A pharmacological investigation of behavioural flexibility in zebrafish *(Danio rerio)*

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“There is a time for everything and a season for every activity under the heavens:

a time to be born and a time to die,
a time to plant and a time to uproot,
a time to kill and a time to heal,
a time to tear down and a time to build,
a time to weep and a time to laugh,
a time to mourn and a time to dance,
a time to scatter stones and a time to gather them,
a time to embrace and a time to refrain from embracing,
a time to search and a time to give up,
a time to keep and a time to throw away,
a time to tear and a time to mend,
a time to be silent and a time to speak,
a time to love and a time to hate,
a time for war and a time for peace.”

- Ecclesiastes 3:1 -8

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Abstract

Human and animal behaviour is ultimately directed at acquiring certain outcomes or achieving certain goals, or alternatively avoiding or mitigating negative outcomes. An intricate interplay between two components of action-outcome processing, namely goal-directed and habitual behaviour constantly surveys and manages behaviour to promote superior outcomes. Goal-directed behaviour is usually employed when behavioural responses are directed towards the completion of a specific, often novel outcome requiring considerable levels of cognitive deliberation. After frequent repetition of said behavioural actions, habitual behaviour begins to develop with the apparent objective of saving cognitive effort. This implies that not all habitual behaviour is entirely without 'goal' since such outcomes normally retain functional value and desirability. However, once the subject demonstrates overreliance on habitual actions, even when the behaviour persists in the absence of a specific functional outcome, psychopathologies, e.g. obsessive-compulsive disorder (OCD)[1] are seen. This interplay between goal-directed and habitual behavioural selection is constantly modulated on a neurocognitive continuum, with cognitive flexibility (CF)[2] on its one end, and cognitive rigidity (CR)[3], on the other. CF can loosely be defined as the ability to appropriately adjust one's behaviour in response to a changing environment, *inter alia* allowing for the selective engagement in tasks of a higher immediate priority. CR, on the other hand, is characterised by slow or even inadequate responses to changing outcomes or a changing environment. As such, when habitual behavioural engagement becomes excessive, an imbalance has formed within the neurocognitive continuum and CR begins to overwhelm CF.

Habitual and goal-directed task execution, hence also cognitive action-outcome processing, is broadly founded upon cortico-striatal-thalamic-cortical (CSTC)[4] signalling at neuroanatomical level. Although this system is differentially modulated by various neurotransmitters, dopamine seems to be of utmost importance. Specifically, it is proposed that excessive dopaminergic signalling, either directly or indirectly via mechanisms of inadequate serotonergic control, is believed to underlie hyperactive CSTC processing. Excessive dopaminergic signalling modulates behaviour by acting on two differentially identified neural pathways in the CSTC circuit, namely the behaviourally activating direct pathway and the behaviourally inactivating indirect pathway. It is further known that excessive dopaminergic activity results in the expression of persistent and repetitive behavioural phenotypes that collectively represent manifestations of CR. Indeed, such an imbalance between CF and CR, and by extension abnormal activity in CSTC circuit, has been demonstrated in a number of psychiatric disorders, e.g. OCD, major depressive

[1] obsessive-compulsive disorder
[2] cognitive flexibility
[3] cognitive rigidity
[4] cortical-striatal-thalamic-cortical

disorder (MDD)[1], and autism spectrum disorder (ASD)[2]. That said, most of these disorders show a suboptimal treatment response to current treatment options, highlighting a need for alternative approaches which may yield superior results. Therefore, it is believed that targeting a common neuropsychological construct, i.e. CR[3], that underlie said conditions, might lead to the development of more promising treatment avenues.

One potential method to pharmacologically interrogate a construct like CR would be to use a reward-feedback learning paradigm, which is often exploited in psychology in the form of conditioned learning, e.g. classic Pavlovian conditioning. Briefly, in a cue-reward contingency perspective, a subject is trained that a specific sensory cue is associated with a tangible rewarding outcome, and thus the presentation of the cue begins to stimulate approaching behaviour. Thereafter, once the contingency has been firmly acquired, but then changed so that the cue no longer predicts the experience of the reward, processes of reversal learning are enacted. Under normal, healthy circumstances, the subject is then able to disengage from the once relevant cue and alter their behaviour accordingly since the presentation of the cue becomes irrelevant to the experience of the reward. However, individuals suffering from CR, seem to be quite resistant to such changes.

Therefore, the aim of the current investigation was to build on previous work done in our laboratory which utilized zebrafish in a cue-reward contingency learning paradigm. In this context, the reward comprised a visual social reward, which was differentially presented with a cue in the form of a monochromatic pattern. Applying this, we aimed to explore the effect of chronic administration of the $D_{1/2}$ receptor agonist, apomorphine (APO)[4], on cue- or reward-directed behaviours and behavioural persistence. We further aimed to establish whether APO-associated behavioural changes would be modified by chronic exposure to levetiracetam (LEV)[5], a potentially novel cognitive enhancer with a mechanism of action that is potentially selective for abnormally active neurons and pathways.

The results obtained from this study confirmed the previous findings that showed zebrafish to be a suitable model system for investigations of reward-directed behavioural responses. Furthermore, we found that long-term dopaminergic potentiation suppressed reversal learning after fish acquired knowledge of a cue-reward contingency. However, with regards to our working hypothesis that LEV, being a putative cognitive enhancer that stabilizes excessive neuronal firing, would attenuate the behavioural effects elicited by APO, our results revealed the opposite. It is likely that the observed effect

[1] major depressive disorder
[2] autism spectrum disorder
[3] cognitive rigidity
[4] apomorphine
[5] levetiracetam

of LEV[1] in the present work, i.e. in blunting reward-directed responses, could be ascribed to the ability of LEV to blunt α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA)[2] receptor specific glutamatergic signalling, which has previously been shown to compromise cue-reward learning. Therefore, future studies are needed to explore this theory.

Keywords

cognitive rigidity; cognitive flexibility; zebrafish; cue-reward learning; obsessive-compulsive disorder; apomorphine; levetiracetam

[1] levetiracetam
[2] α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate

Congress proceedings

The results of the current investigation were presented at the annual South African Society for Basic and Clinical Pharmacology (SASBCP) conference hosted online via Zoom, October 2021. The presenting author is underlined.

- van der Westhuizen, CC; de Brouwer, G; Botha, T; Finger-Baier; K; Wolmarans, PD. "Apomorphine-induced inflexibility in zebrafish (*Danio rerio*) and its response to levetiracetam, a potentially novel cognitive enhancer."

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1 Introduction

1.1 Dissertation layout

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

Chapter 1 presents a brief description of the project problem statement, study questions, study aims, project layout, hypothesis, expected outcomes and ethical considerations. 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 by making overall conclusions, considering potential shortcomings and limitations, and offering potential for follow-up studies. Addendum A contains an expanded description of experimental methodologies followed during the execution of the project.

Since the journal where we intend to submit the scientific manuscript (chapter 3) for publication (*Behavioural Brain Research*) has no specific requirement for a referencing style, we applied the style of *Behavioural Processes* as it is also an Elsevier® journal, with the reference style being concise and easily formatted.

The dissertation is presented in English (UK).

[1] North-West University

1.2 Problem statement

Goal-directed behaviour refers to behavioural responses which are carried out to ensure that a specific outcome is achieved (Balleine and O'doherty 2010). These behaviours normally require significant cognitive effort before they are performed (Ehmer *et al.* 2020; Balleine and O'doherty 2010). After frequent repetition of said behaviours that lead to the achievement of the desired outcome, habitual behaviours develop to save cognitive effort (Ehmer *et al.* 2020; Quinn *et al.* 2010; Tricomi, Balleine, and O'Doherty 2009). Therefore, habitual behaviours are primed by a particular set of circumstances, in a particular environment or context, generally aimed at the completion of goals which remain salient over vast stretches of time, i.e. the need to feed, perform security-related behaviours and many others. The repetitive, yet unchanging nature of such outcomes, explains why habitual behaviours develop—they save valuable time and cognitive effort by largely automating processes that are unchanging, or at least slowly changing (Marien, Custers, and Aarts 2018). However, once a specific context no longer accurately predicts the desired outcome (i.e. there is a change in the contingency between pre-programmed action and outcome), the subject should ideally be able to disengage the unrewarding habitual behaviour and re-establish goal-directed behaviour until updated habits are able to form (Dreisbach and Goschke 2004; Goschke 2003; Hommel 2015). This intricate interplay between goal-directed and habitual behaviour plays a pivotal part in the daily functioning of both humans and animals (Foerde 2018; Graybiel 2008). Dysfunction of said interplay is thought to be caused at least in part by a cognitive construct known as cognitive rigidity (CR)[1] (Schultz and Searleman 2002).

CR can loosely be defined as a cognitive phenotype which is characterised by slow or even inadequate responses to changing—either for better or worse—outcomes or a changing environment (Burguiere *et al.* 2015; Graybiel 2008; Gillan *et al.* 2014; Schultz and Searleman 2002). Furthermore, there is a theoretical link between elevated measures of CR and symptom severity across a range of psychiatric conditions, including obsessive-compulsive spectrum disorders (OCRD[2]s; Schultz and Searleman (2002); Robbins *et al.* (2012)), major depression (MDD[3]; Liknaitzky, Smillie, and Allen (2017); Marazziti *et al.* (2010)) and autism spectrum disorder (ASD[4]; Poljac *et al.* (2017); Watanabe *et al.* (2019)). These disorders also invariably show a suboptimal treatment response to currently used first-line drug interventions, most notably so serotonin reuptake inhibitors (SRIs/SSRIs)[5] and drugs targeting dopaminergic signalling (Bedford, Hunsche, and Kerns 2020; Fineberg *et al.* 2015; Williams *et al.* 2013).

[1] cognitive rigidity
[2] obsessive-compulsive related disorders
[3] major depressive disorder
[4] autism spectrum disorder
[5] selective serotonin reuptake inhibitors

Therefore, by targeting and interrogating a neuropsychological construct such as CR[1] that is common to various disorders, potentially novel pharmacotherapeutic perspectives and an improved understanding of these disorders and their management could be gained (Chamberlain and Menzies 2009; Gottesman and Gould 2003). On the opposite end of what can be termed the flexibility spectrum, stands cognitive flexibility (CF)[2], which allows one to remain sensitive to alternative possibilities, to disengage from inefficient behavioural routines, and to adapt when external or internal states change (Zmigrod *et al.* 2019). It is therefore easy to see how adequate CF is associated with success throughout an individual's lifespan (Engel de Abreu *et al.* 2014; Genet and Siemer 2011; Davis *et al.* 2010)

Considering the complexity of CR, research into the exact neurobiological mechanisms underlying the construct is still inconclusive. However, numerous investigations have implicated the cortical-striatal-thalamic-cortical (CSTC)[3] circuitry in the control of behaviours related to the balance between CF and CR (Buschman and Miller 2014; Stocco, Lebiere, and Anderson 2010; Graybiel 2008). Further, it is proposed that bolstered dopaminergic signalling and/or hypoactivity of the serotonergic pathways in the CSTC circuitry, may contribute to excessively rigid behavioural phenotypes, particularly in the case of obsessive-compulsive disorder (OCD) [4] (Maia, Cooney, and Peterson 2008; Abramowitz and Jacoby 2015; Milad and Rauch 2012; Pauls *et al.* 2014).

Based on this, we propose that by addressing potential perturbations in the balance between CF and CR as regulated by the CSTC circuitry and parallel structures, it might be possible to resist and reverse the development of CR with respect to problematic behaviours, and by extension improve the overall presentation of relevant psychiatric symptomologies. To this end, levetiracetam (LEV)[5], normally prescribed as a supplementary treatment for epilepsy, might be of interest (Lyseng-Williamson 2011; Cramer *et al.* 2000). LEV exerts its effects via several mechanisms including antagonism of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)[6] receptors, *N*-methyl-D-aspartate (NMDA)[7] receptors and inhibition of N-type calcium channels. However, it is the drug's influence on synaptic vesicle release via actions on ubiquitously expressed effectors of vesicular exocytosis (Klitgaard and Verdru 2007) i.e. neuronal synaptic vesicle glycoprotein 2A (SV2A)[8] and synaptotagmin, that are most noteworthy (Nowack *et al.* 2010; Helmstaedter and Witt 2008). The net outcome of the various mechanisms of LEV is believed to be downregulation of the rate of vesicle exocytosis into synaptic

[1] cognitive rigidity
[2] cognitive flexibility
[3] cortico-striatal-thalamic-cortical
[4] obsessive-compulsive disorder
[5] levetiracetam
[6] α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
[7] *N*-methyl-D-aspartate
[8] synaptic vesicle protein 2A

spaces, thereby blunting the activity of neurons (Ciruelas *et al.* 2019; Kaminski *et al.* 2009). However, there is convincing evidence that such regulation only takes place in neurons that are abnormally active, such as nerve fibres that are prone to cause epileptic episodes, or neurons that demonstrate high discharge frequency rates (Surges, Volynski, and Walker 2008; Cortes-Altamirano *et al.* 2016). It is because of such a potentially selective modulatory function, that LEV[1] may act as a cognitive enhancer in cases where there is abnormally elevated CSTC[2] circuit activity that potentially contributes to cognitively rigid or repetitive behaviours. In line with this, LEV improved neuropsychological and cognitive performance in patients with benign epilepsy presenting with centrotemporal spikes (BECTS)[3], high-grade gliomas (HGG)[4], Alzheimer's disease, and even healthy individuals, which resulted in it being labelled as a nootropic drug or so-called 'cognitive enhancer' (Wu *et al.* 2009; Operto *et al.* 2019; de Groot *et al.* 2013; Vossel *et al.* 2021; Magalhaes *et al.* 2015). Furthermore, the antagonistic action of LEV on AMPA and NMDA receptors has been associated with the modification of conditioned behaviours (Ueda *et al.* 2001; Cortes-Altamirano *et al.* 2016; Pignatelli and Bonci 2015). Since the discussed mechanisms could potentially restore balance to perturbed functioning of the CSTC circuits, improved CF[5] may be a resulting outcome in conditions characterised by such CSTC-associated impairments. This research construct will form a major focus of the work presented in this dissertation.

The use of animal models to emulate human psychiatric conditions is a well-known, justified and longstanding practice that aims to improve our understanding of the etiopathological mechanisms and potential cognitive abnormalities of the corresponding human disorders (Nestler and Hyman 2010; Pittenger, Pushkarskaya, and Gruner 2019). That said, selecting and applying an animal model which accurately mimics a human condition in such a way that valuable translational results and knowledge can be gathered, is a challenging task (Robinson *et al.* 2019; Van Dam and De Deyn 2020; Eaton and Wishart 2017). Over the past two decades, zebrafish emerged as a potentially high-throughput, cost-effective and user-friendly model species that may be appropriate for the study of neuropsychiatric traits that are found in human conditions (Khan *et al.* 2017; Vaz, Hofmeister, and Lindstrand 2019). For the purposes of this problem statement, some key aspects of relevance for this study, will briefly be highlighted.

Zebrafish are able to learn in both conditioned (e.g. cue-outcome contingency learning) and unconditioned (e.g. natural habituation to a novel tank) circumstances (Blaser and Vira 2014; Zabegalov *et al.* 2019; Al-Imari and Gerlai 2008; Daggett, Brown, and Brennan 2019). Zebrafish are also highly

[1] levetiracetam
[2] cortico-striatal-thalamic-cortical
[3] benign epilepsy presenting with centrotemporal spikes
[4] high-grade gliomas
[5] cognitive flexibility

social and tend to shoal and actively seek out their conspecifics (Moretz, Martins, and Robison 2007; Saverino and Gerlai 2008). This drive for social interaction has been utilised as a behavioural reinforcer in associative learning paradigms (Al-Imari and Gerlai 2008; Daggett, Brown, and Brennan 2019; van Staden *et al.* 2020). Furthermore, with their sophisticated sensory systems, zebrafish can clearly discriminate between different colours, shapes and patterns (Oliveira *et al.* 2015; May *et al.* 2016; Bruzzone *et al.* 2020). That said, individual fish display different preferences with respect to visual conspecific interaction (Ruhl, McRobert, and Currie 2009; Saverino and Gerlai 2008) as well as colour and pattern choices (van Staden *et al.* 2020; Karnik and Gerlai 2012; Saverino and Gerlai 2008). This is of importance since zebrafish seems to develop and ascribe a context-specific valence to a reward when co-presenting the reward with a demotivating stimulus. In different terms, the actual value of rewarding stimuli, is seemingly clouded by the co-presentation of an aversive stimulus, such as in a preceding study, where red cue cards were unexpectedly found to be aversive (van Staden *et al.* 2020). Thus, using a cue that causes a high degree of aversion in itself, can have a negative effect on the establishment of a cue-reward contingency, thereby not only obstructing the ability of the subject to learn and relearn, but also confounding the study results (Kim *et al.* 2017; Bilotta *et al.* 2005; Peeters, Moeskops, and Veenvliet 2016). The same can be said when a form of reward is used that does not compute a high reward valence value. Therefore, in this work, that broadly aims to investigate goal-directed learning-induced deficits in CF[1] under the influence of dopaminergic potentiation, we opted to use a pattern-, rather than a colour-based cue to accommodate for the possibility of individual preference or aversion to the cue (pattern; conditioned stimulus) used in the cue-reward (sight of social conspecifics; unconditioned stimulus) learning abilities of zebrafish (Bruzzone *et al.* 2020; Avdesh *et al.* 2012). Previous studies done at this and other laboratories have successfully translated the concept of spontaneous alternation in rodents—i.e. the natural propensity of an animal to explore a novel environment and which is a reflection of the degree of CF displayed by the animal—to zebrafish. Further, such alternation can be successfully manipulated to induce persistent arm choices, i.e. being reminiscent of CR[2], under various experimental circumstances, including manipulation of a cue-reward contingency (van Staden *et al.* 2020; D'Amico, Estivill, and Terriente 2015). Here, we will build on these findings by using the same pharmacological manipulation as we have used before, i.e. chronic exposure to apomorphine (APO)[3], to induce behavioural inflexibility—or CR—in zebrafish (van Staden *et al.* 2020).

* * *

[1] cognitive flexibility
[2] cognitive rigidity
[3] apomorphine

In summary, CR[1] is a noteworthy and clinically important construct common to several neuropsychiatric conditions. Most of these disorders show suboptimal response to currently available treatments. There is an increased interest in targeting multi-disease constructs as novel pathways for treatment (Servaas *et al.* 2021). That said, the exact role of cognitive rigidity in the etiopathology of psychiatric illness remains relatively understudied. As such, it would be valuable to extend the current literature base by developing an animal model system in which the constructs of CF[2] and CR can be explored. By using zebrafish, we will attempt to extend our previous work in developing an APO-induced and behaviourally trained zebrafish model of behavioural inflexibility, which might be reminiscent of CR. We will further investigate the potential of LEV[3], an anti-epileptic drug that shows promise as a so-called cognitive enhancer, to modify and improve APO[4]-induced deficits in behavioural flexibility. If successful, both the model and the answers with respect to the effects of LEV that are generated, will deliver valuable insights into the development behaviours that resemble CR. This work will also add to the rapidly expanding pool of knowledge regarding the zebrafish behaviour and drug response in general.

[1] cognitive rigidity
[2] cognitive flexibility
[3] levetiracetam
[4] apomorphine

1.3 Study questions

Based on the current problem statement and considering that this research expands on work that was previously performed in our laboratory, the present investigation will attempt to answer the following questions:

1. Will control, APO[1], LEV[2] and APO + LEV-exposed zebrafish differentially portray associative learning ability in a cue-conditioned learning platform, by associating the presentation of a reward (sight of social conspecifics) with a co-presented cue (visual sight of a monochromatic pattern)?
2. How will zebrafish of the different exposure groups as highlighted in (1), after repeated exposure to the co-presented cue and reward as also described in (1), portray dissociative ability once the patterned cue is presented in the absence of the reward?
3. How will zebrafish of the different exposure groups as highlighted in (1), after reintroduction of the reward in the absence of the cue (where the cue is simultaneously presented in the opposing compartment), portray re-associative learning ability as reflected by their preference for the reward- vs. the cue-containing compartments, respectively?
4. Will APO-exposed zebrafish display a higher degree of behavioural expression akin to CR[3], i.e. persistent cue-directed responses under all three contexts, compared to control and LEV-exposed fish? and
5. Will chronic exposure to LEV (in combination with APO), a drug that shows promise as a novel cognitive enhancer, be able to prevent the effect of APO on the behaviour of exposed zebrafish?

[1] apomorphine
[2] levetiracetam
[3] cognitive rigidity

1.4 Study aims and objectives

The broad aim of this study was to explore the effects of APO[1] and LEV[2] on cognitive-behavioural flexibility in zebrafish under circumstances of cue-reward contingency manipulation. We did so by addressing the following objectives:

i. Forty (40) zebrafish were randomly selected from the population housed in the National Aquatic Bioassay Facility (NABF)[3] of the NWU[4] (with an additional 32 zebrafish used as social conspecifics only; please refer to **paragraph 1.5.2** and **1.5.4** for a full description and breakdown of conspecific numbers);

ii. The individual preference of each zebrafish in terms of visual social interaction (i.e. allowing experimental fish to choose between visual exposure to a single or a group of conspecific fish), and pattern recognition (i.e. assessing preference for stripes or solid dots/circles; see **paragraph 1.5.3**);

iii. Zebrafish selected in (i) were randomly divided into the following exposure groups (*n* = 10 fish per group; **paragraph 1.5.4**):

   a. **Group A**: control-exposed; normal behavioural control group; exposure to normal tank water throughout the investigation only; tested until experimental day 39;

   b. **Group B**: APO-exposed; 39-day 'behaviourally inflexible' group; exposed to APO 100 µg/L per day for 39 days (van Staden *et al.* 2020);

   c. **Group C**: LEV-exposed; 39-day LEV control group; exposed to LEV 750 µg/L per day for 39 days; Sanchez *et al.* (2012) adapted for aqueous immersion as per van Staden *et al.* (2020); and

   d. **Group D**: Combination APO + LEV-exposed; 39-day concurrent experimental group; simultaneously exposed to both APO 100 µg/L and LEV 750 µg/L per day for 39 days;

iv. Cue-reward contingency learning and adaptation thereof to outcome manipulation was investigated in all exposed zebrafish under the most preferred circumstances as determined in (ii), over three separate phases (which commenced after 25 days of drug exposure, i.e. days 29 – 39; please refer to **paragraph 1.5.5** for a detailed methodological description), i.e.

   a. Phase 1: cue-reward contingency learning – 3 days, 6 sessions; reward was co-presented with cue in a rectangular maze;

   b. Phase 2: cue-reward dissociation learning – 3 days, 6 sessions; cue was presented in the absence of reward in a rectangular maze; and

[1] apomorphine
[2] levetiracetam
[3] National Aquatic Bioassay Facility
[4] North-West University

c. Phase 3: re-associative contingency learning – 5 days, 10 sessions; cue and reward were presented at opposite ends in a rectangular maze;

v. All sessions were videotaped throughout Phases 1 – 3 and used videos to score behavioural data, i.e. time spent in the cue - and/or reward-presenting compartments (van Staden *et al.* 2020).

