Changes in Cognitive Function and Behavioural Responses Associated with Neuropsychiatric Disorders.

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Abstract

Neuropsychiatric disorders are on the rise and many are subject to memory and cognitive deficits, which have poor treatment options, generating a great need to better understand the underlying mechanisms to aid future therapeutics. Schizophrenia and drug abuse are neuropsychiatric disorders that are characterised by symptoms of cognitive and behavioural deficits. As with many psychiatric disorders they are the result of genetic and environmental influences that converge on altered behaviour and cognitive processing. Two cognitive domains frequently found to be disrupted in neuropsychiatric disorders are working memory and cognitive flexibility.

Zebrafish are growing in use as a model of complex neuropsychiatric disorders. High genetic homology to humans and ease of genetic manipulation have resulted in a number of zebrafish lines characteristic of many human neuropsychiatric conditions, including schizophrenia and substance abuse. The primary aim of this thesis is the development and validation of a new cross-species task of cognitive assessment. Here I describe the first uses of the Free-Movement Pattern (FMP) Y-maze, a robust and reliable test of cognitive function with both preclinical and clinical applications. The work of this thesis has provided the foundation of future cognitive assessments in zebrafish and paved the way to increased translatability of therapeutic strategies targeting cognitive function from zebrafish models to humans and have been the first to describe comparative deficits in working memory in healthy ageing in zebrafish and humans. I have described the first longitudinal study of cognitive and neuroendocrine development from larvae to young adult and found several biomarkers that could be used to identify critical periods of increased susceptibility to the development of neuropsychiatric disorders. I have also described the first model of cognitive sensitization in zebrafish. Additionally, through the design of a new model of schizophrenia-like phenotypes, I have described a possible genetic link between schizophrenia and nicotine addiction. Finally, this is the first description of cognitive deficits in a zebrafish *disc1* model of schizophrenia.

Declaration by author

I, Madeleine E. Cleal, hereby certify that this thesis, which is approximately 71,000 words in length, has been written by me, and that it is the record of work carried out by me, or principally by myself in collaboration with others as acknowledged. Whilst registered as a candidate for this degree, I have not been registered for any other research award and the results have not been submitted for any other academic award. I was admitted as a research student in September 2016, for the degree of Doctor of Philosophy; the higher study for which this is a record, was carried out in the University of Portsmouth between 2016 and 2020.

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Chapter 5

Dopaminergic Modulation of WM and Cognitive Flexibility in a Zebrafish Model of Aging-Related Cognitive Decline

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Chapter 4

The Cognitive and Behavioural Effects of *D*-Amphetamine and Nicotine Sensitization in Adult Zebrafish

Madeleine Cleal, Barbara. D. Fontana, Matthew. O. Parker

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Abbreviations

AD	Alzheimer's disease
ALIC	Anterior limb of the internal capsule
ASD	Autism spectrum disorder
CF	Cognitive flexibility
CSTC	Cortical-striatal-thalamo-cortical loop
DA	Dopamine
DAT	Dopamine transporter
DISC1	Disrupted in schizophrenia 1
DRD1	Dopamine receptor D1
DRD2	Dopamine receptor D2
DRD3	Dopamine receptor D3
DRD4	Dopamine receptor D4
DRD5	Dopamine receptor D5
DS/VS	Dorsal striatum/ventral striatum
FMP Y-maze	Free-Movement Pattern Y-maze
HPA axis	Hypothalamic-pituitary-adrenal axis
HPI axis	Hypothalamic-pituitary-interrenal axis
LTP	Long term potentiation
MD	Medial dorsal aspect of the thalamus
MDD	Major depressive disorder
NAcc	Nucleus accumbens
NMDA	N-Methyl-D-aspartic acid
NMDA-r	N-Methyl-D-aspartic acid receptor

OCD	Obsessive compulsive disorder
PD	Parkinson's disease
PFC	Prefrontal cortex
PTSD	Post-traumatic stress disorder
SZ	Schizophrenia
THC	Δ9-tetrahydrocannabinol
VTA	Ventral tegmental area
WM	Working Memory
WSCT	Wisconsin card sorting task
WT	Wild type

Part I

Cognition in Health and Disease

Chapter 1

1.0 Introduction

The work described in the first part of this thesis uses a combination of Drosophila, zebrafish, rodents and humans to characterise and validate a new protocol for the assessment of cognitive function, particularly, working memory and cognitive flexibility, in a test designed for preclinical and clinical applications. The aim of this work is to bridge the gap to successfully translate findings affecting cognition, from animal models to humans. Healthy, pharmacological and transgenic models have been utilised to validate this newly developed cognitive test and demonstrate its applicability to the field of neuroscience. The following sections describe molecular mechanisms and integrated neural networks and pathways that are critical to cognitive processing and executive function. Particular attention is paid to neural mechanisms underpinning working memory and cognitive flexibility and the evolutionarily conserved orthologues and homologues that have been identified in two commonly used model organisms: the rodent and the zebrafish. A description of current methods of cognitive assessments in animal models and their translational relevance to clinical assessments are outlined. Subsequent chapters in the first part of this thesis will focus on experimental validation of the Free-Movement Pattern (FMP) Y-maze both as a highly sensitive test for detecting changes in cognitive function and as a clinically and preclinically relevant task, with high translational pertinence.

Working Memory and Cognitive Flexibility in Neuropsychiatric Disorders.

1.1 Introduction

Neuropsychiatric disorders are a global burden that can affect anyone, regardless of socioeconomic status, country of origin, ethnic background, gender or age. It is riff in everyday society and, despite decades of research, is on the rise with an estimated 970 million people globally suffering from mental or behavioural disorders (Vigo et al., 2016; World Health Organisation, 2016). Although there are many identifiable conditions, such as schizophrenia, substance abuse, attention deficit hyperactivity disorder (ADHD), obsessive compulsive disorder (OCD), post-traumatic stress disorder (PTSD), bipolar disorder, depression, anxiety, etc, there is significant overlap in symptoms, such as dysfunctional cognitive processing (Goldberg, 2017; Gould, 2010; Hammar & Årdal, 2009; Kim & Silvestri, 2015; Rădulescu et al., 2017), and triggers, such as stress (Hall et al., 2015; Homberg et al., 2016; Musazzi et al., 2017, 2018). Since the beginning of 2020 the world has been in the grasp of a global pandemic, SARS-CoV-2 (Covid-19), which has caused significant shifts in everyday norms and the way people live their lives (A. Spinelli & Pellino, 2020). This impact is not limited to a single population but affects everyone from the very young to the very old (Daoust, 2020; Götzinger et al., 2020). Covid-19 has brought about unprecedented social isolation, future uncertainty, grave health issues and death. For those with the predisposition to develop neuropsychiatric disorders, any one of these environmental factors could be a trigger for illness-onset (Clay & Parker, 2020; Panchal et al., 2020; Rogers et al., 2020; Steardo et al., 2020). However, combined, there is little doubt of the huge impact that this event will have on the short and long-term mental health of the global population (Nalleballe et al., 2020; Pfefferbaum & North, 2020; Rajkumar, 2020; Troyer et al., 2020). Questions are already being raised as to the steps that can be taken to prevent, reduce or reverse the impact of neuropsychiatric disorders on patients and the society that surrounds them (Tandon, 2020; Torales et al., 2020; Xiong et al., 2020; Zimmer et al., 2020). Understanding the

molecular and neurophysiological mechanisms of neuropsychiatric disorders is critical. Furthermore, having reliable tests that can detect changes in cognitive processing and associated behaviours, that are not only suitable to all age groups, but can robustly translate findings from animal models to humans is going to prove imperative to the future effort of restoring balance to the world's mental health.

Neuropsychiatric disorders are common and complex and often characterised by aberrant cognitive processing which results in altered behaviour (Barnett et al., 2006; Mega & Cummings, 1994; Rao et al., 2019; Sahakian et al., 2015; Sampath et al., 2017). Working memory (WM) deficits are one of the most regularly reported symptoms of many psychiatric conditions and has been reliably found in humans and animal models (Abi-Dargham, 2003; Cargiulo, 2007; Chai et al., 2018; Chamberlain et al., 2006; Chang et al., 2020; Green et al., 2009; Moores et al., 2008; Rapport et al., 2008; Salloway et al., 1994; Schweinsburg et al., 2005; Soraggi-Frez et al., 2017). WM impairments are not only evident in patients diagnosed with psychiatric conditions, but in disorders with a genetic basis, impairment has also been reported in healthy family members (García-Laredo, 2018; Park & Gooding, 2014). Subsequently, it has been suggested that cognitive deficits such as WM could be a potential endophenotypic marker of disorder susceptibility (García-Laredo, 2018; Park & Gooding, 2014). WM has been identified as a critical process, the dysfunction of which underlies multiple cognitive domains and may be at the core of other cognitive deficits associated with neuropsychiatric disease (Baddeley, 2012; Diamond, 2013; Hall et al., 2015; McCabe et al., 2010; Stiles et al., 2015).

Inhibition of cognitive flexibility (CF) is also commonly reported in psychiatric conditions. The ability to adapt to a changing environment is key to selecting appropriate behavioural responses for success and survival (Carruthers et al., 2019; Dajani & Uddin, 2015; Diamond, 2013; Fernández-Serrano et al., 2011; Giraldo-Chica et al., 2018; Orellana & Slachevsky, 2013; Stalnaker et al., 2009; Svoboda et al., 2015). Current treatments target the psychotic symptoms, such as delusions, hallucinations or mood in psychiatric disorders (Buckley, 2001; Ceskova & Silhan, 2018; Patel et al., 2014; Taber et al., 2010). However, little progress has been made in treating the cognitive processes (Wallace et al., 2011). This

highlights a growing need for behavioural and pharmacological strategies targeting cognitive deficits associated with neuropsychiatric disorders. Thus, the aim throughout this thesis is to address this critical need to improve our understanding of the mechanisms that underlie cognitive processes, particularly those associated with neuropsychiatric disorders, that can be severe and potentially debilitating, with substantial impacts on quality of life. However, identification of such mechanisms in animal models alone is not enough. Greater still is the need to improve the translational relevance of such findings to aid in bridging the chasm between preclinical and clinical markers of disease. The lack of fluency between animal models of cognition and human cognition has severely hampered the therapeutic effort in targeting abnormal cognitive symptomology. This fact is addressed throughout this work.

1.2 Working memory and cognitive flexibility.

WM is the process of obtaining and holding small amounts of information in mind, readily available for manipulation and use in other executive functions, such as planning, decision-making and problem-solving (Baddeley, 1992; Baddeley & Hitch, 1974). WM is an essential process to successfully carry out everyday tasks (Baddeley, 2010). For example, when asking directions to the nearest shop, if you are told *take the second left, then the first right and the shop is halfway down the road on the left-hand side,* in order to reach your desired destination, the unique information about the specific stimuli (e.g., the location of the shop) needs to be retained until an appropriate response has been formulated (e.g., planning a route to the shop). Information is held whilst temporal organization of behaviour is carried out to allow planning and execution of the responses necessary to reach the specified goal (e.g., arriving at the shop) (Baddeley, 2012). Properly functioning WM enables the maintenance of information for a period long enough to successfully complete the goal. Impaired WM may limit retainment of information, reducing the amount that can be held (e.g., *take the second left* is stored but the rest is lost before action can be taken). Severe WM deficits could result in the information being lost as soon as it has been relayed, preventing it from being combined into a coherent thought, ultimately leaving the

individual with no information available for processing (Cowan, 2014). This level of impairment can be extremely debilitating with huge consequences on quality of life.

Cognitive flexibility (CF) has been broadly defined as the ability to switch between behaviours, strategies, thoughts and perspectives (Dajani & Uddin, 2015; Diamond, 2013; Klanker et al., 2013; Waltz, 2017). Commonly referred to *set shifting* or *task switching*, it represents the processes of adapting responses in accordance to changing environmental demands or multitasking (Birrell & Brown, 2000; Dajani & Uddin, 2015). Humans, and animals, will maintain a particular behavioural strategy as long it is deemed the optimal strategy for achieving the current goal. However, when the need for change is sufficiently salient and it becomes apparent that the current strategy is no longer working, or there is a more efficient way to achieve the same goal, these strategies can be flexibly updated (R. Cools, 2015). WM has been identified as a crucial process underlying CF, in which continuously maintaining task representations or rules is essential (Diamond, 2013). Impairments in CF are regularly reported in conjunction with impaired WM (Baldacchino et al., 2012; Orellana & Slachevsky, 2013; Rasmussen, 2005; Tanaka et al., 2019; Wobrock et al., 2009). Additionally, many studies have demonstrated that higher WM capacity is strongly correlated with improved CF (i.e. reduced error rates or decreased switching costs) (Blackwell et al., 2009; McCabe et al., 2010).

1.3 The cortical-striatal-thalamo-cortical (CSTC) loop and cognition

The prefrontal cortex (PFC), the striatum and the thalamus form a neural circuitry loop that has been demonstrated to subserve cognitive processes in humans and animals (Floresco et al., 1999; Huang et al., 2018; Peters et al., 2016; Puig et al., 2015; Rădulescu et al., 2017). Executive functions, such as WM and CF, have been assumed to be maintained by persistent neuronal activity in these regions (Furlan et al., 2017). The strength of persistent activity is suggested to represent the degree of information manipulation, which is likely reflected by the complexity of the task (Masse et al., 2019). Lesion and imaging studies have revealed that the PFC is critical to both the holding of information

(short-term memory) and manipulation of information, involved in WM and CF processes. WM assessments, particularly the short-term memory aspect, have revealed a critical role for the reciprocal connectivity between the thalamus and the PFC (Bolkan et al., 2017; Wolff & Vann, 2019). Damage or dysfunction in this circuitry impacts working and short-term memory and associated behavioural performance (Voets et al., 2015). Although some studies have also implicated the thalamus in flexible goal-directed behaviours (Huang et al., 2018; Peters et al., 2016; Wolff & Vann, 2019), a larger body of evidence suggests a key role of the striatum and cortico-striatal loops in controlling CF, rule learning, planning and selecting between competing potential responses or actions (Grospe et al., 2018; Kehagia et al., 2010; Provost et al., 2015; Vaghi et al., 2017; van Schouwenburg et al., 2012). However, overlapping connections and functions mean that dysregulation in any of these regions or the crosstalk between them is likely to result in aberrant cognitive processing with potential behaviour altering effects (Peters et al., 2016).

1.4 The role of dopamine (DA) in cognitive function

Dopamine (DA) has been identified as a key neuromodulator in regulating neuronal activity in the cortical-striatal-thalamo-cortical (CSTC) loop (Carli & Invernizzi, 2014; Dandash et al., 2017; Haber, 2014; Ikemoto et al., 2015; Shipp, 2017). Dopaminergic neurons project from the ventral tegmental area (VTA) into the dorsal and ventral striatum. Input from this region is then received in the globus pallidus which projects into the thalamus. From the thalamus, projections are received in the cortex, mainly the frontal regions, which have output back into the dorsal and ventral striatum. This formulates the direct pathway of dopaminergic communication in the CSTC loop (**Fig. 1**) (Floresco et al., 1999; Haber, 2016; Peters et al., 2016;Puig et al., 2015; Rădulescu et al., 2017). The striatum largely consists of projection neurons, with terminals located in the PFC and the thalamus which are reported to be within ~1 μm of dopaminergic synapses permitting the modulation of information entering the cortex or exerting a significantly sized, general effect on the striatum (Moss & Bolam, 2008). Subsequently, few dopaminergic synapses are required for DA to modulate a substantial cortico-striatal affect.

Figure 1.

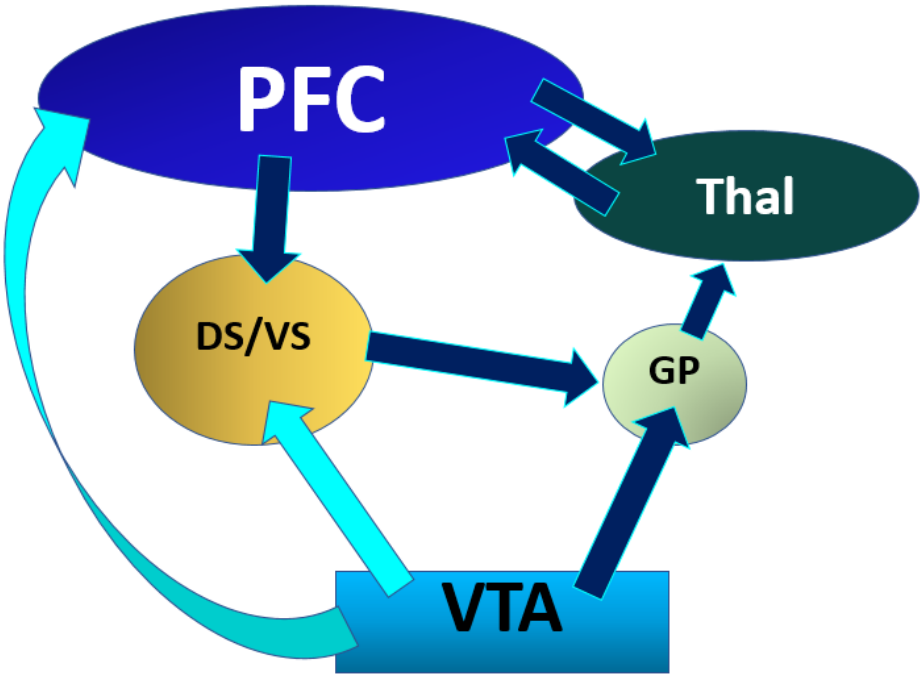


Figure 1. Circuit diagram demonstrating connectivity between dopaminergic projections and the direct pathway of the cortico-striatal-thalamo-cortical (CSTC) loop which subserves executive functions such as working memory (WM) and cognitive flexibility (CF) and plays a critical role in altered cognitive function in neuropsychiatric disorders. Arrows represent direction of information flow, a single headed arrow represents unidirectional information transfer. Dopaminergic projections (light blue), prefrontal cortex (PFC), dorsal and ventral striatum (DS/VS), ventral tegmental area (VTA), globus pallidus (GP), thalamus (Thal).

Adapted from (Haber, 2016)

Historically, the role of persistent neural activity was implemented in maintenance of information used in WM processes (Gazzaley et al., 2004; Goldman-Rakic, 1995; Nyberg & Eriksson, 2016; Srimal & Curtis, 2008). However, this view has since evolved and recent studies using brain imaging and computational models have now revealed a role of persistent neural activity in the processes used to manipulate held information to forge appropriate behavioural responses (Masse et al., 2019; Sreenivasan et al., 2014). Activation patterns of dopaminergic neurons can modulate neural

activity in the CSTC pathway. Varied firing patterns induced by dopaminergic neuron activation, consisting of transient or phasic DA release, caused by DA neuron firing, and sustained tonic DA release, regulated by PFC afferents, are implicated in learning and motivation. A range of dopaminergic firing patterns, from regular pacemaker firing to burst firing, have been related to particular behavioural states (Dreyer et al., 2010; Grace, 1991). Alterations in firing patterns are used to relay an infinite number of 'messages' based on temporal organisation of activity sequences locally and globally, which has demonstrated a robust influence on coordinated ensemble activity (Lohani et al., 2019). In addition to differences in sustained and burst firing patterns, neuronal populations (specifically medium spiny neurons) are subcategorised into excitatory and inhibitory depending on whether neurons are DA D1 receptor (DRD1) or DA D2 receptor (DRD2) expressing (Johnson, 2017; Ott & Nieder, 2019; Rădulescu et al., 2017). Combined, through dopaminergic induced activity patterns, a plethora of messages can be encoded and used to select cue-specific behavioural responses. It is easy then, to imagine how dysregulation in DA availability, resulting in altered firing patterns, could impact the encoded messages sent through to influence behavioural output. In neuropsychiatric disorders, such as schizophrenia and substance abuse, aberrant DA signalling disrupts aspects of CSTC connections, altering informational updates to the detriment of cognitive processing, ultimately influencing long and short-term behavioural responses (Grace, 1991; Rădulescu et al., 2017; Samanez-Larkin et al., 2013; Schultz, 2001; Surmeier et al., 2007; Takahashi et al., 2012).

1.5 Animal models of neuropsychiatric disorders

As outlined above neuropsychiatric disorders are extremely complex and multifaceted conditions that can be constructed of broadly defined symptoms of cognitive and social impairments that can be correlated with animal behaviours (e.g., WM, CF, social interactions), and uniquely human symptoms, such as alterations in thoughts and feelings (e.g., hallucinations, delusions, sadness), that cannot convincingly be replicated in animal models (Nestler & Hyman, 2010). Despite decades of using animals for modelling mental illness, identifying biomarkers, developing novel treatments and general progress

in understanding the underlying pathophysiology has been disappointingly slow, with treatment options representing more a *one size fits all* than a targeted therapeutic approach (Anderzhanova et al., 2017; Bale et al., 2019; Burrows et al., 2011; Gass & Wotjak, 2013; Koob, 2012; Stanford, 2017). Consequently, the discussion has been continuously raised as to the appropriateness, value and reliability of animal models for neuropsychiatric disorders. Although it is clear that not all aspects of the human condition can simultaneously or individually be replicated in animals, there is still an opinion that animal models can be useful tools for basic and translational neuroscience (Anderzhanova et al., 2017; Bale et al., 2019; Gass & Wotjak, 2013). Additionally, as cognitive deficits are such a prevalent and core feature of psychiatric conditions, and represent a subset of symptoms that can be related to a vast repertoire of animal behaviours, their continued use in combination with advancing imaging, molecular, cellular and gene altering techniques may yet prove fruitful in the advancement of treatment strategies and improving the lives of those suffering with what can often be life-long conditions (Baker et al., 2020; Koob, 2012).

To date the vast majority of animal research on neuropsychiatric disorders has been carried out in mammals (Baker et al., 2020; Gururajan et al., 2019; Kokras & Dalla, 2014;McCormick & Green, 2013; Sagvolden et al., 2005; Verbitsky et al., 2020). In possession of homologous brain regions, neural circuitry, chemicals and genes that relate to psychiatric illness in humans, and with many studies showing high face, construct and/or predictive validity to the human condition, rodents have been imperative in elucidating mechanisms of action, development of drug targets and preclinical testing of novel therapeutics (Anderzhanova et al., 2017; Bale et al., 2019; Gass & Wotjak, 2013). Additionally, it is through rodent studies that many *chicken and egg* questions can be answered, for example in understanding the pathological and cognitive changes associated with substance abuse disorders, animal studies have been able to identify if cognitive deficits are a product of those with a pre-existing susceptibility to developing addiction, or as a direct consequence of chronic exposure to addictive drugs (Stalnaker et al., 2009). These are important questions that may otherwise be practically or ethically challenging to answer using patients alone. Considering the long history of using rodents, it is then

perhaps surprising that there has been an uprising in the use of what, on the surface, appears to be a less evolutionarily close animal model: the zebrafish.

The zebrafish has been demonstrated as a powerful model organism for investigating psychiatric disorders (Fontana et al., 2018; Kalueff et al., 2014; Mathur & Guo, 2010;Stewart et al., 2015). High genetic homology to humans and ease of genetic manipulation have resulted in a number of zebrafish lines characteristic of many human neuropsychiatric conditions, including schizophrenia (Eachus et al., 2017; Thyme et al., 2019; Woods et al., 2014; Zimmermann et al., 2016), substance abuse (Bossé & Peterson, 2017; Collier et al., 2014; Gerlai, 2013; Kily et al., 2008; Parker & Brennan, 2012; Parker et al., 2016) and ADHD (Lange et al., 2018; Parker & Brennan, 2016; Parker, Brock, et al., 2014), all of which demonstrate abnormalities in cognitive processing. The well conserved syntenic groups between the human and zebrafish genome (Barbazuk et al., 2000) has resulted in the conservation of evolutionarily ancient pathways such as the reward pathway (Klee et al., 2012), which has orthologues in the zebrafish brain. The neurotransmitters DA (Holzschuh et al., 2001; Kalueff & Cachet, 2011; Lambert et al., 2012), noradrenaline (Stewart et al., 2015), serotonin (Maximino et al., 2013), glutamate (Stewart et al., 2015) and GABA along with their associated receptors (Kalueff, 2017), dopamine receptors (Boehmler et al., 2007; Wendy Boehmler et al., 2004; Lambert et al., 2012; Naderi et al., 2016), opioid receptors (Sanchez-Simon & Rodriguez, 2008), nicotinic acetylcholine receptors (nAChRs) (Klee et al., 2011; Parker, Brock, Walton, et al., 2013) and alcohol dehydrogenases (Tran, Nowicki, Chatterjee, & Gerlai, 2015) all have orthologs in zebrafish.

Despite topographical differences between the zebrafish and mammalian brain, regional homologues have been identified. Equivalent areas to the PFC, striatum, thalamus and VTA in humans and rodents, have been located in zebrafish as the dorsal pallium, ventral and dorsal telencephalic nuclei (part of the subpallium), thalamic nuclei and posterior tuberal nucleus respectively (**Fig. 2**) (Lambert et al., 2012; Mueller et al., 2012; Parker, Brock, Walton, et al., 2013). In addition to possessing the neurotransmitter DA, similar ascending midbrain dopaminergic pathways have been identified with

projections from the posterior tuberal nucleus (VTA) into the subpallium (striatum) that are evident from 96 hours post fertilisation (hpf) (Du et al., 2016). However, as yet there has not been a direct homologue for the CSTC circuitry, despite neurons in the telencephalon being identified as mainly dopaminergic. Highlighting a need to identify the networks between related regions to establish if their relationship is equivocal to that seen in mammals (Du et al., 2016;Parker, Brock, Walton, et al., 2013). These key characteristics, and similarities between teleosts and mammals, suggest that the response of zebrafish to pharmacological treatments and genetic mutations will be similar molecularly and mechanistically to humans. Thus, information gained from studying zebrafish models of neuropsychiatric disorders will likely have bearings on human conditions (Kalueff, 2017; Lieschke & Currie, 2007; Norton, 2013; Parker & Brennan, 2012).

Figure 2.

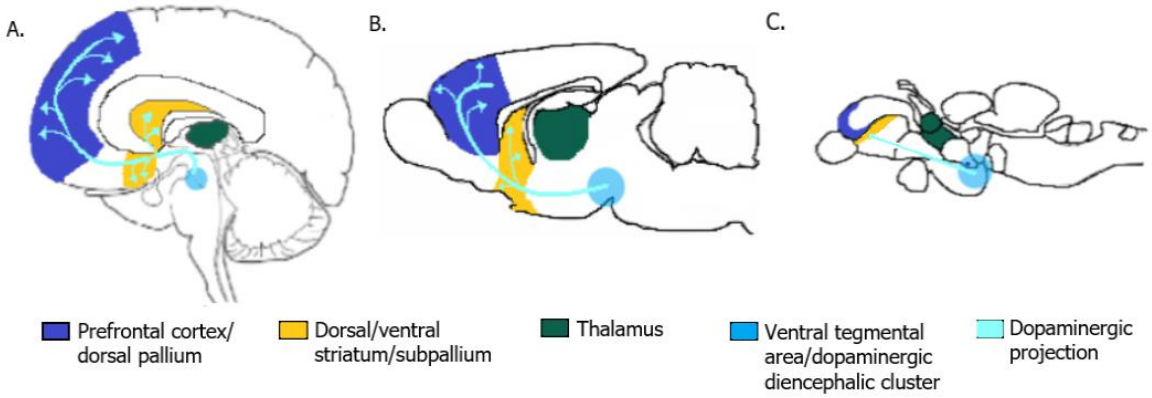


Figure 2. Parasagittal schematic overviews of the dopaminergic projection from the midbrain to the forebrain (light blue) and homologous regions of the cortico-striatal-thalamic network in adult (**A**) humans, (**B**) rodents (mouse) and (**C**) teleosts (zebrafish).

Adapted from (Mueller et al., 2012; Perrone-Capano et al., 2008)

1.5 Assessments of Working Memory and Cognitive Flexibility

There is a vast array of tests available for assessing cognitive functions in humans. As WM has been identified as a central cognitive function, there has been extensive development and validation of WM

tests. The *n*-back test is frequently used as a measure of continuous WM performance. The basic premise is to match the current stimulus to a stimulus *n* number of steps back. Thus, participants are required not only to keep in mind what has gone before but must also be aware of what is currently being presented so that the sequence can progress. The *n*-back test represents a task of both verbal and spatial WM. Despite this being a widely used test for assessing WM in the clinic, its use of both verbal and spatial WM makes this a challenging test to replicate in animal models. Therefore, a substantial number of animal models test WM using spatial WM tasks. Consequently, a plethora of tests have been developed for rodents that are primarily based on mazes. Spatial WM tests can be used to assess WM function by the need to maintain spatial locations, e.g., of a reward, and manipulate this information to ascertain the reward, optimise the strategy to gain maximal reward or to minimise effort in obtaining reward. To test different scenarios a series of maze tasks have been adapted to alter task mechanisms and complexity. Behavioural paradigms for the assessment of spatial WM in rodents include the T and Y-mazes, radial arm maze, Barnes maze, Morris water maze, and others (Sharma et al., 2010; Stewart et al., 2011). All these maze tasks have been assessed using combinations of pharmacological, toxicological and genetic models and have identified critical roles for specific brain regions, such as the PFC and neurotransmitter systems (Carr et al., 2017; Myhrer, 2003; Paul et al., 2009; Ragozzino et al., 1998, 1999; Rich & Shapiro, 2009; Sharma et al., 2010; Stewart et al., 2011; Vorhees & Williams, 2014). Through their use, many insights into the neurobiological mechanisms of psychiatric disorders, including SZ and substance abuse have been elucidated. The variety of labour intensiveness, stimulus use (rewarded versus non-rewarded) and complexity provide a substantial range of assessments that can be readily carried out on models of disease. However, the translation of some of these tasks into an aquatic version for fish has not been straight forward. Discrete-trial tasks, like the T-maze require a substantial amount of animal handling, and although aquatic versions have been developed, there is the risk of stress-induced confounds construing data. One of the most used tasks for assessing WM in rodents, the Morris water maze, cannot be replicated in fish as the water provides a mildly aversive environment that is used to motivate the rodent to find an escape platform. This has obvious difficulties in replication. Several

zebrafish specific tests of WM have been developed to closely match rodent equivalents, however. The plus maze and Y-maze have both been used for assessing WM in zebrafish (Aoki et al., 2015; Cognato et al., 2012; Gaikwad et al., 2011). In addition, there have been some non-maze-based tests developed such as the delayed spatial alternation and the one-trial memory test (Levin & Chen, 2004; Lucon-Xiccato & Dadda, 2014). However, as noted above, these tests have seldom been used to assess WM in zebrafish models of neuropsychiatric disorders.

The above discussions highlight the importance of WM to maintaining good mental health and deviations from properly functioning WM is a major factor underlying numerous neuropsychiatric disorders, as well as neurodevelopmental disorders, such as autism spectrum disorders (ASD), neurodegenerative disorders, such as Alzheimer's disease (AD) and general cognitive dysfunction associated with old age. Considering its critical role, it is interesting, if not a little disappointing, that one of the most frequently used tasks for assessing WM in humans cannot be replicated in animal models. Thus, a divide has clearly emerged between the precise measure of WM assessed in animal models of disease and the associated human condition. Critics of the validity of animal models for assessing human psychiatric disorders have often quoted poor translational relevance and "species specific" tests as a hinderance to the success of developing novel drug targets for treating such disorders. The disparity between the measures of WM may provide a possible route for improvement of the face, construct and predictive validity of cognitive assessments from animals to humans. Some have already attempted to bridge the gap by creating analogous tasks that are used in both rodents and humans, often using a virtual version of the rodent task to replicate the same measure in both species. This is quite possibly the way forward to improve test validity. However, as yet, this does not seem to have to reached mainstream use. Perhaps further development is required until a suitable task has been developed that not only minimises the differences between rodents and humans, but also to other animal models, expanding, instead of limiting, the models that can be used as each model organism surely has an alternative point of view that can be vital in elucidating the mechanisms and potential therapeutic options for treating the human conditions.

1.6 Conclusion

Studies show that there are model appropriate tasks for assessing WM and CF in humans, rodents and zebrafish. Extensive work has been conducted to validate these cognitive tasks in mammals and to identify brain regions, circuitry and chemistry involved in optimal responses and aberrations caused by disease or disorders. However, thus far this information is limited in zebrafish. Having demonstrated a clear ability to complete complex behavioural tasks that can be used to assess cognitive function, it is apparent that further validation, and perhaps a greater number of tasks, are required to fully appreciate the potential of zebrafish for examining cognitive abilities in health and disease. Additionally, it is evident that there is a great need for translationally relevant measures of cognition. The sustained use of animal models of human conditions for the identification and development of novel therapeutic strategies targeting the cognitive deficits that preside over many neuropsychiatric disorders, is reliant on high construct and predictive validity. Without it, as we have witnessed over the last 50 years, progress is slow. Thus, there is need to improve and validate the behavioural tests of cognition that are currently being used, and potentially the development of new cross species assessments, the topic of which is the focus of the first part of this thesis.

Chapter 2

General Materials and Methods

This chapter describes the general protocols used over the course of this thesis and is subdivided into two sections. The first section pertains to animal husbandry, behavioural procedures and apparatus. The second section describes laboratory practices and protocols following optimisation. Specific details of the procedures used for each experiment are outlined in the relevant chapters.

2.1 Experimental Animals

2.1.1 Zebrafish husbandry

Zebrafish adults were housed in ~50:50 male:female groups of 8-10 per 2.8L tank or up to 20 per 6L tank on a re-circulating system (Aquaneering Inc., San Diego, CA, USA) fed from the local water supply (referred to as 'aquarium water' throughout the remainder of this thesis). Room and tank water temperatures were maintained at 25-27°C, pH 8.4 (±0.4), on a 14:10-hour light/dark cycle (lights on 8.15-8.45 am). Fish were fed on ZM fry food from 5 dpf until adulthood when they were moved onto a diet of flake food and live brine shrimp (ZM Systems, UK) 3 times/day (once/day on weekends).

2.1.2 Breeding and embryo collection

Two methods of embryo collection were used, marbling and pair-breeding. Preparation for breeding was set up the evening before embryos were required. Spawning, release of eggs from females and sperm from males, is initiated with first light (when the room lights come on). Breeding is carried out in areas that allow the embryos to filter though (either marbles or a slotted base), to protect them from being eaten by adults.

Marbling: In 6L home tanks, marbling boxes (rectangular boxes filled with marbles) were placed on the bottom of the tank the evening before embryo collection. Eggs were laid over the marbles.

Pair-breeding: The evening before breeding, two females and two males for each pair-breeding set were isolated (by sex) in a separate tank. 15 min before lights on fish were transferred to a breeding tank (a shallow, stand-alone tank with a slotted base), filled with 0.5-1.0L of aquarium water. Males and females were separated by a clear divider. Upon lights on, the divider was removed to allow the fish to mix. Fish were left in the tank for 30-45 min. Once eggs were laid, adult fish were returned to home tanks.

Laid embryos were washed and placed in petri dishes in maximum groups of 50 embryos per dish in aquarium water. Dead embryos were removed regularly, and partial water exchanges were done daily to prevent water contamination from dead embryos, build-up of chorions (casing surrounding embryos) after hatching and to avoid bacterial infections. Fertilised embryos were maintained in a transparent incubator at 28°C on the same light dark cycle as adult fish. Fish were kept in petri dishes until 5 dpf, at which point they were transferred to larger dishes with maximal group sizes of ~30 embryos per dish. Fish were transferred to the recirculating system at 14 dpf.

2.1.3 Zebrafish strains

Wild-type, ageing and pharmacological studies were conducted using AB zebrafish (*Danio rerio*). Live, *in vivo* imaging studies were performed using Tg(*elavl3:*GCaMP6s) on a mixed *casper/nacre/AB* background which produce pigmentless larvae. The mutant line was provided by Dr Matthew Winter (College of Life and Environmental Sciences, University of Exeter). Slit3 mutant line was generated in-house by CRISPR-Cas9 injections on either an Tg(*elavl3:*GCaMP6s) or AB background.

2.1.4 Sample collection for genotyping

Carriers of the Tg(*elavl3:*GCaMP6s) mutation have bright, fluorescent green brains, which were detected using fluorescence detection goggles at 3 dpf. Slit3 mutant adults were identified by fin clipping. From 3 months of age, fish were anaesthetised using Aqua-Sed (100% 2-phenoxyethanol) anaesthetic treatment (Aqua-Sed^{TM}, Vetark, Winchester, UK) in accordance with manufacturer guidelines, in 500 mL of aquarium water. Once fish were fully anaesthetised, checked with a tail pinch, they were removed from the tank and fin clipping was performed using a scalpel and removing tail fin, without damaging the body of the tail. Clippings were either snap-frozen in liquid nitrogen or stored in RNA later solution (Thermofisher, UK) and stored at -20°C until ready for downstream applications. Following fin clipping, fish were placed in a recovery tank, filled with aquarium water until they had fully recovered from the anaesthetic and then returned to home tanks, split into four sections by dividing tanks with clear acrylic dividers, with small holes, to allow fish to remain separate whilst waiting for genotyping results, whilst maintaining visual and olfactory contact. Genotyping was carried out in house. For full details on genotyping protocol see *Laboratory*.

2.1.5 Tissue collection

All fish were culled by submersion in ice cold water followed by severing the spinal cord as a secondary confirmation of death. Required tissue was removed and placed into Eppendorf tubes. Samples were either snap frozen in liquid nitrogen, or placed in RNA later solution (Thermofisher, UK) and stored at -20°C until further use.

2.1.6 Rodent husbandry

Mice were bred in house and reared with littermates (mixed sexes). Mice were housed in Allentown IVC racks and kept at 21°C (±2°C), 55% humidity (±10%) on a 12:12-hour light/dark cycle. Mice were fed on a diet of irradiated SDS RM3 pellets, with food and water available *ad libitum*.

2.1.7 Rodent strains

Wild-type strains were C57BL/6 mice (*Mus musculus).* The APP/PS1 (B6C3-Tg(APPswe,PSEN1sE9) mutant line express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9) gene which result in accelerated production of human Aβ peptide. Breeding pairs were obtained from the Jackson Laboratory (USA) and provided by Professor Jerome Swinny (School of Pharmacy and Biomedical Science, University of Portsmouth). The colony was established by crossing transgenic mice with C57BL/6J mice. Genotyping was carried out by the University of Portsmouth Animal Facility.

2.2 Behavioural testing

All behavioural testing was conducted between 9:00 am and 18:00 pm. All behavioural tests, for larvae and adult zebrafish and rodents were performed using the Zantiks behavioural units (Zantiks Ltd, Cambridge, UK). Descriptions of each behavioural task are detailed in the relevant chapters.

2.3 Laboratory Procedures

2.3.1 gDNA extraction from zebrafish tissues

This method was used to obtain gDNA from fin clippings for genotyping slit3 mutant lines. Tissue was extracted following GeneJET Genomic DNA Purification Kit protocol from ThermoFisher Scientific.

- Tail clippings were homogenized in 180 µL of Digestion Solution using a homogeniser.
- 20 µL of Proteinase K Solution was added to the sample and mixed thoroughly by vortexing.
- Samples were incubated at 56°C for ~1-2 hours, or until tissue was completely lysed and no particles were remaining. During incubation samples were regularly vortexed.
- 20 µL of RNase A Solution was added to samples and mixed thoroughly by vortexing for 15 s.
- 400 µL of 50% ethanol was added and mixed by vortexing or pipetting.
- Samples were transferred to GeneJET Genomic DNA Purification Column inserted in a collection tube. Centrifuge for 1 min at 6000 x g. Discard the collection tube containing the flow-through solution. Place the column into a new 2 mL collection tube.
- Add 500 mL of Wash Buffer I (with ethanol added) to the column. Centrifuge for 1 min at 8000 x g. Discard the flow-through and place the column back in the collection tube.
- Add 500 mL of Wash Buffer II (with ethanol added) to the column. Centrifuge for 3 min at maximum speed (~21000 x g). Discard the collection tube containing the flow-through solution and transfer the column to a sterile 1.5 mL Eppendorf tube.
- Add 50 µL of Elution Buffer to the centre of the column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 2 min at 8000 x g. Discard the column, keep flow-through for downstream applications.
- gDNA concentration was determined using nanodrop ND 100 spectrophotometer.
- Samples were stored at -20°C for short-term storage or -80°C for longer-term storage.

2.3.2 RNA extraction from zebrafish tissues

This method was used to obtain total RNA from zebrafish tissue for generating cDNA stocks. Total RNA was extracted following the RNeasy Mini Kit protocol from Qiagen.

- Up to 20 mg of tissue was homogenised in 350 µL of RTL lysis buffer using a homogeniser (for at least 30 s per sample, timings were consistent across samples).
- Samples were centrifuged at maximum speed (~ 21000 x g) for 3 min to pellet debris.
- Supernatant was transferred to sterile 1.5 mL Eppendorf tubes. 350 µL of 70% ethanol was added and mixed thoroughly by pipetting.
- 700 µL of sample was added to the RNeasy mini column in a 2 mL collection tube and centrifuged for 45 s at 9500 x g.
- The flow-through was discarded and the column placed back in the tube.
- 700 µL of RW1 buffer was added to the column and centrifuged for 45 s at 9500 x g, the flow-through was discarded.
- 500 µL of RPE buffer was added to the column and centrifuged for 1 min at 9500 x g, the flow-through was discarded and the column was placed into a new 2 mL collection tube.
- 500 µL of RPE buffer was added to the column and centrifuged for 2 min at 9500 x g, the flow-through was discarded and the column was placed back into the same collection tube.
- The column was centrifuged empty to dry the column, for 2 min at maximum speed (~21000 x g). The column was placed into a sterile 1.5 mL Eppendorf tube.
- 30-50 µL (typically 40 µL) of RNase free water was added directly to the column membrane and left for 1 min at room temperature.
- The column was centrifuged for 2 min at 9500 x g. The column was discarded and the flow-though was kept.
- Total RNA concentration was determined using nanodrop ND 1000 spectrophotometer.
- Samples, if not used immediately, were stored at -20°C for short-term storage or -80°C for longer-term storage.

2.3.3 Removal of gDNA from RNA samples

This method was used for the removal of gDNA from RNA samples which can contaminate downstream samples and artificially inflate gene expression levels, particularly when using primers that do not span an exon-exon junction. Contaminating gDNA was removed from purified total RNA (from the step above) by RapidOut DNA Removal Kit protocol from Thermo Scientific.

- Up to 8.5 µL (5 pg – 2 µg) of RNA sample was loaded into a 0.2 mL PCR tube.
- 1 µL of 10X DNase buffer with $MgCl_2$ and 0.5 µL of DNase I enzyme was added to RNA sample and mixed by vortexing.
- Nuclease-free water was added to a total volume of 10 µL (this reaction could be scaled up accordingly).
- Sample was incubated at 37°C for 30 min.
- DNase Removal Reagent (DRR) was vortexed prior to use to completely resuspend mixture.
- 2 µL of DRR was added to sample (this could be scaled up accordingly, however, minimum volume of DRR should be equal to or greater than 10% of reaction volume).
- Sample was incubated at room temperature for ~2 min, vortexing 2-3 times to maintaining a homogenous solution.
- Centrifuge sample at ~800 x g for 1 min to pellet the DRR. Remove supernatant (do not transfer any DRR reagent as this can interfere with downstream applications) and transfer to a new PCR tube.
- To determine final RNA concentration following gDNA removal use nanodrop ND 1000 spectrophotometer.
- Samples, if not used immediately, were stored at -20°C for short-term storage or -80°C for longer-term storage.

2.3.4 cDNA synthesis from total RNA

This method was used to convert the mRNA from total RNA into cDNA to be used in qPCR reactions to quantify mRNA expression. Reverse transcription was done using the High Capacity RNA-to-cDNA Kit protocol from Applied Biosystems.

- To ensure the same amount of cDNA was synthesised for each sample a maximum of 1 µg of total RNA was used per reaction (loaded RNA concentration was determined based on the sample with the lowest concentration per experiment. The same total concentration of RNA was loaded into each reaction with variable volumes of water added to make up the total reaction volume).
- 10 µL of RT Buffer Mix and 1 µL of RT Enzyme Mix were aliquoted per reaction.
- Up to 9 µL of RNA, plus nuclease-free water was added to the reaction to a total volume of 20 µL per reaction.
- A thermocycler was used for the reaction, using the following conditions:

  37°C for 60 min

  95°C for 5 min

  4°C for ∞
- Samples that were not immediately used were stored at -20°C for short-term storage or -80°C for longer-term storage.

2.3.5 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

All reagents and templates were stored at -20°C or -80°C. Primer stocks were made up to 100 µM concentration in nuclease-free water. Working aliquots were 10 µM of forward and reverse primers. Two SYBR green master mixes were used throughout depending on the protocol being run. For samples requiring high resolution melt curve analysis the Roche LightCycler 480 High Resolution Melting Master was used. For other applications, the Thermo Scientific PowerTrack SYBR Green PCR Master Mix was used. The basic reaction volumes and cycling conditions are provided for both master mixes, however, some conditions vary depending on samples and primers. The protocol below is the result of extensive optimisation, however, some samples/genes required slightly altered conditions and therefore detailed protocols are provided in the respective chapters.

Roche LightCycler 480 High Resolution Melting Master

- 10 µL of Master Mix (SYBR Green)
- 1 µL of primer pairs (10 µM stock)
- 2.4 µL of $MgCl_2$ (25 mM stock)
- 5 µL DNA template (5 ng/ µL stock-cDNA/gDNA)
- 1.6 µL ddhH_2O

An initial reaction volume of 20 µL was made up. 10 µL was aliquoted per well to provide replicate samples.

Running conditions:

95°C for 600 s - x1

95°C for 20 s
58°C for 25 s } x40
72°C for 35 s

For HRM analysis, high resolution melt curve analysis was completed after the qPCR as follows:

95°C for 60 s - Ramp rate 4.4°C/s

40°C for 60 s - Ramp rate 2.2°C/s

65°C for 1 s - Ramp rate 2.2°C/s

97°C for 1 s - Ramp rate-0.04°C/s, continuous acquisition, 25 readings/°C

Thermo Scientific PowerTrack SYBR Green PCR Master Mix

- 10 µL of Master Mix (SYBR Green)
- 1 µL of primer pairs (10 µM stock)
- 0.5 µL of loading dye
- 2-4 µL DNA template (5 ng/ µL stock-cDNA)
- 6.5 µL ddhH_2O

An initial reaction volume of 20 µL was made up. 10 µL was aliquoted per well to provide replicate samples.

Running conditions:

95°C for 120 s - x1

95°C for 15 s
60°C for 25 s
} X35

2.3.6 PCR

This method was used for amplifying amplicons of >300 bp. The main application of PCR was to amplify samples for genotyping and sending for Sanger sequencing.

- 12 µL of VWR Red Taq Master Mix
- 1 µL of primer pairs (10 µM stock)

- 0.5 µL of $MgCl_2$ (25 mM stock)
- 2 µL DNA template (10 ng/ µL stock-gDNA)
- 9.5 µL ddhH_2O

Total volume loaded per reaction was 25 µL.

Running conditions:

95°C for 2 min - x1

95°C for 20 s
60°C for 20 s } x30
72°C for 30 s

72°C for 5 min - x1

4°C for ∞

2.3.7 Gel electrophoresis

RNA/DNA samples were typically run on a 1.5% (w/v) agarose gel, which was prepared by mixing 0.75g of agarose with 50 mL of 1X TBE buffer in a conical flask. The mixture was heated in the microwave until it was a completely homogenous solution. 5 µL of SYBR safe (Thermo Scientific) was added and gently swirled to mix. The liquid agarose was then poured into a casting tray containing a comb for loading wells and left to set. Once solidified, the ends of the cast were removed and the gel was placed into the gel doc. 1X TBE buffer was added until the entire gel was emersed, then the comb was removed. For loading a 50 bp ladder (1 µL (GeneRuler, Thermo Scientific) + 1 µL of 6X TriTrack DNA Loading Dye + 4 µL nuclease-free water) and sample (5 µL of DNA (~0.5 µg DNA, equal concentration to ladder) + 1 µL of 6X TriTrack DNA Loading Dye) were prepared and a total of 6 µL was loaded onto the gel. The gel was run at 100 V and visualised using a gel imager.

2.3.8 Amplicon purification

This method was used to purify post-PCR reactions for further downstream processing via enzymatic purification in accordance with ExoSAP-IT PCR Product Cleanup protocol from Applied Biosystems. This method was used to purify post-PCR reactions for further downstream processing via enzymatic purification in accordance with ExoSAP-IT PCR Product Cleanup protocol from Applied Biosystems.

- 5 µL of the post-PCR reaction product was combined with 2 µL of ExoSAP-IT reagent (reaction volumes could be scaled up as required).
- Incubate at 37°C for 15 min to degrade remaining primers and nucleotides.
- Inactivate the enzyme by heating the reaction to 80°C for 15 min.

2.3.9 T7 Endonuclease I assay (T7E1)

This method was used to detect heterozygous mutations following CRISPR/Cas9 injections using T7 Endonuclease I Kit protocol from New England Biolabs.

- 200 ng DNA from post-PCR reaction product (amplicon ≥500 bp).
- 2 µL of 10X NEBuffer 2 was added to reaction.
- Nuclease-free water was added to a final volume of 19 µL.

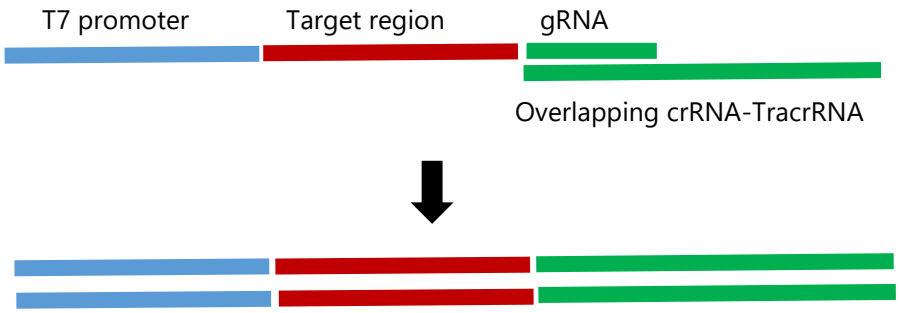
Hybridization was conducted using a thermocycler using the following conditions:

- 95°C for 5 min
- 95-85°C - Ramp rate -2°C/s
- 85-25°C - Ramp rate -0.1°C/s
- 4°C for ∞
- Once the reaction is completed at 1 µL of T7 Endonuclease I and incubate at 37°C for 15 min.
- Stop the reaction by adding 1.5 µL of 0.25 M EDTA.
- Samples are run on an agarose gel for identifying heterozygous mutations.

2.3.10 CRISPR/Cas9 genome editing

This method was used for CRISPR gene editing of specific loci in the genome of zebrafish. Here it was used to create a point mutation in the *slit3* gene.

- 20 nucleotide target sequence was designed using ChopChop web tool for selecting target sites for CRISPR/Cas9 knock-out that result in frameshift mutations in the gene of interest, with no-low predicted off-target binding sites.
- Target sequences were designed using traditional Cas9 PAM sequence (NGG).
- Several target sequences were selected based on off-target sites, mismatches and predicted efficiency.
- Target sequences were validated using NCBI Blast, UCSC genome browser and Ensembl genome browser.
- T7 promoter (NGG) sequence was added at the 5′ of the target sequence and an overlapping crRNA-TracrRNA sequence at the 3′ end of target sequence:



Oligo assembly and preparation of sgRNA

To complete oligo assembly of the sgRNA a T7 promoter (GG) sequence was added to the 5′ end of the *slit-3* specific sequence and at the 3′ end an overlapping crRNA-TracrRNA sequence.

Slit-3 targeting oligo:

TAATACGACTCACTATA GGAGGACTCACATGGTGGAA GTTTTAGAGCTAGAAATAGC

PCR mix: 25 µL (GoTaq) PCR master mix

1 µL Forward primer (slit-3 gRNA stock)

1 µL Reverse primer (crRNA-TracrRNA, universal T7 tail)

1 µL $MgCl_2$

22 µL ddH_2O

Total volume = 50 µL

- PCR run: Denature at 98℃ for 2 minutes

  Anneal at 50℃ for 10 minutes

  Extend at 72℃ for 10 minutes
- NanoDrop 1000 for sample concentration.
- Stored at -20℃ until use

2.3.11 Embryo injections

- Using newly synthesised (guide) gRNA, combine with phenol red, water and Cas9 RNA using the following concentrations: 300 pg of Cas9 RNA, 50 pg of sgRNA
- Vortex injection mix
- Set incubator to 24℃ to slow cell division
- Backfill pulled needle using Hamilton syringe and mineral oil
- Drop sizes are set to 1.4 nL
- Injections are nuclear (or as close the nucleus as possible)
- Upon injection completion embryos are returned to petri dishes filled with aquarium water, dead embryos are removed
- Normal husbandry is resumed.

Chapter 3

A commonly reoccurring theme in the discussion of understanding and treating neuropsychiatric and neurodegenerative disorders is the stall in the development of novel therapeutic strategies, as has been discussed above. A critical issue is that effective treatments in animal models are seldom effective in patients or have unexpected side effects. One regularly quoted problem is the lack of translationally relevant behavioural tasks for assessing analogous domains in animals and humans. The following chapter addresses this translational gap by discussing the development of a simplistic cognitive test aimed at assessing WM and cognitive flexibility. This task has been validated with zebrafish and evaluated for cross-species efficacy, not only to humans, but to other animal models as well. The aim of this work is to develop a task that can improve the success of translating findings from preclinical tests to clinical treatments.

The Free-Movement Pattern Y-Maze: A Cross-Species Measure of Working Memory and Executive Function

This chapter has been published (submitted formatting has been retained):

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3.1 Abstract

Numerous neurodegenerative and psychiatric disorders are associated with deficits in executive functions, such as WM and CF. Progress in developing effective treatments for disorders may benefit from targeting these cognitive impairments, the success of which is predicated on the development of animal models with validated behavioural assays. Zebrafish offer a promising model for studying complex brain disorders, but tasks assessing executive function are lacking. The Free movement pattern (FMP) Y-maze combines aspects of the common Y-maze assay, which exploits the inherent motivation of an organism to explore an unknown environment, with analysis based on a series of sequential two-choice discriminations. We validated the task as a measure of WM and executive function by comparing task performance parameters in adult zebrafish treated with a range of glutamatergic, cholinergic and dopaminergic drugs known to impair WM and CF. We then demonstrated the cross-species validity of the task by assessing performance parameters in adapted versions of the task for mice and *Drosophila*, and finally a virtual version in humans, and identify remarkable commonalities between vertebrate species whilst navigating the maze. Together, our results demonstrate that the FMP Y-maze is a sensitive assay for assessing WM and CF across species from invertebrates to humans, providing a simple and widely applicable behavioural assay with exceptional translational relevance.

3.2 Introduction

Neurodegenerative and neuropsychiatric disorders are widespread causing premature morbidity and increasing social and personal burden (Feigin et al., 2019; Jongsma et al., 2019). These disorders are characterised by diverse cognitive impairments, which can vary significantly within diagnoses, but often have overlapping deficits between disorders (Cope et al., 2016). Impairments in WM and CF are commonly reported in many neurological and neuropsychiatric disorders, such as AD (Guarino et al., 2019a), Parkinson's disease (Handra et al., 2019; Koerts et al., 2011), SZ (Giraldo-Chica et al., 2018; Orellana & Slachevsky, 2013), depression (Darcet et al., 2016; Hammar & Årdal, 2009; Snyder, 2013), substance abuse (Cunha et al., 2010; Gould, 2010), and ASD (Craig et al., 2016; Demetriou et al., 2019). Impairments in WM and CF are well-defined behavioural endophenotypes (Harro, 2019; Parker & Brennan, 2012; Wong & Josselyn, 2016) and combined with animal models have become an integral part of translational research (Fontana et al., 2018). However, animal models and behavioural assays have become increasingly diverse, limiting the behavioural fidelity across model species and in clinical findings in human subjects (Day et al., 2008; Young et al., 2009). Therefore, to improve validity of cross-species paradigms there is a need to design assays of executive function that target the same behavioural dimensions or neurobiological measures in a range of species, including humans, to increase validity and translational relevance (Homberg, 2013; Markou et al., 2009).

There is a diverse array of experiments used for assessing animal cognition, with mazes among the most popular (Paul et al., 2009). Existing in numerous behavioural paradigms, the maze can be designed to vary in complexity and target phenotype depending on the task parameters (Sharma et al., 2010). The Y-maze is one of the simplest methods and has been used extensively in learning and memory paradigms for both rodent (Arendash et al., 2001; Conrad et al., 1997; King & Arendash, 2002; Lainiola et al., 2014; Ma et al., 2007) and zebrafish (Aoki et al., 2015; Cognato et al., 2012) models. There are two commonly used methods, the two-choice task in which there is a 'starting' arm, a 'blocked' arm and the 'other' arm. In the first trial, the animal is free to explore, and upon entry into the unblocked arm, is returned to the starting arm. On the second trial, the previously blocked arm is opened.

Measurements are recorded for time spent exploring the novel arm (Lalonde, 2002). The alternative method is the continuous Y-maze, in which animals are permitted free exploration throughout the trial, typically lasting 5-8 minutes. The sequence of arm entries is recorded and WM capability is determined by the percentage of spontaneous alternation (entry into three different arms in succession) (Hughes, 2004). The Y-maze is proving a useful tool for providing test conditions that do not require rule learning, extensive handling or repeated manipulation (Heredia-López et al., 2016). Other maze tasks, such as the T-maze and radial arm maze require extensive training, high levels of animal handling and, in reward-based trials, food or water deprivation for prolonged periods (Anderson et al., 2000; Bizon et al., 2007; Deacon, Nicholas, et al., 2006; Kotagale et al., 2020; Schmitt et al., 2003; Sharma et al., 2010). Each of these factors can result in potential confounders, leading to high levels of between-subject variability (Sharma et al., 2010)

Although valuable, the Y-maze task has several limitations. Some studies have reported difficulties interpreting results, particularly if models tested had hypo or hyperlocomotion, stereotypic behaviours or anxiety-related novelty avoidance, as a consequence of the test condition or treatment, which could significantly interfere with the measurement of spontaneous alternation. (Herbert & Hughes, 2009; Hughes, 2004; King & Arendash, 2002; Kumar et al., 2015; Miedel et al., 2017; Stewart et al., 2011). A primary issue, as raised by (Stewart et al., 2011) is that a perfect score in the continuous Y-maze, as is currently measured, is a reflection of highly stereotyped behaviour. Therefore interpretation of results can be confusing when test models present with repetitive or perseverative behaviours (Cash-Padgett et al., 2016; Miedel et al., 2017).

To address the limitations of current maze methods, we have designed the Free movement pattern (FMP) Y-maze, a physical maze for animal models and a virtual maze for humans that is analogous to animal versions. The FMP Y-maze is a continuous protocol, run using automated tracking software, with built in data logging of arm entries, aimed at minimising experimenter handling, interference and bias of data interpretation. Our method of data analysis has been developed to allow detail of complex patterns of exploration, using sequences of left and right turns apportioned into 16

overlapping tetragrams (four choices) of left/right combinations ranging from LLLL to RRRR, subsequently shifting the focus away from novelty response to navigational search patterns. Stereotypic responses have been classified as particular search strategies, the presence of which do not overlap with other patterns of normal exploration. Other confounds such as altered locomotor responses are accounted for in the analysis. Use of each sequence pattern is calculated as a proportion of total turns (percentage) and analysed using total turns as a covariate in a general linear mixed model, thus preventing potential inflation of results due to hyper-activity of treatment groups compared to control groups. Prevention of anxiety responses has been diminished by the extension of the run time to 1 h of free exploration. Not only does this permit a habituation period, it removes the need for any pre-trial training and additionally, the extended trial time also allows this method to assess WM and CF in a single paradigm without having to interfere with any of the task parameters during the trial.

To validate the FMP Y-maze as a measure of WM and CF we systemically blocked glutamatergic, cholinergic and dopaminergic pathways, all known for roles in executive function (Blake & Boccia, 2018; Cools & D'Esposito, 2011; Ellis & Nathan, 2001; Ragozzino, 2002; Ragozzino et al., 2002) and dysregulation of these systems has been linked to neurodegenerative and neuropsychiatric disorders (Ballinger et al., 2016; Brisch et al., 2014; Herman & Roberto, 2015; Hindle, 2010; Murueta-Goyena et al., 2017). Additionally, we used time series analysis and autocorrelation to model effects on WM. Zebrafish treated with antagonists compared to control groups, demonstrated decreased WM capacity and changes in search patterns, which were influenced by altered behavioural flexibility. We further validated this task with a range of organisms, including *Drosophila,* mice and humans. Mazes were adapted to suit each organism, but behavioural measures were consistent in all versions. Findings suggested that vertebrate species, zebrafish, rodents and humans, explored in similar patterns, however, invertebrates adopted an alternative search strategy. Combined, our findings validate the FMP Y-maze as a test of executive function in a range of model organisms, including humans, to create a multifunctional task with high cross-species and translational relevance.

3.3 Experiment 1

Experiment 1 was designed to determine the exploration strategy of zebrafish in the FMP Y-maze (**Fig. 1**). Without prior training or habituation, fish freely explored the novel arena for 1 hour (h) with continuous recording of arm entries and exits for the duration of the trial. The absence of reinforcement meant that fish did not require periods of pre-trial food deprivation, therefore fish were directly taken from home tank to test tank, back to home tank, minimising handling and stress in accordance with the 3Rs (Sneddon et al., 2017). Our primary aim was to identify if the FMP Y-maze could be used as a test of memory. Data from the task was output as a discrete time series (Boyce et al., 2010; Mwaffo et al., 2015), from which we mathematically modelled the randomness of serial observations (Robinson, 2003). Using the two-choice guessing task system, introduced by Frith and Done (1983), tetragram analysis was used to identify discernible patterns that departed from a random process (Frith & Done, 1983; Gross et al., 2011). Sequential left and right turns were grouped into overlapping sequences of four turns (tetragrams), giving a total of 16 possible tetragram sequences. The sum of each of 16 overlapping tetragrams of left and/or right turns (e.g. left, left, left, left [L,L,L,L] or right, right, left, left [R,R,L,L]) were analysed to identify strategic search patterns.

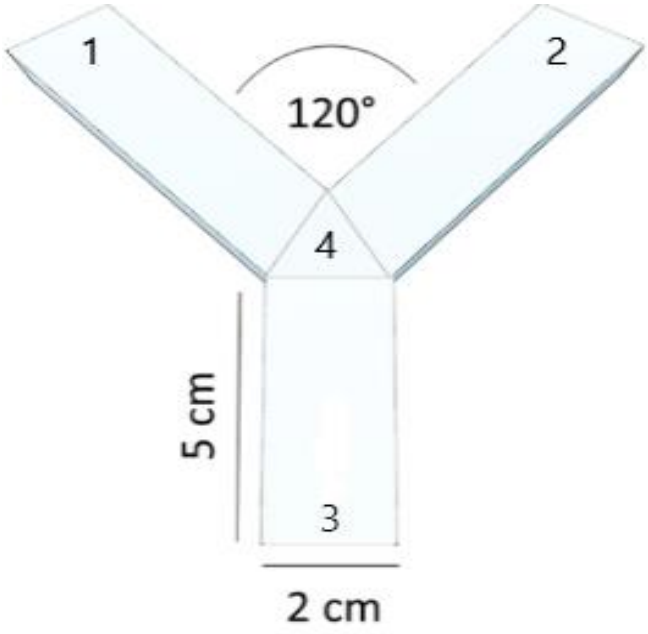

Figure 1. FMP Y-maze diagram depicting maze dimensions and zones used for automated logging of arm entries and exits.

Materials and Methods

Animals and housing

A total of N=18 zebrafish (*Danio rerio*) of AB wild-type strain (4 months-old at time of testing) male and female (~50:50) were bred in house and raised in the University of Portsmouth Fish Facility. Extensive pilot and published work from our lab has revealed no differences in search strategy between male and female zebrafish (Fontana, Cleal, & Parker, 2019). Fish were housed in groups of 8-10 per 2.8L tank on a re-circulating system (Aquaneering Inc., San Diego, CA, USA). Sample sizes were calculated based on power analyses ($\alpha = 0.05$; $\beta = 0.8$) from effect sizes observed in pilot studies, and previous published work from our group (Cleal & Parker, 2018). Room and tank temperatures were maintained at 25-27°C on a 14:10-hour light/dark cycle, water was aquarium treatment (dechlorinated) and pH was 8.4 (±0.4). Fish were fed on ZM fry food from 5 days post fertilisation (dpf) until adulthood when they were moved onto a diet of flake food and live brine shrimp (ZM Systems, UK) 3 times/day (once/day on weekends).

On completion of behavioural testing fish were culled using Aqua-Sed anaesthetic treatment (Aqua-Sed™, Vetark, Winchester, UK) in accordance with manufacturer guidelines.

Apparatus

Behavioural testing was carried out in the Zantiks AD system for adult zebrafish (Zantiks Ltd., Cambridge, UK). Zebrafish were tested in white acrylic Y-maze inserts of two identical mazes (provided with the AD Zantiks base package) fitted into a black water-tight tank with a transparent base (https://www.zantiks.com/products/zantiks-ad) (**Fig 2.**). Maze dimensions were as follows: L50, W20, H140 (mm). Tanks were filled with 3 L of aquarium water. Each system was fully controlled via a web enabled device (laptop, phone or tablet). Filming was carried out from above, which allowed live monitoring within the behaviour system (**Supplemental video 1**). The FMP Y-maze had three equally sized arms which had no intra-maze cues. However, extra-maze (distal) cues were visible from within the maze (e.g., walls and open side of the Zantiks equipment which allowed a small amount of light in). These egocentric cues allow fish to orientate within the maze, but previous studies have demonstrated that these cues do not influence exploratory behaviour (data not shown) (Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). For consistency between tests, light levels were maintained at a consistent level, at a maximum of 2 lux during exploration.

