comparisons of the indices of time spent in the cued vs. non-cued arms.

Exposure groups		Phase 1 Cohen's *d* All *p* values > 0.05	Phase 2 Cohen's *d* All *p* values > 0.05	Phase 3 Cohen's *d* All *p* values > 0.05
Ctrl vs.	**A0.05**	**0.9**	**2.1**	**1.5**
	A0.1	**1.3**	**0.9**	**2.0**
	E0.5	**0.9**	0.5	0.3
	E1.0	**0.8**	**0.9**	0.4
	A0.1E0.5	**1.6**	**2.3**	**1.8**
	A0.1E1.0	**0.9**	**1.5**	**0.8**
A0.05 vs.	A0.1	0.2	0.6	**0.8**
	E0.5	0.3	**1.9**	**1.0**
	E1.0	0.2	**1.1**	0.6
	A0.1E0.5	0.5	0.1	**0.8**
	A0.1E1.0	0.1	0.2	0.5
A0.1 vs.	E0.5	0.6	0.6	**1.6**
	E1.0	0.4	0.2	**1.1**
	A0.1E0.5	0.5	0.6	0.1
	A0.1E1.0	0.01	0.4	**1.1**
E0.5 vs.	E1.0	0.1	0.6	0.2
	A0.1E0.5	**1.1**	**2.1**	**1.4**
	A0.1E1.0	0.4	**1.2**	0.5
E1.0 vs.	A0.1E0.5	**0.8**	**1.2**	**1.0**
	A0.1E1.0	0.3	0.7	0.2
A0.1E0.5 vs.	A0.1E1.0	0.3	0.2	**1.1**

Table 2 – (A) Cohen's *d* values of the within-phase comparisons of the indices of the number of entries made into the cued vs. non-cued arms.

Exposure group	Phase	Level of significance	
		p value	*d* value
Control	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	0.272	**0.8**
	Phase 2 vs. Phase 3	0.391	**1.0**
A0.05	Phase 1 vs. Phase 2	-	0.01
	Phase 1 vs. Phase 3	**0.0009**	**1.6**
	Phase 2 vs. Phase 3	**0.0009**	**2.3**
A0.1	Phase 1 vs. Phase 2	0.716	0.5
	Phase 1 vs. Phase 3	**0.035**	**1.1**
	Phase 2 vs. Phase 3	0.488	0.6
E0.5	Phase 1 vs. Phase 2	0.763	0.8
	Phase 1 vs. Phase 3	**0.0004**	**1.9**
	Phase 2 vs. Phase 3	**0.015**	**2.4**
E1.0	Phase 1 vs. Phase 2	-	0.3
	Phase 1 vs. Phase 3	**0.004**	**1.1**
	Phase 2 vs. Phase 3	**0.022**	**0.9**
A0.1E0.5	Phase 1 vs. Phase 2	0.427	**0.9**
	Phase 1 vs. Phase 3	**0.0495**	**1.0**
	Phase 2 vs. Phase 3	0.997	0.5
A0.1E1.0	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	**0.013**	**0.9**
	Phase 2 vs. Phase 3	**0.007**	1.2

Table 1 – (B) Pairwise between-phase comparisons of the indices of time spent in the cued vs. non-cued arms.

Exposure group	Phase	Level of significance	
		p value	d value
Control	Phase 1 vs. Phase 2	-	0.2
	Phase 1 vs. Phase 3	**0.032**	**0.9**
	Phase 2 vs. Phase 3	0.131	**1.0**
A0.05	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	**0.005**	**1.0**
	Phase 2 vs. Phase 3	**0.001**	**1.9**
A0.1	Phase 1 vs. Phase 2	0.160	0.6
	Phase 1 vs. Phase 3	0.106	**0.9**
	Phase 2 vs. Phase 3	-	0.1
E0.5	Phase 1 vs. Phase 2	0.150	**1.1**
	Phase 1 vs. Phase 3	**0.0003**	**1.9**
	Phase 2 vs. Phase 3	0.095	**1.1**
E1.0	Phase 1 vs. Phase 2	0.632	0.5
	Phase 1 vs. Phase 3	**0.0007**	**1.2**
	Phase 2 vs. Phase 3	**0.031**	**1.0**
A0.1E0.5	Phase 1 vs. Phase 2	0.479	0.7
	Phase 1 vs. Phase 3	**0.013**	**1.1**
	Phase 2 vs. Phase 3	0.387	0.7
A0.1E1.0	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	**< 0.0001**	**1.3**
	Phase 2 vs. Phase 3	**0.0002**	**1.5**

Table 2 – (B) Pairwise between-phase comparisons of the number of entries made into the cued vs. the non-cued arms.

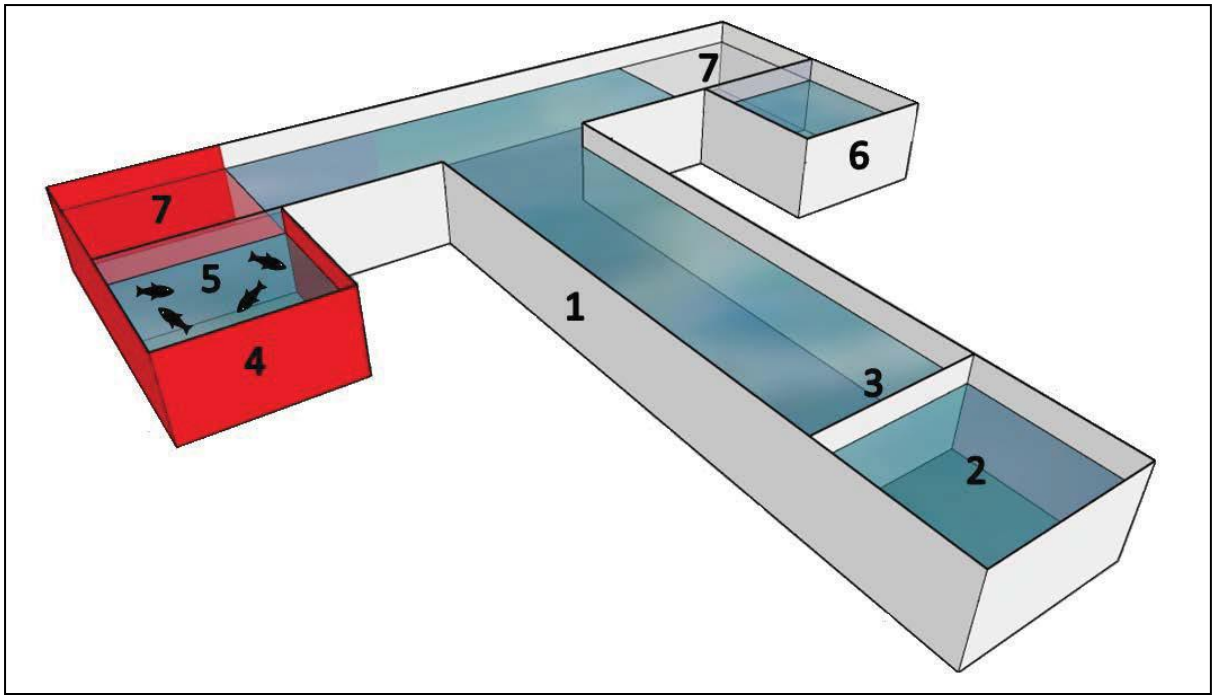


Figure 1

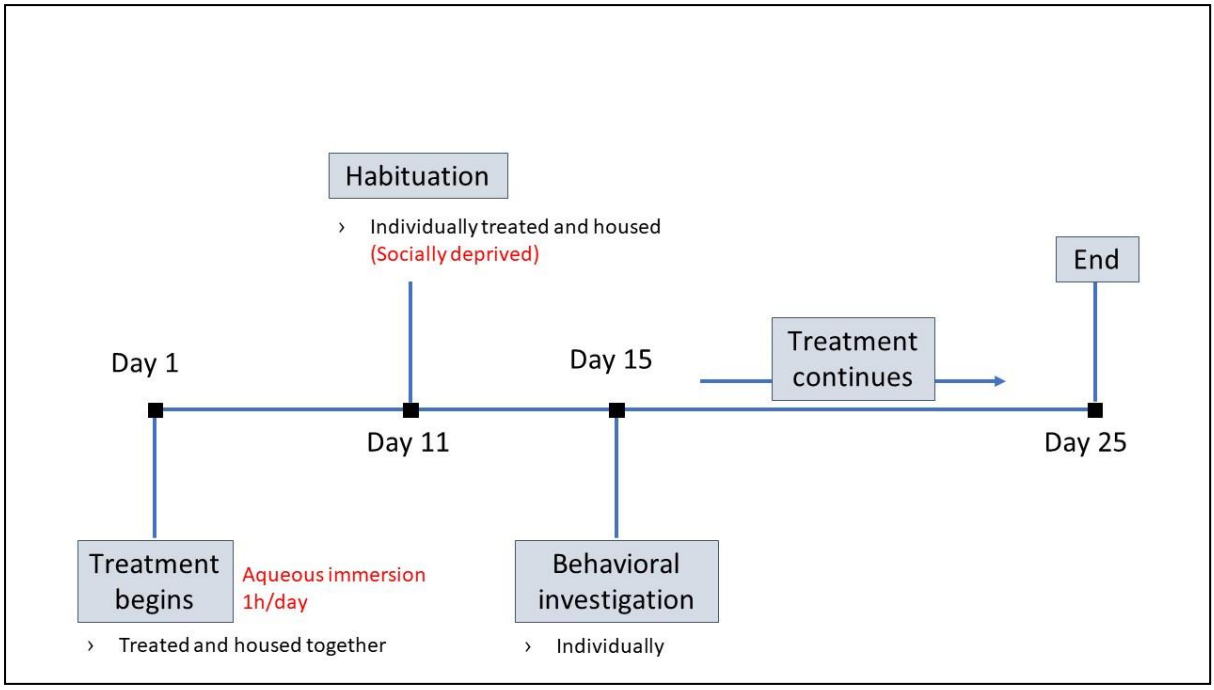

Habituation
› Individually treated and housed
(Socially deprived)
End
Day 1
Day 15
Treatment
continues
Day 11
Day 25
Treatment
begins
Aqueous immersion
1h/day
Behavioral
investigation
› Treated and housed together
› Individually

Figure 2A

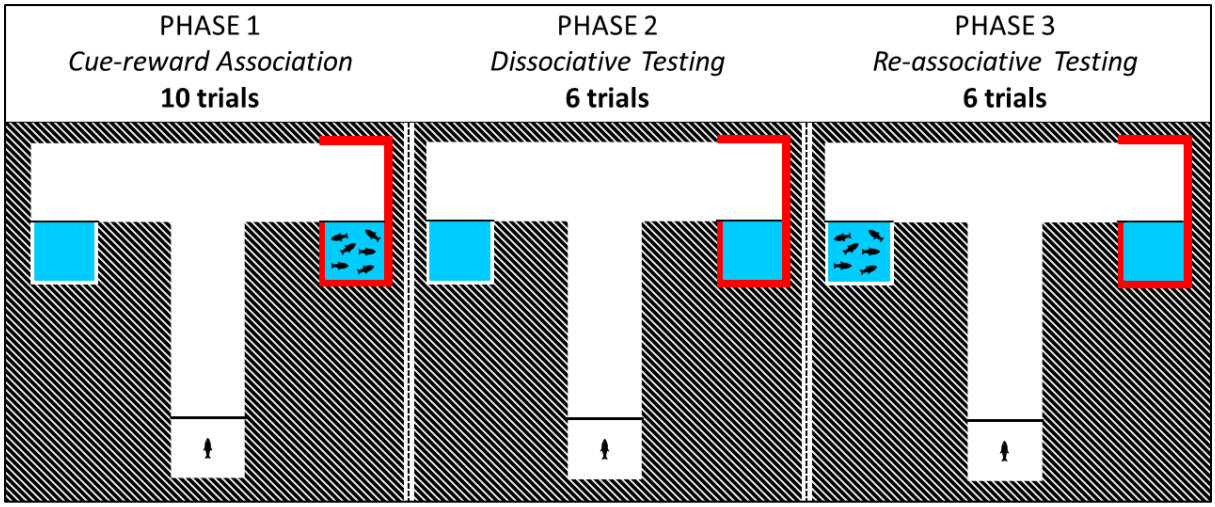

PHASE 1
Cue-reward Association
10 trials
PHASE 2
Dissociative Testing
6 trials
PHASE 3
Re-associative Testing
6 trials

Figure 2B

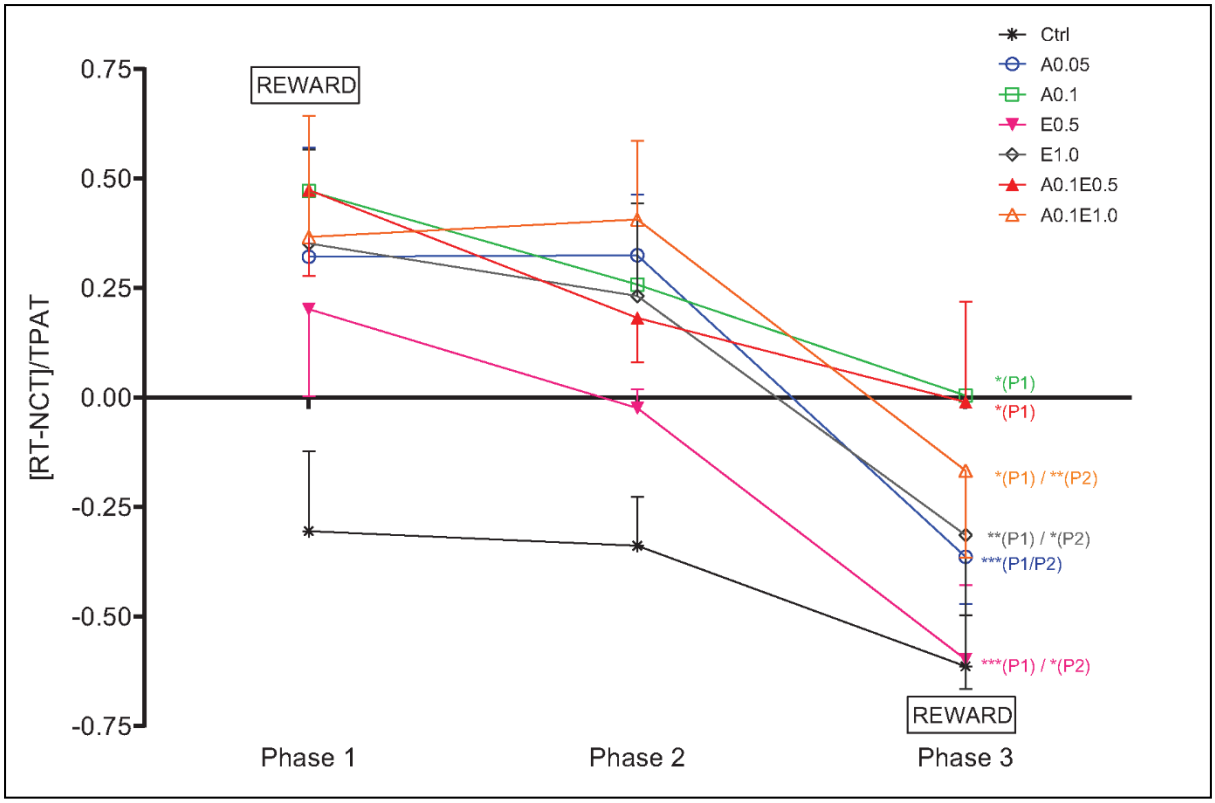

Ctrl
A0.05
A0.1
E0.5
E1.0
A0.1E0.5
A0.1E1.0
REWARD
0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
[RT-NCT]/TPAT
*(P1)
*(P1)
*(P1) / **(P2)
**(P1) / *(P2)
***(P1/P2)
***(P1) / *(P2)
REWARD
Phase 1
Phase 2
Phase 3

Figure 3

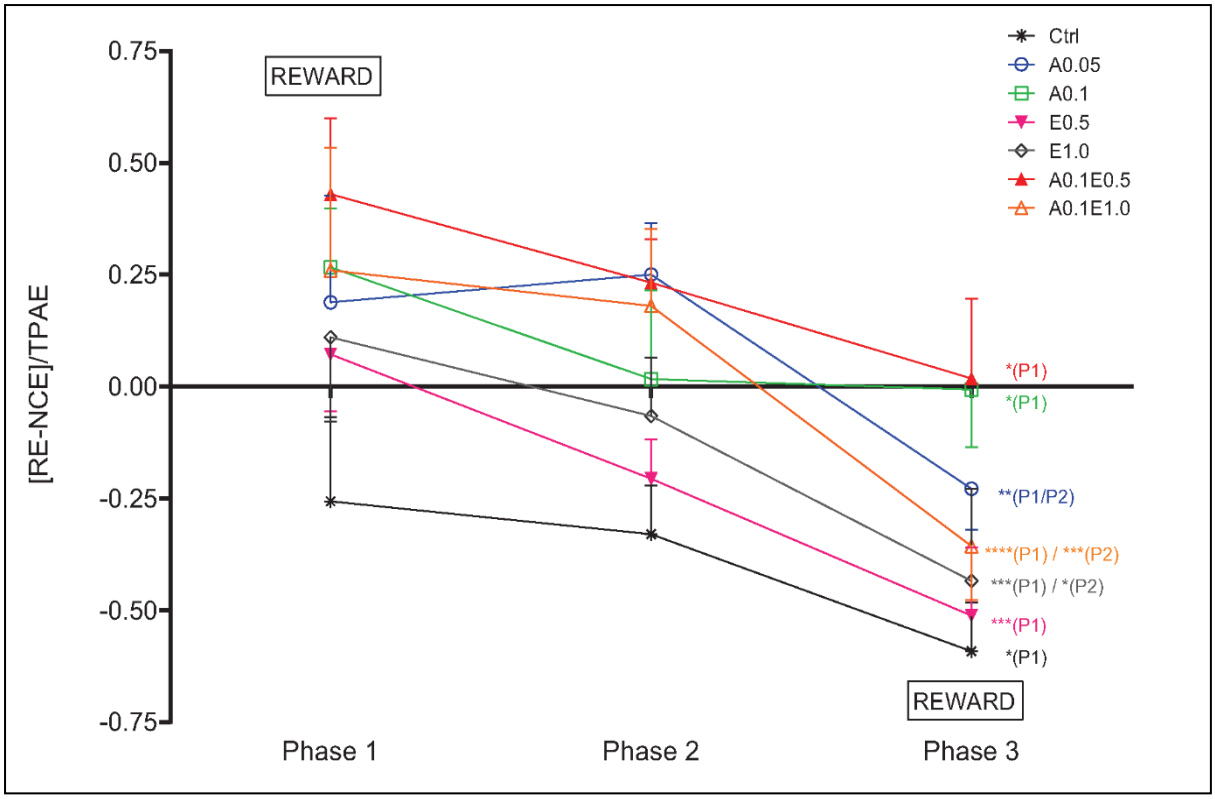

0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
[RE-NCE]/TPAE
REWARD
Ctrl
A0.05
A0.1
E0.5
E1.0
A0.1E0.5
A0.1E1.0
*(P1)
*(P1)
**(P1/P2)
****(P1) / ***(P2)
***(P1) / *(P2)
***(P1)
*(P1)
REWARD
Phase 1
Phase 2
Phase 3