1.5 Study layout

1.5.1 Study timeline

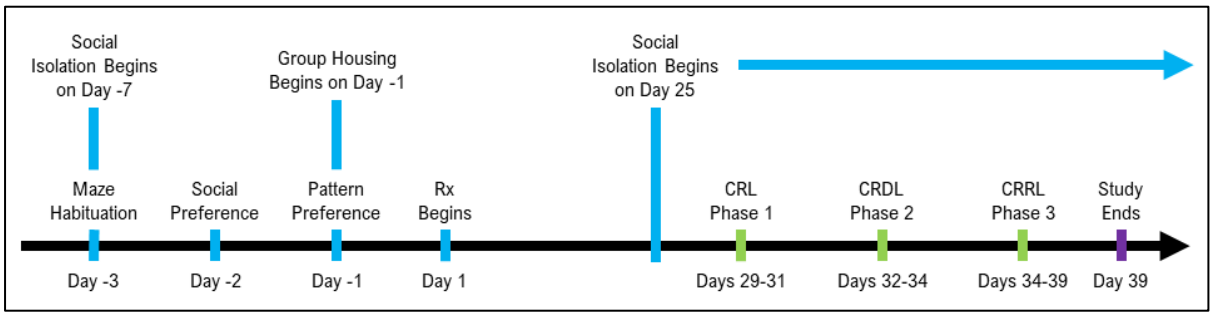


Figure 1-1 *- Graphical representation of the experimental timeline*
Rx: treatment; CRL: cue-reward contingency learning; CRDL: cue-reward dissociative learning; CRRL: cue-reward re-associative learning

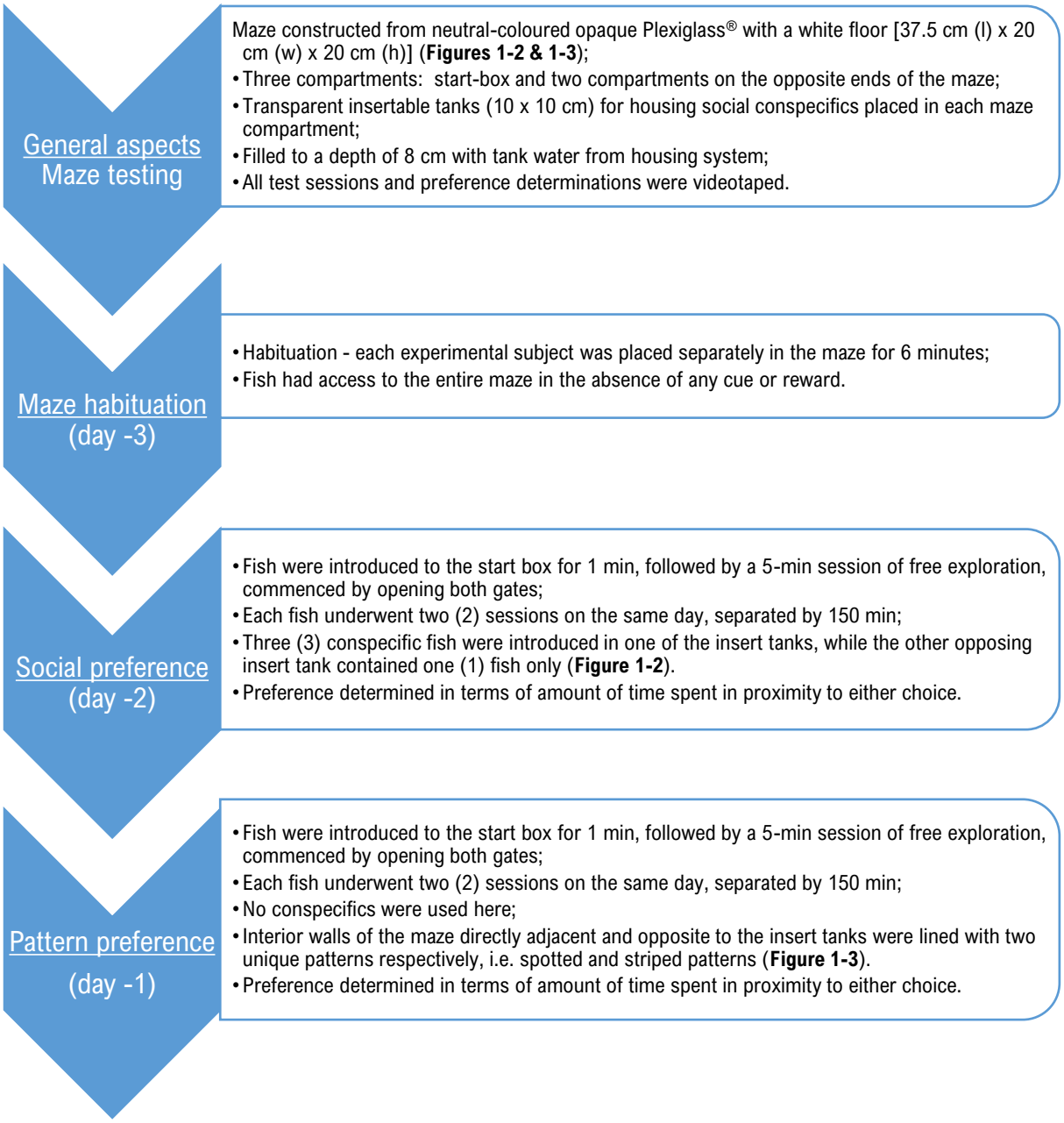
1.5.2 Animals

- Adult, wild-type short-fin zebrafish *(Danio rerio)* of both sexes (3 – 5 months old; ± 40 mm in length) were used.
- Forty (40) experimental and an additional thirty-two (32) social conspecific fish were employed to meet the aims and objectives.
- All subjects were housed at the NABF[1] of the NWU[2], Potchefstroom, South Africa according to standard laboratory conditions as prescribed for zebrafish (Reed and Jennings 2011).
- ZF were deprived of social interaction for four days prior to pattern and social testing to ensure optimal conspecific-directed interaction (**Figures 1-1 – 1-3; paragraph 1.5.3**). This was achieved by single-housing subjects in new home tanks which were separated by opaque dividers.
- Following determination of social and pattern preference, fish were again housed in social, same pattern and social preference groups until day 25 (**Figure 1-1**), from which point onwards they were housed individually throughout the remainder of the study (van Staden *et al.* 2020).

[1] National Aquatic Bioassay Facility
[2] North-West University

- All sessions were video-taped and the amount of time spent in the areas of the maze directly adjacent to the conspecific-tanks in each of the arms (i.e. the proximity areas) was quantified by means of Ethovision® XT14 software (Noldus® Information Technology, Wageningen, The Netherlands). From this data, the personal preference in terms of pattern as well as social interaction was noted for each fish (**paragraph 1.5.3**). Thus, if a particular subject demonstrated preference for the striped pattern and a group of social conspecifics, this combination of cue and reward would be used for that fish for the remainder of the study, as the various phases required.

1.5.3 Baseline determination of social and pattern preference



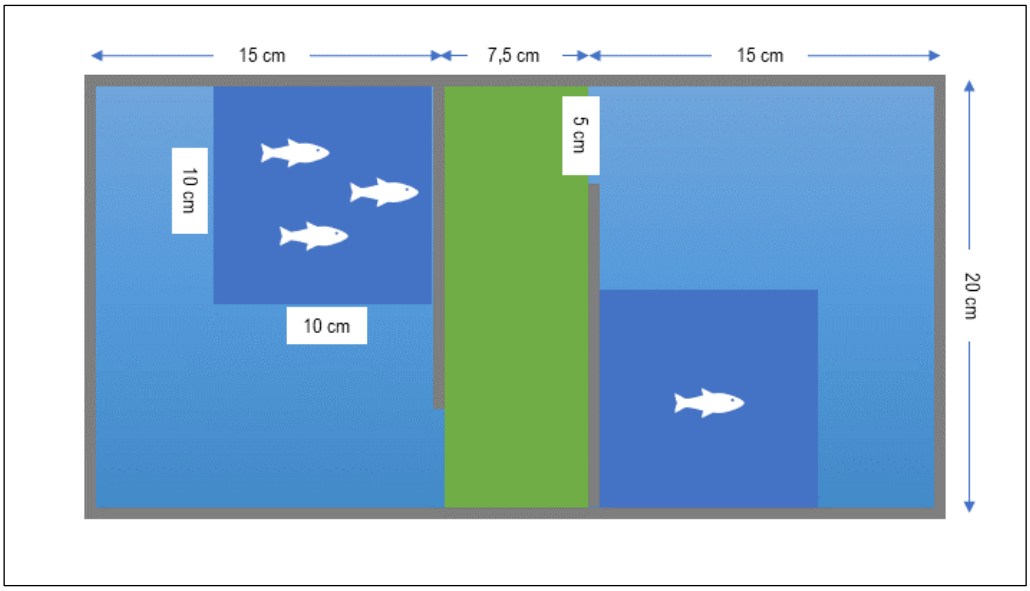


Figure 1-2 *– Top-down view of maze and configuration used for social preference testing*
Green area: start box; Grey lines: opaque walls

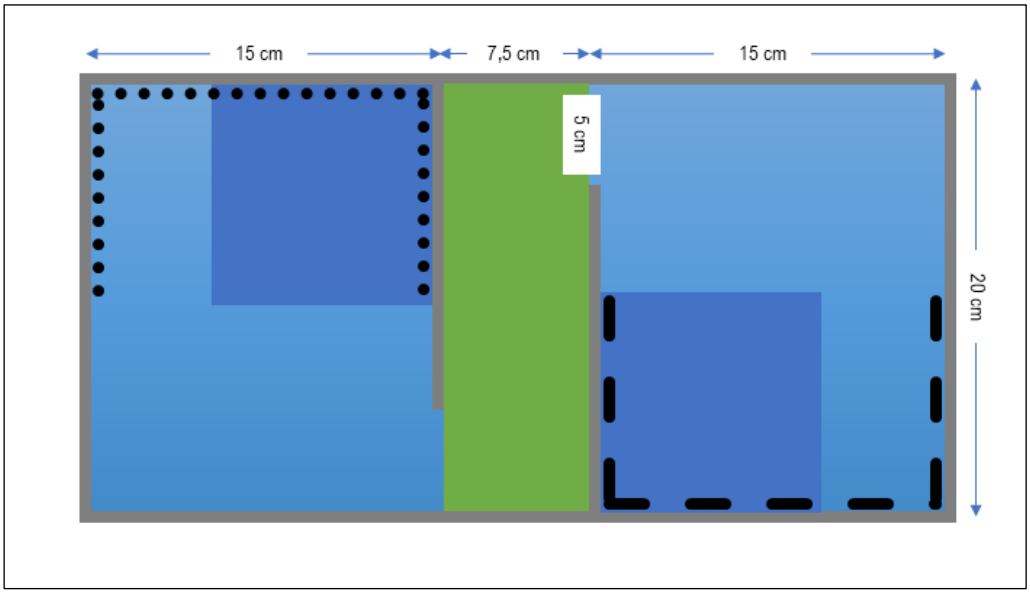


Figure 1-3 *– Top-down view of maze and configuration used for pattern preference testing*
Green area: start box; Grey lines: opaque walls; Dotted and striped lines: pattern inserts

* * *

Important – As explained earlier, following the determination of social and pattern preference, which concluded on day -1, fish were again group housed, albeit clustered in-home tanks according to social and pattern preference.

1.5.4 Drug exposure

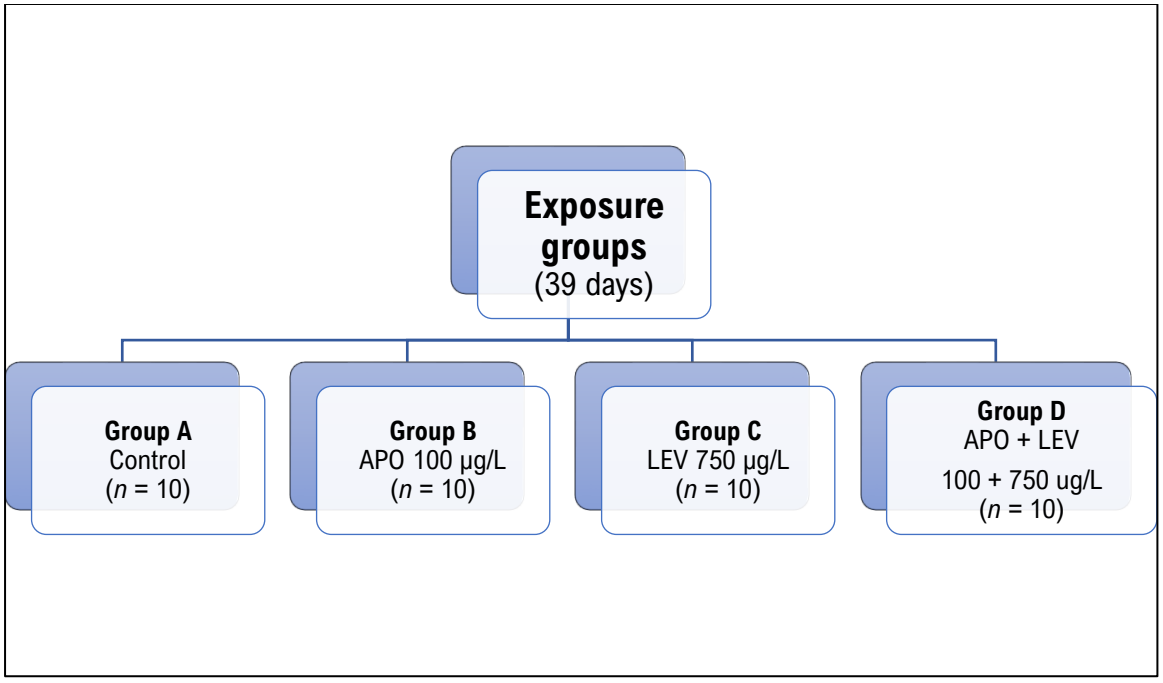


Figure 1-4 *– Layout of experimental drug exposure groups*
APO: apomorphine; LEV: levetiracetam; APO/LEV: combination apomorphine and levetiracetam

For all fish included in experimental groups, exposure began on day 1 and continued until day 39 (**Figure 1-1**). All fish were exposed to drug solutions for one hour per day at approximately 14:00. Both drugs as well as the combination thereof were administered by means of aqueous immersion as previously reported (van Staden *et al.* 2020; Copmans, Siekierska, and de Witte 2017).

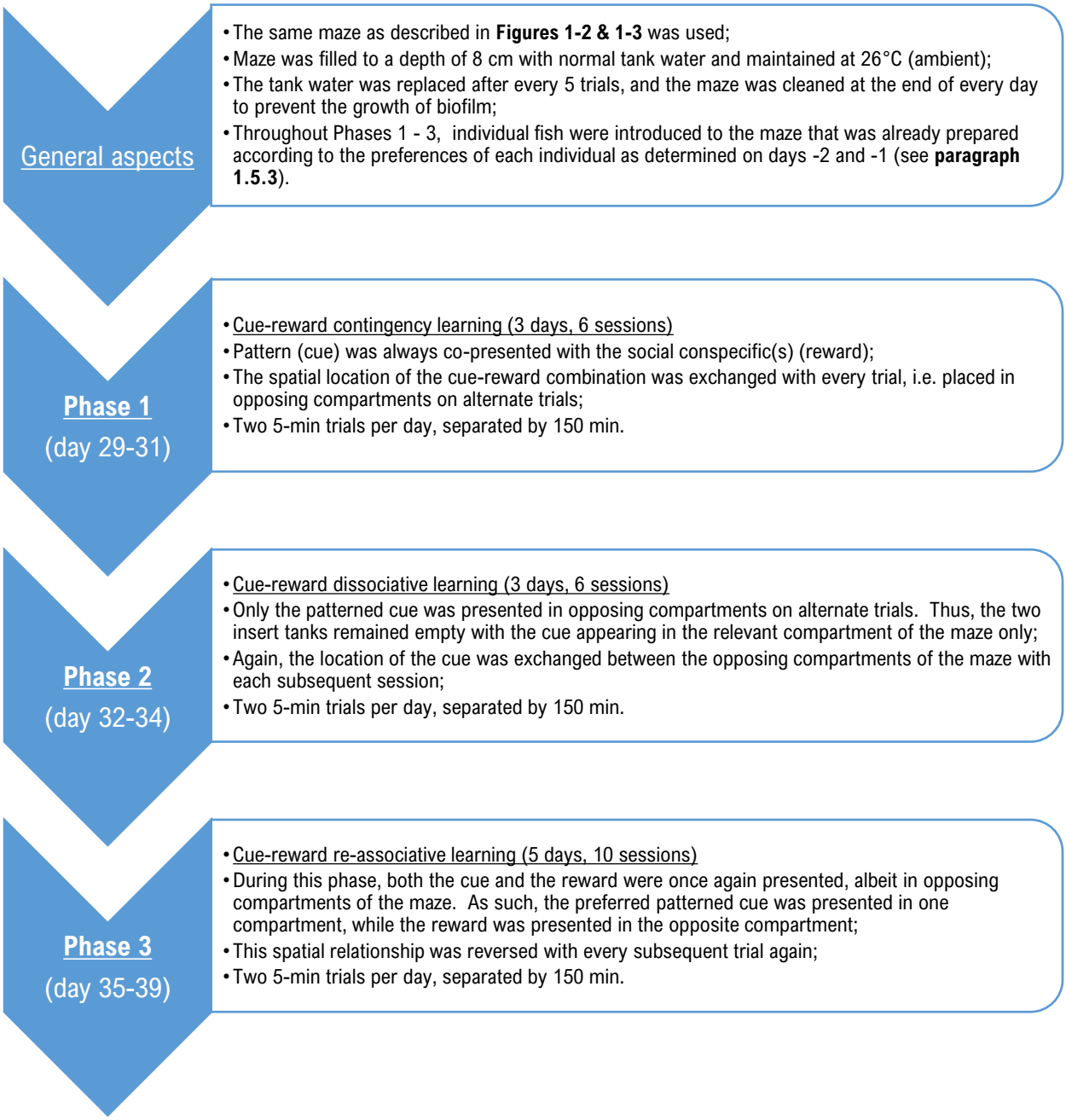
Experimental fish were group-exposed between days 1 – 24. Beginning on day 25, fish were housed and drug-exposed in isolation in preparation for the experimental design. APO[1] and LEV[2] (and the combination) were constituted in normal tank water at concentrations of 100 µg/L (van Staden *et al.* 2020) and 750 µg/L (Sanchez *et al.* (2012); adapted for aqueous immersion according to van Staden *et al.* (2020)), respectively. During group-exposure, fish were exposed to drugs in tanks identical to the home tanks, containing 1.2 L of drug solution constituted in tank water. At the time of individual exposure, fish were individually exposed to drugs in glass beakers, containing 200 mL of drug solution and separated by polystyrene dividers (van Staden *et al.* 2020).

1.5.5 Cue-reward experimentation

From day 29 onwards, all experimental zebrafish underwent experimental testing over three phases defined by unique combinations of cue and reward presentation, namely: cue-reward contingency

[1] apomorphine
[2] levetiracetam

learning (Phase 1), cue-reward dissociative learning (Phase 2) and cue-reward re-associative learning (Phase 3) (**Figure 1-5**).



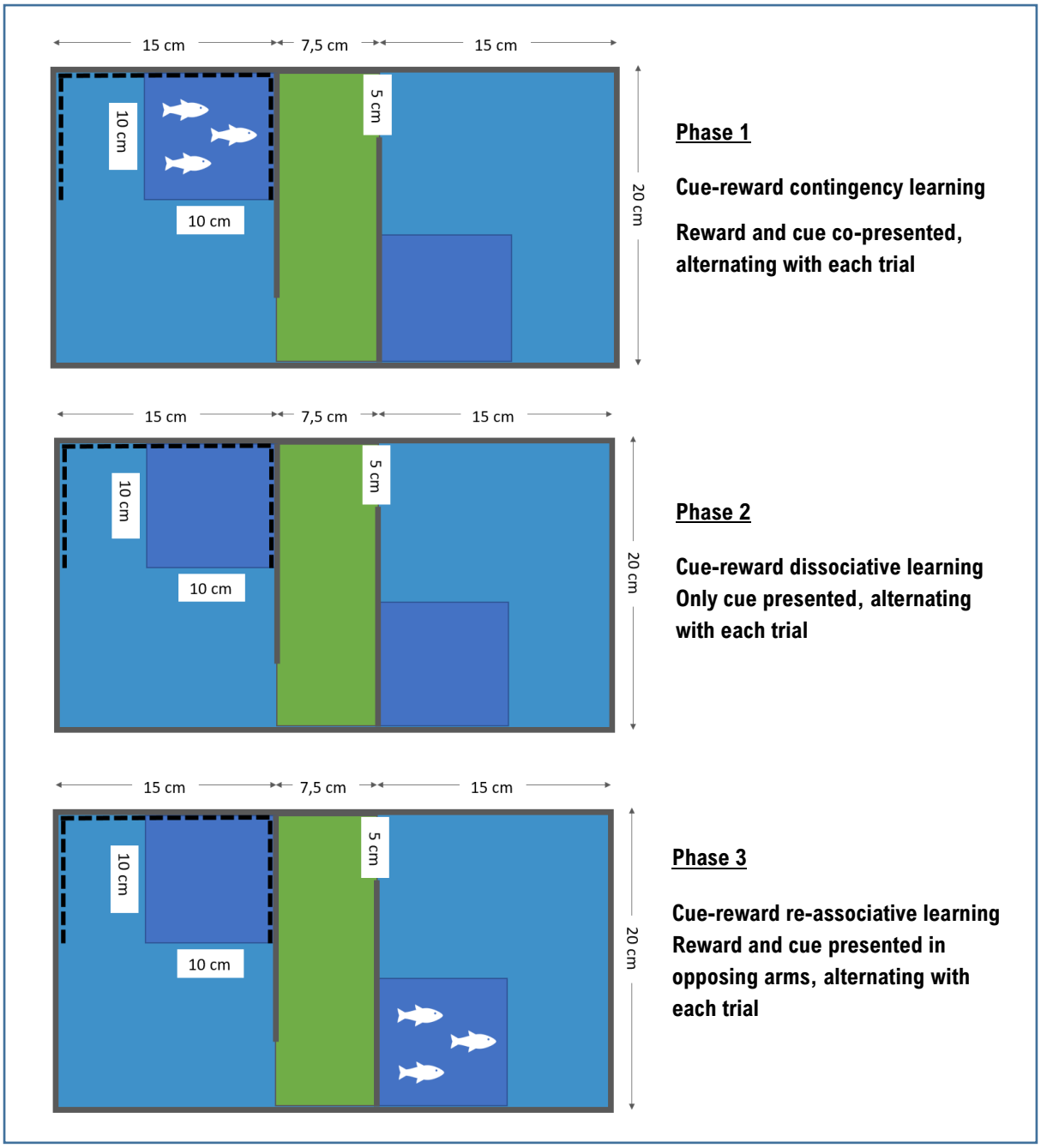


Figure 1-5 *– Schematic representation of maze setup during Phases 1 – 3 of the investigation*

1.6 Research hypothesis

We hypothesize that, in line with previous findings from our laboratory (van Staden *et al.* 2020), both control- and APO[1]-exposed zebrafish will acquire knowledge of a cue-reward contingency. We further posit that chronic exposure to APO, a non-selective dopamine $D_{1/2}$ receptor agonist, but not control or LEV[2] alone, will induce persistent cue-directed, and not reward-directed responses upon cue-reward dissociation in Phase 2 as well as during re-associative learning in Phase 3. In terms of the behavioural effects of LEV, our hypothesis is twofold. First, since LEV acts by modulating synaptic vesicle exocytosis, thought to generally decrease synaptic outflow (Surges, Volynski, and Walker 2008) it is possible that LEV may prevent adequate cue-reward contingency learning; indeed, it is possible that reward-associated dopamine release may be blunted. However, since LEV acts on most neurotransmitter systems, it is also likely that it may elicit a reward-directed behavioural response if serotonergic, but not dopaminergic processes are influenced more (Schultz 2002; Daw, Kakade, and Dayan 2002). Last, we expect LEV to reverse the inflexible, cue-directed responses elicited by APO-exposed fish, since we propose that when administered in combination with APO, LEV will result in balanced synaptic signalling (Helmstaedter and Witt 2008; Wu *et al.* 2009). While this will not be tested on a neuro-molecular level, we believe that the behavioural results obtained from this investigation, will be sufficient to infer a potential mechanism of action or interaction between the experimental drugs.

1.7 Ethical approval

The current investigation was approved by the AnimCare Research Ethics committee (NHREC reg. number AREC-130913-015) of the North-West University (approval number: **NWU-00525-20-A5**). All experimental procedures were conducted by the student researcher, Mr CC van der Westhuizen, under supervision of the study leader and co-study leader. All procedures were performed in accordance with the code of ethics stipulated in the relevant national legislation (South African National Standard for the Care and Use of Animals for Scientific Purposes; SANS 10386:2008). We further strived to adhere to the ARRIVE-guidelines for animal experimentation as closely as possible.

* * *

[1] apomorphine
[2] levetiracetam

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2 Literature review

2.1 Cognitive flexibility and rigidity in psychiatry

2.1.1 General background

Human and animal behaviour is ultimately directed at acquiring certain outcomes or achieving certain goals (Ehmer *et al.* 2020), or conversely performing certain behaviours to prevent or mitigate negative outcomes. Broadly speaking, behaviour as whole can be categorised under two broad classes, namely goal-directed or habitual behaviours (Ehmer *et al.* 2020). Goal-directed behaviour is typically employed when behavioural responses are directed towards reaching very specific and mostly novel tasks; these require a large degree of planning before they are carried out (Balleine and O'doherty 2010). In contrast, habitual behaviours—here referred to as 'functional' habits, e.g. returning milk to the fridge after it has been used to make coffee, or checking that doors are locked before bedtime—are those performed with a certain degree of automation after one has become rather familiar with the outcome (Dickinson 1985; Buschman and Miller 2014). To further illustrate this distinction, it is said that goal-directed behaviours are *reflective*, i.e. requiring consideration, whereas habitual behaviours are *reflexive*, that is to say they performed in a more automated fashion in response to specific contexts or circumstances (Gillan and Robbins 2014).

In a dynamically changing environment, goal-directed behavioural selection is thought to be regulated by two somewhat opposing cognitive constructs, namely persistence and flexibility (Dreisbach and Goschke 2004; Goschke 2003; Hommel 2015). Cognitive persistence, or rigidity, serves to maintain goal-directed processes by preventing irrelevant or distracting executive processes from interfering with successful task completion (Zmigrod *et al.* 2019). In contrast, cognitive flexibility (CF)[1] allows the subject to remain sensitive to alternative possibilities, to disengage from inefficient behavioural routines, and to adapt when external or internal states change (Zmigrod *et al.* 2019). Adequate CF is associated with success throughout an individual's lifespan, e.g. better reading abilities in childhood, greater resilience to negative life events and stress, higher levels of creativity, and higher quality of life (Engel de Abreu *et al.* 2014; Genet and Siemer 2011; Chen *et al.* 2014; Davis *et al.* 2010). While CF is regarded by some as a specific cognitive ability or skill set (Colzato, Huizinga, and Hommel 2009), others view it as a collective of various neuropsychological processes (Blaye and Bonthoux 2001; Plunkett, Munakata, and Johnson 2006; Deak 2003). Suffice to say that CF can be regarded from multiple perspectives and therefore lacks common conceptualization (Ionescu 2012; Cherry *et al.* 2021). Nevertheless, an

[1] cognitive flexibility

attempted unified account considers CF[1] to be dependent on interactions between sensorimotor mechanisms, cognition, and context in a temporal context (Ionescu 2012).