Procedure

The protocol was based on that described in our previous papers (Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Animal handling and experimenter visibility were both kept to a minimum. Fish were netted directly from home tanks into FMP Y-mazes, inserted into test tanks, prefilled with 3L of aquarium water. Test tanks were placed inside the Zantiks behaviour unit. Water was allowed to settle before starting the protocol to ensure accurate tracking of fish. This step is important as the initial detection of the animal is crucial to ensure that tracking is accurate throughout the trial. Once the system has successfully detected the animal a white cross will appear over the animal

which will continuously track its movements and log zone entries and exits. Two fish were tested in each behavioural apparatus. Data were initially output as a time series of arm entries and exits, normalised (proportions of total turns) and analysed according to 16 overlapping tetragrams (RLLR, LLRR, RRRL, etc.) (**Table 1**) of which particular note was taken with regard to search strategies termed alternations (RLRL, LRLR) and repetitions (LLLL, RRRR), having previously seen that these are most notably affected by different treatments. If the fish were adopting a random search strategy, it would be predicted that the distribution of tetragrams over a 1 h period would be approximately stochastic (i.e., the relative frequency of each tetragram ~6.25%), and the data would generate autocorrelation plots equivalent to white noise (all lagged data points would fall below the 95% confidence interval).

Figure 2.

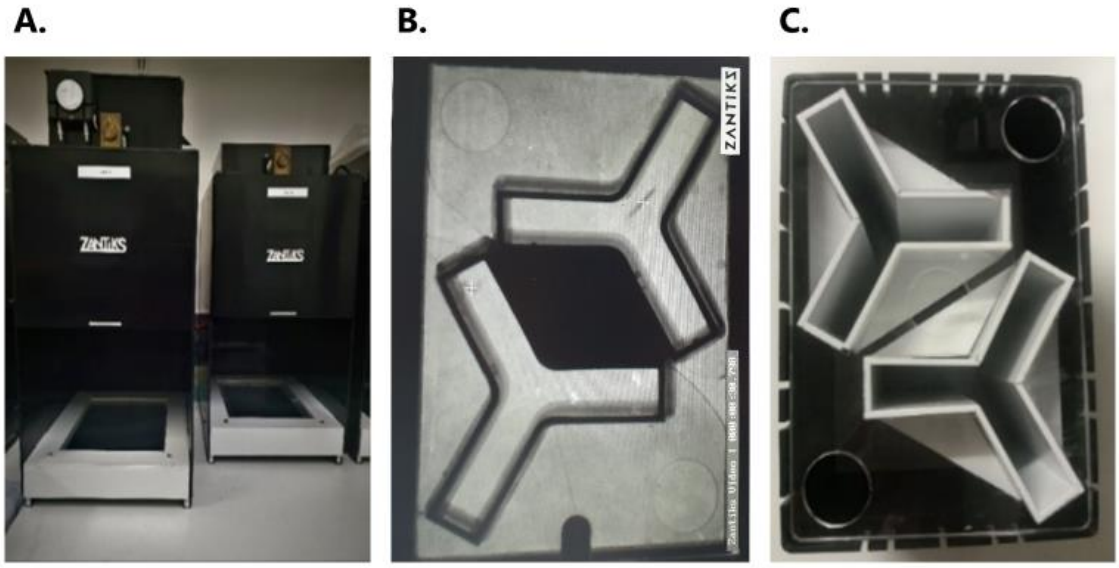

Figure 2. Aquatic FMP Y-maze for zebrafish. (**A**) Zantiks AD behavioural unit for automated animal tracking. (**B**) Top-down view of two FMP Y-mazes for zebrafish inserted into a black water-tight tank, L50:W20:H140mm, filled with 3 L of aquarium water. A mesh lid was used to cover the top of the tank to prevent fish from jumping out during the trial, without interfering with the tracking software. (**C**) In trial image of zebrafish in the FMP Y-maze (n=2).

All experiments conducted for this study were carried out following approval from the University of Portsmouth Animal Welfare and Ethical Review Board, and under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: P9D87106F]

Table 1.

Tetragram analysis was based on a series of 16 unique, overlapping sequences of left and/or right turns. Below is a list of each tetragram used for analysis, with reference to key strategies and the associated term.

Sequence	Term	Step length	Sequence	Term	Step length
LLLL	Repetition	-8	RLRL	Alternation	1
LLLR		-7	RLLR		2
LLRL		-6	RRLL		3
LRLL		-5	LRRR		4
RLLL		-4	RLRR		5
LLRR		-3	RRLR		6
LRRL		-2	RRRL		7
LRLR	Alternation	-1	RRRR	Repetition	8

Data preparation and analysis

Tetragram Analysis. In a test paradigm consisting of two equally likely choice variants; left (L) or right (R) turn, we assume choice selection to be completely random. However, we know from human behaviour in guessing tasks (Paulus et al., 1999; Stroe-Kunold et al., 2009), or animals in choice behaviour tasks, such as rodents in a T-maze, there is a preference to alternate L and R turns. Even in paradigms of equal arm reinforcement choices are never completely random (Deacon, Nick, et al., 2006; Robert

Gerlai, 1998). In a Markov process, a process of completely random events, the probability of choosing L or R depends only on the most recent choice (Grecian et al., 2018). For example, the probability of turning L would be:

$$P(L) = ½,$$

regardless of whether the previous turn had been L or R. Despite the overall process being random, it is possible to detect patterns in large data series by dividing sequences into groups of like-terms and using information theory to detect any departures from randomness (Meehl & Guze, 1993). Let p_i be the probability of event i in a time series, such as the probability of turning L or R. Using general information theory, the first order 'uncertainty' of turning L after previously turning R can be measured using:

$$L = \sum p_i \log_2 p_i,$$

where base 2 for the logarithm stipulates that from two equally likely events (L or R), one choice (one unit of information) is transmitted to resolve the uncertainty of the occurrence of either choice. Relative uncertainty, L_{max}, is the ratio of observed L turns to maximum L turns, for the given number of alternatives, the complement of this is:

$$1\text{-}L/L_{max}$$

Different levels of complexity can be used to determine the probability of turning L based on two previous turns, LR (digram), three previous turns, LRL (trigram), four previous turns, LRLR (tetragram), etc. The larger the number of alternative choices the greater the computational power required. Previous work has demonstrated that in human guessing tasks, examination of past events exceeding four or five choices becomes irrelevant when calculating the probability of a current event (Hochberg & Attneave, 1961; Meehl & Guze, 1993). Therefore, in line with previous two-choice guessing task protocols, we have selected to concentrate on the use of tetragram sequences, limiting the number of alternatives to 2^4 =

16 possible tetragram sequences. The information measure for a sequence of four turn choices for turning L is:

$$L_4 = L(\text{tetragram}) - L(\text{trigram})$$

Tetragram analysis was used to identify patterns over long and short periods of exploration. Tetragram sequences were examined for 'immediate' search strategies, those performed during 10 minutes of exploration and 'global' search strategies that were a consensus of the overall strategy used for the entire hour of exploration. Division of analysis into immediate and global strategies allowed data to be collected on the general exploration strategy and how this strategy was affected by time. This permits examination of multiple characteristics of executive function.

Time series analysis. Time series, $\chi_n = \chi_1, \chi_2 \ldots\ldots\ldots\ldots \chi_k$ were defined as step length, $\omega(k)$, at discrete time-point, *k*, where *k* was representative of equal length time points comprised of tetragram sequences. Therefore, each point in the time series was equal to one tetragram, described as one step. Each experiment was made up of *n* time points. The autocorrelation lag coefficients of steps were calculated for each individual, using step length, $\omega(k)$. ACF was computed in PYTHON using MATLAB (Pal & Prakash, 2017). The lag-1 autocorrelation for the corresponding time lag *k* is:

$$\text{ACF}(k) = \frac{\sum_{s-1}^{T-k}(\omega(s)-\bar{\omega})(\omega(s+k)-\bar{\omega})}{\sum_{s=1}^{T}(\omega(s)-\bar{\omega})^2},$$

where $\bar{\omega}$ is the mean step length for that individuals time series, $\omega(k)$. As the model demonstrated non-stationary and non-random properties, the usual calculation of confidence interval, $\bar{\omega} \pm 2\sigma / \sqrt{n}$, where σ is the standard deviation, was not used. Instead, the 95% confidence interval was based on a moving average calculated using the Bartlett test:

$$T = \frac{(n-k)\, ln\, \sigma_p^2 - \sum_{i=1}^{k}(n_i - 1)\, ln\, \sigma_i^2}{1 + \left(1/\left(3(k-1)\right)\right)\left(\left(\sum_{i=1}^{k} 1 \,/\, (n_i - 1)\right) - 1 \,/\, (n-k)\right)}$$

Where σ_i^2 is the variance of the *i*th group, *n* is the total number of steps, n_i is the step length of the *i*th group, *k* is the number of groups and σ_p^2 is the weighted mean of the group variances, defined as:

$$\sigma_p^2 = \sum_{i=1}^{k}(n_i - 1)\sigma_i^2 \,/\, (n - k)$$

Tetragram sequences were used to define step length and fix time intervals of the discrete time series. Each sequence was arbitrarily assigned a value ranging from 1 to 8. Left-dominant sequences were arbitrarily denoted as negative, whilst right-dominant sequences were positive (**Table 1**), from this point on referred to as 'steps'. Each step was assumed equal time, therefore each observation in the time series was one tetragram sequence or the equivalent of one step. The analysis for zebrafish were based on 1000 arm entries, sequentially divided into overlapping sequences of four arm entries, resulting in a total of 250 steps, *n*=250 time points. The limit was chosen arbitrarily for consistency only as total turns varied between individuals. Animals with more than ten steps of missing data were excluded from subsequent time series analysis. Animals with fewer than ten missing steps had zeros replacing missing values to make up the total number of steps required. The cumulative sum of steps was used to determine the relationship between successive observations and identify if steps were taken randomly and completely independent of one another. This was tested by computing the lag plot and autocorrelation function (ACF) using a custom designed script in MATLAB.

Statistical Analysis

All turn choices recorded in the FMP Y-maze were converted into tetragrams using customised Excel spreadsheets. Each tetragram sequence was reported as a percentage of total turns completed in the allotted trial time. Based on previous research, alternation (LRLR, RLRL) and repetition (RRRR, LLLL) sequences were analysed independently as dependent variables, as these were the most frequently observed amongst all species. Data were fitted to a linear mixed effect model (LMM), with "time" as a within-subjects factor, "total turns" as a covariate, to control for general activity levels in statistical models and "ID" as a random factor. Significant effects were followed by Tukey's *post hoc* multiple

comparison test in which each organism was compared to all other organisms. Alpha values of $p \leq 0.05$ were considered statistically significant. Data were presented as means ± S.E.M.s.

Results and Discussion

Analysis of tetragram sequences used as a global strategy (over the course of the entire trial) revealed that adult zebrafish demonstrated use of a strategy that was significantly dependent on tetragram sequences containing alternating left and right turns (LRLR, RLRL), referred to as alternations (one-way ANOVA: $F_{(15, 272)} = 17.31$, $p<0.0001$; n=21). Although similar to the alternating pattern from the T-maze, in the FMP Y-maze alternations were not used exclusively (which might be consistent with stereotypic behaviour), but instead were distributed regularly throughout the trial (**Fig. 3**). Alternations were used as a search strategy ~26% of the time, regularly dispersed with other combinations of the remaining 14 tetragrams. The regular occurrence of a specific type of tetragram, the alternation, indicates a complex level of behaviour in which the preceding trigram sequences LRL or RLR, are predictors that the following turn choice will be a R or L turn respectively, demonstrating strong intra-sequence dependencies. Thus, despite the overall probability of turning L or R being equally likely, the use of tetragram analysis has revealed the presence of a repeating pattern within the data, resulting in a deviation from complete randomness.

Figure 3.

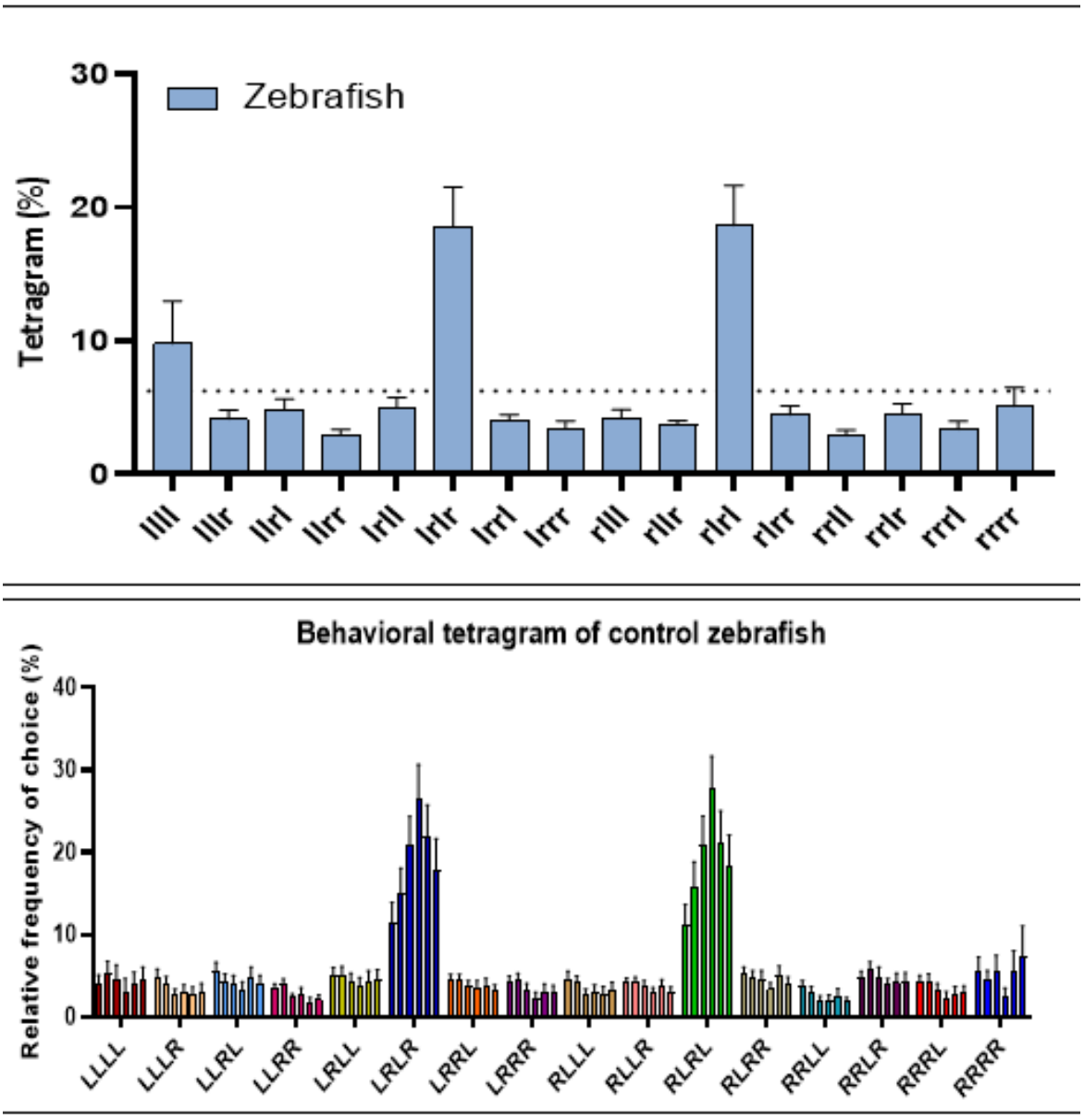

Figure 3. Frequency distribution of global tetragram strategy over the course of 1 h of exploration in the FMP Y-maze (N=18). The dashed line represents random selection, at 6.25%. Dominant strategy preferentially uses alternation patterns (LRLR, RLRL) (**Top**). Percentage use of each tetragram sequence per 10 min of exploration, demonstrating a clear dominant use of alterations throughout the trial, fluctuating over time (**Bottom**). Bars represent mean, error bars are mean ± SEM.

Although tetragram analysis can be used to identify preferential turn choices and dependency of a choice based on the three preceding turns, it cannot be used to determine the persistence of that dependency. Put simply: for a turn choice at position *i*, to what extent are subsequent future turns influenced? Using the lag-1 autocorrelation function (ACF) it is possible to determine the relationship between successive tetragram sequences and identify if dependency lasts beyond the tetragram set (Bailey & Thompson, 2006). ACFs that rapidly decay, fluctuating around zero, are indicative of a completely random, or memoryless process (Stadnytska & Werner, 2006), i.e. a Markovian process (Reynolds, 2010). However, as we have demonstrated strong intra-sequence dependency of specific tetragrams, we know that turn choice is not random. However, there is no indication of whether a tetragram can influence future tetragram sequences.

Our evidence strongly suggests that movement patterns were the result of a global strategy, relying on memory of past turn choices. We therefore hypothesised that subsequent steps (each step representing a tetragram) would demonstrate significant autocorrelation, which would be suggestive of a time series with *memory* of previous events, which exert influence on choice-behaviour for a large number of steps. We found that time series plots for individual zebrafish showed either left or right bias, but the ACF of the cumulative sum of steps showed prolonged autocorrelation, which decayed slowly to zero (**Fig. 4**). These 'long-range correlations' between turn choices reflect a long-lasting effect of previous behaviour on subsequent choice-behaviour. In sum, our data suggest that the generation of the behavioural sequences of turns by wild type adult zebrafish in the FMP Y-maze are characterised by long-range and significant non-random relationships between steps across a large range of responses.

Figure 4.

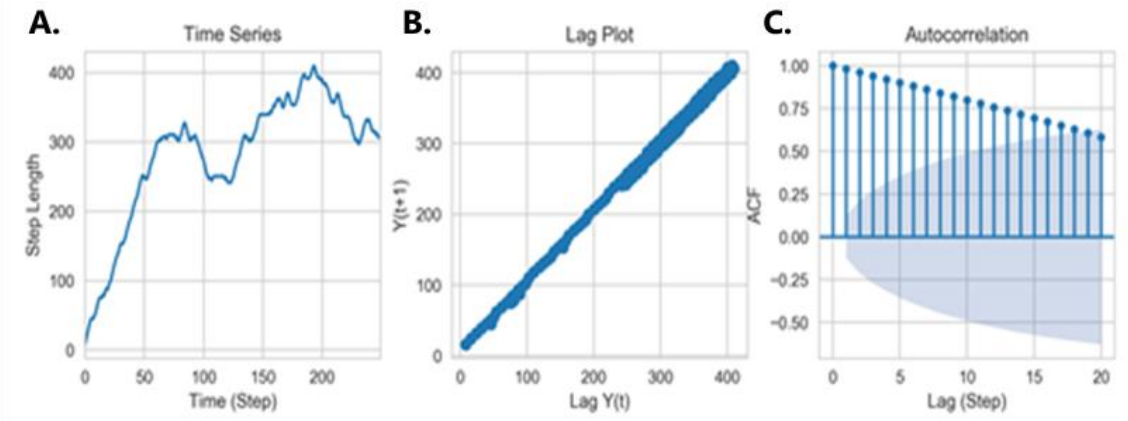

Figure 4. Time series analysis of movement patterns of an individual zebrafish, zf11 (n = 1), showing time series plot of the cumulative sum of step length for n = 250 time points (**A**). Lag plot of data at lag-0 (ω(k)) and lag-1 (ω(k + 1)) demonstrating a positive linear correlation (**B**). Autocorrelation function plot showing the first 20 lags of 250 lag plot (**C**). The plot shows slow decay towards zero, with 18 lag points outside of the 95% CI, depicted by the blue cone. Autocorrelation between data points is indicative of dependency between successive turn choices, demonstrating memory of previous choice events

3.4 Experiment 2

In Experiment 1, we characterised search strategies in the FMP Y-maze and demonstrated that zebrafish rely on WM to formulate search strategies. To further substantiate the use of memory to navigate the FMP Y-maze we pharmacologically targeted neurotransmitter systems involved in memory processing. The glutamatergic, cholinergic and dopaminergic systems are well documented for their roles in executive functions, particularly WM (Ellis & Nathan, 2001; Handra et al., 2019; Myhrer, 2003). Both human and animal studies have demonstrated that pharmacologically blocking these pathways can lead to impairments in WM tasks (Myhrer, 2003). We hypothesis that blocking N-Methyl-D-aspartic acid (NMDA), muscarinic and D1 receptors would lead to a reduction in alternations due to impaired WM. However, as D2 receptors are strongly associated with reward and motivation learning and memory processing (El-Ghundi et al., 2007; Kwak et al., 2014), we expect pharmacologically blocking D2 receptors

will not affect search strategy as exploration is conducted in the absence of reward. To this end, we pre-treated zebrafish with a high, mid and low concentration of four antagonists, inhibiting key receptors in the memory process: MK 801, a non-competitive NMDA receptor (NMDA-r) antagonist known to impair WM by inhibiting long-term potentiation (LTP) (Adler et al., 1998; Lisman et al., 1998; Nam et al., 2004; Nicoll, 2017; Shapiro & Caramanos, 1990); scopolamine, a non-specific muscarinic receptor (M-r) antagonist, similarly to MK-801, reduces LTP in the hippocampus and impairs WM (Ellis et al., 2005; Granon et al., 1995; Hirotsu et al., 1989), SCH-23390, a D1 receptor antagonist and sulpiride, a dopamine D2 receptor antagonist (El-Ghundi et al., 2007; Sylvie Granon et al., 2000; Klanker et al., 2013).

Materials and Methods

Animals

Animals were housed under the same conditions as Experiment 1. A total of N=166 animals were used. Fish were assigned at random to each treatment group from 10 groups of n=15-20 fish per 6 L tank.

Apparatus

The apparatus was identical to Experiment 1.

Procedure

Pharmacological treatments.

To examine the effects of MK801 (Sigma-Aldrich), scopolamine (Sigma-Aldrich), SCH-23390 (Tocris) and sulpiride (Sigma-Aldrich) on performance in the FMP Y-maze, fish were randomly allocated (from >10 groups of age-matched stocks in our fish facility) to a drug treatment group with ~13 fish assigned per group (n=18 control per drug group; MK801: n=13 0.1 mg/L, n=13 0.75 mg/L, n=13 2.0 mg/L; Scopolamine: n=13. 0.25 mg/L, n=13 0.5 mg/L, n=13 1.0 mg/L; SCH-23390: n=12 0.5 mg/L, n=12 1.0

mg/L, n=12 1.5 mg/L; Sulpiride: n=12 5 mg/L, n=11 10 mg/L, n=11 20 mg/L). Concentrations used were based on previously published works as well as range-finding pilot experiments in our laboratory (Blank et al., 2009; Cognato et al., 2012;Ng et al., 2012; Scerbina et al., 2012; Sison & Gerlai, 2011b).

Behavioural procedures.

Fish were netted from home tanks and placed in 400 mL beakers containing 300 mL of either drug or aquarium water for 1 h. During pre-treatment, fish were visually isolated. This avoided impact of conspecifics or experimenters on treatment response. Following treatment fish were immediately placed into the FMP Y-maze. Behavioural procedures were conducted in accordance with Experiment 1.

Statistical Analysis

Tetragram analysis and time series analysis were carried out using the same methods outlined in Experiment 1. In addition, tetragram sequences were fitted to linear mixed effects models, with individual ID as the random effect. Initially, we examined differences in alternations and repetitions. For subsequent analyses, we were interested in putative changes in immediate and global strategies, therefore "time" was included as the within-subjects factor. To control for variations in general activity levels "total turns" were included as a covariate in all analyses. The primary endpoint for analysis was the number of choices for each of the 16 tetragrams as a proportion of total turns. Two-way ANOVA was applied separately to the behavioural data obtained from each drug treated group to examine effect of drug concentration on use of alternations and repetitions. ANOVA was followed by Sidak's *post-hoc* tests (Graphpad 8.4.2). A p-value <0.05 was used as a criterion for significant difference. The data are expressed as mean ± SEM.

Results and Discussion

MK-801 caused a significant decrease in the use of alternations compared to control fish (LMM, $F_{(3, 318)}$ = 34.221, p < 0.0001, 0.1 mg/L n=13, 0.75 mg/L n=13 and 2.0 mg/L n=13, control n= 18) (**Fig. 5A**). Chance selection of each tetragram sequence would be ~6.25%. All concentrations of MK801 reduced alternations to <6%, effectively blocking alternations as a strategy. In mid and high concentrations of MK 801 (0.75 and 2.0 mg/L) the search strategy was inverted, Sidak's *post-hoc* test revealed repetitions were used significantly more than alternations (main effect of drug treatment on strategy, $F_{(3, 110)}$ = 12.01, *p<0.001*; Sidak's *post-hoc* test, 0.1 mg/L alts v reps, *p=0.9994,* 0.75 mg/L alts v reps, *p=0.0028*, 2.0 mg/L alts v reps, *p=0.0182*) **(Fig. 6A)**. Scopolamine similarly decreased alternations, but to a lesser extent than MK-801 (LMM, $F_{(3, 316)}$ = 8.025, p < 0.0001, 0.25mg/L, n=13; p<0.001, 0.5, n=13 and 1.0 mg/L, n=13) **(Fig. 5B)**. *Post-hoc* analysis revealed that alternations were only used significantly more than repetitions in fish treated with 0.5 mg/L (main effect of drug treatment on strategy, $F_{(3, 110)}$ = 5.01, *p=0.0027*; Sidak's *post-hoc* test, 0.25 mg/L alts v reps, *p=0.0728,* 0.5 mg/L alts v reps, *p=0.0408*, 1.0 mg/L alts v reps, *p=0.5443*) **(Fig. 6A)**. Treatment with SCH-23390, caused two major changes in search strategy. At all concentrations, there was a decrease in the use of alternations, similarly to that caused by MK-801. Additionally, the highest concentration caused an increase in the use of repetitions (LLLL, RRRR) (LMM test, $F_{(3, 311)}$ = 19.692, p< 0.0001, 0.5 mg/L, n=12; 1.0 mg/L, n=12; 1.5 mg/L, n=12. LMM test, $F_{(3, 312)}$ = 8.954, p<0.001, 1.5 mg/L, n=12) (**Fig. 5C**). There was no significant difference between the use of alternations and repetitions at 0.5 and 1.0 mg/L, however treatment with 1.5 mg/L resulted in repetitions being used more than alternations (main effect of drug treatment on strategy, $F_{(3, 110)}$ = 6.591, *p=0.0004*; Sidak's *post-hoc* test, 0.5 mg/L alts v reps, *p=0.9060,* 1.0 mg/L alts v reps, *p=0.0993*, 1.5 mg/L alts v reps, *p=0.0002*) **(Fig. 6A)**. No such effect was evident in fish treated with D2 antagonist, sulpiride, which resulted in a search strategy resembling control fish (LMM test, n=33, p=0.622) **(Fig. 5D,6A)**.

Figure 5.

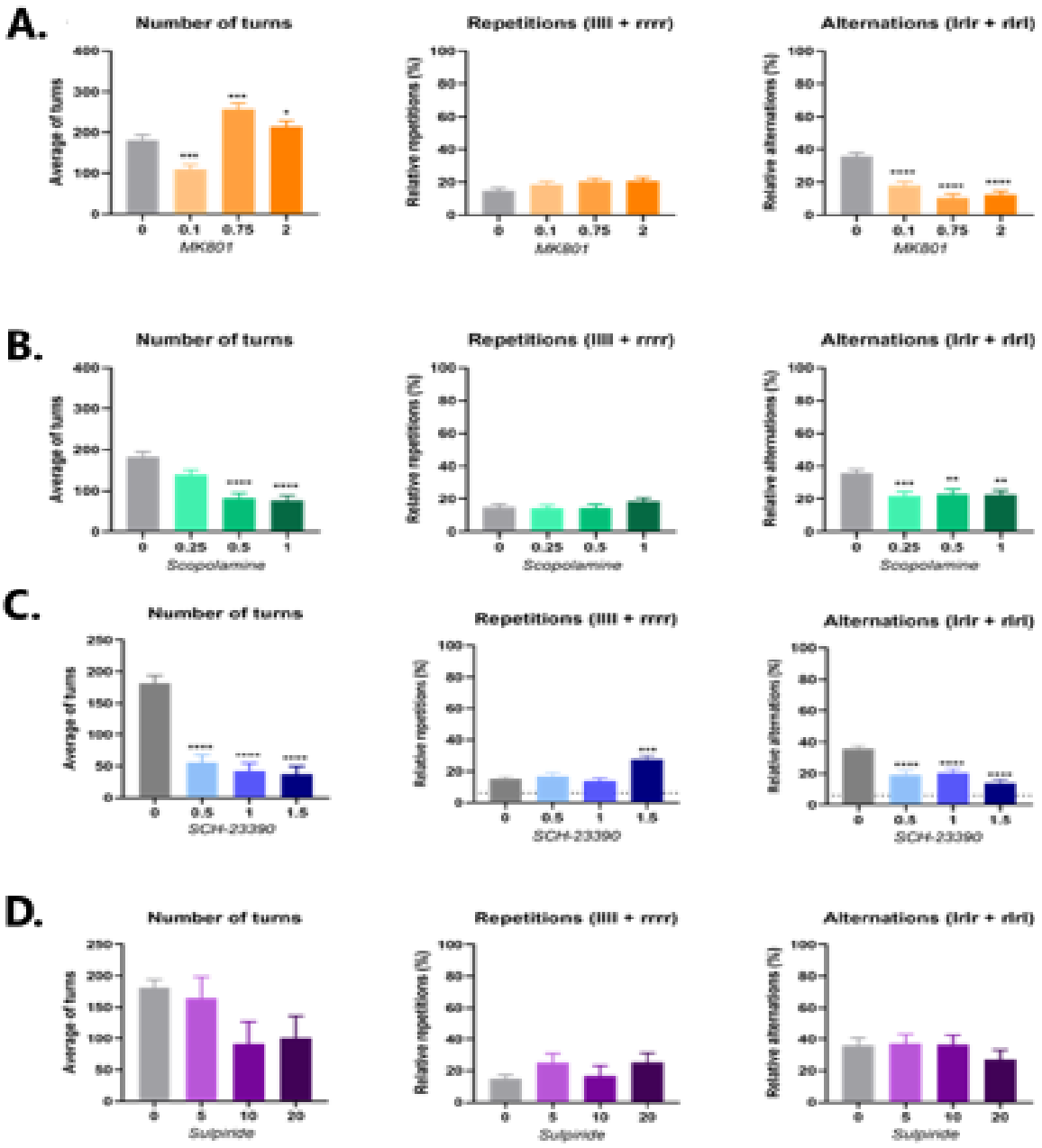

Figure 5. Effects of three concentrations of (**A**) MK-801 (n = 18 control, n = 13 0.1 mg/L, n = 13 0.75 mg/L, n = 13 2.0 mg/L), (**B**) scopolamine (n = 18 control, n = 13 0.25 mg/L, n = 13 0.5 mg/L, n = 13 1.0 mg/L), (**C**) SCH-23390 (n = 18 control, n = 12 0.5 mg/L, n = 12 1.0 mg/L, n = 12 1.5 mg/L) and (**D**) sulpiride (n = 18 control, n = 11 5 mg/L, n = 11 10 mg/L, n = 11 20 mg/L) on locomotor activity, in the form of total turns (**left**), percentage use of repetitions used in the global strategy (**middle**) and the percentage of alternations used as part of the global strategy (**right**). Bars show mean relative frequency of choice, error bars are mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Figure 6.

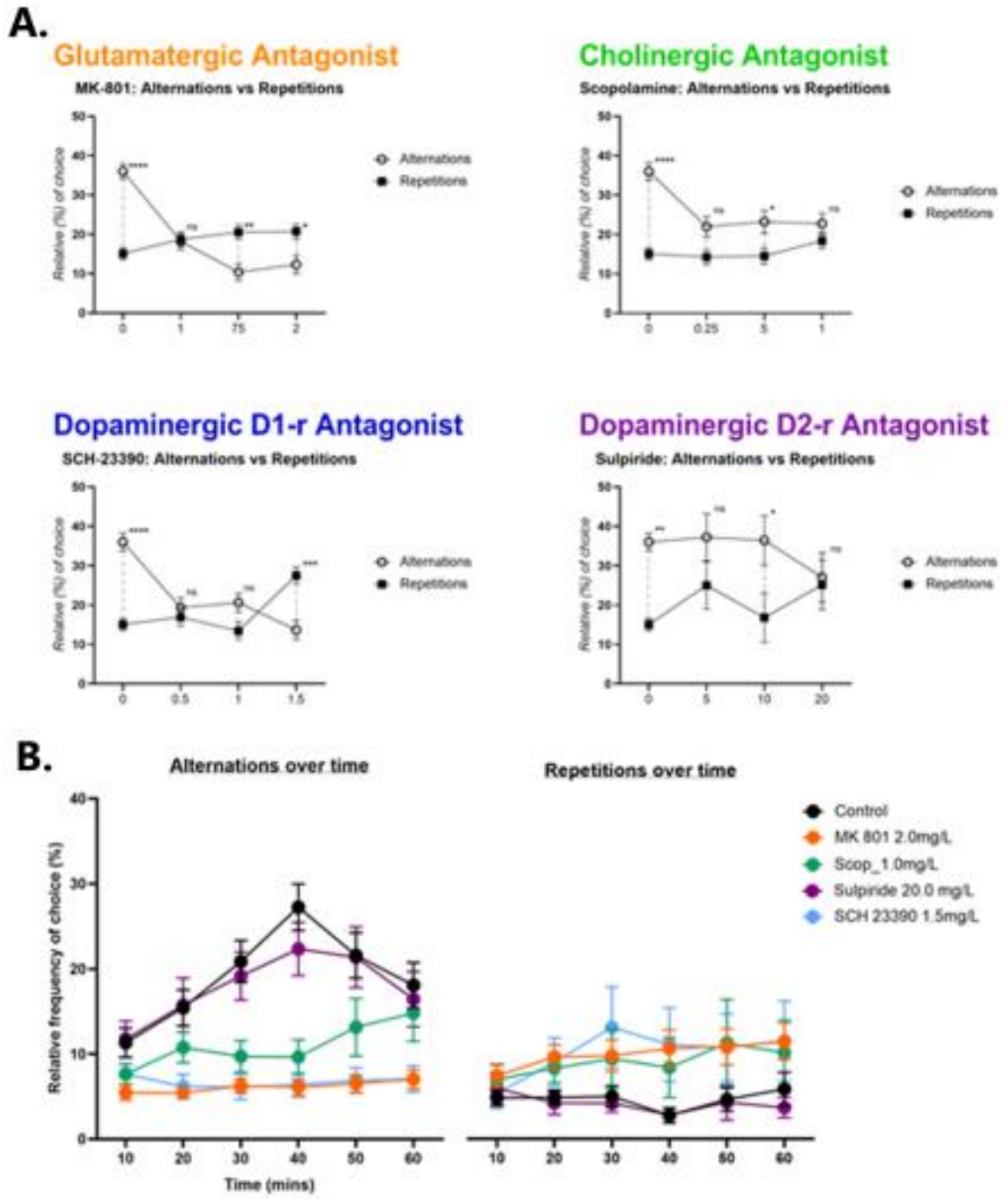

Figure 6. Comparison of total alternations compared to total repetitions for the control fish (0), low, mid and high concentration of antagonist (**A**). Change in total alternations (left) and repetitions (right) during 1 h of exploration divided into 6 equal time bins of 10 min per bin. Graphs representative of control group versus the highest concentration for each antagonist treated group. Points are mean, error bars are mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns – not significant.

The control group showed a clear effect of time on exploration pattern, specifically effecting alternations over successive 10 min search periods (GLMM test, $F_{(5, 186)} = 5.140$, $p=0.0002$). However, there appeared to be a slight decrease in alternations during the last 20 minutes of exploration. There is no obvious reason for this decrease, and further investigation will be required to examine this change in strategy. MK 801 completely blocked changes in alternation-based search strategy, locking animals in an 'immediate' search strategy phase without progression to a global strategy, demonstrating a reduction in behavioural plasticity (GLMM test, $F_{(5, 264.82)} = 1.499$, $p=0.191$). However, this effect was subject to concentration [$F_{(3, 54.33)} = 9.70$, $p<0.001$], concentration by time [$F_{(15, 264.81)} = 2.063$, $p=0.012$] and group interaction [$F_{(1, 54.31)} = 92.628$, $p<0.001$]. Additionally, MK 801 revealed a significant effect on repetitions over time [$F_{(5, 264.53)} = 4.36$, $p=0.001$]. Scopolamine reduced alternations in a manner resembling MK 801 treatment. However, inhibiting muscarinic receptors did not have the same effect on impeding behavioural flexibility. Fish treated with scopolamine maintained a significant effect of time on alternations throughout the trial (GLMM test, $F_{(5, 263.79)} = 4.626$, $p<0.001$), additionally there was an effect of concentration [$F_{(3, 55.41)} = 2.730$, $p=0.05$], a concentration by time interaction [$F_{(15, 263.62)}=1.897$, $p=0.024$] and group interaction [$F_{(1, 55.53)} = 141.43$, $p<0.001$], but, unlike MK 801, there was no effect of time on repetitions [$F_{(5, 263.62)} = 1.936$, $p=0.089$]. Dopamine antagonist SCH-23390 maintained an overall effect of time on strategy [$F_{(5, 259.03)} = 3.785$, $p=0.003$], however, this effect was disrupted at the highest concentration. Similarly to MK 801, 1.5 mg/L of SCH-23390 blocked the effect of time on alternations [$F_{(5, 60)} = 0.514$, $p=0.765$]. SCH-23390 also showed an effect of concentration [$F_{(3, 51.98)} = 5.485$, $p=0.002$], concentration by time [$F_{(15, 259.03)} = 1.791$, $p=0.036$] and interaction [$F_{(1, 51.98)} = 105.217$, $p<0.001$]. Finally, the D2 receptor antagonist sulpiride resulted in exploration behaviour resembling that of the control group, with a significant effect of time on alternations [$F_{(5, 250)} = 5.831$, $p<0.001$] and group interaction [$F_{(1, 50)} = 136.211$, $p<0.001$], but showed no effect of concentration [$F_{(3, 50)} = 0.594$, $p=0.622$] or concentration by time effect [$F_{(15, 250)} = 0.686$, $p=0.798$] (**Fig. 6B**). ACF plots of each concentration of drug resulted in a decrease in the number of significantly correlated lags compared to control fish (One-way ANOVA; $F_{(11, 127)} = 13.94$, $p<0.0001$) **(Fig. 8)**. Thus, memory impaired zebrafish resulted in shorter-

range correlations, limiting the number of steps influenced by choice behaviours showing a reduction in information processing capabilities compared to controls.

Figure 7.

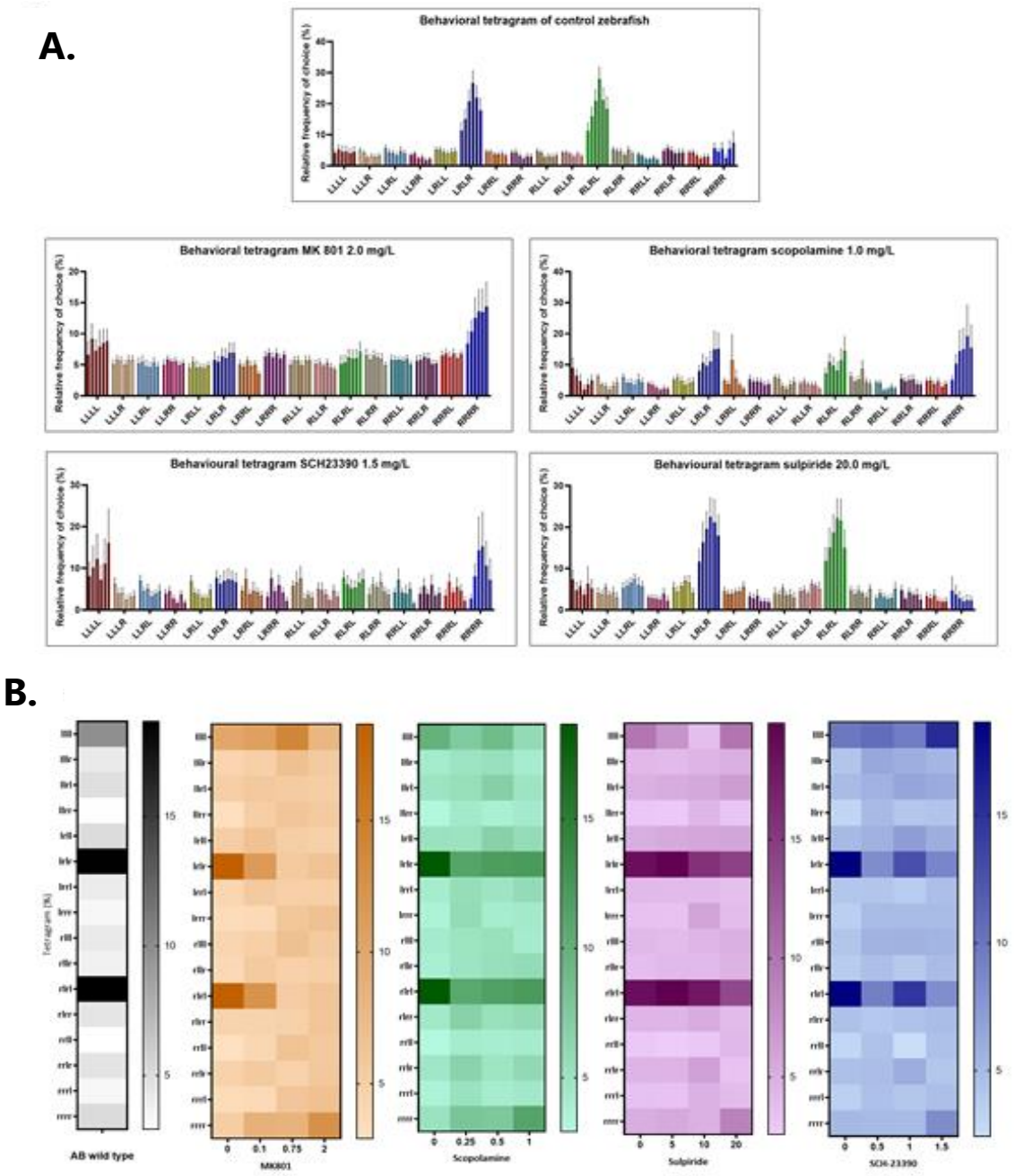

Figure 7. Change in frequency distribution of each of the 16 tetragram sequences as a factor of time, each bar representing a 10 min time bin (**A**). Heat map of changes in global use of all tetragram sequences for each concentration of antagonist compared to control group (**B**).

Figure 8.

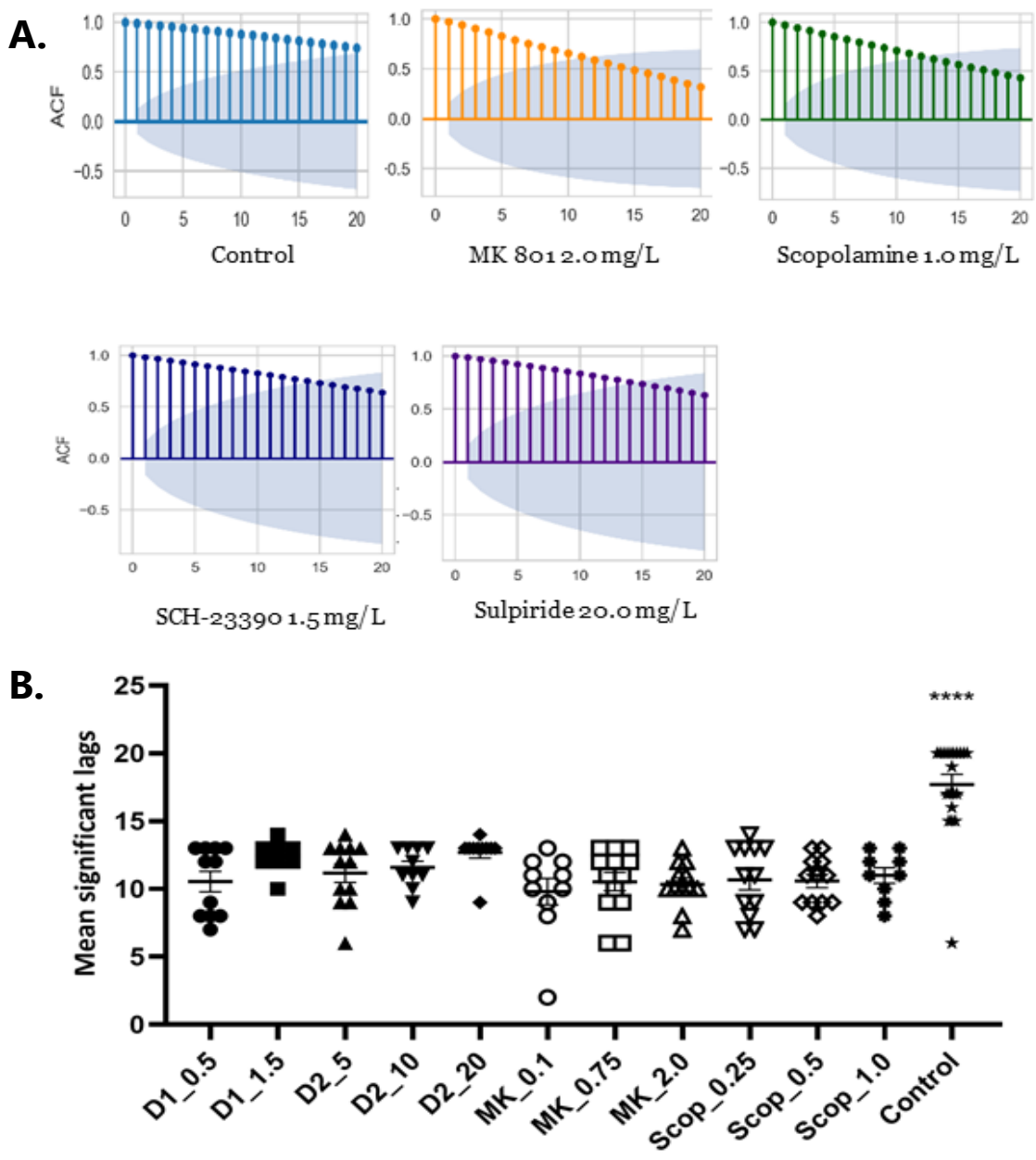

Figure 8. Autocorrelation function plot showing the first 20 lags of 250 lag plot. Each plot shows decays towards zero, with lag points outside of the 95% CI, depicted by the blue cone. ACF plots are of individual animal responses in the FMP Y-maze and are therefore representative of the control group, and drug treatment groups exposure to the highest dose of antagonist for each group for MK-801, scopolamine, SCH-23390 and sulpiride, respectively (**A**). Comparison of the mean significant lags of drug treated groups at low, mid and high concentrations compared to control group. equal time bins of 10 min per bin (**B**). Graphs representative of control group versus the highest concentration for each antagonist treated group. ****$p < 0.0001$.

3.5 Experiment 3

In Experiments 1 and 2, we demonstrated the suitability of the FMP Y-maze for assessing fish. In Experiment 3 we tested the system with other widely used laboratory species (mice and *Drosophila*). Applying an identical protocol to that used with zebrafish, we characterised the exploration strategies of rodents and flies in the FMP Y-maze.

Materials and Methods

Animals

Mice. A total of N=16 C57BL/6 mice (*Mus musculus)* wild types (6-8 weeks old at the time of testing), male and female (50:50) were bred in house and raised in the University of Portsmouth Animal facility. Mice were housed in Allentown IVC racks and kept at 21°C (±2°C), 55% humidity (±10%) on a 12:12-hour light/dark cycle. Mice were fed on a diet of irradiated SDS RM3 pellets, with food and water available *ad libitum*. Following use, mice were retained as breeders in the University facility.

Drosophila. A total of N=30 Canton S wild-type (#64349) *Drosophila melanogaster* (6-7 days old at the time of testing), male and female (50:50), were obtained from Bloomington *Drosophila* Stock Centre, Indiana, USA**.** Flies were kept at 25°C with an average humidity of 60-80% on a 12:12-hour light/dark cycle**.** Flies were housed on ready mixed dried food (Phillip Harris, UK). Flies were collected via light CO_2 anaesthesia and were allowed 48 hours recovery before behavioural testing was conducted. Following completion of the task *Drosophila* were culled using absolute ethanol.

Apparatus

Mice were tested in a stand-alone white acrylic Y-maze insert with transparent base (provided with the LT Zantiks base package) (https://zantiks.com/products/zantiks-lt). *Drosophila* were tested in a clear

acrylic Y-maze insert of 6 identical mazes. Each maze had a sliding cover with a hole which could be moved over the maze as an entry point for introducing the fly (extra for the MWP Zantiks unit) fitted into white opaque holding base for consistent maze alignment (https://zantiks.com/products/zantiks-mwp)**.** Mazes had equal arm length and angle. Maze dimensions were as follows; L152, W50, H155 (mm)-mice, L5, W3, H4 (mm)-*Drosophila*. Mazes were place into their respective Zantiks behaviour units, one maze for mice and 6 mazes for *Drosophila* (**Fig. 9**). Systems used worked on the same basis as the AD system used for zebrafish in Experiment 1 and 2. Distal cues and light levels were constant for all experiments.

Figure 9.

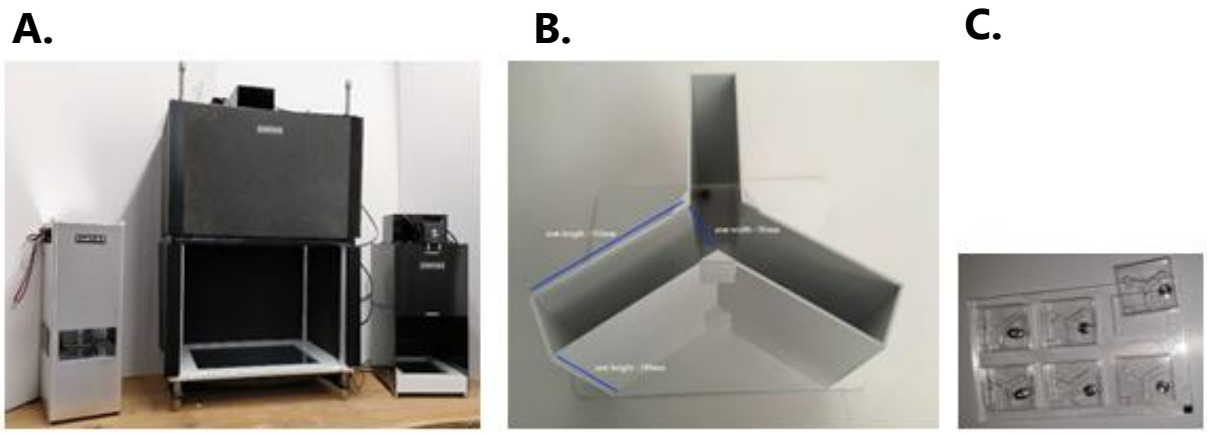

Figure 9. Zantiks behaviour systems, from left to right, MWP system, LT system and AD system, used for *Drosophila*, mice and zebrafish, respectively. Units are completely automated with a computer built into the base, allowing for image/light projection into test arenas, and a camera positioned above, allowing live imaging of animals in the testing unit. This set up reduces experimenter disturbance during testing (**A**). Mouse Y-maze insert. One mouse per maze (**B**). *Drosophila* Y-maze insert, 6 identical mazes with sliding covers (**C**).

Procedure

Mice were transported from home cage to maze using clear, plastic tubes that were kept in their home cages, preventing direct handling prior to the task. *Drosophila* were guided into a pipette tip and tapped gently into the maze through a hole in the lid which could be moved over the maze for entry and once

in the maze, moved away to prevent escape. All animals were recorded for 1 h. As with Experiment 1 and 2, data were output as a time series normalised as a proportion of total turns and analysed using tetragram sequences. The same statistical analysis was applied from Experiment 1 and 2.

Statistical analysis

Two-way mixed design ANOCVA with one between subjects' factor with three-levels (species-zebrafish, mice and flies), and one within-subjects factor with 16 levels (tetragrams), total turns as the covariate, and proportion of choices as the dependent variable, was used to compare global strategies. To examine alternations in more detail, one-way ANOVA determined the difference between tetragram frequencies and as a cross-species comparison of total alternation (LRLR+RLRL) use.

Results and Discussion

Mice navigated the FMP Y-maze using an almost identical strategy to zebrafish, showing dominant use of alternations throughout the task (**Fig. 10B**). There was no significant difference between tetragram frequency distributions for the global strategy (Two-way ANOVA, $F(1, 496)=1.7^{-6}$, $p=0.999$) between mice and fish, however there was a significant difference in alternations, with mice using alternations ~38% compared to ~26% for zebrafish [$F(15, 496)=45.34$, $p<0.001$]. *Drosophila,* however, differed from mice and zebrafish (**Fig. 10A**), characterized by flies employing an exploration strategy reliant on repetitions as opposed to alternations, which accounted for ~38% of their total search strategy (One-way ANOVA, $F(7, 472)=55.12$, $p<0.001$) (**Fig. 10C**). This alternative navigational pattern could be influenced by *Drosophila's* natural tendency to explore using wall-following behaviour (Soibam et al., 2012). Like mice and zebrafish, *Drosophila* displayed the dominant 'repetition' strategy at evenly distributed times throughout the task, regularly interspersed with different sequences of the other 14 tetragram sequences.

Figure 10.

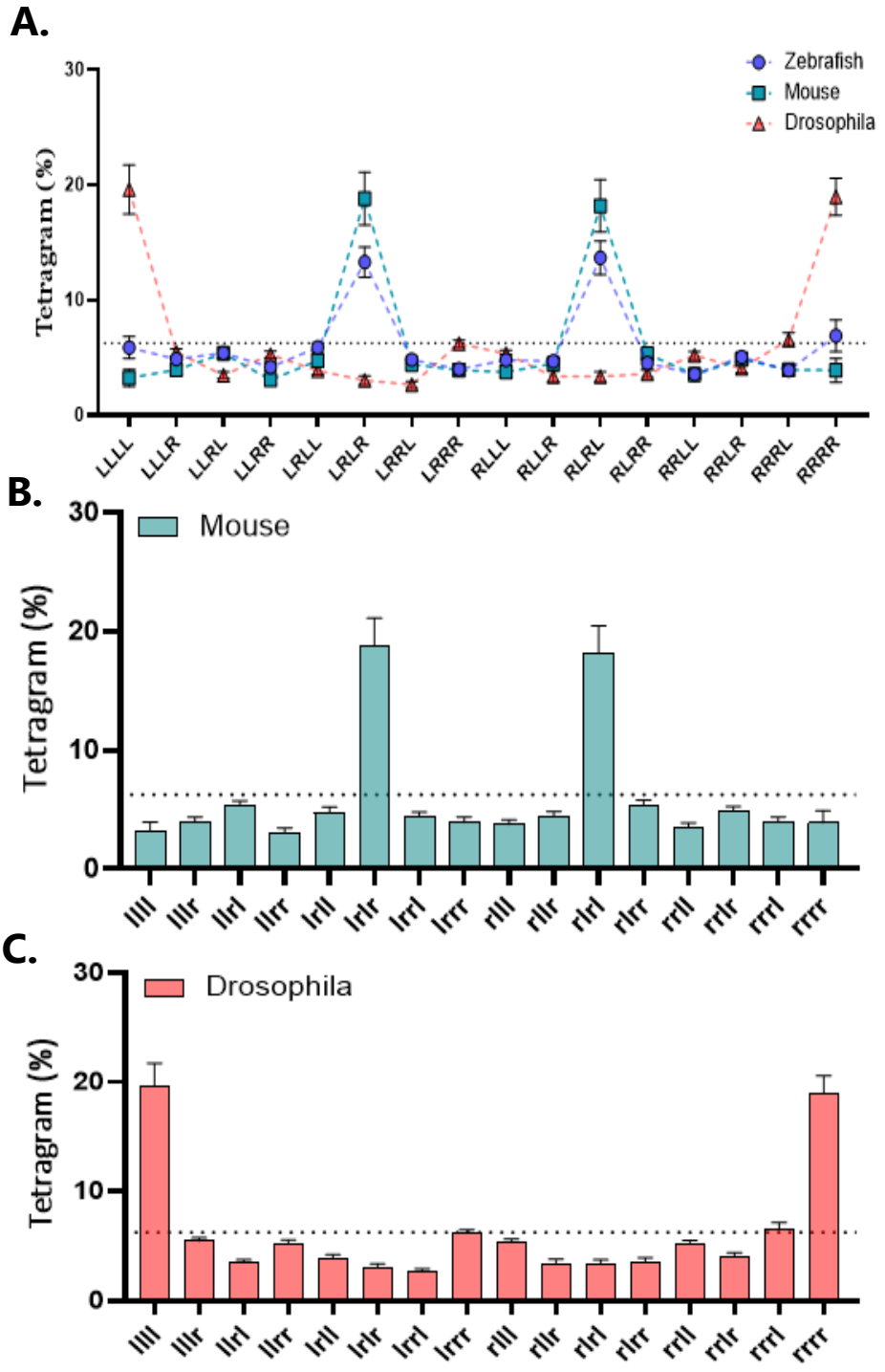

Figure 10. Comparison of zebrafish, mouse and fly global tetragram usage over a 1 h search (**A**). Frequency distribution of global tetragram strategy over the course of 1 h of exploration in the FMP Y-maze for mice (n = 15) (**B**) and *Drosophila* (n = 30) (**C**). Dashed line represents random selection at 6.25%. Dominant strategy uses alternation patterns (LRLR, RLRL) for mice and zebrafish and repetitive patterns (LLLL, RRRR) for *Drosophila*. Points/bars are mean, error bars are mean ± SEM.

Despite the strategic differences used to explore the maze, all organisms tested showed use of a single dominant strategy. Regardless of the search pattern, all species showed similar results in the ACF plots, with persistent, slowly decaying autocorrelation, indicative of long-lasting effect of choice on future choice selections (**Fig. 11**). These data collectively suggest that like zebrafish, mice and *Drosophila* did not search the test arena randomly, but in a systematic and deterministic way, demonstrating use of an underlying process of memory to recall previous turn choices, and guide subsequent turn patterns. This task provides further evidence of the suitability of the FMP Y-maze as a memory test for a range of model organisms (**Supplemental video 2 and 3**).

Figure 11.

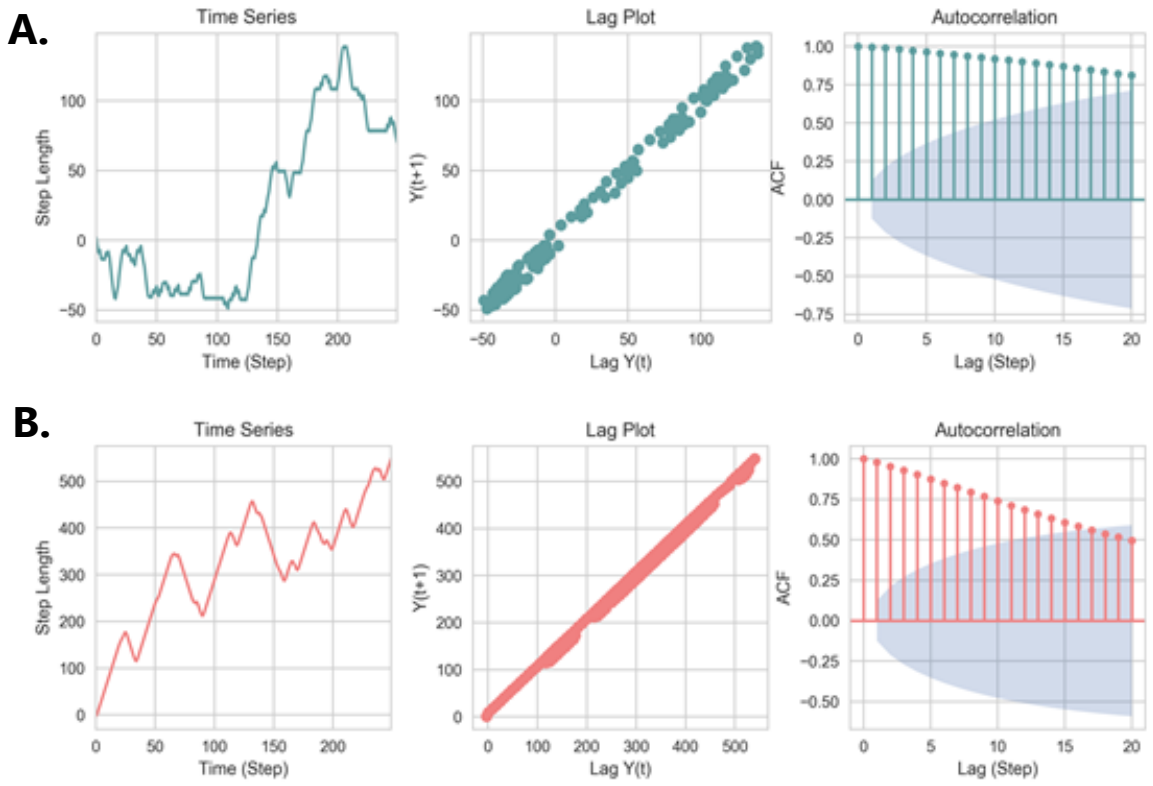

Figure 11. Time series analysis of individual mouse (**A**) and *Drosophila* (**B**) showing time series plot of step length (n = 250 steps) (**left**). Lag plot showing positive correlation for both organisms (**middle**), ACF plot of the first 20 lags, with both organisms demonstrating over 15 lags of significant autocorrelation (**right**).

3.6 Experiment 4

Experiment 1-3 demonstrated the cross-species validity of the FMP Y-maze in laboratory animals; mice, zebrafish and *Drosophila*. In order to test the translational utility of this model, we developed a virtual FMP Y-maze for humans. The maze was based on a honeycomb-layout, requiring participants to navigate a series of 'Y' shaped choice points. In order to make the test clinically relevant and useful for a variety of human testing conditions, we ran the task for 5 minutes, at which point participants were automatically exited from the maze. Previous studies have investigated the relationship between participant response rate and response burden (the perceived effort required by participants to complete an online study, commonly in reference to questionnaires). Increased length of questionnaires has been associated with lower response rates and reduced completion (Presser et al., 2004; Rolstad et al., 2011). In order to increase the translational potential of the virtual FMP Y-maze and suitability to a clinical setting, our aim was to significantly minimise the required participation time to reduce boredom, encourage participants to continually traverse the maze for the allotted time and increase the response rate of participants requested to take part in future studies.

Materials and Methods

Participants

Participants (n=12 male and n=12 female; age range 21-65) were recruited from staff and students at the University of Portsmouth. Following consent, after reading the information form, participants took part in a short task in which they had to 'find the way out' of an online maze. The human experiments were carried out following approval from the University of Portsmouth Science Faculty Ethics Committee (SFEC-2019-062).

Apparatus and Procedure

Human Virtual FMP Y-maze. A honeycomb maze, representing multiple Y-shaped choice points formed the human virtual FMP Y-maze (**Fig. 12**). Participants could initiate the start of the trial when ready and, using the arrow keys on a standard laptop keyboard, navigate their way around the maze (**Supplementary_Video.4**). Participants were free to explore the maze for 5 minutes, after which they were automatically logged out. Turn directions were logged as x,y coordinates which were converted into left and right turns and subsequently transformed into tetragrams.

Figure 12.

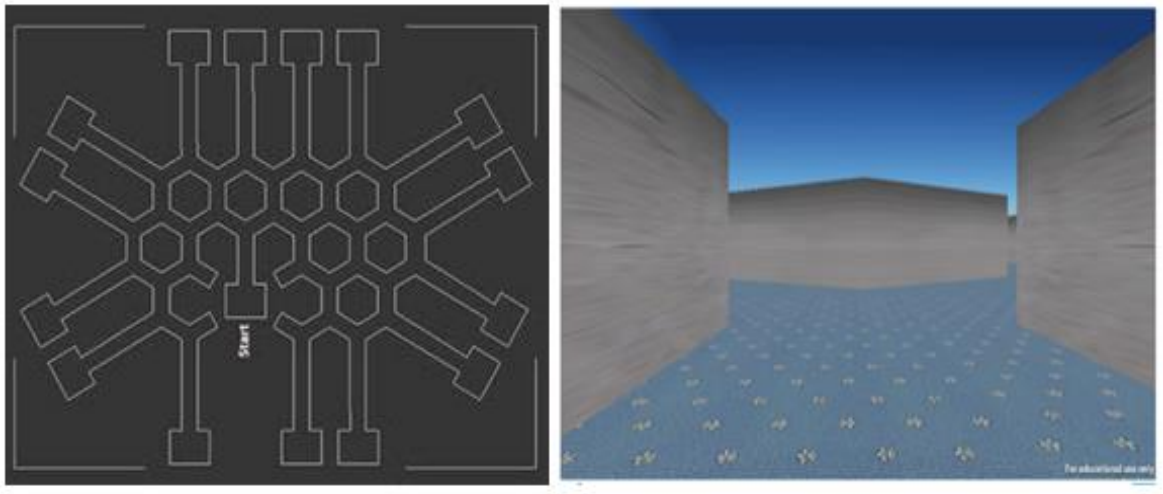

Figure 12. Schematic of maze structure showing interconnected Y-shaped mazes, each of equal length and diameter (**left**). Screen shot taken from the perspective of the participant in the human FMP Y-maze (**right**).

Statistical Analysis

To examine tetragrams, we carried out a one-way within-subjects ANOVA with 'tetragram' as the independent variable and proportion as the DV. To example alternations in more detail, two-way ANOVA determined the difference between tetragram frequencies and as a cross-species comparison of total alternation (LRLR+RLRL) use (between-subjects factor – species; within subjects factor – time).