Figure 4

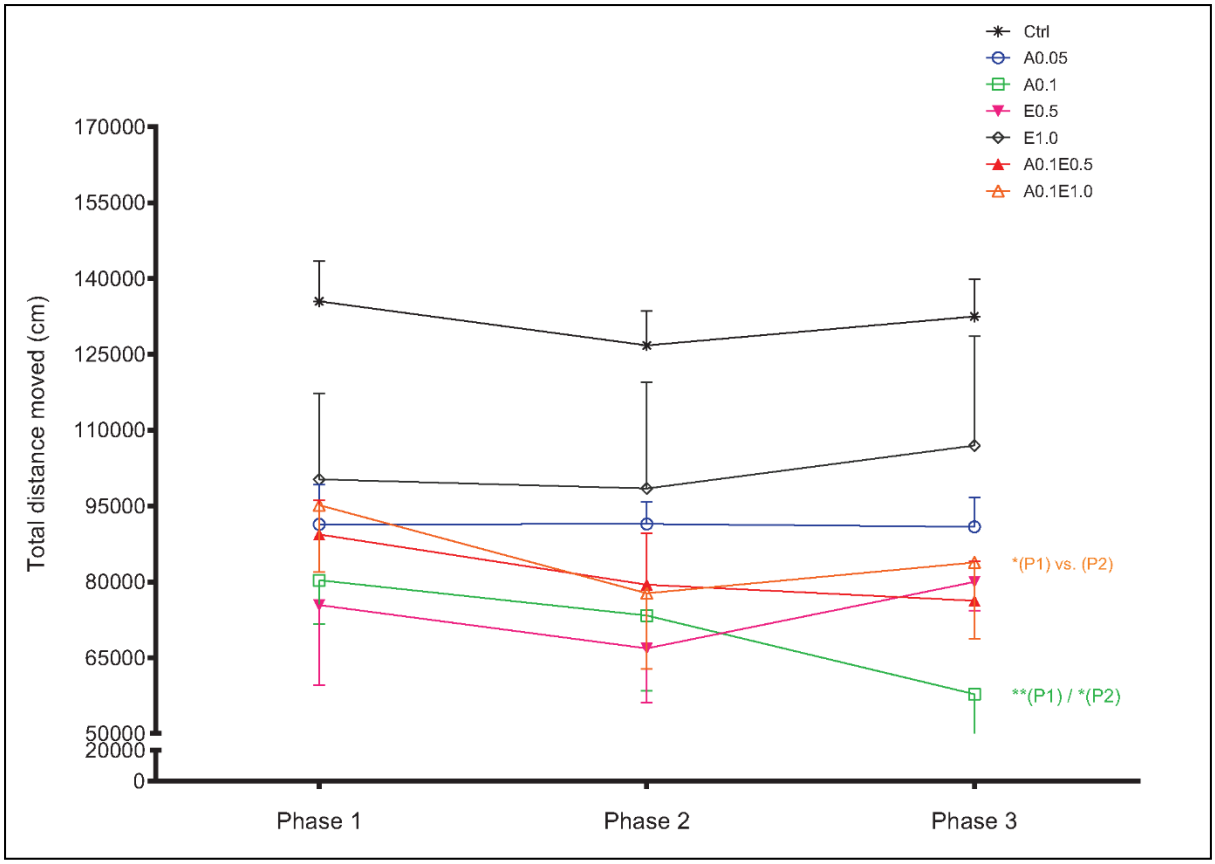
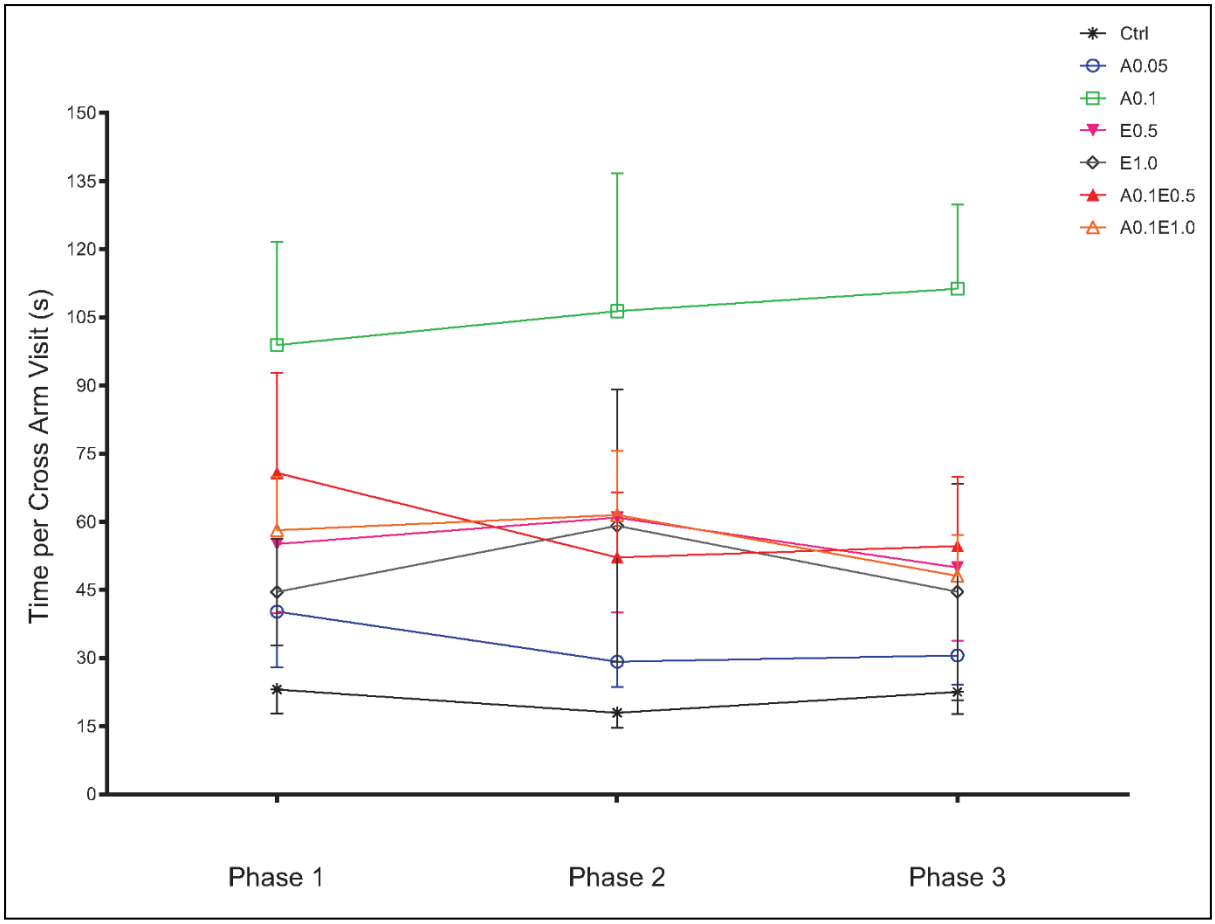
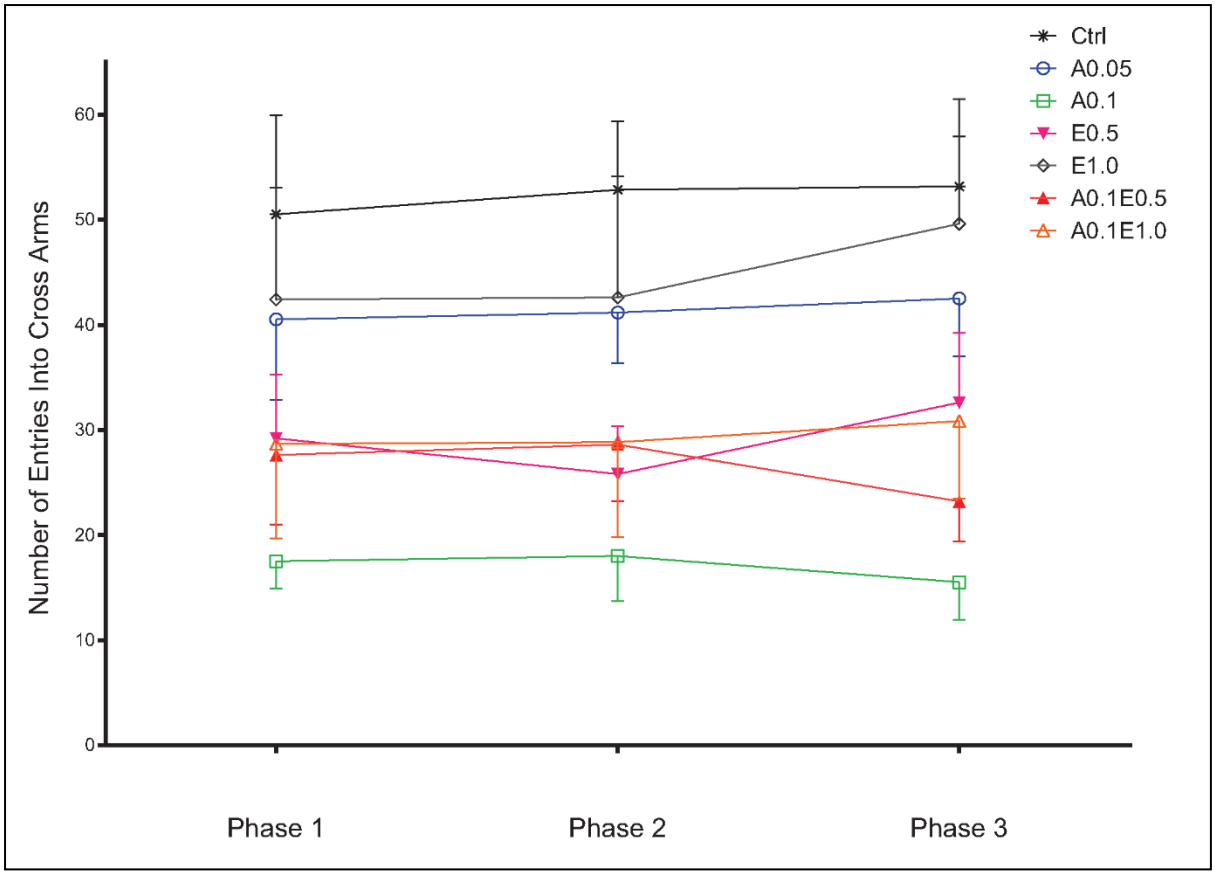

Ctrl
A0.05
A0.1
E0.5
E1.0
A0.1E0.5
A0.1E1.0
Total distance moved (cm)
170000
155000
140000
125000
110000
95000
80000
65000
50000
20000
0
*(P1) vs. (P2)
**(P1) / *(P2)
Phase 1
Phase 2
Phase 3

Figure 5



Supplementary Figure i



Supplementary Figure ii

Figure 1 – Illustration of basic experimental setup. 1) T-maze, 2) starting box, 3) start box door, 4) red cue card, 5) tank containing conspecifics (phase dependent), 6) tank without conspecifics and 7) proximity areas (opposite the two adjacent conspecific tanks).

Figure 2 – **(A)** Schematic layout of the timeline followed for all experimental fish; **(B)** Schematic layout of the phasic setup of the T-maze, indicating the presence of social conspecifics in the vicinity of the red cue card in Phase 1, no social conspecifics presented in Phase 2, and reintroduction of conspecifics in the white arm during Phase 3.

Figure 3 – Comparisons of the average index of time spent in the cued vs. non-cued proximity areas (RT-NCT/[TPAT]; index for time spent in respective arms; see *statistical analysis* for complete explanation) for the different exposure groups over three phases of testing. Positive values on the *y*-axis represent more time spent in the red proximity area, while negative values indicate more time spent in the white non-cued proximity area. *Phase 1* represents 10 trials (5 days), *Phase 2* represents 6 trials (3 days) and *Phase 3* represents 6 trials (3 days). 'Reward' indicates where the sight of social conspecifics was introduced, i.e. either the cued arm (Phase 1), neither of the arms (Phase 2), or the non-cued arm (Phase 3). Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni's multiple comparisons; statistics are mean ± SEM. Significant between-phase differences within the respective exposure groups are indicated on the right side. 'P' indicates the specific phase compared to *Phase 3.* Descriptive statistics provided in **Tables 1A** & **B**, and **supplementary Table i a.**

Figure 4 – Comparisons of the average index of entries made into the cued vs. non-cued proximity areas (RE-NCE/[TPAE]; index for number of entries made into respective arms; see *statistical analysis* for complete explanation) for the different exposure groups over three phases of testing. Positive values on the *y*-axis represent more entries into the red proximity area, while negative values indicate more entries into the white non-cued proximity area. *Phase 1* represents 10 trials (5 days), *Phase 2* represents 6 trials (3 days) and *Phase 3* represents 6 trials (3 days). 'Reward' indicates where the sight of social conspecifics was introduced, i.e. either the cued arm (Phase 1), neither of the arms (Phase 2), or the non-cued arm (Phase 3). Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni's multiple comparisons; statistics are mean ± SEM. Significant between-phase differences within the respective exposure groups are indicated on the right side. 'P' indicates the specific phase compared to *Phase 3.* Descriptive statistics provided in **Tables 2A** & **B**, and **supplementary Table i b**.

Figure 5 – Comparisons of the average total distance moved for the different exposure groups over the different phases. Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni's multiple comparisons; statistics are mean ± SEM. Significant between-phase differences within the respective exposure groups are indicated on the right side. 'P' indicates the nature of specific phase comparisons. Descriptive statistics provided in **supplementary Table ii a** & **b**.

Supplementary Figure i – Comparisons of the time spent per cross-arm visit (seconds) throughout all phases of the investigation for the different exposure groups. Statistics are mean ± SEM. Descriptive statistics provided in **supplementary Tables iii a** & **b**.

Supplementary Figure ii – Comparisons of the number of entries into the cross-arms throughout all phases of the investigation for the different exposure groups. Statistics are mean ± SEM. Descriptive statistics provided in **supplementary Table iv a** & **b**.

Figure 5 – Comparisons of the average total distance moved for the different exposure groups over the different phases. Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni's multiple comparisons; statistics are mean ± SEM. Significant between-phase differences within the respective exposure groups are indicated on the right side. 'P' indicates the nature of specific phase comparisons. Descriptive statistics provided in **supplementary Table ii a** & **b**.

Supplementary Figure i – Comparisons of the time spent per cross-arm visit (seconds) throughout all phases of the investigation for the different exposure groups. Statistics are mean ± SEM. Descriptive statistics provided in **supplementary Tables iii a** & **b**.

Supplementary Figure ii – Comparisons of the number of entries into the cross-arms throughout all phases of the investigation for the different exposure groups. Statistics are mean ± SEM. Descriptive statistics provided in **supplementary Table iv a** & **b**.

4 Conclusion

The aim of the current study was to develop a novel screening test for anti-compulsive drug action by investigating cue-reward contingency learning in zebrafish (*Danio rerio*) and its modification with dopaminergic and serotonergic intervention. The main findings were: 1) sight of social conspecifics, although previously reported to be rewarding for socially deprived zebrafish, was an insufficient reinforcer of cue-reward contingency learning under circumstances of motivational conflict, 2) apomorphine, escitalopram and combinations thereof lessened the negative valence of an aversive scenario, i.e. bolstering reward appraisal and facilitated cue-reward contingency learning, 3) high-concentration apomorphine exposure generally maintained cue-directed responses throughout all testing phases, 4) escitalopram facilitated and maintained reward-directed behavior, and 5) high-, but not low-dose escitalopram intervention reversed apomorphine-induced indiscriminate arm choices during processes of re-associative testing.

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Obsessive-compulsive disorder (OCD)[1] is a severe and disabling neuropsychiatric affliction (Hirschtritt *et al.*, 2017; Abramovitch *et al.*, 2019; Robbins *et al.*, 2019) that is characterized by recurrent, unwanted thoughts and persistent repetitive motor actions (Abramovitch *et al.*, 2019). Chronic high-dose selective serotonin reuptake inhibitor (SSRI)[2] intervention is regarded as the first-line pharmacological treatment for OCD; however, a substantial number of OCD patients remain treatment refractory (Huff *et al.*, 2010; Fineberg *et al.*, 2013). In order to extend the number of currently available treatment options, accurate and robust screening tests for putative anti-compulsive drugs are of great importance.

Over-reliance on habitual responding, which separates actions from clear goals, is believed to be conducive to compulsive routines (Fineberg *et al.*, 2013; Gillan *et al.*, 2016; Vaghi *et al.*, 2017; Gottwald *et al.*, 2018; Robbins *et al.*, 2019). However, any action, be it goal-directed, habitual or otherwise, is preceded by learned associations between the action and its outcome (Seymour *et al.*, 2007; Gillan *et al.*, 2011). In this regard, behavior is governed by two primary learning pathways, i.e. reward and punishment feedback processing (Wise, 2004; Cools *et al.*, 2008; Palminteri *et al.*, 2015) which are regulated by dopaminergic and serotonergic neurotransmission (Wise, 2009; Boureau & Dayan, 2011; Cools *et al.*, 2011; Faulkner & Deakin, 2014). Deficits in both reward and punishment feedback

[1] obsessive-compulsive disorder
[2] selective serotonin reuptake inhibitor

processing result in cognitive rigidity and behavioral inflexibility (Figee *et al.*, 2011; Endrass *et al.*, 2013) as notably observed in patients with OCD[1] (Gruner & Pittenger, 2017; Weisholtz *et al.*, 2017).

Targeted manipulation of the serotonergic and dopaminergic neurotransmission systems has previously been demonstrated to induce ritualistic behaviors reminiscent of compulsive-like rituals in rodents (Yadin *et al.*, 1991; Szechtman *et al.*, 1998). Further, in line with the clinical response of OCD to SSRI[2] intervention, serotonergic interventions have proven useful to attenuate compulsive-like behaviors expressed in pre-clinical models (Fernández-Guasti *et al.*, 2006; Joel, 2006; Albelda & Joel, 2012; Wolmarans *et al.*, 2016). Considering the expensive, time-consuming and labor-intensive nature of rodent models, the work presented here extends this body of research in a putative zebrafish model of compulsive-like persistence.

***

Our findings with respect to the influence of dopaminergic and serotonergic drug intervention on cue-reward contingency learning (Phase 1) is noteworthy. Although the sight of conspecifics has previously been demonstrated to be highly rewarding to socially deprived zebrafish (Saif *et al.*, 2013), control-exposed zebrafish generally avoided cued arm exposure, irrespective of the presentation of conspecifics. As such, it is possible that as far as natural preference is concerned, the negative valence carried by the color red in this investigation outweighed the potential rewarding value of visual interaction with conspecifics. Current research is inconclusive with respect to the color preference of zebrafish (Spence & Smith, 2008; Avdesh *et al.*, 2012; Ahmad & Richardson, 2013; Oliveira *et al.*, 2015; Park *et al.*, 2016). Irrespective, the results reported here indicate that chronically bolstered dopaminergic *and* serotonergic neurotransmission resulted in improved reward appraisal as applied in this investigation, negating the demotivational impact of the red color. Thus, under the influence of both drugs, the red color adopted reward-predicting properties (Schultz, 2002; Wise, 2009; Schultz, 2013) indicating that successful associative learning has indeed been established in exposed fish during Phase 1.

***

In the absence of reward presentation (Phase 2), drug-naive zebrafish continued to display aversion to the red color. However, the behavioral adaptation of apomorphine- and escitalopram-exposed fish seemed to diverge. Both doses of apomorphine resulted in continued dwelling in the cued arm, a finding that points to an overall lack of dissociative ability or a dopamine-facilitated invigoration

[1] obsessive-compulsive disorder
[2] selective serotonin reuptake inhibitor

towards approaching reward predicting cues (du Hoffmann & Nicola, 2014). This is in line with previously reported literature implicating bolstered dopaminergic signaling in persistent reward-*seeking* behavior (Beierholm *et al.*, 2013; den Ouden *et al.*, 2013). We hypothesized that subjects exposed to escitalopram would learn to disengage their cued arm directed behavior during Phase 2, a process governed chiefly by serotonin (Boureau & Dayan, 2011; Faulkner & Deakin, 2014). Although our data is to some extent congruent with previous findings that demonstrated both lower and higher doses of escitalopram to facilitate reversal learning (Brown *et al.*, 2012), the difference observed in the effect of lower and higher dose escitalopram on the time spent in the cued arm, warrants further investigation. Further, it seems that co-exposure to apomorphine and both doses of escitalopram maintained the cue -directed behavior observed in high-dose apomorphine-alone exposed groups. However, the addition of escitalopram, regardless of concentration, seemed to mask the effect of apomorphine and escitalopram exposure alone on exploratory activity. In this regard it is likely that, in the absence of reward, the combined effects of dopamine on reward-seeking behavior and of serotonin on reducing the impact of aversive feedback on decision-making (Bari *et al.*, 2010), converges to facilitate continued exploration of a previously reward-cued context.

***

Considering that zebrafish demonstrated a natural aversion to the color red and that all drug interventions resulted in reward-directed behavior in spite of this, it could have been expected that once introducing the reward to the naturally preferred arm, bolstered reward-directed behavior should be facilitated during Phase 3. This was true for all exposure groups, except the two groups exposed to high-dose apomorphine and a combination of apomorphine and low-dose escitalopram, respectively. From an obsessive-compulsive perspective, the ideal response in the high-dose apomorphine exposed group of fish would have been persistent entries and dwelling time in the cued arm. In light of this, it is likely that the natural aversive properties of the red color as reported in this investigation may have contributed to dampening the effect of high-dose apomorphine on persistent cue-directed responses; this needs further clarification. Nevertheless, our data would suggest that, as opposed to low-dose apomorphine intervention, chronic high-dose apomorphine intervention resulted in a phenotype more akin to behavioral inflexibility. Further, that high-dose apomorphine exposed fish persisted in cue-directed behavior throughout this investigation points to the fact that once a cue-outcome contingency has been learned under the influence of said intervention, such behavior would be more resistant to adaptation over time and irrespective of changing contexts (Boureau & Dayan, 2011; Cools *et al.*, 2011).