With respect to studying cognitive rigidity (CR)[2], it would be important to define what rigidity, as opposed to cognitive flexibility, means (Schultz and Searleman 2002). This might be difficult since, as is true for CF, no true universally accepted definition of CR exists. However, the majority opinion has it that a comprehensive definition should include at least two factors. The first, a prerequisite to the second, involves the formation of mental or behavioural patterns which are acquired by the repetition of a specific behaviour in a particular situation (Luchins and Luchins 1959; Luchins and Luchins 1994; Schultz and Searleman 2002). The second would be the perseveration of the acquired pattern while enduring a certain degree of pressure (internal or external) to change the expression (Schultz and Searleman 2002).

This brings the focus of this review to habitual responding, which also normally emerges after frequent repetition of a goal-directed action. Habits are important for everyday life, since tasks can be completed without much cognitive effort, which in turn, can be recruited for more complex challenges (Ehmer *et al.* 2020; Aarts and Dijksterhuis 2000; Quinn *et al.* 2010; Wood and Neal 2007; Tricomi, Balleine, and O'Doherty 2009). It is important to note though that most 'functional' habits, e.g. are also goal-directed, in that the result of habitual expression is generally also a specific and sought-after outcome. However, it stands to reason that once becoming overly habitual, i.e. when such behaviour becomes less functional and more rigid in its expression, e.g. picking up a cell phone without reason, habit begins to closely resemble CR.

2.1.2 Perturbations in the balance between CF and CR is common to many neuropsychiatric illnesses

The most meaningful way to portray the impact and severity of CR, which is not a disorder itself, would be to delve into the impact and epidemiology of the conditions known to associate with a notable imbalance between CF and CR, e.g. obsessive-compulsive disorder (OCD)[3], major depressive disorder (MDD)[4] and autism spectrum disorders (ASD)[5].

Considering compulsive behaviours, which are expressed in a rigid manner within particular contexts in response to implicitly-generated obsession about specific themes, e.g. excessive hand-washing triggered by irrational contamination fears (Chamberlain *et al.* 2009), the conceptual overlap between such symptoms and CR is apparent. It is thus not surprising that research has identified several

[1] cognitive flexibility
[2] cognitive rigidity
[3] obsessive-compulsive disorder
[4] major depressive disorder
[5] autism spectrum disorders

components of CR[1] that are present in OCD[2] sufferers (Fineberg *et al.* 2018). While cognitive deficits are variably associated with the different obsessive-compulsive (OC)[3] symptom phenotypes, i.e. contamination/washing, symmetry/ordering, safety/checking and covert mental thoughts and routines, it is generally posited that OCD is a condition of inadequate behavioural control which relates to a shift from reward- (or outcome) driven behaviour, to impulsive (rapid, even sometimes reckless), and then compulsive behaviour over time (Everitt and Robbins 2005; Robbins *et al.* 2012). Importantly, since OCD sufferers have insight into the futility of their symptoms, they are unable to disengage from expressing such behaviour, which in itself can be regarded as an artefact of underlying CR.

MDD[4] has been associated with deficits in an individual's response to changing contextual factors and a self-perceived inability to control these factors (Liknaitzky, Smillie, and Allen 2017). According to numerous findings, MDD appears to be associated with an overall negative outlook about oneself, the world, and the future, which is believed to be caused by cognitive biases that are reinforced by rigidly held thought processes (McDermut, Haaga, and Bilek 1997; Deveney and Deldin 2006; Freeman 2004; Pössel 2009). Another hypothesis pertaining to the development of rigidity in MDD patients states that sufferers tend to focus on the completion of a highly specific goal, e.g. cleaning the house, which they often struggle to do, due to a lack of motivation (Smith 2013). This is problematic, since such patients often find it difficult to change their actions to prioritize a more attainable goal (Snyder 2013); indeed, the persistent focus on negative (or unachievable outcomes) subsequently contributes to a negative self-image and state of mind of depressive patients (Ebmeier, Donaghey, and Steele 2006; Philippi *et al.* 2018; Crowe *et al.* 2019). As stated earlier, MDD has been associated with deficits in a person's ability to respond to change, especially on the level of executive functioning, a set of systems and processes that are responsible for the manner in which we approach specific situations that require us to overcome strong habitual responses (Marazziti *et al.* 2010; Stordal *et al.* 2004). As such, perturbation in the balance between CF[5] and CR in MDD, is undeniable.

In the case of ASD[6], which is characterised by a variety of different behavioural symptoms including difficulties in social interactions and having limited interests, and expressing a preference for behavioural monotony (American Psychiatric Association 1980; Poljac *et al.* 2017), it is easy to appreciate efforts to better understand the neurocognitive mechanisms underlying these rigid behavioural expressions (Poljac and Bekkering 2012). One of the leading explanations for these behaviours include the presence

[1] cognitive rigidity
[2] obsessive-compulsive disorder
[3] obsessive-compulsive
[4] major depression disorder
[5] cognitive flexibility
[6] autism spectrum disorders

of CR[1], a theory that is supported by clinical literature (Watanabe *et al.* 2019; Lopez *et al.* 2005; D'Cruz *et al.* 2013; Dajani and Uddin 2015). However, it should be noted that a qualitative literature overview highlighted some contradicting results, and thus was unable to conclude whether CR is indeed central to the expression of autistic symptomology (Geurts, Corbett, and Solomon 2009). That said, more recent studies implicated deficits in a subcategory of cognitive control processing, namely intention, which biases the choice of behavioural goals in patients with ASD[2] (Poljac *et al.* 2017; Poljac and Bekkering 2012; Yeung 2010). It is therefore clear that a bias in favour of CR over CF[3] exists in the presentation of ASD.

2.1.3 Measuring CF and CR

Although more than 3000 research papers have been published since the 1950's with CF and CR as a research theme, a common understanding of what CF and CR really means and how it should be measured, is still lacking (Schultz and Searleman 2002; Cherry *et al.* 2021). This uncertainty transcends into research methodology, for which numerous and vastly different approaches have been applied over time (Lilienfeld and Strother 2020; Cherry *et al.* 2021). Still, most psychiatric assessment scales and personality inventories include sections measuring certain components of rigidity, if not rigidity itself (Schultz and Searleman 2002). These are either based on patient-self reports or experimental tasks.

Self-report questionnaires are widely used, due to their ease of application (Schultz and Searleman 2002). Such questionnaires are designed to interrogate the behaviour of patients as it relates to concepts of CF and CR, often using a numerically rated scale. Examples of self-report-questionnaires include the Cognitive Flexibility Scale (Martin and Rubin 1995), the Cognitive Flexibility Inventory. (Dennis and Vander Wal 2010), and the Cognitive Control and Flexibility Questionnaire (Gabrys *et al.* 2018). Even though these represent frequently used methods to quantify behavioural flexibility, their accuracy remains somewhat questionable (Cherry *et al.* 2021), since patient bias may have an influence on the ultimate scores generated. Thus, the second type of measurement, i.e. experimental task paradigms, is applied. These tests include the Test of Behavioural Rigidity, which is a dual paper- and experiment-based test (Schaie 1975), the Einstellung Water-Jar Task, the Wisconsin Card Sorting Task, and the Stroop Task. Broadly speaking, these tests involve specific tasks that subjects are asked to carry out, which can then be scored by a clinical observer.

[1] cognitive rigidity
[2] autism spectrum disorders
[3] cognitive flexibility

The accurate measurement of CF[1] and CR[2] is of importance not only to detect potential psychobiological processes that may contribute to the observed symptomology, but also to track the effectiveness of its treatment, particularly when the construct in question is the focus of treatment (van Holstein *et al.* 2011). This concept, i.e. targeting dysfunctional underlying psychobiological constructs like CR—which may occur as a component in several psychiatric disorders—is central to the current work, as the potential nootropic drug, levetiracetam (LEV)[3], will be tested in a putative animal model system that presents with behavioural inflexibility, or alternatively behaviour that resembles some components of CR. Although this form of generalised intervention is not yet common practice, the principles thereof are applied to some extent for among others, the treatment of OCD[4]. Here, abnormal psychological processes that lead to habit formation, instead of the biological inhibition of excessive motor routines, are being considered as potential avenues for symptom resolution, or as a manner to better understand the disorder (Gillan *et al.* 2016). As such, we propose that targeting a common neuropsychological construct such as an imbalance between CF and CR, which emerges as a common component in several psychological disorders, could present a novel perspective on understanding said disorders and furthering its treatment (Chamberlain and Menzies 2009; Gottesman and Gould 2003), as recently suggested by other authors (Servaas *et al.* 2021). Therefore, the overarching goal of this dissertation will focus on perturbations in the balance between CF and CR in a putative, pharmacologically-induced zebrafish model of behavioural rigidity so as to provide a platform to further understand the presentation and treatment of CR as a cognitive deficit common to several disorders.

2.2 The neurobiology of cognitive rigidity

2.2.1 Neuroanatomical constructs

Goal-directed and habitual task execution, both intricate components in studies of CF and CR, are governed by the cortical-striatal-thalamic-cortical (CSTC)[5] circuitry, a collective of brain regions that plan, execute and terminate behavioural expression (Buschman and Miller 2014; Stocco, Lebiere, and Anderson 2010; Graybiel 2008). Broadly viewed, the CSTC circuit recruits the actions of three different neuroanatomical regions with the ventral tegmental area (VTA)[6] which triangularly receive and send signals to both the cortex and the striatum. These three neuroanatomical regions are each further subdivided into smaller functional foci (Di Filippo *et al.* 2009).

[1] cognitive flexibility
[2] Cognitive rigidity
[3] levetiracetam
[4] obsessive-compulsive disorder
[5] cortical-striatal-thalamic-cortical
[6] ventral tegmental area

The circuit originates in the orbito-frontal and anterior cingulate cortical regions (Milad and Rauch 2012) from where it innervates specific components of the striatum by means of glutamatergic projections (Di Filippo *et al.* 2009) to the nucleus accumbens, caudate nucleus and putamen (Milad and Rauch 2012). From here, two distinct pathways, mostly consisting of GABAergic interneurons, travel towards the thalamus. These can broadly be described as a behaviourally activating direct, and a behaviourally inactivating indirect pathway (Nambu 2008; Stocco, Lebiere, and Anderson 2010) (**Figure 2-1**). Importantly, both pathways must be simultaneously activated before a cortically planned executive signal will be propagated. This functional conflict between behavioural execution and inhibition is ultimately solved by dopamine, as will be explained later. Suffice to say that an imbalanced, over-activation of the direct pathway is associated with conditions that are more often than not characterised by cognitive rigidity, including OCD[1] and ASD[2] (Maia, Cooney, and Peterson 2008; Abramowitz and Jacoby 2015). However, it remains to be seen whether such an imbalance between the two pathways promulgates persistent symptomology due to its effects on cognitive processing, rather than directly affecting the motor component of the observed behavioural routines *per se*.

Several neurotransmitters, including serotonin, dopamine, gamma-aminobutyric acid (GABA)[3] and glutamate, are involved in the regulation of CSTC[4] signalling. For the purposes of this review, we will focus on the two primary molecules that are targeted by pharmacotherapeutic interventions employed in conditions related to an imbalance between CF[5] and CR[6], i.e. dopamine and serotonin. We will then close by briefly referring to some of the other neurotransmitters which may play a role in the outcomes of treatment with potentially novel cognitive enhancers, e.g. LEV[7].

[1] obsessive-compulsive disorder
[2] autism spectrum disorders
[3] gamma-aminobutyric acid
[4] cortical-striatal-thalamic-cortical
[5] cognitive flexibility
[6] cognitive rigidity
[7] levetiracetam

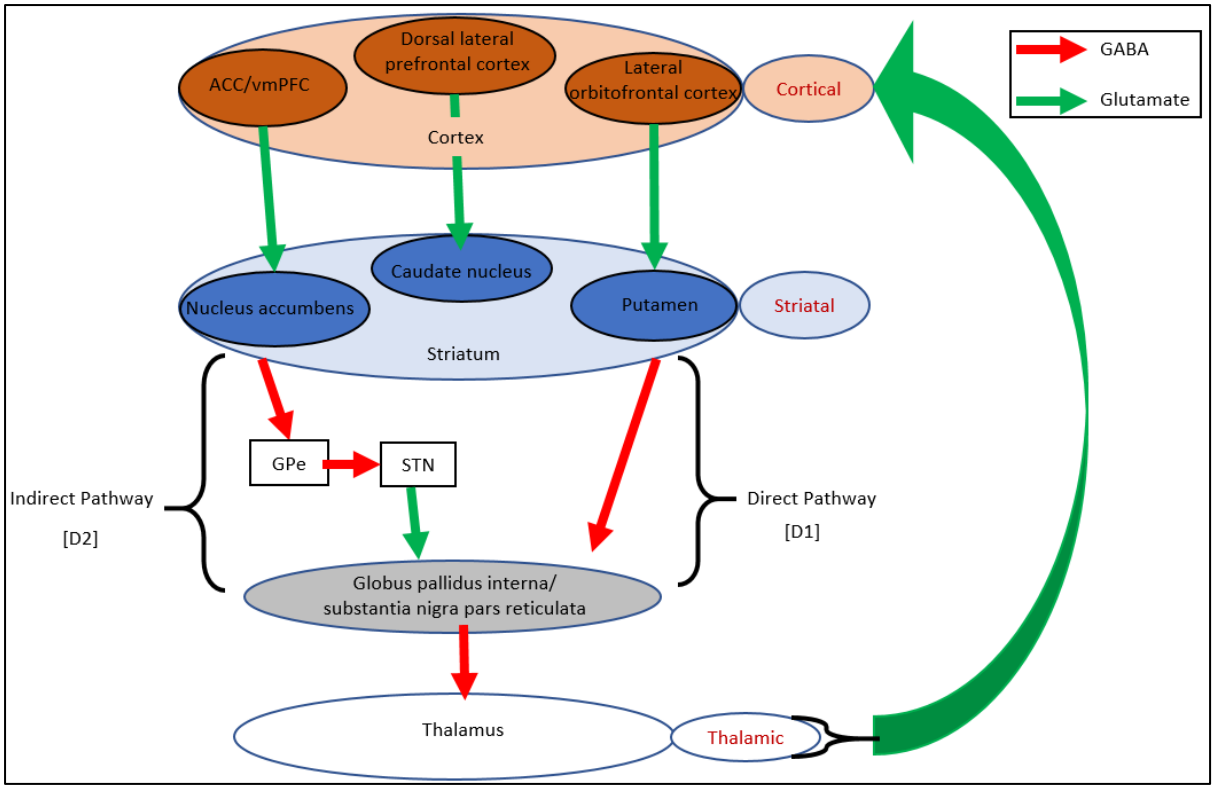

Figure 2-1 *- Schematic simplified representation of the CSTC circuitry.*
ACC - anterior cingulate cortex; vmPFC - ventro-medial prefrontal cortex; GPe - globus pallidus externa; STN - subthalamic nucleus. Adapted from (Di Filippo et al. 2009; Milad and Rauch 2012)

2.2.2 Neurotransmission

2.2.2.1 Dopamine

As alluded to above, dopamine is an important neuromodulator in the CSTC[1] circuit as evinced by its role in goal-directed action motivation and feedback-learning (Klanker, Feenstra, and Denys 2013; Buschman and Miller 2014). In **paragraph 2.2.1** the concept of executive conflict, whereby the direct pathway activates, and the indirect pathway inactivates behavioural engagement, has been touched on. In effect, dopamine acts as the gating go-no-go mechanism which resolves this conflict at the time of behavioural execution. Here, we will briefly afford attention to this mechanism, since it forms a primary foundation for the manner in which we will attempt to induce a model of CR[2] in zebrafish.

The neural communication language within the dopaminergic system, which is said to drive feedback learning, is based on two distinct patterns of firing, tonic and phasic firing (Schultz, Stauffer, and Lak 2017). Under resting state conditions, the dopaminergic system constantly upholds a tonic firing pattern. However, this tonic pattern of firing can be overlaid by a brief phasic firing pattern, depending on the specific reward experience history of the subject (Pignatelli and Bonci 2015). It is believed that

[1] cortical-striatal-thalamic-cortical
[2] cognitive rigidity

the VTA[1], which innervates the nucleus accumbens, is one of the foremost regulators of tonic and phasic dopaminergic firing pattens, and thus also of the CSTC[2] circuit activity (Schultz and Dickinson 2000; Cohen *et al.* 2012; Schultz 1998). A more detailed discussion of these concepts are provided in **paragraph 2.6.1**.

Following from the above, activity within the direct and indirect pathways can differentially be modulated by the activation of striatal and other regional dopamine D_1 and D_2 receptors (Frank and Claus 2006; Winter, Dieckmann, and Schwabe 2009). Whereas the direct pathway expresses D_1 receptors, the indirect pathway expresses D_2 receptors (Groenewegen 2003; Nambu 2008; Stocco, Lebiere, and Anderson 2010). Importantly, activation of D_1 receptors *activates* the direct pathway. However, when D_2 receptors are activated, the behaviourally inactivating indirect pathway, is in itself *inactivated*, hence resulting in a net activating striatal-thalamic signal to be relayed upon a global release of dopamine in the striatal regions (Groenewegen 2003; Nambu 2008; Stocco, Lebiere, and Anderson 2010; Di Filippo *et al.* 2009). Nevertheless, since the affinity of dopamine for D_1 and D_2 receptors differs (Richfield, Penney, and Young 1989), fluctuations in dopamine levels during the different stages of feedback learning may result in unique direct and indirect pathway activation patterns (Klanker, Feenstra, and Denys 2013). For instance, when an unexpected reward, or a reward-predicting stimulus, is presented, the transient and phasic increase in dopamine, which is known to be reward-sensitive (Beierholm *et al.* 2013), results in the activation of low-affinity D_1 receptors, thus stimulating the direct pathway and facilitating behavioural execution. On the other hand, *omission* of an expected reward results in a temporary suppression of dopamine release and hence diminished inhibition of the indirect pathway, resulting in the inhibition of behavioural responding. It has previously been suggested that conditions related to excessive cognitive and motor activation, e.g. OCD[3], may be typified by a hyperdopaminergic state (Westenberg, Fineberg, and Denys 2007; Koo *et al.* 2010; Nikolaus *et al.* 2010). Using OCD, of which cognitive inflexibility is suggested to be a specific endophenotype (Klanker, Feenstra, and Denys 2013), as an example, three questions arise. First, will bolstered dopaminergic signalling prior to and during contextual learning, induce inflexible, goal-dissociated (and likely habitual) behaviour? In extension, and considering the aforementioned time-sensitive nature of dopaminergic activity during processes of reward-feedback learning, can abnormally elevated dopaminergic signalling actually interfere with the normal learning-related functions of phasic dopamine increases? Last, if so, can such changes be reversed by another drug which potentially regulates dopaminergic activity? These questions will be addressed in the present work, where we will aim to induce a repetitive cue- or reward-directed behavioural phenotype by chronically exposing zebrafish to the $D_{1/2}$ agonist, apomorphine

[1] ventral tegmental area
[2] cortical-striatal-thalamic-cortical
[3] obsessive-compulsive disorder

(APO)[1] and determine whether any APO-induced behavioural changes can be attenuated by the neuromodulator, LEV[2].

2.2.2.2 Serotonin

Serotonin, often described as the behavioural opponent of dopamine (Kranz, Kasper, and Lanzenberger 2010; Cools, Nakamura, and Daw 2011; Tops *et al.* 2009), is another important neuromodulator in the CSTC[3] circuit (Simpson *et al.* 2011; Sinopoli *et al.* 2017; Van Der Wee *et al.* 2004). Specifically, serotonin is prominently associated with the processing of aversive stimuli and behavioural inhibition, which can foreseeably play a crucial role in behavioural flexibility, most notably when learning must arise from negative circumstances that require the cessation of certain behaviour (Cools, Nakamura, and Daw 2011; Kranz, Kasper, and Lanzenberger 2010). Since disorders lacking behavioural flexibility involve CSTC circuit dysfunction, the clinical success of selective serotonin-reuptake inhibitors (SSRIs)[4] in these disorders can be appreciated (Fineberg *et al.* 2011; Remijnse *et al.* 2013). In these conditions, a hypo-serotonergic state is believed to play an underlying role in the manifestation of symptoms (Koo *et al.* 2010; Nikolaus *et al.* 2010; Westenberg, Fineberg, and Denys 2007). As alluded to before, in many of these conditions marked reductions in CF[5] are also noted (Linnoila and Virkkunen 1992; Fineberg *et al.* 2011; Hollander and Rosen 2000), pointing to a complex interplay between dopamine and serotonin underlying normal behavioural regulation.

As briefly noted above, serotonin is believed to act as a functional opponent of dopamine, with biological computation by dopaminergic and serotonergic neurons encoding outcome value, thereby gating our approach and avoidance behaviours in response to rewarding or negative circumstances (Boureau and Dayan 2011). That said, the opponency theory seems to be imperfect in some aspects, since the effects of serotonin on dopaminergic neurons is location- and receptor-specific (Alex and Pehek 2007; Gervais and Rouillard 2000). Moreover, aversive stimuli also evoke dopaminergic activity, whereas favourable stimuli also evoke serotonergic activity (Faulkner and Deakin 2014; Lloyd and Dayan 2016); this complicates our understanding of the dopamine-serotonin relationship.

Most research into the role of serotonin in CF and CR[6] originated from animal models of persistent behaviours. The serotonergic system is broadly distributed throughout the brain and exerts its actions via 14 known receptor subtypes (Sharp and Barnes 2020). With respect to the present study topic, the most notable of these are the serotonin 5-$HT_{1A/2A/1B/2C}$ receptor subtypes (Sinopoli *et al.* 2017; Brakoulias

[1] apomorphine
[2] levetiracetam
[3] cortical-striatal-thalamic-cortical
[4] selective serotonin reuptake inhibitors
[5] cognitive flexibility
[6] cognitive rigidity

and Stockings 2019; Murphy *et al.* 2008). However, considering the global and diffuse distribution of the serotonergic system, it is nearly impossible to make definitive deductions about their exact role in specific behavioural anomalies from behavioural experiments. In fact, considering the complex interplay between dopamine and serotonin, as well as that the effects of dopamine on processes of learning, habit and goal-directed approach behaviour are highly context-specific, it is likely that temporal and contextual factors play an important role in the nature of the behavioural outcomes transpiring at any given moment.

It is evident from the above that neither dopamine, nor serotonin alone modulates CF[1]. Hyperactive (or unopposed) dopaminergic neurotransmission is believed to be permitted by a relative reduction in serotonergic signalling (Koo *et al.* 2010; Westenberg, Fineberg, and Denys 2007). Further, changes in the tonic and phasic neurotransmission of both neurotransmitters also play an important role in cognitive control. That said, the fact that the therapeutic value offered by dopaminergic and serotonergic drugs for disorders characterised by deficits in cognitive control potentially function by modulating neurotransmission in the relevant circuits, the current investigation will examine whether CF in a zebrafish model can be influenced by chronic drug intervention with LEV[2], which as mentioned earlier, is believed to stabilise excessive neuronal firing.

2.2.2.3 Glutamate and GABA

Apart from dopamine and serotonin, other neurotransmitters such as glutamate and GABA[3] have been identified as role-players in the modulation of the balance between CF and CR[4] (Logue and Gould 2014; Spurny *et al.* 2021; Moriarty *et al.* 2016). However, there is a paucity of treatment data that indicate successful therapeutic outcomes related to the modulation of these neurotransmitters in conditions related to perturbations in the balance between CF and CR (Peyrovian *et al.* 2020; Ren *et al.* 2016; Rodriguez *et al.* 2013). Thus, potentially novel therapeutic approaches may target the actions of among others, glutamate and GABA, and in so doing, engender balance between excitation and inhibition in dysfunctional states of this intricate neural network (Zhang *et al.* 2021).

Glutamate, the primary excitatory neurotransmitter of the mammalian brain, is important for the efferent activity of neuronal networks, but can have potential excitotoxic effects when overactive (Sattler and Tymianski 2001; Li, Yang, and Lin 2019). Excitotoxicity refers to a neurodegenerative process triggered by unusually high and sustained synaptic glutamate concentrations, which causes neural damage and cell death (Lau and Tymianski 2010; Maragakis and Rothstein 2004; Anderson and Swanson 2000; Danbolt 2001). Glutamate acts on two main receptor classes, i.e. metabotropic G-protein-coupled

[1] cognitive flexibility
[2] levetiracetam
[3] gamma-aminobutyric acid
[4] cognitive rigidity

glutamate receptors (mGluRs), that function by means of the generation of second messengers, and ionotropic receptors (iGluRs) named after the ligands which can selectively activate them, *viz.* the *N*-methyl-D-aspartate (NMDA)[1], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)[2], and kainate (KA)[3] receptors (Willard and Koochekpour 2013) which control the conductance of ions across neuronal membranes. Other important role players in the glutamatergic system include the excitatory amino acid transporters (EAATs)[4], which form part of the solute carrier family 1 (SLC1)[5] protein class, which closely regulate synaptic glutamate reuptake extracellularly, an essential mechanism preventing excitotoxicity.