Results and Discussion

Tetragram analysis revealed that humans used an almost identical strategy to mice and zebrafish, predominately comprising of alternations, which occupied ~50% of the search strategy (One-way ANOVA; $F_{(3, 164)} = 60.88$; $p<0.0001$) (**Fig. 13A**). Humans traversing the virtual FMP Y-maze demonstrated significantly greater use of alternations compared to mice, zebrafish and flies (**Fig. 13C**). Despite, limiting the run time to 5 minutes, this prolific strategy was still detectable. On average, participants completed 39 steps (39 tetragrams) with a maximum of 68 and a minimum of 7 steps. The number of steps completed was substantially lower than any of the other animal models and was therefore based on 100 arm entries compared to 1000 arm entries for zebrafish, mice and *Drosophila*. Humans showed weak correlation in the lag plot and significant autocorrelation lasting only one or two lags, before rapidly decaying to fluctuate around zero (**Fig. 13B**). This indicates that the human FMP Y-maze exploration was characterised by choice selections that were only influenced by the immediate past. Based on the brevity of the trial and the limited number of turns this would be expected as the data set was not large enough to determine longer-term patterns. Additionally, there was a significant effect of time on alternations for all the vertebrate species tested (One-way ANOVA: Humans; $F_{(5, 6)} = 19.48$; $p=0.0012$, mice; $F_{(5, 174)} = 7.635$; $p=0.0002$, zebrafish; $F_{(5, 186)} = 2.369$ $p=0.0002$), but no effect of time on the invertebrate species (*Drosophila;* $F_{(5, 342)} = 1.460$; $p=0.2994$) (**Fig. 13D**). Our results have demonstrated the suitability of the FMP Y-maze as a test of memory, not just for animals, but also for humans, further supporting the theory of a common vertebrate strategy.

Figure 13.

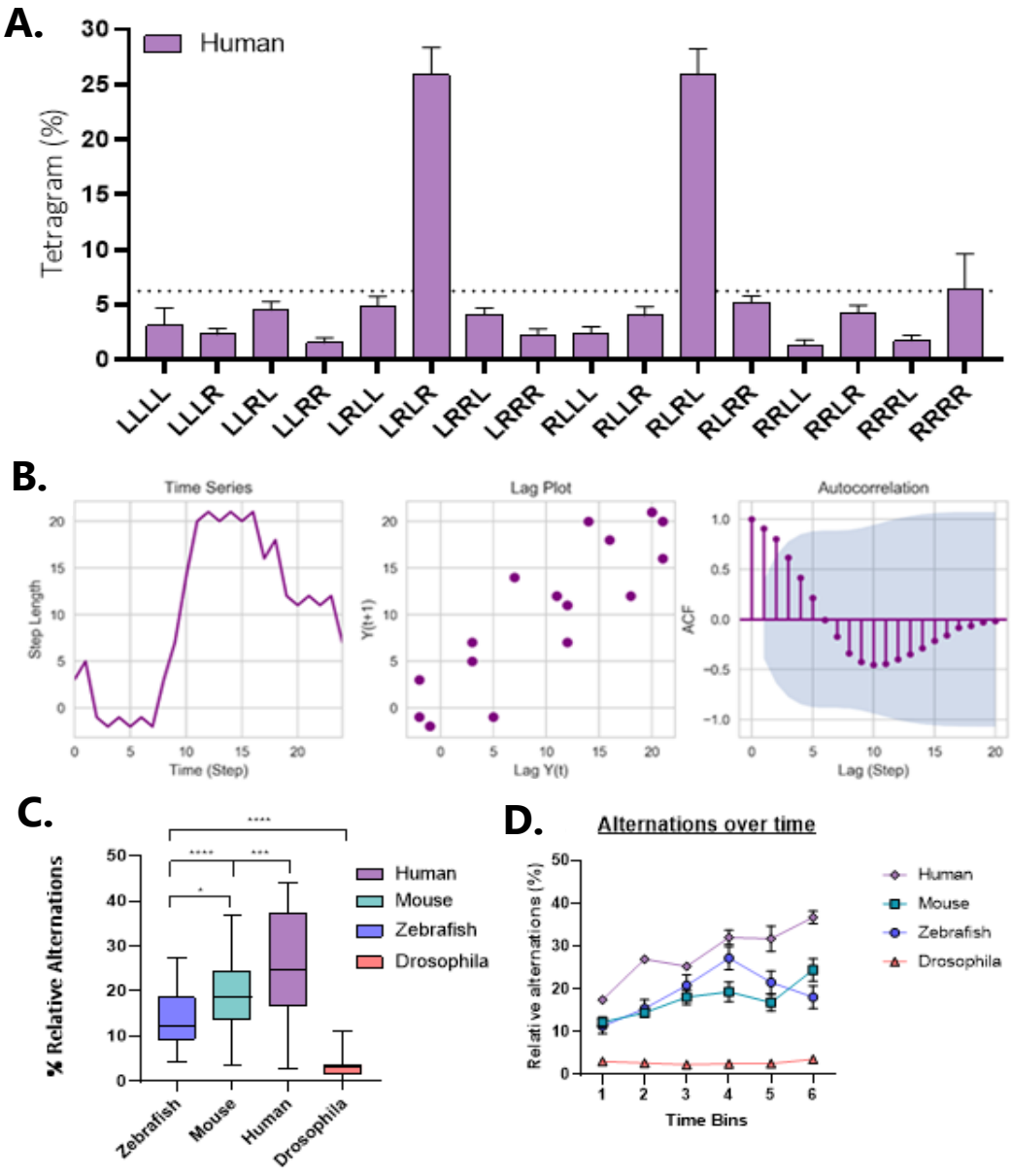

Figure 13. Tetragram frequency distribution of human participants from a 5 min trial (n = 24) (**A**). Time series analysis of an individual participant showing time series plot, lag plot showing weak positive correlation and ACF plot of the first 20 lags, showing significant autocorrelation at lags 1 and 2, which exponentially decay to zero (**B**). Relative means of alternation use in the FMP Y-maze of all organisms, showing an increase in percentage use of alternation with complexity of organism, with *Drosophila* have the lowest alternation use, increasing to humans with the highest (**C**). Alternation use for each time bin (trial time divided into 6 equal time segments) for humans, mice, zebrafish and flies. Points/bars are mean, error bars are mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.7 General Discussion

We demonstrate that the FMP Y-maze, when combined with tetragram analysis, is an effective tool for assessing executive function, particularly WM and CF. The ability to detect cognitive impairment in the absence of training, habituation, reward bias or aversive conditions, creates a reliable test that can be run singly or as part of a battery of behavioural tasks assessing cognition and memory. The non-invasive nature and low-impact on animals, provides a task with a strong '3Rs' justification, with particular emphasis on refinement (Tannenbaum & Bennett, 2015). The conserved response strategies across vertebrates demonstrate exceptional high translational relevance of the task, offering clinical potential.

The FMP Y-maze has implemented use of an extended protocol, which allows 1 h of free exploration, significantly longer than the 5-8 minutes used for the continuous Y-maze task. The increased runtime provides several advantages: firstly, as neither the T- or Y-maze tasks previously included habituation time at the beginning of the trial, it was possible that poor locomotor responses or reduced arm entries were a confound of anxiety in response to a novel environment. The duration of the FMP Y-maze trial permits enough time that persistent behavioural changes can be detected without interference from initial freezing bouts or hypo/hyperactivity. Secondly, exploration patterns more complex than the previously denoted 'alternation' strategy, in the continuous Y-maze, can be identified, without ceiling effects. A perfect score in spontaneous alternation tasks is represented by 100% alternations, therefore, it is only possible to detect improvements with this protocol if there is an initial deficit. In comparison, the detection of complex patterns in the FMP Y-maze allows examination of impairments and improvements with, so far, no detection of ceiling effects. Finally, the role of behavioural plasticity, can be included as a vital part of the analysis to examine how behaviour evolves over time in response to the environment.

We investigated the role of WM in flies, fish, mice and humans, in formulating search patterns used to explore the FMP Y-maze. Tetragram analysis revealed two dominant strategies; a vertebrate strategy used by zebrafish, mice and humans, that largely consisted of alternations (LRLR, RLRL) and an invertebrate strategy used by *Drosophila*, that was reliant on repetitions (LLLL, RRRR). Search behaviour

was the result of complex moves that were highly dependent on past turn choices. Time series analysis and autocorrelation revealed that information of previous turn choices was held for long periods, demonstrated by significant autocorrelation for many steps, and used to influence future movement patterns. The length of time this information was held, was significantly impacted by pharmacological blockade of glutamatergic, cholinergic and dopaminergic, specifically D1, neurotransmitter systems, which showed a decrease in the number of steps with significant autocorrelation. Previous studies in rodents and humans have identified critical roles for each of these systems in maintaining WM (Ellis & Nathan, 2001; Handra et al., 2019; Myhrer, 2003). Zebrafish have homologues of each of these neurotransmitter systems (Horzmann & Freeman, 2016) and results from the present study support findings from human and rodent studies, of impaired WM as a result of pharmacologically blocking glutamatergic, cholinergic and dopaminergic receptors (Ellis & Nathan, 2001; Myhrer, 2003; Shapiro & Caramanos, 1990; Sokolenko et al., 2020; van der Staay et al., 2011). Thus, highlighting the suitability of zebrafish as a behavioural model for assessing WM.

Many conditions that commonly report deficits in WM, such as neurodegenerative or psychiatric disorders, often also present with impaired CF (Pittenger, 2013). This represents a change in cognitive state to allow an organism to adapt their behaviour in response to perceived environmental contingencies (Brown & Tait, 2014). In the wild, animals have been found to alter search patterns in response to resources, using one strategy for food-rich areas, and another for unpredictable environments with patchy prey distributions (Humphries et al., 2010; Sims et al., 2008). The FMP Y-maze represents an unpredictable environment. Therefore, we would expect animals to alter strategy overtime as has been demonstrated by Namboodiri, et al (2016), in birds and humans. Cognitively complex organisms have the ability to learn from their environment and subsequently demonstrate modified search strategies when faced with time costs that can reduce the value of a reward or goal (Namboodiri et al., 2016). Here, we show that healthy fish, mice and humans all demonstrate some degree of behavioural flexibility whilst traversing the maze, by increasing the use of alternations over time. However, flies used a strategy that was static throughout the trial and did not differ significantly from

the first 10 minutes to the last 10 minutes of exploration. This method has demonstrated sensitivity to detect adaptive behaviours in response to time and the environment, in a range of cognitively complex organisms.

Further testing with pharmacological agents, demonstrated the ability of this task to detect drug induced changes in adaptive behaviours. MK-801 has been used in previous studies to model SZ-like behaviours, including deficits in WM and CF (Lobellova et al., 2013; Murueta-Goyena et al., 2017; Svoboda et al., 2015). Here we demonstrate that the FMP Y-maze protocol could detect impaired behavioural flexibility induced by systemic blockade of NMDA-r by acute MK-801 exposure. This task could also detect changes in behavioural adaptability after acute exposure to muscarinic and dopaminergic D1 receptor antagonists, but no effect of systemic D2 receptor blockade, in line with findings from previous rodent studies (Chen et al., 2004; Ragozzino et al., 2002; Winter et al., 2009). These results further support the use of the FMP Y-maze to detect changes in CF and the use of zebrafish to model cognitive impairment.

Deficits in executive functions such as WM or CF are commonly reported in patients diagnosed with neurodegenerative diseases, such as AD (Guarino et al., 2019a) and PD (Handra et al., 2019; Koerts et al., 2011), or as a feature in a variety of neuropsychiatric disorders, such as major depressive disorder (MDD) (Darcet et al., 2016; Hammar & Årdal, 2009; Snyder, 2013), substance abuse (Cunha et al., 2010; Gould, 2010) and SZ (Giraldo-Chica et al., 2018; Orellana & Slachevsky, 2013). As WM and CF can be markers for many complex brain disorders, the FMP Y-maze could be used as a clinical behavioural task for assessing executive function and memory processing as part of a battery of diagnostic tools. The ease and brevity of the human FMP Y-maze task lends itself to testing all age groups, including adolescences that may have increased susceptibility to developing SZ (Bossong & Niesink, 2010; Hollis, 1995). Additionally, the neurotransmitter groups tested here, have been implicated in a number of neurodegenerative and neuropsychiatric disorders and their treatments (Aarsland et al., 2017; Brisch et al., 2014; Francis, 2005; Li et al., 2019; Murueta-Goyena et al., 2017).

Despite the advantages of testing executive function in the FMP Y-maze, there are limitations to the protocol, primarily associated with run time. Animal versions of the FMP Y-maze are run over 1 h. Although this provides some benefits, as outlined above, the time taken to run a full experiment is largely dependent on the resources available to the facility. We operated this task with one MWP unit, one LT unit and four AD units. Thus, we were able to run 8 zebrafish, 6 *Drosophila* and one mouse per hour. In total, it took three days of back-to-back trials to test 166 zebrafish, 5 hours to test 30 *Drosophila* and three days to run 16 mice. Therefore, the level of throughput is dependent on the organism being tested and the number of behavioural units available for simultaneous trials. Additionally, this run time could not be applied to the human maze as the extensive trial time would be expected to have a negative impact on participant recruitment. Therefore, the trial was reduced to 5 min of exploration. However, the time for the online trial may need amending depending on the target group. Preliminary studies showed that younger participants completed sufficient turns in the allotted time, however, older participants completed very few turns, and for some this resulted in exclusion due to insufficient data collection. Therefore, it is suggested that for studies targeting older groups, or treatment groups with cognitive impairments, that run time be increased.

Here, we present a new behavioural task for testing deficits in executive function and WM. We demonstrate the reliability and sensitivity of the FMP Y-maze to alterations in cognition and memory processing in a range of model organisms. Additionally, an online virtual maze has been created as a translational cognitive paradigm for testing humans. This task has the potential to be used either as a diagnostic tool, or as a method for improving drug discovery using animal models of complex brain disorders that report memory and cognitive decline as hallmarks of disease. The FMP Y-maze lays the foundation of future translational research for a range of neurological disorders and could open new avenues of research into cognition and memory, allowing cross-species comparisons with exceptional translational relevance.

Chapter 4

The transition from infancy to adulthood is a period subject to extensive behavioural changes and coincides with development of the majority of psychiatric disorders (Gee et al., 2018; Spear, 2000a). *During these stages is immense neuronal remodelling, developmental plasticity and biochemical fluctuations, particularly pertaining to hormones, which can alter sensitivity to environmental perturbations (Meyer & Lee, 2019). Disturbances during this critical period have been associated with long-term behavioural, cognitive and molecular changes that can maintain throughout adulthood, long after cessation of the disturbing stimuli (Lockhart et al., 2018). It has been reported that of all lifetime mental health conditions, including ADHD, anxiety disorders, SZ, substance abuse and mood disorders, approximately half have first onset during the mid-teens, highlighting the increasing motivation to understand susceptibility during these periods of development* (Gomes et al., 2016; Kessler et al., 2007; Lockhart et al., 2018). *The previous chapter demonstrated the suitability of the FMP Y-maze for assessing changes in cognitive function in adult zebrafish. Thus, the aim of the next chapter was three-fold: 1) to test the suitability of the FMP Y-maze for assessing different age groups, 2) to identify the earliest stage that cognitive function could be assessed in zebrafish and 3) to further our understanding of the relationship between cognitive function and the endocrine system to identify developmental vulnerabilities to onset of neuropsychiatric disorders.*

The Ontogenetic Advancement of Working Memory, Behavioural Flexibility and The HPI Axis in Developing Zebrafish (*Danio rerio*)

4.1 Abstract

Critical periods during development promote the acquisition of necessary skills such as decision-making, novelty seeking and adaptive responses to environmental demands. During these periods, immature organisms are vulnerable to disruptions, which can have life-long behavioural consequences. Zebrafish (*Danio rerio*) are being increasingly used to better understand the long-term behavioural effects of developmental disturbances, due to their high genetic homology to humans. This has highlighted a growing need to understand the life history and formation of executive functions and stress pathology during critical periods of development from larval to adult stages. Cognitive function of pre/postnatal stages in zebrafish (4, 7, 14, 30 and 90 dpf, larvae to young adulthood), were assessed to identify key periods of cognitive and Hypothalamic-Pituitary-Interrenal (HPI) axis development. We examined the co-occurrence of fluctuations in cognitive performance and HPI axis activity to identify potential vulnerability periods. Yolk-dependent and independent feeder were critical stages of cognitive development, demonstrating the transition between basic cue responding to complex behaviour, which steadily improved with age, plateauing from juveniles to young adulthood (30-90 dpf). The HPI axis, however, showed substantial age differences, with a 10-fold up-regulation of cortisol release, following acute stress, from larvae to juveniles (14-30 dpf) which, similar to cognitive performance, plateaued between juvenile and young adult stages (30-90 dpf). *Glucocorticoid receptor* (*gr*) expression equally had a 10-fold increase, but only in young adults (90 dpf). All other age groups had similar expression levels to 4 dpf larvae. Our findings indicate distinct stages of cognitive development and substantial age-related physiological changes in HPI axis activity and regulation, which implicate juvenile stages as a critical period of development due to high stress responsivity whilst maintaining low levels of *gr*, critical to regulating negative feedback of stress responses and maintaining homeostasis.

4.2 Introduction

In humans, the transition from infant to young adult is marked by significant maturation of the central nervous system resulting in altered behaviour and cognitive function (Lupien et al., 2009; Roberts & Lopez-Duran, 2019). Drastic changes in cognitive development have taken place by the time a child reaches the age of 10 years, which likely reflects increases in brain size and weight to near adult proportions (Brenhouse & Andersen, 2011; Cromer et al., 2015; Gogtay et al., 2004). However, development continues into adulthood, and cognitive domain has a substantial impact on the rate of maturation and the age at which adult-levels of ability are achieved (Cromer et al., 2015; Gogtay et al., 2004; Lewis et al., 2010; Luciana & Nelson, 2002). For example, frontal lobe functions, such as WM, have been suggested to reach adult-levels of performance during late-teens to early twenties (Luna et al., 2004; Zald & Iacono, 1998). Cognitive development has largely been reported as a linear progression of increasing ability with increasing age from childhood to adulthood (Brenhouse & Andersen, 2011; Cromer et al., 2015).

Simultaneous to cognitive development, is the maturation of the neuroendocrine system controlling stress responsivity. Unlike cognition, emotional and stress reactivity do not progress linearly, but have been shown to follow an inverted U-shaped response curve, suggested to peak during adolescence (Brenhouse & Andersen, 2011; Spear, 2000b). The impact of HPA axis activity on cognitive function has been well established, with long-term hypo and hyperfunctioning causing impairments in cognitive abilities, including WM, but the reverse may be true for acute increases, which have been reported to positively associate with enhanced WM performance (Romeo, 2013; Stauble et al., 2013). The development of the HPA axis is marked by a significant shift in reactivity during adolescence and is reported in conjunction with increased stress-sensitivity during this period (Romeo, 2013). In humans, studies have suggested that during adolescence there is a greater stress response compared to younger children and increased duration compared to adults (Lupien et al., 2009; Platje et al., 2013; Roberts & Lopez-Duran, 2019). This implicates certain developmental periods with increased psychopathology vulnerability (Cheryl M. McCormick & Mathews, 2010; Platje et al., 2013; Roberts & Lopez-Duran, 2019).

Thus, adolescence is as a critical stage of development and explains adolescents' increased vulnerability to develop stress-induced pathologies, such as SZ and substance abuse.

Activation of the HPA axis, through exposure to stressful stimuli, leads to the rapid release of cortisol, levels of which generally peak between 20-40 min after stressor detection (Dickerson & Kemeny, 2004). Cortisol levels are then decreased through binding to glucocorticoid receptors (GR), which play a critical role in the negative feedback mechanism used to down regulate the HPA axis response and maintain homeostasis (Ramamoorthy & Cidlowski, 2016). The activation of this system has been reported to influence cognition and behaviour (Lupien et al., 2009; Shields et al., 2016; Van Niekerk et al., 2001).

One model organism that has been growing in use in the field of psychiatric research is the zebrafish (*Danio rerio*) (Gerlai, 2011). Providing an alternative vertebrate model to rodents and non-human primates, zebrafish have proved a useful model for prenatal, postnatal and early life, pre-adult investigation into disrupted neural circuitry and resulting behavioural changes that can persist into adulthood (de Abreu et al., 2019; Kozol, 2018; Souza & Tropepe, 2011; Adam Michael Stewart, Nguyen, et al., 2014). Zebrafish possess several advantages for assessing the development of cognitive and biological functions. Firstly, the distinction of *in utero* development of mammals compared to *in vitro* development of zebrafish larvae permits examination of early developmental stages, while avoiding maternal interference (Ge et al., 2019). Therefore, behavioural examination can begin whilst larvae are still yolk-dependent, compared to free-feeding, allowing comparison of cognitive and behavioural abilities and associated biochemistry at distinct life stages.

Much of the zebrafish genome has high genetic homology to human counterparts, including those responsible for regulating stress biology. For example, zebrafish have an analogous system to the human HPA axis, the hypothalamic-pituitary-interrenal (HPI) axis (Derek Alsop & Vijayan, 2009), which, similarly to humans, utilises a single glucocorticoid receptor (*gr*) gene, and several splice variants, to regulate cortisol levels following a stress response (Aponte & Petrunich-Rutherford, 2019; Schaaf et al., 2009; Wilson et al., 2013). Additionally, zebrafish reach sexual maturity in just 12-weeks and can

therefore provide a model of early life, juvenile and young adult stages within just 3 months (Singleman & Holtzman, 2014). Taken together, zebrafish provide an excellent model for understanding cognitive and behavioural abilities and molecular underpinnings of the HPI axis from early development to adulthood in health and disease (Adam Michael Stewart, Braubach, et al., 2014). Although previous studies have investigated aspects of the HPI axis during development, they have been restricted to a specific stage (i.e., multiple stages of larval development) (Derek Alsop & Vijayan, 2009). To our knowledge, this is the first comprehensive examination of cognitive development in zebrafish and the first study to describe HPI axis development from young larvae to young adulthood.

The FMP Y-maze has been used in several studies to investigate changes in WM and cognitive flexibility by analysing sequence strings of left and right turn choices made during one hour (1 h) of free exploration (Cleal, Fontana, et al., 2020; Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). The 'chunking' of turn choices into overlapping sequences of four turns, known as tetragrams, allows the identification of repeating patterns within the data. Previous studies have identified strong correlations between WM performance and changes in global and immediate search strategies, which can be influenced by early developmental drug exposure, neuropharmacological manipulation and ageing (Cleal, Fontana, et al., 2020; Cleal & Parker, 2018). To investigate the impact of developmental stage on cognitive abilities, we tested a cross section of five age groups in the FMP Y-maze, ranging from yolk-dependent larvae to young adult. We further examined whole-body cortisol following an acute stress and basal levels of glucocorticoid receptors (*gr*) to disseminate changes in the HPI axis. We aimed to identify critical stages of development which may highlight periods of increased vulnerability to environmental perturbations.

4.3 Materials and Methods

Animals and housing

Zebrafish were bred in-house at the University of Portsmouth Fish Facility, with eggs collected via the addition of marble-trays to tanks of ~20 adult, AB zebrafish. Offspring were reared in petri-dishes of aquarium-treated water in groups of ~50 embryos from collection until 5 days post fertilisation (dpf) and maintained in a larval incubator at ~28.5°C on a 14/10-hour light/dark cycle. From 5 dpf larvae were transferred into larger dishes in the incubator. From ~20 dpf larvae were split into groups of 20 fish in 6L tanks, until 60 dpf when fish were further split into groups of 8-12 fish in a 2.8L tanks on a re-circulating system (Aquaneering Inc., San Diego, CA, USA). From 5 dpf fish were fed on ZM fry food until 30 dpf and then put on a diet of dried fish flakes and live brine shrimp until 90 dpf, 3 times per day during the week and once a day at weekends (am). Aquarium water was maintained at ~25-27°C, pH 8.4 (±0.4), and fish remained on a 14/10-hour light/dark cycle. All fish used were experimentally naïve. Due to the non-invasive nature and low impact of the behavioural testing, on completion of the FMP Y-maze fish were returned to housing tanks and incorporated into the breeding program.

Ethical statement

Experiments carried out as part of this study were under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: P9D87106F] and with approval from and in accordance with the University of Portsmouth Animal Welfare and Ethical Review Board guidelines.

FMP Y-maze apparatus

For testing larvae, juvenile and young adult zebrafish three different sizes of Y-maze were used, made of multiple, identical Y-mazes (*Figure 1*) with clear acrylic bases and white acrylic walls consisting of 3 arms of equal proportions, at 120° angle. Larval mazes (4-7 dpf): arms were length 1.2 x width 0.4 x depth 0.3 cm; each maze was filled with aquarium water that was changed between larvae. Six mazes were run per test in the Zantiks MWP behavioural unit (Zantiks ltd, Cambridge, UK). Juvenile (14-30 dpf):

a solid Y-maze surround consisting of 3 equal arms of length 2.5 x width 1.0 x depth 0.5 cm, mazes were filled with aquarium water and changed between fish. Two mazes were run per test in the Zantiks MWP behavioural unit. Young adult (90 dpf): arms were length 5 x width 2 x depth 14 cm; mazes were filled with 3 L of aquarium water to allow sufficient swimming depth. To prevent fish from jumping out of the test arena, a fine mesh cover was used during testing. Two mazes were fitted into a water-tight tank and run in the Zantiks AD behavioural unit.

All units were web-enabled and capable of live streaming video feed of in test activity of zebrafish to a laptop or mobile device. Starting and finishing trials was done remotely and logging of arm entries was written into the program script, recorded automatically and output in an excel spreadsheet. Cameras were located above the maze and enable continuous tracking of individual animals throughout the trial. Experimenter visibility and handling were kept to a minimum to reduce stress and distraction whilst animals were exploring the maze. Ambient light was a maximum of 2 lux in each of the testing units.

Figure 1.

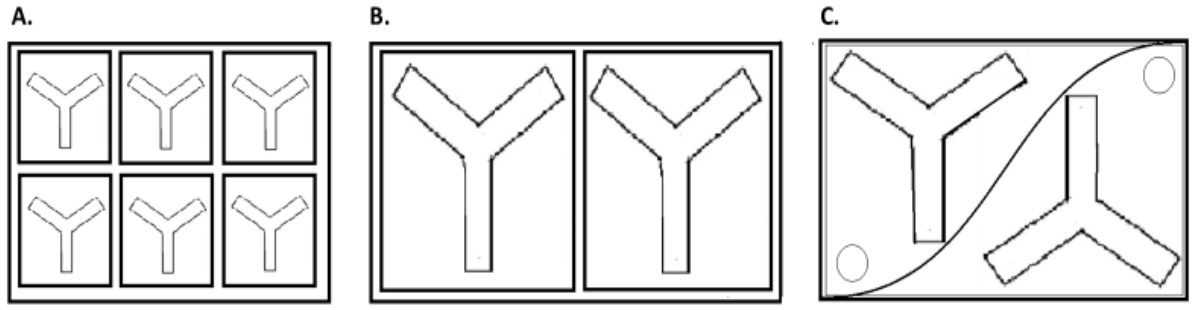

Figure 1. FMP Y-maze. Three different size mazes were used to assess working memory and cognitive flexibility. Each consisted of white acrylic walls and clear acrylic base, inserted into housing blocks and fitted into behavioural testing units to automatically track animal movements and record arm entries and exits. (**A**) Six mazes used for larvae, aged 4-14 dpf (arms; L1.2xW0.4xD0.3 cm). (**B**) Two juvenile mazes, for age 30 dpf (arms; L2.5xW1.0xD0.5 cm). (**C**) Two adult mazes, for age 90 dpf (arms; L5.0xW2.0xD14.0 cm).

Protocol

One-hundred and fifteen larval (4 and 7 dpf, n = 45), juvenile (14 and 30 dpf, n = 52) and young adult (90 dpf, n = 18) AB zebrafish were used for assessing differences in WM and cognitive flexibility in zebrafish developing from larvae to young adults. Sample sizes were calculated based on power analyses and previous studies, and based on α-levels of 0.05 and power (β) of 0.8 (Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Fish were experimentally naïve, and each fish was only used in one trial. There was no training or habituation required prior to testing in the FMP Y-maze. Fish were individually netted or pipetted (dependent on size) directly from home tanks (or dishes) into their respective mazes and placed into behavioural units. Behavioural tests were conducted in the Zantiks, fully automated behavioural testing units for adults [AD unit] and larvae/juveniles [MWP unit] (Zantiks Ltd., Cambridge, UK). Each fish was free to explore the maze for 1 h, no changes in environment, novelty or food rewards were introduced during the trial. Logging data of arm entries were recorded automatically and analysed on completion of all testing groups as performed in previous studies (Cleal, Fontana, et al., 2020; Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019).

Whole-body cortisol

For determination of cortisol levels following an acute stress zebrafish were grouped in 90 mm petri dishes or 2.8L housing tanks for larvae/young juveniles (4 dpf n=50, 7 dpf n=40, 14 dpf n=10) and juveniles/young adults (30 dpf n=10, 90 dpf n=5) respectively. Acute stress was delivered through mechanical stirring of the water using a Pasteur pipette for 4-14 dpf or using a net for 30-90 dpf. Acute stress was delivered for 5-min, after which all fish were left to recover for 10-min, as previous research has shown peak cortisol responses within 3-15 min of receiving a stressor in zebrafish (Ramsay et al., 2009). Following the recovery period, zebrafish were immediately culled by immersion in ice-water and snap-frozen using liquid nitrogen. To ensure sufficient cortisol levels for detection, fish were pooled according to their age group into the following group sizes: 4 dpf n=50, 7 dpf n=50, 14 dpf n=10, 30

dpf n=3, 90 dpf n=1. Cortisol levels were detected using the Salimetrics Salivary Cortisol ELISA kit for human samples (Stratech, Cambridge, UK), which has been found to reliably detect cortisol in zebrafish using the method previously described by (Cachat et al., 2010;Parker et al., 2012). Briefly, samples were homogenised in 5 mL of chilled PBS. Following complete homogenisation, 5 mL of diethyl ether was mixed with the homogenised samples and centrifuged at 7000 x *g* for 15 min. The top, organic layer was removed, and the process was repeated until all the cortisol-containing layer was removed, and then subsequent evaporation of the diethyl ether was continued overnight at room temperature. The resulting cortisol was reconstituted using 1 mL of chilled PBS. The ELISA was carried out in a 96-well plate following the manufacturer's instructions. Standards were used to determine the final cortisol concentration (ng/g^{-1}) using OD readings. Five biological replicates were run for each age group, with each biological replicate being run in duplicate. Inter- and intra-assay coefficients of variation were determined.

RNA extraction and gDNA removal

Fish were culled using ice-water immersion, followed by severing of spinal cord. Whole larvae (4-14 dpf) or whole brains (from 30-90 dpf) were removed and snap frozen using liquid nitrogen. Samples were homogenised and total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's instruction. After extracting and purifying RNA, samples were treated using the RapidOut DNA Removal Kit (Thermo Scientific), following manufacturer's instructions to remove contaminating gDNA from RNA samples prior to conversion into cDNA.

cDNA synthesis

Following removal of gDNA from RNA, sample concentrations were measured using the ND 1000 spectrophotometer. To ensure the same amount of cDNA was synthesised for each sample a maximum of 1 µg of total RNA was used per reaction (the same total concentration of RNA was loaded into each reaction). Reverse transcription of mRNA to cDNA was done using the High Capacity RNA-to-cDNA Kit

(Applied Biosystems) following the manufacturer's protocol. After conversion into cDNA, samples were diluted with ddH_2O to a final concentration of 5 ng/µL. Samples were stored at -20°C until use.

Quantitative Real-Time PCR (RT-qPCR)

Quantitative real-time PCR (RT-qPCR) assays were used to validate relative gene expression based on SYBR green detection. *Gr* primers were predesigned by qPrimerDB (Lu et al., 2018), *eef1a* primers were based on previous studies (Parker et al., 2016; Tang et al., 2007). Primers were synthesised by Invitrogen and are listed in **Table 1**. The Roche LightCycler® 480 High Resolution Melting Master mix and the LightCycler® 96 (Roche Life Science) were used to amplify and detect the transcripts of interest. Thermal cycling conditions were as follows: 95°C for 600s (recommended by manufacturer), 40 cycles of 95°C for 15s, 58°C for 20s and 72°C for 35s followed by melt curve analysis. Melt curve analysis, negative reverse transcription (RT) controls and negative template controls were used to determine specificity and to check for genomic contamination. Elongation factor 1 alpha (*eelf1a*) was used as a housekeeping gene (Tang et al., 2007). Gene-expression levels were calculated using delta-delta CT method and normalised to *eelf1a*. Changes in expression were relative to 4 dpf to demonstrate fold increases during development. Data are presented as means ± SD (n = 4 per group; assayed in duplicate).

Table 1. Primer sets used for qPCR

Gene name	Gene	Primer sequence (Forward)	Primer sequence (Reverse)	Amplicon size
Eukaryotic translation elongation factor 1 alpha	*eef1a*	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGCATTAC	87
Glucocorticoid receptor	*gr*	ACGGTTCTATCAGCTCACTAAG	AAACTCCACGCTCAGAGATTTA	109

Data processing and statistical analysis

Data pertaining to Y-maze performance were collected as a series of binary left and right turns, which in the absence of bias, would be equally likely to be selected (Deacon, Nick, et al., 2006; Frith & Done, 1983; Robert Gerlai, 1998; Stroe-Kunold et al., 2009). In order to discover if turn choice in the FMP Y-maze was a Markov process (overall patterns of turns were chosen randomly) we used a two-choice guessing task analysis (Frith & Done, 1983) to exploit information theory to detect patterns in large data series by 'chunking' turn choices into small groups of information (i.e. groups of four turns, left, left, right, right-LLRR) (Guze, 1993). Previous studies, using two-choice guessing tasks with human participants, have found that examining greater than four or five previous choices results in the calculation of the probability of a current event occurring redundant (Guze, 1993; Hochberg & Attneave, 1961). Therefore, we used a third order uncertainty to chunk consecutive turn choices into overlapping groups of four turns, limiting the number of alternative sequences to 2^4= 16 possible turn configurations, known as tetragrams (**Table 2**). If tetragram selection was completely random, a Markov or 'memoryless' process, the relative frequency of all tetragram sequences would be equal at approximately 6.25% (100% of turns/16 tetragram possibilities = 6.25%). Higher rates would be evidence of strategic tetragram selection (Gross et al., 2011). Using a custom written program we transformed the raw data (arm entry) into tetragram sequences of overlapping choices of turn (i.e. [L,R,{L,R],R,R}L-the first 3 tetragram sequences would be; [LRLR], RLRR, {LRRR}). Previous studies highlighted a role for two particular tetragram configurations, which were alternations (LRLR, RLRL) and repetitions (LLLL, RRRR), therefore particular attention was paid to these patterns (Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019; Gross et al., 2011).

Data were split into two formats for analysis; total percentage use (calculated as a proportion of total turns) of each tetragram sequence for the 1 h of exploration, referred to as 'global' search strategy. This was used as a measure of WM as repetition of previous turn choices must be remembered for patterns of movement to be repeated over 1 h of exploration, requiring memory of arm entries and order of entry. The second type of strategy, known as an 'immediate' search strategy, analysed search

pattern configurations over 10 min time bins throughout the trial, equating to six equal, consecutive time bins. This analysis was used to assess cognitive flexibility. Animals that are not able to update information gained during exploration of the FMP Y-maze will likely perform similar strategies over each time bin. However, those that are able to change behaviour in response to new information would be expected to change movement patterns over time, therefore differences in tetragram usage would be expected with each 10 min time bin.

Data were analysed in IBM SPSS 25.0 and GraphPad Prism 8.4.2. All data were log transformed and a linear mixed model (LMM) was employed to assess alternations and repetitions for all age groups with effect of age and time (as a between subject factors), animal ID (as a random effect) and total turns (as a covariate) to account for the individual variability of movement within and between age groups. One-way ANOVA was used to compare strategies within age groups. Shapiro-Wilk test of normality and boxplot with Tukey confidence interval was used to identify outliers. Tukey's test and Sidak's test for multiple comparisons was used as a *post hoc* analysis. Locomotion was analysed using total turns completed during 1 h of exploration in the FMP Y-maze. Data from total turns, ELISA and qPCR were tested for normality using the Shapiro-Wilk test and analysed using one-way ANOVA followed by Tukey's *post hoc* analysis. Results were considered significant when $p \leq 0.05$. Two adult zebrafish (90 dpf, #5, #7) were excluded from analysis due to tracking errors. Three data points were excluded from locomotion analysis (30 dpf, #2, #18, #26) due to falling outside of the 95% confidence interval. However, as data are normalised as a proportion of total turns for assessing tetragram usage, these animals were not excluded from strategy analysis. No data points were excluded from ELISA or qPCR due to the limited sample size.

Table 2. Tetragram sequences

Tetragram sequence
LLLL #
LLLR
LLRL
LLRR
LRLL
LRLR *
LRRL
LRRR
RLLL
RLLR
RLRL *
RLRR
RRLL
RRLR
RRRL
RRRR #

*** Alternations # Repetition**

4.4 Results

WM from larval development to young adulthood

Search strategies were compared between developing larvae to young adult zebrafish. Our data showed that 4dpf larvae used all tetragram configurations at random. We, therefore, found no evidence of any strategic preference to specific tetragram sequences when exploring the maze. Use of a decipherable strategy is not evident until 7 dpf, at which stage larval zebrafish begin to show signs of a specific search strategy that consisted of non-random selection of alternating patterns of left and right turns (LRLR, RLRL). Alternations dominated exploration patterns and were intermittently used between random selection of each of the other 14 tetragram sequences (**Fig 2A**). This selective use of alternations was evident in 7, 14, 30 and 90 dpf zebrafish which all used alternations above chance (6.25%) and significantly greater than any other tetragram sequence [One-way ANOVA, $F_{(7, 263)} = 27.23$, $p < 0.0001$], only 4 dpf larvae did not have a significant difference between alternations and any other configuration

(**Fig. 2B**). The two main strategy configurations shown to be susceptible to cognitive perturbations are alternations (LRLR, RLRL) and repetitions (LLLL, RRRR). We therefore compared the effect of age, on use of each of these sequences, revealing a significant effect on of age on alternation use (LMM: $F_{(4,116.31)}$ = 3.56, p = 0.009), but no effect on repetitions (LMM: $F_{(4,105.26)}$ = 1.22, p = 0.305) (**Fig. 3**).

Increasing cognitive flexibility with age

We have investigated how developmental stage affects the capability to update information presented in the FMP Y-maze and alter behaviour, as necessary. We examined tetragram use for successive 10-min intervals during the 1 h of exploration for each age group (**Fig. 4**). Due to the greatly reduced locomotor activity of 4 dpf larvae compared to all other age groups, and no evidence of strategy during exploration, this age group was removed from time analyses. Our results show that only 90 dpf adults demonstrated cognitive flexibility by increasing use of alternations over time (7 dpf: $F_{(5,50.69)}$ = 0.97, p = 0.446; 14 dpf: $F_{(5,109.29)}$ = 1.75, p = 0.129; 30 dpf: $F_{(5,103.76)}$ = 0.62, p = 0.682; 90 dpf: $F_{(5,71.08)}$ = 3.37, p = 0.009). Our data demonstrate that age is a critical factor when developing strategy over time (**Fig. 5**).

Figure 2.

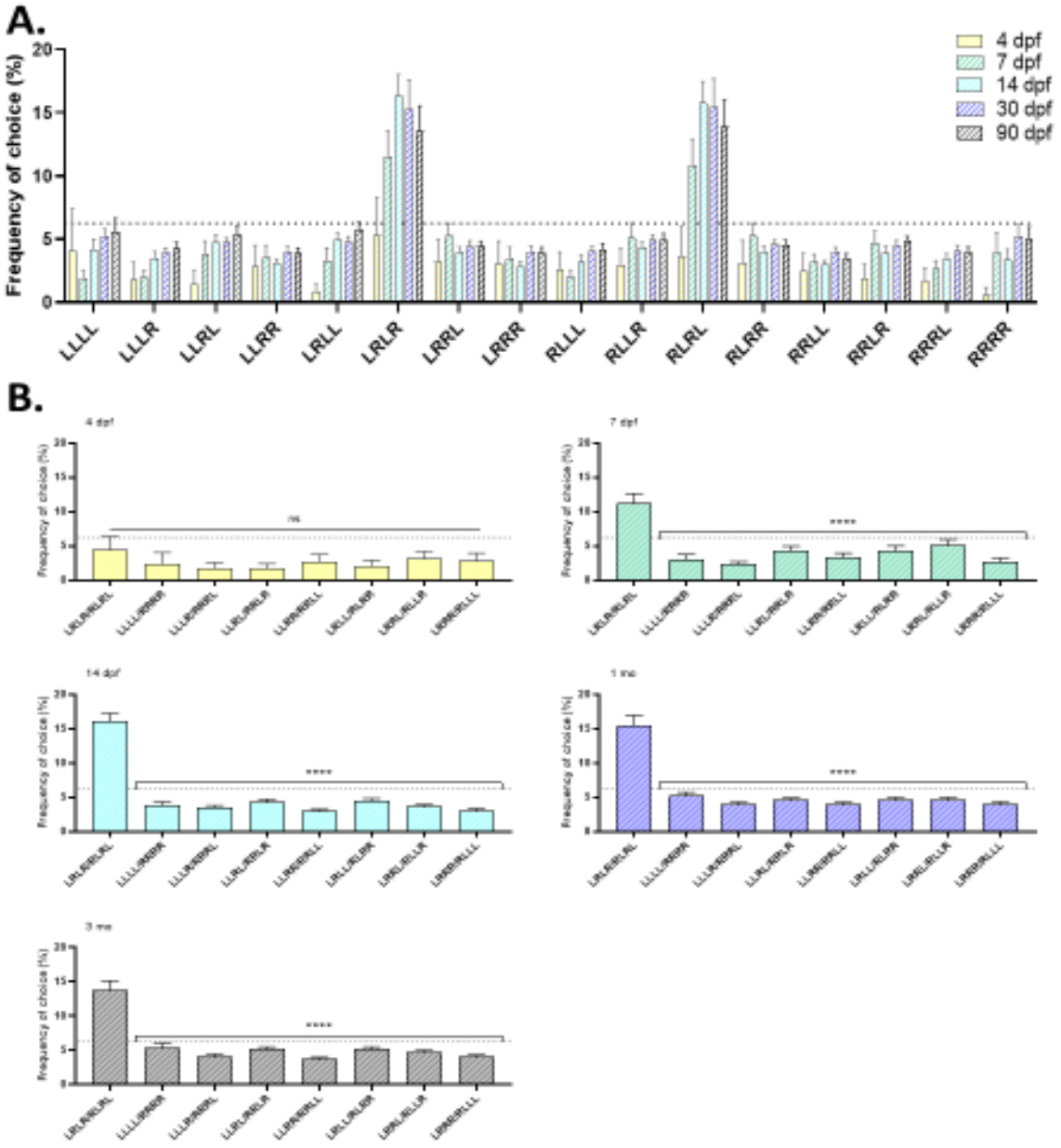

Figure 2. Global search strategy in the FMP Y-maze. (**A**) Frequency distribution of tetragram use after 1 h of free exploration for 4 dpf (n= 24), 7 dpf (n= 21), 14 dpf (n= 25), 30 dpf (n= 24) and 90 dpf (n= 16) zebrafish. Patterns show inflated use of alternations. (**B**) Mean use of each tetragram pair (i.e., LLLR+RRRL, LLRR+RRLL) compared to alternations (RLRL+LRLR). Normality checked using the Shapiro-Wilk test. Normally distributed data were analysed using One-way ANOVA. The dashed line denotes to chance selection (~6.25%). Bars are mean, error bars are mean + SEM. * $p < 0.05$, **** $p < 0.0001$, ns – not significant.

Figure 3.

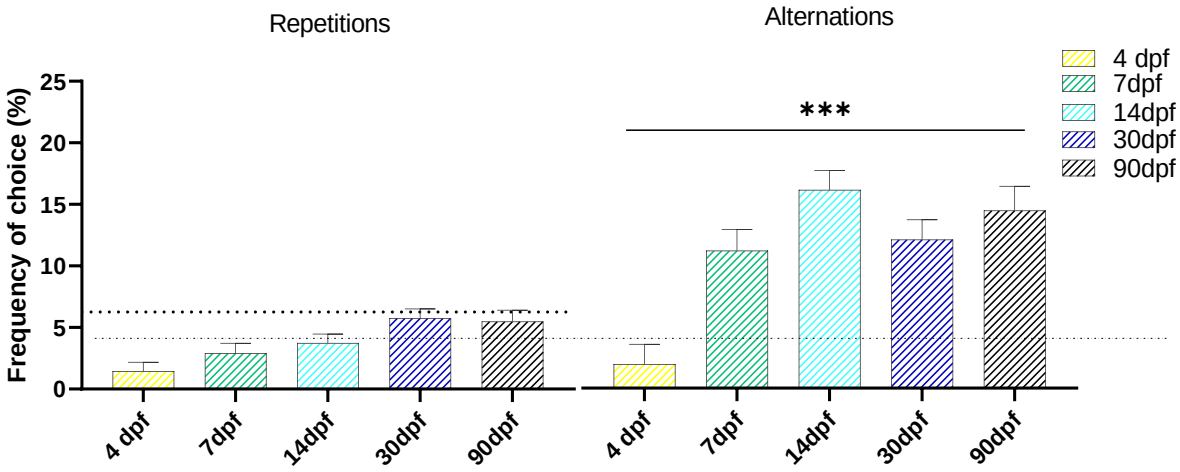

Figure 3. Age comparison of global use of repetitions (LLLL+RRRR) and alternations (LRLR+ RLRL). Data were log transformed and analysed using a LMM. The *dashed line* equates to chance selection of sequences (approximately 6.25%). Bars are mean, error bars are mean + SEM. ***$p \leq 0.001$.

Figure 4.

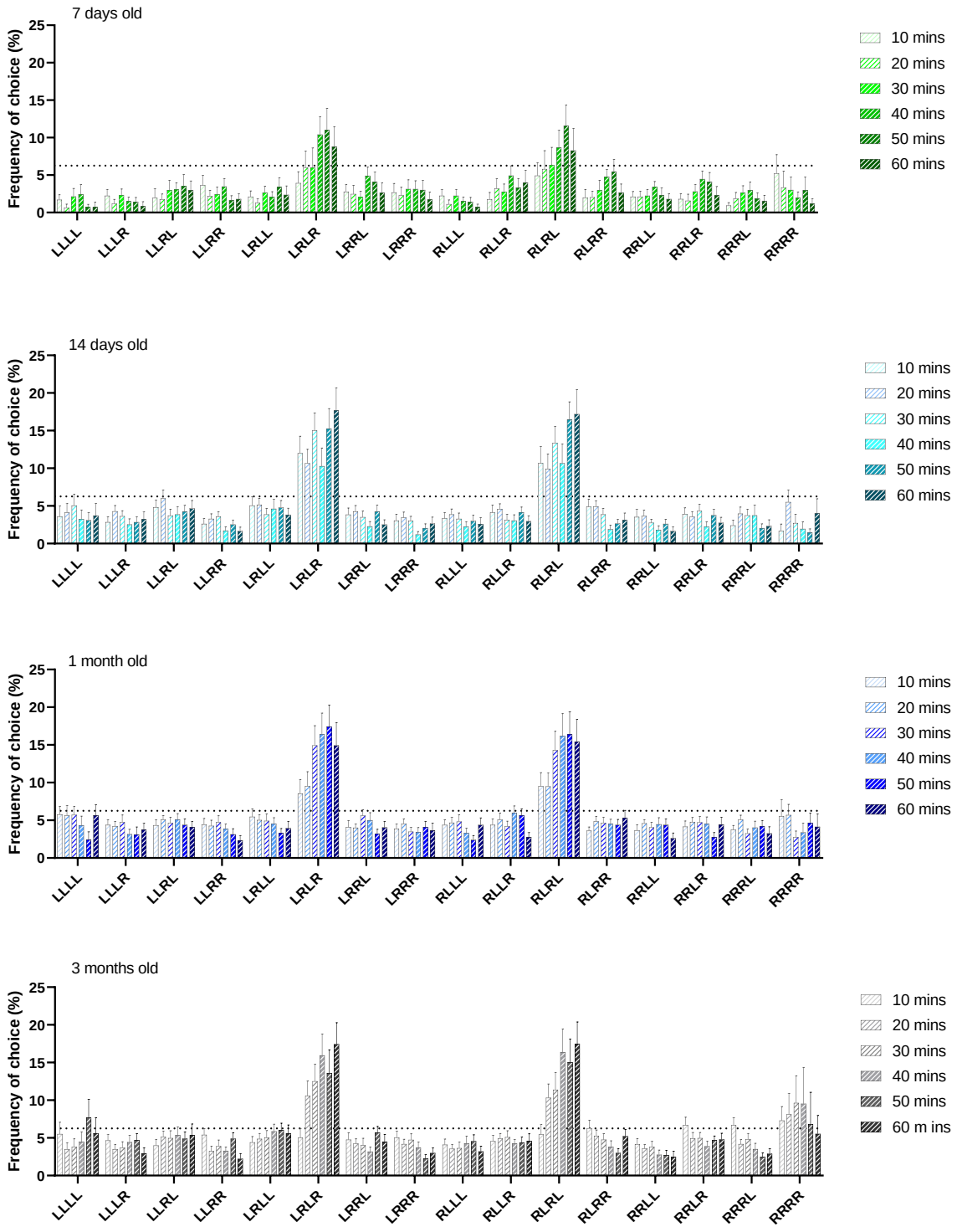

Figure 4. Frequency distribution of each tetragram sequence over successive 10 min intervals over 1 h of exploration in the FMP Y-maze for 7 dpf (n= 21), 14 dpf (n= 25), 30 dpf (n= 24) and 90 dpf (n= 16) zebrafish. The dashed line denotes to chance selection (~6.25%). Bars are mean, error bars are mean ± SEM.

Figure 5.

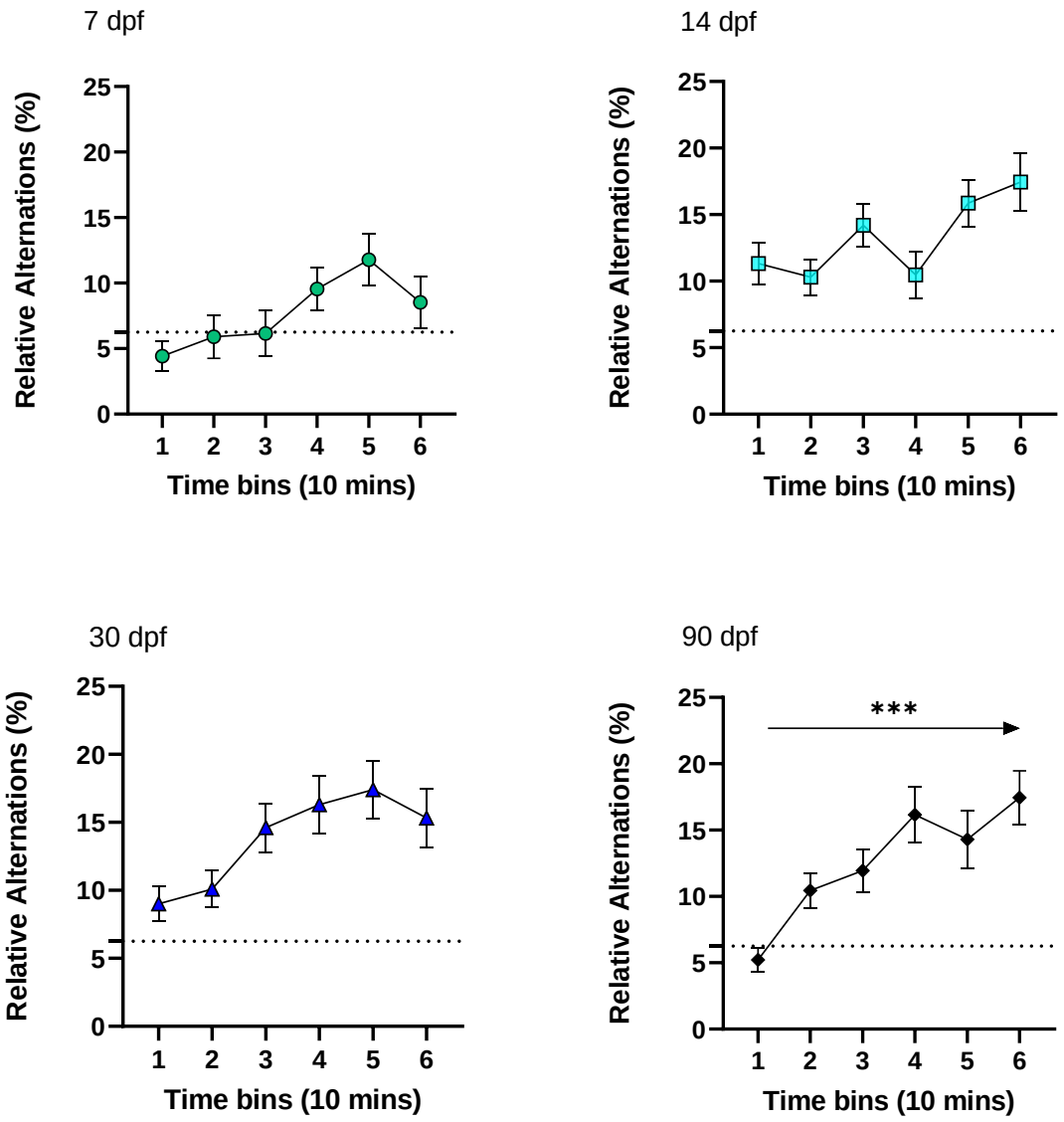

Figure 5. Effect of time on alternation use across six successive 10 min time bins, during 1 h of exploration. Data were log transformed and analysed using a LMM. The *dashed line* equates to chance selection of sequences (approximately 6.25%). Points are mean, error bars are mean ± SEM. ****p ≤ 0.001*.

Effect of age on locomotion

Locomotion was measured by the number of arm entries (denoted by total turns) in the FMP Y-maze. We examined the effect of age on movement during the first 10 minutes and over the full hour of exploration. We found that there was a significant effect of age on total turns for both the first 10 mins [One-way ANOVA, $F_{(4, 109)} = 16.72$, $p < 0.0001$] and 1 h [One-way ANOVA, $F_{(4, 107)} = 47.04$, $p < 0.0001$] of search, demonstrating increased number of turns with increasing age (*Figure 6*). Tukey's *post hoc* analysis revealed that all age groups travelled significantly less than 90 dpf young adults. 4 and 7 dpf larvae performed a similar number of turns, but both were significantly lower than all other age groups for 10 mins and 1 h of exploration (**Table 3**).

Figure 6.

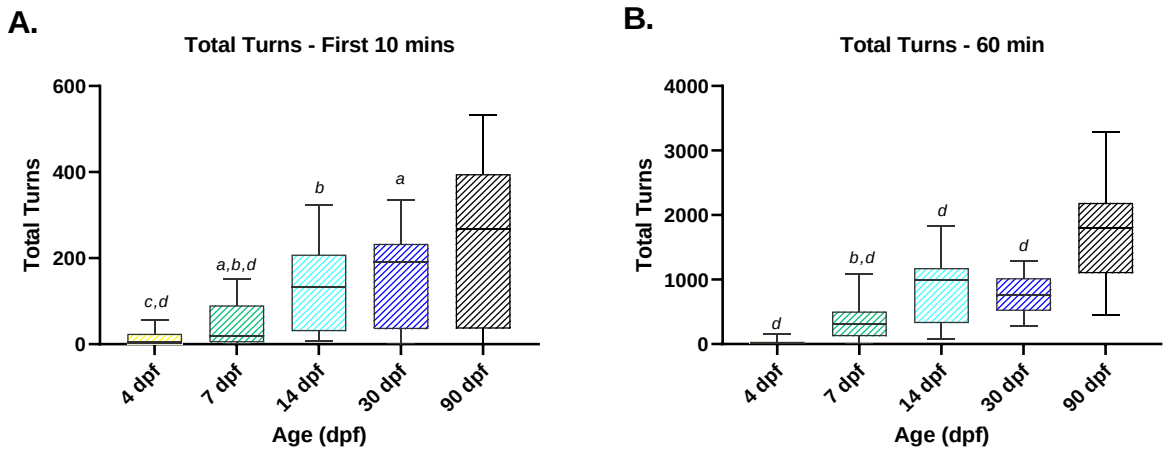

Figure 6. Locomotor activity measured by total turns for each age group for (**A**) the first 10 mins of exploration (4 v 7 dpf = *c;* 4 v 14, 30 and 90 dpf = *d;* 7 v 14 dpf = *a;* 7 v 30 dpf = *b;* 7 v 90 dpf = *d;* 14 v 90 dpf = *b;* 30 v 90 dpf = *a*) and (**B**) the full hour of exploration in the FMP Y-maze (4 v 14 30 and 90 dpf = *d;* 7 v 14 and 30 dpf = *b;* 7 v 90 dpf = *d;* 14 v 30 dpf = *d;* 30 v 90 dpf = *d*). Mean is depicted by the line across the box, whiskers represent 95% confidence interval. *a= * p≤ 0.05, b= ** p≤ 0.01, c= *** p≤ 0.001, d= **** p≤ 0.0001.*

Table 3.

Age Group	95% CI of diff. 10 mins of exploration	P-value	Significance	95% CI of diff. 1 h of exploration	P-value	Significance
4 dpf vs. 7 dpf	-29.49 to 12.88	0.8127	ns	-645.4 to 36.79	0.1040	ns
4 dpf vs. 14 dpf	-49.74 to -9.214	0.0009	***	-1090 to -437.5	<0.0001	****
4 dpf vs. 30 dpf	-54.71 to -14.58	<0.0001	****	-1071 to -412.0	<0.0001	****
4 dpf vs. 90 dpf	-80.37 to -36.16	<0.0001	****	-2024 to -1313	<0.0001	****
7 dpf vs. 14 dpf	-42.16 to -0.1854	0.0469	*	-797.3 to -121.5	0.0024	**
7 dpf vs. 30 dpf	-47.14 to -5.540	0.0057	**	-778.3 to -96.09	0.0050	**
7 dpf vs. 90 dpf	-72.73 to -27.19	<0.0001	****	-1731 to -997.5	<0.0001	****
14 dpf vs. 30 dpf	-25.03 to 14.69	0.9510	ns	-304.0 to 348.5	0.9997	ns
14 dpf vs. 90 dpf	-50.70 to -6.873	0.0037	**	-1258 to -551.9	<0.0001	****
30 dpf vs. 90 dpf	-45.36 to -1.881	0.0261	*	-1283 to -571.1	<0.0001	****

Effect of age on whole-body cortisol and gr mRNA expression

To assess age related changes in HPI axis stress response, whole-body cortisol levels were measured after 5 min acute stress followed by a 10 min rest period. There was a significant effect of age on post stress cortisol levels (One-way ANOVA, $F_{(4,18)} = 5.96$, $p = 0.0031$). Sidak's multiple comparison p*ost-hoc* analysis revealed a significant difference between 30 dpf and 4 ($p = 0.0131$), 7 ($p = 0.0144$) and 14 ($p = 0.0178$) dpf resulting in an almost 10-fold increase in cortisol levels in 30 dpf juvenile zebrafish compared to cortisol responses in larvae. There was no difference between cortisol levels in 30 dpf juveniles and 90 dpf ($p = 0.7272$) young adults. There were no significant differences between any other age group (**Fig. 7A**).

We investigated the effect of age on whole brain *gr* mRNA expression analysing fold change from 4 dpf larvae to 90 dpf young adult zebrafish. (**Fig. 7B**) shows gene expression data of each age group relative

to 4 dpf larvae. We found a significant effect of age on *gr* expression [One-way ANOVA, $F_{(4, 15)} = 138.1$, $p < 0.0001$]. *Post-hoc* analysis revealed that there was no difference between 4, 7, 14 and 30 dpf mRNA expression levels, however, all ages were significantly different to 90 dpf (4 dpf vs. 90 dpf; 95% CI -11.88 to -8.457; 7 dpf vs. 90 dpf; 95% CI -11.87 to -8.450; 14 dpf vs. 90 dpf; 95% CI -12.19 to -8.765; 30 dpf vs. 90 dpf; 95% CI -12.06 to -8.640).

Figure 7.

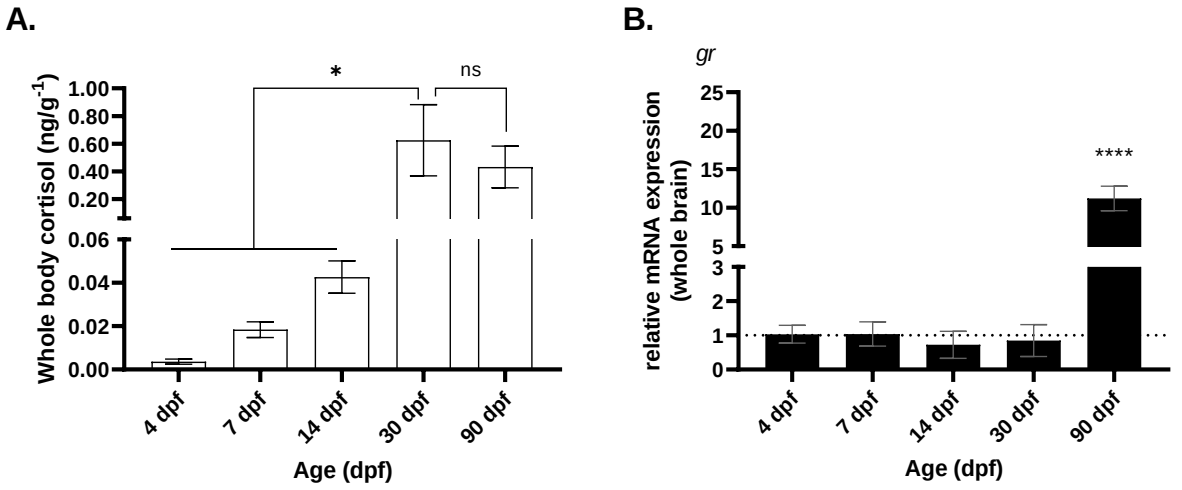

Figure 7. Whole-body cortisol levels for 4, 7, 14, 30 and 90 dpf zebrafish following exposure to an acute stressor (**A**). Relative, whole brain *gr* mRNA expression in 4, 7, 14, 30 and 90 dpf (n=4 per group). Data were normalised to housekeeping gene *eelf1α* and defined as fold change relative to 4 dpf larvae (**B**). Groups were analysed using One-way ANOVA followed by Tukey's *post-hoc* analysis. Data are mean ± SD, ** $p \leq 0.01$, **** $p \leq 0.0001$.

4.5 Discussion

The aim of this study was to understand how executive functions and HPI axis activity in zebrafish are affected by developmental stage. Using the FMP Y-maze, we discovered specific movement patterns used for exploring a novel environment. We observed random search patterns used by larvae dependent on the yolk-sac, but a strategic pattern of exploration in free-feeding larvae, juveniles and young adults, showing preferential use of alternation sequences consisting of consecutive left and right turns (LRLR, RLRL), which has previously been reported to correlate with WM performance (Cleal, Fontana, et al., 2020). Thus, indicating a role for enhancing WM processing in improved strategy development. Through fine-scale analysis we further revealed a changeable strategy that was adapted during trial progression, implicating a role for cognitive flexibility in updating behavioural responses within the maze. In addition to improved cognitive performance with increasing age, we found a significant effect of increased age on increased HPI activation that was evident from 30 dpf. Age was also found to influence *gr* mRNA expression with a sharp rise upon reaching young adulthood, 90 dpf. Combined our data suggest three independent stages for maturation of cognitive processing, stress reactivity and the mechanisms for homeostatic regulation of stress responses.

The ability to learn from experience and to recall information about the surrounding environment is critical to an organism's survival and ability to exploit their natural habitat (Roy & Bhat, 2016). Numerous studies in teleosts and mammals have revealed sophisticated behaviour regarding the use of cognitive control to appropriate action selection in the most efficient manner (Howard et al., 2017; Kottler et al., 2019; Monsell & Driver, 2000). However, in order to achieve adult optimised performance, there is continuous development and improvement throughout larval and juvenile stages (Anderson et al., 2004; Browman & O'Brien, 1992; Grecian et al., 2018; Osborne et al., 2013). In the FMP Y-maze young 4 dpf larvae were the only age group to search the maze completely at random, with all tetragram configurations used at a rate less than or equal to chance. Life history of zebrafish may play a critical role in the stage at which sophisticated search strategies are implicated. For example, zebrafish have very low motility rates during the early stages of development, whilst dependent on the yolk-sac

for survival (Colwill & Creton, 2011). However, free-swimming occurs once larvae are independent feeders and have begun foraging for live prey. This is reported at 5 dpf when resources from the yolk sac have been almost completely consumed and swimming activity and duration have increased in response to the need to search for food (Strähle et al., 2012). Yolk-feeding larvae were comparatively inactive compared to other age groups, raising the question whether hypolocomotion was responsible for the random movement patterns (Selderslaghs et al., 2010; Strähle et al., 2012). Alternatively, dependence on an available food source, and incomplete development of neural structures could result in under-developed cognitive capacity. The lack of systematic search strategy in 4 dpf larvae may be due to the continued development of anatomical structures and axonal pathways. Analysis of dopaminergic development, critical to motor activity and executive function, showed that despite most dopaminergic neurons being present by 4 dpf, many axonal projections, such as those into the telencephalon (equivalent to prefrontal cortex in mammals, and a crucial area for learning and memory) (Salas et al., 2006) are not completed until 5 dpf (Du et al., 2016). This may provide a potential explanation as to why 4 dpf larvae are unable to develop strategies above random choice selection to strategically navigate the maze (Hernandez et al., 2018; Roy & Bhat, 2016). Many studies have relied on external stimuli, such as light/dark changes, to provoke increased movement behaviour in larvae and enable detection of changes in behavioural responses to varying conditions (de Esch et al., 2012). To identify the underlying processes used for exploratory or information seeking behaviour of yolk-dependent larvae, alternative paradigms, either not dependent on locomotion or increasing locomotor activity, would be required.