***

In conclusion, typical theories pertaining to the cognitive, i.e. behavioral inflexibility, and neurobiological, i.e. imbalanced crosstalk between dopamine and serotonin (Markarian *et al.*, 2010), constructs that may underlie OCD[1], provide a useful background for investigating compulsive-like behaviors in animals (Gillan *et al.*, 2016). Not only do the findings reported here confirm the viability of zebrafish as a model species in which to study these processes, it also provides valuable direction for future endeavors toward the development of a novel screening framework that could be sensitive for anti-compulsive drug action.

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[1] obsessive-compulsive disorder

Table 4.1 – Summary of study questions and final outcomes

Study questions	Final outcomes
1) Will zebrafish seek out a social reward and will they associate such reward with a visual cue, thereby demonstrating associative learning?	The color red as applied in this investigation, unexpectedly demonstrated aversive properties. Therefore, control-exposed zebrafish failed to exhibit reward-directed behavior during Phase 1.
2) Will zebrafish, in the absence of reward presentation, display exploratory behavior in the T-maze?	In the absence of reward presentation, control-exposed zebrafish continued to display aversion to red. Hence, instead of exploring the T-maze, they directed their behavior to the non-cued arm.
3) Will zebrafish display reward-orientated behavior upon the reintroduction of the reward in the previously non-cued arm?	Upon reintroduction of the reward in the non-cued arm, control-exposed zebrafish clearly displayed bolstered reward-directed behavior by making more entries into and spending more time in the non-cued arm. This highlights the actual value of conspecifics as a reward when not paired with an aversive color.
4) Will the administration of apomorphine bolster the acquisition of the cue-reward contingency in (1) maintain cue-directed behavior throughout Phases 1 – 3?	While both concentrations of apomorphine improved the positive valence of the social reward and maintained cue-directed behavior in the absence of reward presentation, only high-dose apomorphine intervention resulted in a phenotype more akin to behavioral inflexibility during Phase 3.
5) How will escitalopram alone influence the behavior of zebrafish as per the conditions listed in (1) – (3).	Both concentrations of escitalopram facilitated reward-, and not cue-directed behavior throughout all phases of experimentation.
6) How will escitalopram influence the behaviors observed under the conditions referred to in (4)?	Both concentrations of escitalopram bolstered and maintained the effect of the high apomorphine concentration during Phases 1 and 2. Only the high concentration of escitalopram reversed apomorphine-induced indiscriminate arm choices during Phase 3, thereby eliciting behavior more akin to that observed in control-exposed zebrafish.
7) Do zebrafish hold promise as a potential model of compulsive-like persistent behaviors?	Although the answer to this question may be confounded by the circumstances under which such investigations are performed, zebrafish indeed recognize and respond to social conspecifics while such behavior is governed by processes related to cue-reward contingency learning as well as dopaminergic and serotonergic drug exposure. As such, the model does indeed hold promise for future elaboration.

4.1 Shortcomings, Recommendations and Future Studies

This investigation was a first of its kind in zebrafish and provided significant direction for future research. The study made use of only six zebrafish per group with the aim of establishing putative validity before expanding the group sizes. While Cohen's *d* values revealed differences of very large effect sizes, no statistically significant differences were reported between the different treatment groups within each of the phases. Now that the method has been described and the results of this investigation have been reported, group sizes could be increased to 8 – 10 animals in future investigations.

Given the unexpected aversion to the color red shown by the control-exposed zebrafish, it may have been beneficial to use a more-preferred color that could have resulted in a more robust result. However, current research is inconclusive with respect to the color preference of zebrafish. Therefore, it will be necessary to employ a color preference experiment in individual fish before choosing a different cue color in future investigations.

If possible, it will be advantageous for future studies to use computer-simulated 'fish' as reinforcers as opposed to live conspecifics. Both are effective reinforcers; however, the use of simulated fish would enable the use of fewer fish which would, from an ethical point of view, be beneficial (Saif *et al.*, 2013; Daggett *et al.*, 2019). This rewarding stimulus is further ideal for automated, higher throughput learning tasks that may be of great value in future studies.

In terms of the procedural methodology, the number of trials that were conducted per fish per day could be increased, allowing for a higher throughput results-driven test. The current study made use of two trials per day per zebrafish, with a between trial period of 2.5 hours. Although this approach has been shown to allow adequate learning to take place (Al-Imari & Gerlai, 2008), increasing the trials per day may permit faster acquisition of a cue-reward contingency. In addition, this could ultimately shorten the duration of the behavioral investigation, which will be advantageous for a number of reasons. However, the viability of such an approach, remains to be studied.

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Addendum A

Co-Authors Letters of Permission to Submit Chapter 3 for Examination Purposes

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The Director: Higher Degrees Administration
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SOUTH AFRICA

01 October 2019

Dear Sir/Madam,

I, De Wet Wolmarans, study-leader and senior corresponding co-author of the manuscript first authored by Miss Cailin van Staden titled *"Dopaminergic and serotonergic modulation of social reward appraisal in zebrafish (Danio rerio) under circumstances of motivational conflict: towards a screening test for anti-compulsive drug action"* hereby grant my permission that this work may be submitted for examination purposes as Chapter 3 in the M.Sc. dissertation of Miss Cailin van Staden.

Sincerely,

Dr. De Wet Wolmarans
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The Director: Higher Degrees Administration
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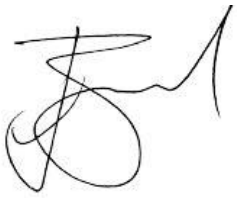
01 October 2019

Dear Sir/Madam,

I, Sarel Brand, co-author of the manuscript first authored by Miss Cailin van Staden titled *"Dopaminergic and serotonergic modulation of social reward appraisal in zebrafish (Danio rerio) under circumstances of motivational conflict: towards a screening test for anti-compulsive drug action"* hereby grant my permission that this work may be submitted for examination purposes as Chapter 3 in the M.Sc. dissertation of Miss Cailin van Staden.

Sincerely,



Dr. Sarel J. Brand
North-West University, Potchefstroom, South Africa

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Higher Degrees Administration
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RE: SUBMISSION OF MISS CAILIN VAN STADEN,
STUDENT NR 25194364

Martinsried, 16.10.2019

To Whom It May Concern:

I, Karin Finger-Baier, co-author of the manuscript titled "Dopaminergic and serotonergic modulation of contingency learning in zebrafish (Danio rerio) under circumstances of motivational conflict: towards a screening test for anti-compulsive drug action" hereby grant my permission that this work may be submitted for examination purposes as Chapter 3 in the M.Sc. dissertation of Miss Cailin van Staden.

Yours sincerely,

K. F-B

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01 October 2019

Dear Sir/Madam,

I, Tarryn Botha, co-author of the manuscript first-authored by Miss Cailin van Staden titled *"Dopaminergic and serotonergic modulation of social reward appraisal in zebrafish (Danio rerio) under circumstances of motivational conflict: towards a screening test for anti-compulsive drug action"* hereby grant my permission that this work may be submitted for examination purposes as Chapter 3 in the M.Sc. dissertation of Miss Cailin van Staden.

I trust that all is in order.

Dr. Tarryn L. Botha
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01 October 2019

Dear Sir/Madam,

I, Geoffrey de Brouwer, co-author of the manuscript first authored by Miss Cailin van Staden titled *"Dopaminergic and serotonergic modulation of social reward appraisal in zebrafish (Danio rerio) under circumstances of motivational conflict: towards a screening test for anti-compulsive drug action"* hereby grant my permission that this work may be submitted for examination purposes as Chapter 3 in the M.Sc. dissertation of Miss Cailin van Staden.

Sincerely,

Mr. Geoffrey de Brouwer
North-West University, Potchefstroom, South Africa

Addendum B

Correspondence Relating to the Submission of the Article (Chapter 3) to *Behavioural Brain Research* and Response to the Reviewer Comments (Rebuttal Letters)

Confirmation of Acceptance for Publication

This document has been formatted in the style of the current dissertation. Extraneous information was removed. Original correspondence available on request.

26 November 2019

Dear Dr. Wolmarans,

You have been listed as a co-author of the following submission:

Submission no: **BBR_2019_1288_R2**

Submission title: Dopaminergic and serotonergic modulation of social reward appraisal in zebrafish (Danio rerio) under circumstances of motivational conflict: towards a screening test for anti-compulsive drug action

Corresponding author: Dr De Wet Wolmarans

Listed co-author(s): Miss Cailin van Staden, Mr Geoffrey de Brouwer, Dr Tarryn Lee Botha, Dr Karin Finger-Baier, Dr Sarel Brand

We are writing to let you know the status of this submission has changed to 'Sent to Production'. The link below takes you to a webpage where you can log in to our submission system using your existing Elsevier profile credentials or register to create a new profile. You will then have the opportunity to view the submission status and see reviewer and editor comments once they become available.

Once again, thank you very much for your submission.

Behavioural Brain Research

Rebuttal to the Reviewer Comments

This section contains the responses to reviewer comments from both the first and second rounds of the review process. All correspondence from the authors of the article is indicated by italic text while the reviewer comments are depicted in normal font. The correspondence was edited to fit the formatting of this dissertation without content changes.

***

Manuscript (BBR_2019_1288) submitted to *Behavioural Brain Research* titled:

"Dopaminergic and serotonergic modulation of contingency learning in zebrafish (*Danio rerio*) under circumstances of motivational conflict: towards a screening test for anti-compulsive drug action"

1 November 2019

Dear Editor,

Following careful consideration of the reviewer comments, for which we are thankful, please find herewith a detailed rebuttal to the comments raised. We sincerely hope and believe that our approach and responses to these issues are addressed to the satisfaction of all the reviewers.

Reviewing comments have, beginning on the next page, been copied over to this rebuttal in black text, each time followed by our detailed responses in italics. An important change that we did not indicate as this was made throughout the text, is having changed 'treatment / treated' with terms more aligned with pre-clinical animal research, e.g. exposure, exposed, intervention. This was an oversight on our part.

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

Warm regards,

CvS; GdD; SJB; KF; TLB; DWW

Reviewer 1

OCD is a very interesting topic that deserves more intense research and a model in zebrafish. However, there are some problems with the manuscript that do not allow it to be published as it is.

Introduction is clear (although too long) and presents the problems, the objectives and the hypothesis properly. The methods section is more problematic. First, training the fish to associate a cue color with social reward has been shown to need a bit more than 10 sections. Usually it takes 12 sections to show association and researchers usually present the fish to the cue 20 times to ensure learning of the task.

- *Thank you for that valuable input. With respect to the Introduction, we have now reworked it slightly to shorten. Please see the revised section in the manuscript.*
- *In terms of the methods, studies pertaining to learning are indeed complex and characterized by significant between-laboratory variance. Considering the data presented, we are confident that successful learning in terms of pairing between the social reward and the red cue card has indeed been established under the influence of dopaminergic and serotonergic intervention. What was surprising, was the significant avoidance behavior shown by control treated fish to the color red. Therefore, irrespective, these fish failed to learn the contingency as they simply did not enter the red proximity area at all. Our assertion is supported by the demonstration that the arm preference exhibited by each group remained relatively stable during phase 2 and yet was modified by changing the reward condition during phase 3. Thus, and considering the natural preference of the Zf used in this investigation in terms of avoiding the color red, the conclusion can be made that the increased dwelling showed by treated fish in the red arm during phase 2, was in fact directed at seeking the paired reward. We also considered the possibility that treatment simply overcame the seemingly negative effect of the color red, without influencing processes of social reward appraisal. However, that treated fish seek out the reward during phase 3, counteracts this notion. Hence, we framed our conclusion as is. We now include more explanation on this matter on* ***page 10, line 252*** *and* ***page 19, line 514 – 515****. With respect to the number of training trials needed, one has to also keep in mind that this work was based on prior experimentation (Al-Imari & Gerlai, 2008) and that different laboratories employ different techniques to this end. It is therefore not entirely possible, given the marked differences in Zf population preferences, to accurately predict the circumstances under which adequate learning becomes successful and as such, a certain degree of experimental deduction will always be incorporated in animal experimentation.*

In the methods, authors say they covered one end of the T maze in red and the rest of the maze in white, thus white is the other color that animals had to learn about. Throughout the text, authors say many times "non-colored arms", what made me confused about whether there was a white cover or no cover (glass only). If so, fish would prefer to be in a closed space than in open space and it interferes in results.

- *The entirety of the maze was white, with the exception of the arm which was covered with the color red in a trial dependent fashion. We have changed the text to refer to the cued or non-cued arms only. Please see* ***changes made throughout the manuscript****. That said, we covered the maze in opaque white sheets as we wanted to entirely prevent any view to the outside environment except through the water surface above; this was needed for video scoring. Fish were habituated in this fashion and were never allowed, except when housed in the home tanks, to view the laboratory.*

Another problem is related to the re-associative contingency learning, and then I would like to know why fish were tested only 3 days. Is it enough to allow learning?

- *Indeed, the question itself provides some direction for thought. The 3 days of testing undertaken during phases 2 and 3 was aimed at testing (and not necessarily to allow for re-learning to take place per se; hence the 3-day testing period;* ***we have not provided more clarity and made changes to this extent throughout the manuscript where referring to Phases 2 and 3****). In fact, we aimed to establish, whether Zf would, if given a choice after Phase 1, engage in persistent cue-directed, reward-seeking responses in Phase 2, and measure whether persistent cued-arm choice (if present) reversed in reaction to the changed position of the social reward (Phase 3). Indeed, the data confirm that the behaviors of all groups exhibited during phase 1, persisted into phase 2, indicating that the association between the color red and the reward was indeed stamped in during the lengthened (10-trail) duration of Phase 1.*

About the statistics, two-way RM ANOVA makes sense to me, but the Cohen's *d* effect seems strange because after showing the ANOVA did not find significance, it seems the authors are sieving for significant results when it does not exist.

- *We reported that there were no significant within phase differences in the time and entry ratios as confirmed by the 2-way RM ANOVA. This is likely due to the sample size employed and the variance observed in the behavior of animals that are characteristic of behavioral assessments. However, since visual inspection of the data indicated clear differences between treatment groups and the control group, Cohen's d values were calculated to assign magnitude*

to these differences (which indeed did miss significance). In fact, the use of Cohen's d is not only appropriate following the observation of significant differences. Rather, it is common practice in psychological science, as we (Wolmarans et al., 2017) and others have previously argued (Cohen, 1988). In fact, that the results from the different groups, especially the control vs. treated fish, seemed to present in exact opposite manners, one would expect Cohen's d to highlight the magnitude of these effects. This contrasts with a demonstration of significant differences, which in reality would not mean much if the averages were nearer one another, and the sample size larger. However, we do concur that demonstrations of significant differences would have strengthened the current work. To this extent and given the relatively unique behavior observed in the control treated cohort, continued investigation with increased sample sizes is underway.

Results section is far long and repetitive. I would not present all the tables with statistical results. Moreover, table 1A and 1B present the same results as figures 2 and 3. tables 2 and 3 are completely unnecessary, as you can say that all post hoc results are above 0.05.

- *We have to concur and fully agree. During the drafting of this work, we went back and forth between the inclusion and omittance of data and statistical descriptors. The big question for us was that if we followed one approach with a specific result or set of data, the logical thing would be to do it for all; in fact, we were concerned of conveying a message of cherry-picking should we fail to report the data in a comprehensive manner. Also, not all of the statistical descriptors can, for simplicity sake, be included in the figures. As such, we felt to rather leave it up to our reviewers for input and guidance, and as such, have now reworked the presentation of the Tables.* ***Please refer to the corrections made throughout the results section as well as the new Table layout now provided. In this respect, we moved Tables 1A and B to supplementary material for purposes of being comprehensive, while Tables 2A and 3A (now Tables 1A and 2A only contains the Cohen's d values of the respective comparisons. This could not be included on Figures 2 and 3 (now Figures 3 and 4). Table 4 has been moved in its entirety to the supplementary material.***
- *In terms of the result section specifically, we find it overly difficult to reduce or narrow down the narrative because the <u>within-</u> and <u>between-phase</u> comparisons are <u>fundamentally different</u> and drives the way in which the discussion is drafted. If at all possible and considering that neither of the other reviewers raised this concern, we would like to humbly request that this be kept as is for the sake of clarity and so as to not confuse the larger readership of BBR.*

Discussion is even more extensive. Many parts of the results are not discussing what the authors found but what they would like to find. For instance, aversive feedback was not tested thus it does not deserve long explanation and comparison with the actual results. Also, the discussion regarding the relationship between OCD and drug addiction is completely wrong, as drug addiction involves other types of brain neurotransmission modifications that induces withdrawal and other behavioral changes.

- *This is valuable input and something we considered in earnest and addressed accordingly. We have reworked both aspects now, having significantly shortened the Discussion while also removing all reference to the potential link between OCD and addiction. Where we refer to aversion and aversive feedback, it is only done so against the background of our data demonstrating that the control treated fish did display avoidance of the cued arm, irrespective of the presence of the reward presented there.*

Finally, I would like to know why the authors did not test a group of fish presenting similar modifications as OCD patients, such as increased DA and reduced 5-HT (by injecting agonists and antagonists), to compare to the control group and the treated fish.

- *This is a valid question! This work represents the first of its kind in our laboratory, i.e. using zebrafish in studies that may potentially relate to. As such, experimentation was aimed at the initial translation of similar models of persistent spatial-choice behavior in rodents into a viable, zebrafish appropriate model, and such steps fell beyond the currently approved research at our facility. That said, that the screening paradigm does indeed depend on dopaminergic potentiation, speaks to the hyperdopaminergic theory already. Continued investigation is underway to assess the influence of amongst others, tryptophan depletion in the species.*

* * *

Reviewer 2

This is a nicely written and comprehensive study examining the role of dopamine and serotonin in contingency learning in zebrafish. The study is soundly designed and carried out and presents novel data with respect to zebrafish performance in this behavioral protocol. I have several concerns about the study in its present form.

Bottom of page 6 - the consensus is that zebrafish associated red with mate and food preference. They generally prefer red over other colors, even to the extent that it is often not used in color preference assessments because they always choose red. The real issue is that no one really does color preference experiments very well, so it is hard to be completely clear! In addition, the authors find that the fish are showing an aversion to red. This seems counter intuitive. Can they attempt to explain?

- *We couldn't have argued better ourselves! In fact, it was difficult to deduce what the actual color preference of zebrafish is, since as mentioned, the literature is conflicted in this regard (please refer to **pages 6-7, lines 145-150** and where we indicated difficulty in ascertaining this). In fact, in a remarkable recent study that was just published in Scientific Reports (Roy et al., 2019), the authors clearly demonstrate that color preference in Zf is not species specific, but individually attuned (we have no included reference to this work in the Discussion (please refer to **page 18, lines 504 – 506**). We also from the outset believed that the Zf used in this investigation would choose to associate sight of conspecifics with the color red. Remarkably, they did not. As such, in terms of this work, our data are clear and we had to make the assertion that the color red indeed had a significantly and natural aversive influence on the behavior of the control-treated group (in this regard, please refer to **Figures 3 and 4**). This assertion is supported by our findings that finally, during phase 3, when the conspecific fish were introduced in the 'favored arm' i.e. the white arm of the maze, control fish spent even more time in the white arm with the presentation of the conspecifics there. Apart from possible differences in the housing conditions, lighting conditions and the breeding colony specific choice of the Zf housed in our facility, we are in fact at a loss of explaining why Zf chose to avoid red in this investigation. That our data clearly indicate this, had to be reported though.*

End of introduction - I am not sure what the rationale of this study is. You are not showing a new mechanism (the role of dopamine-serotonin in OCD and compulsive behaviors are well characterized in rodents), so are you attempting to show that zebrafish are complementary to rodents? You use the term 'high-throughput' (in the introduction, but also in the discussion), but I am not clear how what

you have presented can be described at high-throughput when training takes many weeks to carry out. If anything, this is lower throughput than rodents.

- *Yes, one of the key objectives of the study was to successfully translate the classical rodent models of compulsive-like behavior to fish, given the cost-effective approaches that can be taken with Zf in modern laboratories. This is an important directive for modern translational research so that behaviors may be dissected from as many perspectives as provided by various species (Maximino et al., 2015). Furthermore, we regard Zf as a potential high-throughput model species in terms of its advantages of being a highly social species, capable of high output, low input breeding programs and their cost-effective housing. Considering that drug administration can be induced in home tanks without expensive and time-consuming techniques, does lend some attractive attributes to the species which cannot be replicated in rodents or other mammals. That said, as the test itself may be time-consuming, we rephrased this sentence in the introduction to more accurately reflect what the intention of this work is. Please refer to* ***page 7, lines 151 – 152****. Referral to a high-throughput model in the discussion has been removed.*

The description of the materials and methods has some limitations. Important aspects regarding animal care and randomization were not adequately described. Your most important issue is that of randomization and blinding. Masca et al. (2015; DOI:10.7554/eLife.05519), examining the problems of reproducibility in biomedical research, observed that one of the main reasons for irreproducible research is the lack of blinding/masking and convenience samples. From the text, it is not possible to know whether or not these crucial steps were taken.