Interestingly, functional imaging studies in patients with OCD[6] and in which CR[7] was identified as an endophenotype, showed glutamate-related hyperactivation of the CSTC[8] circuit, which would be in line with a hypothesis of glutamate overactivity in OCD and other related conditions (Grados *et al.* 2013; Rosenberg *et al.* 2000; MacMaster, O'Neill, and Rosenberg 2008). Therefore, while not yet fully translating into robust and effective treatment alternatives, such a neurobiological footprint has been confirmed by studies showing a therapeutic benefit of regulating glutamatergic signalling in treatment-refractory cases of conditions characterised by CR (Pasquini, Berardelli, and Biondi 2010; Stryjer *et al.* 2014). That said, while this approach relies on an overactivity of glutamate-related CSTC processes (Luján, Shigemoto, and López-Bendito 2005), there is also rich expression of GABAergic activity in the interneurons of the CSTC pathways (Nambu 2008; Stocco, Lebiere, and Anderson 2010; Di Filippo *et al.* 2009).

GABA[9], the principal inhibitory neurotransmitter, is released by cortico-striatal interneurons which control the activity of the CSTC circuit as a whole. GABAergic dysfunction can therefore contribute to disrupted functioning of the circuit (Smith-Hicks 2013; Whittington and Traub 2003). However, current views on GABAergic contributions to neuropsychiatric illness, especially in conditions related to CR, still remain inconclusive (Grados *et al.* 2015). GABA exerts its actions via two receptor classes, i.e. $GABA_A$ and $GABA_B$ receptors (Wu and Sun 2015). The $GABA_A$ receptor is complex consisting of four different protein subunits. Activation of this receptor by a suitable ligand opens an integral ion channel, changing the conductance of chloride ions across the neuronal membrane, leading to hyperpolarization of the cell and decreased electrical excitability. $GABA_B$ receptors also inhibit neurotransmission but do so by means

[1] *N*-methyl-D-aspartate
[2] α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
[3] kainate
[4] excitatory amino acid transporters
[5] solute carrier family 1
[6] obsessive-compulsive disorder
[7] cognitive rigidity
[8] cortical-striatal-thalamic-cortical
[9] gamma-aminobutyric acid

of second messenger systems. Because GABA[1] plays a critical role in controlling the excitability of neurons, cortico-striatal GABAergic neurons is implicated in the pathogenesis of several of the pertinent conditions discussed here, i.e. OCD[2] (Richter *et al.* 2012), ASD[3] (Fatemi, Folsom*, et al.* 2009; Fatemi, Reutiman*, et al.* 2009) and MDD[4] (Prevot and Sibille 2021).

2.3 The treatment of conditions characterised by impaired CR

As stated earlier, several psychiatric disorders are to some extent paired with an altered balance between CF[5] and CR[6] (Dennis and Vander Wal 2010; Geurts, Corbett, and Solomon 2009; Palm and Follette 2011; Karvat and Kimchi 2014), while many of these share some commonality with respect to their underlying psychobiological footprint (Poyurovsky, Braverman, and Weizman 2020; Meier *et al.* 2020; Subramaniam *et al.* 2020; Bedford, Hunsche, and Kerns 2020; van Oudheusden *et al.* 2020). It is thus not surprising that a relatively broad degree of conformity is shown with respect to the pharmacological treatment of many of these disorders (Williams *et al.* 2013; Kolevzon, Mathewson, and Hollander 2006; Fineberg *et al.* 2015; Bedford, Hunsche, and Kerns 2020). Since the focus of this review is not to regard each of these conditions on its own, only a broad overview of current and shared treatment approaches for some of these conditions, i.e. OCD, MDD and ASD, and how these might relate to potentially novel treatment alternatives, will be provided.

One of the foremost drug classes used in psychiatric illness, is SRIs/SSRIs[7], not because these drugs necessarily target the constructs of CF and CR, but because they result in symptom improvement, albeit not resolution, in most patients suffering from said disorders (Kraus *et al.* 2019; Fineberg *et al.* 2011; Sinopoli *et al.* 2017). One of the advantages of serotonergic therapy is that dosing regimens can be adjusted according to the clinical objective. In this regard, relatively lower doses are typically used to treat MDD, while higher doses are employed to manage OCD and ASD (Sinopoli *et al.* 2017; Montgomery *et al.* 1993; Hollander *et al.* 2003; McDonough and Kennedy 2002). Considering the wide distribution of the serotonergic system in the mammalian brain, it is likely that a multitude of psychobiological processes are targeted by the administration of SSRIs (Szechtman *et al.* 2020; Fineberg *et al.* 2015; Weitlauf *et al.* 2017; Eissa *et al.* 2018; Kraus *et al.* 2019). For example, while treatment is aimed at restoring serotonergic control over excessive CSTC[8]-promulgated motor behaviours in OCD (Fineberg

[1] gamma-aminobutyric acid
[2] obsessive-compulsive disorder
[3] autism spectrum disorders
[4] major depression disorder
[5] cognitive flexibility
[6] cognitive rigidity
[7] selective serotonin reuptake inhibitors
[8] cortical-striatal-thalamic-cortical

et al. 2015; Katzman *et al.* 2014; Szechtman *et al.* 2020; Geller *et al.* 2003) and ASD[1] (Eissa *et al.* 2018; Soorya, Kiarashi, and Hollander 2008; Lacivita *et al.* 2017), SSRIs[2] act on the limbic system to improve mood in patients with depression (Warren, Pringle, and Harmer 2015; Hensler 2006). Yet, for all these conditions, treatment resistance invariably remains a clinical challenge (Williams *et al.* 2013; Kolevzon, Mathewson, and Hollander 2006; Fineberg *et al.* 2015; Bedford, Hunsche, and Kerns 2020), in which case augmentation strategies are followed.

While different augmentation approaches can be followed, modulation of dopaminergic signalling is one of the foremost. To this end, low-dose anti-dopaminergic drugs, e.g. risperidone, olanzapine and quetiapine, are usually added to current SRI/SSRI regimens in OCD[3] and ASD, but less so for patients with MDD[4], except if there is a need for neuroleptic intervention (Erzegovesi *et al.* 2005; Misri and Milis 2004; Fineberg *et al.* 2015; Dold *et al.* 2015; Kumar *et al.* 2012; Carvalho *et al.* 2007). To the contrary, patients with MDD normally benefit from pro-dopaminergic intervention, e.g. the dopamine and norepinephrine reuptake inhibitor, bupropion and the dopaminergic agonists, bromocriptine and pergolide (Sherdell, Waugh, and Gotlib 2012; Forbes *et al.* 2009; Tremblay *et al.* 2002; Papakostas 2006). While the primary objective of treatment in OCD and ASD is to engender control over a highly dysregulated CSTC[5] circuit pathway (by blocking D_2 receptor activation), the focus of dopaminergic intervention in MDD is to bolster phasic dopamine release in both the CSTC pathways and the limbic system (Tremblay *et al.* 2002; Pruessner *et al.* 2004; Thase *et al.* 2007; Moreines *et al.* 2017), thereby increasing the motivational valence of rewarding outcomes (Salamone *et al.* 2003; Berridge and Robinson 1998; Yadid and Friedman 2008).

Since CF[6] may variably be affected in different phenotypes of these disorders, and since both CF and CR[7] are related to the functioning of the dopamine- and serotonin-regulated CSTC pathways, it would be important to ask how said treatments of the disorders referred to above, influence CF and CR. In fact, it is possible that in patients with no perturbations in the balance between CF and CR, such abnormalities could be elicited by altering CSTC function, while in others already suffering from deficits in CF, could show improvement. Indeed, this possibility has been highlighted in clinical investigations before. Briefly, with respect to the dopaminergic system as example, the relationship between cognitive task execution (used to measure CF) and dopamine modulation is exceedingly complex, due to paradoxical observations of improvement as well as impairment in said tasks through the administration of the same

[1] autism spectrum disorders
[2] selective serotonin reuptake inhibitors
[3] obsessive-compulsive disorder
[4] major depression disorder
[5] cortical-striatal-thalamic-cortical
[6] cognitive flexibility
[7] cognitive rigidity

drug (Cools and D'Esposito 2011; Cools *et al.* 2001; Mehta *et al.* 2004). The findings of these psychopharmacological studies indicate that the effect of the dopaminergic drug is dependent on the baseline performance levels of the individual performing the task (Kimberg, D'Esposito, and Farah 1997; Kimberg and D'Esposito 2003; Gibbs and D'Esposito 2005). For example, administration of bromocriptine to healthy volunteers improved performance in a working memory task of subjects with a lower baseline (before treatment) working memory capacity. However, it worsened the performance of subjects with a higher pre-treatment capacity (Kimberg, D'Esposito, and Farah 1997). Since this phenomenon was observed, it was repeatedly identified in numerous studies using different dopamine-modulating agents and applying various methods that assess cognitive task execution (measures of CF[1]) (Frank and O'Reilly 2006; Cools *et al.* 2007; Cools *et al.* 2009; Sawamoto *et al.* 2008). As such, it was shown that low levels of performance in light of psychopathology could be remedied by agonistic drug therapy, while the same drug could worsen already optimal performance (Cools and D'Esposito 2011). At a simpler level, bolstered dopaminergic signalling can induce rigid and repetitive behaviours that may be reminiscent of compulsive-like behaviours in animals (Cinque *et al.* 2018; Szechtman, Sulis, and Eilam 1998) and humans, presumably in part by modulating CSTC[2] circuit activity (Hassan *et al.* 2011).

Touching on glutamatergic and GABAergic signalling, results are promising, though not yet sufficient to influence currently accepted treatment approaches. Still, altered glutamate and GABA[3] concentrations and signalling intensities have been shown in OCD[4] (Pittenger, Bloch, and Williams 2011; Winter *et al.* 2018), MDD[5] (Sanacora, Treccani, and Popoli 2012; Abdallah *et al.* 2014; Prevot and Sibille 2021) and ASD[6] (Horder *et al.* 2018; Pizzarelli and Cherubini 2011). One of the most investigated glutamatergic modulators, e.g. the NMDA[7] receptor antagonist, ketamine, not only shows promise as a rapid-acting antidepressant (Ionescu and Papakostas 2016), but has also been trialled in OCD and ASD. This makes sense, given a proposed role for heightened glutamatergic activation of the CSTC pathways in these conditions (Chakrabarty *et al.* 2005; Rolls 2012; Duman, Sanacora, and Krystal 2019; Rubenstein and Merzenich 2003). Even though the exact manner in which the drug influences psychobiological processes is still unknown, the general consensus is that ketamine modulates excitatory synapses within affected brain regions, thereby resulting in stabilisation of excessive excitatory signalling, neuroprotection and improved symptomology (Zanos *et al.* 2018; Li *et al.* 2010; Autry *et al.* 2011). It is also believed that ketamine acts via both NMDA receptor-dependent and independent mechanisms

[1] cognitive flexibility
[2] cortical-striatal-thalamic-cortical
[3] gamma-aminobutyric acid
[4] obsessive-compulsive disorder
[5] major depression disorder
[6] autism spectrum disorders
[7] *N*-methyl-D-aspartate

(Kadriu *et al.* 2019; Zanos *et al.* 2018), which also increases GABAergic signalling and reduces glutamate release. Importantly, these mechanisms potentially share some overlap with the actions of LEV[1], which will be investigated in this work (see **paragraph 2.4**; Zanos *et al.* (2018); Kadriu *et al.* (2019)).

Results for GABAergic compounds are less robust. Benzodiazepines allosterically modulate $GABA_A$ receptors, increasing binding of GABA[2] to the complex (Möhler, Fritschy, and Rudolph 2002). While demonstrating acute treatment potential, these agents show little promise in the long-term treatment of conditions characterised by abnormal GABAergic functioning, e.g. OCD[3], ASD[4] and MDD[5] due to their noteworthy side-effect profile and high potential for abuse (Möhler, Fritschy, and Rudolph 2002; Petty *et al.* 1995; Duman, Sanacora, and Krystal 2019; Pehrson and Sanchez 2015). Recently, GABAergic compounds with more acceptable side-effect profiles, including the neuroactive steroids (specifically allopregnanolone and its analogues, brexanolone and SAGE-217, which are also positive allosteric modulators of the $GABA_A$ receptor complex), reached the market (Duman, Sanacora, and Krystal 2019; Fogaça and Duman 2019; MacKenzie and Maguire 2013). The major mechanism of action of these agents is related to restoring the GABA-mediated excitatory-inhibitory imbalance in the CSTC[6]-circuit (Prevot and Sibille 2021; Ren *et al.* 2016; Rodriguez *et al.* 2013; Peyrovian *et al.* 2020).

2.4 A new way forward – beyond the known horizon of treatment

By this point, it is clear that perturbations in the balance between CF[7] and CR[8] is a trait of several psychiatric illnesses. However, since the known treatment interventions alluded to above yield a suboptimal treatment response in many of these conditions, the question can be asked whether distinct pharmacological approaches—perhaps those that selectively target abnormal cognition arising from neural circuit dysfunction—could be employed against this conceptual background. To this end, LEV, clinically used for the treatment of epilepsy, may be of interest.

Interestingly, as opposed to other neuronal stabilizers, e.g. valproate, that act mostly by modulating the actions of voltage-gated ion channels, LEV presents with diverse, unique mechanisms of action which are still rather poorly understood (Steinhoff and Staack 2019). The primary mechanisms of action of LEV appear to involve modulation of synaptic vesicle release via its actions on synaptic vesicle

[1] levetiracetam
[2] gamma amino butyric acid
[3] obsessive-compulsive disorder
[4] autism spectrum disorders
[5] major depression disorder
[6] cortical-striatal-thalamic-cortical
[7] cognitive flexibility
[8] cognitive rigidity

glycoprotein 2A (SV2A)[1] and its interaction with the calcium-sensing protein, synaptotagmin, thereby ultimately regulating the rate of neurotransmitter vesicle exocytosis into synaptic spaces (Surges, Volynski, and Walker 2008). Specifically, impaired synaptotagmin functionality is associated with a reduced ability of calcium to trigger neurotransmitter release (Nowack *et al.* 2010). The fact that LEV[2] does not interact directly with specific neurotransmitters, but rather with SV2A expressed in all neuron types (Janz *et al.* 1999; Klitgaard and Verdru 2007), may be key to its potential as a cognitive enhancer (Helmstaedter and Witt 2008; Wu *et al.* 2009). This potential cognitive enhancing effect is contingent on another rather unique mechanistic effect of LEV; it appears to only exert an effect on abnormally active neurons, leaving typical brain function unaffected (Cortes-Altamirano *et al.* 2016). This forms the basis of our hypothesis that LEV can potentially be of use to attenuate CR[3], with the assumption that CR is to some extent associated with over-activity of specific neuronal bodies in the relevant and identifiable behaviour-controlling circuits. By selectively targeting hyper-firing neurons via its interactions with SV2A, LEV may regulate abnormal CSTC[4] circuit function without disrupting normally functioning synapses in said circuit. This notion is further supported by the fact that SV2A plays an important homeostatic role with respect to typical neurotransmission (Nowack *et al.* 2010). In addition to its effects on SV2A, other known pharmacodynamic effects of LEV include potentiation of GABAergic firing (though only in epileptiform tissues), prevention of excessive neuronal excitation (and thus also excitotoxicity) via its antagonistic effects on NMDA[5] receptors, and inhibition of N-type calcium channels (Ueda *et al.* 2001; Cortes-Altamirano *et al.* 2016; Surges, Volynski, and Walker 2008). Through several of these diverse mechanisms, LEV is thought to have a net *inhibitory* effect on neurotransmitter release, although it must be reiterated that this effect is selective and dependent on the underlying activity levels of the neuron in question. In the present work, we will use APO[6] to hopefully induce a persistent behavioural phenotype founded upon overactive dopaminergic signalling processes. By artificially elevating the firing rate of dopaminergic neurons, dopamine-based learning processes are influenced. Subsequently, by co-administering APO and LEV, we aim to determine whether LEV, through its aforementioned mechanisms, can oppose the behavioural effects of APO administration.

[1] synaptic vesicle glycoprotein 2A
[2] levetiracetam
[3] cognitive rigidity
[4] cortical-striatal-thalamic-cortical
[5] *N*-methyl-D-aspartate
[6] apomorphine

2.5 The zebrafish as a model organism

The use of animal models of human disease is a well-known, justified, and longstanding practice that is enacted to improve our understanding of human illness, especially since a clinical hypothesis, while possibly emerging out of *in-vitro* studies, will remain speculative until it is tested and validated in a whole organism and ultimately translated to clinical studies (Barré-Sinoussi and Montagutelli 2015; Greek and Menache 2013; Chesselet and Carmichael 2012). Even though the contribution of animals to the development of novel therapies and our understanding of disease and disease progression remains invaluable and in many cases irreplaceable, pre-clinical research faces a mounting number of challenges including issues of cost, developing ethical considerations relating to large numbers of animals used and the stress they experience (Freedman, Cockburn, and Simcoe 2015; Rollin 2015; Robinson *et al.* 2019), and because of the potentially limited utility of findings made in a restricted number of experimental species (Van Dam and De Deyn 2020). While the limitations facing pre-clinical research are numerous, short term goals will invariably involve the use of other species, in addition to the already widely employed rodents (Stewart, Ullmann*, et al.* 2015). Such approaches would need to be cost-effective, reproducible, and accurate in emulating specific representative aspects of the human condition, while also coming at a reasonable labour and time cost. However, selecting an animal model which mimics any particular human condition in such a manner that valuable results and knowledge could be gathered, is a challenging task (Eaton and Wishart 2017). This is especially true since it is important to ask whether there is more to be gained from an additional animal model system with respect to our knowledge of human conditions, than what is already known. The answer to this question evades both clinician and pre-clinical researcher, because it is true that not a single psychiatric illness has yet been elucidated in full. Thus, it may be more pertinent to ask whether alternative species to rodents, which may reproduce quicker, have a longer lifespan to enable temporal research, and which may yield a higher throughput framework for investigation, might represent a suitable alternative model. To this end, the zebrafish *(Danio rerio)* appears to be a suitable candidate (Kalueff, Stewart, and Gerlai 2014; Khan *et al.* 2017; Stewart, Grieco*, et al.* 2015). Here a brief overview of relevant aspects of zebrafish physiology and behaviour, which arguably render it a suitable alternative for rodent-based translational models (Kalueff, Echevarria, and Stewart 2014; Stewart, Grieco*, et al.* 2015), will be provided so as to better contextualise the research undertaken in this work.

2.5.1 Developmental background

Zebrafish, which are naturally found in the southern and eastern parts of Asia, undergo rapid reproduction by means of external fertilization, yielding up to 200 offspring per single round of breeding (Goldsmith 2004; Champagne *et al.* 2010; Kalueff, Echevarria, and Stewart 2014; Kalueff, Stewart, and

Gerlai 2014). Moreover, with most basic neurophysiological systems already developed within 72 hours after fertilization, zebrafish makes for an attractive neurodevelopmental animal model (Goldsmith 2004; Champagne *et al.* 2010). Not only do they allow researchers an early-life window onto neurodevelopmental processes, but they also have a long lifespan that provides an opportunity to perform extended investigations of a chronic nature; in fact, zebrafish have a lifespan of 4 – 5 years. Fully grown zebrafish are small in size, approximately 2.5 to 5 cm in length and are small enough to handle with ease (Gerhard 2003; Nabinger, Altenhofen, and Bonan 2020).

2.5.2 Neurobiological overlap between zebrafish and mammals

Zebrafish possess relatively comparable physiological systems and show a high genetic (80%-85%) homology to humans (Kalueff, Echevarria, and Stewart 2014; D'Amico, Estivill, and Terriente 2015; van Staden *et al.* 2020). Moreover, given a high degree of neurochemical similarity between zebrafish and mammals, zebrafish allow for bi-directional translation of biological research findings, i.e. translation from fish to mammals and from mammals to fish (Renier *et al.* 2007; Stewart *et al.* 2014; Khan *et al.* 2017). More specifically, zebrafish demonstrate partial conservation of the dopaminergic, serotonergic, GABAergic and glutaminergic systems, all of which are relevant for studies of CF[1] and CR[2] (D'Amico, Estivill, and Terriente 2015; Stewart, Ullmann*, et al.* 2015).

That said, there are some notable differences between the neurobiological features of zebrafish and mammalian brains; however in such cases, the existence of mammalian-analogous brain structures generally allows for neural circuits—and by extension, similar behaviours—to be conserved (Diotel *et al.* 2020; Stewart, Ullmann*, et al.* 2015; Herculano and Maximino 2014). Zebrafish lack a prefrontal cortex and an expanded telencephalon, brain regions which are partly responsible for executive functioning, e.g. goal-directed action-outcome planning, and by extension also the processes related to CF and CR. However, zebrafish present with similar brain structures, i.e. the ventral and dorsal telencephalic nuclei, posterior tuberous nuclei, thalamic nuclei and dorsal pallium (Panula *et al.* 2010; Mueller *et al.* 2011; D'Amico, Estivill, and Terriente 2015). Here, a brief review of specific neurotransmission systems in zebrafish, and how they converge and diverge with mammalian analogues, will be provided.

2.5.2.1 The dopaminergic neurotransmission system

Only 1% of the neurons in the zebrafish brain are typified as being dopaminergic (Goldman-Rakic 1997; Schultz 2002). In this respect, and relative to mammals, zebrafish express orthologs of the mammalian genes that encode four dopamine receptor types ($D_{1\text{-}4}$); the mammalian D_5 receptor is seemingly not expressed in zebrafish. In terms of receptor homology, mammalian and zebrafish $D_{1/3}$ receptors show

[1] cognitive flexibility
[2] cognitive rigidity

100 % overlap, while there is 80 – 95% homology between the $D_{2/4}$ receptors (Ek *et al.* 2016). Further, the dopamine-synthesizing enzymes, tyrosine hydroxylase (TH)[1] and dopamine beta-hydroxylase (DBH)[2] are also found (Guo *et al.* 1999; Holzschuh *et al.* 2001; Kaslin *et al.* 2004). With regards to the dopamine metabolising enzymes, zebrafish displays only one form of monoamine oxidase (MAO)[3], a closer analogue of monoamine oxidase-A (MAO-A)[4]. However, in zebrafish, this enzyme exhibits functional properties overlapping with both mammalian MAO-A and -B (Aldeco, Arslan, and Edmondson 2011; Arslan and Edmondson 2010).

Notwithstanding the functional similarities between the mammalian and zebrafish dopaminergic systems, some differences regarding its neuroanatomical organization must be noted. First, zebrafish lack dopaminergic neurons in the VTA[5] and substantia nigra, which forms the major dopaminergic nodes of the mammalian CSTC[6] circuitry (Rink and Wullimann 2001; Naderi *et al.* 2016; Mahler, Filippi, and Driever 2010). However, some areas in the zebrafish brain, while not yet confirmed, appear homologous to the mammalian VTA (Rink and Wullimann 2001, 2002; Kaslin and Panula 2001; Naderi *et al.* 2016; Klee *et al.* 2012). These include the posterior tuberculum and/or caudal hypothalamus found in the diencephalon (Rink and Wullimann 2001, 2002; Kaslin and Panula 2001; Naderi *et al.* 2016). Agreement between these and the mammalian structures has indirectly been deduced based on comparisons of similar bio-behavioural traits expressed by both mammal and fish that would normally associate with dopaminergic signalling in the aforementioned mammalian brain structures. These include dopamine-manipulated changes in locomotion, susceptibility to the dopaminergic neurotoxin MPTP, and social-isolation induced changes in dopamine and its metabolites (Ek *et al.* 2016; Naderi *et al.* 2016; Giacomini *et al.* 2006; Shams *et al.* 2018; Echevarria *et al.* 2008).

2.5.2.2 The serotonergic system

Zebrafish only express four serotonin receptors which are encoded by the *htr1aa*, *htr1ab*, *htr1bd* and *htr2c* genes (Panula *et al.* 2010; Stewart, Ullmann*, et al.* 2015). These encode protein expression throughout the zebrafish brain (Panula *et al.* 2010). The orthologs *htr1aa* and *htr1ab* encode for a 5-HT_{1A}-like mammalian receptors, whereas *htr1bd* encodes a receptor that is similar to mammalian 5-$HT_{1B/D}$ receptors. Last, the *htr2c* gene encodes the 5-HT_{2C} receptor (Klee et al., 2012). Further, zebrafish present with three copies of the rate limiting enzyme in the serotonin synthesis pathway, i.e. tryptophan hydroxylase (Stewart *et al.* 2013; Maximino *et al.* 2013). Likewise, two isoforms of the serotonin

[1] tyrosine hydroxylase
[2] dopamine beta-hydroxylase
[3] monoamine oxidase
[4] monoamine oxidase A
[5] ventral tegmental area
[6] cortical-striatal-thalamic-cortical

transporter (SERT)[1] protein encoding gene are present in zebrafish, with pharmacological similarity shared between the two isoforms (*serta* and *sertb*). However, recent research suggests that only *serta,* and not *sertb,* plays a role in the behavioural responses of zebrafish to serotonergic drugs. This is because *sertb* is only distributed in the retina and medulla oblongata, while *serta* is widely expressed in the central nervous system (Maximino *et al.* 2013; Stewart *et al.* 2013; Wang *et al.* 2006). Thus, the zebrafish serotonergic system is a feasible target for the future evaluation of novel serotoninergic and anti-serotonergic pharmacological compounds (Nowicki *et al.* 2014; Stewart *et al.* 2013; Panula *et al.* 2010; Flinn *et al.* 2008).