Older age groups, unlike yolk-dependent larvae, were observed using a specific global search strategy for the full period of exploration in the FMP Y-maze. Although the majority of search configurations were selected randomly, there was evidence of dominant, intermitted use of alternation strategies that relied on equal left and right turns (LRLR, RLRL), performed at a higher frequency than chance. Previous work by our group has demonstrated a correlation between greater use of alternations

and WM performance, thus demonstrating that 4 dpf show no discernible WM use, whilst from 7 to 90 dpf WM is being utilised to enhance exploration strategy.

A key aspect of animals with higher cognitive functions, such as learning and memory, is the ability to update their knowledge of a search area based on recent experiences and adapt their behaviour accordingly (Bracis et al., 2015). The FMP Y-maze protocol provides a negative feed-back task, in which animals exploring the maze are continually presented with an absence of novel stimuli, e.g., food or reward. This would be expected to cause a strategy change, based on an animal's ability to alter behaviour in response to environmental cues. Previous studies from our lab have shown that adult zebrafish at 6 months old are able to use information obtained exploring the FMP Y-maze to alter search strategy by increasing the use of alternations over time (Cleal, Fontana, et al., 2020). However, this ability is strongly affected by senescence or inhibiting memory forming or retrieval pathways (see Part 1: Chpt 5) (Cleal et al., 2020 Aging) (Cleal, Fontana, et al., 2020). The only age group to demonstrate CF in the FMP Y-maze, by significantly up-regulating alternation use with increasing time, were the young adults (90 dpf). No other age showed significant differences between the use of alternations in the first 10 min of exploration compared to the last 10 min, whilst young adult zebrafish linearly shifted alternation-use from below chance, to greater than one fifth of movement patterns from the first to last 10 mins of exploration, demonstrating substantial overlap with the adult strategy. Changes in alternation use during extensive exploration supports a role for experience based, individual learning, influencing search strategy and is reflective of the ability to adapt to environmental cues. The absence of this behaviour in younger age groups is reflective of an immature cognitive performance that has not yet reached adult-level abilities.

Zebrafish whole-body cortisol levels, in response to a mild, acute stressor, remained consistently low in 4, 7 and 14 dpf zebrafish. However, juvenile zebrafish, 30 dpf, showed a significant increase in HPI axis activation resulting in an almost 10-fold greater cortisol level compared to larvae, but no difference between juveniles and young adults. Previous studies in zebrafish have assessed ontogeny of cortisol response to stressful stimuli during embryonic to early larval development and during

adulthood (from young adult, 3 months, to ageing adult, 19 months) (Ramsay et al., 2009; Wilson et al., 2013). No differences during adult development were identified; however, there was a significant effect of age during embryonic/early stage larval development due to the maternal deposit of cortisol, which is depleted prior to hatching and then the larvae begins to synthesis its own cortisol causing an increase after hatching (D. Alsop & Aluru, 2011).

Examination of whole brain *gr* mRNA expression revealed age-related changes in increasing expression levels that were significantly different in young adults (90 dpf) compared to larvae and juveniles. The postnatal ontogeny of GR gene expression has been examined in a number of species including humans, marmosets, rats and mice, similarly with the aim of identifying critical periods of development that represent increased psychopathological vulnerabilities (Perlman et al., 2007; Pryce, 2008; Pryce et al., 2005; Yi et al., 1994). Our findings are slightly at odds with mammalian studies which either show no age-related differences (particularly in the hippocampus) or in regions that do show differences there is generally an increase from infant to adolescence, but no difference between adolescence to adult (Pryce, 2008). In humans there was no discernible difference between age groups (infant, adolescent, young adult, adult, aged) and GR expression in the hippocampus. However, distinct age-related differences were detected in the dorsal lateral prefrontal cortex (dlPFC), which showed an increase in expression in adolescence compared to infants and aged adults in the dlPFC II-VI layers and an increase in adults to infants in the dlPFC IV and VI layers, but no differences between adolescence and adults in any dlPFC layer (Perlman et al., 2007). Two possible reasons for increases being identified in young adult zebrafish, opposed to during the juvenile stage, could be due to the ages tested and examination of whole brain tissue. In zebrafish maturity is better determined by morphological and anatomical features opposed to 'age'. In a study by (Singleman & Holtzman, 2014), they propose the use of factors such as length, pigment pattern and fin morphology to provide a normalised staging of development in maturing zebrafish. Based on rearing at ~28.5°C, the embryonic stage has been classified as ending at ~3 dpf. Following the embryonic stage is the larval stage, which they suggest lasts for ~6 weeks, ending around 45 dpf. This marks the beginning of the juvenile stage. Finally,

zebrafish reared at ~28.5°C reach sexual maturity by ~3 months (90 dpf) at which point they are considered adults (Singleman & Holtzman, 2014). It is therefore possible that at 30 dpf, our 'juvenile' group have not sufficiently matured and thus do not demonstrate the neuronal changes associated with this age group, i.e. increased expression of *gr* mRNA. It is possible that more detailed examination between 30 to 90 dpf may reveal an alternative period of adolescence that demonstrates up regulation of *gr* expression prior to young adulthood. In addition to age considerations, is the broad use of whole brain analysis used in zebrafish. Many mammalian studies have focused on expression changes in specific regions and can thus evaluate regional changes instead of global changes. This is a difficult feat in zebrafish using the method of qPCR, and therefore more in-depth study using alternative measures of *gr* expression, such as whole-mount *in situ* hybridisation, could provide more detailed information of localised, age-related changes.

Regardless of the age of onset of expressional changes, there has been little consistency in understanding the relevance of increases in GR expression in disease pathology. Recently, however, Alder et al (2018) investigated, in humans, the outcome following HPA axis stimulation (following septic shock). They examined not just basal expression levels of GR and cortisol levels following stress, but they furthered their analysis by identifying groups as high or low GR expression and cortisol level. Their findings suggest no direct correlation between general GR expression, cortisol levels and patient outcome. However, when subcategorising patients into groups of high and low levels they identified a pattern of recovery outcome. They identified four groups: high/high, high/low, low/high and low/low (relative cortisol level/relative GR expression level). They found that patients in the group with high cortisol levels following stress and low GR expression at baseline, as those at the greatest risk of complications after septic shock, accounting for 75%. Those in the high/high or the low/low groups had the same outcome likelihood of a complicated course both at 33% and those that had low cortisol levels and high GR expression were the least susceptible, with only 13% having a complicated course (Alder et al., 2018). This study suggests a critical role for the ratio of cortisol to GR expression as an indicator of increased vulnerability to disease following stress-induced activation of the HPA axis. Applying this

theory to the work presented here, identifying fish as low or high expression of cortisol and *gr* expression by designating group means those that fall below the median values of cortisol and *gr* expression levels are "low expression" and those above the median are "high expression" (cortisol: median (IQR) = 0.03 (0.01-0.19), *gr*: median (IQR) = 1.1 (0.61-1.4)). Therefore, 4 and 7 dpf larvae would be in the moderate effect category with low/low expression, as are 90 dpf young adults with high/high expression, but 14 and 30 dpf larvae would be in the highest susceptibility group, both with a high/low cortisol/*gr* expression level. Crucially, this implicates a mechanism of increased vulnerability (i.e., a 'critical period' of development) in late-stage larvae and juveniles. However, further work would be required to identify the relationship between the ratio of stress-induced cortisol levels compared to basal *gr* expression in decreasing the stress response (i.e. how rapidly are cortisol levels returned to baseline following stress in the different expression groups) and how this altered ratio of cortisol to *gr* impacts cognitive function.

4.6 Conclusion

Combined our data identify a single age group of particularly high vulnerability. 30 dpf juveniles demonstrate adult-level WM performance, however, they lack matured CF and show a lack of response to negative stimuli (i.e., the absence of reward or novelty presented in the FMP Y-maze). They are the first developmental stage to exhibit an exaggerated cortisol response following acute stress, causing a 10-fold increase in cortisol compared to larvae. Despite this level of cortisol reactivity being similarly reported in young adults, there is the addition of low levels of *gr* expression that is not evident in young adults, as they show substantial up-regulation of *gr* by this age. Combined with the increased stress responsivity, juveniles demonstrate a significant imbalance in the ratio of post stress cortisol levels and basal *gr*. Human studies of adolescence and patients with neuropsychiatric disorders, such as SZ and substance abuse, have reported prolonged stress responses, i.e., less efficient down-regulation of the HPA axis following a stress, and slower return of cortisol to pre-stress levels (Lupien et al., 2009; Nugent et al., 2015; Platje et al., 2013; A. G. Roberts & Lopez-Duran, 2019). This has often been suggested to be the result of aberrant HPA axis activity in neuropsychiatric disorders. However, this aberrant HPI axis

regulation appears to also be common to healthy juveniles. Thus, a negative feedback loop of poor behavioural responses and increased stress-sensitivity in adolescence becomes apparent: *gr* are a critical mechanism in restoring homeostasis quickly after activation. Low levels of receptors and high levels of circulating cortisol following stress, results in a delay in restoring homeostasis. However, the additional absence of up-dating behaviour in response to negative stimuli (i.e., poor CF) may potentially result in behaviours that cannot appropriately adapt to their environment, which may perpetuate the stress response further. Under extreme circumstances, it could be hypothesised that prolonged elevation of circulating cortisol may ultimately lead to impairments in other cognitive functions, such as WM, which may influence behaviours that perpetuate the stress response further. This hypothesis represents an extreme and simplistic view, but, none-the-less a plausible mode of altered stress responsivity during adolescent stages of development and provide a potential mechanism of increased susceptibility to stress-induced pathologies.

Chapter 5

So far, we have demonstrated the FMP Y-maze as a cognitive task suitable for assessing zebrafish. It has shown sensitivity to alterations in cognitive performance through pharmacological intervention and maturation of cognitive function from larvae to young adulthood. We have shown that this task can be used with larvae as young as 7 dpf. Our aim now, it is to test the applicability of the FMP Y-maze at the opposite end of the age scale. Here, we test the sensitivity of the FMP Y-maze to subtle changes in cognitive function associated with healthy ageing. In humans, even in healthy senescence, there are still reports of cognitive decline compared to younger adults. We thus hypothesised that healthy, ageing zebrafish would similarly show signs of decline in cognitive performance. We therefore anticipate a decrease in the use of the alternation strategy in ageing compared to younger counterparts. To understand the mechanisms involved in altered cognitive performance in ageing fish, we assess the role of the dopaminergic system as both a possible cause and therapeutic strategy to restore cognitive deficits. Furthermore, to assess the translational relevance of these findings, we tested young and ageing humans in the virtual FMP Y-maze to examine 1) task sensitivity to age related cognitive decline in a healthy human cohort, 2) translational use of the FMP Y-maze from zebrafish to humans and 3) the robustness of zebrafish as a model of ageing.

Dopaminergic Modulation of Working Memory and Cognitive Flexibility in a Zebrafish Model of Aging-Related Cognitive Decline

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5.1 Abstract

Healthy aging is associated with a decline in memory and executive function, which have both been linked with aberrant dopaminergic signalling. We examined the relationship between cognitive performance and dopamine function of young, 6-month-old and aging, 24-month-old adult zebrafish (*Danio rerio*), revealing age-related decreases in WM and cognitive flexibility in the Free-Movement Pattern (FMP) Y-maze. Young and older adults received a D1-like agonist (SKF-38393, 35 μM) 30 minutes prior to behavioural assessment. We observed differences in WM performance, but not behavioural flexibility in aged zebrafish. Decline in dopamine transporter mRNA and oxygen metabolism were also evident in older zebrafish. These data suggest a potential role for dopamine via D1-like receptors for restoring deficits in WM during normal aging. The translational relevance of these findings was established by testing young-adult, 18-35-year-olds, and aged adults, 70+ year olds, in a virtual FMP Y-maze, which revealed a similar age-related decline in WM. Thus, strongly supporting zebrafish as a translational model of aging and cognitive decline.

***Keywords*:** FMP Y-maze; zebrafish; memory; dopamine; aging-related cognitive decline

5.2 Introduction

During the process of 'natural' aging, the brain undergoes gradual structural and functional changes that cause deterioration in cognitive ability, even in the absence of neurodegenerative disease (Harada et al., 2013; Salthouse, 2009). The rate of decline is highly variable, with many able to maintain good health and mental ability into their late 80s, whilst others are greatly susceptible to debilitating cognitive impairment and disease (Deary et al., 2012). Individual differences in cognitive aging have driven the identification of underlying biological factors that can predict vulnerability to decline or development of age-related diseases (Berry et al., 2016). Many studies of healthy aging have implicated multiple components of the dopaminergic system in declining cognition, with individual differences in dopamine signalling playing a profound role in performance of cognitive tasks and response to dopamine-altering medications (Cools & D'Esposito, 2011; Kimberg et al., 1997; Volkow et al., 1998). As a result, dopamine has become a focus of research investigating age-associated changes in cognition.

Dopamine plays an important role in many aspects of cerebral functions related to cognition such as attention, learning, WM and mental flexibility (Girault & Greengard, 2004; Naderi et al., 2016). Three key modulators of dopamine function are dopamine synthesis, reuptake and activation of dopaminergic receptors (Klanker et al., 2013). Through pharmacological manipulations, a complex network of interactions involving these modulatory systems are used to maintain dopamine homeostasis (Cools & D'Esposito, 2011), dysregulation of which has been shown to have severe, and sometimes opposing, effects on cognition, particularly WM and cognitive flexibility in health and disease (Brozoski et al., 1979; Cai & Arnsten, 1997; El-Ghundi et al., 2007; Rothmond et al., 2012; Zahrt et al., 1997). In a previous study (see Part 1: Chpt 3), we sought to delineate the role dopaminergic receptor subtypes play in formulating search strategies in zebrafish exploring the FMP Y-maze. We used SCH-23390, a selective D1 and D5 receptor antagonist (from here in referred to as D1-like receptors) and sulpiride, a selective D2, D3 and D4 receptor antagonist (from here in referred to as D2-like receptors) (Neve, 2013). We demonstrated a significant role for D1-like receptors in strategy formation in the FMP

Y-maze, with an associated impact on WM and cognitive flexibility. However, in the absence of reward or motivational learning, no such role was identified for D2-like receptors in exploring the maze (Cleal, Fontana, et al., 2020). Consequently, in models with deficits in WM or behavioural plasticity in the FMP Y-maze, we hypothesised that increasing D1-like receptor activation would enhance these cognitive domains.

Zebrafish (*Danio rerio*) have recently emerged as a promising model of cognitive aging (Gerhard, 2007; Lili Yu et al., 2006). Zebrafish live on average for ~3 years, but can have a life span as long as 4-5 years under laboratory conditions (Kishi, 2011). Changes in cognition are evident from ~2 years, increasing the accessibility of researching gradual senescence in a model of old age (Gerhard et al., 2002; Tsai et al., 2007). In humans, cellular senescence can be detected by a number of age-associated phenotypes using biological and biochemical markers. One such marker is beta-galactosidase, which in humans is only expressed in senescent cells (Dimri et al., 1995). Like humans, zebrafish also demonstrate senescence associated beta-galactosidase activity, with only faint signals detectable at 9-months old, increasing significantly once fish reach 17-months and older (Kishi, 2004). Additionally, like humans and rodents, zebrafish also show age-associated accumulation of oxidative proteins in skeletal muscles. (Kishi et al., 2003), showed higher levels of oxidised proteins in aged fish, at 24-months old compared to younger 3 and 6-month old fish (Kishi, 2004; Kishi et al., 2003; Tsai et al., 2007). We therefore selected 6- and 24-month-old zebrafish as these developmental stages show clear differences in biological markers of aging, both in zebrafish and in humans. Also, like humans and rodents, zebrafish have homologues of neurotransmitters, associated systems and brain regions necessary to assess learning, memory and executive functions such as attention and cognitive flexibility (K. A. Horzmann & Freeman, 2016; Matthew O. Parker, Brock, Walton, et al., 2013; Tufi et al., 2016). Ease of pharmacological manipulation and high throughput behavioural testing, additionally add to the convenience and suitability of zebrafish to model the effects of aging and age-related diseases (Gerhard, 2003; Gerhard & Cheng, 2002; Keller & Murtha, 2004; Kishi, 2004).

The FMP Y-maze has been developed to assess cognition, specifically, exploration, WM and behavioural flexibility, in a range of organisms, including zebrafish, flies, rodents and a virtual task for assessing humans (Cleal, Fontana, et al., 2020; Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Analysis of exploration patterns has led to the observation that vertebrate species are biased towards strategies utilizing alternating left and right turns, known as an 'alternation' (left, right, left, right-LRLR, right, left, right, left-RLRL). Pharmacological blockade of memory forming pathways in zebrafish, revealed a role for memory processing and behavioural flexibility in the formulation of search patterns and updating exploration strategies during task progression (Cleal, Fontana, et al., 2020). In the FMP Y-maze, WM is based on the ability to recall previous arm entries, similar to the alternation task of the T- or Y-maze (Stewart et al., 2011; van der Staay et al., 2011). Recording percentage-use of reoccurring patterns of four consecutive turn choices, known as tetragrams, over one hour of exploration gives rise to a 'global' search strategy. Similar exploration strategies, predominantly reliant on alternations, have been reported in rodents, both in the FMP Y-maze and T-maze using the same analysis (Cleal, Fontana, et al., 2020; Gross et al., 2011). Behavioural assessment of 1 h of free exploration in the FMP Y-maze enables the identification of WM abilities and cognitive flexibility, presenting the FMP Y-maze as a useful tool for assessing memory processing in animal models and potentially patients with disorders characterised by cognitive decline.

We used the FMP Y-maze and pharmacological agonism of D1-like receptors, to understand the role of dopamine in healthy aging in zebrafish. We compared behavioural phenotypes of 6-month-old adults with aged, 24-month-old adults in the early stages of senescence. Our hypothesis was that 24-month-old zebrafish may have begun experiencing mild cognitive decline with the increase of age-associated biological markers. We examined changes in WM and cognitive flexibility in a recently developed behavioural paradigm that can be used to assess both cognitive functions simultaneously with minimal handling and experimenter interference. We furthered our investigation by comparing changes in WM of young and aging zebrafish to changes in WM between young and old, healthy

humans. Our aim was to assess the translational relevance of age-related changes in memory processing in zebrafish compared to humans.

5.3 Materials and Methods

Ethical statement

The University of Portsmouth Animal Welfare and Ethical Review Board guidelines were followed for all experiments carried out as part of this study, and under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: P9D87106F]. Human experiments were conducted following approval from the University of Portsmouth Science Faculty Ethics Committee (SFEC-2019-062).

Animals and housing

71 male and female wild type (AB) zebrafish (*Danio rerio*) aged 6-months old (young adult, n=33) and 24-months old (aged, n=38) were used to assess cognitive aging as a commonly used strain of zebrafish for investigating aging (Kishi, 2004). Previous work in our lab has shown that there are no sex differences in the FMP Y-maze and therefore we did not examine the effect of sex as part of this study (Fontana, Cleal, & Parker, 2019). Zebrafish were bred in-house and raised in the University of Portsmouth Fish Facility. Fish were housed in groups of ~10-12 fish per 2.8L tank on a re-circulating system (Aquaneering Inc., San Diego, CA, USA), aquarium-water was maintained at pH 8.4 (±0.4). Previous work from our group and extensive pilot studies were used to calculate power analysis and inform sample sizes used in this study (Cleal et al., In press; Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Room and tank temperatures were maintained at 25-27°C on a 14/10-hour light/dark cycle. From 5 days post fertilisation (dpf) fish were fed on ZM fry food until adulthood when they were fed on a daily diet of live brine shrimp (maintained at the fish facility) and dried flake food (ZM Systems, UK) 3 times/day (once/day at weekends). All fish used in this study were experimentally naïve. Once behavioural testing had been completed test fish were euthanized by rapid cooling (immersion in 2^{o}C water), followed by decapitation and excision of the brain for downstream processing.

Drugs

To study the effect on WM, cognitive flexibility and mRNA expression of dopamine receptors, adults were incubated for 30 min in 35 µM (10 mg/L) of the selective dopamine D1/D5 receptor partial agonist SKF-38393 hydrochloride (Medchemexpress). Due to the water solubility, drug stock solutions were made by dissolving SKF-38393 in aquarium water. Drug administration was adapted from (Naderi et al., 2016), in which individual fish were entirely immersed in drug solution or aquarium water (control) by gently netting fish from home tank to a 400 mL beaker filled with 300 mL of drug or water and covered with a lid to prevent fish from escaping. Fish were netted into the beaker 30 mins prior to behavioural testing. Treatment time was based on previous studies with dopaminergic agonists, demonstrating that 30 mins of free-swimming during drug immersion was sufficient for drug to influence zebrafish behaviour (Irons et al., 2013; Naderi et al., 2016). Immediately following treatment fish were transferred into the FMP Y-maze.

FMP Y-maze

The protocol was carried out as described in our previous papers (Cleal, et al., in press; (Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Fish were recorded in a Y-maze for 1 hour. If the fish were adopting a random search strategy, it would be predicted that the distribution of tetragrams over a 1-hour period would be approximately stochastic (i.e., the relative frequency of each tetragram would be ~6.25%). *Figure 1* shows an example of a series of movement sequences performed in the FMP Y-maze, a combination of which provide a picture of the global search strategy used. To minimise stress, fish handling and experimenter visibility were both kept to a minimum. Behavioural testing was conducted using a commercially available, fully integrated testing environment, the Zantiks AD system for adult zebrafish (Zantiks Ltd., Cambridge, UK). Tanks were black, opaque acrylic with a transparent base. A white acrylic Y-maze insert was fitted into each tank. Two Y-maze inserts could be fitted per tank. The Y-maze dimensions were as follows: L500 mm x W200 mm x D1400 mm, with a 120° angle between arms. Tanks were filled with 3L of aquarium-water and placed into Zantiks

behaviour units, one tank per unit. Each system was fully controlled via a web enabled device. Filming was carried out from above, which allowed live monitoring of fish within the behaviour system. Data output was automated, preventing any bias in the recording of arm entries.

Figure 1A.

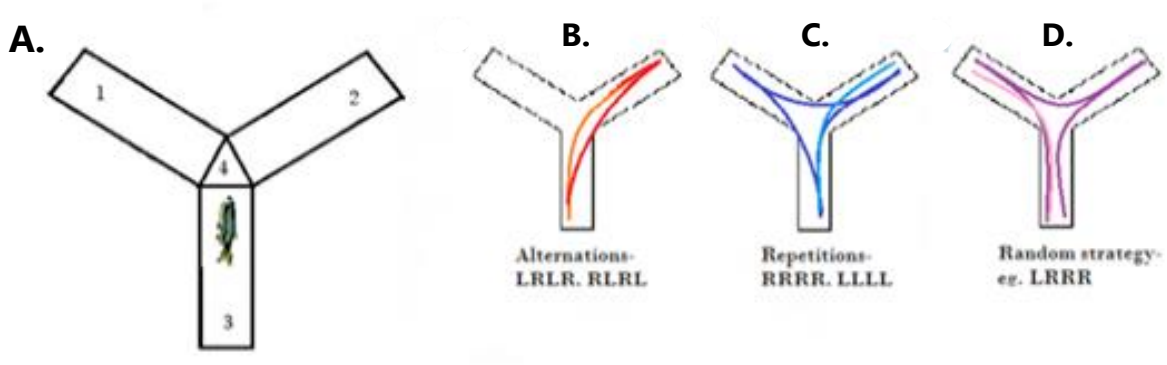

Figure 1A. FMP Y-maze behaviour of free-swimming zebrafish. (**A**) FMP Y-maze arena divided into three arms (1,2,3) and a neutral zone (4). (**B-D**) Examples of movement sequences based on 16 overlapping tetragrams of left and right turns. (**B**) Represents the dominant search strategy of alternations, made up of a series of left-right-left-right or right-left-right-left turn choices. (**C**) Demonstrates repetitions, where an animal turns in a clockwise or anticlockwise rotation for four continuous choices, represented by RRRR or LLLL. (**D**) An example of an alternate strategy possible using tetragram sequences. Here is shown LRRR In on anticlockwise direction, equivalent to RLLL in a clockwise direction.

Virtual FMP Y-maze

The human FMP Y-maze is based on a series of adjoining Y-shaped points, forming a honeycomb maze. At each choice point, participants must choose to turn either left or right (Figure 1A). In order to test for aging related cognitive decline, we recruited N = 80 participants, n = 40 'old' (>70) and n = 40 'young' (18-35). Based on power analyses from our previous studies (Cleal et al 2020), we required a minimum of n = 16 per group. An individual's data were excluded if they completed fewer than 10 turns. Following exclusions, the final sample size for the 'young' group was n = 35 subjects (mean age = 24±4.79 years;

23:12 male:female), and the 'old' group was n = 30 subjects (mean age = 73.83±3.93 years; 18:12 male:female). The maze was uploaded onto the crowdsourcing website Prolific Academic Ltd (https://www.prolific.ac), an online platform which recruits subjects explicitly for participation in research (Palan & Schitter, 2018). Specifically, Prolific can be used to set eligibility criteria, and allows participation of eligible subjects on a first-come, first-served basis until the total number of required participants is fulfilled. We established two studies, with the eligibility criteria based on age only. Once in the maze, participants were free to explore for a total of 5 minutes, after which they were automatically logged out and, via a link, returned to the Prolific website. Turn directions of participants were reported as x, y coordinates which were converted into left and right turns and subsequently transformed into tetragram sequences. Each participant was only permitted to enter the maze once. All participants were compensated £0.65 (rate = £7.20/hour) for taking part.

Figure 1B.

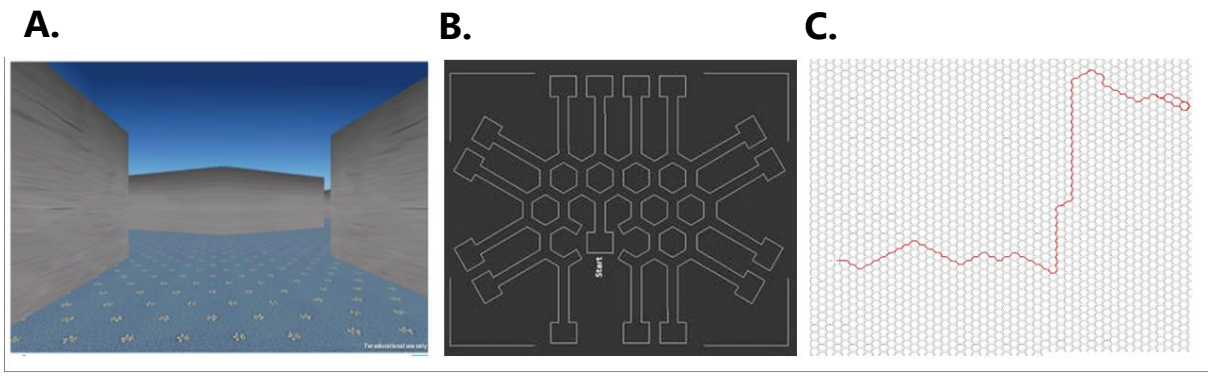

Figure 1B. Virtual FMP Y-maze for human participants. **A**) In maze screenshot taken from the perspective of the participant exploring the maze. **B**) Schematic depicting the honeycomb pattern made up of adjoining Y-shaped mazes. **C**) Overlay of the left and right turns made by a participant in the maze during 5 minutes of free exploration. The left and right turns have been converted from x,y coordinates.

RNA Extraction and cDNA Synthesis

Immediately following behavioural testing, fish were euthanised in ice water, brains were removed, snap-frozen in liquid nitrogen and stored at -80°C until further use. RNA was isolated using the RNeasy Micro kit (Qiagen) as described in the manufacture's protocol. Upon purification, the quality and concentration of all samples were assessed using the NanoDrop ND-1000 (Thermo Scientific). The purities of acceptable RNA samples (as measured by 260:280 and 230:260 absorbance ratios) were equal to or greater than 1.8. All samples were therefore of sufficient quality for expression-level analysis. Total cDNA was prepared using Applied Biosystems High Capacity RNA-to-cDNA Kit for RT-qPCR. Each 20 µL reaction was diluted 10-fold in nuclease-free water and used as the template for the real-time qPCR assays.

Quantitative Real-Time PCR (RT-qPCR)

Quantitative real-time PCR (RT-qPCR) assays were used to validate relative gene expression based on SYBR green detection. Primers used in this study were predesigned primers from qPrimerDB (Lu et al., 2018) or based on previous studies (Parker et al., 2016; R. Tang et al., 2007). Primers were synthesised by Invitrogen and are listed in Table 1. The Roche LightCycler® 480 High Resolution Melting Master mix and the LightCycler® 96 (Roche Life Science) were used to amplify and detect the transcripts of interest. Thermal cycling conditions included an initial denaturation step at 95°C for 600s (recommended by manufacturer), 40 cycles of 95°C for 15s, 58°C for 20s and 72°C for 35s followed by melt curve analysis to confirm product specificity for all transcripts. Primers were tested with melt curve analysis and negative reverse transcription (RT) controls and negative template controls to optimise reaction conditions to generate a single melt peak in control samples, check for genomic contamination in negative RT controls and primer dimers in negative template controls. Elongation factor 1 alpha (*eelf1a*) was used as a housekeeping gene (Tang et al., 2007). Gene-expression levels were calculated using delta-delta CT method and normalised to *eelf1a*. Changes in expression were presented as means ± SD of fold change to control group (n = 4-6 per group; assayed in duplicate).

Table 1. Primer sets used for qPCR

Gene name	Gene	Primer sequence (Forward)	Primer sequence (Reverse)	Amplicon size
Eukaryotic translation elongation factor 1 alpha	*eef1a*	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGC ATTAC	87
Dopamine transporter	dat	GTTGCTGACTTTAGGAATCGAC	GCGTAAACACATAGATTCCACC	179
Dopamine receptor D1	*drd1*	TGGTTCCTTTCTGCAACCCA	AGTGATGAGTTCGCCCAACC	100
Dopamine receptor D2a	*drd2a*	ATACTTCCGCTCTTTGGATGAA	CGTGATGCATTTTCAAAGAAGC	119
Dopamine receptor D2b	*drd2b*	CAAAACCATGAGCAAGAGGAAA	GCAGCCAGCAAATAATGAAAAC	98
Dopamine receptor D5	*drd5*	CGAGAGAAAGATGAACCGTAGA	GTCCAGTTGCTTGTGTTAACC	138
Tyrosine hydroxylase	*th*	ACCGATATTGTCTGATCGAACA	AGTGAACCAGTACATTGTCGAT	112

Oxygen consumption measurements

Standard oxygen consumption rates of individual fish were measured based on wet weight (g) and amount of oxygen consumed during 1 h of free exploration of the FMP Y-maze. Method for measuring oxygen consumption were adapted from (Voutilainen et al., 2011). Briefly, each behaviour tank was filled with exactly 3 L of aquarium-water and an initial reading of oxygen saturation were recorded using a HQ30D portable dissolved oxygen meter. Zebrafish were netted directly into the maze which was covered with parafilm to create a closed system. Tanks were immediately placed into the behaviour unit and the trial started. At the end of 1 h of exploration oxygen readings were recorded for each fish. Zebrafish were then briefly anaesthetised using Aqua-Sed anaesthetic treatment (Aqua-Sed^{TM}, Vetark, Winchester, UK) in accordance to manufacturer guidelines. A wet weight was recorded for each fish in grams. Oxygen consumption was expressed as mg/L O_2 x g^{-1} x h^{-1}, in which g^{-1} is per gram, based on the wet weight of each fish and h^{-1} is per hour, based on 1 hour of exploration. Temperature readings were recorded pre- and post-behaviour to ensure that temperature did not fluctuate significantly over the course of the behavioural task, as this could impact metabolic rate. However, temperature only fluctuated by ±1°C which has been shown to be too small a change to influence oxygen metabolism (Barrionuevo & Burggren, 1999; Okomoda et al., 2020).

Statistical analysis

The primary endpoint for analysis of exploration patterns in the FMP Y-maze was the number of choices for each of the 16 tetragrams as a proportion of total turns (percentage use). Based on previous research we were interested particularly in the proportion of choices that represented alternations (LRLR, RLRL) and repetitions (RRRR, LLLL) as these tetragrams were the most observed. All analyses were carried out using IBM SPSS statistics version 25 and GraphPad Prism version 8, with all graphical representations completed in GraphPad Prism. Analysis of paired tetragram sequences (e.g., LRLR+RLRL, LLLL+RRRR, etc) for a single age group was carried out using a One-way analysis of variance (ANOVA) followed by

Tukey's *post-hoc* test. Comparison of tetragram frequency distributions for age x treatment were analysed using Two-way ANOVA. Data were represented as mean + standard error of the mean (SEM). For subsequent tetragram analyses, we were interested in putative changes in strategy during the search period, and we therefore included "time" as the within-subjects factor. We also included "total turns" as a covariate in all analyses, in order to control for general activity levels in statistical models. For age groups in which drug was added, we included drug (present vs absent) as a between-subjects' factor. Data for time were represented as mean + SEM. For all data sets the Shapiro-Wilk test of normality was conducted. Parametric and non-parametric tests were performed as appropriate for each analysis as detailed below. Comparison of percentage use of alternations and repetitions for each age and treatment group were analysed using the Kolmogorov-Smirnov test for non-normally distributed data sets and the unpaired t-test for normally distributed data sets. Data were represented as the mean + SEM. Oxygen consumption was analysed using a One-way ANOVA for age and Two-way ANOVA followed by Sidak's multiple comparison *post-hoc* test for age x treatment. Data were represented as mean + SEM. Body mass was analysed using unpaired t-test. Data were represented as mean ± SEM. Locomotor data were analysed for age x treatment using Two-way ANOVA followed by Tukey's *post-hoc* test. Data were represented as mean ± SEM. qPCR data were all tested for normality using the Shapiro-Wilk test, as above. Normally distributed data were analysed using unpaired t-test, non-normally distributed data were analysed using Mann-Whitney test. Data were represented as mean ± standard deviation (SD). Alpha values of $P \leq 0.05$ were considered statistically significant.

5.4 Results

Aging zebrafish show mild cognitive decline in the FMP Y-maze

Tetragram analysis revealed that global strategy (percentage use of each tetragram over the entire trial) relied on a similar pattern of turn choice, however, key differences were observed in the use of alternations (LRLR, RLRL) and repetitions (LLLL, RRRR). (**Fig. 2**) shows tetragram frequency distribution for 1 h of free swimming in the FMP Y-maze for 6-month-old v 24-month-old zebrafish. Equal distribution of all possible tetragram configurations would be characteristic of a random search strategy (100% turns completed/16 tetragram configurations = 6.25%), whereas choices made above the 6.25% threshold are considered intentional and part of a global strategy. Most tetragrams were used randomly with bars falling below 6.25% (represented by the dashed line in (**Fig. 2**). 6-month-old fish used alternations significantly more than all other search strategies (One-way ANOVA, F (7, 280 = 36.85, p <0.0001). However, there was a significant interaction between age and turn choice (Interaction, F (1, 68) = 11.94, p = 0.0009) with Tukey's *post hoc* test showing 6-month-old zebrafish used alternations significantly more than 24-month old zebrafish (95% CI diff = 3.32-14.27, p = 0.0004***). This reduction in alternations in 24-month old zebrafish resulted in a change in the global strategy to split dominance between two tetragram sequences, alternations and repetitions, instead of just alternations as seen with the 6-month old zebrafish

Figure 2.

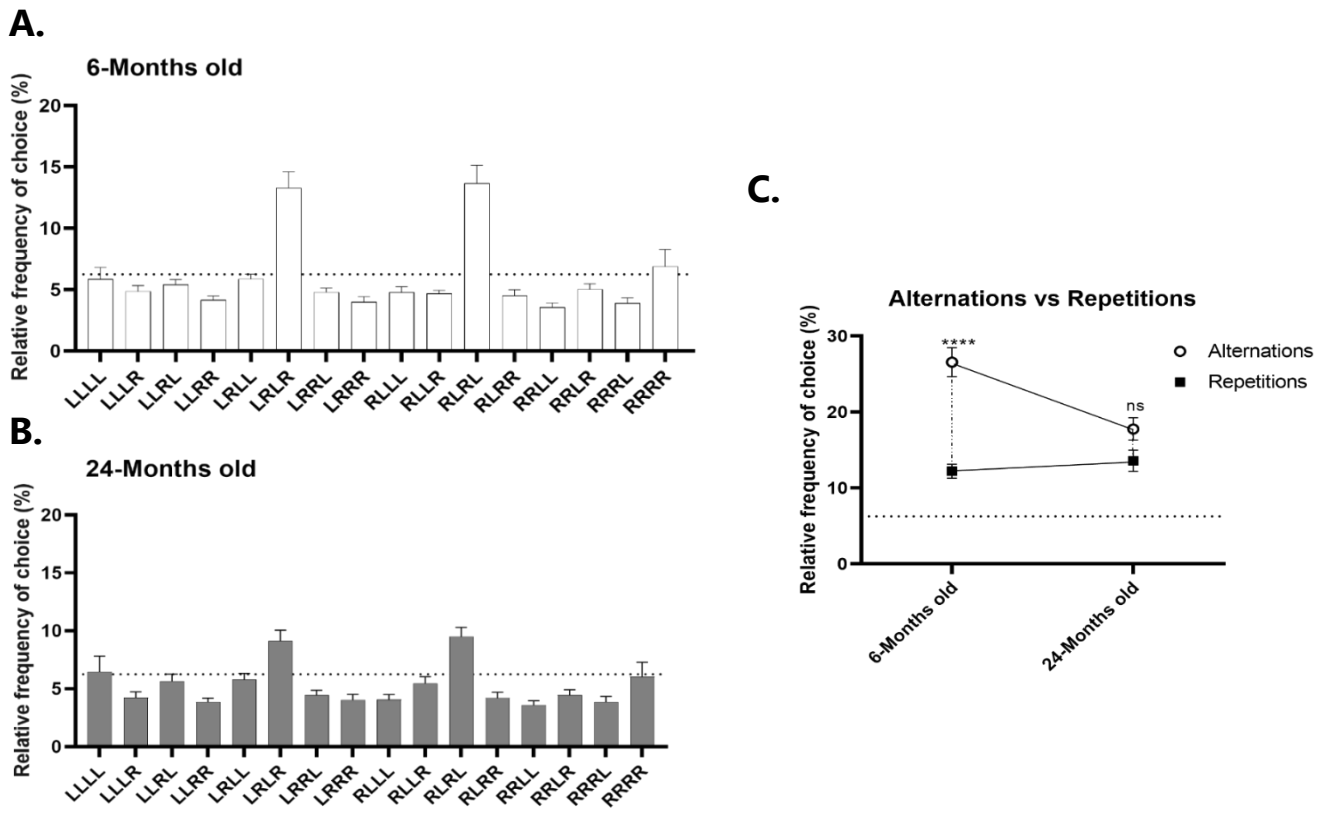

Figure 2. Global search strategy after 1 h of exploration in the FMP Y-maze. (**A**) Percentage use of each tetragram sequence by 6-month old zebrafish in the FMP Y-maze (n = 18), demonstrating clear dominant use of alternations. (**B**) Percentage use of tetragram sequence by 24-month old zebrafish in the FMP Y-maze (n = 20) with comparable use of alternations and repetitions. (**C**) Alternations versus repetitions for 6-month old and 24-month old zebrafish. Normality analysed using Shapiro-Wilk test. Normally distributed data were analysed using One-way ANOVA. The dashed *line* denotes chance performance (approximately 6.25%). *** $p \leq 0.001$, **** $p \leq 0.0001$, ns – not significant. Error bars are mean + SEM. Single data points are mean ± SEM.

Treatment with D1/D5 agonist, SKF-38393, rescues working-memory deficit in aged zebrafish

Having shown a deficit in WM of 24-month old zebrafish compared to 6-month old counterparts, shown by a change in global exploration strategy, we pre-treated zebrafish with D1/D5 agonist SKF-38393 for 30 mins prior to testing in the FMP Y-maze. SKF-38393 treatment had no effect on global strategy in 6-month old zebrafish, with both alternations and repetitions showing no effect between treated (n=15) and untreated (n=18) fish (Two-way ANOVA, F (1, 496) = 0.44, p = 0.5078). However, treatment of 24-month old zebrafish caused a significant increase in the use of alterations (*KS, D* = 0.33, p = 0.0297), without affecting use of repetitions (*KS, D* = 0.14, p = 0.8582) (**Fig. 3**). WM, as measured by global strategy showed a significant effect of age (F (1,69) = 9.037, p = 0.037), but no significant interaction (F (1,69) = 2.546, p = 0.115) and no main effect of treatment (F (1,69) = 1.643, p = 0.204). However, Tukey's *post hoc* tests revealed that treatment with SKF-38393 rescued the deficit in alternations between aged 24-month old and young 6-month old zebrafish (95% CI = -3.74-10.52, p = 0.5969) compared to controls (95% CI = 1.91-16.18, p =0.0073).

Figure 3.

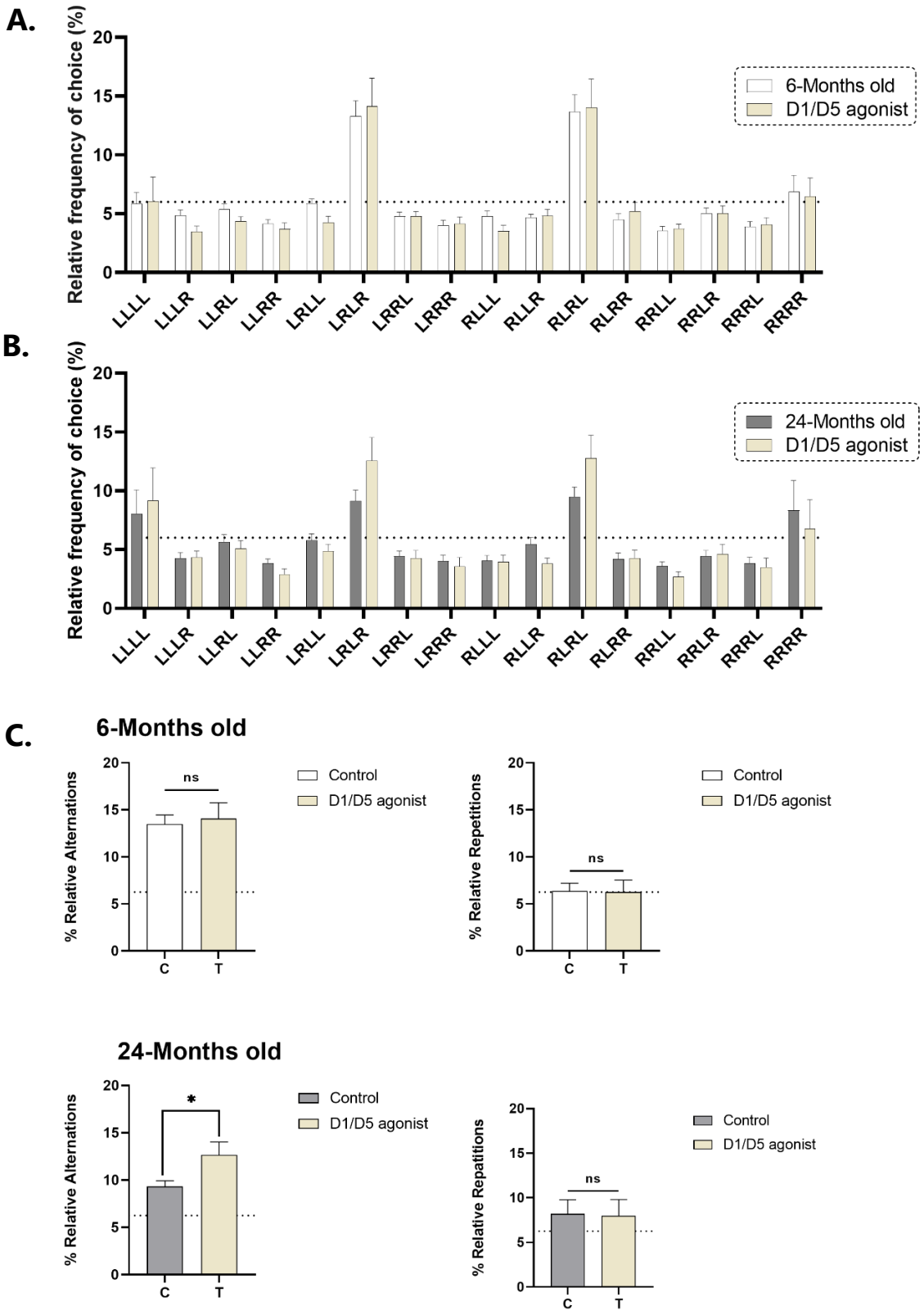
A.
Relative frequency of choice (%)
6-Months old
D1/D5 agonist
LLLL
LLLR
LLRL
LLRR
LRLL
LRLR
LRRL
LRRR
RLLL
RLLR
RLRL
RLRR
RRLL
RRLR
RRRL
RRRR
B.
Relative frequency of choice (%)
24-Months old
D1/D5 agonist
C.
6-Months old
% Relative Alternations
ns
Control
D1/D5 agonist
% Relative Repetitions
ns
Control
D1/D5 agonist
24-Months old
% Relative Alternations
*
Control
D1/D5 agonist
% Relative Repatitions
ns
Control
D1/D5 agonist

Figure 3. Effective pre-treatment with D1/D5 agonist SKF-38393 on global search strategy of zebrafish in the FMP Y-maze. Effect of SKF-38393 on search strategy of (A) 6-month old treated compared to control zebrafish and (B) 24-month old treated compared to control zebrafish after 1 h of exploration. (C) Comparison of alternations and repetitions in 6-month old and 24-month old control v treated zebrafish. C – control, T – treated with D1/D5 agonist SKF-38393. Normality analysed using Shapiro-Wilk test. Normally distributed data analysed using two-way ANOVA and t-test. Non-normally distributed data analysed using Kolmogorov-Smirnov (KS) test. * $p \leq 0.05$, ns – not significant. The dashed *line* denotes chance performance (6.25%). Error bars are mean + SEM.

Healthy aging impacts cognitive flexibility which cannot be recovered by treatment with SKF-38393

The FMP Y-maze is a dual-action behavioural task which enables assessment of WM based on global strategy, but also cognitive flexibility by analysing 'immediate' strategies consisting of exploration patterns for the total trial divided into equal length time bins (six 10 min time bins). This enables the identification of changes in strategy over time. (**Fig. 4**) illustrates the percentage use of each tetragram sequence per 10 mins of exploration clearly denoting differences in the use of alternations over successive 10 min exploration intervals for 6-month old zebrafish, but a diminished effect of time on alternations in the 24-month old zebrafish. As alternations have been revealed as the dominant strategy and prone to change with age and treatment, we further explored the effect of time on alternation use. 6-month old control zebrafish showed the greatest effect of time on alternations as demonstrated in (**Fig 5**). From the initial 10 mins of exploration, alternations were already used above random selection and continue to rise significantly with each successive time bin (One-way ANOVA, F (5, 210) = 4.33, p = 0.009). The maximum mean difference between time bins was 9.7%, indicating that the alternation strategy was not static, but was altered in response to a constant environment. 24-month old aging zebrafish demonstrate a deficit in the ability to adapt their strategy over time, as shown by the stable use of alternations in consecutive time bins (One-way ANOVA, F (5, 234) = 1.35, p = 0.2449), with a maximum mean difference of 3.6% between time bins. Treatment of 6-month old zebrafish with SKF-38393 did not affect global strategy as seen in *Figure 3*, however, it did appear to have a dampening

effect of changes in alternation use over time (**Fig. 5**). Though still significant, the effect was greatly reduced compared to controls (One-way ANOVA, F (5, 164) = 2.73, p = 0.0215). In 24-month old zebrafish treated with SKF-38393 the global use of alternations was increased to a performance level equivalent to 6-month old controls; however, the drug treatment was unable to restore adaptability of search over time, resulting in a stable strategy (One-way ANOVA, F (5, 208) = 0.185, p = 0.9681), which had a similar maximum mean difference of 2.9% between time bins.

Figure 4.

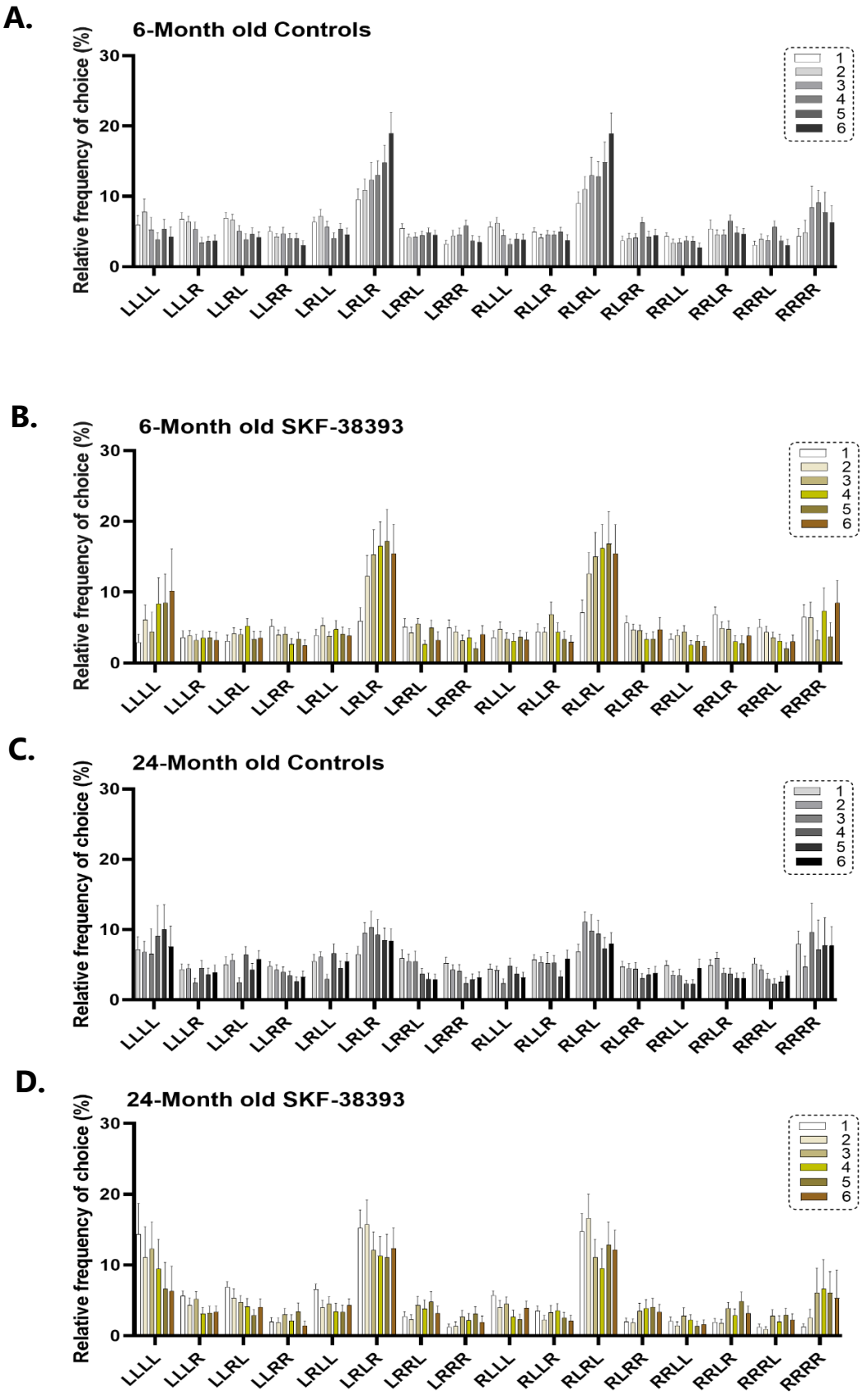
A.
6-Month old Controls
B.
6-Month old SKF-38393
C.
24-Month old Controls
D.
24-Month old SKF-38393
Relative frequency of choice (%)
0
10
20
30
1
2
3
4
5
6
LLLL
LLLR
LLRL
LLRR
LRLL
LRLR
LRRL
LRRR
RLLL
RLLR
RLRL
RLRR
RRLL
RRLR
RRRL
RRRR

Figure 4. Shows the use of each tetragram per 10 min time bin for a 1 h trial of free FMP Y-maze exploration. (**A**) Depicts tetragram use for 6-month old adult controls compared to (**B**) SKF-38393 treated. Both groups clearly illustrate an increasing use of alterations across successive time bins. (**C**) Shows the same frequency distribution for 24-month old aging adult controls versus (**D**) SKF-38393 treated. Although the agonist treated group shows an increased percentage of alternations per time bin compared to controls, both groups have a heavily blunted effect of time, resulting in almost equal use of alternations in each 10 min time bin. Error bars are mean + SEM.

Figure 5.

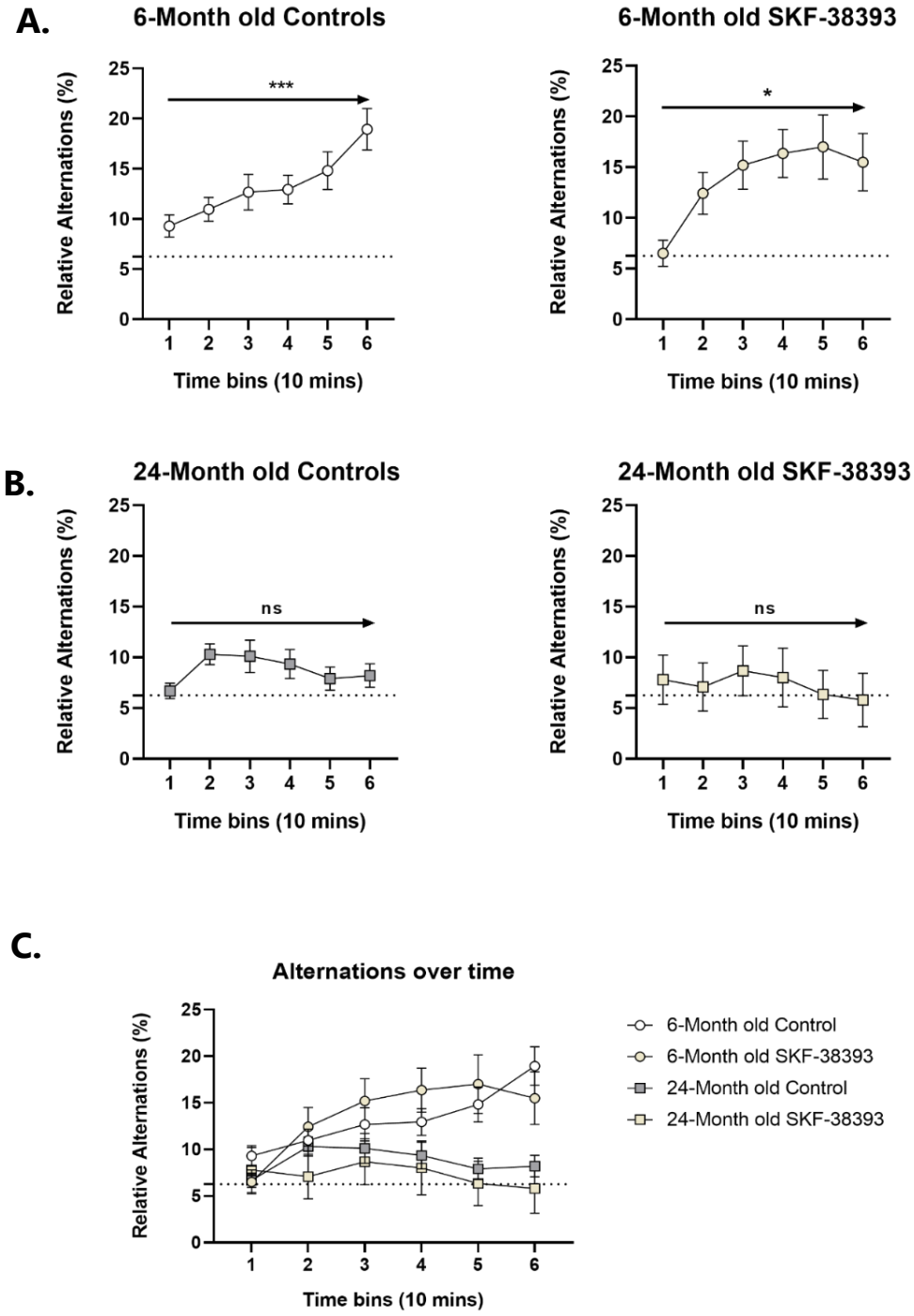

Figure 5. Percentage use of alternations in successive 10 min time bins of exploration (**A**) 6-month old controls (**left**) and treated with SKF-38393 (**right**) and (**B**) 24-month old controls (**left**) and treated (**right**). (**C**) Combined effect of time on percentage use of alternations for all ages and treatment groups. Analysed using one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, ns – not significant. The dashed *line* denotes chance performance (6.25%). Error bars are mean ± SEM.

Metabolic rate is not a factor in changing search strategy with age

There appeared to be a substantial size difference between age groups, therefore, to identify if metabolism played any role in the change in search strategy, wet weight and oxygen consumption over the course of the trial were recorded as an indirect measure of metabolism (Nelson, 2016). We identified a significant difference in wet body mass (t = 7.195, df = 19, p <0.0001) with a mean wet weight of 0.39g for 6-month old and 0.83g for 24-month old zebrafish. We furthered this analysis by comparing oxygen consumption between age groups and found no significant difference (t = 1.660, df = 8, p =0.1356). However, there was an effect of treatment on oxygen consumption (Two-way ANOVA, F(1, 17) = 11.41, p = 0.0036), Sidak's multiple comparison *post-hoc* test revealed that treatment with D1/D5 agonist SKF-38393 caused a significant decrease in oxygen consumption in 6-month old (95% CI = 0.433-1.787, p = 0.0012), but had no effect on 24-month old fish (95% CI = -0.631-0.535, p = 0.9952) (**Fig. 6**).

Figure 6.

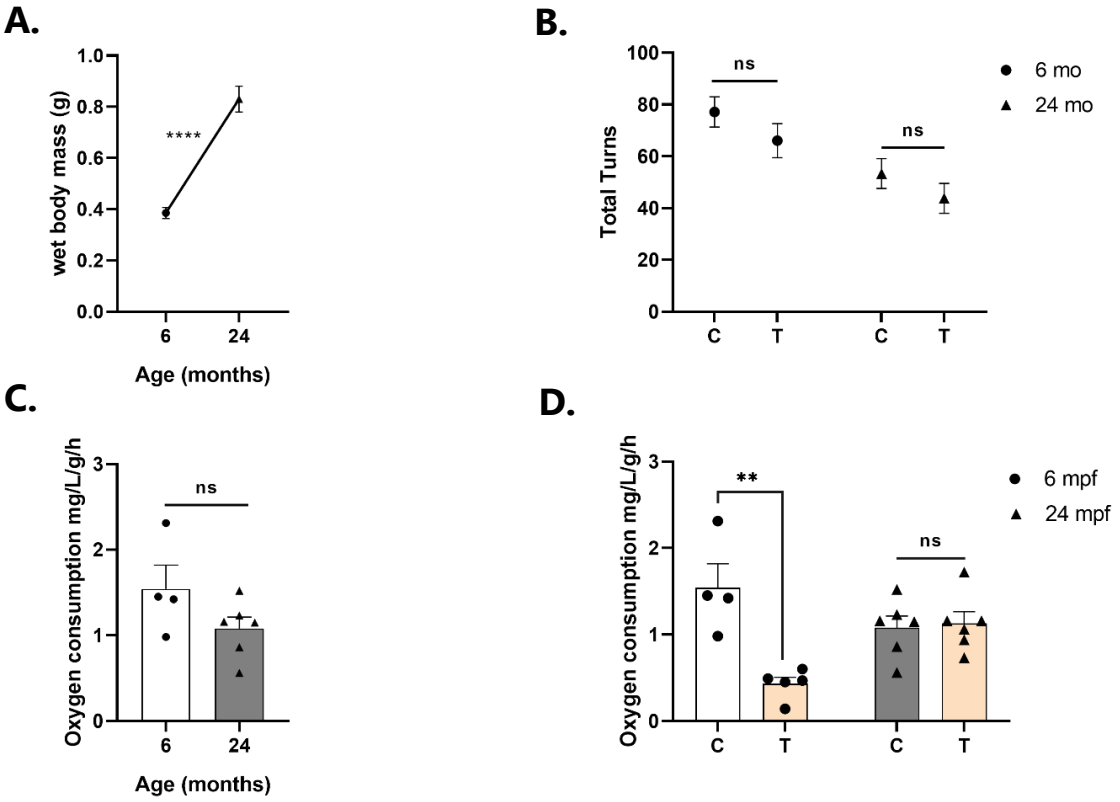

Figure 6. (**A**) The mean difference in body mass between 6-month old (n=10) and 24-month old (n=12) zebrafish. (**B**) Locomotor activity measured by total turns completed in 1 h of exploration. (**C**) Oxygen consumption of 6-month old controls (n=4) compared to 24-month old controls (n=6). (**D**) Effect of SKF-38393 on oxygen consumption over 1 h of exploration in 6-month old and 24-month old control v treated. Normality analysed using Shapiro-Wilk test, normally distributed data analysed by Two-way ANOVA (**B,D**) or unpaired t-test (**A,C**). Data for a and b are mean ± SEM, data for c and d are mean + SEM; * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$, ns – not significant.

Regulation of dopaminergic gene expression by aging

We investigated the effect of age on the dopaminergic system by analysing expression changes between 6 and 24-month old zebrafish. *Figure 7* shows the qPCR data of relative gene expression from whole brain tissue. We found no significant difference between 6 and 24-month old zebrafish for *dat* ($U = 4$, $p = 0.1667$), *drd1* ($t = 1.25$, df = 8 , $p = 0.2460$), *drd2a* ($t = 1.20$, df = 7 , $p = 0.2699$), *drd2b* ($t = 0.21$,

df = 7 , p =0.8434) *or th* (t = 0.55, df = 7 , p =0.5985) mRNA expression levels. However, there was a significant effect of age on *drd5* (t = 2.87, df = 7, p = 0.0239) expression.

Figure 7.

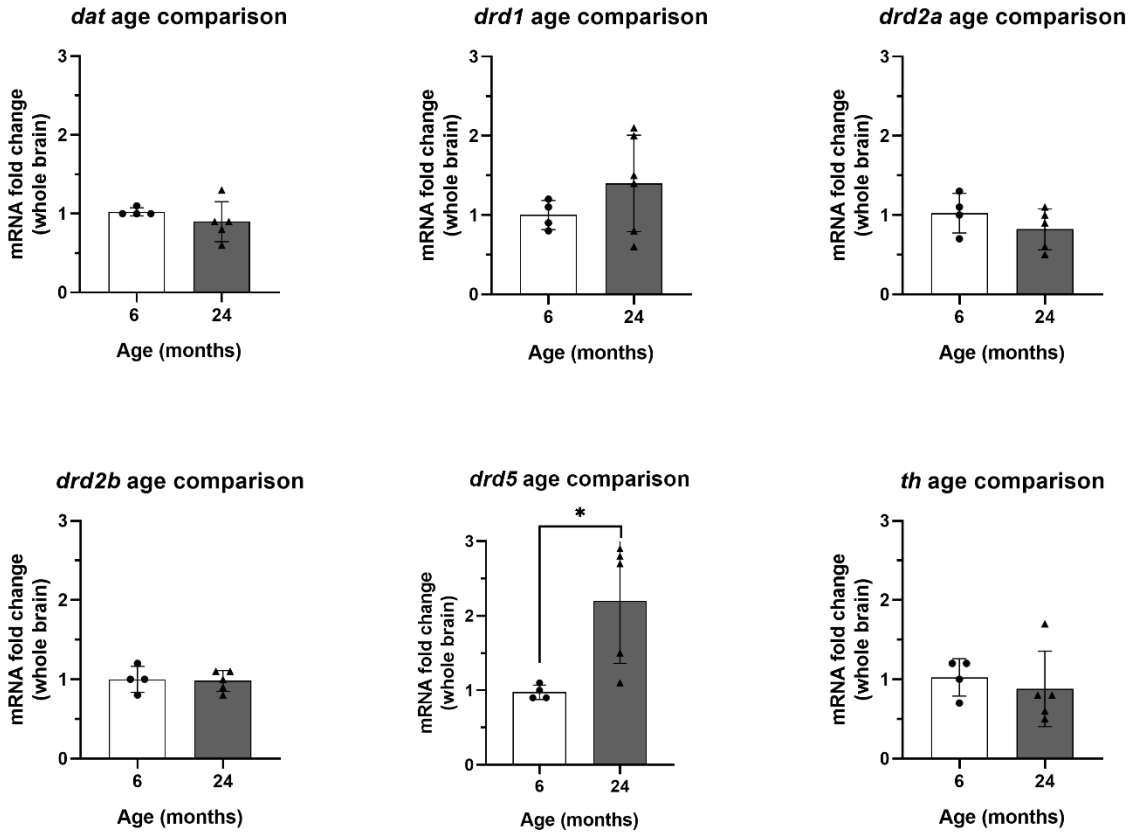

Figure 7. Quantitative real-time PCR analysis showing variations in the relative amounts of *dat, drd1, drd2a, drd2b, drd5* and *th* mRNA in whole brain tissue extracted from 6-month old controls (n=4) and 24-month old controls (n=6). Data were normalised to housekeeping gene e*elf1α* and defined as fold change relative to 6-month old controls. Normality was analysed using Shapiro-Wilk test. Normally distributed data were analysed using unpaired t-test, non-normally distributed data were analysed using the Mann-Whitney test (U). All data are mean ± SD; * $p \leq 0.05$.

Effect of SKF-38393 on dopaminergic gene expression

The qPCR data in *Figure 8* shows that 35µM SKF-38393 was able to decrease the expression of *dat* in 6-month old zebrafish (U = 0, p = 0.0048) but not 24-month olds (U = 9, p = 0.1645). However, 30 min treatment with SKF-38393 did not elicit expression changes in *drd1* (6-months old; t = 1.36, df = 7 , p =0.2170, 24-months old; t = 0.83, df = 10 , p =0.4266), *drd2a* (6-month old; t = 1.33, df = 7 , p =0.2259, 24-month old; t = 1.31, df = 8 , p =0.2265), *drd2b* (6-months old; t = 1.17, df = 8 , p =0.2753, 24-months old; t = 0.18, df = 8 , p =0.8651), *drd5* (6-months old; t = 1.38, df = 8, p = 0.2064, 24-months old; t = 1.62, df = 9, p = 0.1405) or *th* (6-months old; t = 0.72, df = 8 , p =0.4950, 24-months old; t = 0.14, df = 8 , p =0.8894) in either age group.