- *We concur and indeed have broad experience in terms of randomization of model organisms employed in previous work (Wolmarans et al., 2013; Wolmarans et al., 2016a; Wolmarans et al., 2016b; Wolmarans et al., 2017; de Brouwer & Wolmarans, 2018). However, a somewhat different approach was taken with the current work that did not necessitate blinding. While randomization was indeed thoroughly introduced, the matter of experimenter blinding was, as opposed to the above referred to work, not addressed specifically since we employed fully automated and objective video scoring (the raw data from these files are available). Further, all fish in a specific group received treatment in exactly the same manner, while a single experimenter dosed and handled all fish. As such, it was impossible to blind the experimenter to the treatment status of the fish being tested on a given day. That said, all video files were assigned a number and scored in a single session after completion of the experiments,*

therefore excluding potential observer bias. While blinding is paramount when scoring is done manually, this was not the case in the presented work.

Were interventions allocated at random to animals? If so, what was the method of random allocation? If not, why?

- *Yes, they were. Fish were randomly selected from 6 stock tanks each containing between 15 – 20 adult Zf (3.5 L housing tanks; (Reed & Jennings, 2011)). Thus, for each of the 6 fish in a treatment group, one fish was taken from each of the housing tanks and henceforth housed in the newly constituted group tanks for the first phase of treatment, while being socially isolated from day 10 onwards. Please see the restated section on **page 8, lines 203 – 205**.*

Are there any other possible confounders (e.g., testing tanks) to which the units may need to be randomly allocated?

- *No. All treatment groups were housed in identical tanks, while all experiments were conducted in the same T-maze.*

Were care providers (lab technicians or anyone who cares for the animals) blind to treatment? Were experimenters blind to treatment? Were data analysts blind to treatment? If not, why?

How was allocation concealed, and how was blinding maintained? Under what circumstances will the data be unblended? These questions need to be addressed and clearly stated in the methods section.

- *The only person that cared for, handled and treated the experimental fish since their allocation to the different treatment groups, is the experimenter herself. Again, as alluded to earlier, all data were analyzed by means of automated video scoring in a single batch after the completion of all tests, and according to group number. Again, blinding is paramount in experiments of manual scoring. This is indeed a wonderful advantage of software scoring!*

Did the authors take into account the tank effect (i.e. the group from which the fish were originally treated) in the statistical analysis?

- *Due to the reasons explained above, we did not apply tank as a covariate in the analysis of data as fish were randomly allocated from different home tanks, first treated as a group and then separately from which point onwards tank position in the holding racks were rotated.*

How was sample size calculated? Was it based on a pilot experiment to determine effect sizes? How were calculations performed? Describing how the authors reached these n would be helpful in designing future experiments.

- *No pilot study was performed. These group sizes were selected since the present work was designed according to the strict ethical guidelines and criteria set forth by the South African Veterinary Council (SAVC), who advocates meticulous application of the ARRIVE guidelines which advocates no less than 5 animals per group (Kilkenny et al., 2010). This can be elaborated on based on robust evidence only that more animals per group will indeed be necessary to yield clearer findings. Further, since we employed empirical dosing, i.e. specific doses and drug administration for all fish in a given group and taking into account that all fish were from the same progenitor stock (therefore, there being no reason to believe that they would respond differently to the selected interventions) we expected a behavioral response akin to the magnitude of neurobiological intervention. Indeed, this approach seemed to have paid off, except for the fact that we failed to observe statistical significance in terms of the within-phase comparisons. That said, that Cohen's d effect sized revealed differences of very large effect sizes, one has to consider how many fish per group it would necessitate to transform p values of > 0.05 to p values < 0.05 and if possible, what will the ultimate outcome be. Should our reported d values have been lower, we would have expanded on the current sample size. Moreover, this investigation was a first of its kind in Zf, and considering the dose response approach followed here, provided us with significant direction for future research. First, we will employ only the high dose of apomorphine in future studies. Now that the method has been described and the results of this investigation reported, we will be able to defend increasing group sizes to 8 – 10 animals per group in future.*

* * *

Reviewer 3

The MS entitled "Dopaminergic and serotonergic modulation of contingency learning in zebrafish (*Danio rerio*) under circumstances of motivational conflict: towards a screening test for anti-compulsive drug action" shows three phases learning experiment using zebrafish shoaling behavior as reward in a T-maze colored (red) or non-colored (white). Moreover, the authors use anti-compulsive drugs, apomorphine and escitalopram, first dopaminergic and second serotonergic, in different doses and combinate. Although interesting, there are some points that need to be carefully revised or even explained:

- *Thank you for the comments and questions! We appreciate the input!*

According to the authors "The other adjacent arm was covered with the same white plastic sheets as the rest of the maze ('non-colored arm'; Figure 1)", so the non-colored area is not non-colored, it is white. White is usually used as an aversive environment in zebrafish experiments which has not occurred in this experiment. Why white environment does not have aversive properties in this experiment?

- *Indeed, a white environment is indeed often regarded as being anxiogenic. However, with respect to this investigation, all experiments were conducted in T-maze covered with white sheets, apart from the proximity area which was covered in red. We did so as to cover the maze with an opaque surface which have prevented fish from viewing the outside environment. Also, as Zf had no other choice to either be exposed to red or white, the data presented here clearly indicate that the Zf used in this investigation did not find the white coloring that anxiogenic, as control treated fish did not approach the red color, but rather chose to remain in the white colored areas which was also the environment in which fish were habituated.*

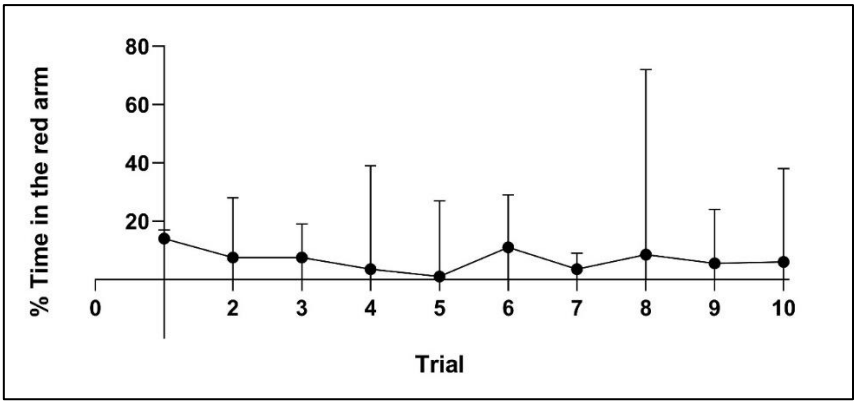
Six subjects per group are not a small number? How this small number could affect your statistical analysis?

- *These group sizes were selected since the present work was designed according to the strict ethical guidelines and criteria set forth by the South African Veterinary Council (SAVC), who advocates meticulous application of the ARRIVE guidelines which advocates no less than 5 animals per group (Kilkenny et al., 2010). This can be elaborated on based on robust evidence only that more animals per group will indeed be necessary to yield clearer findings. Further, since we employed empirical dosing, i.e. specific doses and drug administration for all fish in a given group and taking into account that all fish were from the same progenitor stock*

(therefore, there being no reason to believe that they would respond differently to the selected interventions) we expected a behavioral response akin to the magnitude of neurobiological intervention. Indeed, this approach seemed to have paid off, except for the fact that we failed to observe statistical significance in terms of the within-phase comparisons. That said, that Cohen's d effect sized revealed differences of very large effect sizes, one has to consider how many fish per group it would necessitate to transform p values of > 0.05 to p values < 0.05 and if possible, what will the ultimate outcome be. Should our reported d values have been lower, we would have expanded on the current sample size. Moreover, this investigation was a first of its kind in Zf, and considering the dose response approach followed here, provided us with significant direction for future research. First, we will employ only the high dose of apomorphine in future studies. Now that the method has been described and the results of this investigation reported, we will be able to defend increasing group sizes to 8 – 10 animals per group in future.

Why does the difference of trials between phases (phase 1: 10 trials; phase 2 and 3: six trials. And why there is no analysis of the observed behaviors between the trials. Example: There was a difference in time spent in red between trials 1 and 10 per group in phase one?

- *Considering the first part of the question. The 3 days of testing undertaken during phases 2 and 3 was aimed at testing (and not necessarily to allow for re-learning to take place per se; hence the 3-day testing period);* ***we have now changed the wording relating to Phases 2 and 3 throughout the manuscript****. In fact, we aimed to establish, whether Zf would, if given a choice after Phase 1, engage in persistent cue-directed, reward-seeking responses in Phase 2, and measure whether persistent cued-arm choice (if present) reversed in reaction to the changed position of the social reward (Phase 3). Indeed, the data confirm that the behaviors of all groups exhibited during phase 1, persisted into phase 2, indicating that the association between the color red and the reward was indeed stamped in during the lengthened (10-trail) duration of Phase 1.*
- *This is a valuable question. However, our data analysis revealed that the time spent in and frequency of entries into the respective arms remained stable over the course of a specific phase (data will be made available on request). Considering the already extensive nature of this work, we therefore did not report this finding as well. That said, to illustrate this, we include here the learning data of the control treated group during Phase 1 (data indicated as median with 95% confidence interval).*



About how experimental phases were plan, is there a possibility that the behavior acquired on phase 1 was extinguish in phase 2 and this extinction facilitate the re-associative test in phase 3?

- *Although this may have been a possibility, our data would suggest otherwise. The behavioral performance of all treatment groups during phase 2 indicated a stable behavioral performance, which was still biased in favor of the red arm in the absence of reward, which ultimately speaks against extinction.*

Is it possible to confirm this experiment tests, divided into three phases, as an OCD model? Is it possible to confirm a habit acquired during 10 trials in phase 1 without statistical support?

- *Firstly, it must be noted that there are some significant statistical changes within groups over the course of the three phases. In other words, while different treatment groups did not necessarily differ statistically within-phase, within-treatment group differences were observed over the different phases. Second, our data in terms of the high-dose apomorphine seems to support the idea that at least to some extent, behavioral indecisiveness persists following Phases 1 and 2, even to the extent that the presentation of the reward in the white arm, did not have the motivational approach valence it had for the control, low-dose apomorphine, escitalopram and combination interventions. We also indicate clearly in the title, that we are working towards a model of compulsivity and that this data are indicative of the fact, especially since.*

Add numbers areas in Figure 1 on the description of apparatus

- *This has been done.*

For better procedures (habituation, phases 1, 2 and 3) understanding, insert a schematic figure about it.

- *Thank you for this valuable input. We have now included an additional figure (**new Figure 2A and B**) to elucidate the method.*

Described better your measures in methods.

- *We presume you are referring here to the ratios expressed in **Figures 3 and 4**. We have more clearly explained the meaning of the ratios and how they were calculated. Please see **page 11, lines 295 – 301**.*

The authors described some experiments and the aversiveness or not of the red environment. Is there any study about preference in the red-white environment?

- *There is conflicting data regarding how aversive/appetitive the color red is (Roy et al., 2019). However, careful consideration of our data did indeed indicate that untreated zebrafish did demonstrate a marked avoidance of the red arm. This issue was also raised by Reviewer 2. Please refer to our second response under the comments raised by Reviewer 2 for a detailed explanation of why we made this assertion. To the best our knowledge, we are not aware of data which specifically investigated the influence of the color red within a predominantly white environment.*

We thank the reviewers for all of their valuable input!

CvS, GdB, TLB, KFB, SJB, DWW

Reviewer Second Round

I really appreciated the author's effort to change or respond to my points, but the learning behavior is still not clear as the authors affirmed to. Even with Cohen's *d* size effect data, there were no significative differences between control and experimental groups and there was no difference between trials in the phases: Line 321 "*Phase 1:* Although no statistically significant differences were demonstrated between any of the exposure groups during Phase 1 with respect to either the indices of time spent in (Figure 3), or entries made into (Figure 4) the cued- vs. the non-cued arm"; Line 333 "*Phase 2:* No statistically significant differences were found between exposure groups. In the absence of reward presentation in either arms, control-exposed fish maintained their preference for the non-cued arm, demonstrating continued cued arm avoidance"; Line 347: "*Phase 3:* Again, no statistical differences were observed with respect to any of the pairwise comparisons between the different exposure groups". And when it was requested to the analyses of learning behavior between trials in each phase, the graphic sent show no learning curve between the trials.

- *We thank the author once again for the detailed oversight of our work! It is true that we struggled with the same concepts as those raised by the reviewer and would like to provide improved feedback to these concerns in this version of the rebuttal.*
- *At face value, it seems that two issues remain here, i.e. 1) the lack of significant differences between the exposure groups within each phase and 2) the lack of a clear and decisive demonstration with respect to learning.*
- *On the first topic, we have previously (in the prior rebuttal round) alluded to the fact that our p values were > 0.99 for most of the within-phase comparisons of the different exposure groups. We explained that this is a really challenging aspect of the work reported here, as it is clear that it would take a significant elaboration on sample size to reduce these p values to < 0.05. It would not have been ethically or practically appropriate to increase sample sizes ad infinitum, as clearly expanding to even 12 fish per group would not have made a significant difference on the findings reported here. Given this, we had to work around the problem to establish, in a preliminary study, if dopamine and serotonin would influence the social reward appraisal behavior of zebrafish. The question then simply became "Do exposed fish show a higher interest in social conspecifics and do they continue to seek out these conspecifics in a previously cued context, compared to control treated fish?" We then attempted to answer this question by means of calculating indices, which would be indicative of time spent in and number of entries made into the respective arms. Now, given the large p values we observed and taking into account that only a very high number of fish per exposure group would yield lower p values, the interpretation of Cohen's d becomes paramount. Indeed, and especially*

also when working with lower sample sizes, Cohen's d considers the average standard deviation of the behaviors of a specific group when comparing differences in group behavior. Therefore, in instances where fish, in this case, present with highly individualized behavioral responses, the SD would be greater, yielding smaller d values. Therefore, while the lack of extensive sample sizes could account for the lack of statistical significance, the meaning of the very large d values presented here, cannot be ignored and have to be appreciated for what it is intended to show.

- *Against this background, the second problem we had to overcome was to interpret our findings with respect to 'learning' against the background of the behavior of control-exposed fish. We believe we understand better now what the reviewer refers to as 'learning' and will attempt to provide clarity. <u>First</u>, what we meant when speaking of learning is simply the successful acquisition of the knowledge that, during Phase 1, the conspecifics are presented in the red arm. Therefore, irrespective of the chronological demonstration of such learning over the course of Phase 1 (which we did not find as per the figure above), we simply alluded to whether successful cue-contingency knowledge has been gained during Phase 1, and tested this during Phases 2 and 3. <u>Second</u>, considering the data presented, we could not come to any other conclusion than the fact that only dopamine and serotonin-exposed fish, valued the conspecifics in the presence of the color red. We coined this as 'learning' and can see here why the reviewer are uncertain as to whether this is indeed the correct approach as no chronology to the adap. <u>Third</u>, and this is important for the interpretation of our results. Zebrafish were allowed interaction with conspecifics and the color red for the first time only after 14 days of uninterrupted drug exposure. Hence, learning could not have adapted as function of treatment and time, as by the time we initiated Phase 1, fish already had bolstered dopaminergic and serotonergic responses. It was therefore likely more a matter of appreciating the conspecifics and disregarding the negative valence of the color red, than it was a matter of learning. Our findings would therefore indicate that 1) dopamine and serotonin did indeed highlight the value of social conspecifics and that fish successfully associated this with the color red and 2) that where motivational conflict does indeed exist as demonstrated here by fish in the control-exposed group, the presentation of social conspecifics is not sufficient as a rewarding stimuli and that the 'negative valence' of a potentially demotivating scenario, would carry the highest influence. We have now reworded and reworked several aspects of the manuscript to better reflect and convey what our data demonstrates, including the title. Please see the **Title, keywords, highlights** and **Figure 2B***

('learning' removed) *and* ***lines 32, 36 – 39, 88, 99, 152, 205, 216, 246 – 248, 441, 443 – 444, 467 – 475, 488, 490, 510 – 515, and 528.***

It was a great experiment, well written and experimental controlled, the preference of red is a relevant found, but I am not fully convinced about the drug effects in learning, dissociative and re-associative behavior because of the lack of any change in behavior in the phases. No learning curve was found, so it seems that in all phases Zf behavior was maintained by the environment (no difference between trials in the phase) and it changed when the environment change (different exploratory pattern among phases).

- *To answer this, please referred to the context provided for the aforementioned comment. We have now reworked several sections of the manuscript where we have referred to 'learning' in the line numbers indicated, as we agree, this may have been confusing.*
- *With respect to the behavior being influenced by the environment, this is indeed what was shown, and we apologize for not making this clearer. We used environmental manipulation to assess the effects of dopaminergic and serotonergic drug intervention on the initial association made by exposed fish between the color red and the conspecifics. However, it needs to be clear that environment on its own could not have controlled the data shown here as we have shown significant main effects of Phase and exposure on the behavior of the fish. Therefore, it is likely that dopaminergic and serotonergic intervention actually contributed meaningfully to the responses to environmental manipulation shown here and that the association made during Phase 1, was maintained during Phase 2. Only when the social conspecifics was introduced in the non-cued arm, did the behavior of exposed fish change, albeit markedly less so in high concentration apomorphine exposed fish, which supports the conclusions drawn in this work. To better explain this, we have now reworked different sections of the paper. Please refer to* ***lines 545 – 551****.*

And a minor question, it still not clear if the social reward is not efficient to modulate behavior in Zf, or if the contrast od color and non-colored environment impaired the social reward effectivity.

- *In short, our data would indicate that the presentation of a social reward is not sufficient as a motivational reinforcer of behavior under circumstances of motivational conflict. Given the findings presented here, we cannot come to any other conclusion. Therefore, it must be considered that should the environmental conditions have been different, we would have observed even more robust cue-reward contingency associative patterns (please see* ***lines 587 – 589*** *and* ***618 – 621****). Work to establish this, is currently under way in our laboratory.*

Again, we thank the reviewer for the valuable input! We truly hope that based on the answers provided here, we have now explained ourselves better.

CvS, GdB, TLB, KFB, SJB, DWW

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Addendum C

Supplementary Tables Not Included in Chapter 3

Supplementary Table i a

Treatment groups	Mean ± SEM		
	Phase 1	Phase 2	Phase 3
Control	-0.31 ± 0.18	-0.34 ± 0.11	-0.61 ± 0.12
A0.05	0.32 ± 0.25	0.33 ± 0.14	-0.36 ± 0.12
A0.1	0.47 ± 0.16	0.26 ± 0.17	0.01 ± 0.20
E0.5	0.20 ± 0.20	-0.02 ± 0.04	-0.60 ± 0.17
E1.0	0.35 ± 0.21	0.23 ± 0.21	-0.31 ± 0.35
A0.1E0.5	0.47 ± 0.20	0.18 ± 0.10	-0.01 ± 0.23
A0.1E10.	0.37 ± 0.28	0.41 ± 0.18	-0.17 ± 0.20

Supplementary Table i a: Mean ± SEM of the indices of time spent in the cued vs. non-cued arms

Supplementary Table i b

Treatment groups	Mean ± SEM		
	Phase 1	Phase 2	Phase 3
Control	-0.26 ± 0.19	-0.33 ± 0.11	-0.59 ± 0.11
A0.05	0.19 ± 0.24	0.25 ± 0.12	-0.23 ± 0.09
A0.1	0.27 ± 0.13	0.02 ± 0.20	-0.01 ± 0.13
E0.5	0.07 ± 0.13	-0.21 ± 0.09	-0.51 ± 0.15
E1.0	0.11 ± 0.19	-0.07 ± 0.13	-0.43 ± 0.21
A0.1E0.5	0.43 ± 0.17	0.23 ± 0.20	0.02 ± 0.18
A0.1E10.	0.26 ± 0.27	0.18 ± 0.17	-0.36 ± 0.12

Supplementary Table i b: Mean ± SEM of the indices of the number of entries made into the cued vs. non-cued arms.