2.5.2.3 The glutamatergic system

As in mammals, glutamate is the primary excitatory neurotransmitter in the zebrafish brain (Braitenberg and Schüz 2013) and extensive homology of the spatial development of glutamatergic releasing neurons is shown (Stewart, Ullmann*, et al.* 2015). Recent research showed that long-term potentiation (LTP)[2] is modulated by NMDA[3] receptors in the telencephalon of zebrafish, the brain area proposed to parallel to the hippocampus and amygdala in mammals (Nam, Kim, and Lee 2004; Blank *et al.* 2009; Portavella *et al.* 2002; Rodrıguez *et al.* 2002). This is of importance because, LTP refers to processes of synaptic pruning that are necessary for learning and memory consolidation (Nam, Kim, and Lee 2004).

In zebrafish, vesicular glutamate transporters (VGLUTs) [4], which are involved in the release of glutamate from glutamatergic neurons, are already found in embryos at 20 – 24 hours post fertilization (hpf)[5] (Higashijima, Mandel, and Fetcho 2004). As zebrafish develop, the glutamatergic system expands with both VGLUT1 and VGLUT2 being expressed in most of the midbrain, hindbrain and spinal cord by 4 – 5 days post fertilization (dpf) [6] (Higashijima, Mandel, and Fetcho 2004). The expression of glutamate receptors displays a comparable pattern to that of mammals, with metabotropic glutamate receptors found in the olfactory bulb, optic tectum, hypothalamus, cerebellum and retina of both adult and larval zebrafish (Yang *et al.* 2009). Ionotropic glutamate receptors are similarly found throughout the brain and spinal cord, and are also expressed in the telencephalon, olfactory bulb, retina and hindbrain (Mueller and Wullimann 2015; Nam, Kim, and Lee 2004; Ali, Buss, and Drapeau 2000). Further, zebrafish have eight paralogous genes that code for AMPA[7]-like receptor subunits, 13 genes coding for NMDA-type receptors, six genes that code for KA[8]-type subunits and 12 genes coding for metabotropic

[1] serotonin transporter
[2] long-term potentiation
[3] *N*-methyl-D-aspartate
[4] vesicular glutamate transporters
[5] hours post fertilization
[6] days post fertilization
[7] α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
[8] kainate

receptors which are similarly categorised as those expressed in the mammalian brain (Dhanasiri, Fernandes, and Kiron 2012; Lin *et al.* 2006; Hoppmann *et al.* 2008; Cox, Kucenas, and Voigt 2005; Haug *et al.* 2013).

Functional overlap between the mammalian and zebrafish glutamatergic systems can also be inferred from behavioural expression. Zebrafish have been utilised in numerous studies involving pharmacological manipulation of glutamatergic neurotransmission with similar behavioural results shown in both mammal and fish, thus emphasising the sensitivity and possible utility of the species in further neuropharmacological research concerning excitatory neurotransmission (Herculano *et al.* 2015; Riehl *et al.* 2011).

2.5.2.4 The GABAergic system

Zebrafish display a comparable spatial and temporal distribution of GABAergic neuronal activity to that of mammals (Delgado and Schmachtenberg 2008; Horzmann and Freeman 2016; Stewart, Ullmann*, et al.* 2015). Both the $GABA_A$ and $GABA_B$ receptor classes are found in zebrafish (Renier *et al.* 2007). There are however certain differences in the subunit configuration of zebrafish, compared to mammalian GABA[1] receptors (Sadamitsu *et al.* 2021; McCarroll *et al.* 2019). Still, sufficient functional conservation of receptor characteristics and functions, that enables relevant translational research in zebrafish, has been proposed (Sadamitsu *et al.* 2021).

In zebrafish, GABA is widely produced in the brain and spinal cord (Higashijima, Schaefer, and Fetcho 2004). The production of GABA is detectable at 3 dpf[2] in the subpallium, pre-optic region, ventral sections of the dorsal thalamus, and the hypothalamus (Mueller, Vernier, and Wullimann 2006; Doldan *et al.* 1999). Further GABA-expressing neurons are known to develop in the telencephalic nucleus, the ventral diencephalons, tectum stratum, the midbrain, and the olfactory bulb (Kim *et al.* 2004; Mueller and Guo 2009; Doldan *et al.* 1999; Sandell, Martin, and Heinrich 1994).

Even though there are minor differences to be noted between the zebrafish and mammalian GABAergic systems (such as the fact that not all zebrafish GABA receptor subunits have human analogues), the species still displays several neuro-behavioural abnormalities associated with a dysfunctional GABAergic system and which can be pharmacologically manipulated to study mammalian-like phenotypes, e.g. sedation and behaviours related to sedative withdrawal (Bencan, Sledge, and Levin 2009; Cachat *et al.* 2010).

* * *

[1] gamma-aminobutyric acid
[2] days post fertilization

Taken as a whole, it is evident that the classic neurotransmitter systems implicated in higher order executive functions and cognitive control are also present and functional in zebrafish (D'Amico, Estivill, and Terriente 2015). While differences between the mammalian and fish brain are noteworthy, this may also present preclinical research with an advantage, since it allows an opportunity to determine how unique neurobiological architectures differentially or similarly contribute to behaviour (D'Amico, Estivill, and Terriente 2015; Klanker, Feenstra, and Denys 2013; Gruner and Pittenger 2017). Collectively, neurobiological findings from the literature base reviewed above, lay a robust foundation for the present study in which the underpinnings of cognitive flexibility in zebrafish will be investigated.

2.5.3 Sensory ability, association, and learning

Regarding the sensory abilities of zebrafish, colour perception is comparable and even superior to that of humans (Kaiser and Boynton 1996). This is because zebrafish vision is tetrachromatic, implying the expression of four types of retinal cone cells that allow for light detection in both the typical visible light spectrum (blue, green, red), but also in the ultraviolet spectrum (Robinson *et al.* 1993; Hughes *et al.* 1998). Thus, zebrafish have been employed in studies involving conditioning-based colour-dependent learning (Ahmad and Richardson 2013; van Staden *et al.* 2020). That said, caution is required when using colour preference as a cornerstone of a study design; colour preference of individual zebrafish seems to be highly varying (Roy *et al.* 2019; Siregar *et al.* 2020; Avdesh *et al.* 2012), an aspect that may become a potential confound to be accounted for in the methodological planning of studies, such as when certain colour cues appear to induce avoidance behaviour (van Staden *et al.* 2020).

Zebrafish are a highly social species and are known to shoal and actively seek out conspecifics (Saverino and Gerlai 2008; Al-Imari and Gerlai 2008; Daggett, Brown, and Brennan 2019; Saif *et al.* 2013), a characteristic which is easily leveraged when designing behavioural studies. Zebrafish are also capable of observing and interacting with conspecifics over a relatively large distance (Pather and Gerlai 2009). Simple observation of conspecifics has been shown to be highly rewarding to zebrafish, especially following social deprivation (Al-Imari and Gerlai 2008; Saif *et al.* 2013). That said, the valence of social reward seems to be context specific, as conflicting stimuli, i.e. when co-presenting conspecifics and a demotivating (or aversive) stimulus, potentially diminish the valence of social reward (van Staden *et al.* 2020). Nevertheless, zebrafish do not seem to develop tolerance to social reward, at least not rapidly, positioning social rewards as an ideal reinforcer in learning based tasks (Daggett, Brown, and Brennan 2019). Also, zebrafish are able to combine their socially driven exploratory behaviours with spatial learning, since fish trained that social conspecifics will be presented in a particular location in a maze, will continue to return to that location, long after the conspecifics stop appearing in the learned location (Al-Imari and Gerlai 2008). While zebrafish are also capable of spatial and outcome-feedback learning (Blank *et al.* 2009; Braubach *et al.* 2009; Pather and Gerlai 2009; Eddins *et al.* 2009; Williams, White, and

Messer Jr 2002), caution should be applied in maze construction and experimental setups, especially when zebrafish are expected to execute right-or-left choices, since zebrafish seemingly show individual preference for going 'left' or 'right' (Miklósi, Andrew, and Gasparini 2001). Indeed, the study of lateralisation in zebrafish has recently received substantial attention (Horstick, Bayleyen, and Burgess 2020; Fontana *et al.* 2019; Bisazza, Dadda, and Cantalupo 2005), especially if one considers that such behaviour might have a potential impact on the interpretation of experimental results (Gómez-Laplaza and Gerlai 2010). Brain-behaviour lateralisation refers to the tendency of showing 'side bias', which often manifested in the motor dimension, e.g. being left- or right-handed (Brown and Taylor 1988; Fontana *et al.* 2019). Neuroanatomical asymmetries within the brain, particularly that of the habenula and the epithalamus (a region of the dorsal diencephalon residing in the pineal complex), is said to be associated with the presentation of this phenomenon (Facchin, Argenton, and Bisazza 2009; Dadda *et al.* 2010; Duboc *et al.* 2015). Indeed, lateralisation has been observed with respect to various aspects of zebrafish behaviour, e.g. as seen in biased C-start direction (measuring fast escape response), slow-turning swimming direction and visual eye preference; such forms of lateralisation is likely individual, rather than species specific (Heuts 1999; Sovrano, Bisazza, and Vallortigara 2001; Duboc *et al.* 2015).

2.5.4 Behavioural parallels between zebrafish and human behaviour

Zebrafish can portray complex, context-dependent behavioural responses (Agetsuma *et al.* 2010; Ahmed, Seguin, and Gerlai 2011; Blaser and Gerlai 2006; Gerlai 2010). For example, they display a wide variety of conceptually relevant and quantifiable behaviours when exposed to anxiogenic stimuli. These include increased scototaxis (preference of dark areas), geotaxis (diving response or bottom-dwelling), freezing (being immobile), thigmotaxis (dwelling near the walls of a tank) and erratic movements such as darting and rapid turning (Cachat *et al.* 2011; Egan *et al.* 2009; Wong *et al.* 2010; Shams, Chatterjee, and Gerlai 2015). Further, these behaviours can be convincingly exacerbated or attenuated by pharmacological means such as anxiogenic (Nowicki *et al.* 2014; Maximino *et al.* 2013) or anxiolytic drug intervention, respectively (Bencan, Sledge, and Levin 2009; Connors *et al.* 2014; Sackerman *et al.* 2010; Kalueff, Echevarria, and Stewart 2014).

Importantly, zebrafish also portray behaviours associated with CF[1] and CR[2] in their responses to both reversal learning and intra-dimensional set-shifting tasks (Zabegalov *et al.* 2019; Colwill *et al.* 2005; Parker *et al.* 2012). In terms of the current investigation, we have previously shown that zebrafish are prone to pharmacologically-induced behavioural persistence and impaired outcome-feedback

[1] cognitive flexibility
[2] cognitive rigidity

processing, with respect to the association of social conspecifics with a colour cue in a T-maze (van Staden *et al.* 2020). This concept will be further explored here.

2.6 Perspectives on the current work – towards a novel pharmaco-behavioural model of CR in zebrafish

The present investigation will seek to develop a pharmaco-behavioural model of CR[1] in zebrafish. As such, a brief background on the behavioural constructs that will be exploited in this work, is necessary. Furthermore, and important to note, all reference to an unconditioned stimulus (US[2]), will henceforth be made in the light of it being a rewarding outcome, except if stated otherwise (van Staden *et al.* 2020).

2.6.1 Feedback learning and conditioning

One of the primary drivers of feedback learning is evaluating the *difference* between what is *expected* and the *actual* outcome (Schultz, Dayan, and Montague 1997; Schultz 2002, 2017, 2013). The ability to predict a positive or negative outcome guides better future decision-making. When an action and its outcome is repeatedly paired, contextual circumstances begin to predict a particular outcome. For example, when entering a coffee shop, the smell of coffee predicts that a cup of coffee is about to be enjoyed. This principle is often exploited in psychology in the form of conditioned learning, e.g. classic Pavlovian conditioning (Gould 2002; Overmier 2002). Briefly, subjects are trained that a specific signal, i.e. the 'conditioned stimulus' (CS[3]; normally a sensory cue), is associated with a specific tangible outcome, i.e. the (US) (normally a rewarding or punishing outcome) (Pezzulo, Rigoli, and Friston 2015; Blaser and Vira 2014). Successful learning of such an association enables the subject to predict the relevant outcome following presentation of the CS, thereby provoking a suitable approach or evasion response. In such a scenario, the subject considers this memorised, predictable value assigned to a certain outcome, and compares it to the actual outcome obtained. The *difference* between these two conceptual values is termed the 'reward prediction error'(RPE)[4] and may be positive (if the reward is greater than expected), negative (in the case of a less-than-predicted reward), or neutral (representing an accurate prediction of the US) (Bayer and Glimcher 2005). As described in **paragraph 2.2.2**, tonic and phasic firing of dopaminergic neurons are responsible for the coding of prediction errors (Steinberg *et al.* 2013; Romo and Schultz 1990; Schultz and Dickinson 2000; Redish 2004), i.e. increased dopaminergic firing in the case of positive prediction errors, and vice versa. In this manner, presentation

[1] cognitive rigidity
[2] unconditioned stimulus
[3] conditioned stimulus
[4] reward prediction error'

of a "greater-than-expected" reward, elicits a phasic dopaminergic response which drives behavioural engagement (Wise and Kiyatkin 2011; Stuber *et al.* 2008). Another interesting characteristic of RPE[1]-encoding dopaminergic neurons is the progressive transfer of the neural activation so that a cue, or a CS[2], that was repetitively paired with the presentation of an US[3], becomes the driver or inhibitor of dopamine (Bayer and Glimcher 2005; Schultz and Searleman 2002; Cohen *et al.* 2012). A cue-reward association (CS-US pairing) is then "learned" when the phasic burst response is fully transferred from the reward (US) to the cue (CS), and the neuronal response to the reward (US) is only of a tonic nature (Steinberg *et al.* 2013). Under normal circumstances, changes to RPE value provides crucial information to the subject that enable it to alter its behaviour to promote superior outcomes if the relationship between the CS and US changes. However, in individuals suffering from conditions characterised by CR[4], behavioural adaptation seems to be quite resistant to the effects of RPEs (Palminteri *et al.* 2012), resulting in persistent engagement in functionally outdated behaviours, irrespective of changing outcomes (Palminteri *et al.* 2012; Vaghi *et al.* 2017). It is thus not surprising that various drugs, especially dopaminergic drugs, are able to modify reward-directed processes at both a cognitive and behavioural level (Schultz, Stauffer, and Lak 2017), as evinced in patients with Parkinsonism, who often develop impulse control disorders, arguably originating from the use of dopamine bolstering therapies (Antonini and Cilia 2009; Hassan *et al.* 2011).

In extension of the aforementioned concepts of conditioned learning, reversal learning speaks to the ability to modify an automated behavioural response to a specific CS in the event where the US is no longer realising as the predicted outcome (Jocham *et al.* 2009; Cools *et al.* 2002). For reversal learning to be successful, the previous CS-US pairing needs to be unlearned or extinguished (Klanker, Feenstra, and Denys 2013). Marked deficits in reversal learning have been demonstrated in a number of conditions, including OCD[5] (Remijnse *et al.* 2006) and ASD[6] (McDougle *et al.* 1995). Adequate reversal learning ability is crucial, since it serves as an indicator of overall CF[7], which itself is essential for optimal learning (Izquierdo *et al.* 2017).

[1] reward prediction error'
[2] conditioned stimulus
[3] unconditioned stimulus
[4] cognitive rigidity
[5] obsessive-compulsive disorder
[6] autism spectrum disorders
[7] cognitive flexibility

2.6.2 Applying learning and conditioning in zebrafish to investigate cognitive flexibility

Zebrafish are able to learn in both conditioned (as explained in **paragraph 2.6.1**) and unconditioned (e.g. non-cued adaptation to a novel environment) circumstances (Blaser and Vira 2014). This has been demonstrated in tests of conditioned place preference (CPP[1]) (Tzschentke 1998; Vaz, Hofmeister, and Lindstrand 2019), stimulus learning (Wong *et al.* 2010), cued conditioning (van Staden *et al.* 2020), and habituation to a novel tank (Wong *et al.* 2010). With respect to the current work, we will build on prior research from our laboratory that utilised social reward as a positive reinforcer of APO[2]-induced cue-directed behaviour (van Staden *et al.* 2020). Indeed, our research as well as that of others have shown that presentation of conspecifics to socially isolated zebrafish could be effectively used as an US to fortify cue- (van Staden *et al.* 2020) or location-directed behaviour (Sison and Gerlai 2011; Karnik and Gerlai 2012).

The aforementioned study, which inspired the current study design (van Staden *et al.* 2020), used a traditional T-maze, pairing social rewards and cues in opposing arms of the maze, in a similar phase-based cue/reward schedule as applied here. APO was successfully in said work to induce an inflexible cue-directed behavioural phenotype. In extension of these findings, the current study inherits the basic study design, while introducing some important modifications. First, patterned shapes (spots and stripes) are used instead of coloured cue cards. The use of pattern was necessary, since the use of coloured cues (red in the aforementioned work) revealed a robust aversion to the colour red demonstrated by the majority tested zebrafish. Zebrafish are indeed able to distinguish between different shapes and patterns (Rosenthal and Ryan 2005; Engeszer, Ryan, and Parichy 2004; Stach, Benard, and Giurfa 2004). Considering the use of a social reward, the present study design was expanded by first determining the individual social preference of each fish prior to the onset of the cue (pattern)-outcome (conspecific) learning phase. This was done by offering zebrafish a choice between a single conspecific of the opposite sex, or a group of conspecifics. This was important, as several authors have shown that the nature of social preference may differ for each fish (Ruhl, McRobert, and Currie 2009; Etinger, Lebron, and Palestis 2009). Last and importantly, the design of the rectangular maze used here, though inspired by the fundamental concept of the T-maze—presenting fish with a choice to move left or right—introduces some important changes. By removing a separate and distinct starting stem and instead fusing the start box between the two choice arms, the maze appears symmetrical to the subject when facing inward from either of the terminal ends of the start box. This was done to minimise the potential influences of naturalistic or trained 'right or left handedness', as discussed earlier (Fontana *et al.* 2019; Miklósi, Andrew, and Gasparini 2001; Gómez-Laplaza and Gerlai

[1] conditioned place preference
[2] apomorphine

2010). Further, since the walled-off choice areas are only accessible through gated areas, the fish cannot see the other choice area once they have made a decision to enter a specific compartment (refer to **Chapter 3** for details). We believe this approach to be conceptually superior, since it necessitated the experimental subject to make a determined movement to investigate the other choice arm.

2.7 Summary of chapter 2

In this review, we briefly highlighted the concepts of CF[1] and CR[2] and how perturbations in this balance, associate with or contribute to neuropsychiatric illness. We further explained that many disorders that associate with such disturbances, show suboptimal treatment response to current pharmacotherapeutic agents. We therefore posit that by targeting an overarching construct in psychiatry, i.e. an altered balance between CF and CR, novel avenues for pharmacotherapeutic research in psychiatry might be highlighted. To this end, we frame the present research against the background of the role of potential cognitive enhancers, e.g. LEV[3], as such an alternative.

The balance between CF and CR is largely regulated by the CSTC[4] circuitry. With respect to this project, it is important to note that excessive stimulation of dopamine D_1 and D_2 receptors may contribute to persistent and repetitive behaviours. Indeed, chronic administration of the $D_{1/2}$ receptor agonist, APO[5], was shown to induce behaviours that are reminiscent of CR. Since LEV, clinically prescribed as a supplementary treatment for epilepsy, appears to only exert its net inhibitory effects on abnormally active neurons, where the over-activity of certain neurons in behaviour controlling circuits, i.e. the CSTC circuit, might be associated with the presentation of CR, we propose that this agent might be useful to attenuate behaviours related to CR. While the exact mechanism of action of LEV is not entirely known, its main effect is believed to be related to preventing synaptic vesicle release.

To test the validity of the above-mentioned proposal, that is that LEV might reverse APO-induced behavioural persistence, the current investigation will employ an APO-induced zebrafish (*Danio rerio*) model of behavioural inflexibility as described and applied in a novel test of cue-reward contingency learning.

* * *

[1] cognitive flexibility
[2] cognitive rigidity
[3] levetiracetam
[4] cortical-striatal-thalamic-cortical
[5] apomorphine

2.8 References

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3 Journal Article

Cue-associated reward appraisal in zebrafish and its modulation by apomorphine and levetiracetam

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Abstract

Cognitive rigidity (CR)[1] is a psychological construct which broadly refers to a pattern of slowly adapting thought processes in the face of changing circumstances. Cognitive flexibility, which essentially stands as the antithesis of CR, is necessary for organisms to be adaptable and function optimally. Accordingly, elevations in CR are correlated with a number of psychiatric disorders. A novel treatment strategy may be to target constructs like CR, which may promulgate said disorders. Levetiracetam (LEV)[2] is an anti-epileptic drug with potential cognition-enhancing effects. This pro-cognitive effect is largely ascribed to LEV's unique mechanism which appears to selectively regulate excessively active neurons by targeting vesicle release mechanism systems expressed ubiquitously in all neuron types. By targeting such neurons in relevant behaviour controlling circuits, such as the cortico-striatal-thalamic-cortical (CSTC)[3] circuit, LEV may be able restore balance to abnormal action-outcome contingencies favoured by abnormally active CSTC circuit pathways. To test this hypothesis, we applied a novel model of cue-reward conditioned-based learning in zebrafish which involves three unique phases of cue-reward pairing. During Phase 1, reward (social conspecifics) and cue (monochromatic pattern) were co-presented in the arms of a novel rectangular maze. During Phase 2 only the cue was presented and during Phase 3 the reward and the cue were presented in opposing arms. Herewith, 4 groups (n = 10) of different drug exposures i.e. control (CTRL), apomorphine (APO[4]; 100 µg/L), LEV (750 µg/L) and APO + LEV (100 µg/L + 750 µg/L) were employed. APO, a dopamine receptor $D_{1/2}$ agonist was used to induce a cognitive rigid cue-directed response which we theorised would be opposed by LEV co-administration. The main findings of this experiment were that all four exposure groups performed similarly with respect to reward- and cue-directed learning over the first two study phases. Compared to the CTRL group, all drug interventions, but especially the APO + LEV combination, lowered the degree of reward-directed behaviour during Phase 3. Future studies are needed to explore the relevance of these findings for our understanding of neuropsychiatric illness.

Keywords

Cognitive flexibility, cognitive rigidity, apomorphine, levetiracetam, zebrafish

[1] cognitive rigidity
[2] levetiracetam
[3] cortico-striatal-thalamic-cortical
[4] apomorphine

3.1 Introduction

Cognitive flexibility (CF)[1] can be loosely defined as the ability to appropriately adjust one's behaviour in response to a changing environment (Zmigrod *et al.* 2019; Dajani and Uddin 2015). This allows the subject to remain sensitive to alternative possibilities, to disengage from inefficient behavioural routines and to adapt when external or internal states change (Dajani and Uddin 2015; Zmigrod *et al.* 2019; Ionescu 2012). As such, CF is associated with general success throughout an individual's lifespan, as it is associated with, among others, better reading ability in childhood, greater resilience to negative life events and stress, higher levels of creativity, and higher quality of life (Engel de Abreu *et al.* 2014; Genet and Siemer 2011; Chen *et al.* 2014; Davis *et al.* 2010). On the opposite side of what can be termed a flexibility spectrum, is another construct, i.e. cognitive rigidity (CR)[2], which is characterised by slow or even inadequate responses to changing outcomes or a changing environment (Schultz and Searleman 2002; Graybiel 2008; Gillan and Robbins 2014). This balance between CF and CR is vital for many executive processes, including the extent to which an individual can quickly switch between goal-directed and habitual responding (Ehmer *et al.* 2020; Dreisbach and Goschke 2004; Goschke 2003; Hommel 2015). Goal-directed behaviour is typically employed when behavioural responses are directed towards the completion of a very specific, often novel outcome that requires a considerable level of cognitive deliberation (Tricomi, Balleine, and O'Doherty 2009). In contrast, habitual behaviours are those performed with a certain degree of automation after becoming rather familiar with the outcome (Ehmer *et al.* 2020; Aarts and Dijksterhuis 2000; Quinn *et al.* 2010; Wood and Neal 2007); however, most habits still have 'goal', in that its outcomes retain functional value, even over extended periods of time. However, it stands to reason that when habits persist in the absence of a specific functional outcome, i.e. when such behaviour becomes less functional and more rigid (and thus, when CR begins to overwhelm CF), habit begins to resemble a form of compulsivity which manifests as a repetitive engagement in behaviours that are unnecessary, inappropriate to the context, and no longer as productive as they once were (Gillan *et al.* 2016; Everitt and Robbins 2005).