Figure 8.

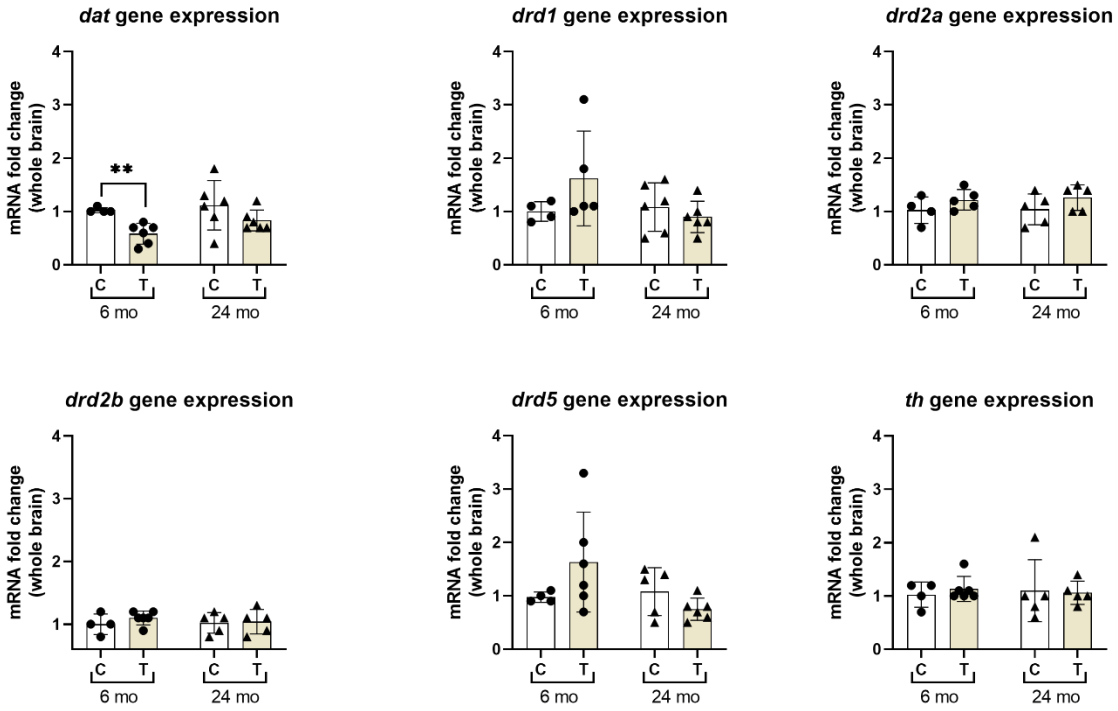

Figure 8. Quantitative real-time PCR analysis showing variations in the relative amounts of *dat, drd1, drd2a, drd2b, drd5* and *th* mRNA in whole brain tissue of controls and treated groups exposed to 35 µM SKF-38393 in 6-month old controls (n=4) and treated (n=5) and 24-month old controls (n=6) and treated (n=6) zebrafish. Data were normalised to housekeeping gene e*elf1α* and defined as fold change relative to controls. Normality was analysed using Shapiro-Wilk test. Normally distributed data were analysed using unpaired t-test, non-normally distributed data were analysed using the Mann-Whitney test (U). All data are mean ± SD; ** $p \leq 0.01$.

Healthy aging humans show mild cognitive decline

Having shown a deficit in WM of old versus young zebrafish, to understand the translational relevance of these findings, we assessed WM in healthy populations of young and old humans in a previously developed virtual FMP Y-maze, which is analogous to the animal version (Cleal, et al., 2020). To establish clinically relevant findings, we ran participants in the FMP Y-maze for 5 minutes of free exploration. Like zebrafish, humans showed dominant use of the alternation strategy and similarly there appeared to be a significant reduction in the use of alternations in older (70+ year-olds) compared to younger (18-35 year-old) adults (F (1, 65) = 7.175, p = 0.009) (Figure 9). Thus, we demonstrated a deficit in WM in healthy aged adults compared to their younger counterparts.

Figure 9.

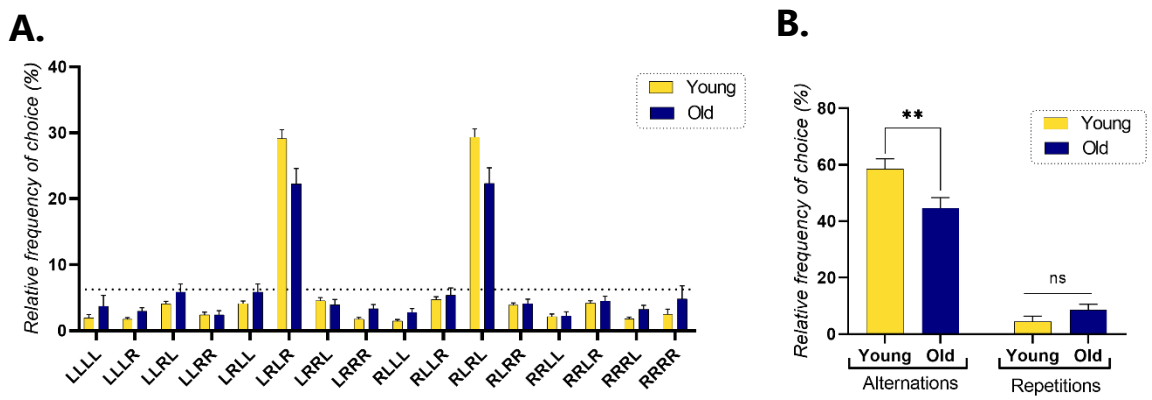

Figure 9. Global search strategy after 5 minutes of exploration in the human virtual FMP Y-maze. **A**) Percentage use of each tetragram sequence by young adults aged 18-35 years old (n=35) compared to older adults aged 70+ years old (n=32). Both demonstrating dominant use of the alternation strategy. **B**) Comparison of young and old aged groups and the use of total alternations (LRLR+RLRL) and total repetitions (LLLL+RRRR). Analysed using LMM. The dashed *line* denotes random choice selection at 6.25%. Error bars are mean + SEM. ** $p < 0.01$.

5.5 Discussion

Using a zebrafish model of aging we have investigated changes in WM and cognitive flexibility between young adulthood and aging, suggesting mild cognitive decline. The present findings indicate that WM and strategy changes are both impaired in aged zebrafish; however, acute exposure to SKF-38393, a selective dopamine D1/D5 receptor partial agonist, enhanced WM in aging, but had no detectable effect on behavioural flexibility. Real-time qPCR analysis identified an up-regulation of *drd5* in 24-month old zebrafish compared to 6-month old counterparts. Treatment with SKF-38393 caused a down regulation of *dat* mRNA expression in 6-month old adults, but no effect on aged adults, supporting the role of *dat* in regulating dopamine availability and cognitive flexibility. These findings provide characterisation of cognitive changes in healthy aging which can be partially rescued by activating D1-like receptors, promoting the role of maintaining WM, but not behavioural flexibility. This study, to our knowledge, provides the first examination of changes in dopaminergic gene expression in the aging brain of zebrafish. To address the translational relevance of the zebrafish model of aging, we examined WM of healthy adults using an analogous version of the FMP Y-maze adapted for humans. We identified a similar reduction in alternations in old versus young adults, comparable with the changes seen in zebrafish. Thus, our findings demonstrate for the first time that zebrafish faithfully replicate aspects of healthy aging seen in humans, that results in a decline in WM with old age.

Changes in WM in healthy aging zebrafish

Younger adult zebrafish have demonstrated a very specific global strategy used to explore the FMP Y-maze. Using tetragram sequence analysis, we identified patterns of choice selection of left and right turns in 6-month old adults that use alternation sequences (LRLR, RLRL) for more than 25% of the global search strategy, four times the use if selected randomly. All other sequences were used at a level equivalent to chance selection. Prior work has demonstrated that changes in global spatial activity patterns, particularly those relating to alternations and repetitions, are representative of changes in WM

processing (see Part 1: Chpt 3) (Cleal, et al., 2020). Previous work has also demonstrated similar patterns of alternations in young adult zebrafish ranging from 3-6-month old (see Part 1: Chpt 4) (Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Aging zebrafish at 24-month old have, however, demonstrated a marked change in global spatial activity patterns reducing the use of alternations by ~8%, bringing the use of alternations and repetitions almost in line at 18% and 16% respectively. This deficit in alternations may represent an inability to recall which arms of the maze have previously been entered and/or the order of entry, a process which has been shown to be dependent on WM (Lalonde et al., 1986; Moran et al., 1995; Myhrer, 2003). These findings further support that zebrafish, like humans, have a natural decline in cognitive abilities as part of healthy aging, resulting in deficits in WM (Berry et al., 2016; Dreher et al., 2008; Goldberg, 2017; Salthouse, 2009).

The role of aging in cognitive decline has been well documented in humans, with many animal models replicating similar deficits in cognitive performance (Gerhard, 2007; McQuail & Nicolle, 2012). Human and animal studies, have implicated disruption of the dopaminergic system in age-related decline in executive functions such as WM (Castner & Goldman-Rakic, 2004; Costa, 2014; Decker & McGaugh, 1991; Dreher et al., 2008; Godefroy et al., 1989). To this end we pre-treated adult and aging zebrafish with the partial dopamine D1/D5 receptor agonist to identify what role D1-like receptors play in the cognitive decline in healthy aging zebrafish. Similar to animal and human studies (Castner & Goldman-Rakic, 2004; Hemby et al., 2003; Molloy & Waddington, 1988; M. Wang et al., 2019), we found that treatment with a partial D1/D5 agonist enhanced WM in aging zebrafish, resulting in a rise in the use of alternations as part of the global strategy. 24-month old treated zebrafish increased alternations by nearly 7%, bringing alternation use to a level comparable with 6-month old zebrafish. The effect of SKF-38393 was age specific, as no such changes in WM were observed in treated 6-month old zebrafish. Our findings support work from (Part 1: Chpt 3), indicating that boosting D1-like receptor activation is only beneficial if there is pre-existing dysregulation within the dopaminergic system (Cools & D'Esposito, 2011).

The role of metabolism and the effect of SKF-38393 on cognitive flexibility

Fine-scale analysis revealed that not only do fish use an overall, global strategy of alternations and repetitions to explore the FMP Y-maze, but this strategy is subject to change over time. We observed a pattern of increasing use of alternations throughout the trial in young, 6-month old adults. However, this natural tendency to modify search strategy with time ceases in aged adults. Throughout the life-span of an organism there is a continuous, dynamic equilibrium between goal stabilised and destabilised behaviours, in which an individual is required to balance focus on the current task against new information altering current goal perceptions (Cools, 2016). This cognitive flexibility has been strongly associated with the dopaminergic system and WM (Cañas et al., 2003). In the FMP Y-maze the current goal could be perceived as foraging for potential food sources, or information seeking. This behavioural paradigm works on the basis of negative feedback (a continuous lack of food or reward) to update knowledge of the environment and inform decisions to select appropriate behaviours, in this task reflected as search strategy. In young, healthy adult zebrafish the negative feedback loop dictates an increasing use of alternations over time. However, in line with previous studies in humans (Harada et al., 2013), aged zebrafish could not utilise negative feedback to update behaviour in response to the lack of environmental change and thus relied on the 'immediate' strategy, that was employed within the first 10 mins of novel exploration, as the only strategy used to explore the maze. This strategy was used regardless of how familiar the environment had become or in light of continuous lack of reward or novelty whilst exploring the FMP Y-maze. Changes in cognition is a normal aspect of healthy aging and similarly to humans, zebrafish exhibit age-related deficits in cognitive abilities (Adams & Kafaligonul, 2018). It is therefore unsurprising that in aging adults there is no longer an effect of time on alternations. The ability to adapt behaviour based on new information is fundamental to healthy cognitive processing, the disruption of which is common to psychiatric illness and neurodegenerative diseases (Pittenger, 2013; Waltz, 2017). The inability to adapt behaviour in response to the environment is of critical importance in aging conditions, and thus highlights the suitability of zebrafish to aid in informing human conditions of cognitive decline in aging.

An interesting finding from this study was that treatment with SKF38393 caused a reduction in the effect of time on search strategy in younger adults. However, aged adults showed no effect of time on search strategy, regardless of whether they were treated with SKF-38393. Dopamine acts as a neuromodulator, which is essential for achieving and maintaining cognitive control functions such as flexibly adjusting goal-directed behaviours (Ott & Nieder, 2019). Via D1-like receptor activation, dopamine can modulate WM performance and sustain goal-focused behaviour by stabilising neuronal activity and generating a high signal to noise ratio, reducing the influence of interfering (off target or distracting) stimuli (Durstewitz et al., 2000). However, in order to achieve goal-orientated behaviour with flexible adaptations a balance is required between a D1 (including both D1 and D5 receptors)- and D2- (including D2, D3 and D4 receptors) dominant state, known as Dual state theory (Cools, 2016). Dual state theory implicates intermediate neural levels of dopamine, primarily acting via D1-like receptors, resulting in low firing rate (lower energy use) and increased goal-directed behaviour as the D1-dominant state. The D2-dominant state, activation via D2-like receptors, is characterised by fast firing rates (high energy use) and behavioural flexibility, e.g. set shifting or behavioural adaptations (Cools, 2016). A shift to the D1-dominant state by pharmacological intervention, for example following pre-treatment with a selective D1/D5 receptor agonist, would result in system bias in favour of goal stabilisation, which is good for achieving the current goal (e.g. searching for food), but bad for adapting behaviour in response to new information (e.g. no food and a constant, unchanging environment, as presented in the FMP Y-maze) (Cools, 2016; Lianchun Yu & Yu, 2017).

We measured oxygen consumption in control and treated groups to identify if changes in metabolic activity played a role in the change in behaviour. Supporting the theory of dual state action between D1-like - goal-directed behaviour and D2-like - flexible behaviour. We found that 6-month old controls showed the greatest behavioural flexibility, but also had the highest mean oxygen consumption of 1.54 mg/L O_2 over 1 h of exploration. Treatment with SKF-38393 caused a significant decrease in oxygen consumption and a reduction in adaptive search behaviour over time. However, no such effect was evident in aged adults which had similar search strategies over time and similar oxygen

consumption in control and treated groups. Our findings support over activation of the D1-like receptor pathways, in 6-month old adults' treated with SKF-38393, potentially biasing the dopaminergic system in favour of a goal stabilised state, which decreased the amount of flexibility and reduced energy consumption by switching to a state with a lower firing rate in dopaminergic neurons.

The lack of change in search patterns over time in aged adults and the accompanying lack of change in oxygen consumption between treated and control groups, further supports this hypothesis. Examples of the Dual state theory have been evidenced in human, primate and computational studies (Durstewitz et al., 2000; Durstewitz & Seamans, 2008; Fallon & Cools, 2014; Ott & Nieder, 2019). Here we see that increasing D1-like receptor signalling interferes with cognitive flexibility in younger adults and is unable to restore it in aged adults. Thus, biasing away from flexible behaviour appears to be a robust mechanism; however, as we have outlined above, in order to positively influence goal-directed behaviour a specific level of receptor activation is required. It is also noteworthy that these changes do not appear to be related to locomotion as there are no changes in total turns between controls and treated fish of either age group, despite changes in metabolism in 6-month old adults and changes in global strategy in 24-month old fish in response to SKF-38393 treatment. This further supports the hypothesis that changes are mechanistic and dependent on age, opposed to physical, i.e. increased number of turns resulting in an increased number of alternations, or decreased activity decreasing oxygen consumption. Here we provide the first evidence that the Dual state theory may also apply to zebrafish. However, the modulatory effect of dopamine is complex, influenced by dopamine receptor activation and age. These findings provide further understanding of the role of the dopaminergic system in age-related changes in cognition, but more work is necessary to fully elucidate the mechanisms and their potential manipulation to improve executive function in aging adults.

Molecular changes in dopaminergic gene expression

To further understand the role of dopamine in healthy aging we investigated the expression of genes critical to the dopaminergic system including tyrosine hydroxylase (*th*), dopamine transporter (*dat*) and the dopamine receptors (*drd1, drd2a, drd2b* and *drd5*). Contrary to previous studies, we did not observe any age-dependent alterations in expression levels of *th, dat, drd1* or *drd2* receptor subtypes, however, there was a significant increase in *drd5* gene expression in aged compared to young adult zebrafish. Few studies have investigated gene expression of D5 receptors in aging in the healthy brain of mammals, and to our knowledge this has not previously been investigated in zebrafish. Our finding of increased *drd5* expression is somewhat at odds with the few mammalian studies of D5 receptors in aging (Hemby et al., 2003; Rothmond et al., 2012). Rothmond, et al, found that D5 receptor mRNA expression was negatively correlated with age from neonate (6 weeks old) to adult (up to 49 years old), however they noted that there was no significant difference between the age groups examined, including young adults (20-25 years old) and adults (35-50 years old). Hemby, et al, examined age-related changes in mRNA expression of dopamine receptor subtypes. They found a significant age-related decline in relative abundance of D5 receptors in CA1 pyramidal neurons, but no change in the entorhinal cortex layer II stellate neurons. Despite a negative correlation of D5 receptor expression in the CA1 pyramidal neurons with increasing age, from 19-92 years, there was greater variability in expression of D5 receptor expression (R = 0.533) compared to the other four dopamine receptor subtypes (D1 R = 0.764; D2 R= 0.775; D3 R = 0.895; D4 R = 0.789) (Hemby et al., 2003). Additionally, the author's noted that age-related changes in D5 receptor expression were only possible due to the ability to examine specific neuronal populations. Both studies found overall negative correlations with D5 receptor mRNA expression and increasing age, however, this was over multiple age groups differing by ~49 years and ~70 years respectively (Hemby et al., 2003; Rothmond et al., 2012). The difference in *drd5* receptor mRNA expression between 6 and 24-month old zebrafish may be a consequence of analysing whole brain expression instead of specific regions or neuronal populations. The high variability of samples may also be reflective of sex differences or different rates of aging between individuals,

potentially demonstrating high sensitivity of *drd5* receptors to these changes. Kishi, et al, noted that in aging zebrafish there was significant variation in the levels of biochemical and biological markers used as an indicator for the onset of senescence (Kishi et al., 2003) and this may be the case for *drd5* expression.

Similarly, the absence of differences in the other dopaminergic mRNA expression levels may be due to the use of region-specific tissue for analysis in mammalian studies, e.g. the hippocampus, prefrontal cortex, ventral or dorsal regions of the striatum (Araki et al., 2007; Godefroy et al., 1989; Hemby et al., 2003). The asymmetric distribution of dopamine receptors throughout the brain strongly correlates localization with functional specificity (El-Ghundi et al., 2007). It is therefore, a possibility, that regional differences in expression may counteract one another when the system is subject to subtle changes in expression. It is also possible that differences in expression are not typical between 6 and 24-month old zebrafish as, to our knowledge, this is the first work to examine age-associated differences in dopaminergic gene expression in aging zebrafish. Therefore, our initial findings should be interpreted with care and further clarity is needed to understand the species-specific changes in the dopaminergic system during healthy aging in zebrafish and what potential role sex differences may play.

Large or regionally uniform changes may be detectable with whole brain gene expression analysis. To this end we examined if there were any detectable changes in expression related to fish treated with SKF-38393. We found a significant reduction in expression of *dat* in 6-month old treated adults compared to controls, an effect that was not replicated in 24-month old treated fish, suggesting an age specific effect. *Dat,* a plasma membrane protein exclusively expressed in dopamine synthesising neurons, plays a crucial role in regulating the amplitude and duration of dopamine-mediated neurotransmission by clearing dopamine from the synaptic cleft (Bannon, 2005; Bannon et al., 2001; Mortensen & Amara, 2003). The constitutive process of transporter trafficking of dopamine allows for rapid up- and down- regulation of cell surface transporter expression and, thus, transport activity (Gulley & Zahniser, 2003). Down-regulation of *dat,* thus resulting in an increase of synaptic dopamine availability by reducing reuptake, is a likely explanation for the reduction in cognitive flexibility over time

in the treated 6-month old zebrafish. Studies investigating dopamine system function modulating behaviour, have identified a role for presynaptic dopamine transporters (*dat*). Studies examining cognitive flexibility in patients with PD have found that patients taking dopamine-enhancing medication (e.g. dopamine receptor agonists or L-DOPA) perform poorly on reversal or reinforcement learning tasks compared to patients that are not receiving medication (Cools et al., 2001; Rutledge et al., 2009; Waltz, 2017). Additionally it was noted that learning rates that were enhanced by dopaminergic medications only impacted positive reward learning and had no effect on negative outcome learning (Rutledge et al., 2009). These findings are consistent with other studies using methylphenidate or cocaine- both substances blocking dopamine reuptake by inhibiting *dat* (Gatley et al., 1999). (Clatworthy et al., 2009) found that young healthy subject's orally administered methylphenidate had the least cognitive flexibility in reversal learning when associated with the greatest amount of striatal dopamine release; however, spatial WM performance was improved with increasing amounts of striatal dopamine. Similarly studies of cocaine-use found that cognitive flexibility was selectively impaired, with some studies showing that WM remained unaffected (Colzato et al., 2009; Stalnaker et al., 2009). Combined, these experiments support the findings from this study that increased synaptic dopamine, either by reducing dopamine transporters or treatment with dopamine-enhancing drugs, can impair behavioural flexibility in healthy subjects, or patients treated with enhancing drugs, by causing an 'over-dose' of dopamine in regions with optimal dopamine levels, in relation to other dopamine depleted regions resulting in distinct functional changes, i.e. no effect on WM, but a decrease in behavioural flexibility as seen here in 6-month old treated zebrafish compared to controls. This hypothesis would also explain the lack of effect of SKF-38393 on *dat* expression in 24-month old adults, which had already shown a deficit in cognitive flexibility in the control group, therefore pre-existing dysregulation of the dopamine system as a result of aging, prevented any 'over-dosing' effect causing changes in *dat* expression.

Changes in WM in healthy aging humans

Deficits in WM found in aging zebrafish compared to younger counterparts, support findings from human studies (Berry et al., 2016; Dreher et al., 2008; Goldberg, 2017; Salthouse, 2009). However, to fully appreciate the translational relevance of zebrafish as a model of aging and the FMP Y-maze as a sensitive measure of cognition, we used a previously developed virtual version of the FMP Y-maze with healthy human subjects to assess WM in aging. Previous tests in the FMP Y-maze found that humans, like zebrafish, relied on alternations as the dominant strategy for exploration in the maze (Cleal, et al., 2020). However, the sensitivity of this task to subtle changes in cognitive decline have yet to be examined. We used a virtual version of the FMP Y-maze, run using a clinically relevant protocol requiring only 5 minutes of subject participation, to assess exploration strategies. We found that young adults, aged 18-35, used alternations more than adults aged 70 and above. Demonstrating an age-related deficit in WM, in line with previous studies (Glisky, 2019; Goldberg, 2017; Pliatsikas et al., 2019). Our findings in humans, replicate those seen in zebrafish, supporting their use as a model of aging and additionally demonstrating the suitability of the FMP Y-maze as a measure of cognition that can be used to assess the same neurobiological measures in humans and fish.

5.6 Conclusion

Our study shows that mild cognitive decline is a common component of healthy aging in both humans and zebrafish. Using the FMP Y-maze, subtle changes in WM can be detect in aged compared to young adults. Our results are consistent with previous findings that the dopamine system plays a vital role in maintaining normal cognition and consequently appropriate behaviour selection in natural, healthy aging. We found that aged adult zebrafish have impaired WM and cognitive flexibility compared to their younger counterparts, however, treatment with a D1/D5 agonist could improve WM performance in the FMP Y-maze. We also identified a role for 'over-dosing' of dopamine and regulation of *dat* expression in subjects without dopamine depletion, which consequently resulted in reduced cognitive flexibility in

healthy treated adults compared to controls. Further study is required to fully elucidate the mechanisms underlying responses to dopamine-enhancing drugs, with particular focus on regional brain changes and specific behavioural impairment and enhancement. Our work further supports the use of zebrafish as a model organism for studying behavioural changes and cognitive decline in aging, with significant translation relevance to humans.

Chapter 6

The FMP Y-maze has now been used to assess cognitive function in zebrafish from 4 dpf larvae to 2 year old adults. We have identified critical periods during development and ageing that impact on cognitive performance. The flexible use of the same task for assessing cognition across all ages makes it possible to map cognitive development. The lack of recurrent cognitive tests for children and adults has been raised as a potential caveat in investigations of cognitive maturation and identification of abnormal cognitive trajectories, such as those observed in neuropsychiatric disorders and neurodegenerative disease (Cromer et al., 2015). *This highlights another positive application of the FMP Y-maze as a cognitive task that has the capability to assess cognitive performance at a broad range of ages.*

When considering diseases which are characterised by impaired memory performance AD is one of the most well-known. During development of new behavioural tasks it is important to ensure that they have of a combination of face, construct and predictive validity, particularly when using them as measures for translational research. The previous three chapters have detailed use of the FMP Y-maze with a range of organisms, including humans, a broad spectrum of ages and sensitivity to pharmacologically induced cognitive alterations, designed to test the uses and limitations of the FMP Y-maze. However, investigations completed so far have all utilised healthy animals and humans. An important test of validation is with the use of a genetic model of known cognitive impairment. To this end, we have utilised one of the best characterised models of one of the most well-known diseases to cause memory impairment: AD. We are using the rodent APP/PS1 double transgenic model of AD. Deficits in WM in this model have been well characterised and extensively reported in the literature. The next chapter describes cognitive performance of transgenic AD mice in the FMP Y-maze.

Validation of The FMP Y-maze as a Simple, non-invasive Test to Detect Deficits in WM in APP/PS1 Transgenic Alzheimer's Disease Model

6.1 Abstract

To date, cognitive tests in mouse models of Alzheimer's disease (AD) have provided challenges in translating findings to the clinic, due to the animal specific nature of many behavioural tasks. Improving face, construct and predictive validity of animal models is greatly needed to better recapitulate symptoms associated with the human condition. The Free Movement Pattern (FMP) Y-maze has been demonstrated as a reliable measure of cognitive function that can not only be translated to humans, but also to multiple animal models. Thus, a larger breadth of model can be utilised for the assessment of human conditions, such as AD, and can be retrospectively combined to provide a broader, more informed view of the mechanisms and treatments that contribute to disease onset, progression and improvement. Here we further validate the FMP Y-maze as a measure of cognitive function using the rodent APP/PS1 model of AD to verify the detection of cognitive deficits, specifically WM, demonstrating sensitivity to changes in cognitive function and suitability to testing rodent models of AD.

6.2 Introduction

Cognitive impairments are a core feature of neurodegenerative disorders, such as AD, which is most notably characterised by a loss of memory function (Hugo & Ganguli, 2014). Several types of memory loss are well established in patients with AD, including episodic memory, semantic memory, recognition memory and working memory (WM) (Baddeley et al., 1991; Jahn, 2013). Previous work has highlighted WM capabilities as a central cognitive function which underlies the processing of other complex executive functions, such as planning, decision-making and cognitive flexibility (Baddeley, 1992, 2012).

Therefore, impairment of WM performance has the capacity to impact a subset of executive functions, deficits of which are regularly reported in AD patients (Guarino et al., 2019b; Kirova et al., 2015).

WM assessments in rodents are commonly carried out using maze-based tasks, such as the T and Y-mazes, radial arm maze, Morris water maze (MWM) and Barnes circular maze (Dudchenko, 2004; Shepherd et al., 2016; Stewart et al., 2011). WM impairments have been identified using a variety of maze tests, for assessing rodent models of AD, including the APP/PS1 mouse line (Bayer & Wirths, 2008; Ferguson et al., 2013; Wirths et al., 2008). However, discrepancies in findings are not uncommon with some investigators reporting deficits whilst others testing the same strain-matched model find no differences between transgenic mice and controls (Puzzo et al., 2015). A critical factor in determining WM deficits is the method of assessment used, with significant variation in impairment detection between tasks (Stewart et al., 2011). These discrepancies may arise as a consequence of confounding factors that often involve non-cognitive processes, such as locomotor and visual impairments, anxiety, fear and environmental disturbances, which can mask or exaggerate inferred cognitive dysfunction (Kapadia et al., 2016; Shepherd et al., 2016).

To successfully translate findings from animal models to humans, methodologies used for testing rodents need to minimise the effects of confounding variables and generate tasks that, as closely as possible, resemble clinical assessments (Bussey et al., 2008). To this end the FMP Y-maze has been designed as a task with improved translational relevance, that targets analogous cognitive domains across a range of species. The FMP Y-maze has been shown to be a reliable test of exploration strategy and WM performance in humans, rodents and zebrafish, demonstrating sensitivity to drug-induced cognitive changes (Cleal & Parker, 2018), ageing humans and zebrafish (Cleal, et al., under review) and is sensitive to perturbation in glutamatergic, cholinergic and dopaminergic systems (see Part 1: Chpt 3 & 5) (Cleal, Fontana, et al., 2020). Although the effectiveness of the FMP Y-maze has been tested pharmacologically and in healthy humans and animals, it has not yet been assessed using a transgenic model of cognitive impairment. To this end we tested the transgenic APP/PS1 rodent model of AD which has been used in a number of maze paradigms to assess WM deficits (Arendash et al., 2001; Bayer &

Wirths, 2008; Ferguson et al., 2013; Huang et al., 2011; Zhang et al., 2019; Zhu et al., 2017). We compared search strategies employed by carriers versus noncarrier littermate controls. Using tetragram sequence analyse we identified differences in exploratory behaviour between the two groups and demonstrate sensitivity of the FMP Y-maze to cognitive differences in rodents during the early stages of amyloid plaque formation (Zhu et al., 2017). Here, we further validate the FMP Y-maze as a method for assessing cognitive deficits, particularly WM, using a rodent model of AD.

6.3 Materials and Methods

Animals

Five double-transgenic and five noncarrier male mice (B6C3-Tg(APPswe,PSEN1sE9) at 7 months of age were assessed for cognitive deficits in WM in the FMP Y-maze. APP/PS1 mice express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9) gene which result in accelerated production of human Aβ peptide. Breeding pairs were obtained from the Jackson Laboratory (USA). In the University of Portsmouth Animal Facility, a colony was established by crossing transgenic mice with C57BL/6J mice. Controls used were noncarrier littermates for the APP/PS1 subjects. Mice were group housed and provided food and water ad libitum and maintained on a 12h:12h light/dark cycle (7 a.m.-7 p.m.) at 21 ± 2°C and 45-55% humidity. Procedures were carried out in accordance with the Animals (Scientific Procedures) Act, 1986 (UK) and the University of Portsmouth Animal Welfare and Ethical Review Board.

Apparatus

Behavioural testing was carried out in the Zantiks LT unit and rodent Y-maze (Zantiks Ltd., Cambridge, UK). A single, white acrylic Y-maze, L152, W50, H155 (mm) was used per mouse, with a clear plastic base and top to prevent mice from escaping the maze during testing. Live tracking, incorporated into the Zantiks unit, was used to record movement patterns by automatically logging arm entries and exits. Light levels in the apparatus were constantly maintained at a maximum of 2 lux during testing.

Protocol

The protocol used was based on previous work (Cleal, Fontana, et al., 2020). Briefly, mice were habituated to the behaviour room the day before testing. Animal handling was kept to a minimum by using a clear plastic tube, that was kept in the home cage, to transfer mice into the FMP Y-maze. One mouse was assessed per test. Once in the maze, mice were permitted free exploration for 1 hour (1 h). Arm entries and exits were automatically logged and exported as an excel. Arm entries are converted into a time sequence of left and right turns, which are subsequently 'chunked' into overlapping tetragram sequences (four consecutive turn choices, e.g. right, left, left, left (RLLL)). There are 16 possible tetragram sequences. Exploration strategies are analysed for patterns in sequence selection. The number of arm entries were additionally recorded to monitor locomotor response in the maze.

Statistics

Analysis were carried out using IBM SPSS (v25) and GraphPad Prism (v8). Previous studies have identified alternations (LRLR+RLRL) as the dominant strategy used by rodents, zebrafish and humans (Cleal, Fontana, et al., 2020). Variations in the use of alternations have been associated with changes in WM processing (Cleal, Fontana, et al., 2020; Cleal & Parker, 2018; Fontana, Cleal, Clay, et al., 2019). Residuals were taken of total percentage use of alternations for the duration of the trial. Residuals were plotted to assess normality, using Shapiro-Wilk test, and to screen for outliers. Extreme values (>3*IQR)

were removed from further analysis. Linear mixed model (LMM) analysis was carried out using "total turns" as the dependent variable, "condition" (control, APP/PS1), "time" and "condition*time" as the within-subjects factor and "ID" as a random effect. Analysis was followed by Bonferroni-corrected *post-hoc* test. Comparison between total strategy use for each pair of tetragram sequences (e.g. RRRR+LLLL, LLRR+RRLL) was analysed using One-way ANOVA followed by Dunnett's multiple comparison test comparing each tetragram pair to alternations. Locomotion was analysed using unpaired t-test. Data are represented as mean + standard error of the mean (SEM). Alpha values of $p \leq 0.05$ were considered statistically significant.

6.4 Results

WM performance was analysed using relative percentage use of alternations during 1 h of free exploration in the FMP Y-maze. There was a significant effect of condition (control v APP/PS1) ($F_{1,45}$ = 4.60, p = 0.037) and time ($F_{5,45}$ = 4.96, p = 0.001) on alternations, but no effect of condition*time ($F_{5,45}$ = 0.71, p = 0.621) (*Figure 1A*). Relative to noncarrier control mice, the transgenic mice exhibited a decrease in global alternation use showing a decrease in WM performance. Analysis of strategy use revealed a significant difference between tetragram sequences in control mice, showing a clear preference for alternations compared to all other strategies (One-way ANOVA, $F_{7,469}$ = 4.69, p < 0.0001) (*Figure 1B*); however, preferential strategy use is lost in transgenic mice that show equal use of all tetragram strategies with approximately chance selection of each tetragram sequence (One-way ANOVA, $F_{7,472}$ = 0.96, p = 0.459) (*Figure 1C*). Transgenic mice have shown a complete loss of strategy formation and instead explore the maze at random.

Figure 1.

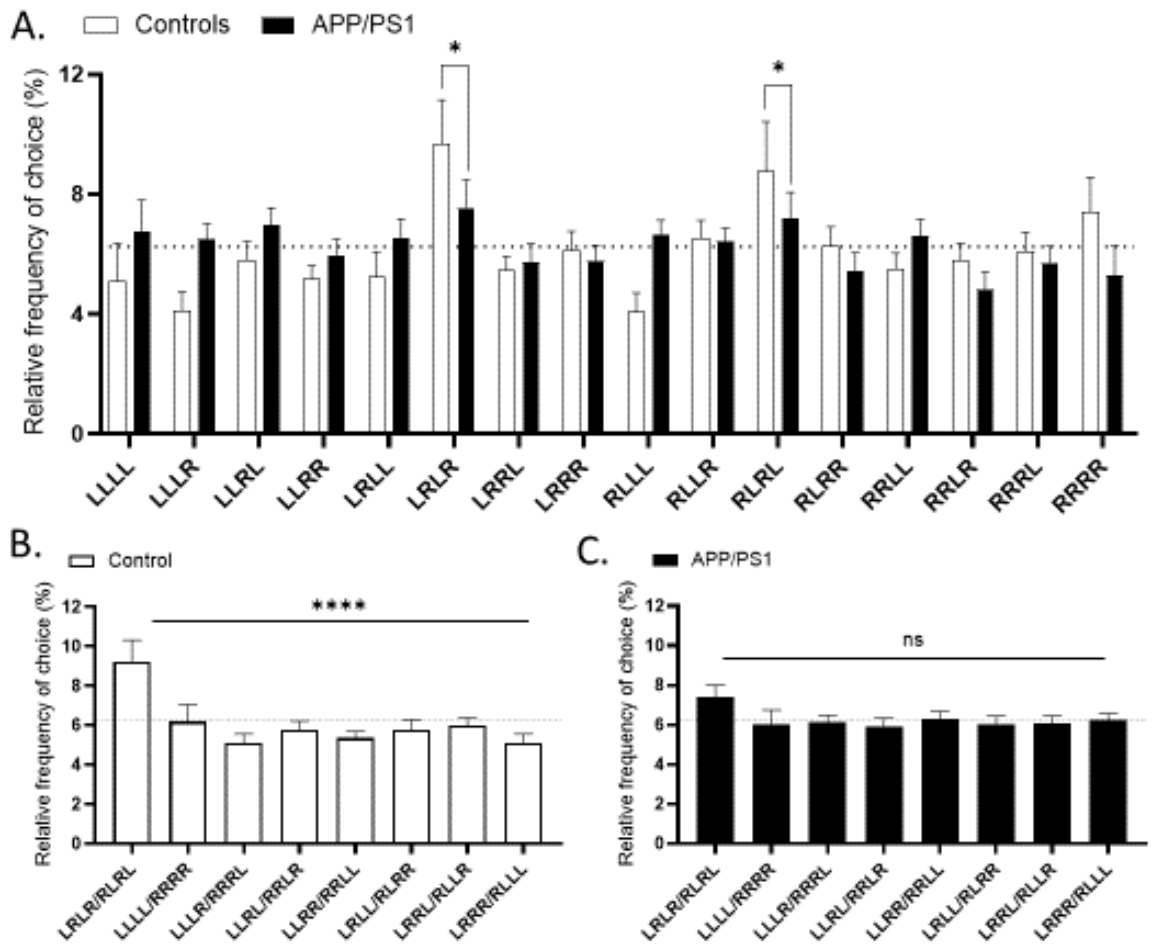

Figure 1. Free exploration in the FMP Y-maze for noncarrier control mice and transgenic APP/PS1 mice (n = 5/group). Comparison of search strategy based on 16 overlapping tetragram sequences used by control compared to littermate transgenic mice. **A**) shows the relative percentage use of each tetragram sequence. Comparison of paired tetragram sequences for **B**) control and **C**) APP/PS1 mice to identify preferential search strategy. Data represent mean + SEM, ns = not significant, * $p \leq 0.05$, **** $p \leq 0.0001$. The dashed line denotes chance selection (6.25%).

6.5 Discussion

The FMP Y-maze has been shown as an effective method for analysing WM performance through examination of search strategy use during 1 h of free exploration. Assessment of the APP/PS1, double transgenic mouse model of AD showed deficits in WM performance compared to littermate controls. Deficits resulted in the loss of strategic planning to the extent that exploration by transgenic mice was performed completely at random. Wild type mice, however, maintained a specific strategy, consisting of preferential use of alternations, throughout exploration of the maze.

Several studies have previously assessed WM performance of the APP/PS1 model and have demonstrated deficits starting from 5-months-old when Aβ plaques have started to develop and the number and area of plaques progresses in a near linear fashion up 22-months-old (Zhu et al., 2017). However, there are inconsistencies in detecting WM deficits at 5-months-old (Kelly et al., 2017). Mice from 6-months-old have been repeatedly shown to have deficits in WM when assessed in the T-maze, Y-maze and MWM, therefore, to ensure that amyloid plaque pathology and cognitive deficits had suitably progressed we used mice at 7-months-old. As with previous studies investigating WM in APP/PS1 transgenic mice we found a significant decrease in WM performance in the FMP Y-maze which resulted in the random, non-strategized exploration of the maze, compared to the alternation driven search strategy employed by littermate controls.

Previous studies using AD mouse models have found differences in the deficits reported for WM performance between studies, even when using age-matched and strain-matched comparisons (Puzzo et al., 2015). As noted above, previous studies investigating APP/PS1 do not confer on age of onset of cognitive impairment, with some studies reporting deficits from 5-months-old and others finding no differences at this stage compared to controls (Kelly et al., 2017; Zhu et al., 2017). A critical factor in determining WM deficits is the method of assessment used, with significant variation in impairment detection between tasks (Stewart et al., 2011). These discrepancies may arise as a consequence of confounding factors that often involve non-cognitive processes, such as locomotor impairments, or those arising from other cognitive domains, such as fear and anxiety. Impairments in

locomotion are commonly reported in rodent models of AD, which may result in ceiling effects in some WM tasks such as the MWM where swimming velocity may impact the route or latency to platform and could impact progression in subsequent trials (Kapadia et al., 2016). Hypo and hyperlocomotion caused by environmental factors such as novelty-induced anxiety, fear or fatigue, can also influence the outcome of maze-based tasks. Short, one-trial tests, such as the T and Y-mazes have been identified as particularly susceptible to altered locomotion as the brevity of these tasks does not permit a habituation time to allow for the subsidence of anxiety (due to entering a novel environment) or stress (due to experimenter handling) which may impact results (Hughes, 2004; Shepherd et al., 2016; Stewart et al., 2011). Many of these tasks additionally have long training periods required to learn task rules and are often motivated by rewards, as in some versions of the T- or radial arm maze, or aversive environments, such as the water in the MWM (Sharma et al., 2010; Stewart et al., 2011). Rewarding and aversive stimuli can both influence how attention is allocated, resulting in the diversion of resources either away from or in favour of the current task, thus influencing behavioural performance (Steel et al., 2016; Yokoyama et al., 2015). Another drawback to maze tasks is that once probe tests have been completed, the same animals cannot repeat the task. This significantly limits re-run facilities (Shepherd et al., 2016) which substantially impacts following disease progression or treatments that aim at slowing cognitive decline.

The FMP Y-maze task addresses several issues associated with traditional rodent tests of WM. Firstly, to avoid locomotor induced biases in task completion, the number of turns completed by each individual is used as a covariate in the analysis so that differences in alternations are not the result of hypo or hyperlocomotion of the model or environmental factors. Secondly, allowing animals to freely explore the arena for 1 h both minimises experimenter interference and incorporates sufficient time for a habituation period without affecting the overall results of the trial, i.e. if rodents were to freeze on first entry into the maze, but after a short period, 5-10 min, begin exploring, the prolonged trial time will still enable exploration pattern analysis based on the movements completed over the rest of the trial. Thirdly, there is no instruction, learning or correct way to explore the maze. Although previous studies have demonstrated a common search strategy employed by healthy rodents, humans and zebrafish,

there were no cues or prior training to achieve this method of exploration, it is a natural, instinctive mechanism (Cleal, Fontana, et al., 2020). Therefore, there is no need for extensive pre-trial training regimes, or the need for prolonged periods of food or water withdrawal as if often required to motivate rodents to respond to reward based learning trials. Although not demonstrated here, previous zebrafish trials have shown that the FMP Y-maze can be used as a repeated measures test, maintaining its sensitivity to detect differences in WM performance (see Part 2: Chpt 3) (Cleal, et al., under review). Finally, and most importantly, the FMP Y-maze has direct translational relevance to humans. Previous studies in healthy adults have identified analogous search strategies to rodents and reported decline in WM performance in aged (70+ year-old) compared to young (18-35-year-old) adults (see Part 1: Chpt 5) (Cleal, et al., under review). The similar search strategies and findings in ageing compared to animal models suggests that the FMP Y-maze animal task is measuring similar cognitive domains to the human virtual task. High sensitivity to age-related cognitive decline and reliable and robust cross-species protocol, suggests the FMP Y-maze is a task with potential to identify preclinical, therapeutic strategies and translate them to humans.

6.7 Conclusion

Assessment of the APP/PS1 transgenic mouse model in the FMP Y-maze reliably recapitulates cognitive deficits found in previous studies using rodent tests of memory. Exploration in the FMP Y-maze was characterised by an overall reduction in the use of alternations, the dominant strategy in healthy rodents and humans, and a loss of strategic exploration, which was instead replaced by random investigation of the maze. Prior studies have examined the exploration patterns of zebrafish, rodents and humans in the FMP Y-maze and have identified a specific strategy of increased alternation use that was evident in all three species. Thus, highlighting the relevance and potential that the FMP Y-maze has to reliably translate findings from animal models to humans. The use of a transgenic mouse model of AD, which has been extensively assessed in other, rodent specific, memory tasks and has reliably reported deficits in learning and memory, further validates the specificity of the FMP Y-maze for detecting WM deficits.

Chapter 7

7.1 General discussion

The studies covered in this portion of the thesis describe the development of a new assessment of cognitive function, with a particular focus on WM and cognitive flexibility. Initial studies successfully demonstrated that with the application of species-specific modifications, the FMP Y-maze could be used to assess cognition in *Drosophila,* zebrafish and rodents using a physical maze, in which animals freely explore for 1 h, and a virtual maze for humans, in which participants explore for 5 min, making the FMP Y-maze a short and simple task that could be applied in the clinic. Pharmacological manipulation of adult zebrafish suggested roles for the glutamatergic, cholinergic and dopaminergic systems in the maintenance of normal, healthy explorative behaviour and the associated executive functions. Studies of zebrafish from early larval stages through to senescence have not only demonstrated the sensitivity of the FMP Y-maze to subtle age-related changes in cognitive processing, but also provides a more comprehensive understanding of the ontogeny of advancingly complex behaviours and the gradual decline in these abilities during ageing in zebrafish. With the growing use of zebrafish to model complex neuropsychiatric disorders, understanding of species-specific learning and memory processes can aid in identifying commonalities with humans that may possibly mark evolutionarily conserved mechanisms. Thus, highlighting specific areas that have high translational relevance and may prove useful in future investigations of human disorders associated with these analogous areas. Commonalities such as altered vulnerabilities due to learning, memory and stress processing in juveniles/adolescence and the effects of senescence on decreasing cognitive capacity, which have been demonstrated here. These processes are similar in humans and zebrafish, and the preliminary findings discussed in the first part of this thesis highlight research areas that could greatly benefit from further work utilising zebrafish as a model organism.

The utility of the FMP Y-maze has been extended by examination of a rodent model of AD which had identifiable cognitive deficits compared to littermate, non-carrier controls. Thus, showcasing the FMP Y-maze as a possible assessment for cognitive impairments associated with AD. Combined this subset of studies provide strong evidence as to the suitability of the FMP Y-maze as a preclinical and clinical method for assessing subtle changes in cognitive processing in health and disease. However, further studies fully characterising the underlying pathways and processes used for developing particular strategies in the FMP Y-maze would greatly benefit both animal and human studies, providing a more comprehensive knowledge of the mechanisms detected in the maze and how these relate to behavioural changes associated with neuropsychiatric disorders and other conditions that affect cognitive processing.

Having extensively assessed cognitive function in health, the next two parts of this thesis will focus on aberrations in cognitive performance associated with disease. Part II will investigate the role of drugs of abuse on cognitive control using a prenatal and sensitization paradigm. The final section will build on the impacts of drugs of abuse and investigate their effects and relationship in SZ-like models. Combined, these two sections will provide a comprehensive view of zebrafish as a model of neuropsychiatric disorders and provide further evidence for the utility of the FMP Y-maze as a measure of cognitive performance.

Part II

Effect of Drugs of Abuse on The Healthy Mind

Chapter 1

1.1 Addiction – How do drugs of abuse alter cognitive state in health?

"Addiction represents a pathological usurpation of the neural mechanisms of learning and memory that under normal circumstances serve to shape survival behaviours related to the pursuit of rewards and the cues that predict them." (Hyman, 2005)

Addiction has only relatively recently been recognised as a *disease of the brain* and is now one of the most pressing health concerns we encounter today. Drug use disorders are reported to affect 35 million people using opioids, psychostimulants (such as cocaine, amphetamine, methamphetamine and opiates) and cannabis (United Nations Office on Drugs and Crime, 2019), a further 107 million people are estimated to have an alcohol dependence disorder (Ritchie & Roser, 2019) and a staggering 1.3 billion people use tobacco products worldwide (World Health Organization, 2015, 2020). Addiction to drugs of abuse is characterised by a compulsive-seeking and taking of a drug through a cycle of occasional intoxication which becomes increasingly chronic and uncontrolled, developing into withdrawal and anticipation/preoccupation in the absence of drug taking, culminating in physical and psychological dependency: addiction (Herman & Roberto, 2015). The neurological source of these symptoms is dysregulation of the brain's reward system, which encompasses the VTA, striatum

(particularly the ventral striatum which includes the nucleus accumbens) and the PFC (both the ventral and dorsal aspects) (Arias-Carrián et al., 2010), altering cognitive processing through DA availability, to favour drug-seeking and taking behaviour over normal and essential life-sustaining activities such as eating and procreating (Gould, 2010; Hyman, 2005). Early stages of drug taking alters synaptic plasticity to foster maladaptive drug-associated cues, often taking previously irrelevant features (such as being outdoors) and forging strong connections with the thoughts and feelings associated with a drug (e.g. nicotine-smoking) (Gould, 2010). Combined, this connection results in the user craving or taking a drug when in the presence of the drug-associated cue (e.g. on entering an outdoor space immediately craving a cigarette) (Baker et al., 2004; Hyman, 2005). As anything can become a drug-associated cue, avoiding environments or circumstances that elicit drug cravings can be difficult, with sufficient drug taking episodes having the potential to ultimately result in physical dependence which can disrupt cognitive processes essential to maintain abstinence.

WM deficits are a less pronounced deficit in substance abuse than other executive functions such as CF, planning, decision-making and risk taking (Baldacchino et al., 2012; Cousijn et al., 2014; Kelley et al., 2004; Stavro et al., 2013; Sen Yan et al., 2014). Although there is extensive literature examining the cognitive effects of substance abuse disorder on WM performance (Baldacchino et al., 2012; Butler & Le Foll, 2019; Edwards & Koob, 2012; Krueger et al., 2009; Patterson et al., 2010; Ramey & Regier, 2019). Several studies have reported WM deficits associated with chronic alcohol, cocaine, methamphetamine, opioid and nicotine abuse (Bechara & Martin, 2004; Goldstein et al., 2004; Hester et al., 2010; Liang et al., 2016). Additionally, WM performance has been suggested to be linked with smoking relapse, with poorer WM during abstinence predicting more rapid smoking resumption (Patterson et al., 2010). In alcoholics deficits in WM were not just evident during periods of alcohol abuse, but have been shown to last, on average, for a year of sobriety before deficits start to abate (Stavro et al., 2013). However, there has been a strong link between baseline WM performance (e.g. WM capacity) and abuse potential (Ramey & Regier, 2019). A recent study by (Soutschek et al., 2020) has demonstrated a critical role for baseline WM functioning in the effect of increased DRD1 activation

(using a D1 agonist) on reactivity to reward-associated cues and flexibility of cue reward-associations (i.e. the ability to flexibly update stimulus-reward associations). Further demonstrating the relationship between WM and CF processing for normal behavioural responding. Additionally, WM performance has been implicated as a potential therapeutic target (Bickel et al., 2011). WM training has been suggested as a possible therapeutic mechanism for improving impulse control and self-regulation in substance abuse disorders. This method is based on the premise of encouraging alternative neural plasticity networks to formulate within targeted neural systems by repetitive and increasingly difficult cognitive training to improve cognitive processing. As WM is a central cognitive function that underlies multiple other executive functions is it hoped that by targeting such as crucial system that other cognitive processes, such as aberrant decision-making, may also improve (Bickel et al., 2011, 2014; Brooks et al., 2016; Herold et al., 2012; Stavroulaki et al., 2017).

Aberrant CF has been well established as a core feature of substance abuse disorders (Butler & Le Foll, 2019). Addiction itself can be considered as an inability to alter responses when presented with a cue that has previously been associated with a drug reward. Such changes in CF can often be long-lasting and may play a critical role in enhanced vulnerability to relapse and compulsive drug-seeking and taking (Stalnaker et al., 2009). Thus, poor adaptability and response rigidity to specific drug-related cues, could contribute an important underlying behavioural pattern that perpetuates addiction (Day et al., 2015). Humans addicted to cocaine, alcohol, methamphetamine, Δ 9-tetrahydrocannabinol hydrochloride (THC) and opioids present with impaired CF, which is particularly evident on reversal learning and WCST tasks (Baldacchino et al., 2012; Butler & Le Foll, 2019; Colzato et al., 2009; Fillmore & Rush, 2006; Kroener et al., 2012a; Rogers & Robbins, 2001; Thomson et al., 2020). There is significant overlap in the brain regions associated with the reward system and those involved in control of executive functions. The PFC and striatum, as outlined above are central to the mesolimbic reward system and the CSTC network, critical for cognitive processing, including CF. Imaging studies have identified the PFC as a critical region associated with tracking and anticipating outcome reward values, represented by changes in neuronal firing (Blair et al., 2006; Dolan, 2007; Gottfried et al., 2003). The lack of this altered

neuronal activity demonstrates an absence of information updating, without which it becomes difficult to recognise changes in condition contingencies and thus, causes deficits in identifying the correct behavioural response under the new conditions (Schoenbaum et al., 2009; Stalnaker et al., 2009). This deficit has been shown following chronic cocaine exposure using predicted versus unpredicted outcome variables. Controls demonstrated the ability to recognise differences (in neural activity patterns) associated with the predicted outcome compared to the unpredicted outcome. However, the cocaine exposed group failed to achieve this (Stalnaker et al., 2009). Additionally, cocaine addicts show diminished feedback mechanisms that are equally important for information updating, but with regard to negative outcomes, i.e. when outcomes do not match expectations. Therefore, this potentially inhibits the mechanism used to inform us of behaviours that have negative implications, such as the impact of chronically seeking and taking drugs of abuse (Stalnaker et al., 2009). Changes are not only seen in the PFC of addicts, but additionally the striatum and the thalamus have also been reported to have structural and functional changes in response to chronic drug taking. Imaging studies in humans have shown reduced functional connectivity between the thalamus, the striatum and frontal cortices (PFC) as well a reduction in white matter tracts, as is similarly found in SZ patients. Additionally, correlations have been found between heaviness of smoking, alcohol, cocaine and heroin addiction and reduced connectivity between regions of the CSTC network (Huang et al., 2018).

DA is known for its role in the reward pathway, which is essential for rewarding us for carrying out crucial activities for survival, such as eating, drinking and reproducing. When these tasks are carried out our brain sends messages of pleasure to reinforce these actions and encourage us to seek out the stimulus again and again (Di Chiara & Bassareo, 2007). When we engage a naturally rewarding stimuli, dopaminergic neurons in the VTA are stimulated to release DA. This reward signal is emitted to the PFC, the nucleus accumbens (NAcc), amygdala and the hippocampus: the mesolimibic pathway (Di Chiara & Bassareo, 2007; Parker, Brock, Walton, & Brennan, 2013). Drugs of abuse act by *hijacking* this system and causing large and rapid surges in DA availability, that greatly exceed, in duration and intensity, those usually caused by natural rewards. Different drugs increase DA via different routes, for example

alcohol, heroin and nicotine act by increasing the amount of DA released into the synaptic cleft by exciting DA neurons in the VTA to produce more action potentials, thus increasing the amount of DA released. Whilst amphetamine and cocaine inhibit mechanisms of removing DA from the synaptic cleft by blocking DAT, causing a rapid and persistent rise in synaptic DA levels. Opioid-dependent individuals have demonstrated impaired WM and CF, with increased errors and perseverative responses compared to controls. Repeated opioid exposure was also found to reduce striatal DAT density which negatively correlated with WM performance (Liang et al., 2016). Methamphetamine and cocaine similarly have demonstrated reduced DAT levels in the striatum, an effect which has been shown to reverse after prolonged periods of abstinence (Berke & Hyman, 2000). Regardless of the mechanism to achieve increased levels of DA availability, continued upsurges in DA has the ultimate consequence of blunting the DA system to everyday stimuli. This is often associated with increased anhedonia, which is a frequent feature in addiction and has significant correlation with drug craving, withdrawal and relapse (Hatzigiakoumis et al., 2011).

One of the major difficulties in creating animal models of addiction, as with many neuropsychiatric disorders, is being able to extract a relevant sample of conditions that relate to a disorder that is extremely complex and multifaceted both in its pathology and the circumstances required to develop the condition (Edwards & Koob, 2012). Similarly to SZ, there is no single gene responsible for the development of addiction. Instead, there are a series of interactions between genetic traits, behaviours, environmental exposures and experiences that culminate in the physical and psychological dependence on a drug. There are two main types of animal model of substance abuse, non-contingent models in which animals are passively exposed to drugs. This can be via intravenous injection, provided in food or water for rodents or put into the tank water of zebrafish. The other model is the contingent model which commonly involves animals self-administering rewarding stimuli, relying on the self-motivation of the animal to seek out the drug (Kuroda et al., 2017; Markou et al., 1999). The most commonly used method in zebrafish models is the non-contingent model as drugs can be administered to zebrafish easily, with minimal handling and stress by simple addition to tank water.

Therefore, the focus here will be on non-contingent models of drug abuse that used measures of executive function, particularly WM and CF. Many substance abuse models have used chronic drug exposure to model long-term effects of drugs of abuse. Rodent studies have shown WM and CF deficits following chronic alcohol, cocaine, morphine, methadone and methamphetamine exposure in line with human studies (Barron et al., 2005; Brockett et al., 2018; DePoy et al., 2013; Dougherty et al., 1996; Kroener et al., 2012b; Miladi-Gorji et al., 2011; Spain & Newsom, 1991; Stalnaker et al., 2009; Tramullas et al., 2007; W. Sen Yan et al., 2014). Structural and functional changes in the PFC and striatum, caused by chronic drug taking, have been implicated for their role in priming the brain, causing alterations in connectivity that potentially underlie the development of persistent drug taking and addiction witnessed in humans (Herman & Roberto, 2015; Hyman, 2005; Koob & Volkow, 2010; Ramey & Regier, 2019). The use of zebrafish as a model of addiction has grown dramatically over the last two decades (Maximino et al., 2013). Extensive studies on nicotine (Gómez, Carrasco, & Redolat, 2008; Levin, Bencan, & Cerutti, 2007; Levin, Limpuangthip, Rachakonda, & Peterson, 2006), ethanol (Miller & al., 2013; Parker, Annan, et al., 2014; Parker, Evans, et al., 2014), cocaine (Darland & Dowling, 2001; López-Patiño, Yu, Cabral, & Zhdanova, 2008; Mersereau, Poitra, Espinoza, Crossley, & Darland, 2015) and morphine (Bretaud et al., 2007; Khor, Jamil, Adenan, & Shu-Chien, 2011; Fatima Macho Sanchez-Simon, Zhang, Loh, Law, & Rodriguez, 2010), have been carried out using zebrafish. Similar to humans, chronic drug exposure in zebrafish has demonstrated altered dopaminergic parameters. However, by far the most commonly used measure for assessing addiction phenotypes in zebrafish is the use of the conditioned place preference (CPP) task, in which animals are non-contingently administered a drug that is associated with a specific cue, such as striped background. Fish are trained to expect drug reward in the presence of the reward-associated cue, and therefore in the absence of drug still show a preference of the drug paired side. This demonstrates the drug-induced reinforcement pathway that is strongly implicated in addiction. This method is commonly used in rodents and has been demonstrated with a vast array of drugs in zebrafish, including cocaine, amphetamine, nicotine and opiates (Bretaud et al., 2007; Alistair James Brock et al., 2017; Darland & Dowling, 2001; Ninkovic & Bally-Cuif, 2006). However,

there are comparatively few studies in zebrafish that specifically target cognitive processes such as WM and CF and the few that have generally assess cognition only after acute exposures (Edward D. Levin & Chen, 2004; Luchiari et al., 2015). This further highlights a great need to improve and increase testing of executive functions in zebrafish following chronic periods of drug exposure.

Chapter 2

The first chapter of this section on drugs of abuse is focused on a technicality of drug administration in zebrafish. Although not striking in its contribution to addiction pathology, it is a much overlooked consideration in zebrafish studies of drugs of abuse (or any drug for that matter). It is important to understand the chemistry of drugs, their biological targets and the interface between them. Environmental influences can be a substantive factor in drug affect. This is particularly important in non-contingent drug exposure models, in which drugs are passively, opposed to actively, administered. One positive often cited by researchers using zebrafish is the ease of drug administration - simply add to tank water. However, several factors are generally overlooked by researchers when conducting such studies. Here we discuss the importance of one of them: pH. Preliminary and pilot work in our group had shown unexpected differences in responses to drugs, and drug toxicity, and we decided to test the hypothesis that pH might impact on the effects of the drugs on the fish. To date, few studies report the pH of drugs and, as described in this chapter, this could be impacting interpretation and replicability of findings.

The Importance of pH: How Aquarium Water is Affecting Behavioural Responses to Drug Exposure in Larval Zebrafish.

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2.1 Abstract

There has been rapid growth in the use of larval zebrafish as a complementary vertebrate model for drug discovery, abuse liability and pharmacological toxicology, resulting in a huge increase in zebrafish facilities worldwide. However, many research groups working with zebrafish do not typically report the pH of husbandry conditions in methodologies, nor are the pH of drug treatments reported in many research articles. This unknown factor can be a major contributor in the differential effects of drug treatments. Therefore, as a case study, we tested the impact of altering pH of several drugs of abuse and assessed locomotor changes associated with a single drug concentration delivered at different pHs. We found that a change of a single pH unit, within the pH ranges commonly used in zebrafish husbandry, was enough to alter locomotor activity at a fixed drug concentration. Many pharmacological agents are dependent on environmental factors, such as pH, to determine bioavailability. Efficaciousness for many classes of drug is dependent on their ionization state in which shifts towards uncharged species can influence the easy of a drug crossing biological membranes. Thus, we urge users to report pH in husbandry methods and drug treatments to improve replicability and inter-study comparisons.

2.2 Introduction

Zebrafish have been rapidly growing in popularity as a model organism (Kalueff et al., 2014; Parker, Brock, Walton, et al., 2013; Stewart et al., 2015), lending themselves exceptionally well to studies of environmental toxicity, teratogenesis, neurotoxicity, and preclinical drug discovery (Chapela et al., 2019; Gehrig et al., 2018; Kalueff et al., 2014; Charles Ben Lovely et al., 2016). The high numbers of offspring, external fertilisation, transparent eggs, cost-effective maintenance and ease of genetic and pharmacological manipulation have resulted in what can now be considered as one of the most versatile and well characterised vertebrate models (Kalueff, 2017). Currently used in over 1,200 labs worldwide (*zfin.org*), there is growing use of zebrafish as an alternative model for both environmental and translational models. Aided by advancements in technology, zebrafish embryos and larvae have been gaining ground as a model for drug discovery due to the ease of drug administration and high-throughput whole-animal assays (; Gehrig et al., 2018; Lieschke & Currie, 2007; Strähle et al., 2012). Despite the success of mammalian pharmacokinetic and efficacy models, they are often expensive, time consuming, require large quantities of compounds and can be extremely invasive, particularly for dosing (Zon & Peterson, 2005). Zebrafish, on the other hand, offer a cost-effective alternative which has low compound requirement and an easy method for drug delivery which is facilitated through the aqueous environment (Vaz et al., 2018a; Zon & Peterson, 2005). Thus, zebrafish are an invaluable species for screening the pre-regulatory and preclinical phases of drug discovery and pharmacological toxicology (Basnet et al., 2019; Chapela et al., 2019; Gehrig et al., 2018; Norton, 2013; Stewart, Braubach, et al., 2014).

Understanding the pharmacology of compounds is crucial to interpreting the interaction between chemicals and organisms. This is of particular importance when screening novel drugs for therapeutic treatments or for abuse liability, as biochemical function can be altered by temperature and pH, critically influencing pharmacokinetics (Dowd, 2017; Mitra & Kesisoglou, 2013; Stevens & Balahura, 2007). Currently, 'Materials and Methods' sections in publications report general husbandry parameters, including a description of water temperature, light-dark cycles and feeding routines; however, it is not

mandatory to report pH. Subsequently, many studies do not report either environmental aquarium water (the main system water used for the majority of fish husbandry) or pH values of drug containing solutions. An article by Aleström, et al, outlines updated recommendations for the husbandry and care of zebrafish in line with the EU Commission Recommendation 2007/526/EC and the EU Directive 2010/63 concerning care and well-being of experimental animals (Aleström et al., 2019). This article recommends pH levels between 6.5-8.0. This range is inclusive of weakly acid to weakly basic conditions. Of the 154 articles examined here, only 34.4% of them reported pH, with a range of 5.8-9.0, representing a maximal change of 2.2 units. These changes in pH may be accounted for by the water source for recirculating systems. This can either be from local water supplies (e.g. tap water), which is subject to effects of the local environment, such as hard versus soft water, or RO systems which rely on pre-filtration. The initial water source may influence overall pH levels. For example, hard water areas have increased concentrations of multivalent cations, most commonly calcium or magnesium ions, which consequently increase water pH. Thus, with the global distribution of zebrafish facilities, fed off a vast range of water sources, it is reasonable to expect that baseline pH levels of aquarium water will vary from lab to lab. Although many facilities that have reported pH fall within the recommended pH levels, we demonstrate that some facilities operate outside of these parameters, thus, extending the pH range at which drug studies are conducted.

Here, we varied the pH of the test environment by a single unit, within the pH range recommended by Aleström, et al. We selected pH 7.0 and 8.0 to identify if this small change can influence survival or locomotor responses of larvae exposed to fixed concentrations of drug treatments. The drugs tested have been regularly used to investigate mechanisms underlying neuropsychiatric disorders, such as substance abuse, and have been selected from the same family of organic bases, to identify if pH is a significant factor which can alter behavioural responses to compounds that share similar chemical structures. Our findings suggest that at the concentrations selected, there were no detrimental effects on survival. However, slight structural differences between compounds, resulting in variable pK_a values, are significant predictors of altered organism-drug interactions that potentially

result in modified locomotor responses to the same dose of drug when exposed at different pH conditions. Our study suggests that, when using locomotor activity as a measure of behavioural response to a drug, pH is a critical factor. The growing use of zebrafish as a model for screening compounds or testing novel therapeutics and many studies using locomotor activity as a behavioural end point, further supports the need to accurately report environmental conditions in which test compounds are introduced.