Supplementary Table ii a

Treatment groups		Phase 1		Phase 2		Phase 3	
		p	Cohen's *d*	*p*	Cohen's *d*	*p*	Cohen's *d*
Ctrl vs.	A0.05	0.164	**2.3**	0.671	**2.6**	0.249	**2.6**
	A0.1	**0.021**	**2.7**	**0.029**	**2.0**	**0.0003**	**3.4**
	E0.5	**0.013**	**2.2**	**0.014**	**2.9**	0.056	**3.4**
	E1.0	0.866	**1.2**	-	**0.9**	-	**0.8**
	A0.1E0.5	0.168	**2.7**	0.137	**2.4**	**0.028**	**3.2**
	A0.1E1.0	0.309	**1.6**	0.098	**1.8**	0.072	**1.8**
A0.05 vs.	A0.1	-	0.5	-	**0.8**	0.927	**1.6**
	E0.5	-	0.6	-	**1.4**	-	**0.8**
	E1.0	-	0.3	-	0.2	-	0.5
	A0.1E0.5	-	0.1	-	0.7	-	**0.9**
	A0.1E1.0	-	0.1	-	0.6	-	0.3
A0.1 vs.	E0.5	-	0.2	-	0.2	-	**1.1**
	E1.0	-	0.7	-	0.6	0.100	**1.3**
	A0.1E0.5	-	0.5	-	0.2	-	**0.8**
	A0.1E1.0	-	0.6	-	0.1	-	**0.8**
E0.5 vs.	E1.0	-	0.7	-	**0.9**	-	**0.9**
	A0.1E0.5	-	0.6	-	0.5	-	0.2
	A0.1E1.0	-	0.6	-	0.4	-	0.2
E1.0 vs.	A0.1E0.5	-	0.4	-	0.5	-	0.5
	A0.1E1.0	-	0.1	-	0.5	-	**0.9**
A0.1E0.5 vs.	A0.1E1.0	-	0.2	-	0.1	-	0.3

Supplementary Table ii a: Pairwise within-phase comparisons of the total distances swam.

Supplementary Table ii b

Treatment group	Phase	Level of significance	
		p value	*d* value
Control	Phase 1 vs. Phase 2	0.482	0.5
	Phase 1 vs. Phase 3	-	0.2
	Phase 2 vs. Phase 3	-	0.3
A0.05	Phase 1 vs. Phase 2	-	0.005
	Phase 1 vs. Phase 3	-	0.03
	Phase 2 vs. Phase 3	-	0.05
A0.1	Phase 1 vs. Phase 2	0.780	0.2
	Phase 1 vs. Phase 3	**0.002**	**0.9**
	Phase 2 vs. Phase 3	**0.041**	0.5
E0.5	Phase 1 vs. Phase 2	0.623	0.3
	Phase 1 vs. Phase 3	-	0.2
	Phase 2 vs. Phase 3	0.165	0.7
E1.0	Phase 1 vs. Phase 2	-	0.04
	Phase 1 vs. Phase 3	0.975	0.2
	Phase 2 vs. Phase 3	0.636	0.2
A0.1E0.5	Phase 1 vs. Phase 2	0.435	0.5
	Phase 1 vs. Phase 3	0.166	**0.8**
	Phase 2 vs. Phase 3	-	0.2
A0.1E1.0	Phase 1 vs. Phase 2	**0.018**	0.5
	Phase 1 vs. Phase 3	0.209	0.3
	Phase 2 vs. Phase 3	0.979	0.2

Supplementary Table ii b: Pairwise between-phase comparisons of the total distances swam.

Supplementary Table iii a

Treatment groups		Phase 1		Phase 2		Phase 3	
		p	Cohen's *d*	*p*	Cohen's *d*	*p*	Cohen's *d*
Ctrl vs.	A0.05	-	**0.8**	-	**1.0**	-	0.6
	A0.1	**0.021**	**2.2**	**0.003**	**2.1**	**0.003**	**3.1**
	E0.5	-	**1.4**	-	**1.6**	-	**1.1**
	E1.0	-	**1.1**	-	**1.1**	-	0.7
	A0.1E0.5	0.930	**1.5**	-	**1.7**	-	**1.4**
	A0.1E1.0	-	**1.7**	-	**2.0**	-	**1.5**
A0.05 vs.	A0.1	0.210	**1.4**	**0.017**	**1.8**	**0.010**	**2.6**
	E0.5	-	0.5	-	**1.0**	-	0.7
	E1.0	-	0.2	-	0.7	-	0.4
	A0.1E0.5	-	**0.8**	-	**1.0**	-	**1.0**
	A0.1E1.0	-	0.6	-	**1.3**	-	**0.9**
A0.1 vs.	E0.5	-	**1.0**	-	**0.8**	0.214	**1.5**
	E1.0	0.470	**1.3**	0.975	0.7	0.112	**1.4**
	A0.1E0.5	-	0.5	0.476	**1.0**	0.366	**1.4**
	A0.1E1.0	-	**1.0**	0.990	**0.8**	0.118	**1.9**
E0.5 vs.	E1.0	-	0.3	-	0.03	-	0.1
	A0.1E0.5	-	0.4	-	0.2	-	0.1
	A0.1E1.0	-	0.1	-	0.01	-	0.1
E1.0 vs.	A0.1E0.5	-	0.7	-	0.1	-	0.2
	A0.1E1.0	-	0.5	-	0.05	-	0.1
A0.1E0.5 vs.	A0.1E1.0	-	0.3	-	0.3	-	0.2

Supplementary Table iii a: Pairwise within-phase comparisons of the time spent per crossarm visit.

Supplementary data Table iii b

Treatment group	Phase	Level of significance	
		p value	*d* value
Control	Phase 1 vs. Phase 2	-	0.5
	Phase 1 vs. Phase 3	-	0.04
	Phase 2 vs. Phase 3	-	0.5
A0.05	Phase 1 vs. Phase 2	0.940	0.5
	Phase 1 vs. Phase 3	-	0.4
	Phase 2 vs. Phase 3	-	0.1
A0.1	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	0.773	0.2
	Phase 2 vs. Phase 3	-	0.1
E0.5	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	-	0.1
	Phase 2 vs. Phase 3	-	0.3
E1.0	Phase 1 vs. Phase 2	0.676	0.3
	Phase 1 vs. Phase 3	-	0.001
	Phase 2 vs. Phase 3	0.680	0.2
A0.1E0.5	Phase 1 vs. Phase 2	0.368	0.5
	Phase 1 vs. Phase 3	0.544	0.4
	Phase 2 vs. Phase 3	-	0.1
A0.1E1.0	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	-	0.4
	Phase 2 vs. Phase 3	0.667	0.5

Supplementary Table iii b: Pairwise between-phase comparisons of the time spent per crossarm visit.

Supplementary Table iv a

Treatment groups		Phase 1		Phase 2		Phase 3	
		p	Cohen's *d*	*p*	Cohen's *d*	*p*	Cohen's *d*
Ctrl vs.	A0.05	-	0.5	-	**0.8**	-	**0.8**
	A0.1	**0.021**	**2.2**	**0.011**	**2.6**	**0.004**	**3.7**
	E0.5	0.817	**1.2**	0.194	**2.1**	0.965	**1.6**
	E1.0	-	0.3	-	0.5	-	0.2
	A0.1E0.5	0.56	**1.2**	0.403	**1.7**	0.085	**3.0**
	A0.1E1.0	0.56	**1.0**	0.317	**1.3**	0.492	**1.5**
A0.05 vs.	A0.1	0.414	**1.8**	0.396	**2.1**	0.136	**2.4**
	E0.5	-	0.7	-	**1.4**	-	0.7
	E1.0	-	0.1	-	0.1	-	0.4
	A0.1E0.5	-	**0.8**	-	**1.1**	-	**1.7**
	A0.1E1.0	-	0.6	-	0.7	-	0.7
A0.1 vs.	E0.5	-	**1.2**	-	**0.8**	-	**1.5**
	E1.0	0.34	**1.7**	0.367	**1.4**	**0.024**	**1.9**
	A0.1E0.5	-	**1.0**	-	**0.9**	-	**0.9**
	A0.1E1.0	-	**0.8**	-	0.7	-	**1.1**
E0.5 vs.	E1.0	-	0.7	-	**0.9**	-	**0.8**
	A0.1E0.5	-	0.1	-	0.3	-	**0.8**
	A0.1E1.0	-	0.03	-	0.2	-	0.1
E1.0 vs.	A0.1E0.5	-	**0.8**	-	0.7	0.308	**1.5**
	A0.1E1.0	-	0.6	-	0.6	-	**0.8**
A0.1E0.5 vs.	A0.1E1.0	-	0.1	-	0.01	-	0.6

Supplementary Table iv a: Pairwise within-phase comparisons of the number of crossarm entries.

Supplementary Table iv b

Treatment group	Phase	Level of significance	
		p value	*d* value
Control	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	-	0.2
	Phase 2 vs. Phase 3	-	0.02
A0.05	Phase 1 vs. Phase 2	-	0.04
	Phase 1 vs. Phase 3	-	0.1
	Phase 2 vs. Phase 3	-	0.1
A0.1	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	-	0.3
	Phase 2 vs. Phase 3	-	0.3
E0.5	Phase 1 vs. Phase 2	-	0.3
	Phase 1 vs. Phase 3	-	0.2
	Phase 2 vs. Phase 3	0.412	0.5
E1.0	Phase 1 vs. Phase 2	-	0.01
	Phase 1 vs. Phase 3	0.348	0.3
	Phase 2 vs. Phase 3	0.379	0.3
A0.1E0.5	Phase 1 vs. Phase 2	-	0.1
	Phase 1 vs. Phase 3	-	0.4
	Phase 2 vs. Phase 3	0.71	0.5
A0.1E1.0	Phase 1 vs. Phase 2	-	0.01
	Phase 1 vs. Phase 3	-	0.1
	Phase 2 vs. Phase 3	-	0.1

Supplementary Table iv b: Pairwise between-phase comparisons of the number of crossarm entries.

Addendum D

Description of Supplementary Methods

The methods discussed in this addendum are supplementary to the detailed methods reported in Chapter 3. During preparation of the manuscript for submission to *Behavioural Brain Research*, detailed descriptions of methodological approaches were included (please refer to both Chapter 3 and Addendum B for background). As such, Addendum D only contains additional information that was not required by the journal.

Layout of Groups for the Behavioral Investigation

During each of the 25 days of the behavioral investigation, two groups of zebrafish (*n* = 6 per group) were exposed to the respective treatments and both were tested on the same days according to the experimental setup. The control group was exposed to treatment and tested alone (see **Table D1**).

Table D1 - Layout of groups and dates

Start date	End date	Groups tested	Groups description
01/10/2018	25/10/2018	Group 1	Control
26/10/2018	19/11/2018	Groups 2 and 3	Apomorphine
20/11/2018	14/12/2018	Groups 4 and 5	Escitalopram
22/01/2019	15/02/2019	Groups 6 and 7	Apomorphine +Escitalopram

Daily Routine During the Behavioral Investigation (Table D2)

- Each day started at 08h00 and included the monitoring of the following: 1) the temperature of the room as well as the water in the T-maze to ensure both were consistently 26˚C, 2) the control unit of the automated ZebTec® housing system to ensure that the readings, i.e. water quality (pH: 7; conductivity: 600 μS), temperature (26 ± 0.1 ˚C), and aeration (7.2 mg O_2/L) were correct, and 3) zebrafish in their home tanks to ensure that they appear healthy and display normal behavior.
- Thereafter (at approximately 08h15), the first trial of the day was initiated (see **Table D2**). Only one trial was conducted on habituation days (days 11 – 14).
- Upon completion of each trial, fish were gently placed back in their home tanks and fed. Where applicable, i.e. during Phases 1 – 3, a between-trial period of 2.5 hours elapsed before the second trial of the day. Fish were left undisturbed in their home tanks during this time.
- Following the completion of the second daily trial, zebrafish were again gently placed back in their home tanks until drug exposure (14h00).
- Upon completion of the drug exposures, zebrafish were for a final time placed back in their home tanks and left undisturbed until the next day's trials started (i.e. approximately 18 hours post exposure).

Table D2 - Daily routine for experimental days (day 15 - 25)

Group (any)	Trial 1	Between trial period	Trial 2	Drug exposure (Group or individual)
1st group	08h15 – 09h15	2.5 hours →	10h45 – 11h45	14h00
2nd group	09h15 – 10h15	2.5 hours →	11h45 – 12h45	14h00

Additional Information

- Prior to the start of the study, zebrafish were bred and housed in the National Aquatic Bioassay Facility (NABF) and fed once daily from the larval to adult (3 months old) stages according to the same schedule used during this study (Chapter 3).
- Two days prior to initiating the study, zebrafish were moved to an exposure room in the NABF where experiments would be conducted in order to habituate to the experimental conditions.
- All tanks used in the exposure room were properly cleaned before a new group of fish were introduced.
- All tanks and beakers used for drug exposure were cleaned immediately after zebrafish exposures were completed. The T-maze was also cleaned every day and filled with fresh tank water to a depth of 9 cm, as condensation took place throughout the night and ±1 cm of water was subsequently lost. The final depth of water in the maze was 8 cm when experiments commenced on the following day (Figures D1 – D3).
- All drugs used in this investigation were weighed on a microbalance, added to the appropriate volume of diluent (Milli-Q® ultrapure water) and subsequently sonicated to ensure the optimal dissolution of particles. The appropriate volumes of drug solution were measured by a micropipette and added to water either in the tank or beakers (phase dependent) where zebrafish would undergo drug exposure by means of aqueous immersion.
- Double LED fluorescent tube lights (400 lux) were used in the behavioral room as the lighting source.
- Zebrafish were euthanized in a different room than the exposure room.

Supplementary Images

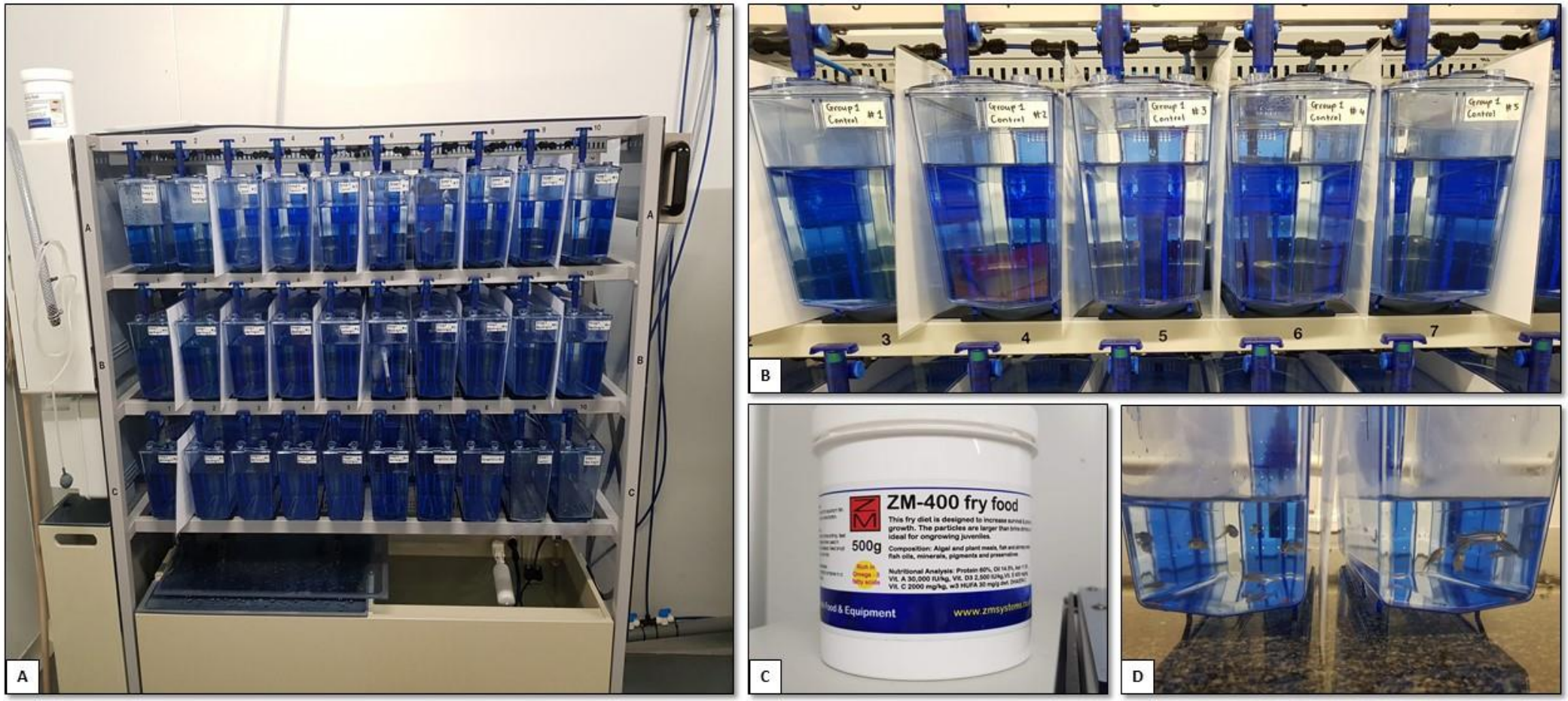

Figure D1 – ZebTec® Zebrafish Housing System (housed zebrafish according to standard laboratory conditions). **B)** Zebrafish acrylic tanks in the ZebTec® Housing System. Each tank was marked according to the group and fish number so that individual fish could be identified. White separators were placed between tanks to occlude the view of zebrafish in the adjacent tanks. **C)** Food provided to zebrafish throughout the investigation (ZM-400 fry food). **D)** Group exposure in tanks identical to home tanks. White separators were placed between the two tanks to occlude the view of fish from adjacent tanks.

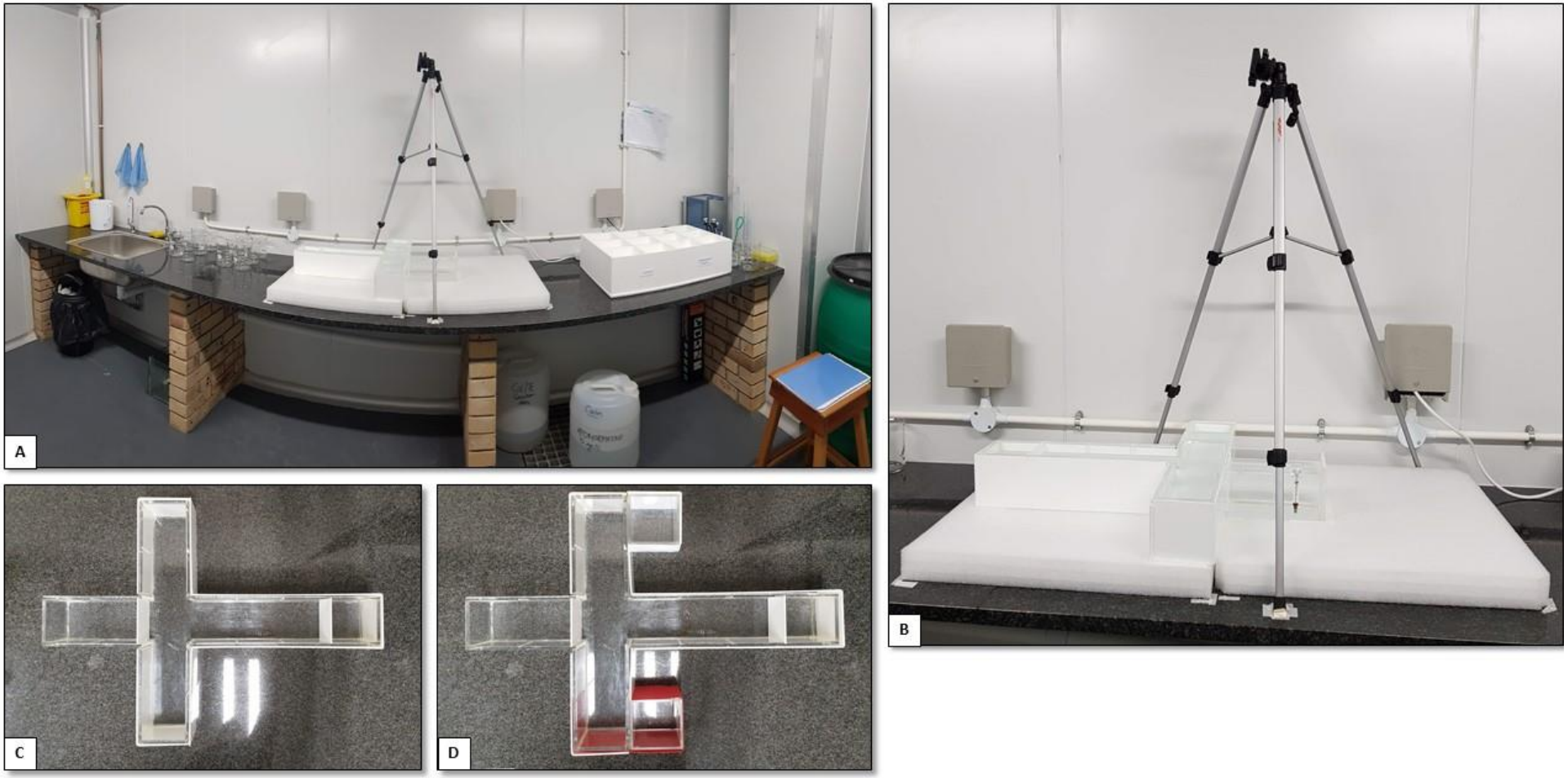

***Figure D2* – A)** Setup of experimental room; Left: waste disposal, basin and beakers. Middle: T-maze and video camera setup. Right: polystyrene separator box used for individual treatment exposures. **B)** Experimental setup; T-maze (without conspecifics tanks) on white polystyrene (i.e. to create contrast for fish recognition by software) and camera mounting. **C)** T-maze used for experiments (without conspecifics tanks). Important: the top short section of the stem was never used; we formed a T-maze by blocking the top section off with a white Perspex® separator. **D)** T-maze used in experiments with conspecifics tanks and red cue card.