Habitual and goal-directed task execution, hence also cognitive action-outcome processing, is broadly founded upon cortico-striatal-thalamic-cortical (CSTC)[3] signalling (Buschman and Miller 2014; Stocco, Lebiere, and Anderson 2010; Graybiel 2008). This circuitry consists of three different neuroanatomical regions (Di Filippo *et al.* 2009; Milad and Rauch 2012), i.e. the frontal cortex, striatum and thalamus. Briefly, frontal cortical (where actions are planned) afferents project to the striatum, through which two distinct pathways, i.e. the behaviourally activating direct, and the behaviourally inactivating indirect

[1] cognitive flexibility
[2] cognitive rigidity
[3] cortical-striatal-thalamic-cortical

pathways, exert actions on the thalamus, (Stocco, Lebiere, and Anderson 2010; Nambu 2008). Finally, efferent neurons linking the thalamus to the cortex close the circuit (Di Filippo *et al.* 2009; Stocco, Lebiere, and Anderson 2010), resulting in action termination. The cortex and striatum are also linked via the ventral tegmental area (VTA)[1], which both send and receive signals to and from those mentioned regions outside the boundaries of the typical CSTC[2] circuit (Di Filippo *et al.* 2009). The CSTC circuit and its connections via the VTA express several neurotransmitter systems, i.e. the dopaminergic, serotonergic, GABAergic and glutamatergic systems (Pignatelli and Bonci 2015). Particularly, it is important to note that dopamine D_1 and D_2 receptors are expressed on the direct and indirect pathways respectively and that stimulation of both these receptors is required to activate the CSTC circuit (Maia, Cooney, and Peterson 2008; Abramowitz and Jacoby 2015; Groenewegen 2003). The binding of dopamine to D_1 receptors (which promotes neural excitability) in the direct pathway, activates the behaviourally activating direct pathway, whereas binding of the D_2 receptor leads to the inactivation of the *behaviourally inactivating* indirect pathway, therefore resulting in the net activation of the circuit, and successively, the execution of behaviour (Beaulieu, Espinoza, and Gainetdinov 2015). Therefore, in certain contexts, dopamine mediated CSTC activation can result in an excessive, i.e. rigid and repetitive, performance of behaviours (Gillan *et al.* 2016; Maia, Cooney, and Peterson 2008; Abramowitz and Jacoby 2015).

Apomorphine (APO)[3], a non-selective D_1 and D_2 receptor agonist, can be used to induce behavioural inflexibility, presumably by binding to the dopamine receptors in the CSTC circuit (Frank and O'Reilly 2006; Winter *et al.* 2018; Klanker, Feenstra, and Denys 2013). Chronic administration of APO leads to a biobehavioural state that resembles hyperdopaminergic signalling, by over activating both pathways of the CSTC circuitry (Westenberg, Fineberg, and Denys 2007; Kool *et al.* 2010). The other principal agent used in this study is levetiracetam (LEV)[4], which is clinically prescribed as a supplementary treatment for epilepsy but has since demonstrated promise as a drug that may enhance cognitive performance (Helmstaedter *et al.* 2008; Helmstaedter and Witt 2010; Wu *et al.* 2009). While its exact mechanism of action is not entirely understood, it is believed that LEV mainly influences synaptic vesicle release via its action on synaptic vesicle glycoprotein 2A (SV2A)[5] and its interaction with the calcium-sensing protein, synaptotagmin (Surges, Volynski, and Walker 2008; Nowack *et al.* 2010). Impaired synaptotagmin functionality compromise the ability of calcium to trigger neurotransmitter release (Surges, Volynski, and Walker 2008; Nowack *et al.* 2010; Cortes-Altamirano *et al.* 2016). In addition,

[1] ventral tegmental area
[2] cortical-striatal-thalamic-cortical
[3] apomorphine
[4] levetiracetam
[5] synaptic vesicle glycoprotein 2A

LEV[1] also appears to potentiate the GABAergic inhibition of post-synaptic neurons in epileptiform tissues and prevents excessive neuronal excitation by showing antagonistic effects on direct N-methyl-D-aspartate (NMDA)[2]-type receptors and opposing the excitatory effects of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)[3] receptor activation through secondary mechanisms involving calcium channels (Cortes-Altamirano et al. 2016; Ueda *et al.* 2001). Moreover, the fact that LEV only selectively exerts effects on abnormally active neurons, leaving typical brain function unaffected, could explain its potential as a cognitive enhancer, since it likely restores balance to neurochemically unbalanced circuits and neural pathways (Cortes-Altamirano *et al.* 2016; Helmstaedter and Witt 2008; Wu *et al.* 2009; Klitgaard and Verdru 2007; Janz *et al.* 1999). As such, we propose that LEV may have the potential to restore excessive CSTC[4] circuit functioning, by exerting a net inhibitory effect on abnormally active neurons known to be associated with the presentation of CR[5], especially in behaviour controlling circuits like the CSTC, and could aid in combatting CR.

Animal models of human disease is a well-known, justified, and longstanding practice that is used to improve our understanding of the human illness (Barré-Sinoussi and Montagutelli 2015; Greek and Menache 2013; Chesselet and Carmichael 2012). Over the past few decades, zebrafish (*Danio rerio*) appeared as a suitable candidate for this purpose, in some respects (Stewart *et al.* 2014; Khan *et al.* 2017; Stewart *et al.* 2015), particularly for investigations of the present nature. Zebrafish exhibit several quantifiable behaviours which foreseeably mirror specific human states, traits, and behaviours (Zabegalov *et al.* 2019; Colwill *et al.* 2005; Blaser and Vira 2014). In addition, they possess relatively comparable physiological systems and a high genetic (80% - 85%) homology to mammals (Stewart *et al.* 2014; D'Amico, Estivill, and Terriente 2015). Furthermore, since there is a high degree of conservation of neurotransmission systems and neural pathways between zebrafish and mammals, bi-directional translation of relevant research findings is possible (Renier *et al.* 2007; Khan *et al.* 2017). Regarding response to known pharmacological agents, zebrafish show sensitivity to centrally active drugs including but not limited to APO[6] and anti-epileptic drugs, e.g. valproate and LEV (Nishimura *et al.* 2016). Furthermore, zebrafish are employed in studies involving cue-reward contingency learning-dependent learning (van Staden *et al.* 2020), an experimental paradigm that will also be exploited here. The current study represents an elaboration of previous work from our laboratory (van Staden *et al.* 2020) which tested how zebrafish explored a differentially cued and rewarded T-maze under the

[1] levetiracetam
[2] N-methyl-D-asparate
[3] α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
[4] cortical-striatal-thalamic-cortical
[5] cognitive rigidity
[6] apomorphine

influence of APO[1]. While the core concepts pertaining to cue-reward learning will still be applied here, the present work was adapted to include a rectangular maze to account for potential left-right arm choice preponderance. We also introduced the use of individualised cues and social reward paradigms, in an attempt to prevent behavioural conflict during the acquisition of cue-reward contingency.

Drawing from the above, we hypothesized that chronic, (28-day) APO administration in zebrafish would induce persistent cue-, instead of reward-directed behaviour when tested in a phased cue-reward contingency. Furthermore, we hypothesized that co-administration of APO and LEV[2] would result in attenuation of APO-like behaviour, with LEV, in so doing, demonstrating potential utility as an effective pharmacological intervention against behavioural manifestations that may be the result of underlying CR[3].

3.2 Materials and methods

3.2.1 Study layout

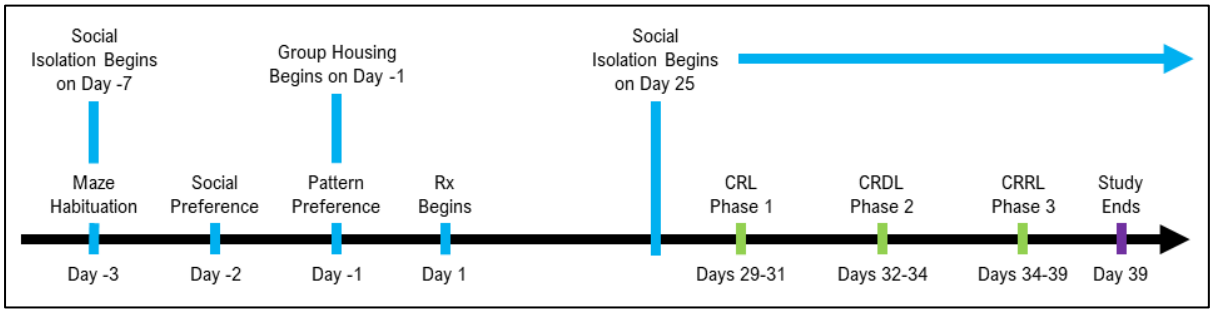

Figure 3-1 – Schematic representation of study layout.

The experimental timeline (**Figure 3-1**) for all subjects started on day -7, when subjects were selected and randomly assigned to their exposure groups. On days -3, -2 and -1 subjects were individually habituated to the maze, tested for social preference and pattern preference, respectively. Follow this last screening test, fish were group housed (see below). Daily drug exposure commenced on day 1 and continued until day 39, i.e. study endpoint. Fish were re-isolated from day 25 onwards to prepare for the onset of behavioural experimentation that commenced on day 29.

3.2.2 Animals and housing overview

A total of 72 randomly chosen adult wild-type short-fin zebrafish (*Danio rerio*) of both sexes were used in this study. Of these, 40 fish were employed as experimental subjects (3-5 months old; ± 30 - 40 mm

[1] apomorphine
[2] levetiracetam
[3] cognitive rigidity

in length; 10 fish per exposure group; refer later). The remaining 32 fish were used as social conspecifics (see below, **paragraph 3.2.6.4**). 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 in the study were bred from this stock and housed in the National Aquatic Bioassay Facility (NABF)[1], North-West University, Potchefstroom, South Africa. The study was approved by the AnimCare Research Ethics Committee (Reg. Nr. AREC-130913-015), of the North-West University; approval nr. **NWU-00525-20-A5**). 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) which regulated water quality (pH: ± 7; conductivity: ≈ 600 µS, and oxygenation: 7.2 mg O^2/L; temperature: 26 ± 1 ˚C. The ambient temperature of the experimental room was set at 28 ˚C at all times.

Four days prior to the onset of individual preference testing on day -3, experimental zebrafish were randomly selected from the quarantine room housing tanks and singly rehoused in 3.5 L acrylic tanks in the exposure room according to the conditions stated above. Tanks were carefully handled, while dividers were used to prevent fish from seeing one another at any time, ensuring complete social isolation for the four prior to preference testing (**paragraph 3.4.4**). After the determination of social and pattern preference, which concluded on day -1 (**Figure 3-1)**, fish with the same pattern and social preference combinations were group housed in the 3.5 L tanks, up to a maximum of 4 fish per tank, in such a manner to ensure that each experimental group consisted of n = 10 fish. Fish were group housed for 25 days in these same preference tanks before once again being housed in isolation for the remainder of the study (beginning on experimental day 25). Food (ZM-200 fry food, Zebrafish Management® Ltd., Twyford, United Kingdom) was provided once daily between 10h00 – 11h00 on drug exposure/non-experimental days, or between the first and second behavioural trials during phase 1 - 3 testing. Conspecific fish were group-housed, separated by sex for ease of experimentation (max n = 8 per tank) for the duration of the study.

[1] National Aquatic Bioassay Facility

3.2.3 Apparatus and configurations

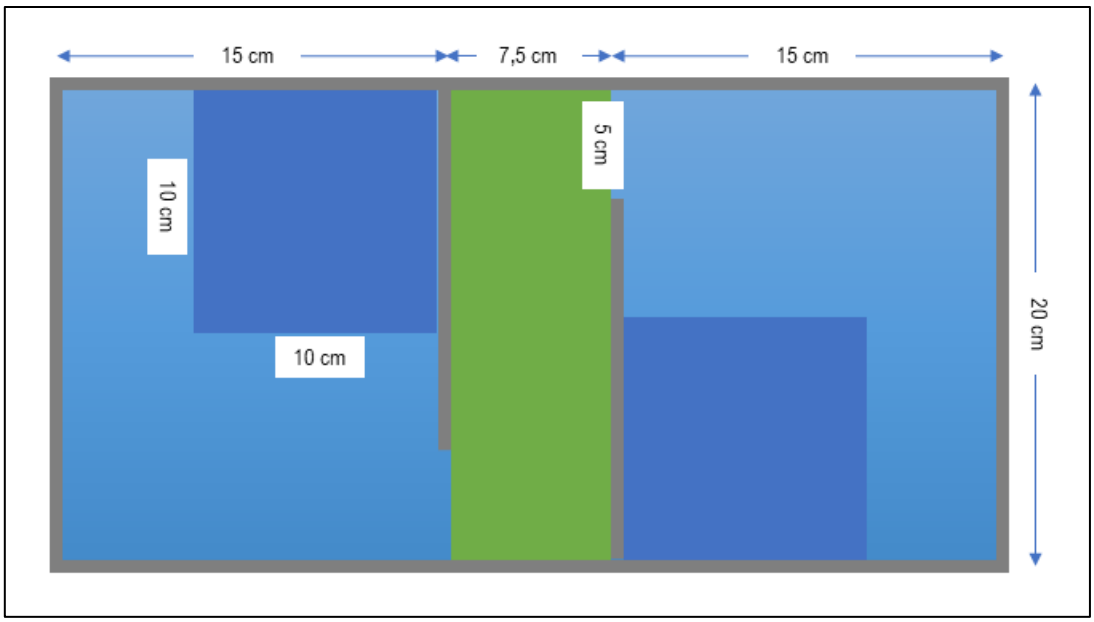

Figure 3-2 - Behavioural apparatus employed in the present investigation.

All behavioural testing procedures involved the use of a white, opaque rectangular maze, constructed from Plexiglas® (**Figure 3-2**) with a white floor which measured 37.5 cm (l) x 20 cm (w) x 20 cm (h). To form a start box, the maze was divided into three compartments, with a central starting compartment measuring 7.5 cm (l) x 20 cm (w) located between two, walled off, identically sized compartments on the opposite ends of the maze, each measuring 15 cm (l) x 20 cm (w). Two 5-cm openings with guillotine doors were made into the walls separating the three compartments. One separate 10 cm (l) x 10 cm (w) x 20 cm (h) insert tank of clear Plexiglas® was placed in each of the larger compartments which were used for the housing of social conspecifics (**Figure 3-2**). Depending on the phase of investigation and the individual social preference of the test subject, the insert tanks held either one or three (phases 1 and 3) or no (phase 2) conspecific fish. For the determination of pattern preference which was further used as the conditioned stimulus (CS/cue), laminated paper cards showing either spotted or striped patterns, were placed along the interior walls directly behind the insert tanks (please see **section 3.2.6** as well as **Addendum A**). The design of the maze ensured that the test subjects could only see into the insert tanks after completely entering the side compartments (**Figure 3-2**). The maze was filled with water from the home tank system to a depth of 8 cm and since the experimental room was temperature controlled, this water temperature was also maintained at approximately 26˚C, identical to that of housing conditions. Total ammonia and nitrate levels were monitored by using a manual testing kit (see also Addendum A), with water changes occurring after every 5 trials. The maze was cleaned at the end of every experimental day to prevent the growth of biofilm. A digital video-camera was positioned 90 cm above the maze and all trials were digitally recorded. Recordings were

subsequently analysed using EthoVision® XT 14 (Noldus® Information Technologies, Wageningen, The Netherlands) digital tracking software.

3.2.4 Baseline social and pattern preference testing

3.2.4.1 Habituation

Prior to the onset of preference determination, zebrafish were individually habituated to the rectangular maze for a single session on day -3 (**Figure 3-1**). During habituation, fish were allowed access to the entire maze in the absence of both social conspecifics and visual cue and were left to explore the maze freely without initially being confined to the start box. Before the onset of the habituation session, each fish was gently netted from their socially isolated home tanks and introduced to the start box area, though not confined to it. Fish were left to explore the maze freely for 6 min. Upon completion of the 6-min session, fish were gently netted out of the maze and placed back into their home tanks. This procedure was repeated for all the experimental fish.

3.2.4.2 Determination of social preference

For the determination of social preference on day -2, experimental zebrafish were again introduced to the start box of the maze; however, subjects were now confined to the start box for 1 min before the guillotine doors were manually removed and the fish allowed to explore the maze for a duration of 5 min. Each fish underwent two (2) sessions on the day, separated by 150 min (van Staden *et al.* 2020). However, on this day, three (3) conspecific fish were introduced to the one insert tank, while a single conspecific was introduced to the other (**Figure 3-2**). Conspecifics (always the opposite sex of the test subject) were introduced into the insert tanks in an alternating fashion during the first and second session, i.e. the group of conspecifics on the right, and the single conspecific on the left during the first trial, and vice versa for the second trial. Both sessions were videotaped, and the time spent in the area directly adjacent to the conspecific-tanks in each compartment measured and quantified. Importantly, only the data from the second session were used to determine the personal social preference of each fish, allowing the first trial to act as a brief learning and/or habituation period. The social preference of each fish was then used for their subsequent contingency experimentation according to the procedures described below.

3.2.4.3 Determination of pattern preference

For the determination of pattern preference on day -1, the same procedures as explained in paragraph 3.2.4.2 were followed. However, in this instance, the insert tanks contained no conspecifics, while the interior walls of the maze directly behind and opposite to the insert tanks were lined with two unique patterns respectively, *viz.* black round dots and stripes. The patterns were presented in an alternating fashion by trial as above. As before both sessions were videotaped, however, only the second trial of the day was scored to determine pattern preference.

3.2.5 Drug exposure

After determination of social and pattern preference, the 40 experimental zebrafish were divided into four drug exposure groups (n = 10 per group) and exposed as follows: 1) control exposed, 2) APO[1] (100 μg/L), 3) LEV[2] (750 µg/L) and 4) a combination of APO and LEV (100 µg/L + 750 µg/L). Each group was exposed for 1 h per day for 39 days, beginning on experimental day 1 (**Figure 3-1**). All drug exposures were performed by means of aqueous immersion. Fish were exposed in accordance with the social housing conditions of the relevant stage of the experiment i.e. group-housed exposure (days 1 - 25) and isolated exposure (days 26 - 38) (**Figure 3-1**). With respect to group exposures, grouped fish were placed into tanks identical to those in which they were housed, containing 1.2 L of the respective drug solutions whereas during individual exposures, the 1.2 L drug solutions were divided amongst beakers containing 200 mL of drug solution. To ensure social isolation when applicable, non-reflective white polystyrene separators were placed in between the exposure beakers. To deliver the desired concentrations of drugs, drug solutions were prepared by dissolving 6.8 mg APO hydrochloride hemihydrate (Sigma-Aldrich®, South Africa) or 45 mg LEV (BLD Pharm®, China) in 10 mL Milli-Q ultrapure water. Solutions were then sonicated for 1 min (additional detail provided in **Addendum A**). On both non-experimental and experimental days, fish were exposed for 1 h between 13h00 and 14h00, to allow for the timely completion of behavioural experiments prior to being drug exposed.

[1] apomorphine
[2] levetiracetam

3.2.6 Cue-reward contingency testing

3.2.6.1 Phase 1 – Cue-reward learning (CRL)

In building on previous work done in our laboratory, and to assess whether zebrafish that have been chronically exposed to each of the respective exposure interventions would value and associate the presentation of a reward (sight of social conspecifics) with a patterned cue, Phase 1 was executed over three consecutive days, with two 6-min trials conducted per fish per day, separated by 150 min. During all trials of Phase 1, one compartment of the maze was lined with the individual pattern preference (cue) as well the individual social preference of each fish; presentation of these combinations alternated from trial to trial. At the start of each trial, the test subject was introduced to the start box for 1 min, with the maze already having been set up according to the individual preferences of the fish being tested. Subsequently, the start box guillotine doors were raised, and the fish left to explore the maze freely for a period of 5 min, while being videotaped. After the 6-min trial, the fish were gently netted from the maze and transferred back to their socially isolated home tanks. This procedure was repeated for each experimental fish with the first trial of the day beginning at approximately 8h00, followed by the second trial 150 min, i.e. being the inter-trial period, later. This order of events applied throughout the remainder of the study.

3.2.6.2 Phase 2 – Cue-reward dissociative learning (CRDL)

To establish whether zebrafish engaged in cue-directed responses where the prior cue-reward paired and initially preferred pattern acts as the visual cue, fish were assessed in Phase 2 over three consecutive days of testing, again with two trials conducted per fish per day, separated by 150 min, beginning on the day following the completion of Phase 1 (**Figure 3-1**). In this instance, the cue was presented in alternate compartments of the maze during each successive trial. However, during this Phase, no social reward (sight of conspecifics) was presented at any time; the insert tanks thus only contained water.

3.2.6.3 Phase 3 – Cue-reward re-associative learning (CRRL)

Phase 3 was conducted over 5 days of testing, with two daily 6-min trials, separated by 150 min, being conducted per day. Each fish was therefore assessed across 10 trials during Phase 3 (van Staden *et al.* 2020), with the procedural course and the experimental setup exactly the same as described for Phases 1 and 2. Now, the presentation of cue and reward was split so that the reward was presented on one side of the maze and the cue on the other, which alternated with each trial.

3.2.6.4 The use of conspecifics in social preference determination and Phases 1 + 3 of Cue-reward contingency testing

The 32 conspecific fish were divided into 4 groups (A – D) of four male and four female fish and employed as follows: Groups A and B were used for experimentation with fish from the control- and APO[1]-exposed groups. Groups C and D were used for experimentation with fish from the LEV[2] and APO/LEV groups.

3.2.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.3.0. To analyse the average performance per trial (**Figures 3-3A** and **3-4A**), two-way repeated measures analyses of variance (2-way RM ANOVA) were applied. For these comparisons, the independent variables were set as trial and drug exposure. The dependant variable for both comparisons was set as the amount of time spent by fish in the proximity of a specific target zone, depending on Phase, as denoted on the respective figures. Similarly, to compare the average performance of each group of fish per Phase (**Figure 3-3B** and **3-4B**), 2-way RM ANOVA was also applied. Here, the independent variables were set as drug exposure and Phase. The dependant variable was the *average* time spent by fish of the various drug exposure cohorts in the proximity of a specific target zone, depending on Phase, as denoted on the respective figures. All graphical representations of data are represented as mean ± standard error of the mean (SEM)[3], while all statistics appearing in the text are represented as mean ± SD[4]. All 2-way RM ANOVA analyses were followed by Bonferroni's multiple comparisons tests. Where significant differences were noted in post-hoc tests, or where other noteworthy trends were observed, effect size calculations using Cohen's *d* were carried out. A single fish each from the CTRL, APO and APO+LEV groups were excluded from statistical analyses as they failed to engage in begin tested by residing in the start box for the full duration of testing. These groups were thus all (n = 9).

3.3 Results

3.3.1 Table 3-1 and Figure 3-3: Reward-directed behaviour

With respect to the average performance of zebrafish exposed to the different control or drug interventions over all individual trials (**Figure 3-3A**), a significant two-way trial-drug-exposure interaction was shown [$F(63,693) = 2.63$, $p < 0.0001$]. Further, both trial [$F(6.2,204.7) = 14.55$, $p < 0.0001$] and

[1] apomorphine
[2] levetiracetam
[3] standard error of the mean
[4] standard deviation

drug exposure [$F(3,33) = 3.08$, $p = 0.041$], had significant main effects on zebrafish behaviour. Subsequent analysis of the average reward-related behaviour of zebrafish within each of the experimental Phases, i.e. CRL, CRDL, and CRRL, but irrespective of trial (**Figure 3-3B**), revealed a significant two-way Phase-drug interaction [$F(6,66) = 3.38$, $p = 0.006$]. Here, Phase [$F(1.8,59.14) = 26.01$, $p < 0.0001$], but not drug [$F(3,33) = 1.84$, $p = 0.159$], significantly impacted overall Phase performance. Statistical descriptors of significant pairwise differences are provided in **Table 3-1**.

Table 3-1 - Descriptive statistics and pairwise comparisons with respect to the average reward-related behaviour of zebrafish exposed to the various control or drug interventions.
Data are represented as mean ± SD and are shown for significant pairwise comparisons only.