2.3 Materials and Methods

Systematic Review

We carried out a systematic analysis of the reporting of pH in zebrafish behavioural experiments over the past 20 years. Using PubMed, we inserted the search terms "zebrafish" AND "behavio*" AND "locomoto*" AND "drug". This returned 465 results, of which 154 had full free text available. **Table 1** displays a summary of the data from these studies relating to reporting of pH.

Breeding and husbandry

Adult, AB wild-type zebrafish (*Danio rerio*) were retained as breeders in the University of Portsmouth Fish Facility. Housing conditions were maintained on a re-circulating system (Aquaneering Inc., San Diego, CA, USA), with aquarium water at pH 8.4 (±0.4), temperature 25-27 °C, on a 14H: 10H light/dark cycle. The day prior to embryo collection male and female breeders were housed in separate tanks overnight. On the day of embryo collection, 15 mins prior to light onset, all adults were placed in a breeding tank, with males and females separated by a transparent divider. Upon light onset, the divider was removed. One hour after light onset, the adults were returned to the colony, and embryos were collected from the breeder tank. The embryos were pooled, and placed in petri dishes of aquarium water, in groups of ~50 embryos per petri dish and reared at 28 °C in aquarium water at pH 8.4, to mimic adult conditions, in a clear incubator for three days (toxicity assessment) or seven days

(behavioural assessment) on a 14H:10H light/dark cycle. All studies were conducted following the guidelines of the University of Portsmouth Animal Welfare and Ethical Review Board and under licence from the UK Home Office.

Pharmacological agents

To study the effect of pH on different drug conditions, four commonly used drugs of abuse were selected for this study. Nicotine (Sigma-Aldrich) was administered at a concentration of 5 μM (García-González et al., 2020; Petzold et al., 2009; Yoo et al., 2018), morphine sulphate (Sigma-Aldrich) was administered at 2.6 μM (Lopez-Luna et al., 2017), *d*-amphetamine (Sigma-Aldrich) was administered at 22 μM (Irons et al., 2010), and caffeine (Tocris) was administered at 200 μM (Gutiérrez et al., 2020). Concentrations were based on a combination of larval and adult zebrafish studies investigating effects on locomotor activity.

Toxicity assessment

For control groups, aquarium water was buffered with citric acid to either pH 7.0 or 8.0. For treatment groups, respective concentrations of drug were added to aquarium water and then buffered using either $NaHCO_3$ or citric acid to bring the final pH to either pH 7.0 or 8.0. The final solution pH values were reported at 26°C. From the final buffered solution, 0.9 mL was added to each well of a 48-well plate. Individual, 3 dpf larvae were transferred into each well for 24H. Tail-touch-evoked motor (TEM) responses were used to assess survival at 0.5H, 1H, 1.5H, 2H, 3H, 4H, 5H and 24H intervals. Plates were maintained at room temperature, 26°C. Each drug challenge/control was conducted in separate plates with n = 8 larvae/dose/pH. 48-well plates were used due to the well sizes minimising volume of drug required per test and providing enough space for larvae to swim and for TEM to be conducted with ease. Pilot work was conducted demonstrating that TEM was a suitable and effective method for assessing survival with the drugs tested as part of this study. However, it is worth noting here that when

testing drugs with analgesic properties or causing extreme hypolocomotion, TEM may be an unsuitable method for assessing survival. Under these circumstances it would be recommended that heartbeat is used as an alternative method for assessing survival. During the transfer of larvae to individual wells and the use of TEM have the potential to inflict a low level of mortality due to regular handling of larvae. To account for this, a significant effect of drug on survival was determined by treatments that caused more than 50% mortality.

Larval locomotor response

Following toxicity assessments, drugs that did not cause a significant impact on survival were used for subsequent detailed behavioural analysis. The same method of buffering drug treated, and control groups were applied as above. 7 dpf larvae show spontaneous movement and were used to assess locomotor changes in response to drug and pH challenges. 7dpf larvae were placed in individual wells of a 48-well plate. Movement was tracked by placing plates immediately into the Zantiks MWP larval behavioural unit. Each larva was tracked individually using automated tracking software (Zantiks MWP, Cambridge, UK). Locomotor assays were run in the dark as previous studies have demonstrated that larvae move more in dark than light environments (Burgess & Granato, 2007). Movement data were logged after 0.5H (acute) and 4H (prolonged) exposure, larvae were recorded for 30 mins. Any larvae that displayed severe immobility behaviour, defined as less than 10 mm travelled during each assessment, were not included in any data analyses or figures. Variance in immobility was due to individual larval differences (i.e. not overrepresented in any drug or pH condition), and from the total animals tested represented less than 2% of the total population.

Statistical analyses

All data were analysed using GraphPad Prism (version 8.4.2). Toxicity was assessed using Kaplan-Mayer survival curves, proportion of survival was compared to pre-drug exposure viability. Outliers from behavioural groups were identified using boxplot with Tukey analysis and excluded from subsequent

analysis. Comparison of each pH condition was tested for normality using the Shapiro-Wilk test. Groups that were normally distributed were analysed using the unpaired *t*-test on data after outliers were excluded. Groups with non-normally distributed data were analysed using the Mann Whitney test, after the exclusion of outliers. Within each treatment group, an interaction was considered significant when $p \leq 0.05$. All data are presented as mean ± standard error of the mean (SEM).

2.4 Results

Systematic Review

To identify the extent to which pH of either husbandry or drug treatment conditions were reported in research articles relating to zebrafish behaviour, we conducted a systematic analysis of a population of published research articles in the fields of toxicology, neuroscience, drug discovery, drug testing, genetics or other. **Table 1** provides a summary of our findings. We found that for each research field, 50% or less studies reported pH, and for toxicology, neuroscience, drug discovery and drug testing, the pH range was greater than one unit between studies.

Table 1.

Research field	Behavioural endpoint (e.g. locomotion)	N. articles with pH reported/Total articles	pH value (or range)
Toxicology	Locomotion, aggression, exploration, predator interactions, social interactions, shoaling	10/21	6.5-7.7
Neuroscience	Locomotion, anxiety, aggression, exploration, escape, predator interactions, social interactions, shoaling, cognition	23/80	6-9
Drug discovery	Locomotion	7/14	7-9
Drug testing	Locomotion, anxiety, freezing, exploration, predator interactions, shoaling, stress	9/26	6.5-9
Genetics	Locomotion, anxiety, shoaling	3/9	7.2-7.7
Other	Locomotion	1/4	7.9-8.3

Water challenge

The survival curve for larvae exposed to aquarium water, buffered with citric acid to either pH 7.0 or 8.0, is shown below in (**Fig. 1A**). 3 dpf larvae showed no adverse effects on survival when exposed to altered pH conditions for 24H. (**Fig. 1B**) shows the effect of pH on the total activity levels of 7 dpf larvae during the first 0.5H and after a further 4H of exposure to their respective pH conditions. In the absence of drug, changes of ± 1.0 pH was not sufficient to evoke changes in locomotor activity (0.5H: t = 1.719, df = 84, p = 0.089; 4H: t = 1.213, df = 84, p = 0.229).

Figure 1.

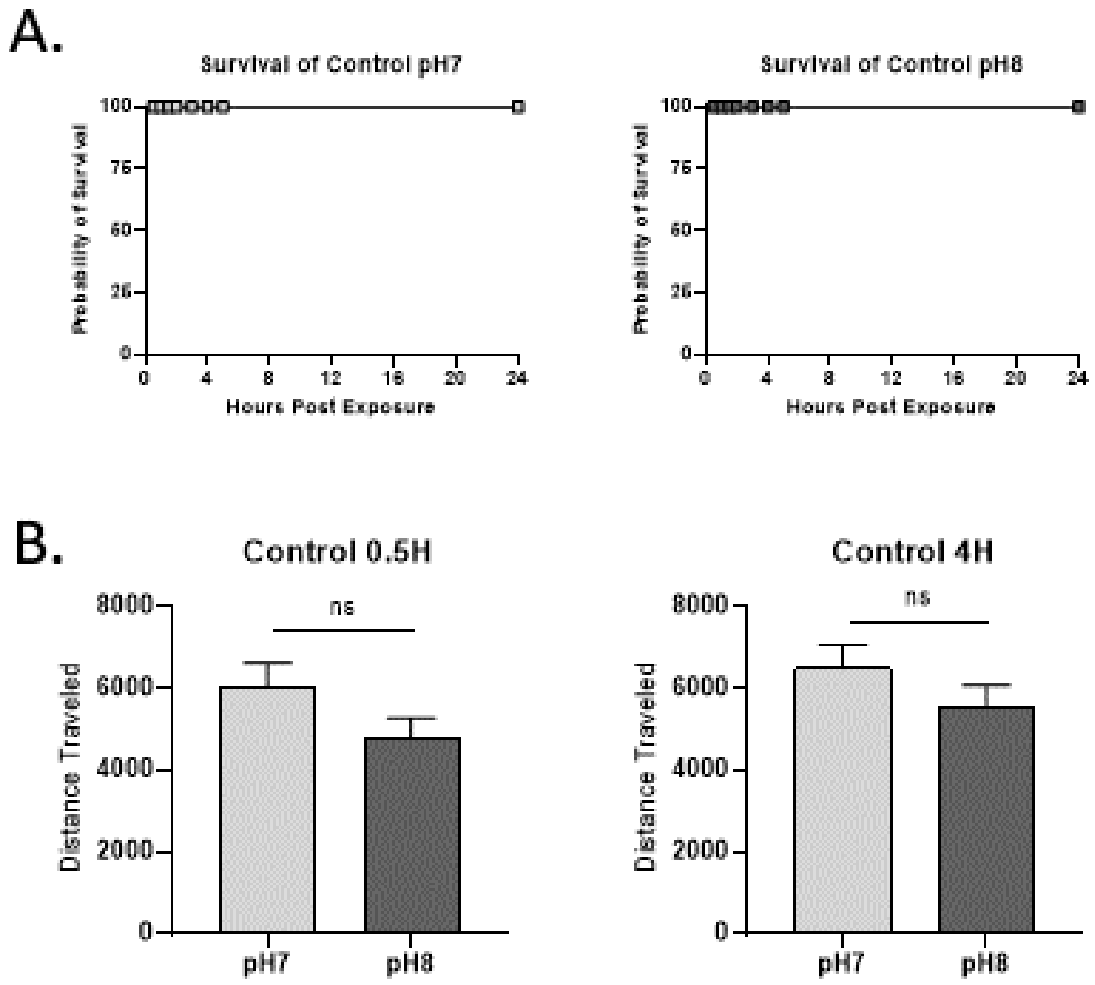

Figure 1. The effect of pH controlled aquarium water on (**A**) survival of 3 dpf larvae over 24 hours, assessed using tail-touch-evoked (TEM) motor response at 0.5H, 1H, 1.5H, 2H, 3H, 4H, 5H and 24H intervals. Analysis was carried out using the Kaplan-Mayer survival curve, with proportion of survival compared to pre-drug exposure viability. Curves show 100% survival for both pH conditions. pH7, n=8; pH8, n=8 (**B**) The effect of pH controlled aquarium water on locomotor behaviour in 7 dpf larvae during the first 0.5H of exposure, and at 4H of exposure was analysed for outliers using boxplot with Tukey analysis. Following removal of outliers, Shapiro-Wilk normality test was conducted. Samples that were not normally distributed were analysed using Mann Whitney test. pH7 n=46; pH8 n=46. There was no significant difference in distance travelled between the two groups. Bars represent mean, error bars are SEM; ns – not significant.

Drug challenge

Larvae at 3 dpf were treated with four commonly used drugs of abuse; nicotine, morphine sulphate, *d*-amphetamine and caffeine. Treatment water was buffered to either pH 7.0 or 8.0 and the effect on survival was monitored over 24H of exposure (**Fig. 2**). Like control animals, nearly all treatment groups at both pH 7.0 and 8.0 had 100% survival after 1 day of continuous treatment. The only exception was in the nicotine treated group at pH 7.0, in which a single larva was reported to have died at the 24H time point, which, as an isolated death, it is unlikely to be the result of the treatment on survival. The survival curve analyses, demonstrated that at each of the tested concentrations, the drugs and pH conditions used were safe to conduct behavioural analysis on older larvae.

Following survival curve analysis, acute (0.5H) and prolonged (4H) exposures to a single concentration of drug under two pH conditions were conducted using 7 dpf larvae. (**Fig. 3A**) shows the response of larvae exposed to 5 µM of nicotine, which caused an increase in locomotor activity in larvae treated at pH 8.0 compared to those treated at pH 7.0. This increased activity level was evident after acute and prolonged exposure (0.5H: t = 2.175, df = 13, p = 0.0487; 4H: t = 2.446, df = 13, p = 0.0294). Treatment with 2.6 µM of morphine sulphate, showed no locomotor difference after an acute exposure (0.5H: t = 0.314, df = 14, p = 0.758). However, differences in activity levels were evident after prolonged treatment, resulting in a significant increase in the distance travelled by larvae in the group buffered to pH 8.0 (4H: t = 2.990, df = 14, p = 0.0098) (**Fig. 3B**). Larvae treated with 22 µM of *d*-amphetamine showed no differences between pH groups after acute exposure (0.5H: t = 1.984, df = 13, p = 0.0688). However, after prolonged exposure there was a significant effect of pH 8.0 causing a decrease in locomotor activity compared to larvae treated at pH 7.0 (4H: t = 2.883, df = 13, p = 0.0128) (**Fig. 3C**). Caffeine caused no detectable differences between pH groups either at the acute or prolonged treatment stages (0.5H: t = 1.183, df = 13, p = 0.258; 4H: t = 0.835, df = 14, p = 0.418) (**Fig. 3D**).

Figure 2.

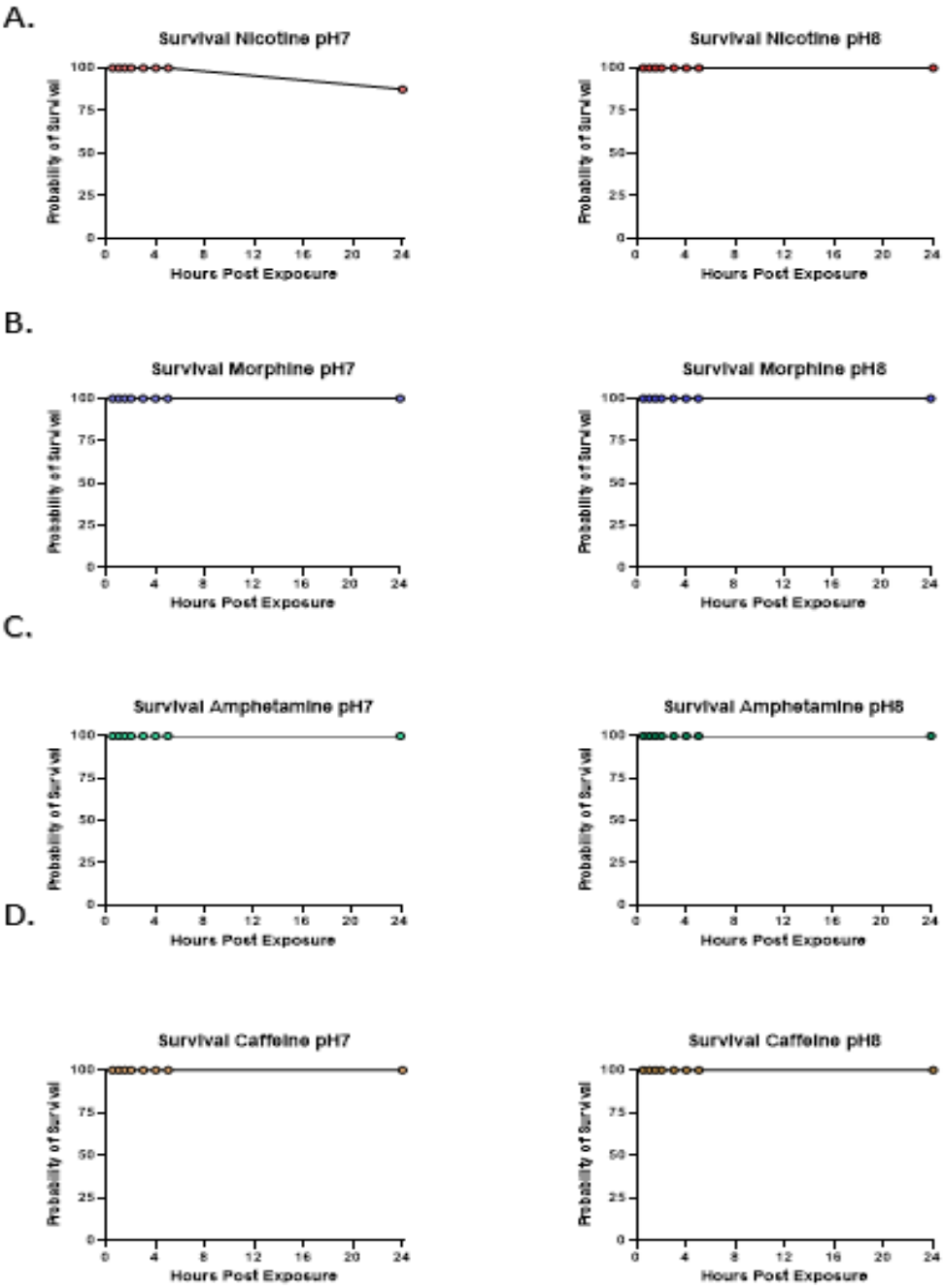

Figure 2. The effect of pH-controlled drug treatment on the survival of 3 dpf larvae over 24 hours, assessed using tail flick at 0.5H, 1H, 1.5H, 2H, 3H, 4H, 5H and 24H intervals for **A**) 5 µM nicotine, **B**) 2.6 µM morphine sulphate, **C**) 22 µM of *d*-amphetamine and **D**) 200 µM caffeine. Analysis was carried out using the Kaplan-Mayer survival curve, with proportion of survival compared to pre-drug exposure viability. Survival curves show 100% survival for both pH conditions for all drug exposures, except pH7 nicotine, which reported a single death. pH7 n=8; pH8 n=8 for each drug condition.

Figure 3.

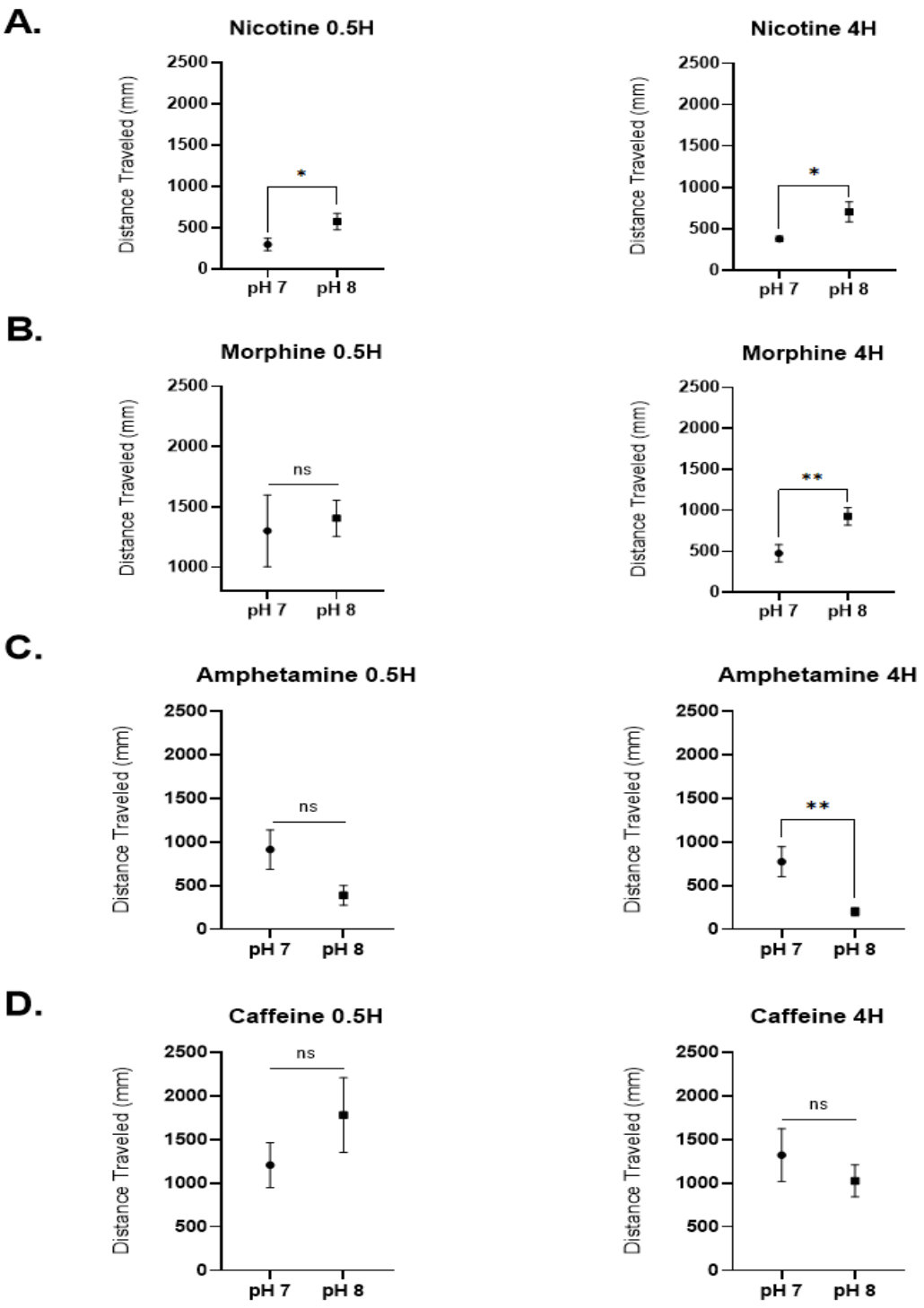
A.
Nicotine 0.5H
Nicotine 4H
B.
Morphine 0.5H
Morphine 4H
C.
Amphetamine 0.5H
Amphetamine 4H
D.
Caffeine 0.5H
Caffeine 4H
Distance Traveled (mm)
pH 7
pH 8
ns
*
**

Figure 3. The effect of pH controlled drug treatments on locomotor activity of 7 dpf larvae assessed using automated tracking software for the first 0.5H of exposure (acute) and after 4H of exposure (prolonged). Treatments were buffered to either pH 7.0 or pH 8.0 (±0.1) using citric acid. A) 5 µM nicotine, B) 2.6 µM morphine sulphate, C) 22 µM of *d*-amphetamine and D) 200 µM caffeine. Analysis for outliers was carried out using boxplot with Tukey analysis. Following removal of outliers, Shapiro-Wilk normality test was conducted. Samples that were normally distributed were analysed by unpaired t-test, not normally distributed samples were analysed using Mann-Whitney test. Nicotine, 0.5H: pH7 n=7, pH8 n=8, 4H: pH7 n=7, pH8 n=8. Morphine, 0.5H: pH7 n=8, pH8 n=8, 4H: pH7 n=8, pH8 n=8. Amphetamine, 0.5H: pH7 n=8, pH8 n=7, 4H: pH7 n=8, pH8 n=7. Caffeine, 0.5H: pH7 n=8, pH8 n=8, 4H: pH7 n=8, pH8 n=8. Bars represent mean, error bars are SEM; ns – not significant, * $p < 0.05$, ** $p < 0.01$.

2.5 Discussion

The aim of this study was to assess if a change of environmental pH was substantive enough to cause a change in behavioural activity when larvae were exposed to the same concentration of drug at different pH's. We found that a change in pH of ± 1.0 unit significantly impacted locomotor activity of larvae for three of the four test drugs. Nicotine was found to have a pH-dependent effect that immediately impacted locomotor activity. This effect was sustained over both acute and prolonged exposure. Morphine and *d*-amphetamine both showed altered locomotor responses based on pH, but only after prolonged exposure. Caffeine was the only drug tested that did not cause different activity levels between pH groups. Our findings suggest that the chemical composition of a drug can be a strong determinant in the effect of pH on behavioural response, potentially due to altered bioavailability. Our results thus highlight the differential effects that small changes in pH can have on behavioural output in response to drug exposure.

Zebrafish have been found to live in bodies of water with pH varying from 6 to 10, demonstrating suitability to a wide range of environments (Aleström et al., 2019; Arunachalam et al., 2013). However, in laboratory settings, the aim has been to provide stable husbandry conditions, and

thus zebrafish housing systems have been recommended at pH 6.5-8.0 (Aleström et al., 2019). Analysis of 154 studies assessing effects of drugs on zebrafish behaviour showed the most widely used pH levels fell between pH 7.0-8.0, found in 77% of studies reporting pH. We assessed if aquarium water buffered to either pH 7.0 or 8.0, reducing our normal aquarium water levels by 1.4 or 0.4 respectively, caused differences in toxicity to 3 dpf larvae or baseline locomotor activity of 7 dpf larvae. We found that there was no difference in the survival rate over 24H, in which both pH conditions had 100% survival. There was also no evidence of pH related changes in locomotion, as both groups showed similar distances moved after acute and prolonged exposure to pH altered aquarium water. Similar to control groups, toxicity studies of test drugs at pH 7.0 and 8.0, revealed that over 24H of exposure, the selected concentrations did not cause any toxic effects, thus resulting in 100% survival in all conditions. However, behavioural assessment of locomotion at 7 dpf revealed significant pH-dependent differences in response to a fixed dose of drug with variable pH conditions.

Here, we demonstrated that within the first 0.5H of exposure of 7 dpf larvae to 5 µM nicotine, there was an immediate difference in the locomotor activity of larvae at pH 7.0 compared to those treated at pH 8.0. Nicotine is a weakly basic drug and can exist in three forms in aqueous solution: the univalent (pyrrolidinium) cation, divalent cation and the unionized 'free base' (Barlow & Hamilton, 1962). At physiological pH (ca. 7.4), 69% of nicotine is present in the ionized form (predominantly the univalent cation) (Pogocki et al., 2007). The rest remains unionized (uncharged) and it is in this form that molecules are easily absorbed, as the free base can readily pass across biological membranes (Benowitz et al., 2009; Pogocki et al., 2007). Absorption in this state is rapid, and in conjunction with moderate hydrophobicity and low polarity (due to the presence of the pyridine and pyrrolidine rings) nicotine can easily cross the blood-brain barrier to access the brain (Oldendorf, 1974;Oldendorf et al., 1993; Spector & Goldberg, 1982; Tega et al., 2018). When nicotine is present in aqueous solution at 25 °C, it has two pK_a values; 8.02 and 3.12. If the environmental pH, that the drug is dissolved in, is equal to the pK_a value, then there are equal quantities of drug in the ionized and nonionized forms (50:50) (Dowd, 2017). Therefore, when nicotine is solubilized in pH 8.0, equal to the pk_a value, ~50% of the nicotine must be

in the unionized state. Many studies have reported that at physiological pH, 31% of nicotine is the free base species. Therefore, at pH 7.0, it would be expected that an even higher degree of ionization would occur, thus reducing the total amount of free base available to less than 30% (Pogocki et al., 2007). This represents a difference of greater than 20% nonionized, biologically available nicotine between pH 7.0 and 8.0. Zebrafish larvae treated at pH 8.0 had a mean of 572.4 mm travelled during acute exposure to nicotine, while larvae treated at pH 7.0 on average only travelled 294.2 mm, almost half the distance travelled by the pH 8.0 treated group. These findings of increasing locomotor activity with increasing nicotine concentration is supported by a previous study from Petzold, et al, investigating the effect of nicotine concentration on zebrafish larvae locomotion. They demonstrated that increasing concentrations of nicotine from 2.5 μM to 50 μM resulted in increases in movement (Petzold et al., 2009). Therefore, it is likely that hyperlocomotion of larvae treated at pH 8.0 are the result of increased nicotine availability (effectively increasing brain concentration of nicotine) by altering the amount of unionized nicotine in solution.

Morphine and *d*-amphetamine have similar chemical properties to nicotine. In aqueous solution, morphine can be found in three forms: two ionized forms (one with a charge on the nitrogen atom and the other lacking a proton on the phenolic hydroxyl group at carbon 3) and an uncharged, free base (Stevens & Balahura, 2007). *D*-amphetamine is only found in two forms: an ionized cation and the free base (Pubchem). Morphine, like nicotine, has two equilibrium constants (pK_a) of 8.00 and 9.75, whilst *d*-amphetamine has one of 9.9 at 25 °C (Heal et al., 2013). Due to the weakly basic properties of both drugs, like nicotine, there is consequently more of the drug present in the lipid soluble, free base form when in more alkali pH conditions, thus increasing absorption and brain concentration of drug (Dowd, 2017). Acute exposure to both morphine and *d*-amphetamine, had no pH-induced differences in motor activity. However, the effect of altered pH condition became evident after prolonged exposure (4H of treatment), which caused significant differences in the distances travelled in the drug treated groups at pH 7.0 compared to pH 8.0. Morphine treated larvae behaved similarly to nicotine treated larvae, both groups demonstrating hyperlocomotion in pH 8.0 compared to pH 7.0. Almost identical

pK_a values of nicotine (8.02) and morphine (8.00) may be a principle factor in the similar behavioural responses of increased locomotion with increasing brain concentrations of available drug (Barlow & Hamilton, 1962; Stevens & Balahura, 2007; Tomar, Scott & Henningfield, Jack, 1997).

The percentage of the ionized versus nonionized form depends on the number of pH units above or below the pK_a (Bhagavan, 2002). As morphine and nicotine have pK_a values of ~8.0 (±0.2), the degree of ionization at pH 7.0 would be expected to be similar, ~20% lower than at pH 8.0. Therefore, the increase in bioavailability of morphine would be expected to cause a similar response to nicotine based on the same degree of ionization at their respective pHs. *D*-amphetamine, however, caused the opposite effect in the prolonged treated group, resulting in hypolocomotion of larvae treated at pH 8.0 compared to pH 7.0. Previous studies of zebrafish larvae exposed to *d*-amphetamine reported an "inverted U" dose-response in locomotor activity dependent on drug concentration (Irons et al., 2010). Irons, et al, reported that low doses, up to 2.2 µM caused hyperlocomotion, whilst doses from 6.6 µM began to cause increased hypolocomotion, with the maximal dose tested (20 µM) having the greatest effect on hypoactivity, similarly found here with a dose of 22 µM (Irons et al., 2010). Thus, in line with this study, we found that the pH with the highest level of *d*-amphetamine in the free base form, pH 8.0, caused the greatest reduction in distance moved in 7 dpf larvae.

Differences in the onset of pH-dependent locomotor changes found between amphetamine and morphine compared to nicotine, could be the result of relative absorption times. Oral administration of nicotine has been reported to have an almost immediate effect, whilst previous studies using amphetamine have reported that peak responses from oral administration was 1-3H post exposure (Schepers et al., 2003). It is therefore possible, that the lack of effect seen in the acute dose of morphine and *d*-amphetamine was a result of incomplete or limited absorption at that stage, thus only after sufficient exposure time is there an effect of the drug on behavioural output. These three drugs each have similar chemical compositions which include an alkaloid amine group which is deprotonated forming the free base (nonionized) species (Völgyi et al., 2010). The weak alkalinity and the similar pK_a values result in drugs that are readily absorbed under more basic conditions (Bhagavan, 2002).

The only drug tested that did not follow this trend of pH-dependent locomotor activity was caffeine. Although, like the other test drugs, caffeine also has an alkaloid amine group and is weakly basic, it has two pK_a values of 0.7 and 14.0. Subsequently, caffeine is found in a neutral state at pH 5-9 (National Center for Biotechnology Information, 2020; Švorc, 2013). Consequently, as the test environments both fall within this range, there is no difference in the percentage of ionized/nonionized caffeine in solution, thus behavioural changes as a result of exposure to 200 µM caffeine, would be expected to affect larvae in both pH groups equally. Therefore, as expected, there were no detectable changes in locomotor activity between pH groups treated with caffeine after acute or prolonged exposure. Previous studies investigating the effect of different concentrations of caffeine on locomotion have also reported no difference between doses (Gutiérrez et al., 2020).

To our knowledge this is the first systematic investigation of the effects of varying pH conditions on the biological activity of drugs, using locomotion as a behavioural endpoint. The aim of this study was to highlight the importance of reporting this environmental condition, demonstrating that changes of a single pH unit can be sufficient to influence locomotor responses to a fixed concentration of drug. We appreciate that the controlling factor of pH conditions in aquatic facilities is the water source used to feed the system. Changing this system is impractical and, in many cases, can be cost prohibitive. We are therefore not suggesting that facilities attempted to alter husbandry conditions to fit within the range recommended by (Aleström et al., 2019). Zebrafish can be found living under a broad range of conditions in the wild, and as part of the literature review, we found no study that conducted work outside of the natural range that zebrafish have been found to live in. Although it has not been systematically assessed how long-term exposure to varying pH conditions affects behaviour in a laboratory setting, it is our belief that as long as fish are kept within the natural environmental range this should be acceptable. However, future investigations would be of great use as a resource to the zebrafish community. Based on our findings here, we suggest carrying out drug treatments at a similar pH to the housing conditions to prevent any shock responses from rapidly changing environmental conditions, which could require additional habituation periods or cause behavioural artefacts. Instead,

we propose that when carrying out drug treatments the pH value is taken into consideration as part of the experimental design. To prevent the addition of drug induced fluctuations to test conditions, we recommend either neutralising or matching drug solutions to aquarium water pH. Reporting of pH conditions and awareness of pKa values of test drugs when designing experiments and drawing conclusions will greatly aid in improving replicability between laboratories and provide a potential mechanism for interlab differences when testing the same drug at what appears to be the same concentration. Our understanding of how environmental conditions can influence experimental design, particularly in light of toxicology studies, drug discovery and drug testing using aquatic organisms is in its infancy. Future work investigating the effects of altered environmental conditions in the presence of a drug on behaviour and bioavailability and how drug x pH interactions apply to adult zebrafish behaviour would provide invaluable information for those testing zebrafish with drugs or toxins. Understanding how environmental conditions can influence experiments may prove a valuable tool in optimising zebrafish as a model organism.

2.6 Conclusion

With the increasing use of high-throughput behavioural screening of zebrafish larvae for assessing pharmacological agents, the need to improve inter-lab reproducibility is of critical importance. This study demonstrates that four drugs from the same family of organic bases can all exert different effects on locomotor responses by altering environmental conditions by a single pH unit. We have reported that even small changes can cause significant effects and could result in substantial differences in the way larvae, and potentially adult zebrafish respond to drug exposure. It is currently not mandatory to report the pH of aquarium water or of drug treated water in which fish are immersed. We demonstrated that from a sample of studies less than 35% reported pH. We therefore propose that the zebrafish community start to report pH values of drug conditions and general husbandry so that results can be more accurately replicated and compared between studies.

Chapter 3

This chapter represents the first use of the Y-maze as a test of cognitive performance, utilising the more complex tetragram configuration of pattern examination. Here we describe the first evidence that the Y-maze could detect subtle differences in cognitive performance, demonstrating a reduction in the use of the alternation strategy. The findings from this chapter were the instigation of further investigation of the function of the Y-maze as a test of WM and lead to characterisation of the FMP Y-maze described in Part 1, Chapter 3. Additionally, this chapter demonstrates the far-reaching effects that even low concentrations of alcohol can have on long term cognitive function. Exposure during the early stages of development to low concentrations of alcohol have been demonstrated to persist into adulthood and have been shown here to be replicable in zebrafish and highlights the acute impact that drugs of abuse have in influencing cognitive trajectories.

Moderate developmental alcohol exposure reduces repetitive alternation in a zebrafish model of fetal alcohol spectrum disorders

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3.1 Abstract

The damaging effects of alcohol on a developing fetus are well known and cause a range of conditions known as fetal alcohol spectrum disorder (FASD). High levels of alcohol exposure lead to physical deformity and severe cognitive deficits, but more moderate exposure leads to a range of subtle cognitive effects such as reduced social behaviour, higher propensity to develop addictions, and reduced spatial WM. Previous studies have demonstrated that following exposure to relatively low levels of ethanol during early brain development (equivalent in humans to moderate exposure) zebrafish display a range of social and behavioural differences. Here, our aim was to test the hypothesis that moderate developmental ethanol exposure would affect aspects of learning and memory in zebrafish. In order to do this, we exposed zebrafish embryos to 20 mM [0.12% v/v] ethanol from 2 to 9 dpf to model the effects of moderate prenatal ethanol (MPE) exposure. At 3 months old, adult fish were tested for appetitive and aversive learning, and for spatial alternation in a novel unconditioned y-maze protocol. We found that MPE did not affect appetitive or aversive learning, but exposed-fish showed a robust reduction in alternations in the y-maze when compared to age matched controls. This study confirms that moderate levels of ethanol exposure to developing embryos have subtle effects on spatial WM in adulthood. Our data thus suggest that zebrafish may be a promising model system for studying the effects of alcohol on learning and decision-making, but also for developing treatments and interventions to reduce the negative effects of prenatal alcohol.

3.2 Introduction

Consumption of alcohol by women during pregnancy can result in a range of physical and behavioural abnormalities in the fetus, symptoms which are collectively known as fetal alcohol spectrum disorders (FASDs) (Dörrie et al., 2014; van Wieringen et al., 2010). The most severe and easily diagnosed disorder is fetal alcohol syndrome (FAS), which is characterised by craniofacial malformations, central nervous system dysfunction, growth retardation and reduced intellectual abilities (Archibaldma et al., 2001; Mattson, 1998). Although FAS is an extreme case caused by high levels of chronic alcohol abuse, lower levels of alcohol intake have also been shown to cause a range of milder, less obvious symptoms including deficits in social behaviour (Fernandes & Gerlai, 2009), decision-making and planning (Berman & Hannigan, 2000; Sood et al., 2001) and an increased susceptibility to substance abuse in later life, even following adoption (i.e., controlling for environmental effects (Cadoret et al., 1995)).

Though heavy chronic abuse of alcohol by a pregnant woman leads to obvious symptoms in the child, behavioural symptoms of milder cases of FASD are rarely accompanied by physical deformities and are thus problematic to diagnose (Astley & Clarren, 2000). As a result, the number of children affected by milder forms of FASDs is likely to be much higher than those reported (May et al., 2014; Stoler & Holmes, 1999). In the absence of physical symptoms, details of any alcohol consumption must derive from self-report and is open to response biases (Jacobson et al., 1994). Thus, to better understand the effects of amount, frequency and timing of exposure of the embryo to alcohol, animal models have been used to bridge the gap (Patten et al., 2014). Traditionally most animal models of FASDs have been carried out in rodents. However, recently zebrafish have come to light as an alternative model for neurobehavioral research, striking a balance between similarities with human and rodent models, complex behavioural interactions, ease of genetic manipulation, low cost of maintenance and high throughput (Kalueff, 2017; Kalueff et al., 2014; Kalueff & Cachat, 2011).

In rodents, the effects of moderate prenatal ethanol exposure on learning have been mixed and unclear, with some conflicting reports of effects on some aspects of learning (Abel, 1979; Carvan et al., 2004; Patten et al., 2014). This lack of consistency may be due to the complexities associated with rodent

models of prenatal exposure, such as dosing regimen (injection vs gavage vs voluntary drinking), maternal effects (i.e. during gestation) and effects of rearing (e.g., cross-fostering vs maternal rearing (Marquardt & Brigman, 2016; Valenzuela et al., 2012). It is critical, therefore, to get a more developed understanding on the effects of moderate exposure to ethanol during early brain development, and zebrafish may offer a useful complementary model organism in which to achieve this.

Since the pioneering work from the Gerlai (Fernandes & Gerlai, 2009) and Carvan III (Carvan et al., 2004) groups, zebrafish have been proving to be excellent models for examining the effects of low-to-moderate concentrations of ethanol exposure on the developing embryo on behavioural endpoints. For example, previous work has shown that zebrafish exposed to moderate developmental alcohol exposure display alterations in adulthood of social and anxiety behaviour, an increased propensity to develop habits, and this corresponded to changes in mRNA expression of genes typically associated with the reward pathway, including dopamine, serotonin, μ-opioid and nicotinic acetylcholine receptors (Parker et al., 2016; Parker, Annan, et al., 2014). Despite some evidence that exposed embryos show reductions in ability to learn a spatial two-choice guessing task (Carvan et al., 2004) no studies have carried out a full assessment of the effects of moderate exposure to ethanol during early brain development on different aspects of learning and memory. The aim of this paper was therefore to characterize aspects of learning and memory in adult zebrafish that have been exposed to moderate levels of ethanol during early brain development. We approached our aim by examining appetitive and aversive learning, and repetitive alternation in a novel unconditioned search protocol using a Y-maze. Y-maze tests are widely used to measure exploratory behavior in rodents (Dember & Fowler, 1959; Hughes, 2004; Roberts et al., 1962) and spatial memory in zebrafish (Cognato et al., 2012).

3.3 Materials and Methods

Subjects and ethanol treatment

Embryos (AB wild-type strain) were collected from multiple individual pairings, sorted and cleaned, and placed at random (fish from each individual pairing mixed into final groups) in groups of ~40/petri dish in a translucent incubator (28°C) on a 14/10-hour light/dark cycle. The concentration of ethanol used was based on our previous research, and the ethanol treatment protocol was as previously described (Parker et al., 2016;Parker et al., 2014). Briefly, at 48 hours post-fertilization, embryos were visually inspected and sorted to ensure all were at the same developmental stage (long-pec phase), then transferred into multiple replicates (5/concentration) of either 20 mM (0.12 percent [v/v] ethanol in aquarium water (ethanol-treatment), or to fresh aquarium water with no alcohol (control). Our previous work, and that of others, has shown that 20 mM ethanol gives a final alcohol concentration of ~0.04 g/dl blood alcohol [BAC]) (Fernandes et al., 2014; Parker et al., 2014; Parker & Brennan, 2012). The reason for choosing 48 h to start treatment, is that by this stage all embryos have emerged from the chorion, so we can be sure the concentration of ethanol getting into the embryos is uniform. In addition, 48 hpf represents the long-pec phase of development, when the main catecholaminergic neural development takes place (Guo et al., 1999). At 5 dpf, embryos were transferred, still in their treatment medium, into larger containers (10 × 10 × 20 cm [depth × width × length]) containing 500 ml solution (ethanol or aquarium water) and remained in the incubator. During treatment, water/ethanol media were changed daily. Fish remained in the treatment solution for 7 days, until 9 dpf, after which all fish were transferred within their initial treatment groups into fresh aquarium water and placed on our re-circulating system, initially in groups of 40 in 1.4 L tanks (Aquaneering Inc., San Diego, CA, USA). Juvenile zebrafish (30 dpf) moved to groups of ~20 in 2.8 L tanks on the re-circulating system, on a 14/10-hour light/dark cycle, at ~28.5°C, pH 8.4 ± 0.5. Fish were tested on behavioral procedures at 3 months of age. Fish were fed a mixture of live brine shrimp and flake food 3 times/day (once/day at weekend). No fish was used for multiple protocols, and following the experiment, all ethanol-exposed fish were euthanized (Aqua-Sed™, Vetark, Winchester, UK). We used a mixture of male and female fish for all behavioral testing.

Previous research with zebrafish has not revealed sex effects for developmental alcohol exposure, and sex was not evaluated as a variable in this study. Finally, there were no differences in mortality or in gross morphology in any of the groups, although specific data are not reported here.

Ethical Statement

All experiments were carried out following scrutiny by the University of Portsmouth Animal Welfare and Ethical Review Board, and under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: P9D87106F].

Randomization and blinding

All experiments were carried out under the ARRIVE guidelines (Kilkenny C, Browne WJ, Cuthill IC, Emerson M, 2010). First, all embryos were randomly allocated to treatment from multiple pair-breeding's. During treatment, experimental staff carried out ethanol treatment (see above) but technical staff were not aware of treatment allocation (blinded). This was achieved by putting the name of the experiment on treatment dishes and an individual dish identifier, but not indicating which level of treatment on the dish. This protocol was continued throughout development (i.e., when fish were on the housing rack). When testing was carried out, when fish were individually housed (for the appetitive learning protocol), they were numbered, but the treatment group was not known by either the experimenter or the technical staff. This was achieved by creating an excel sheet with identities in a hidden column. Identity was not revealed until data had been examined for outliers and analyses were carried out. For protocols where the fish came from a group (aversive learning and y-maze), fish were taken from housing tanks, but the treatment level was not revealed until after data had been examined for outliers and was ready for final analysis. Finally, sample sizes for all experiments were determined by initial pilot studies or previous research (details in sections below).

Materials

Figure 1.

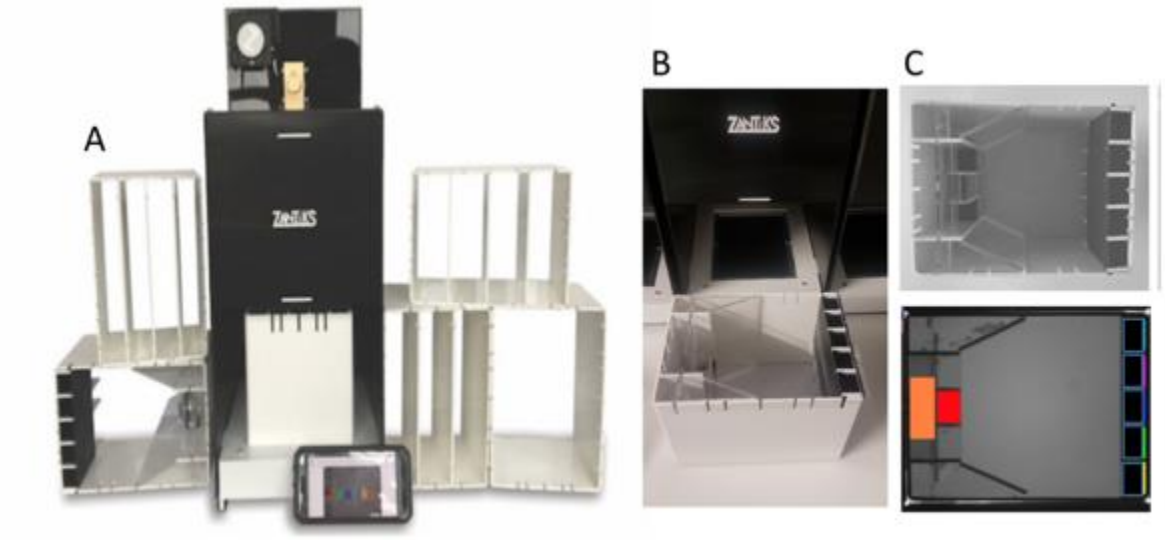

Figure 1. (**A**) Zantiks AD testing system used for behavioural testing of adult zebrafish. This a completely automated system with built in computer for image projection, camera for live imaging and feeder mechanism. This allows for minimum experimenter disturbance during trials. (**B**) Behavioural tanks for measuring appetitive learning. (**C**) (**top**) 5-choice inserts, these can be fitted into each tank and were used in the appetitive learning task. (**bottom**) shows the trigger areas for fish during the 5-choice task. The initiator light (red*) comes on first, fish have to swim into the light to initiate the trial. Following initiation, a food reinforcer (ZM300 zebrafish food) was delivered into the feeding zone (orange*). When the fish passes through the feeding zone the light is turned off and the initiator light turns back on, ready for the next trial. Tanks are filled with 3 L of aquarium water during testing.

*In trial lights are all white, colours have been added for the benefit of the diagram.

Behavioral testing of adults was carried out using the Zantiks (Zantiks Ltd., Cambridge, UK) AD system (https://www.zantiks.com/products/zantiks-ad), a commercially available, fully integrated behavioral testing environment for adult zebrafish (Brock et al., 2017a) (**Fig 1**). All tank inserts were acrylic, with opaque sides and a transparent base. The test tanks were placed into the Zantiks AD system (**Fig. 1A, B**) one tank/time. Each Zantiks AD system was fully controlled via any mobile/web enabled device. (**Fig. 1B, C**) display the tanks used to measure appetitive learning. This Zantiks AD unit is designed to carry

out multiple learning protocols in zebrafish but in the present experiment, fish were trained to swim into an initiator zone **(Fig. 1C**, red area) in order to receive a food reinforcer (ZM300 zebrafish food) in the food delivery zone (**Fig. 1C**, orange area). Tanks were filled with 1 L water during testing. The testing environment was L20xW14 cm.

Figure 2.

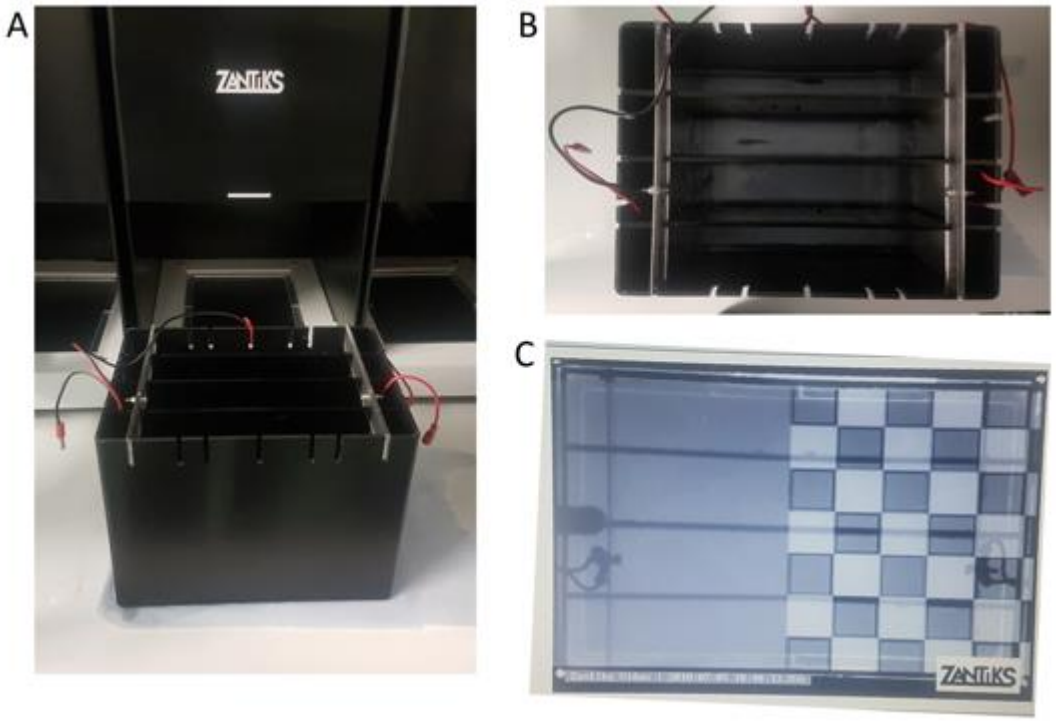

Figure 2. (A-B) Tank and shocking plates used for Pavlovian fear conditioning (aversive learning). (C) Stimuli used were based on previous work and comprised either a checker-board design ('check', black and white alternating squares) or a dark grey ('grey') background. Each tank comprised of four lanes, separated by opaque acrylic dividers. At each end of the tank was located a steel plate capable of passing a mild electric current through the tanks (9V). Tanks were filled with 1 L of water.

(**Fig. 2**) displays the tank equipment used for Pavlovian fear conditioning. The stimuli used were based on previous work (Brock et al., 2017; Valente et al., 2012), and comprised either a checker-board design ('check') (black/white alternating squares) or a dark grey ('grey') background. Each tank comprised four

lanes (L16xW32 cm), separated by opaque acrylic dividers. At each end of the tank was located a steel plate, capable of passing a mild electric current through the tank (9V). Tanks were filled with 1 L of water, with ~4 cm water at the base. Pilot studies found this amount of water to be optimal for both tracking and conditioning.

Figure 3.

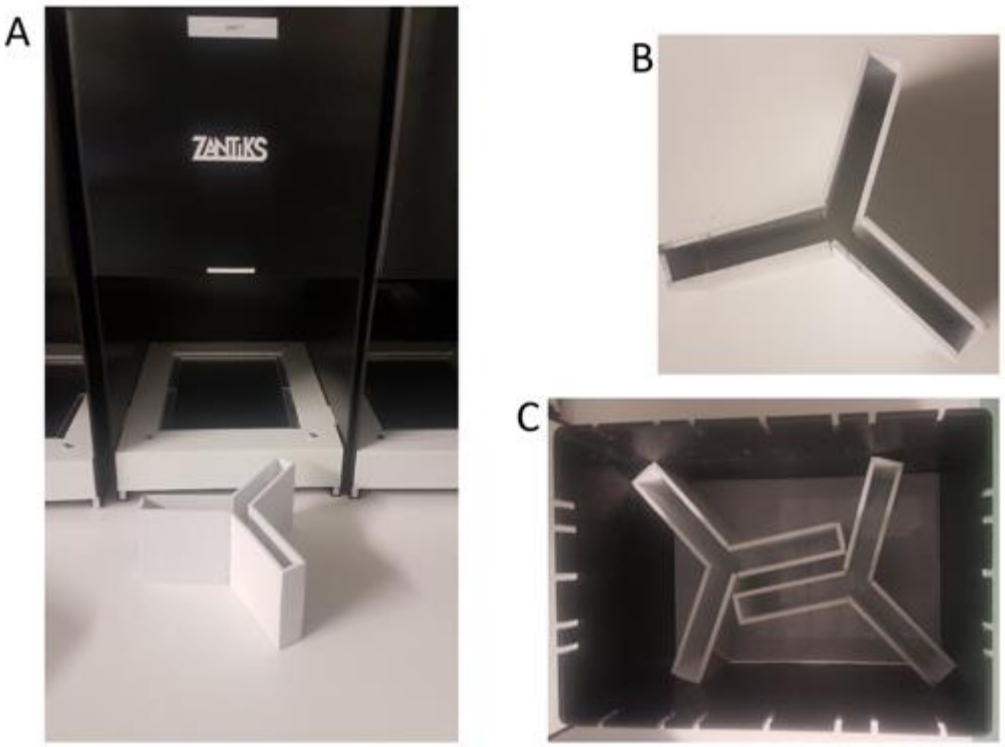

Figure 3. (A-B) FMP Y-maze insert used to assess alternation use. (C) Tank set up for FMP Y-maze. Fish are placed into one of two removable Y-maze inserts in 3 L of aquarium water.

(**Fig. 3**) displays the tank set up for the y-maze alternation test. Fish were placed into one of two removable acrylic y-mazes (L7x W1.5 cm) in 3 L aquarium water (~40mm depth of water). Fish were filmed from above for a period of 1 h.

Procedure

Appetitive conditioning

Initially 20 fish were selected (n = 10 from each treatment) to allow for attrition. Any fish that did not respond (i.e., did not swim into stimulus areas or 'froze' and did not carry out the task) was excluded. Following attrition, a final sample of N = 16 adult (n = 8 control, n = 8 20 mM ethanol) zebrafish were tested on the appetitive conditioning protocol at 3-months of age. Final sample size was determined based on previous research examining appetitive learning in developmentally exposed fish (Fernandes et al., 2014). During appetitive conditioning training, fish were housed in pairs in 2.8 L tanks (divided breadthwise with transparent mesh dividers). The training was divided into two phases, and learning was assessed following the second phase. Initially, fish were shaped for one week to associate the noise of the feed dispenser with delivery of a small amount of ZM300 (~2 mg). In the second phase, fish were trained that swimming into an initiator area (**Fig. 1**, red zone) resulted in food delivery. They were trained on this protocol for 3 days (~30-trials/day). Following completion of training, we tested learning with a series of probe trials, during which fish were exposed to 5 trials of initiator light "on" (30-sec), or "off" (30-sec) and measured total number of entries to the initiator during light on/light off.

Pavlovian fear conditioning

N = 44 adult (n = 22 control, n = 22 20 mM ethanol) zebrafish were tested on the Pavlovian fear conditioning protocol at 3-months of age. Fear conditioning was based on a protocol developed based on published data (Valente et al., 2012); sample size was based on a power calculation of previous research (Valente et al., 2012) and following a pilot assessment of change in preference following mild shock (effect size [d] = 0.9, power = 0.8, alpha = 0.05, required sample = 22/treatment). First, fish were placed, individually, into one of four lanes in each Zantiks tank. Into each tank was placed n = 2 ethanol and n = 2 control fish (the experimenter was blinded at this stage to the treatment of the individual fish, and the position of fish from either treatment [a or b] counterbalanced between trials). Initially, fish were

habituated to the test environment for 30 mins, during which the base of the test tank was divided in half, lengthways, with the 'check' and 'grey' stimuli occupying half of the base each (**Fig 2c**), switching position every 5 min. Baseline preference was ascertained over 10 min. Following baseline preference assessment, conditioning was carried out, during which the conditioned stimulus (CS+; full screen of 'check' or 'grey', randomized between subjects) was presented for 1.5 sec, at the end of which was delivered the unconditioned stimulus (US) a brief, mild shock (9V DC, 80ms), followed by an 8.5 sec inter-trial interval (ITI), during which the non-CS (CS-) exemplar was presented at the bottom of the tank. The CS+/US was presented nine times. Following conditioning, avoidance of CS+ was ascertained by repeating the baseline, presenting both CS+ and CS- simultaneously for 1 min, and switching positions after 30 sec.

Y-maze test

N = 28 adult (n = 14 control, n = 14 20 mM ethanol) zebrafish were used for the Y-maze test, at 3 months of age. This was a novel protocol, and sample size was based on pilot data (effect size [d] = 1.2, power = 0.8, alpha = 0.05, required sample = 14/treatment). In order to characterize perseveration and alternation in zebrafish, we designed a simple Y-maze, with three identical arms. Although memory in zebrafish using a Y-maze has been previously evaluated (i.e., by blocking one arm, and exploring use of the novel arm once it is opened during training), simple unconditioned Y-maze search patterns and performance has yet to be evaluated in zebrafish, and this study represents the first reports of this protocol. There were no intra-maze cues, but extra-maze (distal) cues were visible from each maze (e.g., the walls and open side of the Zantiks equipment), providing egocentric cues and allowing the fish to orient within the apparatus. The experimenter was not visible to the fish at any point during the protocol. Fish were recorded in the Y-maze apparatus for up to 1 h, or until they performed 100 arm-entries, whichever the sooner (here, all fish performed 100 entries in the allowed h). This allowed for 97 overlapping series of four choices (tetragrams), of which there were a total of 16 manifestations possible. Two tetragrams (RRRR and LLLL) represented pure repetitions, and two (RLRL, LRLR) pure alternations.

A completely random search strategy would be to choose every potential tetragram equally (97/16 = 6-times). However, perseverant response sequences may encompass above-average use of alternations or of repetitions. Previous research using a T-maze in which each arm was baited with an equal probability reinforcement, has demonstrated that mice tend to show generally higher levels of alternations between arms (LRLR, RLRL) than other alternatives (Gross et al., 2011).

Data analysis

Data were analyzed in IBM SPSS® Statistics for Macintosh (Version 24). Appetitive conditioning was measured by examining both acquisition data, and a series of probe trials to test learning. Acquisition data was compared between control and 20 mM ethanol treated fish using a general linear mixed model, with fixed factors as treatment (2-levels: control, 20 mM ethanol) and day (3-levels), and their interaction, and fish ID nested in tank as the random effect (to account for non—independence of replicates). Denominator degrees of freedom were estimated using the Satterthwaite approximation. Probe trials comprised count data and were fitted to a generalized linear mixed effects model (Poisson distribution, log link function), with fixed effects as treatment (control, 20 mM ethanol) and light status (light on, light off) and the random effect as fish ID nested in tank (to account for non-independence of replicates). Denominator degrees of freedom were estimated using the Satterthwaite approximation. Fear conditioning was assessed by comparing change in preference for a stimulus following conditioning with 9 x 9V shocks. A two-way, mixed design analysis of variance (ANOVA) was applied, with proportion of CS+/CS- preference as the dependent variable, ethanol treatment as the between-subjects factor (2-levels; control, 20 mM ethanol) and conditioning stage as the within-subjects factor (2-levels; pre- and post-conditioning). 'Tank' was added as a covariate into the initial model, but removed from the final model owing to lack of effect ($F < 1$). Finally, in order to examine perseveration in the Y-maze, we carried out two analyses. In the first, we considered whether there were differences in the frequency of each of the 16 tetragrams as a function of treatment. We fitted frequency data to a generalized linear mixed effects model (Poisson distribution, log link), with treatment (control, 20 mM ethanol) and tetragram

(16-levels) as fixed factors, and ID as the random effect (to account for non-independence of replicates). Denominator degrees of freedom were estimated using the Satterthwaite approximation. Next, to assess whether there were any effects of developmental ethanol on frequency of pure alternations (LRLR + RLRL) or pure repetitions (RRRR + LLLL). This was assessed by fitting generalized linear models (Poisson distribution, log link function) to alternation and repetition frequency data. In both models, fixed factor for each was ethanol treatment (2-levels: control, 20 mM ethanol). Again, 'Tank' was added as a covariate into the initial model, but removed from the final model owing to lack of effect ($F < 1$).

3.4 Results

Moderate developmental ethanol exposure does not affect appetitive learning in zebrafish

Acquisition of learning data for the 20 mM ethanol treated and control fish (**Fig 4A**). A GLMM revealed a significant main effect of day, F (2,28) = 9.15, $P < .01$ (Day 1 vs Day 2, $P = .9$; Day 1 < Day 3, $P = .001$; Day 2 < Day 3, $P = .001$), but no significant effect of treatment, F (1,14) = 1.17, $P = .3$, or day × treatment interaction, $F < 1$. Figure 4b displays the probe trial following appetitive conditioning, in which fish were presented with the stimulus light 5-times (10 sec), interspersed with non-light presentations (10 sec). A GLMM (Poisson distribution, log link function) was fitted to the data, with number of entries to the stimulus zone as the response variable, treatment and lights-on/off as the fixed factors, and fish ID nested in tank as the random effect. There was a main effect of lights on/off, F (1,18) = 9.41, $P < 0.01$ (Lights ON > Lights OFF), but not effect of ethanol treatment or lights on/off × treatment interaction, Fs < 1.

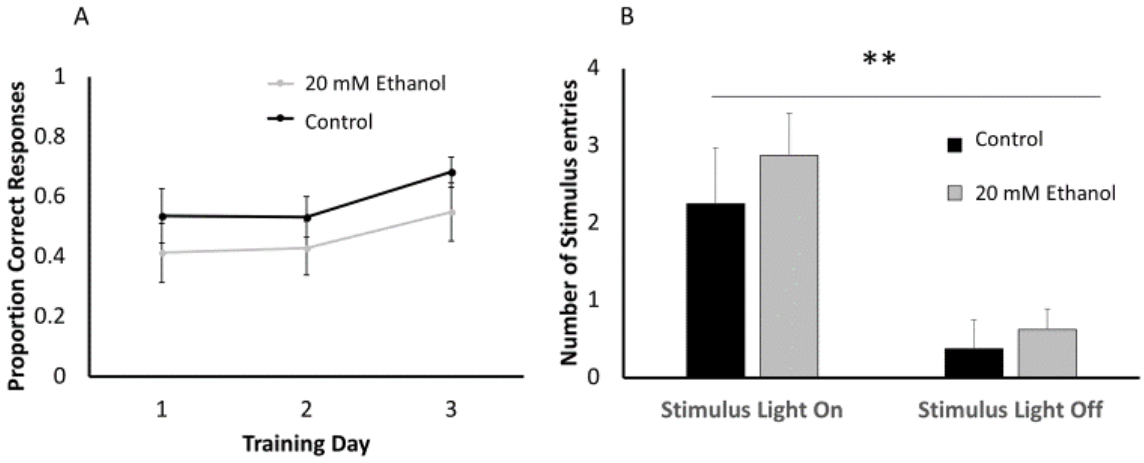

Figure 4. (A) Training data (appetitive condition) for 20 mM treated fish compared to control fish. (B) Probe trial data (appetitive conditioning) for 20 mM treated fish compared to controls.

Moderate developmental ethanol exposure does not affect fear conditioning in zebrafish

Mean preference for the conditioned stimulus following Pavlovian fear conditioning, during which fish were given 9 CS + US (shock) pairings with either a checker-board or grey image (**Fig. 5**). A Two-way ANOVA revealed a significant main effect of conditioning, F (1,42) = 62.79, P < .001, with fish showing a robust reduction in preference for the conditioned stimulus. There was no main effect of ethanol treatment (F < 1) nor conditioning*ethanol treatment interaction (F < 1).

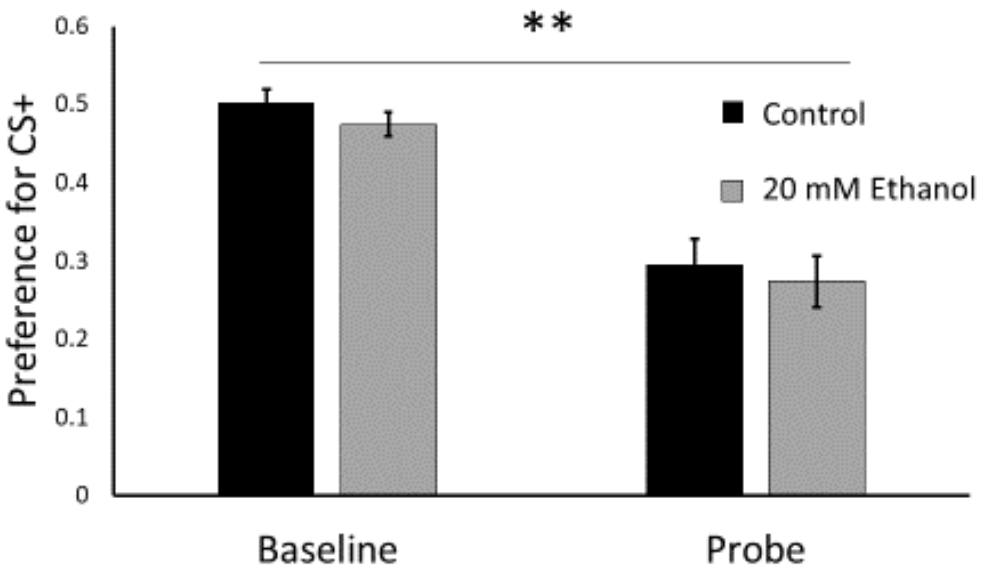

Figure 5. Preference for conditioned stimulus (CS+) prior to conditioning (baseline) and following pairing with 9 x 9V shocks (probe). Bars are mean, error bars are mean ± SEM ** $p < 0.001$.