***Figure D3* – A)** Side view of polystyrene box used for individual drug exposure. Individually exposed groups (*n* = 6 per group) could be identified by a label on the front of the box. Each compartment (6 on the left and 6 on the right) were also labelled individually from 1 - 6 to allow easy identification of zebrafish. **B)** Top view of polystyrene box used for individual drug exposure (12 compartments allowing for 2 groups of fish (*n* = 6 per group) to be individually exposed simultaneously). **C)** Zebrafish exposed individually in 600 ml beakers that were placed in their individual compartments in the polystyrene box. Each beaker was numbered from 1 – 6 according to the number allocated to each individual zebrafish in their respective groups (as labeled on the home tanks) in order to ensure that each fish could be correctly identified and prevent confusion

Addendum E

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Research report

Dopaminergic and serotonergic modulation of social reward appraisal in zebrafish (*Danio rerio*) under circumstances of motivational conflict: Towards a screening test for anti-compulsive drug action

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ABSTRACT

Cognitive flexibility, shown to be impaired in patients presenting with compulsions, is dependent on balanced dopaminergic and serotonergic interaction. Towards the development of a zebrafish (*Danio rerio*) screening test for anti-compulsive drug action, we manipulated social reward appraisal under different contexts by means of dopaminergic (apomorphine) and serotonergic (escitalopram) intervention. Seven groups of zebrafish ($n = 6$ per group) were exposed for 24 days (1 h per day) to either control (normal tank water), apomorphine (50 or 100 µg/L), escitalopram (500 or 1000 µg/L) or a combination (A100/E500 or A100/E1000 µg/L). Contextual reward appraisal was assessed over three phases i.e. *Phase 1* (contingency association), *Phase 2* (dissociative testing), and *Phase 3* (re-associative testing). We demonstrate that 1) sight of social conspecifics is an inadequate motivational reinforcer under circumstances of motivational conflict, 2) dopaminergic and serotonergic intervention lessens the importance of an aversive stimulus, increasing the motivational valence of social reward, 3) while serotoninergic intervention maintains reward directed behavior, high-dose dopaminergic intervention bolsters cue-directed responses and 4) high-dose escitalopram reversed apomorphine-induced behavioral inflexibility. The results reported here are supportive of current dopamine-serotonin opponency theories and confirm the zebrafish as a potentially useful species in which to investigate compulsive-like behaviors.

1. Introduction

Obsessive-compulsive disorder (OCD), like several other neuropsychiatric disorders, e.g. autism [1], gambling disorder [2], eating disorders [3], and schizophrenia [4], is often associated with behavioral inflexibility and dysfunctional reward-feedback processing [5]. OCD is primarily characterized by persistent and anxiety-provoking thoughts (obsessions) which are typically associated with rigid, repetitive behavioral routines (compulsions), often initiated to alleviate the symptoms of anxiety [6–8]. Sensations of relief generated by such obsession-compulsion loops are typically temporary [9] leading to worsening cycles of repetitive behavior.

Although the exact etiology and pathophysiology of OCD remains unclear, the condition is often associated with cortico-striatal-thalamic dysregulation [10–13,8]. The prefrontal cortex, striatum and thalamus are all involved in the planning, execution and termination of goal-rewarded behavior [12,14,15]. Dysfunction within these regions is similarly associated with changes in the expression of goal-directed behavior and abnormal reward feedback processing [12,15]. From a neurobiological perspective, cortico-striatal interactions between dopaminergic and serotonergic signaling are pivotal in the regulation of these processes. Indeed, in line with the proposed role of dopamine in reward processing [16–18] and the opponency theory, describing the motivational dopamine-serotonin interactions under circumstances of rewarding and negative feedback respectively [19–21], it is believed that a bias in favor of behaviorally activating dopaminergic signaling

Abbreviations: OCD, obsessive-compulsive disorder; SSRIs, selective serotonin reuptake inhibitors; 5-HT, serotonin, 5-hydroxytryptamine; NCT, time spent in the proximity area of the non-cued arm; RT, time spent in the proximity area of the red-colored cued arm; NCE, number of entries made into the proximity area of the non-cued arm; RE, number of entries made into the proximity area of the red-colored cued arm; TPAT, total time spent in the proximity areas of both arms; TPAE, total number of entries made into the proximity areas of both arms; 2-way RM ANOVA, two-way repeated measures analysis of variance; 8-OH-DPAT, 8-hydroxy-2-[di-n-propylamino] tetralin

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[22,23] exists [24–26]. Although the neurobiology of OCD and other disorders of behavioral persistence is more complex [27], this theory provides a rationale for the use of selective serotonin reuptake inhibitors (SSRIs) as a first-line intervention for the treatment of OCD [28,29]. Briefly, chronic administration of SSRIs, e.g. escitalopram, increases the concentration of synaptic serotonin [24], thereby presumably attenuating excessive behavioral activation, a theory which is supported by data from a number of pre-clinical investigations [30–33]. However, not all patients respond to SSRI monotherapy, in which case treatment is often augmented with low-dose dopamine receptor antagonists [34,35].

Observations of dopamine-serotonin interactions are critical when explaining animal behavior since it is often functionally directed at obtaining a reward or avoiding potential aversive outcomes. More specifically, a novel rewarding scenario would initially provoke mass phasic dopaminergic activation [16,36,37]. Although repeated presentation of the same rewarding scenario within a specific context is, over time, associated with a diminishing dopaminergic response [16,18]—thereby preventing engagement in persistent reward-seeking behaviors—it also enables an organism to acquire knowledge of cue-outcome contingencies, whereby the presentation of specific contextual cues *predicts* potential rewarding outcomes [16,37,38]. Conversely, serotonergic signaling is typically activated during the experience of aversive feedback and is integral in the learning of future avoidance behaviors [39]. As alluded to earlier, OCD patients often present with dysfunctions related to such constructs, including abnormal reward feedback processing [7], altered neurobiological reward anticipatory responses [7], overinflated threat estimation [40,41] and often also being relatively insensitive to punishment [42,43]. It has also been proposed that individuals suffering from OCD may be indifferent to the external feedback cues associated with adequate task completion, which may ultimately manifest as repetitive behavioral engagement [44,45].

The present investigation attempts to investigate how natural reward-seeking behavior in the zebrafish (*Danio rerio*), i.e. the motivational drive to engage in social interaction [46–48], can be modified by the pharmacological manipulation of dopaminergic and serotonergic neurotransmission. Taken from the literature summarized here, it is possible that bolstered dopaminergic signaling would be associated with persistent reward-seeking behaviors and that such behaviors would be subject to attenuation by serotonergic agents. Although this concept has been investigated in rodent studies [49], promising zebrafish models of neuropsychiatric conditions may present a unique avenue to complement rodent models [50]. Indeed, the numerous advantages of zebrafish as a model species, including their prolific nature and rapid development [51,52], lengthy lifespan [53,54], ease of drug administration through aqueous immersion [53–56] and that it enables cost-effective high-throughput results-driven screening [57], explain why zebrafish are rapidly emerging as a complementary pre-clinical framework for neurobiological research [58–60]. With respect to the focus of this work, the species shows a strong homology to rodent and human neurobiology [53,54], presenting with almost fully conserved dopaminergic and serotonergic systems [48,61–64]. In addition, zebrafish demonstrate both associative and non-associative cognitive ability, including adequate reversal learning, appetitive reinforcement-based learning and avoidance learning [46,47]. Moreover, zebrafish are highly social and are known to shoal and seek out conspecifics, constituting the basis of the present investigation [46–48,65]. Indeed, it has been shown that simple observation of conspecifics is highly rewarding to zebrafish [46,48]. A further advantage of a social, as opposed to a food-based, reward construct is that zebrafish do not seem to develop tolerance to social interaction [47]. This is of particular value for the present work, which attempts to employ social reinforcement as a suitable reward stimulus for socially deprived zebrafish. Further, in terms of ensuring robust acquisition of cue-reward contingencies, adult zebrafish can clearly distinguish between different colors and are therefore capable of conditioning-based color-dependent learning [66]. In addition, color perception in zebrafish influences learning, decision-making and memory formation [67]. Although it has been demonstrated that zebrafish seemingly prefer red (as used in this investigation) and green equally over no color, yellow and blue [68], findings with respect to color perception remain largely inconclusive [67].

Currently, no rapid and cost-effective screening test exists that can detect potential anti-compulsive drug action. Hence, to this extent and considering the literature summarized here, the current investigation will elaborate on previous work [46,69] by interrogating contextual reward appraisal in zebrafish exposed to either no drug, the dopaminergic $D_{1/2}$ receptor agonist, apomorphine, the SSRI, escitalopram, and combinations of both.

2. Experimental procedures

2.1. Animals and housing

A total of 42 randomly chosen adult wild-type long-fin zebrafish (*Danio rerio*) of both sexes (experimental fish; 3–5 months old; ± 30 − 40 mm in length; 6 fish per exposure group; refer to '*Exposure groups and drug administration*') were assessed in this investigation. A further 36 fish were used as social conspecifics (see below). The progenitor stock was originally obtained from Aquaworld Tropical Fish (Singapore) via a national South African importing supplier (WCB Imports, Pretoria, South Africa). All fish used were subsequently bred and housed in the National Aquatic Bioassay Facility (NABF), North-West University, Potchefstroom, South Africa. All procedures and experimental methods were approved by the AnimCare Research Ethics Committee (approval number NWU-00161-18-A5), North-West University; Committee Registration Nr. AREC-130913-015. Subjects were housed according to standard laboratory conditions as prescribed for zebrafish [70] and maintained on a 12-h light/dark cycle (06h00/18h00) in a fully automated system (ZebTec® Zebrafish Housing System, Techniplast®, Varese, Italy) that regulated water quality (pH: 7; conductivity: 600 μS), temperature (26 ± 0.1 °C), and aeration (7.2 mg O_2/L). Experimental zebrafish were group-housed (n = 6 per group) in 3.5 L acrylic tanks for the first 10 days of the investigation and then individually allocated to replicas of the group tanks for the remainder of the study (15 days). Food (ZM-400 fry food, Zebrafish Management Ltd, Twyford, United Kingdom) was provided once daily after the first trial or at 10h00 on exposure days (days 1–10). Conspecifics were group-housed (n = 6 per tank) for the duration of the study.

2.2. Apparatus

Testing procedures involved the use of a transparent Plexiglas® T-maze, the stem of which measured 50 cm × 10 cm × 10 cm. To form a start box, an area (10 cm × 10 cm × 10 cm) was closed off at the foot of the stem. Each arm of the maze measured 20 cm × 10 cm × 10 cm. The cross-arm is defined as the entire arm perpendicular to the arm containing the start box. One separate 10 cm × 10 cm × 10 cm tank of clear Plexiglas® was placed adjacent to the extreme end of each arm of the T-maze (Fig. 1). Depending on the specific phase of investigation, six (6) conspecific fish were placed into the tank adjacent to one arm of the T-maze. The tank located at the other arm contained only water. At no time were conspecifics introduced to both adjacent tanks simultaneously. The terminal 10-cm end of one of the arms (cued arm) was colored red by covering all relevant surface areas (including that of the adjacent tank, but excluding the adjoining surface between the T-maze and the adjacent tank) as well as those areas of the arm that were located directly opposite the adjacent tank, with a red-colored cue card ('cued arm'; Fig. 1). The other adjacent arm was covered with the same white plastic sheets as the rest of the maze ('non-cued arm'; Fig. 1). The resulting setup ensured that the experimental fish could only see into the adjacent tanks by swimming to and entering the proximity area

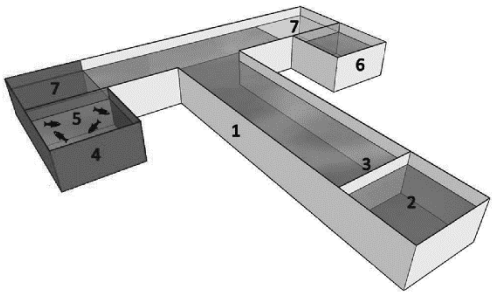

Fig. 1. Illustration of basic experimental setup. 1) T-maze, 2) starting box, 3) start box door, 4) red cue card, 5) tank containing conspecifics (phase dependent), 6) tank without conspecifics and 7) proximity areas (opposite the two adjacent conspecific tanks).

directly in front of the adjacent tank (Fig. 1). The maze was filled with water from the home tank to a depth of 8 cm and maintained at 26 °C, with the water changed and the maze cleaned every day to prevent excessive growth of biofilm. A digital video-camera (Panasonic® HC-V180) was positioned 150 cm above the T-maze and all trials were digitally recorded. Recordings were subsequently analyzed at the same time by means of EthoVision® XT 14 (Noldus® Information Technologies, Wageningen, The Netherlands) digital tracking software.

2.3. Exposure groups and drug administration

Experimental zebrafish were randomly divided into 7 groups (n = 6 per group). The different groups were constituted by randomly allocating 6 fish, each from a different home tank to the respective groups. Each group was exposed for 1 h per day for 24 days to one of the respective drug exposures (Fig. 2A). First, we aimed to establish what the effect of a lower (50 µg/L) and a higher (100 µg/L) dose of apomorphine (Sigma-Aldrich®, Johannesburg, South Africa) would be on contextual reward appraisal. These dosages were based on data from rodent studies [71] and adapted for translational application in zebrafish according to previously reported guidelines [72,73]. As such, the first three exposure groups consisted of 1) control (normal tank water), 2) apomorphine 50 µg/L, and 3) apomorphine 100 µg/L. Subsequently, we aimed to establish the effect of escitalopram (Lundbeck A/S®, Copenhagen, Denmark) alone at both a lower and a higher dose, *viz.* exposure groups 4) escitalopram 500 µg/L and 5) escitalopram 1000 µg/L. Based on earlier work in studies of neuropsychiatric states in rodents and zebrafish, escitalopram oxalate equivalent to 50 mg/kg/day [74] roughly equates to 500 µg/L for aqueous immersion studies [56,75–77]. Last we aimed to establish the effect on contextual reward appraisal following exposure to a combination of apomorphine (at the optimal dose established, i.e. 100 µg/L; refer to *'Results'*) and escitalopram at both doses, *viz.* apomorphine/escitalopram 100/500 µg/L (group 6) and 100/1000 µg/L (group 7). All exposures were administered by means of aqueous immersion throughout the study. During the first ten days of the study, zebrafish were group-housed and group-exposed (Fig. 2A). For the remainder of the study (days 11–25), zebrafish were individually housed, drug exposed and tested, therefore being socially isolated throughout the behavioral testing phase (Fig. 2A). Drug solutions were constituted by dissolving appropriate quantities of drug in a volume of 1.5 L (for group exposure) or 1.2 L (for individual exposure) tank water. Group-exposed fish were placed into tanks identical to the housing tanks and contained 1.5 L of the relevant drug solutions. During individual exposure, fish were placed into 600 mL beakers containing 250 mL of drug solution. During individual exposure and social isolation, non-reflective white polystyrene separators were

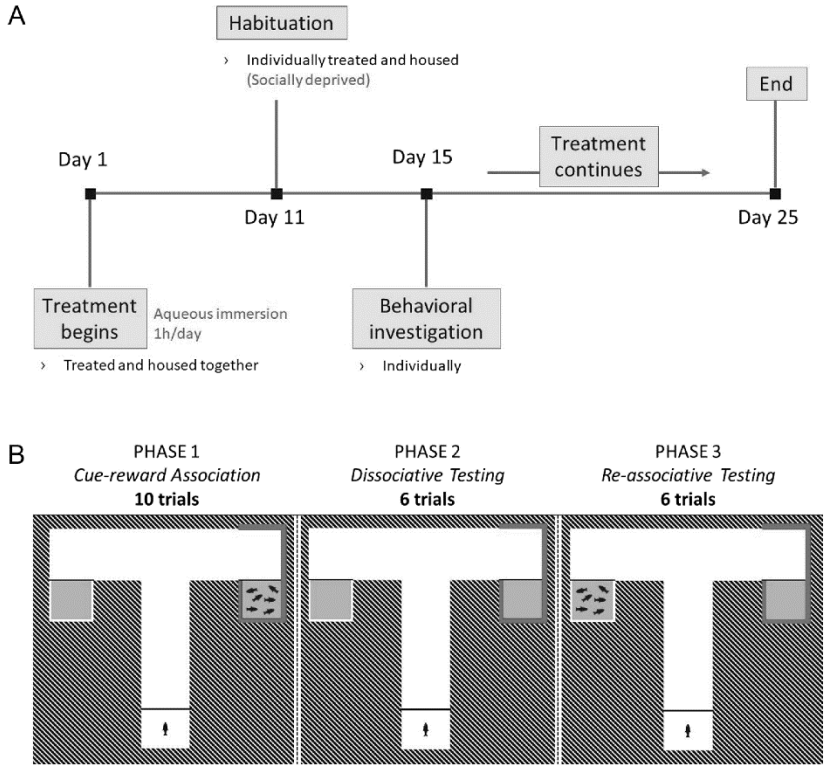

Fig. 2. (A) Schematic layout of the timeline followed for all experimental fish; **(B)** Schematic layout of the phasic setup of the T-maze, indicating the presence of social conspecifics in the vicinity of the red cue card in Phase 1, no social conspecifics presented in Phase 2, and reintroduction of conspecifics in the white arm during Phase 3.

placed between exposure beakers and tanks to prevent zebrafish from seeing one another.

2.4. Procedures

2.4.1. Habituation

Prior to the start of Phase 1, zebrafish were habituated individually in the T-maze during a single daily 5-min session repeated over four consecutive days (Fig. 2A; days 11–14). During habituation, neither conspecifics nor a red cue card was presented, with all of the T-maze outside walls being covered with white plastic sheeting. Each habituation session on each of the four respective habituation days consisted of a 7-min exposure session adapted from literature [46]. For each of the habituation sessions, a single fish was gently introduced to the start box in the stem of the T-maze for 2 min. Next, the start box door was manually raised and lowered again once the fish exited the start box. Each fish was then left to explore the T-maze without restriction for a duration of 5 min. Upon completion of the 5-min session, fish were netted out of the T-maze and placed back into the home tanks. This procedure was repeated for all the fish in the group. Following the completion of all the habituation trials, experimental fish were tested individually in the T-maze according to the procedures explained below (Fig. 2B).

2.4.2. Phase 1: cue-reward association

To establish whether fish already chronically exposed to each of the respective interventions would value and appraise the presentation of social conspecifics in the red-colored arm, Phase 1 was executed over 5 days, with two 5-min T-maze trials per fish per day, separated by 2.5 h. As such, each fish was assessed across 10 trials during Phase 1 [46]. During all trials of Phase 1, one arm of the maze was colored with the red cue card as described under *'Apparatus'*, while a shoal of 6 conspecific fish (social reward) was always presented adjacent to the proximity area of this cued arm. That said, to prevent the potential influence of place preference on arm choice, the cue-reward presentation (red-colored cued arm with conspecific shoal) alternated, i.e. left or right, with each successive trial. Each trial was started by introducing a single zebrafish to the start box for 2 min. Subsequently, the start box door was raised and then lowered after the fish exited. Free exploration of the T-maze was allowed for a 5-min, videotaped session. After the 5-min trial, the fish was gently netted and placed back into its home tank. This procedure was repeated for each fish. Each trial began at roughly the same time each day, approximately 08 h00 for the first trial, followed by the second trial after the inter trial period (2.5 h), starting at approximately 10h30. This procedural flow was maintained throughout all phases.