Average performance over individual trials (Figure 3-3A)					
Trial	**Comparison**	**Difference**	***p***	***d***	***d*CI**
7	Apo vs Apo+Lev	40.63 ± 11.08 vs. 18.28 ± 12.6	0.0079	1.838	-2.937, -0.699
13	Ctrl vs Apo+Lev	70.36 ± 28.31 vs. 35.79 ± 14.25	0.0409	1.542	-2.589, -0.459
	Apo vs Apo+Lev	64.53 ± 22.04 vs. 35.79 ± 14.25	0.0333	1.549	-2.597, -0.465
14	Ctrl vs Apo+Lev	78.79 ± 21.80 vs. 34.63 ± 12.96	0.0010	2.464	-3.696, -1.190
	Lev vs Apo+Lev	69.21 ± 23.99 vs. 34.63 ± 12.96	0.0191	1.560	-2.583, -0.503
15	Ctrl vs Apo+Lev	69.21 ± 23.99 vs. 38.55 ± 9.23	0.0287	1.687	-2.759, -0.578
17	Ctrl vs Apo	75.65 ± 22.87 vs. 36.77 ± 14.66	0.0047	2.024	-3.161, -0.848
	Ctrl vs Lev	75.65 ± 22.87 vs. 37.82 ± 22.96	0.0136	1.651	-2.689, -0.578
	Ctrl vs Apo+Lev	75.65 ± 22.87 vs. 40.91 ± 19.19	0.0188	1.646	-2.711, -0.544
19	Ctrl vs Apo+Lev	69.63 ± 27.88 vs. 35.86 ± 6.67	0.0389	1.666	-2.734, -0.560
20	Ctrl vs Apo+Lev	74.91 ± 17.56 vs. 49.22 ± 15.98	0.0306	1.531	-2.576, -0.450
21	Ctrl vs Apo+Lev	63.24 ± 23.94 vs. 33.95 ± 9.15	0.0372	1.616	-2.676, -0.520
Average performance per phase (Figure 3-3B)					
Phase	**Comparison**	**Difference**	***p***	***d***	***d*CI**
3	Ctrl vs Apo+Lev	72.04 ± 20.35 vs. 41.67 ± 8.85	0.0081	1.936	-3.055, -0.778
1 vs 3	Ctrl only	40.65 ± 27.02 vs. 72.04 ± 20.35	0.0324	1.313	0.268, 2.325
2 vs 3	Ctrl only	24.64 ± 12.17 vs. 72.04 ± 20.35	0.0011	2.827	1.466, 4.147
2 vs 3	Lev only	29.22 ± 12.70 vs. 50.81 ± 16.39	0.0368	1.473	0.459, 2.455
2 vs 3	Apo+Lev only	26.08 ± 7.01 vs. 41.67 ± 8.85	0.0200	1.953	0.791, 3.075

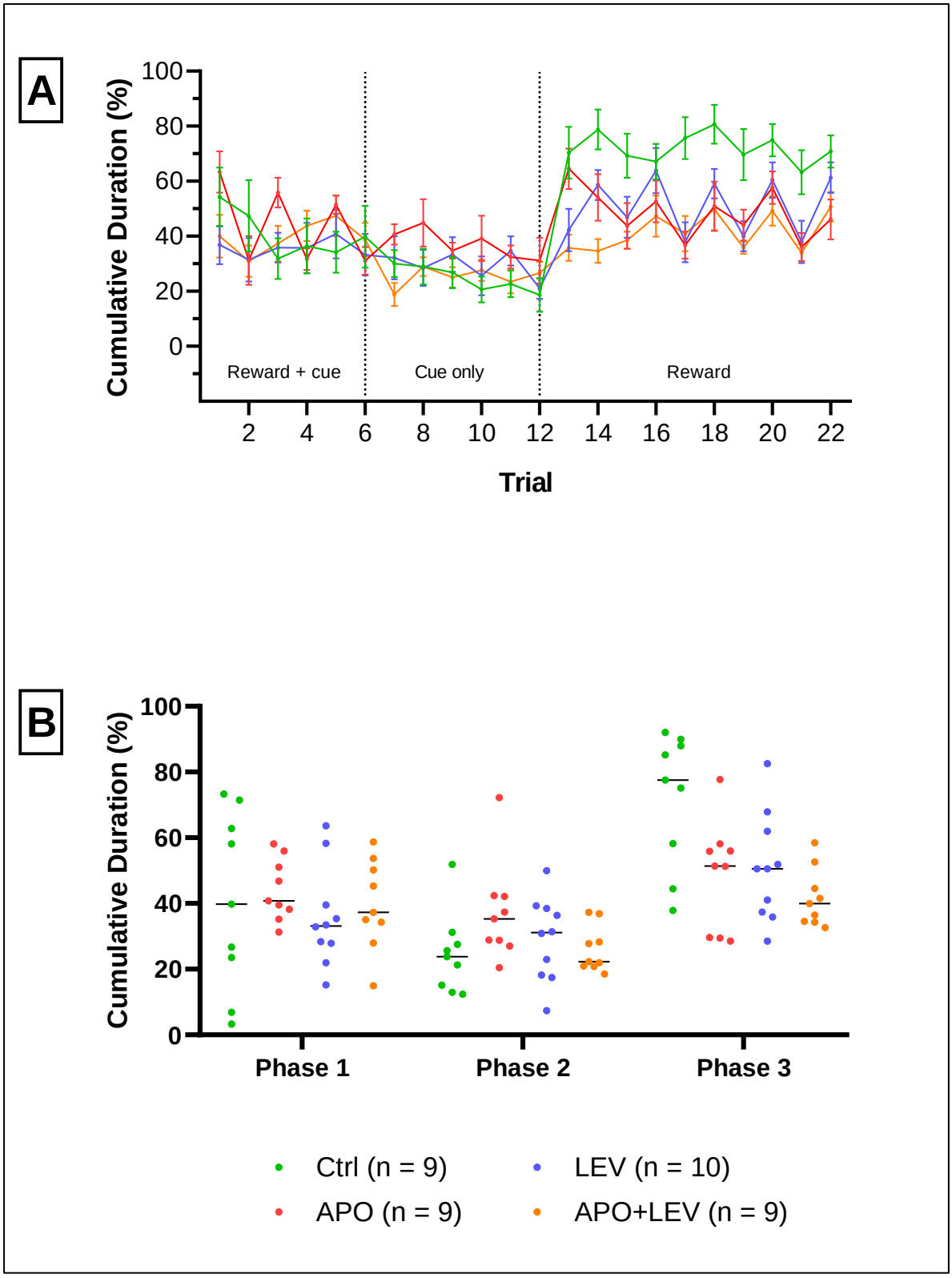

Figure 3-3 – Reward-directed behaviour of zebrafish during phased behavioural experimental testing. *Data are represented as means of the percentage of time subjects spent in proximity to the relevant target areas per trial (A) and per phase (B)* ***A****: Average performance per trial; data represented as mean ± SEM.* ***B****: Average performance per phase; data represented as group means plus individual values. 2-way RM ANOVA; significant differences for both figures are provided in Table 3-1. Ctrl: control; APO: apomorphine; LEV: levetiracetam.*

3.3.2 Table 3-2 and Figure 3-4: Cue-directed behaviour

The average cue-directed behaviour of zebrafish in the respective exposure groups (**Figure 3-4A**), was impacted by both trial and drug exposure, as revealed by a significant two-way interaction [$F(63,693) = 1.44$, $p = 0.0172$] as well as a significant main effect of trial [$F(6.1,201.1) = 14.93$, $p < 0.0001$]. Analysis of the average cue-directed behaviour of zebrafish within each of the experimental Phases (**Figure 3-4B**), revealed a significant main effect of Phase only [$F(1.38,45.52) = 34.16$, $p < 0.0001$]. Statistical descriptors of significant pairwise differences are provided in **Table 3-2**.

Table 3-2 — Descriptive statistics and pairwise comparisons with respect to the average cue-directed behaviour of zebrafish exposed to the various control or drug interventions. Data are represented as mean ± SD.

Table 3-2 - Descriptive statistics and pairwise comparisons with respect to the average cue-directed behaviour of zebrafish exposed to the various control or drug interventions.
Data are represented as mean ± SD and are shown for significant pairwise comparisons only.

Average performance over individual trials (Figure 3-4A)					
Trial	**Comparison**	**Difference**	***p***	***d***	***d*CI**
7	Apo vs Apo+Lev	40.63 ± 11.08 vs. 18.79 ± 12.65	0.0079**	1.836	-2.936, -0.698
14	Ctrl vs Apo+Lev	6.68 ± 7.93 vs. 27.06 ± 13.56	0.0113*	1.835	0.697, 2.934
17	Ctrl vs Apo	6.78 ± 9.97 vs. 24.02 ± 12.58	0.0336*	1.519	0.440, 2.563
	Ctrl vs Lev	6.78 ± 9.97 vs. 25.92 ± 14.93	0.0267*	1.491	0.446, 2.503
	Ctrl vs Apo+Lev	6.78 ± 9.97 vs. 23.21 ± 11.77	0.0347*	1.507	0.430, 2.548
Average performance per phase (Figure 3-4B)					
Phase	**Comparison**	**Difference**	***p***	***d***	***d*CI**
1 vs 3	Apo only	44.11 ± 9.37 vs. 22.06 ± 15.19	0.0187*	1.75	-2.83, -0.63
2 vs 3	Apo only	37.16 ± 15.03 vs. 22.06 ± 15.19	0.0008***	1.000	-1.972, 0.000
1 vs 3	Lev only	35.67 ± 15.06 vs. 16.28 ± 6.82	0.0069**	1.658	-2.669, -0.613
2 vs 3	Lev only	29.22 ± 12.70 vs. 16.28 ± 6.82	0.0124*	1.269	-2.223, -0.286
1 vs 3	Apo+Lev	39.71 ± 13.70 vs. 21.96 ± 6.11	0.0252*	1.673	-2.742, -0.566

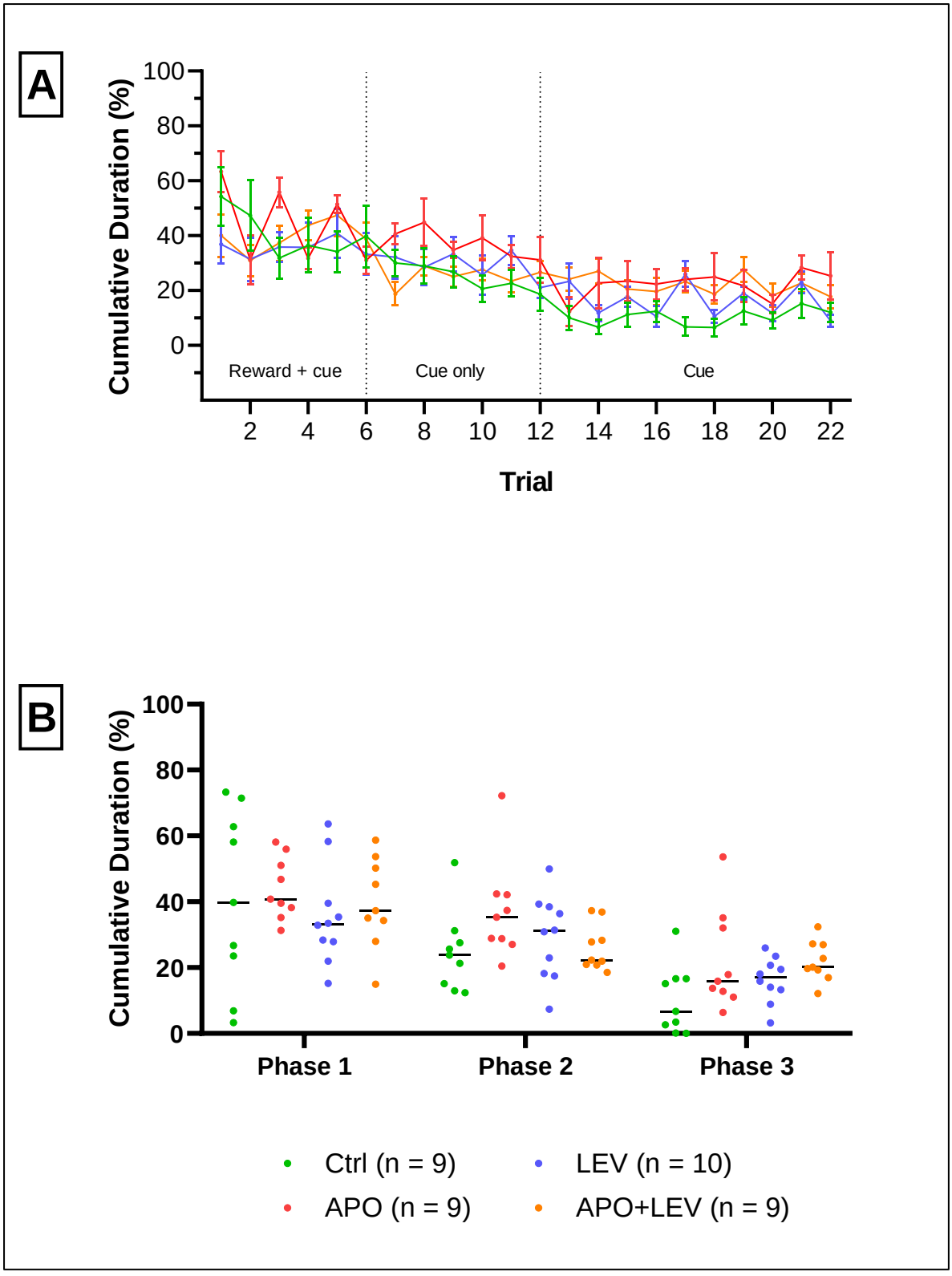

Figure 3-4 - Cue directed behaviour of zebrafish during phased behavioural experimental testing. *Data are represented as means of the percentage time subjects spent in proximity to the relevant target areas per trial (A) and per phase (B).* ***A:*** *Average performance per trial; data represented as mean ± SEM. B: Average performance per phase; data represented as group means and individual values. 2-way RM ANOVA; significant differences for both figures are provided in Table 3-2. Ctrl: control; APO: apomorphine; LEV: levetiracetam.*

3.4 Discussion

In this work, we explored the effect of the $D_{1/2}$ receptor agonist, APO[1], on reward-directed behaviours and behavioural persistence in zebrafish. We further aimed to establish if APO-associated behaviours would be modified by chronic exposure to LEV[2], a potentially novel cognitive enhancer with a mechanism of action that is potentially selective for abnormally active neurons and pathways. The three main findings from this experiment are that 1) all zebrafish, irrespective of drug cohort, performed similarly with respect to reward- and cue-directed learning over the first two study Phases, 2) compared to control-exposure, all drug interventions, but especially the APO+LEV combination, lowered the degree of reward-directed behaviour upon split reward- and cue presentation, and 3) that, compared to the behaviour of control-exposed fish, all drug-exposed groups demonstrated an alternating pattern of increased and decreased interest in social reward with successive trials during Phase 3.

Considering the behaviour of fish with respect to reward-directed (**Figure 3-3A**; Phase 1) and cue-directed responses (**Figure 3-4A**; Phase 2), little difference is demonstrated between the performance of the differentially exposed fish (**Tables 3-1** and **3-2**). It is further interesting to note that all fish, regardless of drug exposure, showed a rather indifferent responsivity to the presence of a social reward during Phase 1, as revealed by the similar mean percentage of times spent in close proximity of the conspecifics, which was without exception, lower than 50% (Ctrl: 40.65 ± 27.02, APO: 44.11 ± 9.37, LEV: 35.67 ± 15.06, and APO +LEV: 39.71 ± 13.7). During Phase 1 (**trials 1 - 6**), conspecifics were paired with the cue in a trial-alternating fashion, so this lack of heightened sociability is striking, considering that subjects were socially deprived at this time and that the individual social preference of each subject was attempted to be accounted for. This finding is contrary to what we expected, particularly with respect to APO-exposed fish, since the dopaminergic action of this drug (a $D_{1/2}$ receptor agonist) is expected to promote reward seeking responses, and which was shown in our previous work (van Staden *et al.* 2020). Still, the APO-exposed group did demonstrate high interest (relative to the remainder of Phase 1) in the reward during **trial 1**, while their reward-directed behaviour followed a waxing and waning pattern for the remainder of the Phase; the same pattern of response was shown again in the behaviour of all treatment groups during Phase 3 (see later).

With respect to Phase 2 (**trials 7 - 12**; **Figure 3-3A** and **Figure 3-4A**), a general continuation of the Phase 1 behavioural pattern is noted. Considering the experimental context of Phase 2, i.e. that only the preferred cue was presented in an alternating fashion, the general lack of cue-directed behaviour was expected for the control-exposed fish. However, the lack of such a response in drug-exposed fish,

[1] apomorphine
[2] levetiracetam

could suggest that fish failed to acquire knowledge of the co-presented cue and reward contingency during Phase 1 (however, see discussion on Phase 3, below). Again, this finding contrasts our previous work, in which we showed that APO[1] exposure indeed facilitates a bolstered cue-seeking response, albeit in a Phase of which the method corresponded to Phase 3 of this work (van Staden *et al.* 2020). Nevertheless, it must be noted that in our previous work, fish were allowed 10 trials to acquire knowledge of the cue-reward contingency instead of the six employed here, which potentially facilitated a more robust association to be established. Second, only one significant difference in both the reward- and cue-directed behaviour, respectively, i.e. between the CTRL and APO + LEV[2] groups during the first trial of Phase 2 - **trial 7** (**Tables 3-1** and **Table 3-2**) was found. Given the long duration of this experiment, this lone difference fades into obscurity in the context of Phase 2 as a whole. Thus, we conclude that no significant changes to behaviour, compared to the behaviour of control-exposed fish, were induced by any of the drugs with respect to Phase 2.

With respect to the second main finding of this work, the behaviour of control- and drug exposed fish began to separate during Phase 3 (**trials 13 - 22; Figures 3-3** and **3-4**). Here, a marked increase in the reward-directed responsivity of all fish at the first trial of this phase is noted against the background of the fact that social conspecifics were now reintroduced to the maze. This can be seen in **Figure 3-3A,** where at **trial 13**, the CTRL and APO groups demonstrated a marked increase in their interest towards the conspecifics, relative to **trial 12**, the greatest magnitude of reward interest demonstrated in the experiment thus far. Considering this uptick in reward-driven behaviour at the start of Phase 3, Cohen's *d* calculations were carried out for all groups between the last trial of Phase 2 and the first of Phase 3 (**trials 12** and **13**). Accordingly, large effect sizes were found for CTRL: (2.177) and APO (1.420) and moderate effect sizes for LEV (1.114) and APO + LEV (0.694). Thus, and to some extent refuting our earlier notion that fish may not have acquired sufficient knowledge of the cue-reward contingency in Phase 1, all fish acquired some degree of knowledge about the possible presentation of conspecifics inside the experimental maze; this knowledge was seemingly sensitized by the short-term period of 'social isolation' that occurred during Phase 2. However, since the behaviour of fish in Phase 3 demonstrated that social reward did eventually come to represent some degree of importance to all subjects (when compared to the overly indifferent behaviour observed during Phase 1), there is little evidence that any meaningful association was made with the cue. In other words, the behaviour of the fish appeared to be purely reward-driven, as evidenced by the fact that none of the groups showed bolstered cue-seeking during Phase 2 after the removal of social conspecifics at the site of the cue. Following the first few trials of Phase 3, an interesting 'sawtooth pattern' of reward preference emerged

[1] apomorphine
[2] levetiracetam

in all groups, most notably so in the three drug-exposed groups. This interesting finding is discussed in greater detail below. At a statistical level, the behaviour of fish in the APO[1] + LEV[2] group most frequently differed from that of fish in the CTRL group (**Figure 3-3A**) at trials **13**, **14**, **15**, **17**, **19**, **20** and **21** (**Table 3-1**). APO alone and LEV alone also occasionally resulted in significant differences in behaviour compared to CTRL but, these were too few to indicate any meaningful effect considering the phase as whole (**Table 3-1**). Expectedly, analysis of the average performance per phase (**Figure 3-3B**), revealed a significant difference between the reward-directed behaviour of CTRL and APO + LEV groups only. It is also noteworthy that bolstered dopaminergic signalling as elicited here, significantly bolstered neither reward- (**Figure 3-3A** and **B**), nor cue- (**Figure 3-4A** and **B**) seeking behaviour. However, a significantly increased cue- and/or reward-directed behaviour could have been expected in fish under the influence of dopaminergic potentiation, since dopamine is crucially involved in processes of reward learning and vigour of reward engagement (Pignatelli and Bonci 2015); our present findings with respect to APO showing the contrary, are perplexing. A possible explanation for the fact that APO lowered reward responsivity (**Figure 3-3A** and **B**), without increasing cue-directed behaviour (**Figure 3-4A** and **B**, Phases 2 and 3), may be the lengthy exposure period involved, i.e. 28 days compared to the 14 days used previously. It is likely that APO administration induced a downregulatory effect on $D_{1/2}$ receptor expression, as has been shown with the administration of other compounds to zebrafish before (Castner, Williams, and Goldman-Rakic 2000; Arnsten *et al.* 2017; Souders *et al.* 2019; Ashok *et al.* 2017). Considering the role of phasic dopamine release in generating positive reward-associated prediction errors, such a phenomenon could in fact contribute to the findings reported here. This may be especially true in the current circumstance, since testing took place approximately 17 hours after drug exposure and not sooner.

Given our knowledge of how LEV regulates the activity of hyperactive neurons, i.e. by means of interaction with SV2A[3] and synaptotagmin, as well as the context-specific suppression of GABAergic activity and antagonism of NMDA[4] and AMPA[5] receptors (Surges, Volynski, and Walker 2008; Cortes-Altamirano *et al.* 2016), it was rather unexpected that the drug would induce a behavioural signature during Phase 3 three that was highly similar to that of APO-exposed fish. Further, considering the reported selectivity of LEV for the regulation of high frequency neurotransmission (Cortes-Altamirano *et al.* 2016; Surges, Volynski, and Walker 2008), we expected that LEV might oppose any behavioural effects of APO, the administration of which could presumably result in sustained, abnormally active

[1] apomorphine
[2] levetiracetam
[3] synaptic vesicle glycoprotein 2A
[4] N-methyl-D-aspartate
[5] α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

dopaminergic neurotransmission. Thus, it was also interesting that the behaviour of APO[1] + LEV[2]-exposed fish differed most significantly from CTRL behaviour, rather than from the behaviour of APO-alone-exposed fish. Indeed, given the robust differences observed in the behaviour of CTRL vs. APO + LEV-exposed fish, it would appear as though the effects of APO and LEV is synergistic; however, further investigation into this potential interaction is needed. Importantly, there is express evidence that ionotropic glutamate receptors, specifically AMPA[3] receptors, play a vital role in the maintenance of behavioural sensitization (Pignatelli and Bonci 2015). Specifically, blockade of AMPA receptors (which is one of the mechanisms of LEV), specifically those in the VTA[4], can disrupt the establishment of classically conditioned Pavlovian-type associations (Pignatelli and Bonci 2015; Stuber *et al.* 2008; Kelley *et al.* 2003; Harris and Aston-Jones 2003). It is therefore entirely plausible that this mechanism may have accounted for the unexpected behavioural data generated by the LEV and APO + LEV groups.

In terms of our third main finding, which showed a temporal variance in the reward-directed behaviour of zebrafish, it is likely that the observed behavioural fluctuation may be related to 'behavioural laterality', which is prevalent in most vertebrates, notably also in zebrafish (Horstick, Bayleyen, and Burgess 2020; Bisazza, Dadda, and Cantalupo 2005; Facchin, Argenton, and Bisazza 2009; Fontana *et al.* 2019). Brain and behavioural laterality refers to a tendency of animals towards a side bias (either in terms of neural networks or actual behavioural responses), which is often manifested in motor responses as left or right 'handedness', 'footedness' and 'eyedness' (Brown and Taylor 1988; Fontana *et al.* 2019). In zebrafish, visual lateralization also exists, since fish presented with their own reflection, tended to prefer the use of their left-eye for close observation (Sovrano, Bisazza, and Vallortigara 2001; Sovrano *et al.* 2016). Further, the lateralization behaviour of zebrafish has been convincingly correlated with neuroanatomical asymmetries between the brain hemispheres, particularly in the epithalamus, a region of the dorsal diencephalon which represents the most widely studied neuroanatomical example of lateralization in vertebrates (Facchin, Argenton, and Bisazza 2009; Dadda *et al.* 2010; Duboc *et al.* 2015; Miletto Petrazzini *et al.* 2020). In a study evaluating spontaneously occurring motor left-right bias in a continuous free movement pattern Y-maze task, researchers have demonstrated that some zebrafish exhibit naturalistic motor lateralization which has the potential to alter learning responses (Fontana *et al.* 2019). Here they showed that biased and non-biased fish display differentiated seeking patterns with regards to a repetitive and alternating behavioural ratio and that this ratio could be manipulated by stress, pharmacological manipulation, and excessive training (Kool *et al.* 2010; Rodriguez *et al.* 1992). Considering this, an understanding of laterality bias potentially comes into play when explaining the saw-

[1] apomorphine
[2] levetiracetam
[3] α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
[4] ventral tegmental area

tooth pattern. In fact, it should be reiterated that the experimental fish (during Phase 1) were always introduced to the maze from the same side of the maze, and that the cue-reward pairing was always presented on the left compartment of the maze in the first trial of the day during Phase 1 (i.e. **trials 1**,**3**,**5**; **Figure 3-3** and **3-4**). However, during Phase 3, where the reward and cue were presented at opposing sides of the maze, the location of the reward on the first trial of the Phase, was reversed relative to Phase 1 i.e. the reward was now on the right during the first trial of the day and vice-versa. Bearing these Phase-specific considerations in mind, careful examination of the data indicates that a type of spatial place preference originated during Phase 1, a tendency of zebrafish previously described in a social learning paradigm (Al-Imari and Gerlai 2008). In line with our findings, zebrafish examined in a latent reward-learning paradigm, showed an asymmetrical response pattern to training (Gómez-Laplaza and Gerlai 2010). Briefly, fish that were trained to find a reward via a specific route (right-side tunnel in this instance), tended to prefer swimming via the same route to obtain reward, even if they were allowed to choose the tunnel on either side of the maze; the same tendency was shown for fish trained on the other side of the maze. Although both side biases corresponded to the training location, the right side-trained fish showed a more robust directional bias, compared to left side-trained fish only, thus confirming a role for side-preference in zebrafish.