Moderate development ethanol exposure reduces alternations in a Y-maze

Total choices for each tetragram during a 100-trial search period for zebrafish in the FMP Y-maze (**Fig. 6A**). A GLMM, with Poisson distribution specified and a log link function, revealed a significant main effect for 'tetragram', F (15, 416) = 8.74, $P < .001$, characterized as significant increases in frequency of alternations (LRLR, RLRL; $P < .001$). There were no main effects of ethanol treatment ($F < 1$), nor tetragram*ethanol treatment interaction ($F < 1$). (**Fig. 6B**) displays the frequency of pure alternations, and (**Fig 6C**) pure repetitions. GLMM (Poisson regression) revealed a significant effect of ethanol treatment on alternations (Figure 6b; χ^2 [df = 1] = 3.98, P =.046), but not on repetitions (Figure 4c; χ^2 [df = 1] = .3, P = .58).

Figure 6.

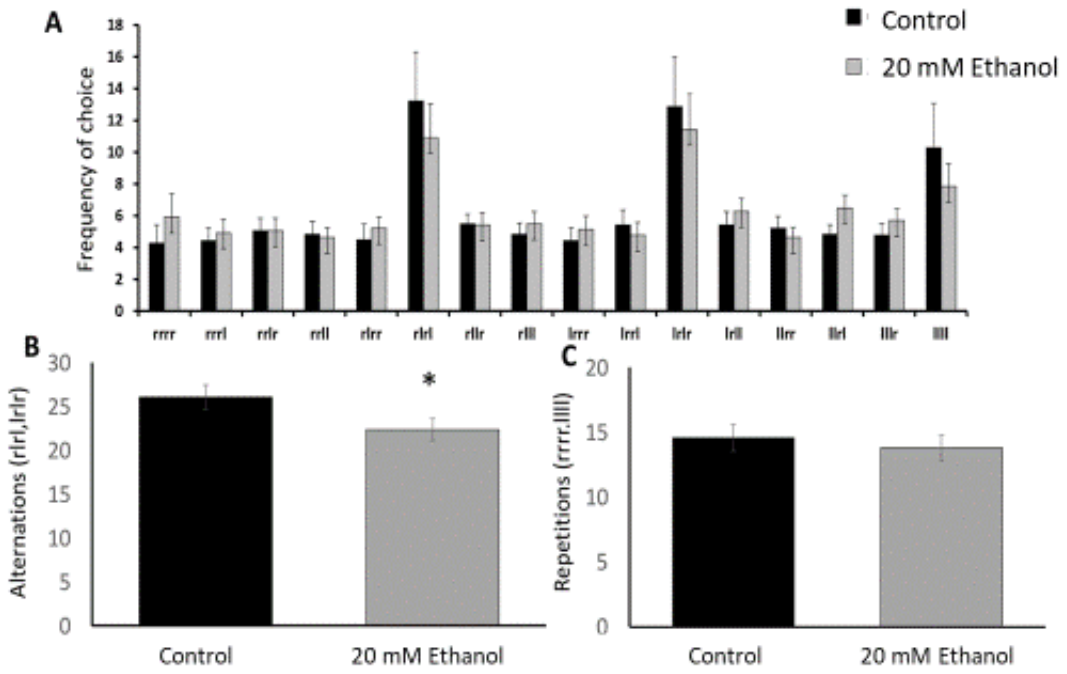

Figure 5. (**A**) Frequency distribution of tetragrams during unconditioned search in the Y-maze (Ethanol treated, n=14; control, n=14) comprising of mean (±SEM) choices during 100 trials. (**B**) Mean (±SEM) total pure alternations for control compared to 20 mM ethanol treatment. (**C**) Mean (±SEM) total pure repetitions for control and 20 mM ethanol treated fish. * $p < 0.05$.

3.5 Discussion

In this study we combined operant and Pavlovian learning tasks with a spatial memory task to test whether moderate levels of ethanol exposure during early brain development in zebrafish affected aspects of learning and WM. We found that adults developmentally treated with 20 mM ethanol (the equivalent of 2-3 drinks in a single sitting) performed equally well as control groups in Pavlovian and operant learning tasks, but in a spatial memory task had a decreased frequency of pure alternations (LRLR, RLRL) in a Y-maze. These observations indicate that MPE can cause disruption in specific aspects of learning that persist into adulthood even when executive function is not obviously affected. This highlights the difficulty in diagnosing milder forms of FASD, but further supports the growing body of

evidence implementing low to moderate levels of alcohol exposure in cognitive and behavioral abnormalities.

Appetitive and aversive learning tasks

Operant learning tasks are used to measure executive functions involved in learning, planning, motivation and memory (Valente et al., 2012), higher functions that are often impaired in children with FASD (Green et al., 2009; Rasmussen, 2005). Here we found that exposure of larvae to 20 mM ethanol [0.12% v/v] from 2-9 dpf had no detectable differences in executive function in adulthood when tested in appetitive or aversive learning tasks. This was irrespective of whether the task was under a positive (food reward) or negative (shock) stimulus control. These findings are in contrast to models of high ethanol exposure which have reported marked deficits in behavioral tasks of exposed groups (Abel, 1979; Brown et al., 2008; Fernandes et al., 2014). Although these findings may seem at odds with our own results, this disparity was not unexpected. Obvious deficits in executive function are found in humans with severe forms of FASD which are associated with periods of binge drinking during pregnancy (Bailey et al., 2004). The effects of high concentrations of ethanol exposure have been replicated in a number of primate (The effects of prenatal alcohol exposure on behavior: Rodent and primate studies, 2011), rodent (Riley et al., 1979; Wagner et al., 2014) and zebrafish models (Carvan et al., 2004; Fernandes & Gerlai, 2009), many showing a concentration-dependent effect on learning (Carvan et al., 2004). Children also show a concentration-dependent effect of alcohol exposure resulting in extreme variability between mild and severe cases (Sood et al., 2001). Those exposed to moderate intrauterine alcohol levels may not display obvious deficits in executive function, but have subtler effects that may not be evident until later in life. A study by Carvan III, et al (2004) looked at learning and memory in low to high concentrations of ethanol exposure in zebrafish and found a concentration-dependent effect on learning and memory when embryos were exposed to low concentrations ranging from 10 mM-30 mM ethanol (Carvan et al., 2004). The disparity between our study showing no learning deficits at 20 mM could be explained by differences in the timing of concentrations. In their study dosing

began at 4 hpf compared to 48 hpf in this study. Studies in other species have also found discrepancies in concentration amount and effects on learning and memory tasks, a key difference between them being the timing and duration of concentration (Lovely et al., 2014;Lovely et al., 2016). At 48 hpf zebrafish larvae are almost completely developed, the brain has developed into 5 distinct lobes, the circulatory system has developed and the heart is beating, fins develop and there is sensitivity to the environment and uncoordinated movements (Kimmel et al., 1995). This is the equivalent to late stages of development in the human fetus. We suspect that full brain development at the time of dosing may be a reason for executive functions still being intact and showing no obvious signs of dysfunction.

Our findings highlight two major influences on severity and variability in FASD. Firstly, from findings of higher-concentration animal models (Abel, 1979; Carvan et al., 2004; Fernandes et al., 2014), we can draw the conclusion that concentrations higher than 0.12% v/v of ethanol exposure [up to 3% v/v] are required to cause functional deficits in executive function, specifically in goal-orientated reward tasks. Secondly, dosage is not the only key factor causing developmental defects, timing and duration of exposure are also crucial and can cause marked differences in cognitive and behavioral abilities even at lower concentrations of ethanol exposure (Carvan et al., 2004; Lovely et al., 2014). This ability to mirror the variability seen in human forms of FASD further strengthens the use of zebrafish as a model of complex neurological disorders.

Y-maze Spontaneous Alternation Task

The Y-maze has been established as a reliable protocol for testing spatial WM in zebrafish (Cognato et al., 2012). For the first time, here we use unconditioned free-search of the Y-maze as a behavioral test for fish prenatally exposed to moderate levels of ethanol. We observed that all fish had a tendency for highly alternating sequences (pure alternations, LRLR or RLRL). However, 20 mM ethanol treated fish used pure alternation as a search strategy significantly less than their untreated counterparts. Using tetragram configurations, similar to those used by (Gross et al., 2011), allowed us to compare random

or specific search strategies employed by fish when swimming freely in a Y-maze. We would expect that in a reward-absent maze, with no highly salient intra or extra-maze cues, that fish would choose each search configuration equally (i.e. in 100 trials each tetragram should be chosen n=6 times). However, for both treated and nontreated groups this was not the case. Our findings that all subjects have an increased preference for pure alternations was previously seen by (Gross et al., 2011) with rats and (Neuringer, 1992) with pigeons. Both studies similarly reinforced each option equally (here this was done by an absence of reward, opposed to associating each arm with a reward an equal number of times). This observation could be explained by the '*law of least mental work*', an adaptation from Hull's '*law of less work*' (Hull, 1943), which suggests that in a non-reinforced task subjects will opt for the behavior that is least cognitively demanding. Simplified strategies, like repeating the last action, may be favored in non-discrete behavioral tasks, possibly identifying repetition of pure alternations as the least cognitively demanding strategy. The random incorporation of the other 14 possible search strategies could be influenced by information seeking or a desire for change or novelty (Kool et al., 2010), but in the absence of any new information being presented, the organism goes back to the least mentally demanding search pattern.

The reduction of pure alternations in ethanol treated fish is contradictory to what we would expect. Several animal models have used a form of spontaneous alternation to measure WM, whether it is in a T-maze (Deacon, Nicholas, et al., 2006; Gross et al., 2011), Y-maze (Dobson et al., 2012) or plus maze (Sison & Gerlai, 2010), or the use of two-choice guessing tasks in humans (Frith & Done, 1983). Over a range of conditions frequency of pure alternations has normally been reported as higher in the treated group than in the control groups (Frith & Done, 1983; Gross et al., 2011). However, the mechanisms affected by moderate ethanol exposure that result in the deficit that we see here, are not completely clear. It is possible that these differences are due to damage to the hippocampus, effecting WM. Rats exposed to ethanol within the first 2 weeks of neonatal life, the rodent equivalent of the third trimester in humans, have been reported to have functional impairments of hippocampal neurons, specifically in the CA3 region (Baculis et al., 2015; Caldwell et al., 2015; West et al., 1984). This could

potentially explain deficits seen here, as rodents with hippocampal lesions also perform poorly in spatial memory tasks, specifically with familiarization (Roberts et al., 1962). However, if there were hippocampal impairments, we would expect to see some evidence of this during either of the other two operant learning tasks used. Even though these trials require extensive training and rely on associative memory to be successfully performed, they also require the hippocampus, and during the early process of learning, spatial WM, short-term memory and the ability to convert experiences into long-term memories. The combination of learning and memory tasks used and the paralleled ability of treated and control groups in the operant learning tasks thus suggest that memory may not be a fault for the changed behavioral pattern seen in the Y-maze by treated fish.

An alternative explanation is the theory of "choice hysteresis". Choice hysteresis is the tendency of animals and humans to repeat recent choices. (Bonaiuto et al., 2016) describe a virtual model in which activity decay leftover from recently activated neural circuits increased repetition biases. They also describe how depolarizing or hyperpolarizing the dorsolateral prefrontal cortex (dlPFC) could increase or suppress choice hysteresis, respectively. Children born with FASD are often reported to have deficits in the dlPFC as is established by the Wisconsin Card Sorting task (neuropsychological test for executive function which is used to determine damage to the dlPFC) (VAURIO et al., 2008). Again, this is another area in which severity and variability are in strong correlation with concentration of alcohol exposure, with high, chronic concentrations of alcohol exposure, (i.e., those born to alcoholic mothers) performed the poorest on this task compared to those exposed to lower concentrations of alcohol and control groups.

This is the first time that the Y-maze has been used in the context of FASD and there are evident differences in the search strategies employed by treated and nontreated groups. However, the lack of comparative studies carried out at other concentrations limits what can be inferred from this data and therefore mechanisms and meanings are limited to theoretical ideas. Although many other studies have investigated the effects of PNEE on spatial WM (Berman & Hannigan, 2000; Hamilton et al., 2014; Vicari et al., 2004; Zimmerberg et al., 1991), the lack of standardized measures of spontaneous alternation

makes it difficult to compare findings from one study to another unless they have used similar data analysis. Also, significant differences can be seen between rewarded and reward-absent tasks. From this study we can concluded that moderate PNEE does alter behavior in a spontaneous alternation task. However, at this stage we can only hypothesize about possible mechanisms responsible for this change. It is also difficult to judge how this may relate to human behavior, thus, further work would be required to draw out anything conclusive.

Limitations and future directions

There are limitations to this study. First, only one concentration and one exposure time were used. In light of differences on learning and memory seen in other behavioral studies (see review: (Patten et al., 2014)) future studies could use variations of these two factors to test if there are any behavioral changes in the tasks performed here. Most interestingly would be the effect on spontaneous alternations in the Y-maze. We predict that exposure to high concentrations of ethanol, to the extent that it causes cognitive impairment, would cause an increase in pure repetitions (LLLL ,RRRR) and pure alternations would occur at a rate equal to that of controls. Secondly, we used a mixture of male and female fish for our experiments. Many studies have found effects of sex in some species following developmental ethanol exposure (Zimmerberg et al., 1989, 1991). This may represent a limitation of the fish model in terms of translational relevance, as it appears at odds with other vertebrates in this regard. A further limitation could be that we did not measure locomotion in the study. Some have found that locomotor differences can lead to false results if not carefully controlled. However, because all fish were tested for the same period of time, and because the tests rely on movement (i.e., swimming in and out of the arms of the maze) we are not concerned that locomotion differences would lead to confounding results. Finally, our limited repertoire of behavioral tasks may result in other behavioral abnormalities being missed. Future studies should incorporate more tasks involving complex cognitive functions to fully elucidate the effect of moderate PNEE.

3.6 Conclusion

In regard to the primary aim of this study we found that MPE caused marked behavioral changes in the search strategy employed by treated fish in a Y-maze. However, the concentration and timing of exposure did not impact on executive functions required for Pavlovian operant learning tasks. Thus, we can conclude that even in the absence of physical malformations, developmental exposure to moderate levels of ethanol can cause subtle behavioral and cognitive changes that persist into adulthood.

Despite decades of investigation and a mountain of data examining the toxic and deleterious effects of alcohol on the developing fetus, the number of children born with FASD is on the rise (Mattson & Riley, 1998). Being the leading form of preventable mental retardation and having lifelong effects that can severely reduce the quality of life of those affected (Sokol et al., 2003), it is critical that we fully understand the impact that all levels of ethanol exposure can have. The effect of moderate levels of alcohol exposure are becoming even more crucial with the number of women having unplanned births and pregnancies being as high as 23% and 40% respectively (Bearak et al., 2018; Sedgh et al., 2014), worldwide. Therefore, the chance of drinking before becoming aware of being pregnant is a huge risk factor. Concentrations as low as 10 mM have been reported to affect cognitive abilities, this is the equivalent of 1-2 drinks in one sitting (Carvan et al., 2004). This is also the current UK recommend 'safe level' of consumption during pregnancy ((UK), 2008). With guidelines like these in place and a growing body of evidence suggesting detrimental effects of low and moderate levels of alcohol exposure, research in these areas is becoming even more important to help change health advice and the way society see maternal drinking.

Chapter 4

The literature is clear that the developing brain is particularly vulnerable to exposure to drugs of abuse. Modulation of developing neural networks and neurotransmitter systems can be severely impacted by prenatal exposure to legal drugs such as alcohol and nicotine. Such exposures can have long lasting implications for brain structure and function. In the previous chapter, we describe such altered functioning, even after a relatively low concentration of drug exposure, resulting in poorer cognitive performance, particularly pertaining to WM. These effects on the developing nervous system, may be heightened in sensitivity due to the lack of a fully matured homeostatic regulatory system, that is better placed to counteract such toxicological perturbations in adults. In this next chapter we investigate how legal drugs of abuse, nicotine and amphetamine, alter cognitive control. We use a sensitization model to assess the impact of chronic exposure, withdrawal and repeat exposure on cognitive performance and describe the first model of cognitive sensitization in zebrafish. We further implicate differences in stimulation of the dopaminergic and cholinergic systems in cognitive domain dependent shifts in cognitive function and impacts on behavioural output.

The Cognitive and Behavioural Effects of *D*-Amphetamine and Nicotine Sensitization in Adult Zebrafish

This chapter has been accepted for publication (formatting has been retained):

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4.1 Abstract

Background: Zebrafish are growing in use as a model for understanding drug dependence and addiction. Sensitization paradigms have been a useful tool in identifying mechanisms involved in drug-induced behavioral and neurological changes, but in zebrafish have tended to focus on locomotor, rather than cognitive, endpoints.

Methods: Here, we used a novel method, the FMP Y-Maze, which measures continuous performance through a series of repeated binary choices (L vs R), to establish a model for assessing parameters associated with psychostimulant-induced behavioral and cognitive sensitization in adult zebrafish.

Results: Repeat, intermittent exposure to *d*-Amphetamine (AMPH) for 14 days increased alternations (LRLR) in the maze, suggesting improved working memory, which was enhanced further following drug challenge after a short withdrawal period, suggesting behavioral sensitization. However, this cognitive enhancement coincided with a reduction in the use of other exploration strategies, hypolocomotion and inhibition of cognitive flexibility. Like AMPH, exposure to nicotine (NIC) increased alternations following drug challenge after chronic treatment. Repeat NIC exposure appeared to induce both cognitive and psychomotor sensitization, as evidenced by increased working memory performance

(alternations) and locomotor activity, without negatively impacting other search strategies or cognitive flexibility.

Conclusion: Chronic treatment with AMPH or NIC boosts cognitive performance in adult zebrafish. Cognitive sensitization occurred with both drugs, resulting in enhanced working memory, however, repeat AMPH exposure, following a withdrawal period, resulted in inhibited cognitive flexibility, an effect not evident with repeat NIC exposure. Cognitive and behavioral sensitization paradigms in zebrafish could serve as a useful tool for assessing cognitive states which result in cognitive enhancing or impairing effects of drugs.

4.2 Introduction

Psychostimulants, such as amphetamine (AMPH), nicotine (NIC), and cocaine, are known to increase extracellular synaptic dopamine (DA) concentrations and activate DA neurotransmission, resulting in altered behavioral and locomotor responses (Brown & Kolb, 2001; Cunningham et al., 1997; Dela Peña et al., 2015; Di Chiara & Bassareo, 2007; Niculescu et al., 2005; Volkow et al., 1999). Repeated administration of a psychostimulant drug enhances psychomotor responsiveness to the drug. This is mediated by enhancement of dopaminergic activation and modification of dendritic morphology, which can last for extended periods (Robinson & Berridge, 1993). This long-term ability of previously encountered drugs to activate DA neurotransmission and modify behavioral outputs is known as drug-induced sensitization. Sensitization to repeated, intermittent drug exposure in humans is theorised to be a critical driver in maintaining drug use and escalation from casual experimentation to craving and abuse. Further, those attempting to withdraw from their habit can relapse following long periods of abstinence (Robinson & Berridge, 2008; Vezina, 2007). The psychostimulants AMPH and NIC have been well document for modulating locomotor responses (Buenrostro-Jáuregui et al., 2016; Cunningham et al., 1997; Pisera-Fuster et al., 2019; Valjent et al., 2010), as well as cognitive altering effects in both animals and humans (Kuhn et al., 2019; Steketee & Kalivas, 2011; Vezina, 2007; Vezina & Leyton, 2009).

Expanding our understanding of the mechanisms underlying drug-induced sensitization may boost the efficiency of therapies used to treat substance abuse and reduce the probability of relapse.

Conservation of the ascending midbrain dopaminergic pathways between mammals and teleost fish, such as zebrafish, has resulted in the increasing use of zebrafish as a neurobehavioral model for assessing the effects of pharmaceutical and illicit drugs (Antunes & Biala, 2012; Barros et al., 2008; Goldsmith, 2004; Kalueff et al., 2014). Behavioral responses of zebrafish to psychostimulant drug exposure (e.g. amphetamine (Parker et al., 2012), cocaine (Lopez Patino et al., 2008) or nicotine (Suen et al., 2013)) have corroborated findings in rodents and humans , thus supporting the suitability of zebrafish as a translational model of psychostimulant abuse (Parker & Brennan, 2012; Stewart et al., 2015). There have been several studies that have demonstrated drug-induced sensitization in zebrafish (Blaser et al., 2010; Pisera-Fuster et al., 2019). Although these studies have been a useful starting point, they have yet to further our understanding of the mechanisms underpinning sensitization, and the relationship between sensitization, addiction and relapse. In addition, despite extensive investigation into psychomotor sensitization, few studies have investigated the role of cognitive sensitization (Castner & Goldman-Rakic, 2004; Muhammad & Kolb, 2011; Peleg-Raibstein et al., 2009) and none, to our knowledge, have done so in zebrafish.

Here, we aimed to establish whether young-adult zebrafish develop cognitive or psychomotor sensitization to repeated administrations of AMPH and NIC, as assessed through the impact on working memory, locomotor activity and cognitive flexibility. To do this, we used an established behavioral assay for zebrafish that has high translational relevance to humans, the FMP Y-maze (Cleal et al 2020). We assessed locomotor activity, working memory and behavioral flexibility at four time points: baseline (prior to any drug exposure), chronic exposure (14 days of drug exposure), withdrawal (2 days without drug) and finally following drug challenge (single, repeat drug exposure). Changes in cognitive performance and motor activity were determined relative to baseline, pre-drug exposure levels. To our

knowledge this is the first study to examine the cognitive effects of psychostimulant sensitization in zebrafish.

4.3 Materials and methods

Subjects and drugs

Thirty-six adult, AB wild-type, mixed sex (50:50 M:F) zebrafish (*Danio rerio*), aged 3 months old at the start of treatment, were used for this study. Sample size (N = 36 (n=12/treatment group) [50:50 M:F]) was determined by power analyses based on large effect sizes (f^2 > .3) observed in extensive previous experiments in our group using the FMP Y-maze. Housing conditions were maintained on a re-circulating system (Aquaneering Inc., San Diego, CA, USA), tank water was maintained at pH 8.4 (±0.4), temperature 25-27 °C, on a 14h:10h light/dark cycle. From free-feeding (5 days post fertilisation (dpf)) fish were fed on ZM fry food until adulthood when they were fed on a daily diet of live brine shrimp (maintained at the fish facility) and dried flake food (ZM Systems, UK) 3 times/day during the week and once/day at weekends. All fish used in this study were experimentally naïve. Ethical approval was granted by the University of Portsmouth Animal Welfare and Ethical Review Board and conducted in accordance to the Animals (Scientific Procedures) Act 1986. Drugs were obtained from Sigma-Aldrich (Dorset, UK). Drug solutions were made up in aquarium water at pH 8-9. Moderate doses of AMPH and NIC were based on previous studies which found these concentrations to have reinforcing effects on zebrafish, assessed in the conditioned placed preference (CPP) task (Brock et al., 2017; Kalueff, 2017; Kily et al., 2008; Ninkovic & Bally-Cuif, 2006; Parker et al., 2013). All experiments were carried out in line with ARRIVE guidelines (NC3Rs, UK).

FMP Y-maze

The FMP Y-maze has been previously shown to be a useful method for assessing working memory, behavioral flexibility and locomotion, and has been described in detail elsewhere (Cleal, et al., 2020;

Cleal & Parker, 2018; Fontana et al., 2019). Briefly, fish were placed individually in white acrylic maze inserts (arms: L50 x W20 x D140 mm). Each arm of the maze was set at 120° angles. There were two inserts per tank, allowing two individuals to be tested simultaneously. The inserts were fitted into a tank containing 3L of aquarium water (**Figure 1**). The FMP Y-Maze comes as a standard protocol within the commercially available automated behavioral tracking unit (Zantiks AD system for adult zebrafish, Zantiks Ltd, Cambridge, UK). Fish were free to explore all arms of the maze for 1 hour (h). Entries and exits from each arm were automatically recorded and logged in an excel file for the duration of the task. Arm entry data was extrapolated to obtain a time series of left and right turns, which were subsequently divided into 16 overlapping sequences of tetragrams (four consecutive turn choices, e.g. left, left, right, right [LLRR] or right, left, right, left [RLRL]; for details of analysis of tetragrams, see Data analysis section below). Analyses of temporal dynamics of search patterns have demonstrated that zebrafish show flexibility in their behavior during the search parameters. Pharmacological analyses have demonstrated that alternations (LRLR, RLRL) are reduced by memory-blocking drugs in zebrafish (Cleal et al 2020), suggesting that alternations are linked to working memory. Locomotion was measured by the total number of arm entries.

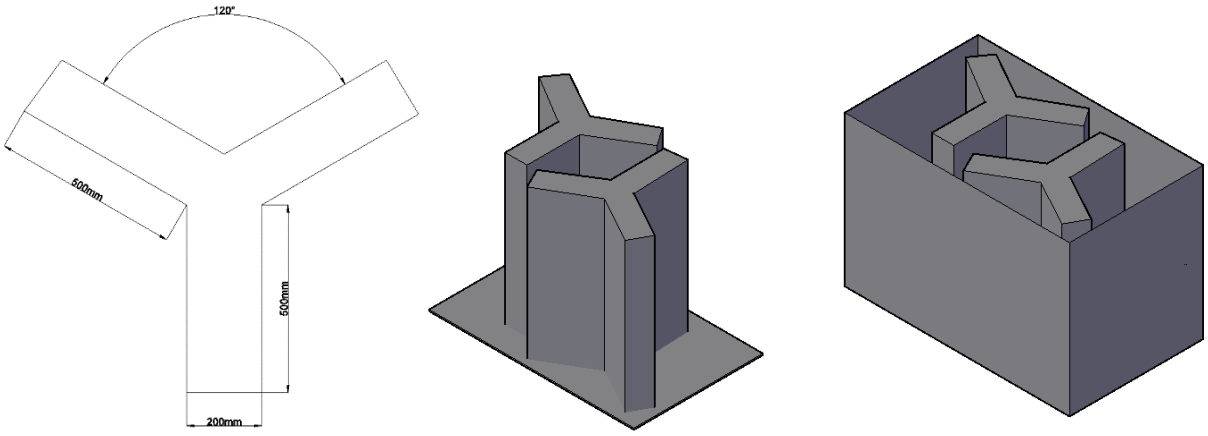

Figure 1. Schematic showing dimensions of individual Y-mazes *(left)*, two Y-maze inserts *(middle)* and two Y-mazes inserted into a watertight tank *(right)*. One zebrafish per Y-maze.

Experimental design

First, all fish were screened for baseline performance in the FMP Y-maze for 1-h of free exploration. Zebrafish were then allocated randomly into treatment groups (n = 12 AMPH [25μM]; n = 12 NIC [5μM]; n = 12 control) and pair-housed in treatment groups for identification purposes. Experimenters were blinded to treatment-group allocation. We pair-housed the fish four days prior to the start of repeated intermittent exposure to drugs in order to ensure they had habituated to the new housing system, and during this time, technical staff were blinded to treatment allocation. Acquisition of AMPH or NIC sensitization was established over three treatment phases (**Figure 2**). Phase I: each fish was given 14 consecutive 30-min daily sensitizing exposures to their respective treatment. On day 14, following their final 30-min exposure, fish were immediately placed into the FMP Y-maze for 1-h of free exploration. Phase II: zebrafish had two days without treatment wherein they remained in their home tanks, in pair-housed conditions. After 48-h of withdrawal zebrafish were recorded in the FMP Y-maze for 1-h. Phase III: each group received a challenge-dose of their respective treatment for 30-min. Immediately following drug challenge, zebrafish were recorded in the FMP Y-maze for 1-h.

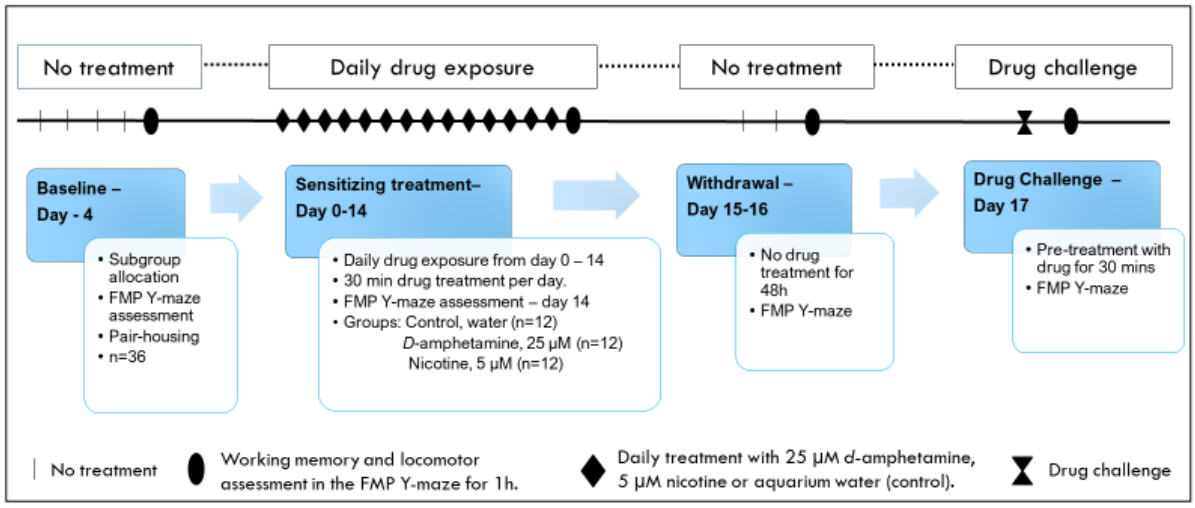

Figure 2. Schematic representation of working memory, cognitive flexibility and locomotor assessments during pre-treatment phase to establish baseline performance, following 14 days of daily drug treatment (intermittent exposure), after two days of rest (withdrawal) and following repeat drug treatment (drug challenge) to examine AMPH and NIC sensitization.

Data analysis

All analyses were carried out using IBM SPSS Statistics (v26) and graphical representations were completed using GraphPad Prism (v8). Studentized residuals were generated from statistical models and screened for outliers. Any data point identified as an extreme value (>3*IQR) was removed from further analysis. Previous work from our lab has identified two regular tetragram patterns (alternations [LRLR+RLRL] and repetitions [RRRR+LLLL]) that appear disproportionately regularly (compared to other sequences) throughout the duration of the trial. In order to study locomotor responses, we analysed each treatment stage based on the total number of turns completed during 1-h of exploration in the maze. LMM analysis was carried with "total turns" as the dependent variable, "treatment (AMPH, NIC, control)" and "treatment stage" (baseline, sensitizing, withdrawal, challenge) as the within-subjects factor and "ID" as a random effect. Analysis was followed by Bonferroni-corrected *post-hoc* test comparing each stage to baseline.

Cognitive flexibility was based on changes in alternation strategy during trial progression. The percentage of alternations used during each 10 min time bin for six successive time bins was analysed using LMM with "alternations" as the dependent variable, "time" (6 levels, 10-min time bins), "treatment" and "treatment stage" as the within-subjects factors, "total turns" as a covariate and "ID" as a random effect. Analysis was followed by Bonferroni-corrected *post-hoc* analysis comparing each time bin to every other time bin for each treatment group. Data are represented as mean + standard error of the mean for bar charts and mean ± SEM for scatter plots. Alpha values of $p \leq 0.05$ were considered statistically significant.

4.4 Results

Development of sensitizing effects of AMPH and NIC on working memory and stereotypic behavior in adult zebrafish.

Use of alternation strategy in the FMP Y-maze has been linked with changes in working memory. Adult zebrafish were assessed at pre-treatment stage, prior to drug exposure and then subsequently after 14-day intermittent exposure, withdrawal and finally, following drug challenge, to assess sensitization effects in water (n=12), AMPH (n=12) and NIC treated (n=12) adult zebrafish. LMM showed a significant effect of treatment stage ($F_{3, 683}$ = 10.23, p < 0.001), and a significant treatment x treatment stage interaction ($F_{3, 684}$ = 6.84, p < 0.01). There was no effect of treatment alone ($F_{2, 30}$ = 1.16, p = 0.33). Control fish treated with water showed no difference in alternation use between any treatment stage (p = 0.12). AMPH-treated fish demonstrated a significant effect of treatment stage with increased alternations after sensitization (95% CI, 12.24-30.60, p < 0.001), returning to near baseline levels after withdrawal (although this effect fell short of significance: 95% CI,-.141-16.75 p = 0.06). Drug challenge with AMPH caused alternation levels to increase significantly above baseline level (95% CI, 18.87-37.48, p < 0.001), demonstrating an increase in alternation use by ~11% after sensitization and ~19% following acute drug exposure. NIC has a significant effect on treatment stage (p = 0.03). Similarly to AMPH, NIC-treated fish showed no difference between sensitization and baseline (95% CI, -.814-16.45, p = 0.09) or withdrawal and baseline (95% CI, -1.98-14.46, p = 0.21) alternation levels, but demonstrated a significant increase in alternations from ~25% at baseline to ~36% following sensitization (95% CI, 1.889-20.376, p = 0.01) compared to pre-treatment levels (*Figure 3A*). Neither controls (p = 0.92) nor NIC treated fish (p = 0.29) showed changes in repetition use at any treatment stage; however, AMPH treated fish showed a significant effect of repetitions on treatment stage following drug challenge compared to baseline (95% CI, -10.64— -2.67, p < 0.001) (*Figure 3B*).

Locomotor sensitization induced by AMPH and NIC.

Locomotor activity was assessed by number of turns completed during 1 h of exploration. LMM revealed a significant effect on locomotor activity of treatment ($F_{2, 122} = 4.47$, $p = 0.01$) and treatment * treatment stage interaction ($F_{3, 122} = 4.0$, $p < 0.01$), but no main effect of treatment stage ($F_{3, 122} = 1.15$ $p = 0.33$). NIC demonstrated psychomotor sensitization following acute NIC challenge, resulting in hyperlocomotion compared to baseline (95% CI, -4.41—16.13, $p = 0.03$) (*Figure 3C*), while AMPH challenge caused significant hypolocomotion compared to controls (95% CI, 99.99-539.8, $p < 0.001$) (*Figure 3D*).

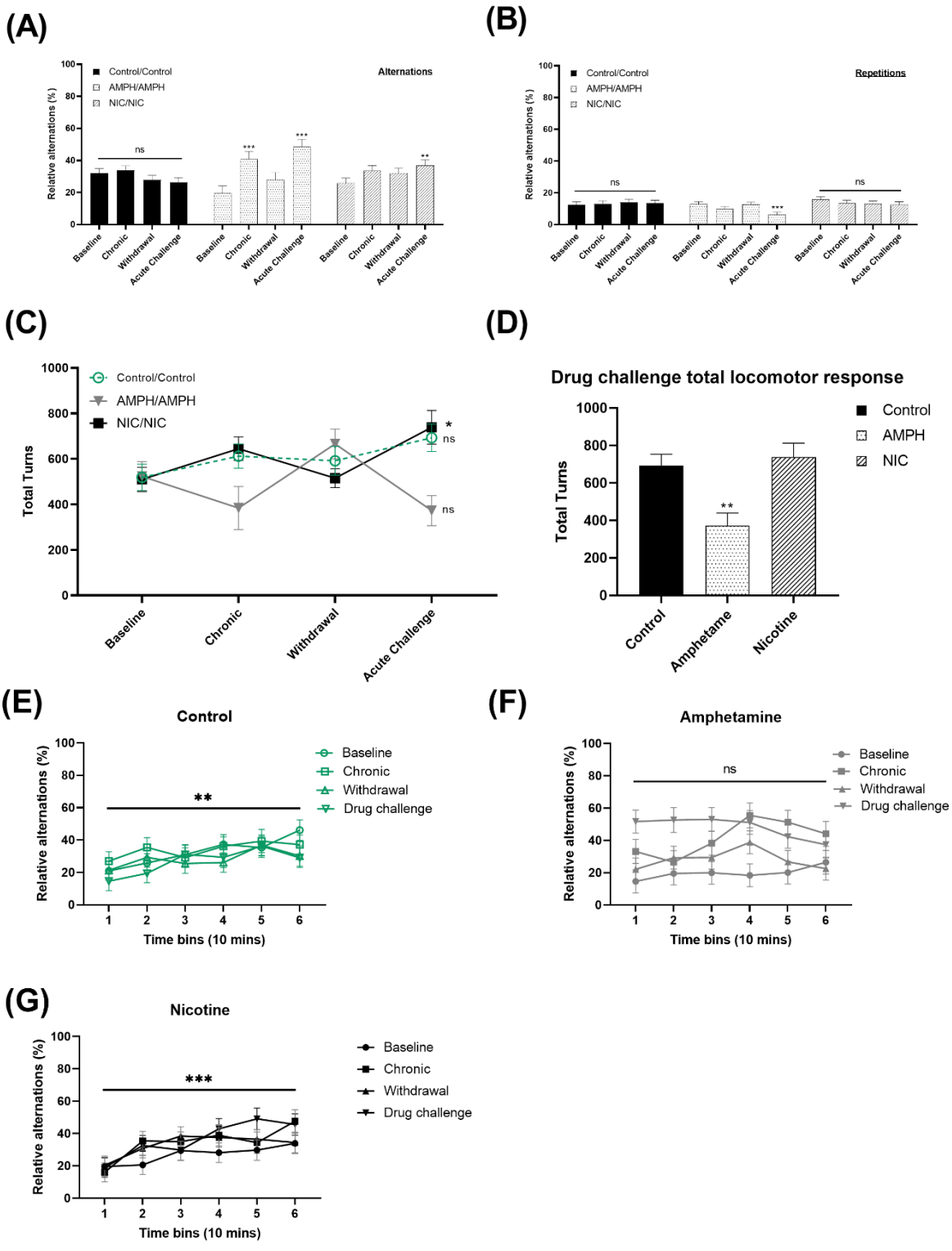
(A)
Control/Control
AMPH/AMPH
NIC/NIC
Alternations
Relative alternations (%)
ns
***
***
**
Baseline
Chronic
Withdrawal
Acute Challenge
(B)
Repetitions
(C)
Total Turns
*
ns
ns
(D)
Drug challenge total locomotor response
Control
AMPH
NIC
Amphetame
Nicotine
(E)
Control
Drug challenge
Time bins (10 mins)
(F)
Amphetamine
(G)
Nicotine

Fig 3. Behavioral response to repeat drug exposure in adult zebrafish treated with NIC or AMPH. Analysis of drug-induced sensitization of AMPH- and NIC-induced cognitive enhancement. Working memory and stereotypic behavior were determined during several stages of drug exposure using a repeated measure analysis of pre-treatment (*baseline*) abilities compared to cognition following 14 days of sensitization to either water (*control/control*), AMPH (*AMPH/AMPH*) or NIC (*NIC/NIC*), 2 days of withdrawal and after challenge with either water, AMPH or NIC (n=11 control, n=11 AMPH, n=12 NIC treated). All animals were given an acute dose of the same treatment that they were sensitized to. Data are expressed as mean + SEM of total percentage use of (A) alternations for assessment of working memory, (B) repetitions for the assessment of stereotypic behavior and mean ± SEM of (C) locomotor activity based on total turns completed during 1 h of exploration. Data were analysed using a LMM analysis followed by Bonferroni *post-hoc* analysis comparing each stage to baseline. (D) Analysis of total locomotor activity after repeated administration of water, AMPH or NIC. Analysis of percentage use of alternations over time for each treatment stage per treatment group was analysed using LMM followed by Bonferroni *post-hoc* analysing using pairwise comparison of each 10 minute time bin for a total of 6 time bins for (E) controls treated with water, (F) conditioned to AMPH followed by acute AMPH challenge and (G) conditioned to NIC followed by acute NIC challenge. Data are expressed as mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns- not significant.

AMPH treatment inhibits cognitive flexibility

To assess cognitive flexibility the percentage use of alternations was analysed as a factor of time. For each treatment group LMM was used to examine the effect of treatment stage on alternations per time bin for six successive 10 min time bins. For controls, there was a significant effect on alternations of time ($F_{5, 208} = 4.06$, $p < 0.01$), but no main effect of treatment stage ($F_{3, 210} = 2.12$, $p = 0.10$), or treatment stage x time interaction ($F_{5, 208} = 0.55$, $p = 0.91$) (*Figure 3E*). NIC caused a significant effect on alternations of time ($F_{5, 222} = 7.01$, $p < 0.01$) of treatment stage ($F_{3, 223.7} = 2.70$, $p = 0.05$), but no effect of treatment stage x time interaction ($F_{5, 222} = 0.70$, $p = 0.79$) (*Figure 3G*). AMPH treated fish showed a significant effect on alternations of treatment stage ($F_{3, 191} = 20.91$, $p < 0.001$), but no effects of time ($F_{5, 190} = 1.42$, $p = 0.22$) or a treatment stage x time interaction ($F_{5, 190} = 1.35$, $p = 0.18$). Collectively, this demonstrated a significant impairment to alter use of alternations over time in fish treated with AMPH (*Figure 3F*).

4.6 Discussion

Here, we investigated the sensitizing effects of the psychostimulants AMPH and NIC on both cognitive abilities and locomotor responses in zebrafish. Psychostimulants have performance-enhancing effects, best characterised in subjects with low baseline levels of cognitive abilities or pre-existing disorders that cause cognitive deficits, such as schizophrenia or attention deficit hyperactivity disorder (ADHD) (for reviews see: Valentine & Sofuoglu, 2017; Wood et al., 2014). AMPH is therefore the front-line pharmaceutical treatment for ADHD, with many studies reporting improvements in attention and concentration (Faraone & Biederman, 2002; James et al., 2001; Spencer et al., 2006). Nicotine has similarly been found to ameliorate cognitive impairment in ADHD (Levin et al., 1996; Levin et al., 2006; Liebrenz et al., 2014; Rezvani & Levin, 2001; Wilens, 2003). Although less conclusive, improvement in certain cognitive domains has also been reported in healthy subjects treated with nicotine or 'clinically relevant' doses of AMPH (Bagot & Kaminer, 2014; Barch & Carter, 2005; Heishman et al., 2010; Jasinska et al., 2014; Mattay et al., 2000; Turner et al., 2003; Valentine & Sofuoglu, 2017; Wood & Anagnostaras, 2009). Here, we demonstrate that administering a moderate dose of AMPH, but not NIC, directly into the water of healthy adult zebrafish, for 14 days of intermittent, daily exposure, improved working memory compared to baseline levels. In addition, unlike AMPH, NIC did not cause degradation of cognitive flexibility, suggesting that it might have fewer unwanted negative effects compared to AMPH.

Repeat administration of psychostimulants induces behavioral sensitization when exposed to a subsequent drug challenge, and this often manifests as an enhancement of cognitive function (Berridge et al., 2012). Following repeated intermittent exposure, both AMPH and NIC-treated adults showed cognitive sensitization, as evidenced by enhancement of performance, with alternation use increasing by ~11% for AMPH and ~8% for NIC (compared to baseline) immediately following chronic dosing. A further increase of ~8% and ~3%, respectively, was reported following the challenge dose. Decreased alternations in the FMP Y-maze have been pharmacologically characterised as a reduction of working memory, suggesting that increased alternations may be indicative of improved working memory (Cleal

et al., 2020). The sensitization of certain cognitive domains, such as working memory, are likely due to altered neurotransmission in ascending DA pathways from the striatum (Berke & Hyman, 2000). Previous studies have shown that after chronic administration of a psychostimulant, subsequent doses cause increased DA release (Herman & Roberto, 2015). The duration of this increased DA response can be several weeks, or as demonstrated here, can be evoked after 48 hours of withdrawal from sensitization treatment.

AMPH and NIC increase striatal DA release (Krause et al., 2002), which can enhance striatum-dependent tasks, such as those requiring working memory and memory consolidation (Brown et al., 2000; Landau et al., 2009), and subsequently potentiate performance of a previously established pattern of behaviors; in this case, the increased use of alternations. Elevated striatal DA increases the use of 'habits', blocking the extinction of established behaviors following devaluation of the goal (Berke & Hyman, 2000). This may provide a possible mechanism for the persistent use of alternations, at the expense of other strategies, after AMPH-induced sensitization. This response strategy may also represent persistent behavioral changes that are associated with drug addiction, in which the persistent seeking and taking of drugs of abuse come at the expense of other normal and necessary behaviors. The fact that this habitual use of alternations, in favour of other search strategies, is only evident in AMPH-treated fish, could be suggestive of the different mechanisms of increasing extracellular DA through dopaminergic or cholinergic pathways. Alternatively, the differences we observed between AMPH and NIC could be a result of the concentration used, and thus future studies should examine the sensitizing effects of a range of drug concentrations.

Psychomotor stimulants are known for their motor activity altering effects (Cunningham et al., 1997). Repeated, intermittent exposure to psychostimulants causes progressive enhancement of locomotor activity with subsequent challenge exposures (Adriani et al., 2006; Kuczenski & Segal, 2002; Peleg-Raibstein et al., 2009; Pisera-Fuster et al., 2019). Here, NIC-treatment caused sensitized

hyperlocomotion, whereas AMPH-treated fish demonstrated hypolocomotion. Treatment with AMPH has been reported to have a paradoxical effect of cognitive enhancing attentional increases and behavioral-calming reductions of hyperactivity. Many studies have shown that these effects are not only evident in patients diagnosed with ADHD, but also in healthy subjects (Bagot & Kaminer, 2014; Holze et al., 2020; Roberts et al., 2020). Human studies with healthy participants have shown that dosing with AMPH can cause an overall decrease in activity levels in healthy boys and adults and similar decreases in hyperactive boys. At low-moderate concentrations of AMPH (as used here), increased hypoactivity is correlated with improved memory performance in cognitive tasks (Rapoport et al., 1980; Spencer et al., 2015). Here, we demonstrate improved cognitive performance corresponding to hypolocomotion in zebrafish.

NIC has regularly been reported as causing hyperlocomotion. Progressive long-lasting increases in locomotor and DA activating effects are generally associated with moderate exposure regimens, whereas high doses of NIC for prolonged periods are more closely associated with signs of withdrawal and typically do not demonstrate locomotor sensitization (Pisera-Fuster et al., 2019). Several studies have shown that a single low dose of NIC is ineffective at inducing locomotor or dopaminergic activation; however, daily low doses can induce locomotor sensitization after days of withdrawal from drug treatment (Fennell et al., 2020; Pisera-Fuster et al., 2019). These conclusions are in line with our findings here, demonstrating no differences in locomotor effects following intermittent dosing; however, after just two days of withdrawal, drug challenge resulted in psychomotor sensitization, demonstrated by a significant rise in motor activity from baseline to repeat drug challenge.

The goal of exploring the FMP Y-maze could be perceived as information seeking or foraging behavior. As fish become familiar with and continue to explore the test arena, they are continuously updating their knowledge of the environment and this can subsequently be used to alter search strategies. In the FMP Y-maze the absence of reward during the 1 h of exploration has been shown to instigate a change in alternation use in healthy adult zebrafish. Many studies have investigated the relationship between

DA agonists, such as AMPH and methylphenidate (MPH), and the relationship between different cognitive domains such as working memory, attention and cognitive flexibility (Bagot & Kaminer, 2014). The inverted-U shaped response to psychostimulants has often been reported to result in the simultaneous enhancement and impairment of multiple cognitive functions (Wood et al., 2014). Studies investigating effects of psychostimulants on cognitive function have reported that high levels of sustained attention correlate with lowered levels of cognitive flexibility, assessed using set shifting tasks (Berridge et al., 2012).

The PFC has been found to be a key region for the regulation of cognitive flexibility (Rougier et al., 2005). Both high and low prefrontal D1 receptor stimulation has been linked with impaired flexibility responding in humans, using the Wisconsin Card Sorting Task (WCST) (Klanker et al., 2013; Takahashi et al., 2008, 2012). In a previous study conducted by our lab, we administered a serial dilution of acute doses of the D1-like antagonist SCH-23390 prior to testing in the FMP Y-maze. We found that SCH-23390 severely impaired cognitive flexibility in the FMP Y-maze at the highest dose (1.5 mg/L), but not at the lower doses (0.5-1.0 mg/L) which still maintained an effect of time on alternations. Additionally, all doses of SCH-23390 caused a significant reduction in overall use of alternations, hypolocomotion and, at the highest dose, an increase in stereotypic behavior, shown by a significant increase in repetitions (Cleal et al., 2020). These findings, in conjunction with the present work, demonstrate a complex role for D1 in modulating cognitive flexibility. Further work is required to disentangle the relationship between DA receptor activation and extracellular levels of DA to fully understanding the mechanisms involved in maintaining cognitive flexibility and other cognitive functions.

4.7 Conclusion

We investigated the sensitizing effects of AMPH and NIC on working memory, locomotion and cognitive flexibility. We demonstrated that non-contingent exposure to the psychostimulants AMPH, but not NIC, improved working memory performance in the FMP Y-maze after 14 days of intermittent dosing,

returning to basal levels after two days of abstinence. Subsequent challenge resulted in sensitization of both drugs, resulting in greater use of alternations, demonstrating an increase in the preference for this exploration strategy. In AMPH this increase in alternation use was accompanied by a decrease in the use of other strategies, demonstrating a focused increase, bordering on stereotypic behavior. Only NIC demonstrated increased locomotor sensitization compared to pre-drug exposure levels. AMPH caused significant hypolocomotion during challenge. Additionally, our findings suggest a role for dopaminergic, but not cholinergic, overactivation in the inhibition of cognitive flexibility. Although the protocol for psychostimulant-induced motor sensitization is substantially less cumbersome to establish than other models of addiction, it is not a precise homologue for psychostimulant addiction in humans (Berke & Hyman, 2000). Understanding drug-induced changes in neurotransmitter systems and neural plasticity by studying cognitive sensitization may provide a useful model with high face validity. These results demonstrate that previous exposure of fish to AMPH or NIC can enhance cognitive performance, but extreme levels of improved performance in one cognitive domain can result in disruption in another domain. This may demonstrate the first signs of drug-induced maladaptive activities. The distinction between cognitive enhancement and inhibition of normal behaviors, may be dependent on cognitive flexibility, the loss of which has been strongly linked with substance abuse disorders which are often characterised by deficits in cognitive flexibility and may underlie the persistence of harmful, drug seeking and taking behaviors.

Finally, this paper is the first to demonstrate robust test-retest reliability for the FMP Y-Maze. In previous studies we have only looked at single exposures of animals to the maze. With the demonstration from our control animals here that there are no significant changes in total turns, alternations, repetitions or general search patterns in the maze during the hour test between the four test periods, suggesting that the FMP Y-maze can be used longitudinally to examine changes in memory or behavioral flexibility over time.

Chapter 5

5.1 General discussion

This section has focused on the effects of legally obtainable drugs of abuse on cognitive performances at two distinct developmental stages: prenatal and adult. Our initial study investigating the effects of low concentrations of prenatal alcohol exposure reported causal alterations in cognitive trajectory, resulting in poorer cognitive performance than their non-drug exposed counterparts in the FMP Y-maze. Early developmental exposures have been well documented as being highly susceptible to environmental perturbations, even in the absence of any known genetic susceptibilities. However, this study highlights just how sensitive the developing brain is, as even low alcohol exposures, the equivalent of moderate drinking in humans, is sufficient to cause life-long disparities in cognitive function compared to unexposed controls. Although effects of low/moderate alcohol exposure during early brain development has been shown previously in humans and in other species, we demonstrated that the FMP Y-maze is sufficiently sensitive to detect even very small differences in cognition that are often observed.

The next section focused on the effect of drugs of abuse on cognitive performance following adult drug exposure regimes. Quite the reverse story was true for adult drug exposure compared to prenatal drug exposure. Both NIC and AMPH resulted in cognitive enhancing effects after 2 weeks of daily treatment. Not only did low doses improve WM after the initial exposure regime, but repeat exposure, after a short withdrawal period, demonstrated cognitive sensitization for both drugs. However, these pro-cognitive outcomes were not completely without negative 'side effects'. AMPH exposure was also associated with significant deficits in CF. Findings that may have identified the first signs of drug induced maladaptive behavioural responses. These negative 'side effects' were not, however, evident in NIC treatment, as fish repeatedly exposed to NIC maintained behavioural flexibility

throughout. This study raises an interesting question as to the inequality in the modulation of cognitive functions through the activation of the dopaminergic system directly, through DA receptors, and indirectly through activation of the cholinergic system.

In addition, although not in the same light of cognitive effects of drugs of abuse, we put a call out to zebrafish researchers to be mindful of potentially confounding variables when considering studies on non-contingent drug exposure in zebrafish. Our brief study effectively demonstrated the impact that even small changes in environmental conditions, such as water pH, could have on experimental design and output. In an attempt to improve inter-lab consistency and replicability of findings we suggest the regular reporting of pH conditions of zebrafish husbandry and, more importantly, pH of drug exposure conditions. This is especially important when immersing zebrafish in drug solutions as this can significantly impact up-take and ultimately influence final drug concentrations in the brain. This could potentially result in misleading data due to disparities in perceived dose and actual dose administered. Since identifying the susceptibility of up-take mechanisms to fluctuations in environmental pH conditions, we have endeavoured to consistently report pH levels of drug treatments in all of our studies.

Finally, this section provides further validation of the FMP Y-maze as test of cognitive abilities, demonstrating the use not only with pharmacological models investigating the impact of drugs of abuse on cognition, but additionally highlights a role for repeat testing. This is a particularly interesting application of the FMP Y-maze as to date there are few quick, simple and reliable tests of cognitive function in zebrafish that have suitable retest potential. This potentially offers a strategy for longitudinal monitoring of therapeutic strategies targeting cognition.

Part III

Cognition in Schizophrenia-like models: Effects of Drugs of Abuse in Disease

Chapter 1

Introduction

Schizophrenia

Schizophrenia is a chronic mental health disorder affecting 20 million people worldwide (James et al., 2018). It is characterised by positive symptoms (hallucinations, delusions, confused and disorganised thoughts), negative symptoms (anhedonia, reduction in emotional expression, motivation and social interactions) and cognitive symptoms (deficits in executive functions such as WM, CF, processing speed, inhibition and general intelligence) (Blanchard & Cohen, 2006; Dickinson et al., 2008; Heinrichs & Zakzanis, 1998; Peralta & Cuesta, 2001; Rector et al., 2005). Cognitive deficits are regarded as a core feature of SZ (Lett et al., 2014). Clinical studies have repeatedly found evidence of impaired executive function, particularly in WM and CF in patients diagnosed with SZ (Dickinson et al., 2008; Eryilmaz et al., 2016; Frydecka et al., 2014; Giraldo-Chica et al., 2018; Grover et al., 2019; Heaton et al., 2001). Studies have identified the PFC, specifically the dorsal lateral aspect, as a region associated with dysfunction in SZ patients (Bolkan et al., 2017; Giraldo-Chica et al., 2018; Orellana & Slachevsky, 2013). Functional imaging studies and post-mortem examinations have reported volume deficits in the PFC, the MD

(medial dorsal thalamus) and the anterior limb of the internal capsule (ALIC) (the white matter tract that provides reciprocal connections between the cortex, striatum and thalamus) (Beasley et al., 2009). Additionally, the length and density of the ALIC were also reduced in SZ patients compared to controls (Beasley et al., 2009; Lang et al., 2006; Zhou et al., 2003), as well as a reduction in the total connectivity between the MD linking to the lateral PFC, which has been correlated with impaired WM (Giraldo-Chica et al., 2018).

The dopaminergic system has been significantly implicated in the pathology of SZ (Brisch et al., 2014; Kokkinou et al., 2020; Peleg-Raibstein et al., 2009; Rao et al., 2019). Abnormalities in DA synthesis, availability and receptor activation have all been linked to aberrant functioning and connectivity in the CSTC circuitry. All aspects of SZ have been associated with dysregulation of the dopaminergic system (Brisch et al., 2014). Hyperactivity of DRD2 neurotransmission in subcortical regions, including the thalamus and striatum, are associated with the positive, psychotic symptoms of SZ (Alfimova et al., 2013; Noble, 2003; Ripke et al., 2014). Whilst hypoactivation of DRD1 in the PFC have been implicated in negative symptoms and cognitive deficits (Rao et al., 2019). Patient studies have further implicated dopamine-related genes, such as dopamine transporter (DAT) (Rybakowski et al., 2006; Szekeres et al., 2004; Xu et al., 2010), DRD3 (Szekeres et al., 2004), DRD4 (Alfimova et al., 2006; Hwang et al., 2012) and DRD5 (Golimbet et al., 2008) in neurocognitive deficits in SZ.

Substantial progress has been made in validating the aetiology, brain pathology and behavioural abnormalities in animal models of SZ. Drug-induced and genetic models of SZ have been extensively studied in rodents (for reviews see: Jones et al., 2011; Lee & Zhou, 2019; Neill et al., 2010) and are growing in use in zebrafish (Demin et al., 2019; Gawel et al., 2019). The most commonly used pharmacological model of SZ, pre-treatment with MK-801, captures aspects of positive (psychosis), negative (social interaction deficits) and cognitive symptoms (memory impairment) that present in the human disorder (Andiné et al., 1999; David et al., 2008; Lim et al., 2012; Maaswinkel et al., 2013; Rung et al., 2005; Sison & Gerlai, 2011a; Zimmermann et al., 2016). The effect of MK-801 on cognitive impairment has been assessed in zebrafish (Cleal, Fontana, et al., 2020; Cognato et al., 2012; M.-C. Ng et al., 2012;

Seibt et al., 2011; Sison & Gerlai, 2011b; Swain et al., 2004) and it is for this reason that we included it a pharmacological model for validating the FMP Y-maze as a test of WM (see Part 1: Chpt 3). It has also been tested in rodents (Abraham & Mason, 1988; Manahan-Vaughan et al., 2008; Neill et al., 2010; van der Staay et al., 2011) and humans (Thomases et al., 2013). It is an NMDA receptor (NMDA-r) antagonist that causes cognitive deficits by impairing long term potentiation (LTP) (Kovacic & Somanathan, 2010; Song et al., 2018; Wieschollеck & Manahan-Vaughan, 2012; Yoshiyama et al., 1991). LTP is the mechanism used to store information by activity-based modification of synapses, which has been associate with learning and memory formation (Aigner, 1995; Bliss & Lomo, 1973). NMDA-r are highly concentrated in the telencephalon in both mammals and zebrafish, and have been strongly implicated in memory formation (**Fig. 1**) (Aigner, 1995; Nam et al., 2004). NMDA-r antagonists act by modulation of voltage-gated currents, thus decreasing neuronal excitability by hyperpolarizing the affected cells, consequently decreasing the role of the neuron within a neural circuit, e.g. by impairing memory formation (Brosnan, 2011). Glutamatergic and dopaminergic pathways interdependently modulate one another. NMDA-r activation alters firing patterns of DA neurons, whilst dopamine receptors directly (DRD1) or indirectly (DRD2) regulate NMDA-r function (Chergui et al., 1993, 1994; Mereu et al., 1997; Overton & Clark, 1992; M. Wang et al., 2012). Taken together, the literature supports the use of MK-801 as a model with high face and construct validity for mimicking the cognitive symptoms of SZ by evoking dopaminergic dysregulation resulting in altered DA neuron activity and behavioural endophenotypes both in rodents and zebrafish.

Figure 1.

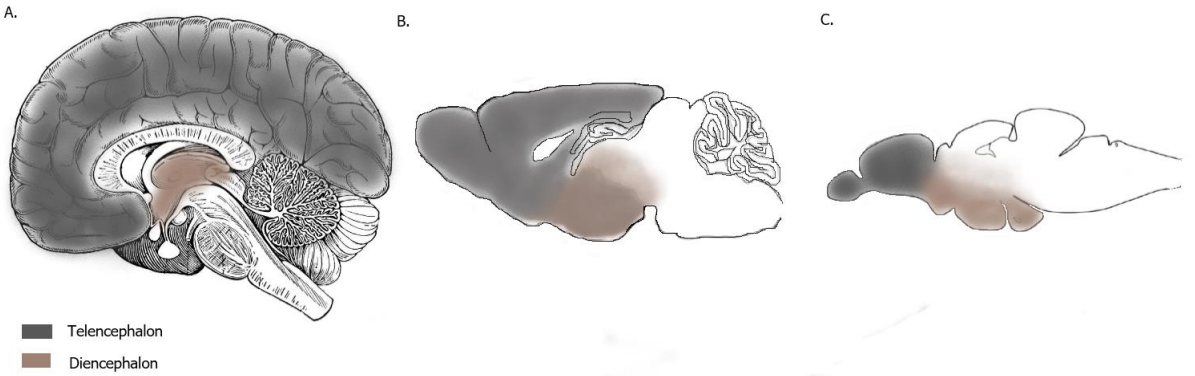

Figure 3. Brain divisions of the telencephalic and diencephalic regions in adult (A) humans, (B) rodents (mouse) and (C) teleosts (zebrafish).

(adapted from (Perrone-Capano et al., 2008; Vaz et al., 2018b)

Numerous candidate genes have been identified as markers for increased susceptibility for developing SZ (Ripke et al., 2014). Many of these genes have provided targets for generating animal models with SZ-like phenotypes including cognitive deficits (Papaleo et al., 2012; Winship et al., 2019). The leading risk-associated gene, and most extensively studied, is Disrupted in Schizophrenia-1 (Disc1). Several rodent models have been developed, targeting different mutation variations in Disc1 (Papaleo et al., 2012). Through this mutant line, a direct link was identified between Disc1 and NMDA-r functions, providing further support of the validity of the MK-801 pharmacological model of SZ (Ramsey et al., 2011; Z. Yan et al., 2014). Models of Disc1 were found to have reduced cortex volume, as reported in humans, and a range of cognitive deficits, including WM and CF. One study by (Gamo et al., 2013) demonstrated exacerbated cognitive impairments with stress, specifically affecting WM performance. Recently, a role for an excitatory-inhibitory imbalance in the PFC in Disc1 locus impaired mice, revealed a reduction in inhibition, causing altered synaptic communication between the MD and the PFC circuitry (Delevich et al., 2020). This demonstrates a possible mechanism for the cognitive deficits identified in SZ patients. Rodent models additionally found deficits in WM and CF and implicated a role for the PFC

in models of Dysbindin-1 (Dtnbp1) , Neuregulin 1 (Nrg1), Reelin and Proline dehydrogenase (Prodh), all genes that have been associated with increased risk of developing SZ (Papaleo et al., 2012).

There are several genetic models of SZ-related phenotypes in zebrafish. Susceptibility genes associated with SZ, such as *disrupted in schizophrenia-1* (*disc1*) (Eachus et al., 2017; Singh et al., 2011; Wood et al., 2009), *neuregulin 1* (*nrg1*) (Wood et al., 2009) and *regulator of G-protein signalling 4* (*rgs4*) (Cheng et al., 2013) have all been knockdown in zebrafish, and although some SZ-like phenotypes, mostly associated with positive or negative symptoms, have been assessed, none of these models to date have conducted comprehensive cognitive assessments, despite cognitive deficits being a core feature of SZ. A recent study by (Thyme et al., 2019) has completed a comprehensive genomic study of 132 zebrafish orthologs of human SZ-associated genes, demonstrating the potential of zebrafish for conducting large-scale genetic mutation and screening of phenotypes associated with a specific disorder, a feat unimaginable in rodents. However, the lack of cognitive testing in assessment of SZ models highlights a huge gap in the use of zebrafish as a model of SZ and potentially raises the need to develop more reliable, robust and high-throughput cognitive tests available for assessing zebrafish at larval and adult stages.

Chapter 2

As has been described above, there are only a few zebrafish models of SZ, compared to an extensive range of rodent lines. In addition, the few that have been developed in zebrafish have yet to investigate cognitive performance of these models, thus lacking description of one of the most prevalent features of SZ in humans. As we have suggested, this may be due to a lack of high throughput, robust methods for assessing cognition in zebrafish. The early sections of this thesis have described the use of the FMP Y-maze as behavioural task that can be used to fulfil these criteria. In addition, as discussed above, one of the most efficient pharmacological models for inducing SZ-like phenotypes is MK-801, which has been tested in humans, rodents and zebrafish. In conjunction with previous zebrafish models, we treated zebrafish with MK-801 to validate the FMP Y-maze as a test of memory, as MK-801 is well known for disrupting WM and cognitive flexibility. In Part 1, Chpt 3 we describe the cognitive impairing effects of MK-801 on zebrafish exploration patterns in the FMP Y-maze. This study has provided preliminary data on exploration patterns of zebrafish exhibiting SZ-like phenotypes in the FMP Y-maze.

The following chapter is concerned with the development of a new zebrafish line targeting another susceptibility gene for SZ: SLIT-3. Several genome association studies have now implicated dysfunction of this gene not only in SZ, but in several other neuropsychiatric disorders. The next chapter describes the characterisation and validation of this new zebrafish line exhibiting a broad spectrum of behavioural and biochemical abnormalities that are characteristic of patients diagnosed with SZ.

CRISPR/Cas9-Induced *Slit-3* Mutant Zebrafish (*Danio rerio*) Display Schizophrenia-Like Behaviours

2.1 Abstract

Human genetic studies have suggested a causal role of *SLIT-3* deficiency in schizophrenia (SZ) and altered smoking behaviour potentially indicating a link between the high smoking-SZ comorbidity. However, the molecular mechanisms underlying *SLIT-3* deficiency resulting in SZ and modified smoking behaviour are not fully understood. Recently, ENU-mutagenized zebrafish carrying a loss of function mutation in *slit-3* have replicated modified smoking behaviour in humans, by demonstrating an increased preference for nicotine in the conditioned place preference (CPP) task. However, as of yet, no evidence of *slit-3*-induced SZ-like behaviour has been modelled using zebrafish. Here, we report the generation and characterisation of a *slit-3* mutant zebrafish at adult stages using the CRISPR/Cas9 genome editing technique. *Slit-3* zebrafish exhibited abnormal anxiety responses, cognitive function, drug preference and hypothalamic-pituitary-interrenal (HPI) axis activation, which are partially rescued by treatment with nicotine but not ethanol. We generated the first *slit-3* zebrafish model to exhibit robust SZ-like behaviours and demonstrate a therapeutic role for nicotine. Zebrafish models of complex neuropsychiatric disorders will likely have a significant contribution to future studies of human *SLIT-3* function and SZ and may play a critical role in identifying potential therapeutic strategies.