2.4.3. Phase 2: cue-reward dissociation testing

To establish whether zebrafish engaged in cue-directed responses, fish were assessed in Phase 2 over 3 days of testing, with two trials per fish per day, separated by 2.5 h, beginning on the day following the last Phase 1 trial. Phase 2 proceeded identically to Phase 1 in that the red cue card was once again presented in alternate arms of the maze during each successive trial. However, during this phase, *no social reward (conspecifics) was presented* at any time, hence only filling the adjacent tanks with water.

2.4.4. Phase 3: re-associative contingency testing

Phase 3 was, as Phase 2, conducted over another 3 days of testing with two daily 5-min trials, separated by 2.5 h, completed in a single day. During Phase 3, the experimental setup was applied exactly as in Phases 1 and 2. However, here the presentation of the conspecific-containing tank was paired with the non-cued arm.

2.4.5. The use of conspecifics in Phases 1 and 3

The 36 conspecific fish were divided into 6 groups (A–F) of 6 fish each and used as follows: Groups A and B were used for exposure groups 1–3. Groups C and D were used for exposure groups 4 and 5. Groups E and F were used for exposure groups 6 and 7. During each of the phases, the first group of conspecifics was used during the first trial of the day, and the second group of conspecifics, during the second trial of the day.

2.5. Statistical analyses

Statistical analyses were performed with GraphPad Prism® 8.0.1 software. Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni post-hoc testing was applied to evaluate the arm-choice behavior of fish in the different exposure groups over the three separate phases. As behaviors relating to arm-choice were primarily expressed as the indices of entry frequency and time spent in the red-colored cued vs. the non-cued arm (see below), these were set as between-subjects (dependent) factor, and phase and exposure as within-subjects (independent) factors, except if stated otherwise. Statistical significance was set at $p < 0.05$ for all analyses. All comparisons, irrespective of being statistically significant or not, were informed with calculations of Cohen's *d* effect size to establish the magnitude of the effects observed. Effect sizes are considered large at values between 0.8 and 1.29 and very large at values higher than 1.3 [78].

The values expressed on the Y-axis of Figs. 3 and 4 are representative of an index describing the time spent in ([RT-NCT]/TPAT; Fig. 3) and entries made into ([RE-NCE]/TPAE; Fig. 4) the cued compared to the non-cued arm of the maze. These indices were calculated by subtracting the time spent in (NCT) and the entries made into (NCE) the non-cued proximity area from the time spent in (RT) and entries made into (RE) the red-colored proximity area of the cued arm divided by the total time spent in (TPAT) and the number of entries made into (TPAE) the proximity areas.

3. Results

3.1. Comparisons of the effect of phase and drug exposure on the indices of time spent in and number of entries made into the arms

Two-way RM ANOVA did not reveal significant interactions between phase and exposure for either the index of time spent in [$F(12,64) = 0.7910$, $p = 0.658$] or the index of entries made into the cued vs. the non-cued arm [$F(12,64) = 1.211$, $p = 0.296$]. However, with respect to the index of time spent in the cued vs. the non-cued arm, significant main effects of phase [$F(2,64) = 35.27$, $p < 0.0001$] and exposure [$F(6,32) = 2.421$, $p = 0.048$] were demonstrated (Fig. 3). In terms of the index of entry frequency into the cued vs. the non-cued arm, a significant main effect of phase [$F(2,64) = 43.18$, $p < 0.0001$], but not exposure [$F(6,32) = 2.176$, $p = 0.072$] was shown (Fig. 4).

3.2. Establishing the most appropriate dose of apomorphine

Based on the observation that apomorphine 100 μg/L, and not 50 μg/L induced *both* a higher degree of cued-arm persistence during Phase 2, *as well as* indiscriminate arm-choice behavior during Phase 3 compared to control exposure (Figs. 3 and 4; descriptive statistics provided in Tables 1A, 1B and 2A, 2B), this dose was chosen for combined exposure with escitalopram in exposure groups 6 and 7.

3.3. Within phase comparisons of the indices of time spent in and number of entries made into the arms

3.3.1. Phase 1

Although no statistically significant differences were demonstrated between any of the exposure groups during Phase 1 with respect to either the indices of time spent in (Fig. 3), or entries made into (Fig. 4)

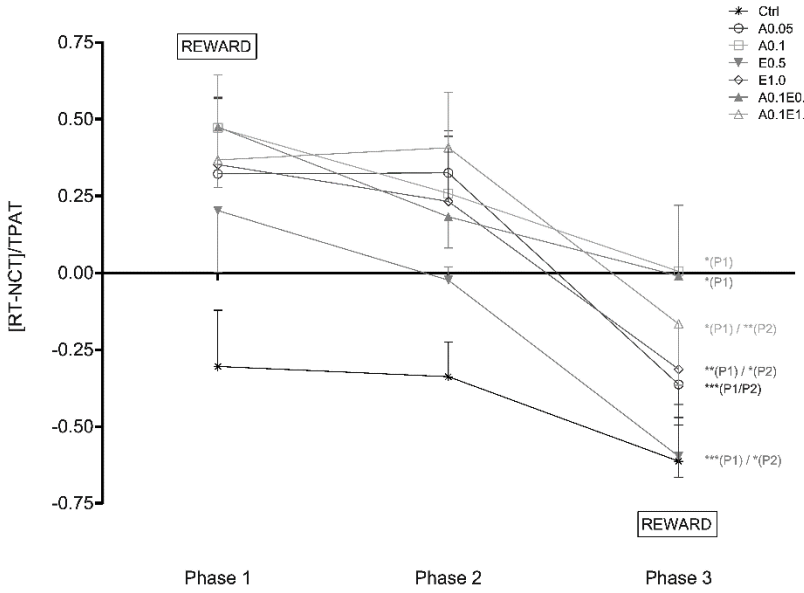

Fig. 3. Comparisons of the average index of time spent in the cued vs. non-cued proximity areas (RT-NCT/[TPAT]; index for time spent in respective arms; see *statistical analysis* for complete explanation) for the different exposure groups over three phases of testing. Positive values on the *y*-axis represent more time spent in the red proximity area, while negative values indicate more time spent in the white non-cued proximity area. *Phase 1* represents 10 trials (5 days), *Phase 2* represents 6 trials (3 days) and *Phase 3* represents 6 trials (3 days). 'Reward' indicates where the sight of social conspecifics was introduced, i.e. either the cued arm (Phase 1), neither of the arms (Phase 2), or the non-cued arm (Phase 3). Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni's multiple comparisons; statistics are mean ± SEM. Significant between-phase differences within the respective exposure groups are indicated on the right side. 'P' indicates the specific phase compared to *Phase 3*. Descriptive statistics provided in Tables 1A & 1B, and Supplementary Table i a.

the cued vs. the non-cued arm, differences with large effect sizes were observed between the control group and all other exposure groups (Tables 1A and 2A). Further, the difference in the index of time spent in the cued vs. the non-cued arm between the control-exposed fish and group exposed to 100 μg/L apomorphine only narrowly missed statistical significance ($p = 0.076$). Moreover, despite conspecific presentation in the cued arm, control-exposed fish avoided the color red, which was reversed by all the exposure groups, as indicated by the negative, as opposed to the positive index values calculated for the control-exposed and all other exposure groups, respectively. This trend was demonstrated with respect to both the indices of time spent in (Fig. 3) and the number of entries made into (Fig. 4) the cued vs. the non-cued arm.

3.3.2. Phase 2

No statistically significant differences were found between exposure groups. In the absence of reward presentation in either arm, control-exposed fish maintained their preference for the non-cued arm, demonstrating continued cued arm avoidance (Figs. 3 and 4; Tables 1A and 2A). In contrast, all other exposure groups, except for fish exposed to escitalopram 500 μg/L, spent more time in the cued arm compared to the non-cued arm (Fig. 3; Table 1A). However, considering the number of entries made into the arms, escitalopram-alone exposed groups trended toward showing a higher interest for exploring the non-cued arm, as evinced by the negative index of entry frequency into the cued- compared to the non-cued arm of the maze (Fig. 4, Table 2A). Moreover, while fish exposed to apomorphine 50 μg/L and 100 μg/L spent an

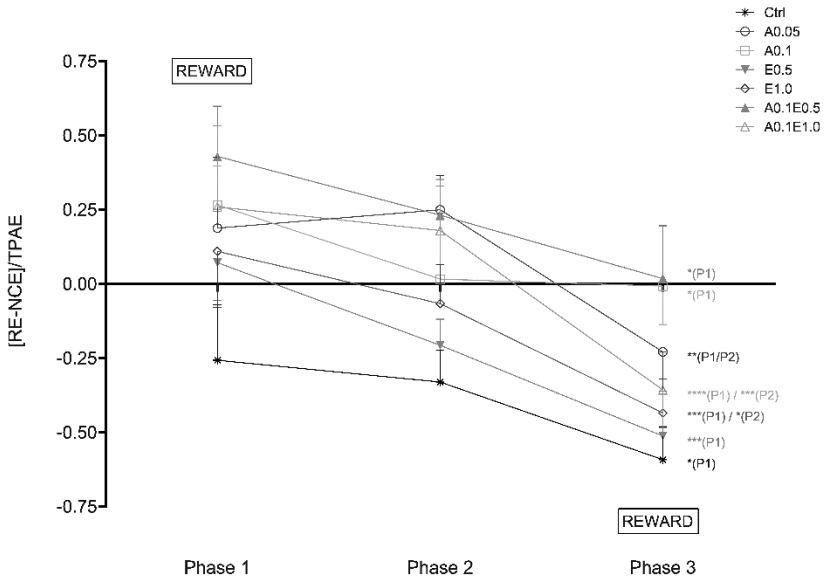

Fig. 4. Comparisons of the average index of entries made into the cued vs. non-cued proximity areas (RE-NCE/[TPAE]; index for number of entries made into respective arms; see *statistical analysis* for complete explanation) for the different exposure groups over three phases of testing. Positive values on the *y*-axis represent more entries into the red proximity area, while negative values indicate more entries into the white non-cued proximity area. *Phase 1* represents 10 trials (5 days), *Phase 2* represents 6 trials (3 days) and *Phase 3* represents 6 trials (3 days). 'Reward' indicates where the sight of social conspecifics was introduced, i.e. either the cued arm (Phase 1), neither of the arms (Phase 2), or the non-cued arm (Phase 3). Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni's multiple comparisons; statistics are mean ± SEM. Significant between-phase differences within the respective exposure groups are indicated on the right side. 'P' indicates the specific phase compared to *Phase 3*. Descriptive statistics provided in Tables 2A & 2B, and Supplementary Table i b.

Table 1A
Cohen's *d* values of the within-phase comparisons of the indices of time spent in the cued vs. non-cued arms. Cohen's *d* values are considered large at values between 0.8 and 1.29 and very large at values higher that 1.3 (indicated in bold).

Exposure groups		Phase 1 Cohen's *d* All *p* values > 0.05	Phase 2 Cohen's *d* All *p* values > 0.05	Phase 3 Cohen's *d* All *p* values > 0.05
Ctrl vs.	A0.05	**1.2**	**2.2**	**0.9**
	A0.1	**1.9**	**1.7**	**1.6**
	E0.5	**1.1**	**1.7**	0.1
	E1.0	**1.4**	**1.5**	0.6
	A0.1E0.5	**1.8**	**2.1**	**1.5**
	A0.1E1.0	**1.2**	**2.1**	**1.2**
A0.05 vs.	A0.1	0.3	0.2	**1.0**
	E0.5	0.2	**1.6**	0.7
	E1.0	0.1	0.2	0.1
	A0.1E0.5	0.3	0.5	**0.9**
	A0.1E1.0	0.1	0.2	0.5
A0.1 vs.	E0.5	0.7	**1.1**	**1.4**
	E1.0	0.3	0.1	0.5
	A0.1E0.5	0.002	0.2	0.03
	A0.1E1.0	0.2	0.3	0.4
E0.5 vs.	E1.0	0.3	**0.9**	0.5
	A0.1E0.5	0.6	**1.3**	**1.3**
	A0.1E1.0	0.3	**1.6**	**1.0**
E1.0 vs.	A0.1E0.5	0.3	0.1	0.5
	A0.1E1.0	0.02	0.4	0.2
A0.1E0.5 vs.	A0.1E1.0	0.2	0.7	0.3

Table 1B
Pairwise between-phase comparisons of the indices of time spent in the cued vs. non-cued arms. Statistical significance was set at $p < 0.05$ (indicated in bold). Cohen's *d* values are considered large at values between 0.8 and 1.29 and very large at values higher that 1.3 (indicated in bold).

Exposure group	Phase	Level of significance	
		p value	*d* value
Control	Phase 1 vs. Phase 2	–	0.1
	Phase 1 vs. Phase 3	0.272	**0.8**
	Phase 2 vs. Phase 3	0.391	**1.0**
A0.05	Phase 1 vs. Phase 2	–	0.01
	Phase 1 vs. Phase 3	**0.0009**	**1.6**
	Phase 2 vs. Phase 3	**0.0009**	**2.3**
A0.1	Phase 1 vs. Phase 2	0.716	0.5
	Phase 1 vs. Phase 3	**0.035**	**1.1**
	Phase 2 vs. Phase 3	0.488	0.6
E0.5	Phase 1 vs. Phase 2	0.763	0.8
	Phase 1 vs. Phase 3	**0.0004**	**1.9**
	Phase 2 vs. Phase 3	**0.015**	**2.4**
E1.0	Phase 1 vs. Phase 2	–	0.3
	Phase 1 vs. Phase 3	**0.004**	**1.1**
	Phase 2 vs. Phase 3	**0.022**	**0.9**
A0.1E0.5	Phase 1 vs. Phase 2	0.427	**0.9**
	Phase 1 vs. Phase 3	**0.0495**	**1.0**
	Phase 2 vs. Phase 3	0.997	0.5
A0.1E1.0	Phase 1 vs. Phase 2	–	0.1
	Phase 1 vs. Phase 3	**0.013**	**0.9**
	Phase 2 vs. Phase 3	**0.007**	1.2

Table 2A
Cohen's *d* values of the within-phase comparisons of the indices of the number of entries made into the cued vs. non-cued arms. Cohen's d values are considered large at values between 0.8 and 1.29 and very large at values higher that 1.3 (indicated in bold).

Exposure groups		Phase 1 Cohen's *d* All *p* values > 0.05	Phase 2 Cohen's *d* All *p* values > 0.05	Phase 3 Cohen's *d* All *p* values > 0.05
Ctrl vs.	A0.05	**0.9**	**2.1**	**1.5**
	A0.1	**1.3**	**0.9**	**2.0**
	E0.5	**0.9**	0.5	0.3
	E1.0	**0.8**	**0.9**	0.4
	A0.1E0.5	**1.6**	**2.3**	**1.8**
	A0.1E1.0	**0.9**	**1.5**	**0.8**
A0.05 vs.	A0.1	0.2	0.6	**0.8**
	E0.5	0.3	**1.9**	**1.0**
	E1.0	0.2	**1.1**	0.6
	A0.1E0.5	0.5	0.1	**0.8**
	A0.1E1.0	0.1	0.2	0.5
A0.1 vs.	E0.5	0.6	0.6	**1.6**
	E1.0	0.4	0.2	**1.1**
	A0.1E0.5	0.5	0.6	0.1
	A0.1E1.0	0.01	0.4	**1.1**
E0.5 vs.	E1.0	0.1	0.6	0.2
	A0.1E0.5	**1.1**	**2.1**	**1.4**
	A0.1E1.0	0.4	**1.2**	0.5
E1.0 vs.	A0.1E0.5	**0.8**	**1.2**	**1.0**
	A0.1E1.0	0.3	0.7	0.2
A0.1E0.5 vs.	A0.1E1.0	0.3	0.2	**1.1**

Table 2B
Pairwise between-phase comparisons of the number of entries made into the cued vs. the non-cued arms. Statistical significance was set at $p < 0.05$ (indicated in bold). Cohen's *d* values are considered large at values between 0.8 and 1.29 and very large at values higher that 1.3 (indicated in bold).

Exposure group	Phase	Level of significance	
		p value	d value
Control	Phase 1 vs. Phase 2	–	0.2
	Phase 1 vs. Phase 3	**0.032**	**0.9**
	Phase 2 vs. Phase 3	0.131	**1.0**
A0.05	Phase 1 vs. Phase 2	–	0.1
	Phase 1 vs. Phase 3	**0.005**	**1.0**
	Phase 2 vs. Phase 3	**0.001**	**1.9**
A0.1	Phase 1 vs. Phase 2	0.160	0.6
	Phase 1 vs. Phase 3	0.106	**0.9**
	Phase 2 vs. Phase 3	–	0.1
E0.5	Phase 1 vs. Phase 2	0.150	**1.1**
	Phase 1 vs. Phase 3	**0.0003**	**1.9**
	Phase 2 vs. Phase 3	0.095	**1.1**
E1.0	Phase 1 vs. Phase 2	0.632	0.5
	Phase 1 vs. Phase 3	**0.0007**	1.2
	Phase 2 vs. Phase 3	**0.031**	**1.0**
A0.1E0.5	Phase 1 vs. Phase 2	0.479	0.7
	Phase 1 vs. Phase 3	**0.013**	**1.1**
	Phase 2 vs. Phase 3	0.387	0.7
A0.1E1.0	Phase 1 vs. Phase 2	–	0.1
	Phase 1 vs. Phase 3	**< 0.0001**	**1.3**
	Phase 2 vs. Phase 3	**0.0002**	**1.5**

equal period of time in the cued arm (Fig. 3, time index 0.33 ± 0.34 vs. 0.26 ± 0.43; Table 1A; supplementary data), the group exposed to 100 μg/L also trended towards increased exploration of the non-cued arm in the absence of reward presentation (Fig. 4, entrance index 0.02 ± 0.49 (A0.1) vs. 0.25 ± 0.28 (A0.05); Table 2A; supplementary data).

3.3.3. Phase 3

Again, no statistically significant differences were observed with respect to any of the pairwise comparisons between the different exposure groups. However, in Phase 3, where social conspecifics were presented in the non-cued arm, all exposure groups, except for fish exposed to apomorphine 100 μg/L and a combination of apomorphine and low-dose escitalopram (100/500 μg/L) spent more time in (Fig. 3) and made more entries into (Fig. 4B) the non-cued arm, compared to the cued arm (Tables 1A and 2A, respectively). However, only low-dose escitalopram administered alone, induced behavior akin to that observed in the control-exposed fish with respect to both arm choice parameters (Figs. 3 and 4; Tables 1A and 2A). Apart from the near-zero indices calculated with respect to both the time spent in (Fig. 3; Table 1A) and the number of entries made into (Fig. 4; Table 2A) the cued and non-cued arms respectively, the behavior of animals in the apomorphine 100 μg/L and combination 100/500 μg/L groups differed

to the greatest extent from that of control-exposed fish (Tables 1A and 2A), demonstrating indiscriminate arm-choice behavior.

3.4. Between-phase comparisons of the indices of time spent in and number of entries made into the arms

3.4.1. Control group

Control-exposed fish demonstrated a persistent preference for the non-cued arm throughout all phases of the investigation, irrespective of reward presentation in the cued arm during Phase 1, as measured by both the time spent in (Fig. 3; Table 1B) and the number of entries made into (Fig. 4; Table 2B) the arms. Moreover, once sight of conspecifics was introduced in the non-cued arm, control-exposed fish made more entries into the non-cued arm compared to Phase 1 (0.59 ± 0.27 vs. 0.26 ± 0.46, $p = 0.032$, $d = -0.9$) and Phase 2 (0.59 ± 0.27 vs. 0.33 ± 0.27, $p = 0.131$, $d = -1.0$). Further, although not statistically significant, a difference with a large effect size was observed between the time spent in the non-cued arm during Phase 3, compared to Phase 1 (-0.61 ± 0.29 vs. -0.31 ± 0.45, $p = 0.272$, $d = -0.8$).

3.4.2. Apomorphine-alone exposed groups

Considering the time spent in the respective arms, both 50 μg/L and 100 μg/L induced persistent cued arm choice in the absence of reward presentation during Phase 2, compared to Phase 1 (Fig. 3; Table 1B). However, the behavior of 50 μg/L exposed fish was once again more orientated towards the reward as evinced by both the time spent in (Fig. 3, Table 1B; Phase 3 vs. Phase 1: 0.36 ± 0.26 vs. 0.32 ± 0.61, $p = 0.0009$, $d = -1.6$; Phase 3 vs. Phase 2: 0.36 ± 0.26 vs. 0.33 ± 0.34, $p = 0.0009$, $d = -2.3$) and the number of entries made into (Fig. 4, Table 2B; Phase 3 vs. Phase 1: -0.23 ± 0.22 vs. 0.19 ± 0.58, $p = 0.005$, $d = -1.0$; Phase 3 vs. Phase 2: -0.23 ± 0.22 vs. 0.25 ± 0.28, $p = 0.001$, $d = -1.9$) the non-cued, compared to the cued arm.