During Phase 1, APO[1]-exposed fish (**Figure 3-3A**, red line) continued to interact with conspecifics at the site of first introduction (on the left) for the remainder of the phase. Moving to Phase 3, the pattern is broken at first, since all groups showed relative preference to the reward when it was presented on the righthand side of the maze. This short-term occurrence could potentially be explained by an overwhelming social drive at the onset of Phase 3, induced by the social deprivation of Phase 2. However, after a few trials in Phase 3, the pattern of relative preference of all treatment groups began to mirror that observed in Phase 1, beginning at **trial 16** (**Figure 3-3A**). Thus, although the maze used in this study was designed to offer a mirror image, regardless of the four cardinal directions faced in the start box, we cannot rule out the possibility that experimental fish acquired some manner to orientate themselves relative to the start box. By extension it is possible that fish "stamped in" the initial location of reward presentation, creating the subsequent pattern of behaviour observed (Nabinger *et al.* 2021).

Another plausible, though less likely explanation for the temporal variance in behaviour noted might relate to research demonstrating increased acquisition of learned contingencies in animals after moderate food restriction. The theory underlying this effect is based on the potential of moderate food restriction to enhance motivational and hedonistic states, both of which play a pivotal part in the learning and acquisition of behavioural tasks (Pignatelli and Bonci 2015; Branch *et al.* 2013). Throughout

[1] apomorphine

literature there is ample evidence pointing to altered behavioural processes involving the dopaminergic system due to different feeding states and diets (Sevak *et al.* 2008; Baladi and France 2009; Carr 2002; Thanos *et al.* 2008). For example, food restriction is used as a method to enhance the reinforcement efficacy of drugs of abuse in rodent models (Carroll, France, and Meisch 1979; Pignatelli and Bonci 2015). Since the experimental design employed in the current study involved a single daily feeding session timed in between trials, it is possible that the relative unfed state of the fish in the first trial, induced a more robust reward-directed response. That said, although this is plausible, data from Phase 3 contradicts such a conclusion, since the behaviour of fish during Phase 3, indicated enhanced reward during the second trial of the day post feeding (**Figure 3-3A**, **trials 16**, **18**, **20** and **22**), a finding which we explained above. Therefore, it is possible that the fed state of the animals in some way modified their behaviour. However, the experiment was not designed to control for this, and this is considered a potential limitation of the presently employed study design.

While notable findings were made with respect to the behaviour of APO[1]- and LEV[2]-exposed zebrafish, the proposed hypotheses inspiring the study design were not supported by the data. Also, there were several unintended shortcomings incorporated in the study design which were considered after study completion and that can potentially be addressed in future work. First, during Phases 1 and 2, the behaviour of zebrafish was only assessed at one location i.e. in the cue/reward zone during Phase 1, and in the cued zone during Phase 2. Scoring activity at both ends of the maze during all phases, as was done for Phase 3, could arguably have resulted in better insights into the behavioural expressions of the zebrafish employed here. Second, the insert tanks which contained conspecifics inside the maze were not entirely separated from the rest of the maze water, i.e. there were dividers inserted into the maze, rather than being fully contained and confined in itself. Due to this, it is possible that olfactory cues from the conspecifics could have diffused into the maze water and guided the behaviour of experimental fish (Suriyampola *et al.* 2020). Since we highlighted the possibility that feeding fish between trials might change their reward-driven behaviour, it might be prudent to move the feeding time later in the day, well removed from the experimental period. Lastly, given the possibility that fish demonstrated place preference, it might be useful to use a less predictable schedule of reward/cue presentation, i.e. R-L-L-R-L-R-R vs. the presently employed L-R-L-R-L-R schedule.

[1] apomorphine
[2] levetiracetam

3.5 Conclusion

The data from the present investigation confirm previous findings that demonstrated zebrafish to be a suitable model system for investigations of reward-directed behavioural responses and subsequent application to investigate cognitively rigid behaviour. We also demonstrated that long-term dopaminergic potentiation, suppresses reversal learning after fish acquired knowledge of a cue-reward contingency. While our working hypothesis with respect to the potential action of LEV[1] was founded upon the notion that LEV, being a putative cognitive enhancer that stabilizes excessive neuronal firing, would attenuate the behavioural effects elicited by APO[2], our data revealed the opposite. Indeed, it is likely that the observed effect of LEV in the present work, i.e. in blunting reward-directed responses, could be ascribed to its ability to blunt AMPA[3] receptor specific glutamatergic signalling, which has previously been shown to compromise cue-reward learning. On the other hand, the overly long periods of APO administration applied in the current study may contribute to a loss of intended behavioural rigidity, possibly due to receptor regulatory effects. Future studies are therefore needed to explore these ideas further and refine the model.

[1] levetiracetam
[2] apomorphine
[3] α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

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4 Conclusion

An intricate interplay between two components of action-outcome processing is said to be representative of all human and animal behaviour, namely goal-directed and habitual behaviour (Goschke, 2003; Dreisbach and Goschke, 2004; Hommel, 2015; Ehmer et al., 2020). Goal-directed behaviour is usually employed when behavioural responses are directed towards the completion of a very specific, often novel outcome that requires a considerable level of cognitive deliberation (Tricomi et al., 2009). In comparison, habitual behaviours are those performed with a certain degree of automation after becoming rather familiar with the outcome (Aarts and Dijksterhuis, 2000; Wood and Neal, 2007; Quinn et al., 2010; Ehmer et al., 2020). Habitual behaviour often develops as a result of frequently repeating goal-directed behaviour with the apparent objective of saving cognitive effort (Everitt and Robbins, 2005; Wood and Neal, 2007; Tricomi et al., 2009; Gillan and Robbins, 2014). That said, habitual behaviour is not entirely without 'goal', since its outcomes normally retain functional value and desirability, even over extended periods of time. However, overreliance on habitual behaviours, even when the behaviour persists in the absence of a specific functional outcome, resembles a form of compulsivity which manifests as repetitive engagement in behavioural repertoires that are unnecessary, inappropriate within the specific context, and no longer as productive as they once might have been (Everitt and Robbins, 2005; Gillan et al., 2016). It is believed that this interplay between goal-directed and habitual behavioural selection is constantly under the influence of a neurocognitive continuum, with cognitive flexibility (CF)[1] on its one end, and cognitive rigidity (CR)[2], on the other (Wood et al., 2014; Hommel, 2015). CF can loosely be defined as the ability to appropriately adjust one's behaviour in response to a changing environment, *inter alia* allowing for the selective engagement in tasks of a higher immediate priority (Dajani and Uddin, 2015; Zmigrod et al., 2019). CR, on the other hand, is characterised by slowed or even inadequate responses to changing outcomes or a changing environment (Schultz and Searleman, 2002; Graybiel, 2008; Gillan and Robbins, 2014). This balance between CF and CR is vital for many executive processes, including the extent to which an individual can quickly switch between goal-directed and habitual responding (Goschke, 2003; Dreisbach and Goschke, 2004; Hommel, 2015; Ehmer et al., 2020). As such, when habitual behavioural engagement becomes excessive, CR begins to overwhelm CF (Everitt and Robbins, 2005; Gillan et al., 2016).

Habitual and goal-directed task execution, hence also cognitive action-outcome processing, is broadly founded upon cortico-striatal-thalamic-cortical (CSTC)[3] signalling (Graybiel, 2008; Stocco et al., 2010; Buschman and Miller, 2014). Specifically, it is proposed that excessive dopaminergic signalling, either

[1] cognitive flexibility
[2] cognitive rigidity
[3] cortical-striatal-thalamic-cortical

directly or indirectly via mechanisms of inadequate serotonergic control, is believed to underlie hyperactive CSTC[1] processing, resulting in the persistent and repetitive behavioural phenotypes that collectively represent manifestations of CR[2]. Indeed, an imbalance between CF[3] and CR has been demonstrated in a number of psychiatric disorders, e.g. obsessive-compulsive disorder (OCD)[4], major depressive disorder (MDD)[5], and autism spectrum disorder (ASD)[6], all of which are treated with either serotonergic potentiators, e.g. the selective serotonin reuptake inhibitors (SSRIs)[7], or dopamine antagonists, or a combination of both. Moreover, most of these disorders show a suboptimal treatment response to these treatment options, highlighting a need for alternative approaches which may yield superior results. To this end, targeting a common neuropsychological construct (like CR) underlying these conditions, may be promising (Servaas et al., 2021).

Therefore, the aim of the current investigation was to build on previous work done in our laboratory by exploring the effect of chronic administration of the $D_{1/2}$ receptor agonist, apomorphine (APO)[8], on reward (as a behavioural goal) -directed behaviours and behavioural persistence in zebrafish. We further aimed to establish whether APO-associated behaviours would be modified by chronic exposure to levetiracetam (LEV)[9], a potentially novel cognitive enhancer with a mechanism of action that is potentially selective for abnormally active neurons and pathways.

The main findings from this experiment were that 1) all zebrafish, irrespective of drug cohort, performed similarly with respect to reward- and cue-directed learning over the first two study phases, 2) compared to control-exposure, all drug interventions, but especially the APO+LEV combination, lowered the degree of reward-directed behaviour upon split reward- and cue presentation, and 3) that compared to the behaviour of control-exposed fish, all drug-exposed groups demonstrated an alternating pattern of increased and decreased interest in social reward with successive trials during Phase 3. **Table 4-1** contains the study questions (as stipulated in Chapter 1) matched with the outcomes of this investigation.

[1] cortical-striatal-thalamic-cortical
[2] cognitive rigidity
[3] cognitive flexibility
[4] obsessive-compulsive disorder
[5] major depressive disorder
[6] autism spectrum disorder
[7] selective serotonin reuptake inhibitors
[8] apomorphine
[9] levetiracetam

Table 4-1 Summary of main findings

Study questions	Final outcomes
1) Will control, APO[1], LEV[2] and APO/LEV-exposed zebrafish differentially portray associative learning ability in a cue-conditioned learning platform?	As opposed to control-exposed fish, APO, LEV and APO + LEV-exposed fish displayed no particular preference towards either the cue or reward during any of the phases. All exposure groups resulted in a reduction of socially driven behaviour compared to unexposed fish.
2) How will zebrafish of the different exposure groups as highlighted in (1), after repeated exposure to the co-presented cue and reward as also described in (1), portray elevated cue seeking during Phase 2?	All experimental zebrafish demonstrated a notable lack of interest in the cue during phase 2. This represents a poor establishment of cue-reward association in all groups.
3) How will zebrafish of the different exposure groups as highlighted in (1), after reintroduction of the reward in the absence of the cue, portray re-associative learning ability?	All experimental groups, except for the APO+LEV-exposed group, showed a temporarily heightened response to the reintroduction of social reward. Still, all exposure groups demonstrated reduced social interest at this time point relative to the CTRL group.
4) Will APO-exposed zebrafish display a higher degree of behavioural expression akin to CR[3] compared to control and LEV-exposed fish?	APO-exposed zebrafish behaved similarly to CTRL and LEV exposed throughout Phases 1 and 2. During Phase 3, APO- and LEV-exposed fish presented with an overall reduction in reward/socially driven behaviour.
5) Will chronic exposure to LEV (in combination with APO), be able to prevent the effect of APO on the behaviour of exposed zebrafish?	No, the combination of APO + LEV did not reverse any behavioural alterations induced by APO. In fact, the opposite was seen, as these two drugs appeared to act synergistically in blunting the reward-directed behaviour of zebrafish.

[1] apomorphine
[2] levetiracetam
[3] cognitive rigidity

4.1 Shortcomings and recommendations for future studies

This investigation represents an extension of our previous work, and as such still requires development and improvement. In line with this, we considered several potential limitations of the current work and suggest possible ways in which these can be addressed in future.

* * *

First, from a practical perspective, the separation of visual conspecifics was accomplished by using dividers which lacked a closed-ended floor to completely seal off the conspecific fish. Thus, the same water filled the maze and the conspecific holding tanks which could potentially have led to detrimental effects on the experimental outcomes, since zebrafish have an highly sophisticated olfactory system which could have sensed olfactory cues diffusing from the conspecific compartment to the test environment (Braubach et al., 2009; Suriyampola et al., 2020). However, since olfactory cues diffuse quickly, this is unlikely but should be considered, especially since the conspecific tanks in our earlier investigation, where APO[1] elicited a significant degree of cue-directed behaviour, were located entirely outside of the maze.

In line with zebrafish showing an ability to express conditioned place preference behaviour (Gómez-Laplaza and Gerlai, 2010), our findings showing a clearly alternating behavioural response in most drug-exposed fish were interesting, though unintentional. While we employed an—in hindsight, predictable—alternating pattern of reward/cue presentation, it would be advantageous to present the cue in more randomized, less predictable pattern, i.e. L-R-R-L-L-L-R, to account for the effects of place preference, as opposed to reward or cue preference, on the observed behaviours.

* * *

In terms of conceptual considerations, both Phase 1 (cue-reward co-presentation) and Phase 2 (cue only presentation) consisted of six trials performed over the course of three days, while Phase 3 (dissociated cue/reward presentation) consisted of 10 trials performed over five days. It might be advantageous to increase the duration of the initial learning phase as it is uncertain whether the six trials used, resulted in sufficient cue-reward association (van Staden et al., 2020). We followed this route from a practical point of view since our previous work showed adequate learning already by trial six. However, we also changed the nature of the cue, i.e. using a pattern instead of colour, which could have contributed to the results reported here. This may be especially true, since zebrafish prefer some

[1] apomorphine

colours vastly more compared to others, which may introduce a rewarding component of colour cues in itself, a possibility that may arguably fortify processes of cue-reward learning.

Last, based on theories of how reward-prediction errors are generated, we speculate that it would be necessary to design future mazes in such a manner that the presentation of the cue precedes the later presentation of a reward so that robust reward prediction errors can be formed. Indeed, some research indicate that simultaneous pairing of a cue and reward, is inadequate for robust contingency learning to be consolidated (Keiflin and Janak, 2015).

* * *

— THE END —

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Addendum A

Description of supplementary methods

The information contained in this addendum is intended to be read alongside the primary results of this investigation reported in *Chapter 3*. However, since the article presented in that chapter is intended for publication in *Behavioural Brain Research*, certain details were considered excessive for publication. As such, any such additional methodological details are provided here for the sake of completeness of this dissertation.

Layout of groups for behavioural investigation and time frames

This study was designed so that 2 experimental groups could be run simultaneously. Therefore, two groups of zebrafish (n = 10) began experimentation simultaneously following the experimental procedure as described in Chapter 3 and as laid out below.

Table A-1 *- Layout of groups and relevant dates*

Group start date	Group	Social preference test	Pattern preference test	Behaviour tests	Drug Exposure	End date
05/03/21	1	10/03/21	11/03/21	8/04/21 to 18/04/21	Control	18/04/21
05/03/21	2	10/03/21	11/03021	8/04/21 to 18/04/21	Apomorphine	18/04/21
22/04/21	3	27/04/21	28/04/21	29/05/21 to 8/06/21	Levetiracetam	8/06/21
22/04/21	4	27/04/21	28/04/21	29/05/21 to 8/06/21	Apomorphine + Levetiracetam	8/06/21

General routines and procedures

Health and welfare monitoring routine

Each day of the entire process, regardless of phase, the daily routine began at approximately 08h00 with the monitoring of the following parameters:

- Prior to the onset of experimentation, zebrafish were bred and housed in the National Aquatic Bioassay Facility (NABF)[1] of the North-West University (NWU)[2], where they were monitored for health and fed once daily until the start of the investigation.
- Room temperature was controlled using wall mounted digital thermometers (28 ± 1 ˚C).
- The control unit of the automated ZebTec® housing system was examined to ensure that the relevant parameters (temperature, pH, conductivity) were within the accepted ranges as stipulated in Chapter 3.

[1] National Aquatic Bioassay Facility
[2] North-West University

- Zebrafish were also visually inspected for healthy swimming behaviours. Tanks were checked for water levels, biofilm growth and excessive left-over food.

Feeding procedures

- During experimentation (Phase 1 – 3 of testing), fish were tested twice daily, with the first trial beginning at approximately 08h00 and the second after 11h00. Fish were fed during part of the inter-trial period of 150 minutes between the two trials. This time was between approximately 10h00 and 11h00.
- At times when experimental groups were not actively undergoing experimentation, i.e. behavioural testing, whether single or group housed, fish were fed at approximately 10h00 to align with the feeding time during experimentation.
- Regardless of phase or type of housing (grouped or social), feeding simply entailed the provision of small quantities of granular food (ZM-200 fry food, Zebrafish Management Ltd, Twyford, United Kingdom) into the housing tanks.
- Once fed, the feeding response was observed to ensure fish reacted normally as an indicator of wellbeing.

Drug exposure protocols

- The preparation for drug exposure was done by:
    1. Defrosting a pre-prepared Eppendorf© Safe-Lock tube containing a concentrated solution of the relevant drug for dilution (since apomorphine (APO)[1] degrades rapidly in light conditions, defrosting and all subsequent procedures were carried out under dark conditions).
    2. Pipetting exactly 200 µL of the concentrated drug solution (in line with pre-calculated concentration of the frozen solutions; into a measuring cylinder containing 1.2 L of water obtained from the housing system cistern, resulting in a diluted solution containing the required concentrations [for apomorphine (100 µg/L) and levetiracetam (LEV[2], 750 µg/L)].
    3. Pouring the drug solution with the required concentration into tanks identical to those used for the housing of zebrafish (3.5 L tanks).
    4. Immersing the grouped fish in the solutions for 1 h.
    5. Upon completion of the drug exposures the zebrafish were gently returned to their home tanks for a final time, therefore being undisturbed until the initiation of the next daily routine (approximately 18 hours later).

[1] apomorphine
[2] levetiracetam

- Single-housed drug exposure followed the same procedure as described above; however, the 1.2 L of drug solution was divided into 6 beakers containing 200 mL of drug solution, separated by polystyrene dividers. More drug solution was constituted as necessary to ensure all fish were treated in 200 mL of the respective drug solutions.
- The same daily drug exposure routine was followed during the experimental testing phase (days 29-39) as in periods of non-experimentation; however drug exposure was always performed under isolated conditions and was applied after experiments were completed for the day, at approximately 13h30 – 14h00.

Experimental procedures

Social/pattern preference determination

- Performed on day -2 and -1 respectively (see **Table A-2**).
- Fish were introduced to the maze for two 5-min trials, separated by 150 min. All trials were videotaped for later analysis.
- For social preference testing, the maze was prepared with three social conspecifics in one holding tank on one on side and one conspecific in the tank on the opposite side. Social conspecifics were always of the opposite sex.
- For pattern preference testing, the maze was prepared with a black and white striped pattern around the holding tank on one on side and a black and white spotted pattern in the tank on the opposite side.
- The position of the cues/rewards was reversed for the second trial of the day.
- The amount of time spent in proximity to each cue was measured using Noldus® Ethovision XT 14 softward (Noldus® Information Technology, Wageningen, The Netherlands).
- Only the data of the second trial was scored and used to assign preference to each subject, allowing the first trial to act as a short-term habituation session.
- Since two identical mazes existed, fish could be trialled in pairs.

Daily routine during phased experimental trials (**Table A-2**)

- Daily checks were performed as described under the above section 'Health and welfare monitoring routine'
- Thereafter at approximately 8h30, the first trial of the day was initiated (see **Table A-2**). Important to note, on the day of habituation only one trial was conducted.
- Fish were gently netted from their home tanks (housed in isolation) and placed into the start box of the maze, which was already set up for the particular investigation of the day depending on the

phase of experimentation and the preferences of the test subject in question. Fish were left in the start box for approximately 1 min to settle and allow the water surface to calm, before the start gates were removed and the fish was allowed to explore the maze freely for 5 min.

- After 5 min, the fish were gently netted from the maze and placed back into their home tanks. A period of 150 min elapsed before the initiation of the second trial of the day for each individual. It was during the first few minutes of the inter-trial period when fish were fed in their respective home tanks and left undisturbed for the remainder of the time once a positive feeding response was observed.
- The process described in the two previous bullets was repeated for the second trial of the day, after which fish were once again gently returned to their home tanks awaiting drug exposure according to the procedure already described (approximately 13h30).

Table A-2 *Daily routine for experimental days preference testing (days -2 & -1) and phased experimentation (day 29 - 39).*

Group	Experimental phase	Trial 1	Inter-trial period	Trial 2	Drug exposure (individual)
1st group (*n* = 10)	Preference determination	08h30 - 09h30	150 min →	11h00 – 12h00	None
	Behavioural testing	08h30 – 09h30	150 min →	11h00 - 12h00	Approximately 13h30
2nd group (*n* = 10)	Preference determination	09h30 - 10h30	150 min →	12h00 - 13h00	None
	Behavioural testing	09h30 - 10h30	150 min →	12h00 - 13h00	Approximately 13h30

Miscellaneous information

- Prior to the start of the investigation and before the introduction of each new experimental group, all the tanks used in the exposure room were properly cleaned.
- Housing tanks were cleaned approximately every 4 days and also as needed, to prevent the growth of biofilm while the fish underwent experimentation or drug exposure.
- All tanks and beakers used for drug exposure were cleaned after exposures, at the end of each experimental day.
- The behavioural maze was cleaned after five fish had undergone experimentation and filled with fresh water from the housing system to a depth of 8 cm before experimentation continued. Thus, the water was replenished four times during morning testing (for 20 fish) and four times during afternoon testing.

- This was based on the measurement of parameters including oxygenation, nitrate and ammonia levels in the water using purpose made probes supplied by Extech©, by taking water samples after five fish underwent habituation in the maze to ensure the water parameters fell within an acceptable range without potentially altering the fish's behaviour.
- To constitute the ampules of drugs used in this investigation, the correct masses of drug were weighed using a microbalance, then added to the appropriate volume of solvent (Milli-Q® ultrapure water) and sonicated to ensure the full dissolution of drug particles.
- The lighting source in the behavioural room consisted out of 2 double fluorescent tube lights (400 lux).
- Zebrafish were euthanized on the final behavioural investigation day, in a separate room by using a dual tactic to ensure swift and effective euthanasia. Herewith, fish were rendered unconscious with swift finger flick to the head, and the spinal cord was severed with a scalpel blade.

Supplementary images

Figure A-1 *- Supplementary Images. A) Zebrafish housed according to standard laboratory conditions during experimentation. B) ZebTec® Zebrafish Housing System. C) ZM-200 fry food used throughout experimentation. D) Experimental room.*

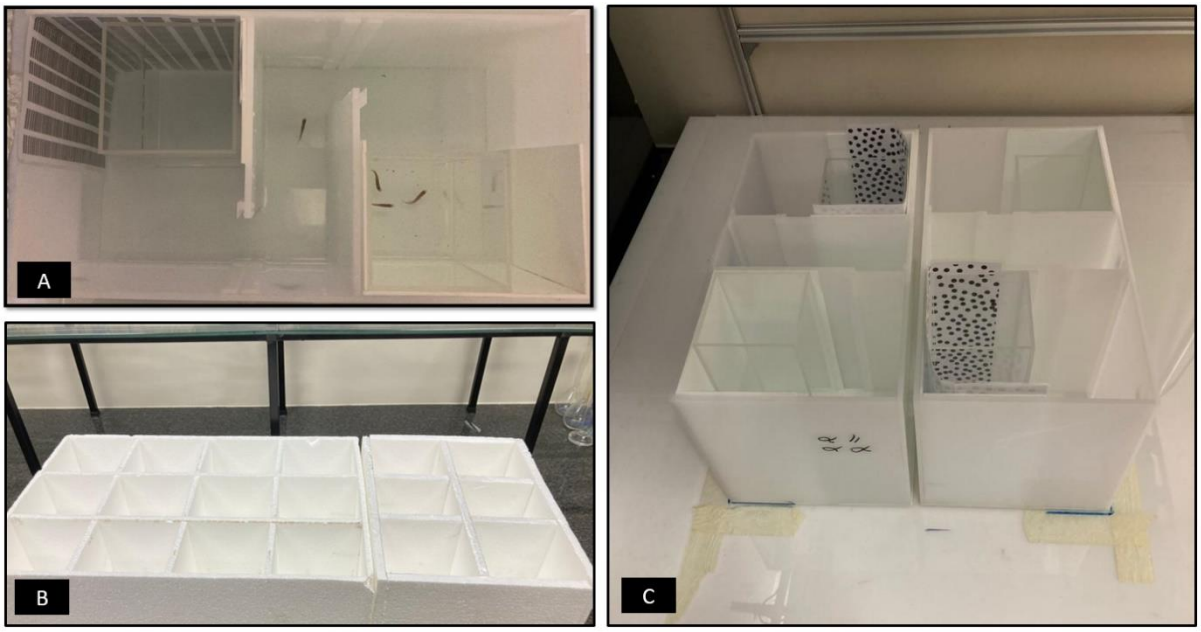

Figure A-2 *- Supplementary images. A) Phase 3 experiment taking place. B) Polystyrene separator used for individual drug exposures. C) Experimental set-up with 2 mazes.*