In contrast, although spending significantly less time in (Fig. 3, Table 1B; 0.01 ± 0.48 vs. 0.47 ± 0.38, $p = 0.035$, $d = -1.1$) and making fewer entries, albeit not significantly so, into the cued arm (Fig. 4, Table 2B; -0.01 ± 0.32 vs. 0.27 ± 0.32, $p = 0.106$, $d = -0.9$) during Phase 3, compared to Phase 1, fish exposed to apomorphine 100 μg/L expressed indiscriminate arm choice behavior during Phase 3 as indicated by near-zero discrimination indices calculated for both time and entry frequency (Tables 1B and 2B).

3.4.3. Escitalopram-alone exposed groups

In the absence of reward presentation (Phase 2 vs. Phase 1), chronic intervention with lower dose escitalopram tended to reduce the index of time spent in (-0.02 ± 0.10 vs. 0.20 ± 0.45, $d = -0.8$) and the number of entries made into the cued vs. the non-cued arm (-0.21 ± 0.20 vs. 0.07 ± 0.29, $d = -1.1$). In contrast, compared to Phase 1, high-dose escitalopram exposed fish failed to modify their behavior in the absence of reward presentation (Figs. 3 and 4; Tables 1B and 2B).

However, upon reintroducing sight of conspecifics in the non-cued arm, both groups of escitalopram-exposed fish directed their behavioral response toward the reward. Compared to both Phase 1 and Phase 2, both groups spent significantly more time in [Fig. 3, Table 1B; Phase 3 vs. Phase 1: (500 μg/L: 0.60 ± 0.38 vs. 0.20 ± 0.45, $p = 0.0004$, $d = -1.9$; 1000 μg/L: 0.31 ± 0.78 vs. 0.35 ± 0.48, $p = 0.004$, $d = -1.1$); Phase 3 vs. Phase 2: (500 μg/L: 0.60 ± 0.38 vs. 0.02 ± 0.015, $p = 0.015$, $d = -2.4$; 1000 μg/L: 0.31 ± 0.78 vs. 0.23 ± 0.47, $p = 0.022$, $d = -0.9$)] and trended towards making more entries into the non-cued, compared to the cued arm [Fig. 4, Table 2B; Phase 3 vs. Phase 1: (500 μg/L: 0.51 ± 0.34 vs. 0.07 ± 0.29, $d = -1.9$; 1000 μg/L: 0.43 ± 0.46 vs. 0.11 ± 0.42, $d = -1.2$); Phase 3 vs. Phase 2: (500 μg/L: 0.51 ± 0.34 vs. 0.21 ± 0.20, $d = -1.1$; 1000 μg/L: 0.43 ± 0.46 vs. 0.07 ± 0.29, $d = -1.0$)].

3.4.4. Combination exposed groups

Compared to the behavior observed in fish exposed to 100 μg/L apomorphine only, the addition of the lower dose of escitalopram had no overall effect. In fact, the behavior of fish exposed to combined apomorphine and escitalopram (100/500 μg/L) remained analogous to the behavior of apomorphine-alone exposed fish (Figs. 3 and 4, Tables 1A, 1B and 2A, 2B). In contrast, the combination of apomorphine and high-dose escitalopram, although not affecting cue-directed reward-seeking behavior in the absence of reward presentation compared to apomorphine 100 μg/L alone-exposed fish (*Phase 2*, A0.1E1.0 vs. A0.1: Fig. 3, Table 1A, $d = 0.3$; Fig. 4, Table 2A, $d = 0.4$), trended towards attenuating the indiscriminate arm choice during Phase 3 by modifying the behavior of apomorphine-alone exposed fish to a presentation more akin to escitalopram-alone- and control-exposed fish (*Phase 3*, A0.1E1.0 vs. A0.1: Fig. 3, Table 1A, 0.17 ± 0.49 vs. 0.01 ± 0.48, $d = -0.4$; Fig. 4, Table 2A, 0.36 ± 0.30 vs. 0.01 ± 0.32, $d = -1.1$).

3.5. Total distance moved, total arm entries, and time per cross-arm visit

A statistically significant interaction was demonstrated between phase and exposure with respect to the average total distance moved, an indication of general locomotor activity ([F (12.64) = 1,925, $p = 0.048$]), with significant main effects of phase [F (2, 64) = 5,327, $p = 0.007$], as well as exposure [F (6, 32) = 3,509, $p = 0.009$]. Indeed, drug exposure induced significant within-phase differences between the distances swam by the control group and the apomorphine 100 μg/L and escitalopram 500 μg/L exposed groups (Phase 1 and 2) and the apomorphine 100 μg/L and escitalopram 500 μg/L exposed groups (Phase 3) (See Fig. 5, and supplementary data). Further, with respect to between-phase behavior, pairwise comparisons revealed apomorphine 100 μg/L-exposed fish to present with shorter distances swam in Phase 3 compared to Phase 1 ($p = 0.002$, $d = -0.9$), and Phase 3 compared to Phase 2 ($p = 0.041$, $d = -0.5$) (Fig. 5 and Supplementary data). However, said reduction in the total distance swum, neither confounds nor undermines the results reported in the results, as fish exposed to apomorphine (100 μg/L) made an equal number of entries into the cross-arms throughout all phases of the investigation (supplementary data). Furthermore, although the average total distance moved by apomorphine 100 μg/L-exposed fish did not differ significantly from any other exposure group, with the exception of control-exposed fish (Fig. 5), apomorphine 100 μg/L exposure was associated with an increased time spent per cross-arm visit during each of the phases, compared to all other exposure groups (Supplementary data).

4. Discussion

In an effort towards developing a novel screening test for anti-compulsive drug action, the present work investigated cue-reward contingency learning in zebrafish and its modification with dopaminergic and serotonergic intervention. Our main findings were that 1) sight of social conspecifics, although previously reported to be rewarding for socially deprived zebrafish, was an insufficient behavioral reinforcer under circumstances of motivational conflict, 2) apomorphine, escitalopram and combinations thereof lessened the negative valence of an aversive scenario, i.e. bolstering reward appraisal and facilitated reward valuation, 3) high-concentration apomorphine exposure generally maintained cue-directed responses throughout all testing phases, 4) escitalopram facilitated and maintained reward-directed behavior, and 5) high-dose, but not low-dose escitalopram intervention reversed apomorphine-induced indiscriminate arm choices during processes of re-associative testing.

Repetitive behavioral rituals such as hand washing, compulsive checking of locks or re-arranging of items, among others, are characteristic of OCD [79]. By themselves, these actions often demonstrate a clear goal-directed outcome; however, in OCD, they are performed beyond any functional value [6,80]. Chronic high-dose SSRI

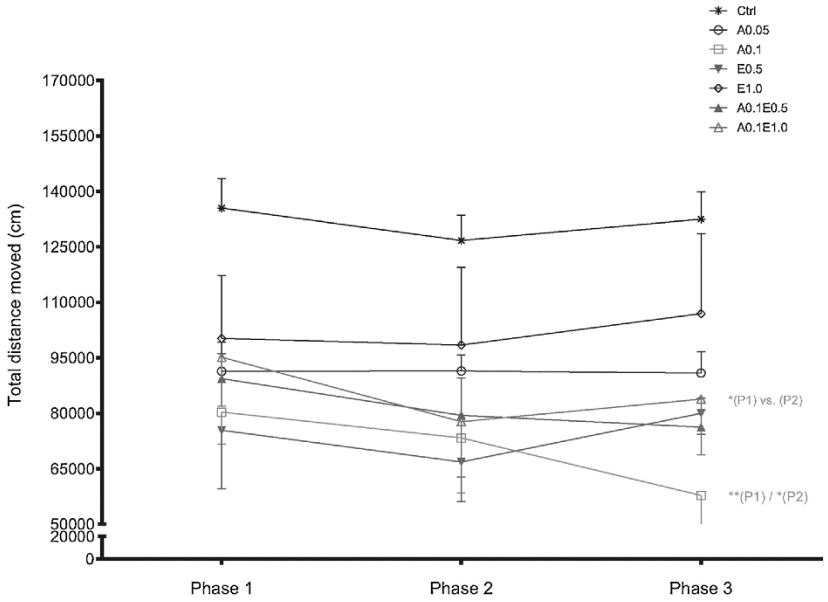

Fig. 5. Comparisons of the average total distance moved for the different exposure groups over the different phases. Two-way repeated measures analysis of variance (2-way RM ANOVA), followed by Bonferroni's multiple comparisons; statistics are mean ± SEM. Significant between-phase differences within the respective exposure groups are indicated on the right side. 'P' indicates the nature of specific phase comparisons. Descriptive statistics provided in Supplementary Table ii a & b.

intervention is regarded as the first-line pharmacological treatment for OCD; however, a substantial number of OCD patients remain treatment refractory [81,82]. In order to extend the number of currently available treatment options, accurate and robust screening tests for putative anti-compulsive drugs are of great importance.

Irrespective of the manner in which persistent repetitious behavior arises, organisms normally expend effort to perform actions that are often, but not always, aimed at achieving specific goal-directed outcomes [83,84], e.g. seeking out a food reward [85,86], constructing a nest [87] or grooming [88]. However, if such actions are frequently repeated, they become *habitual*, i.e. expressed in an automated fashion which reduces the need for intensive cognitive decision-making efforts [83]. That said, over-reliance on habitual responding styles is viewed by contemporary literature as being conducive to compulsive routines [15,81,89–91]. Any action, be it goal-directed, habitual or otherwise, is preceded by associating certain actions with specific outcomes [92,93]. In this regard, behavior is governed by reward and punishment feedback processing [94–96] which are regulated by dopaminergic and serotonergic neurotransmission [39,97–99]. Disruption of both systems within the neurobiological architecture that regulates voluntary and planned behavior, i.e. the cortico-striatal-thalamic circuitry [38,89,92,100] is often characteristic of OCD [101,102].

Targeted manipulation of the serotonergic and dopaminergic neurotransmission systems has previously been shown to induce ritualistic behaviors reminiscent of compulsive-like rituals in rodents [103,104]. Further, in line with the clinical response of OCD to SSRI intervention, SSRIs have proven useful to attenuate compulsive-like behaviors expressed in pre-clinical models [105,106,85,86,107]. Considering the expensive, time-consuming and labor-intensive nature of rodent models, the work presented here extends this body of evidence in a putative zebrafish model of compulsive-like persistence. As alluded to earlier, zebrafish demonstrate significant potential as a model species for cost-effective, high-throughput drug screening tests.

4.1. Phase 1 - cue-reward association

Our findings with respect to the influence of dopaminergic and serotonergic drug intervention on reward appraisal during Phase 1, must be regarded against the background of the behavioral response of control-exposed animals. Although sight of conspecifics has previously been shown to be highly rewarding to socially deprived zebrafish [48], control-exposed zebrafish generally avoided cued arm exposure, irrespective of the fact that the red color was cued with the presentation of conspecifics. As such, it is possible that as far as natural preference is concerned, the negative valence carried by the color red outweighed the potential rewarding value of visual interaction with conspecifics. Current research is inconclusive with respect to the color preference of zebrafish [66–68,108,109]. For example, Avdesh et al. [68] reported zebrafish to display a natural preference for reds and greens, while demonstrating strong aversion towards blue. However, in accordance with our findings, Oliveira et al. [67] reported that zebrafish display an aversion towards red and yellow while preferring blue and green. That said, the collective of research seems to point to color preference in zebrafish being subject, rather than species-specific [110].

Irrespective, the results reported here show that chronically bolstered dopaminergic *and* serotoninergic neurotransmission resulted in improved reward appraisal as applied in this investigation, outweighing the negative impact of the red color. Indeed, irrespective of being administered alone or in combination, both apomorphine and escitalopram increased the time spent in and the number of entries made into the cued arm compared to the non-cued arm during Phase 1. Thus, under the influence of both drugs, the motivational value of social conspecifics was highlighted over and above the potential negative valence carried by the color red. In fact, considering the data presented with respect to Phase 2 (refer to *'Phase 2 – Cue-reward dissociation testing'*) we can with some certainty conclude that zebrafish associated the presentation of conspecifics with the color red. Although our data pertaining to the influence of apomorphine on reward appraisal are in line with the well-known role of dopamine in reward-orientated behavior [16,36,111–114], it is the actions of serotonin that are noteworthy. Low-dose acute SSRI intervention has been shown to increase the effect of aversive feedback on behavioral outcomes, i.e. resulting in avoidance behavior [115], a finding that is supported by the results reported here. Recent evidence also points to the long-term importance of serotonin in the appraisal of rewards and decision making with respect to learned cue-reward contingencies (126). Furthermore, SSRI-bolstered serotonergic tone also promotes increased effort cost towards achieving rewarding results [116], possibly explaining the currently observed behaviors.

4.2. Phase 2 – cue-reward dissociation testing

In the absence of reward presentation, cued arm approach bias would confirm successful cue-reward association during Phase 1, manifesting as reward-seeking behavior. However, behavioral flexibility, if intact, should also quickly promote reversal learning and favor exploration of the alternate, non-cued arm. Indeed, considering the apparent aversive nature of the color red, the *absence* of social reward presentation when it was *expected*, should prompt rapid dissociation between the cue and its conditioned outcome and facilitate an alternative behavioral choice, an ability which is impaired in OCD [42,117]. Drug-naive zebrafish continued to display aversion to the red color. However, the behavioral adaptation of apomorphine and escitalopram seemed to diverge, not only as a function of drug but also of dose. First, both doses of apomorphine resulted in continued dwelling in the cued arm, a finding that points to an overall lack of dissociative ability or dopamine-facilitated invigoration towards reward predicting cues [118]. This is in line with previously reported literature implicating bolstered dopaminergic signaling in persistent reward-seeking behavior [119,120]. Further, our data seem to support literature in that transiently depressed, not increased, dopaminergic signaling has been shown to act in concert with serotonin when learning from worse than expected outcomes [18], a process that should assist with cognitive flexibility. This may have been prevented by the administration of apomorphine. Still, considering the fact that the two arms of the T-maze differed significantly in presentation, we cannot rule out the potential influence of possible persistent spatial memory, a behavioral effect also fortified by bolstered dopaminergic signaling [121]. However, this seems less likely than a reward-seeking response per se, as zebrafish exposed to higher dose apomorphine demonstrated increased exploration of the non-cued arm as reflected by the equal number of entries made into both arms of the maze by this group. Irrespective, regarded against the background of the red color aversion shown by drug-naive zebrafish, it can be concluded that the presentation of social conspecifics in the cued arm during Phase 1 resulted in an initial preference for this arm by exposed groups. As such, spatial memory, if playing a role in the data presented here, was primarily founded in the co-presentation of the red color and the reward.

We hypothesized that subjects exposed to escitalopram would learn to disengage their cued arm directed behavior during Phase 2, a process governed chiefly by serotonin [39,97]. Although our data is to some extent congruent with previous findings that demonstrated both lower and higher doses of escitalopram to facilitate reversal learning [122], the difference observed in the effect of lower and higher dose escitalopram on the time spent in the previously cued arm, warrants further investigation. Sadly, most investigations into serotonergic influences on behavioral flexibility and reversal learning, employ serotonin depletion as the primary intervention [123,124], with very little data existing that divulge the role of different serotonin concentrations in cognitive flexibility within analogous experimental paradigms as followed here [115].

Our findings related to combination intervention are interesting. Indeed, it seems that combined intervention with both doses of escitalopram maintained the cued arm-directed behavior observed in high-dose apomorphine-alone exposed groups. The addition of escitalopram, regardless of dose, seemed to increase the number of entries made into the cued arm, i.e. masking the effect of apomorphine and escitalopram exposure alone on exploratory activity. In this regard it is likely that, in the absence of reward, the combined effects of dopamine on reward-seeking behavior and that of chronic serotonin in as far as reducing the impact of aversive feedback on decision making [115], converges to facilitate continued exploration of a previously reward-cued context.

4.3. Phase 3 – re-associative contingency testing

Considering that zebrafish demonstrated a natural aversion to the color red and that all drug interventions resulted in reward-directed behavior in spite of this, it could have been expected that once introducing the reward to the naturally preferred arm, bolstered reward-directed behavior should be facilitated during Phase 3. This was true for all exposure groups, except the two groups exposed to high-dose apomorphine and a combination of apomorphine and low-dose escitalopram. From an obsessive-compulsive perspective, the ideal response in the high-dose apomorphine exposed group of fish would have been persistent entries and dwelling time in the cued arm, an effect that would have been indicative of the effect of apomorphine previously shown in other species, e.g. pigeons [125]. It is likely that the natural aversive nature of the red color as reported in this investigation may have contributed to dampening the effect of high-dose apomorphine on persistent cue-directed responses; this needs further clarification. Nevertheless, our data would suggest that, as opposed to low-dose apomorphine intervention, chronic high-dose apomorphine intervention resulted in a phenotype more akin to behavioral inflexibility. Further, that high-dose apomorphine exposed fish persisted in cue-directed behavior throughout this investigation points to the fact that once a cue-outcome contingency has been acquired under the influence of said intervention, such behavior would be more resistant to adaptation over time and irrespective of changing contexts, albeit being reversible by high-dose SSRI intervention [97,98].

Importantly, it is unlikely that any of the results obtained and conclusions drawn here were confounded by the locomotor ability of the animals (Fig. 5). Although the total distance moved was the highest in drug-naive fish over the course of the investigation, all exposure groups displayed relatively similar locomotor activity during each phase, apart from the high-dose apomorphine exposed group, which demonstrated a slight reduction in locomotor activity over time. However, rather than being indicative of a general motor inability, this points to an increased interest in cued arm exploration. This is true as the data presented here are expressed as indices of the time spent in and number of entries made into the cued vs. the non-cued arm. Indeed, the number of entries made into the respective arms did not change in parallel with a reduction in the total distance swum (Supplementary Figs. i & ii).

The present investigation was conceptualized to provide a foundation for establishing a novel, high-throughput screening test for anti-compulsive drug action using zebrafish as a model organism. Considering the current theories describing the roles of dopamine and serotonin in OCD, we aimed to induce compulsive-like persistence with the dopaminergic agonist, apomorphine, and further investigated if such persistence, if present, would be reversed by chronic escitalopram. To this extent, the results reported here provide sufficient grounds for further investigation. Although compulsive-like persistence toward habitual, cue-directed behavior was not induced by either dose of apomorphine, fish exposed to high-dose apomorphine did, in fact, present with behavior more akin to behavioral inflexibility compared to their counterparts in all other exposure groups; this was reversed by chronic high, but not lower dose escitalopram, a finding that is supportive of current dopamine-serotonin theory. The apparent aversion shown by drug-naive subjects to the color red was unexpected and has complicated the interpretation of our results. Indeed, it is likely that the use of a more-preferred color in this population may yield a more robust result, a possibility that we will investigate in future.

In conclusion, typical theories of neurotransmitter involvement in OCD, i.e. imbalanced crosstalk between dopamine and serotonin [102], provide a useful background for investigating compulsive-like behaviors in animals [89]. Not only do the findings presented here confirm the viability of zebrafish as a model species in which to study the neurobiological and cognitive processes underlying dopamine-serotonin interactions under circumstances of motivational conflict, it also provides valuable direction for future endeavors towards the development of a novel screening framework that could be sensitive for anti-compulsive drug action.

Author's statement

CvS designed the experiments in consultation with DWW and SJB and conducted the experimental work. She performed all data and statistical analyses with DWW and GdB and wrote the first version of the manuscript. She also contributed to the revision of the manuscript following input from all co-authors.

GdB assisted in the interpretation of data and the statistical analyses of this work. He was instrumental in the writing of the final version of this paper.

TLB provided extensive insight into the animal work performed during this investigation. She was responsible for the appropriate breeding, housing and maintenance of all zebrafish used prior to the onset of experimentation. She also provided valuable advice regarding the exposure protocols and experimental application of zebrafish as reportedhere. She also contributed to the drafting of the manuscript.

KFB was instrumental in the interpretation of data and pointing out potential alternative perspectives for interpreting the work reported here. She contributed to both the original and revised versions of the current paper.

SJB and DWW contributed equally to this work. They funded, designed and conceptualized the investigation in consultation with CvS, GdB, TLB and KFB. They contributed to both versions of the manuscript. DWW acted as corresponding and editing author following the input from the reviewers.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bbr.2019.112393.

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