2.2 Introduction

Schizophrenia (SZ) is a severe mental health disorder that globally affects over 20 million people (James et al., 2018). It is an extremely debilitating condition that is characterised by an extensive, heterogeneous combination of features that have been subcategorised into positive (hallucinations, thought disorders

and delusions), negative (anhedonia, emotional blunting and social withdrawal) and cognitive symptoms (impaired WM, attention and executive function) (Andreasen, 1995; Blanchard & Cohen, 2006; Dickinson et al., 2008; Peralta & Cuesta, 2001). Cognitive deficits have been well documented as a core characteristic of SZ, with both patients (Dickinson et al., 2008; Eryilmaz et al., 2016; Frydecka et al., 2014; Giraldo-Chica et al., 2018) and animal models (Castner & Williams, 2007; Neill et al., 2010; Papaleo et al., 2012; Young et al., 2009) exhibiting deficits in memory processing, particularly WM. In addition to altered behavioural outcomes, there is substantial evidence for molecular changes particularly affecting the hypothalamic-pituitary-adrenocortical (HPA) axis activity in many psychiatric disorders, including SZ (Bradley & Dinan, 2010; Jacobson, 2014). Both hyper- and hypo-functioning of the HPA axis have been linked to altered stress response and have been suggested to contribute to poor physical health and premature mortality associated with SZ (Pruessner et al., 2013; Van Venrooij et al., 2012). There are a number of additional comorbidities, such as anxiety and substance abuse (Goodwin et al., 2003), that are frequently reported in SZ patients, in conjunction to the above behavioural and biological mechanisms.

The most well documented comorbidity with SZ, is the extremely high prevalence of tobacco smoking (D'Souza & Markou, 2012). Altered smoking behaviour in patients diagnosed with SZ is widely reported, with higher frequency and intensity of smoking and difficulties in smoking cessation when compared to the general population and to other serious mental illnesses (De Leon & Diaz, 2012; Hartz et al., 2018; Lyon, 1999). There are several theories posited to explain the increased incidence of nicotine addiction in SZ patients which include genetic susceptibility to addiction, increased craving, self-medication, modulation of negative symptoms and cognitive enhancing effects of nicotine (De Leon & Diaz, 2012; Lucatch et al., 2018; Wing et al., 2012). However, to date, the precise role of nicotine in SZ is still unclear.

Extensive genome-wide association studies (GWAS) and genetic modelling studies have investigated increased incidence of specific SZ-risk genes that occur in the population. A genetic association study of the Han Chinese population, conducted by (Shi et al., 2004), identified *SLIT*-3 as a

susceptibility gene associated with SZ. The Slit family consists of large secreted proteins that act as guidance cues to regulate neuronal orientation, axon pathfinding, cell migration and differentiation during embryonic development (Brose et al., 1999; Little et al., 2002; Long et al., 2004). There are three Slit proteins that have been identified in vertebrates so far, Slit-1, 2 and 3 (Brose & Tessier-Lavigne, 2000). Previously, dysregulation of *SLIT-3* has been extensively associated with cancer development and progression (Dickinson et al., 2004; Jiang et al., 2019; Ng et al., 2018; Tong et al., 2019; Zhang et al., 2015). However, more recently genetic association studies have supported the original findings of (Shi et al., 2004), identifying *SLIT-3* as a SZ-susceptibility gene (Ma et al., 2019; Vieland et al., 2014; Wang et al., 2018), but it has also been identified as a risk gene for autism spectrum disorder (ASD) (Park et al., 2018; Perez et al., 2016), major-depressive disorder (MDD) (Glessner et al., 2010), substance abuse (García-González et al., 2020) and Alzheimer's disease (AD) (Antonell et al., 2013) and has been linked with intelligence pathways in humans (Najafi et al., 2018).

A recent study by (García-González et al., 2020) identified single-nucleotide polymorphisms (SNPs) in the *SLIT-3* gene in two human cohorts that associated with modified smoking behaviour, including nicotine dependence symptoms, age at onset of weekly smoking, number of cigarettes smoked per day and time to first cigarette after waking in the morning. The latter two have been suggested to be the most predictive measures used in the Fagerström Test for Nicotine Dependence (FTND) (De Leon & Diaz, 2012). They also reported increased sensitivity to the rewarding effects of nicotine, identified by increased preference for a nicotine-paired environmental cue in the conditioned place preference (CPP) task, using an N-ethyl-N-nitrosourea (ENU) mutagenized zebrafish model exhibiting a *slit-3* loss of function mutation (García-González et al., 2020). This study not only supported the role for forward genetic screening and modelling of complex neuropsychiatric disorders in zebrafish, but additionally highlighted altered response to nicotine in *SLIT-3* mutants, potentially identifying a genetic link between modified smoking behaviour and SZ that could aid in elucidating the comorbidity so frequently encountered between the two.

Zebrafish are growing in use as a model of neuropsychiatric disorders and thus far, several genetic models have been generated examining SZ-risk genes, the most notable of which is disrupted-in-schizophrenia-1 (*disc1*) (see Part 3: Chpt 1) (De Rienzo et al., 2011; Eachus et al., 2017; Singh et al., 2011) Although these models have faithfully replicated aspects of SZ-like phenotypes found in humans and rodents, they are mostly associated with either the positive or negative symptoms of SZ and as yet there are no models that have validated cognitive symptoms, despite these being a core feature of SZ (Cheng et al., 2013; Eachus et al., 2017; Singh et al., 2011; Wood et al., 2009). With the identification of *SLIT-3* as a SZ-susceptibility gene and the discovery of altered nicotine preference in carries of *SLIT-3* SNPs, in both humans and zebrafish, we aimed to use CRISPR/Cas9 genome editing to generate a new zebrafish *slit-3* mutant line to assess SZ-like phenotypes, including cognition and anxiety, and assess the impact of anxiolytic drugs of abuse to identify a causal link between SZ and substance abuse, particularly nicotine.

Our findings support the role for *slit-3* mutations in the development of SZ-like endophenotypes, including increased anxiety, attenuated cortisol levels following acute stress and impaired cognitive function, particularly relating to WM, increased stereotypic behaviour and altered drug preferences, which has been replicated together in a single model for the first time in a zebrafish model of SZ. Treatment with anxiolytic concentrations of two drugs of abuse, nicotine and ethanol, demonstrate increased preference for drug-paired cue compared to controls in the CPP task, but only nicotine exerted an anxiolytic effect on *slit-3* mutants. Additionally, nicotine also rescued previously observed *slit-3* mutant cognitive deficits. We have demonstrated the first *slit-3*-induced SZ animal model which recapitulates cognitive deficits associated with SZ.

2.3 Materials and Methods

Generation of slit-3 mutant zebrafish

The detailed procedure for CRISPR/Cas9 editing in zebrafish was described previously (Irion et al., 2014; Mali et al., 2013). The *slit-3* target in this study was 5′ – CAGGCGCACAGAGCTCAAGCCGG - 3′. Injection mixtures included 300 pg of Cas9 mRNA and 50 pg of gRNA. Embryos were injected with 20 ng of Cas9-gRNA mixture at the single-cell stage. Fish were grown until adulthood and fin clipped for genotyping using T7 endonuclease assay, high resolution melt (HRM) curve analysis and confirmed with sanger sequencing. Mutant sites were verified by comparison to the WT unaffected sequences.

Animals and housing

Zebrafish were bred in-house at the University of Portsmouth Fish Facility, with eggs collected from pair-breedings of adult Tg(*elavl3*:GCaMP6s) on a mixed *casper/nacre/AB* background. Females and male fish (two females and one male for each pair-breeding) were isolated (by sex) in a separate tank the evening before breeding. 15-min before lights on fish were transferred to a breeding tank (a shallow, stand-alone tank with a slotted base), filled with 0.5-1.0L of aquarium water. Males and females were separated by a clear divider. When lights were turned on, the divider was removed to allow the fish to mix. Fish were left in the tank for 30-45 min. Once eggs were laid, adult fish were returned to home tanks. Laid embryos were washed and placed in petri dishes in maximum groups of 50 embryos per dish in aquarium water and immediately used for CRISPR/Cas9 injections at single-cell stage then returned to the fish facility. Larvae were reared in the fish facility until adulthood. Fish were maintained on a re-circulating system (Aquaneering Inc., San Diego, CA, USA). From 5 dpf fish were fed on ZM fry food until 30 dpf and then put on a diet of dried fish flakes and live brine shrimp, 3 times per day during the week and once a day at weekends (am). Aquarium water was maintained at ~25-27°C, pH 8.4 (±0.4), on a 14/10-hour light/dark cycle. All fish used were experimentally naïve.

Ethical statement

Experiments carried out as part of this study were under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: P9D87106F] and with approval from and in accordance with the University of Portsmouth Animal Welfare and Ethical Review Board guidelines.

Behavioural analysis

Adult zebrafish, at ~3-5 months old, were used throughout this study. Fish were transferred into the behavioural room for 30 min prior to analysis to allow for acclimatization. A total of N=120 fish (~12 per group) were used for all behavioural procedures. Novel tank diving tests were always run prior to cognition tests. All behaviour was carried out in the Zantiks adult behavioural unit (AD unit) (Zantiks Ltd, Cambridge, UK), which tracked fish continuously whilst in the test arena. Units were web-enabled allowing live streaming video feed of zebrafish during testing, to a laptop or mobile device. Starting and finishing trials was done remotely. Pre-determined protocols were used that depicted specific zones and data was automatically logged and output into an excel file. Experimenter visibility and handling were kept to a minimum to reduce stress and distraction whilst animals were performing behavioural tasks. Ambient light was a maximum of 2 lux in testing units.

Novel tank diving apparatus

All fish were run individually in a clear rectangular tank, 20 cm length x 5 cm width x 17.5 cm depth and filled with 1 L of aquarium water. Fish were permitted free swimming for 6 min, starting from first entry into the tank. The test arena was divided into three equal, horizontal zones (bottom, middle, top) to allow evaluation of vertical activity. Anxiety behaviour was analysed using time spent in each zone and number of entries into the top zone as behavioural endpoints and distance travelled was used to assess locomotor activity.

Free-Movement Pattern (FMP) Y-maze apparatus

All fish were run individually in a white acrylic Y-maze with clear base and mesh top (to prevent escape), maze dimensions are: 5 cm length x 2 cm width x 14 cm depth and filled with 3 L of aquarium water to allow sufficient swimming depth. Two mazes were fitted into a water-tight tank and run in the Zantiks AD unit. Logging of arm entries was written into the program script, recorded automatically and output in an excel spreadsheet. Arm entries and exits were continuously logged throughout the trial and converted into a series of left and right turns. Search strategy was based on overlapping sequences of four turn choices, giving rise to 16 possible tetragram configurations. Protocols for the FMP Y-maze have been described in detail elsewhere (Cleal et al., 2020).

Conditioned place preference (CPP)

CPP was conducted as previously described (Brock et al., 2017; Parker, Brock, Millington, et al., 2013). Adult zebrafish were singly isolated in groups of four (fish maintained visual and olfactory contact with conspecifics but were kept separate for identification) for 4 days prior to habituation. Fish were habituated to the test arena, four fish per arena, for 20 min per day for two consecutive days prior to baseline testing. All four stages of the CPP (habituation, baseline, drug treatment and probe trial) were conducted in a black acrylic tank (14 cm width x 20 cm length x 15 cm height), longitudinally separated with black acrylic dividers, into four equally sized lanes, filled with 2 L of aquarium water. A clear acrylic base permitted the projection of visual cues (grey/black and white check squares). Following habituation was five days of drug testing consisting of: day 1 – baseline. Tanks were split vertically, half with grey and the other half with black and white checks. Fish were in the test arena for 10 min, the first 5 min were habituation with no light cues, the second 5 min had light cues and fish were tracked for the duration of test, basal preference was determined by the percentage of time spent in each half of the tank (% time spent grey v % time spent check). Day 2-4 - fish were conditioned to the least preferred side (i.e., fish that spent <50% on the grey side would receive drug treatments in the presence of the grey background). Fish were placed into the test arena with the favoured light cue for the first 20 min.

Then the least preferred light cue would be used for the second 20 min in conjunction with the addition of the drug at the beginning of the second 20 min. Following conditioning fish were returned to home tanks. Day 5 – fish tested in the probe test, using the same procedure as the baseline test. Testing was conducted in the absence of drug. Change in preference was assessed by deducting the time spent on the drug-paired cue minus baseline preference to the same cue prior to drug exposure (i.e., for a fish with grey as the least preferred cue change in preference was calculated as: % time grey probe test - % time grey baseline).

Drugs and concentrations

Two commonly used drugs of abuse with anxiolytic properties, nicotine and ethanol, were selected for this study (Gebauer et al., 2011; Levin et al., 2007; Parker et al., 2012a). Nicotine (Sigma-Aldrich) was administered at a concentration of 5 μM (Cleal, Gibbon, et al., 2020; García-González et al., 2020; Petzold et al., 2009) and absolute ethanol (Thermo Scientific) was administered at a concentration of 171 mM (Brock et al., 2017). Both drugs were delivered as a stock to make up the final concentration in 2 L of aquarium water. Stock concentrations were ~pH 8.0 ± 0.5. Stock concentrations were divided into four equal parts and administered to each lane to ensure drug was evenly distributed throughout the test chamber. Concentrations were based on previous work by (Brock et al., 2017) assessing different concentrations of drug in the CPP using adult zebrafish. The concentrations selected here reflected concentrations that exhibited a positive, drug-induced change in the CPP.

Acute stress

To study the effect of acute stress on cognitive performance in WT and *slit-3*$^{+/-}$ mutants, fish were removed from home tanks and placed into 2.8 L tanks (5 fish per tank). Acute stress was delivered by net chasing for 5 min, then all fish were left to recover for 10 min, as previous research has shown peak cortisol responses within 3-15 min of receiving a stressor in zebrafish (Ramsay et al., 2009). Following

the rest period, zebrafish were immediately placed into the FMP Y-maze for behavioural testing or culled by immersion in ice-water and snap-frozen using liquid nitrogen for whole-body cortisol analysis.

Whole-body cortisol

Following acute stress, cortisol levels were detected using the Salimetrics Salivary Cortisol ELISA kit for human samples (Stratech, Cambridge, UK), which has been found to reliably detect cortisol in zebrafish using the method previously described by (Cachat et al., 2010; Parker et al., 2012). Briefly, samples were weighed and then homogenised in 1 mL of chilled PBS. 5 mL of diethyl ether was mixed with the homogenised samples and centrifuged at 7000 x g for 15 min. The top, organic layer was removed, and the process was repeated until all the cortisol-containing layer was removed, and then subsequent evaporation of the diethyl ether was continued overnight at room temperature. The resulting cortisol was reconstituted using 1 mL of chilled PBS. The ELISA was carried out in a 96-well plate following the manufacturer's instructions. Standards were used to determine the final cortisol concentration using OD readings. Weights were used to calculate cortisol concentration per gram (ng/g^{-1}). Inter- and intra-assay coefficients of variation were determined. Data are represented as mean ± SD (n = 4 per group; assayed in duplicate).

gDNA extraction

Adult fish were anaesthetised using Aqua-Sed (100% 2-phenoxyethanol) anaesthetic treatment (Aqua-Sed™, Vetark, Winchester, UK) in accordance to manufacturer guidelines, in 500 mL of aquarium water. Fin clippings were taken and stored in absolute ethanol at -20 °C until use. Following recovery from anaesthetic, fish were returned to home tanks. Genotyping was either performed significantly in advance of behavioural testing (i.e., with sufficient time for the tail to grow back or following behavioural testing to prevent confounding effects). Samples were homogenised and gDNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific) following the manufacturer's instruction.

Sample concentrations were analysed using the ND 1000 and diluted to 10 ng/µL in DNase and RNase free water and stored at -20°C until use.

High resolution melt curve (HRM) analysis

Quantitative real-time PCR (RT-qPCR) followed by HRM analysis was used to genotype samples (Norambuena et al., 2009; Słomka et al., 2017; Wojdacz & Dobrovic, 2007). Primers were designed using NCBI blast and Primer3 and synthesised by Invitrogen (**Table 1**). The Roche LightCycler® 480 High Resolution Melting Master mix and the LightCycler® 96 (Roche Life Science) were used to amplify and detect the transcripts of interest. Thermal cycling conditions included an initial denaturation step at 95°C for 600s (recommended by manufacturer), 40 cycles of 95°C for 25s, 58°C for 25s and 72°C for 35s followed by 25 readings of -0.1°C/s melt curve analysis for genotyping gDNA samples. Negative template controls were used for checking primer specificity. Differences in high resolution melt peaks were manually identified.

Table 1. Primer sets used for HRM analysis

Gene name	Gene	Primer sequence (Forward)	Primer sequence (Reverse)	Amplicon size
Slit homolog 3 protein	*Slit-3*	GATCAACCTGCCTGTGTGTTT	TGGTATGTGCATGTGTTTGTGT	220

PCR

Genotyping was confirmed using PCR to amplify a ~600 bp amplicon. Primers were designed using NCBI blast and Primer3 and synthesised by Invitrogen (**Table 2**). The VWR Red Taq Master Mix and BioRad T100 Thermal Cycler were used to amplify transcripts. Thermal cycling conditions included an

initial denaturation step at 95°C for 2 min (recommended by manufacturer), 30 cycles of 95°C for 20s, 60°C for 20s and 72°C for 30s and one cycle of 72°C for 5 min and held at 4°C.

Table 2. Primer sets used for PCR

Gene name	Gene	Primer sequence (Forward)	Primer sequence (Reverse)	Amplicon size
Slit homolog 3 protein	*Slit-3*	TGTTGAGCACTGTATATCCCTCA	AATAGGCGCTTGATTGGTGG	595

T7 endonuclease assay/gel electrophoresis/purification

Following PCR, amplicons were treated with the T7 Endonuclease I Kit (New England Biolabs) following the manufacturer's instructions, to identify heterozygous mutations following CRISPR/Cas9 injections. Following the enzyme digest samples were immediately run on a 1.5% (w/v) agarose gel with 5 µL of SYBR safe and visualised using a gel imager. Samples were purified using the ExoSAP-IT Product Cleanup Kit (Applied Biosystems) in accordance to manufacturer's instructions and outsourced for Sanger sequencing.

RNA extraction and gDNA removal

Fish were culled using ice-water immersion, followed by severing of spinal cord. Whole brains were removed and snap frozen using liquid nitrogen. Samples were homogenised and total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's instruction. After extracting and purifying RNA, samples were treated using the RapidOut DNA Removal Kit (Thermo Scientific), following manufacturer's instructions to remove contaminating gDNA from RNA samples prior to conversion into cDNA.

cDNA synthesis

Following removal of gDNA from RNA, sample concentrations were measured using the ND 1000 spectrophotometer. To ensure the same amount of cDNA was synthesised for each sample a maximum of 1 µg of total RNA was used per reaction (the same total concentration of RNA was loaded into each reaction). Reverse transcription of mRNA to cDNA was done using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) following the manufacturer's protocol. After conversion into cDNA, samples were diluted with ddH_2O to a final concentration of 5 ng/µL. Samples were stored at -20°C until use.

Quantitative Real-Time PCR (RT-qPCR)/

Quantitative real-time PCR (RT-qPCR) assays were used to validate relative gene expression based on SYBR green detection. S*lit1-3* and *gr* primers were predesigned by qPrimerDB (Lu et al., 2018), *eef1a* and *bdnf* primers were based on previous studies (Parker et al., 2016; Tang et al., 2007). Primers were synthesised by Invitrogen and are listed in **Table 3**. The Roche LightCycler® 480 High Resolution Melting Master mix and the LightCycler® 96 (Roche Life Science) were used to amplify and detect the transcripts of interest. Thermal cycling conditions included an initial denaturation step at 95°C for 600s (recommended by manufacturer), 40 cycles of 95°C for 15s, 58°C for 20s and 72°C for 35s. Negative reverse transcription (RT) controls and negative template controls were used to determine specificity and to check for genomic DNA contamination. Elongation factor 1 alpha (*eelf1a*) was used as a housekeeping gene (Tang et al., 2007). Gene-expression levels were calculated using delta-delta CT method and normalised to *eelf1a*. Changes in expression were relative to controls to demonstrate fold changes in *slit-3* mutants. Data are presented as means ± SD (n = 6 per group; assayed in duplicate).

Table 3. Primer sets used for qPCR gene-expression analysis

Gene name	Gene	Primer sequence (Forward)	Primer sequence (Reverse)	Amplicon size
Eukaryotic translation elongation factor 1 alpha	*eef1a*	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGCATTAC	87
Slit homolog 1 protein	*slit-1*	TAAATGCCGCTGTGAGTCTAAT	TAAGAGTCTTGAAAGCACCCAT	149
Slit homolog 2 protein	*slit-2*	GGACTTACTGAAATACCCACCA	TATTCTTCGGAGTCGCTTGTAA	120
Slit homolog 3 protein	*slit-3*	ACTGACACTCCTAGACCTTAGT	AACAGTTGAAAGGTCATTTCCG	187
Glucocorticoid receptor	*gr*	ACGGTTCTATCAGCTCACTAAG	AAACTCCACGCTCAGAGATTTA	109
Brain-derived neurotrophic factor	*bdnf*	AACTCCAAAGGATCCGCTCA	GCAGCTCTCATGCAACTGAA	262

Statistical analysis

Data were analysed in IBM SPSS 25.0 and GraphPad Prism 8.4.2. Data from the FMP Y-maze were analysed in IBM SPSS, studentized residuals were generated from statistical models and screened for outliers. Any data point identified as an extreme value (>3*IQR) was removed from further analysis. Residuals were analysed for normality using histograms and Shapiro-Wilk normality test. All data were log transformed and a linear mixed model (LMM) was employed to assess alternations and repetitions, which, from previous work from our lab, have been identified as strategies susceptible to change with changing cognitive performance (Cleal et al., 2020; Cleal & Parker, 2018; Fontana, Cleal, Clay, et al., 2019). LMM analysis was carried out with "total turns" as the dependent variable, "condition" and "time" as the within-subjects factor and "ID" as a random effect. Analysis was followed by

Bonferroni-corrected *post-hoc* test. Locomotion was analysed using total turns completed during 1 h of exploration in the FMP Y-maze.

All other data were analysed using Graphpad prism, normality analysis used the Shapiro-Wilk test and screened for outliers using the ROUT outlier analysis (Q = 1%) and boxplot with Tukey whiskers. Outliers were removed from further analysis. Zone data from the novel tank diving test were analysed using two-way ANOVA followed by Sidaks multiple comparison test, distance travelled in the novel tank, total turns in the FMP Y-maze, ELISA and qPCR data were analysed using *t*-test for normally distributed data and Mann-Whitney U test for non-normally distributed data. Results were considered significant when $p \leq 0.05$.

Due to a tracking issues with the software, several fish were missing data from the behavioural tests. No data points were removed from ELISA or qPCR analysis due to small sample size.

2.4 Results

Genotyping *slit-3* mutation

The single guide RNA (sgRNA) targets exon 18 and overlaps the exon-intron boundary (**Fig. 1A**). Engineered nuclease-induced mutations are detected by various methods, including mismatch-sensitive T7 endonuclease I (T7E1) assay, DNA high resolution melt curve (HRM) analysis and Sanger sequencing. The T7E1 assay is used to detect heteroduplexes (formed by the hybridization of either two different mutant sequences or a wild type and mutant sequence) (**Fig. 1B**). Testing of *slit-3* samples with the T7E1 assay shows four samples with heterozygous mutations, as shown by a single band at ~600 bp and two other bands just below and just above 300 bp, all of reduced intensity compared to WT. All other *slit-3* samples and WT samples show a single band, of increased intensity, at ~600 bp. However, this method has the limitation that only heterozygous mutations can be detected, thus homozygous biallelic mutations cannot be distinguished from WT samples. Therefore, to distinguish between samples that did not carry a heterozygous mutation, as noncarriers or carriers of homozygous mutations, HRM

analysis was performed. This post-PCR analysis is used to identify variations in nucleic acid sequences, by detecting small differences in the melt curves of PCR amplicons. *Slit-3* and WT samples analysed using HRM showed three distinguishable melt curves representing homozygous WT, homozygous mutant and heterozygous mutant (**Fig. 1C**). As expected, samples with the heterozygous melt curve (red) corresponded with samples showing heteroduplexes in the T7E1 assay, further confirming correct identification of heterozygous *slit-3* mutations. Interestingly, the HRM method is a more efficient method for detecting homozygous mutations and differences between homozygous mutants and homozygous WT. To check for transcriptional differences and compensatory mechanisms, we assessed mRNA expression of *slit-1, 2* and *3*, but found no differences in *slit-3* mutants compared to WT controls (**Fig. 1D-F**).

Figure 1.

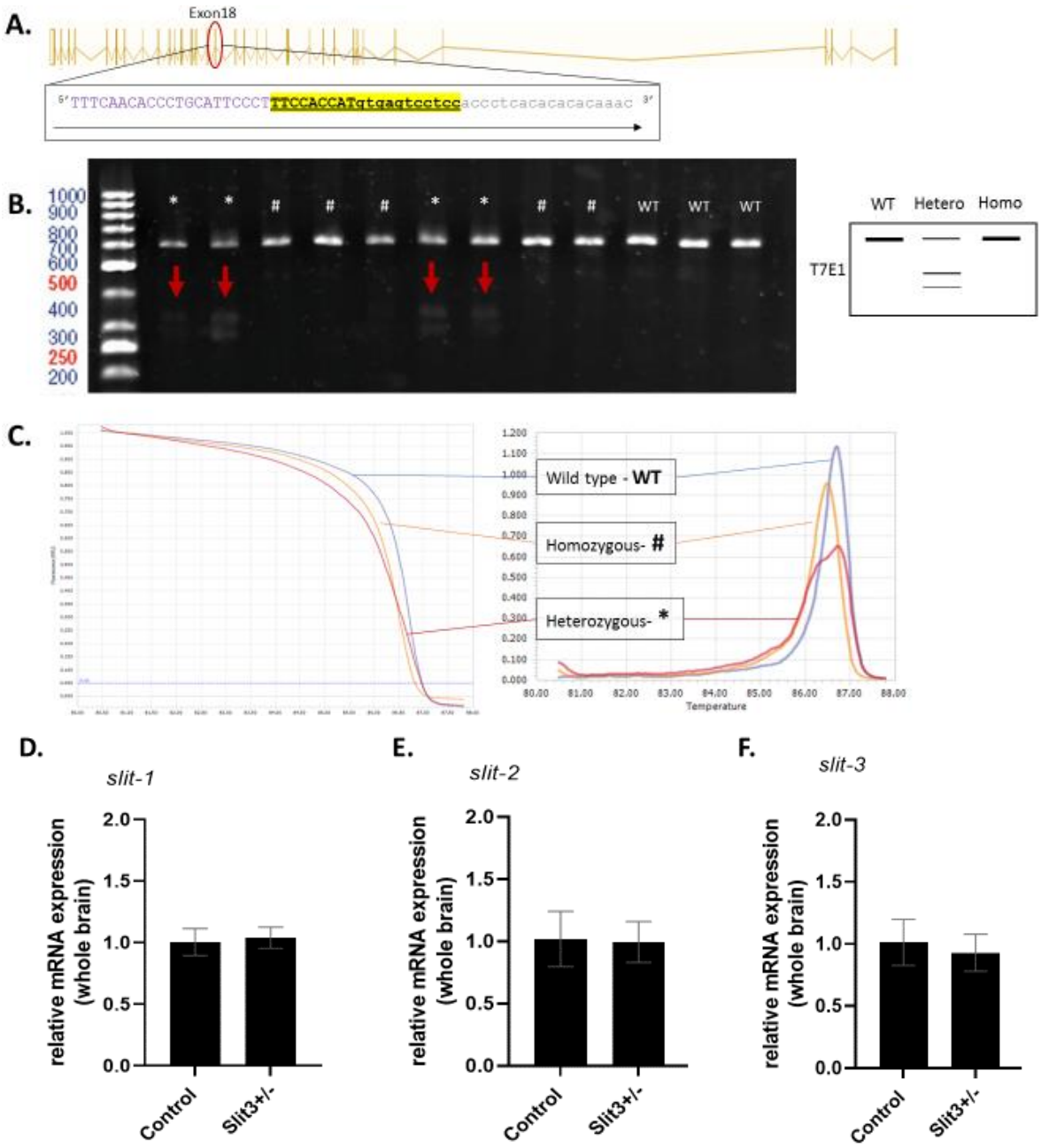
A.
Exon18
5'TTTCAACACCCTGCATTCCCTTTCCACCATgtgagtcctccaccctcacacacacaaac 3'
B.
1000
900
800
700
600
500
400
300
250
200
* * # # # * * # # WT WT WT
WT Hetero Homo
T7E1
C.
Wild type - WT
Homozygous- #
Heterozygous- *
Temperature
D.
slit-1
relative mRNA expression (whole brain)
2.0
1.5
1.0
0.5
0.0
Control
Slit3+/-
E.
slit-2
relative mRNA expression (whole brain)
Control
Slit3+/-
F.
slit-3
relative mRNA expression (whole brain)
Control
Slit3+/-

Figure 1. (A)(upper) Schematic of *slit-3* exons and introns in zebrafish with identification of exon 18 sgRNA target and target sequence (lower). Capital letters represent exon sequence, lower case letters represent intron sequence, highlighted area represents sgRNA sequence. (B) agarose gel with T7E1 assay of *slit-3* injected and WT samples. * = heterozygous mutation, # = homozygous mutation, WT = wild type control. Red arrows indicate heteroduplexes (left). Schematic of T7E1 results with WT, heterozygous and homozygous mutations (right). (C) HRM analysis of heterozygous (red), homozygous mutant (orange) and homozygous wild type (purple) represented in normalised melt curves (left) and normalised melt peaks (right). (D-F) whole-brain mRNA expression of *slit-1* (D), *slit-2 (*E) and *slit-3 (*F) (n = 6 per group). All data were analysed for normality using Shapiro-Wilk test. Normally distributed data were analysed using *t*-test, non-normally distributed data were analysed using Mann-Whitney U test. Bars are mean and error bars are mean ± SD, **p*<0.05, ***p*<0.01.

Slit-3 mutation impairs WM, enhances stereotypic behaviour and increases anxiety responses

To understand the impact of this mutation on adult behaviour, we first analysed baseline cognitive function. WM performance was analysed using relative percentage use of alternations (RLRL+LRLR) during 1 h of free exploration in the FMP Y-maze. There was a significant effect of condition (control v *slit-3*$^{+/-}$) ($F_{1,134.36}$ = 5.278, p = 0.023) on alternations (**Fig. 2A**). Relative to noncarrier control fish, the transgenic fish exhibited a decrease in global alternation use showing a decrease in WM performance. Stereotypic behaviour was analysed using relative percentage use of repetitions [RRRR+LLLL], which revealed a significant increase in repetitions in *slit-3* compared to WT controls ($F_{1,129.10}$ = 10.046, p = 0.002), demonstrating an increase in stereotypic behaviour. Having demonstrated altered WM processing and increased stereotypic behaviour in *slit-3* mutants, we analysed anxiety responses using the novel tank diving test. *Slit-3* mutants showed a significantly increased anxiety response, with more time spent bottom dwelling compared to WT (Mann-Whitney U = 62, n_1 = 18 n_2 = 19, *p* = 0.0039, two-tailed) (**Fig. 2E**). However, unlike in the FMP Y-maze the *slit-3*$^{+/-}$ mutants showed significant hyperlocomotion in the novel tank compared to WT controls. Suggesting that hyperlocomotion may be

part of the increased anxiety response that is not evident after substantial habituation to a test arena. (**Fig. 2F**).

Figure 2.

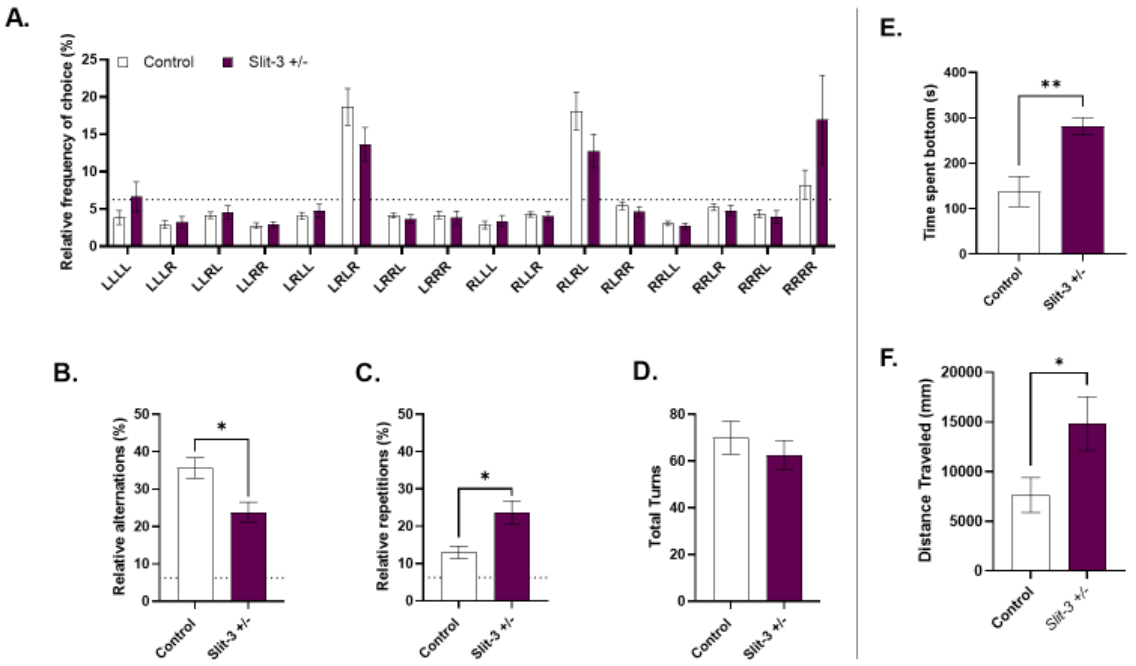

Figure 2. Effect of *slit-3* mutation on global search strategy of adult zebrafish in the FMP Y-maze compared to WT controls after 1 h of free exploration **A**). Comparison of relative percentage use of alternations (mean RLRL+ mean LRLR) **B**) and repetitions (mean RRRR+ mean LLLL) **C**) and total turns of *slit-3* mutants compared to controls. Data were log transformed and analysed using LMM (n = 18 controls, n = 11 *slit-3*). Analysis of anxiety response measured as time spent in the bottom in the novel tank diving test **E**) and locomotor response **F**) of *slit-3* versus controls. Data were checked for normality using the Shapiro-Wilk test. Normally distributed data were analysed using the paired t-test, non-normally distributed data were analysed using the Mann-Whitney U test (n = 18 controls, n = 19 *slit-3*). * $p \leq 0.05$, ** $p \leq 0.01$. The dashed *line* denotes chance performance (6.25%). Bars are mean, error bars are mean ± SEM.

We assessed the effect of acute stress on cognitive performance of control and *slit-3* fish on global search strategy (**Fig. 3A**). We found a trend towards significance of treatment group on percentage use of alternations ($F_{3,143.84}$ = 2.57, p = 0.056), with *slit-3* and *slit-3*+stress showing a slight decrease in the use of alternations compared to controls (**Fig. 3B**). Analysis of stereotypic behaviour showed a significant effect of treatment on relative repetitions ($F_{3,168.16}$ = 3.892, p = 0.010), Bonferroni *post-hoc* analysis revealed a significant difference between controls compared to *slit-3* (CI, -0.417 - -0.038, *p* = 0.009), however, there was no difference between WT controls and control +stress (CI, -0.366 - -0.273, *p* = 1.000) or *slit-3*+stress (CI, -0.456 - -0.057, *p* = 0.235) (**Fig. 3C**). Our data demonstrate that acute stress attenuates stereotypic behaviour of the *slit-3* mutation.

Figure 3.

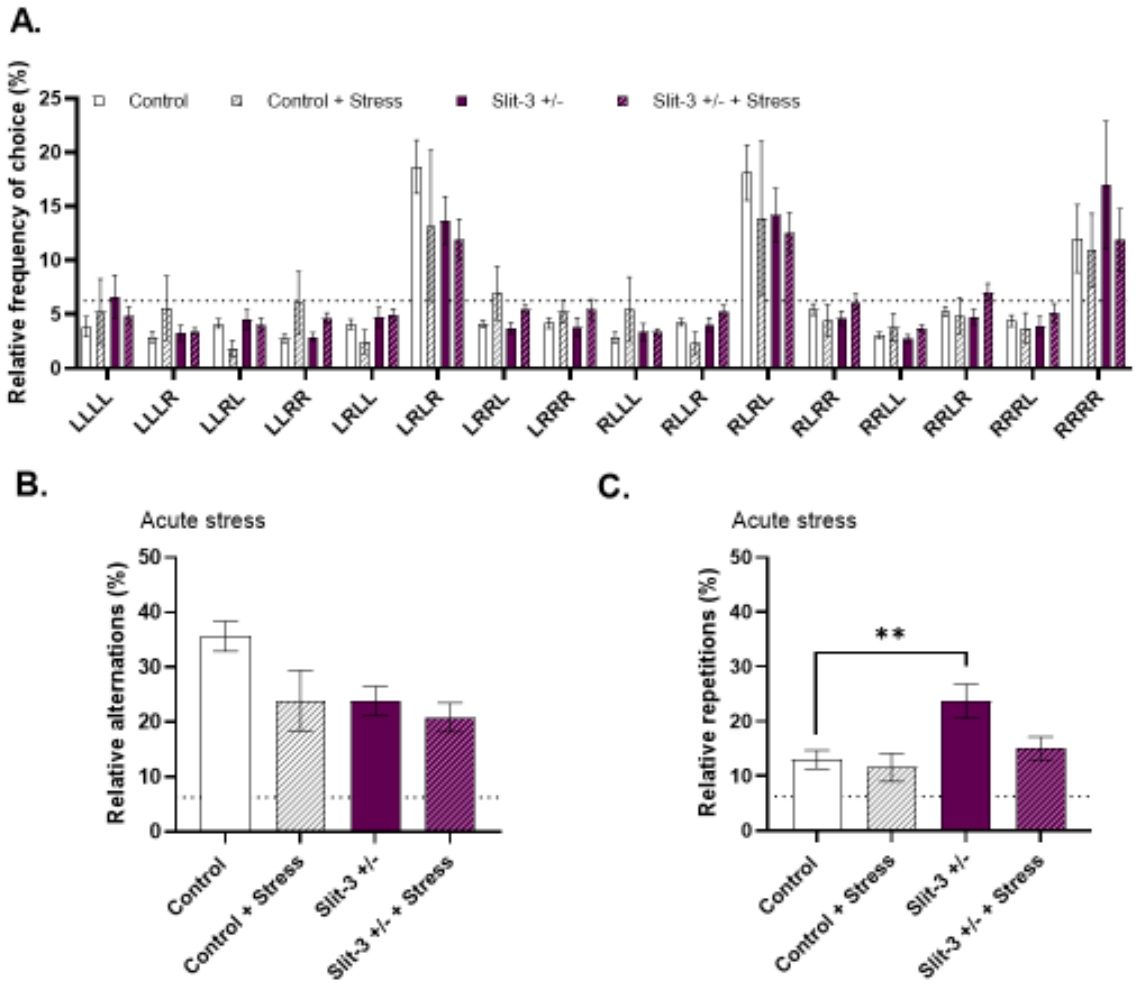

Figure 3. Effect of acute stress on control and *slit-3* transgenic fish on global search strategy in the FMP Y-maze after 1 h of free exploration **A**). Comparison of relative percentage use of alternations **B**) and repetitions **C**) of controls compared to control +stress, *slit-3* and *slit-3* +stress. Data were log transformed and analysed using LMM (n = 18 controls, n = 4 control +stress, n = 11 *slit-3*, n = 9 *slit-3* +stress). ** $p \leq 0.01$. The dashed *line* denotes chance performance (6.25%). Bars are mean, error bars are mean ± SEM.

Slit-3 mutant exhibit aberrant HPI axis activation

The HPA axis in humans and the HPI axis in zebrafish has been found to be dysregulated in patients with schizophrenia and in some zebrafish schizophrenia-like models. Having identified that *slit-3*$^{+/-}$ mutants exhibit an increased anxiety response in the novel tank and having shown that acute stress decreases the alternations in the FMP Y-maze, we analysed whole-body cortisol following acute stress. We found that in comparison to WT there was a significant decrease in cortisol levels in *slit-3*$^{+/-}$ mutants (*t*-test; $t = 3.49$, df = 5, $p = 0.018$) (**Fig. 4A**). In addition, qPCR analysis revealed a significant upregulation of basal levels of *bdnf* (*t*-test; $t = 2.79$, df = 6, $p = 0.032$) in *slit-3*$^{+/-}$ mutants compared to WT (**Fig. 4B**), but no difference in *gr* expression (Mann-Whitney U = 1.0, $n_1 = n_2 = 2$, $p = 0.667$, two-tailed) (**Fig. 4C**).

Figure 4.

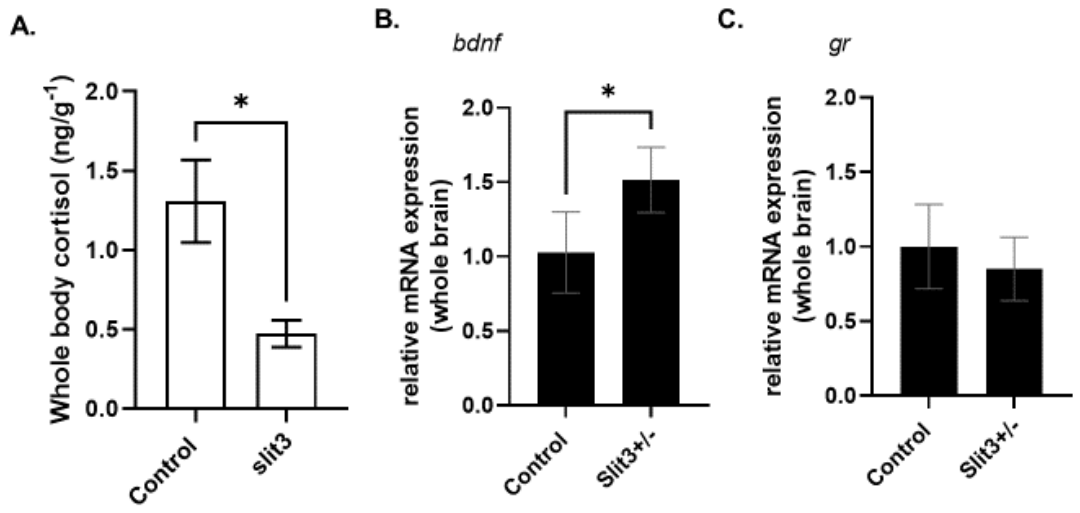

Figure 4. **A**) Whole body cortisol following acute stress showed significant down regulation of cortisol levels in *slit-3*$^{+/-}$ mutants compared to WT (n = 4 per group). Quantitative RT-PCR for *slit-3*$^{+/-}$ mutants shows an increase in *bdnf* expression (n = 4 per group) **B**) but no difference between mutants and WT in *gr* whole brain mRNA expression (n = 2 per group) **C**). All data were analysed for normality using Shapiro-Wilk test. Normally distributed data were analysed using *t*-test, non-normally distributed data were analysed using Mann-Whitney U test. Bars are mean and error bars are mean ± SD, *$p<0.05$, **$p<0.01$.

Nicotine alleviates cognitive deficits and anxiety responses in *slit-3*$^{+/-}$ mutants

The comorbidity between high smoking and schizophrenia is well documented and is hypothesised to ameliorate the underlying neurochemical deficits associated with schizophrenia (D'Souza & Markou, 2012; De Leon & Diaz, 2012; Hartz et al., 2018; Lucatch et al., 2018). We investigated if *slit-3*$^{+/-}$ mutants exhibited changes in cognitive or anxiety responses. Our findings suggest that a single 20 min pre-treatment with nicotine was sufficient to enhance cognitive performance to control levels, resulting in no significant difference between *slit-3* and control groups on the percentage use of alternations ($F_{1,112}$ = 3.26, p = 0.074) (**Fig. 5A**). Nicotine pre-treatment also alleviated stereotypic behaviour, by decreasing repetitions in *slit-3*, whilst maintaining control repetitions to near random selection ($F_{1,111}$ = 1.878, p = 0.173) (**Fig. 5A**). Additionally, nicotine also exhibited an anxiolytic effect on *slit-3* fish, decreasing anxiety responses in the novel tank (*t*-test; *t* = 1.036, df = 18, *p* = 0.314) (**Fig. 5B**), resulting in no difference between *slit-3*$^{+/-}$mutants and WT fish.

Figure 5.

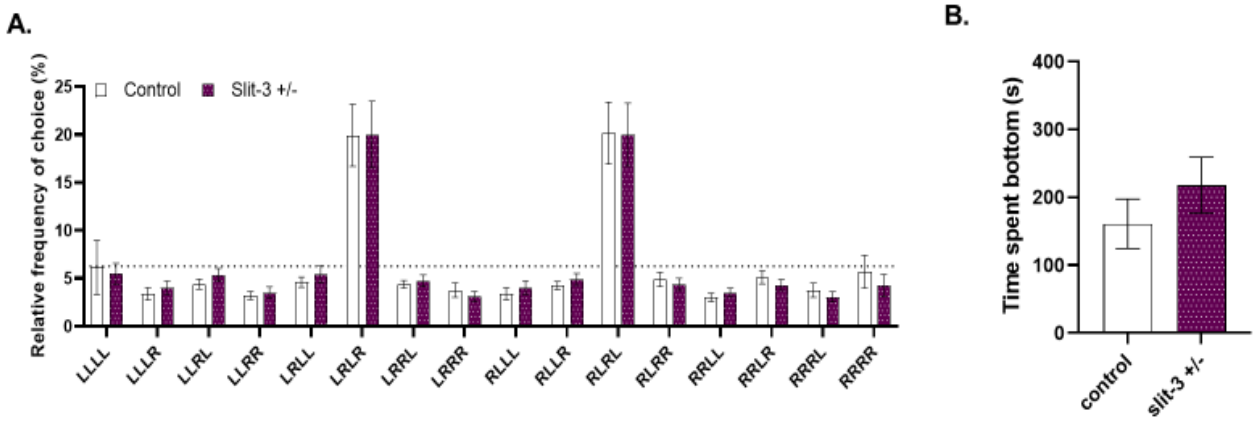

Figure 5. Effect of 5 µM nicotine on global search strategy of control and *slit-3* mutant fish in the FMP Y-maze **A**). Data were log transformed and analysed using LMM (n = 13 controls, n = 10 *slit-3*). Analysis of anxiety response measured as time spent in the bottom in the novel tank diving test **B**) of nicotine treated *slit-3* mutants compared to nicotine treated controls. Data were checked for normality using the Shapiro-Wilk test. Normally distributed data were analysed using the paired t-test, (n = 13 controls, n = 10 *slit-3*). The dashed *line* denotes chance performance (6.25%). Bars are mean, error bars are mean ± SEM.

Ethanol induces an increased CPP response, but has no effect on anxiety

Like nicotine, ethanol is known to have anxiolytic properties. We therefore hypothesised that ethanol would have a similar effect on anxiety responses to nicotine in *slit-3*$^{+/-}$ mutants. To make the study comparable we tested a concentration of nicotine and ethanol that both were reported to induce an increased preference for the drug-paired side in the CPP. As previous studies have investigated the effects of 5 μM nicotine on inducing increased CPP response in different *slit-3* variants, we tested ethanol with our *slit-3*$^{+/-}$mutant line. Our findings showed that even with low doses of ethanol, *slit-3* fish showed a greater than 10% increased preference for the drug-paired side compared to controls (*t*-test; $t = 2.80$, df = 16, $p = 0.013$) (**Fig. 6A**). Having identified that 171 mM of ethanol was sufficient to increase *slit-3* ethanol preference in the CPP, we tested the effect of this concentration of ethanol on anxiety responses on *slit-3* mutants in the novel tank diving test. We found that an effective dose of ethanol in the CPP was not sufficient to alleviate anxiety responses of *slit-3* mutants in the novel tank (*t*-test; $t = 4.28$, df = 22, $p = 0.0003$) (**Fig. 6B**). To assess treatment effects on anxiety (*slit-3* mutation, *slit-3*+nicotine, s*lit-3*+ethanol) we pooled control data and compared tank diving responses of treated and untreated *slit-3* mutants compared to WT controls. There was an overall effect of condition (Kruskal-Wallis test; Kruskal-Wallis statistic = 17.67, $p = 0.0005$) on tank diving response, with *post-hoc* analysis revealing a significant effect of *slit-3* with and without ethanol on increased anxiety responses compared to controls (control vs. *slit-3;* $p = 0.0013$: control vs. *slit-3*+ethanol; $p = 0.026$), but no difference between each other (*slit-3* vs. *slit-3*+ethanol; $p > 0.999$). There was no difference between *slit-3*+nicotine and WT controls (control vs. *slit-3*+nicotine*;* $p = 0.850$) and no difference between *slit-3*+nicotine and *slit-3* or *slit-3*+ethanol (*slit-3* vs. *slit-3*+nicotine*;* $p = 0.921$: *slit-3*+ethanol vs. *slit-3*+nicotine; $p > 0.999$) (**Fig. 6C**). Thus, our data suggests that reinforcing concentrations of nicotine and ethanol do not have equivalent anxiolytic effects on anxiety responses in *slit-3* mutants.

Figure 6.

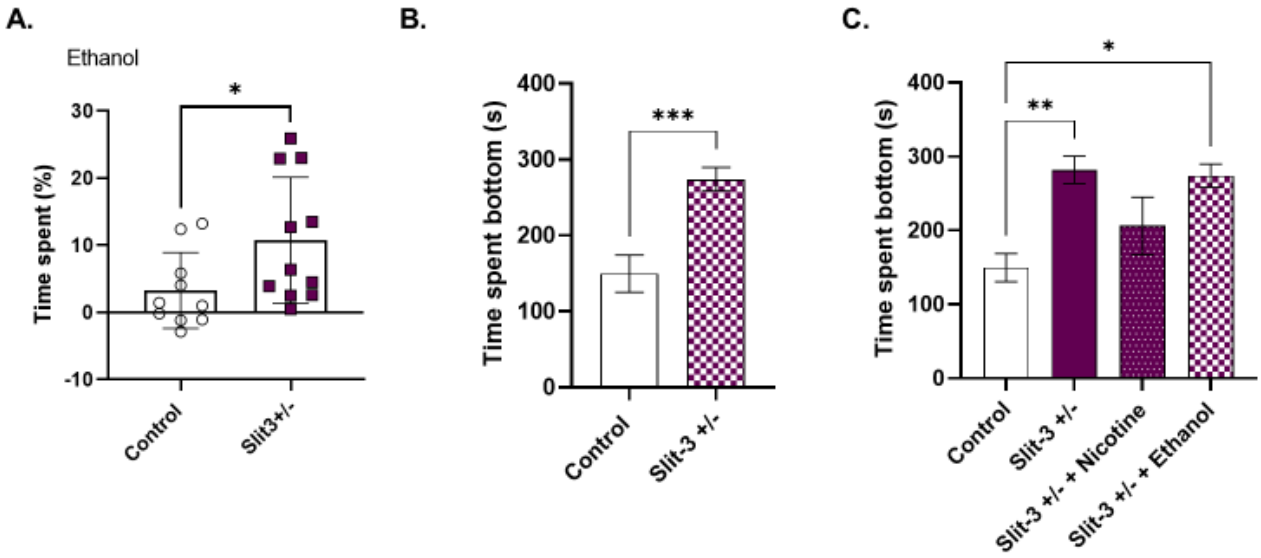

Figure 6. Effect of 171 mM ethanol on CPP of control and *slit-3* mutant **A**). Analysis of anxiety response measured as time spent in the bottom in the novel tank diving test of ethanol treated *slit-3* mutants compared to ethanol treated controls **B**). Comparison of novel tank response, based on time spent in the bottom zone, of WT, *slit-3, slit-3* + nicotine and *slit-3* + ethanol **C**). Data were checked for normality using the Shapiro-Wilk test. Normally distributed data were analysed using the paired t-test, (n = 13 controls, n = 10 *slit-3*) or One-way ANOVA, non-normally distributed data were analysed using Kruskal-Wallis test. Bars are mean, error bars are mean ± SEM. *p<0.05,

2.5 Discussion

There is evidence that SLIT-3 is related to SZ in humans (Shi et al., 2004), but as yet there are no animal models to investigate these findings. Here we generate a *slit-3* mutant line in zebrafish to examine SZ phenotypes. This study provides evidence of a role of mutations in the gene *slit-3* in aberrant SZ-like behaviours which recapitulates multiple characteristics of human SZ patients, including cognitive deficits, increased stereotypic behaviours, increased anxiety responses, attenuation of cortisol levels following acute stress and increased sensitivity to drugs of abuse. Additionally, we demonstrate a therapeutic role for nicotine in reducing anxiety behaviours and enhancing cognitive performance, providing a possible explanation for high prevalence of tobacco smoking in SZ patients.

Characterisation of CRISPR/Cas9 genome editing of the *slit-3* gene in zebrafish detected two mutant populations, a heterozygous and homozygous mutation, identified using multiple methods for genotyping. Due to time restrictions, all experiments were conducted on mixed populations of heterozygous and homozygous mutations as test populations were generated directly from CRISPR/Cas9 injected embryos (f0) to provide pilot data on the behavioural and biochemical phenotypes associated with the mutation. We analysed mRNA expression of *slit* genes and revealed no differences in whole-brain mRNA expression of *slit-1*, *2* or *3*, between WT and *slit-3* injected groups. Although some mutations can cause changes in mRNA expression levels of the target gene (Eachus et al., 2017), many functional mutations do not affect mRNA expression and rather, result in altered protein expression. Here, however, lack of mRNA expression changes may be due to the analysis of whole-brain tissue, opposed to regional amplification, as previous studies have identified localised regions of *slit-3* expression in the brain, particularly in the frontal cortex, dentate gyrus and hippocampus (Piper et al., 2000; Sasaki et al., 2020). Additionally, *slit-3* has been shown to be differentially regulated during embryonic compared to adult stages, implicating a role for spatiotemporal pattens affecting gene expression (Marillat et al., 2002). Therefore, further characterisation of the *slit-3* mutation in zebrafish to assess regional expression changes in the brain, mRNA and protein expression during multiple stages of development, as well as differences between homozygous and heterozygous mutations is required.

Despite the need of further characterisation of functional changes of *slit-3* following point mutations, clear and obvious behavioural differences were evident between *slit-3* mutants and WT controls. Primarily, there were distinct exploratory differences pertaining to WM performance and stereotypic behaviours that were evident when tested in the FMP Y-maze. *Slit-3* mutants demonstrated impaired WM performance and increased stereotypic behaviour compared to controls. WM deficits are regularly reported in patients and animal models of SZ-like behaviour (Carless et al., 2011; Castner & Williams, 2007; Eryilmaz et al., 2016; Frydecka et al., 2014). However, despite cognitive deficits being described as a core characteristic, no zebrafish model of SZ has to date reported cognitive function. Here, we describe the first SZ-like model to demonstrate cognitive deficits in zebrafish, in line with human performance. Like cognitive impairment, stereotypy is also a distinct feature of SZ and has been defined as a repetitive motor action (Bush et al., 1996; Morrens et al., 2006). In the FMP Y-maze, stereotypic behaviour has been attributed to increased use of the strategy termed 'repetitions', which is circling behaviour consisting of the repetitive use of left or right turns (LLLL, RRRR) to an extent greater than chance selection. In SZ patients, studies examining stereotypies have reported a correlation between stage of illness and stereotypic behaviour, suggesting that stereotypic behaviour progresses with illness stage, and is not present during early/prodromal stages (Morrens et al., 2006). It would, therefore, be interesting to investigate if stereotypic responses seen here are also evident during adolescence, as behavioural markers of disorder progression in an animal model of SZ would prove invaluable in understanding illness trajectory.

Abnormal stress sensitivity is thought to be a major contributing factor to illness onset, exacerbation and relapse in SZ (Brunelin et al., 2008). Aberrant activation of the HPA axis has not only been associated with psychosis risk, but it has also been found to play a role in cognitive function (Baumeister et al., 2014; Cherian et al., 2019; McCormick & Mathews, 2010; van Looveren et al., 2019). We examined the effects of acute stress on cognition in *slit-3* mutants and found a trend towards significance of lowered WM performance of stressed and non-stressed *slit-3* mutants and stressed controls compared to non-stressed controls. Post-hoc analysis revealed a greater reduction in

alternations in stressed and non-stressed *slit-3* mutants compared to non-stressed controls than stressed versus non-stressed control fish. Unexpectedly, the stressed *slit-3* mutants showed a reduced effect of stereotypies, whilst non-stressed *slit-3* fish maintained a much higher rate of repetitions compared to non-stressed controls. The trend in reduced alternations of *slit-3* fish, but lack of significance, suggests that stressed groups may be underpowered and therefore, both WM performance and repetitive behaviours should be interpreted with care.

It has been progressively recognised that there is an inter-play between genetic vulnerabilities and acute and chronic stress exposures which interact to cause emergence and maintenance of neuropsychiatric disorders (Gomes & Grace, 2017; Howes et al., 2017; Howes & Murray, 2014). With the suggestion of maintained WM deficits in both stressed and non-stressed *slit-3* mutants, we investigated the underlying activity of the HPI axis based on cortisol levels and mRNA expression of *glucocorticoid receptors (gr)* and *brain-derived neurotrophic factor* (*bdnf*), all critical elements of the HPI axis. Acute environmental stress not only failed to up-regulate cortisol levels in *slit-3* mutants, but actually showed a significant down-regulation of whole-body cortisol levels following acute stress, compared to control levels. Dysregulation of HPA axis function is one of the most consistent biological findings in SZ patients, and has been linked with altered stress-vulnerability and behavioural phenotypes in SZ and other serious mental illnesses (Baumeister et al., 2014). Evidence suggests that abnormalities in HPA axis activation may manifest as either hyper- or hypo-functioning, both conditions occurring in SZ and correlating with cognitive impairment (Baumeister et al., 2014; Shah & Malla, 2015). Studies have shown that SZ patients and patients that are clinically high risk for the development of psychosis or those having experienced their first episode of psychosis have all demonstrated a blunting effect of acute cortisol response following an acute stressor (Shah & Malla, 2015). Cortisol has shown an inverted U-shaped dose-response curve with respect to cortisol levels and cognitive function, with both high and low concentrations resulting in deficits in cognitive performance (Cherian et al., 2019; Holtzman et al., 2013; Lupien & McEwen, 1997). Here, we demonstrate that the *slit-3* mutation causes hypoactivation of the HPI axis, which coincides with cognitive impairment.

BDNF and *GR* both play critical roles in the activation and modulation of the stress response via control of HPA axis reactivity. Aberrant genetic and functional activity of both regulators have been implicated in SZ (Garabedian et al., 2017; Jeanneteau et al., 2012). *BDNF* has been identified as a crucial neurotrophin in the regulation of synaptic plasticity, learning and memory, the dysregulation of which can have detrimental effects on cognition. Rodent models of BDNF overexpressing mice and genetic variations that increase BDNF levels, result in cognitive impairment and increased anxiety-related phenotypes (Jeanneteau et al., 2012; Lu & Martinowich, 2008; Papaleo et al., 2011), as seen in *slit-3* mutant fish. Additionally, up-regulation of BDNF has been implicated in SZ patients and, in combination with attenuated cortisol levels, implicate dysfunctional HPI axis reactivity in the SZ-like phenotypes identified in *slit-3* mutant zebrafish. However, we did not detect any differences in *gr* expression in *slit-3* compared to WT fish. Although *gr* have also been implicated as an important regulator of HPI axis functioning (Derek Alsop & Vijayan, 2009; Liu et al., 2020; van Looveren et al., 2019). Previous studies of SZ models have identified sex differences in *gr* expression levels following acute stress (Desbonnet et al., 2019), therefore, differences could potentially be masked by combined sex groups or by the small sample sized used for assessing *gr* expression compared to *bdnf* and should therefore be repeated with increased power and potentially investigating sex differences. However, identification of sex differences would also be of interest in stimulated cortisol responses and *bdnf* expression levels and should be investigated further.

Having identified impaired cognition, increased stereotypies and increased anxiety responses, potentially resulting from aberrant HPI axis activity, we investigated the effect of nicotine on these behaviours, to identify a relationship between the effect of nicotine exposure on SZ-like behaviours and the extremely high prevalence of tobacco smoking the SZ population. Theories of nicotine abuse in SZ have been attributed to several factors including self-medication, alleviation of negative symptoms and cognitive enhancing effects of nicotine. Although the 'self-medication' hypothesis has been met with much scepticism (De Leon & Diaz, 2012; Prochaska et al., 2008; Ziedonis et al., 2008), there have been several studies in animals and humans, and in both SZ and non-SZ patients/models that have reported

anxiolytic and pro-cognitive effects of nicotine (Bencan & Levin, 2008; Brielmaier et al., 2008; D'Souza & Markou, 2012; Ghatan et al., 1998; Gómez et al., 2008; Heishman et al., 2010; Jasinska et al., 2014; Levin et al., 2007; Patterson et al., 2010; Rezvani & Levin, 2001; Valentine & Sofuoglu, 2017). Previous work has demonstrated that in WT adult zebrafish, nicotine sensitization can have pro-cognitive effects on increased WM performance in the FMP Y-maze, depicted by increased alternations, that was evident even in the absence of baseline impairment (see Part 2; Chpt 4). Therefore, we hypothesised that in a SZ-like model, that demonstrates baseline cognitive impairment, acute pre-treatment with nicotine may improve cognitive performance in the FMP Y-maze.

We found that even a low concentration of nicotine had cognitive enhancing effects and was sufficient to increase WM performance of *slit-3* mutants to control levels. Not only did nicotine improve WM, but it also attenuated stereotypic behaviours, reducing the use of the repetition strategy to random selection and reduced anxiety responses to WT levels. Since the identification of increased smoking in SZ patients, there have been many studies conducted to identify the therapeutic potential of nicotine and nicotinic agonists in attenuating aberrant behaviours and dysregulation of underlying biochemical processes associated with SZ (Dondé et al., 2020; Evins et al., 2019; Jacobson, 2014; Parmar et al., 2020; Potasiewicz et al., 2019; Tregellas & Wylie, 2019). However, to our knowledge this is the first experiment examining nicotinic effects in a zebrafish SZ-like model of hypo-functioning HPI axis activity. The large number of human studies reporting pro-cognitive effects of nicotine-based treatments in SZ and the replication of these findings in *slit-3* mutant zebrafish provides evidence of a promising model for examining comorbidities between SZ and substance abuse. Recent human studies have shown that nicotine improves WM performance by enhancing activation and functional connectivity between brain regions that mediate performance, such as the prefrontal cortex and the thalamus (Dondé et al., 2020; Evins et al., 2019; L. Jacobson, 2014; Parmar et al., 2020; Potasiewicz et al., 2019; Tregellas & Wylie, 2019). As previously discussed altered functional connectivity in the cortical-striatal-thalamo-cortical (CSTC) network can significantly impair cognition and has been particularly noted for WM deficits (Floresco et al., 1999) (see Part 1: Chpt 1).

Additionally, many SZ studies, in rodents and humans, have identified altered connectivity in the CSTC loop and it is, therefore, interesting to note that aberrations in *slit-3* functioning may disrupt CSTC networks, particularly as spatiotemporal studies of *slit-3* expression have identified frontal cortices as a significant region of *slit-3* expression (Berni, 2015; Chisholm et al., 2016; Sasaki et al., 2020). It is also interesting to note that decreased gray matter has been associated with loss of structural and functional integrity of aspects of the CSTC loop resulting in impairment of cognitive control and has been implicated in a number of psychiatric disorders, including SZ (Peters et al., 2016). A recent study by (Ma et al., 2019) has identified *SLIT-3* as a risk gene for altered gray matter volumes in the frontal cortex. Thus, potentially identifying a mechanism through which *slit-3* affects cognition and nicotine restores it. This avenue of enquiry requires further investigation and may provide novel insights into the mechanisms underlying SZ-induced cognitive impairment and a potential therapeutic target.

Substance abuse has high comorbidity with SZ (Goodwin et al., 2003), particularly, as noted above, with nicotine. Increased prevalence and sustainment of smoking behaviour has been suggested to be resultant of difficulties in cessation of smoking (D'Souza & Markou, 2012; Evins et al., 2019). Having identified that this may in part be due to attenuation of negative symptoms and cognitive enhancing effects, it is possible that other drugs of abuse with anxiolytic properties may exert similar beneficial effects on SZ behaviours and thus perpetuate continued use. We therefore assessed effects of ethanol on alterations in drug preference and anxiety response in fish carrying the *slit-3* mutation. We did not investigate cognition as ethanol, unlike nicotine, is not a cognitive enhancer, therefore any benefits would likely be derived from its anxiolytic or rewarding effects. We firstly identify if *slit-3* mutants have increased sensitivity to the reinforcing properties of ethanol, as has previously been described by (García-González et al., 2020) with nicotine, in the CPP task. As with nicotine, *slit-3* mutants showed, on average, a 10% increase to the drug-paired side in the CPP compared to non-mutant controls. Having identified the reinforcing effects of ethanol on *slit-3* fish, we tested the effect of pre-treatment on anxiety responses and, surprisingly, found that despite many reports of the anxiolytic properties of ethanol, pre-treatment did not alter anxiety response compared to untreated *slit-3* mutants, and

demonstrated significantly greater anxiety in the novel tank test than WT controls. Alleviation of anxiety responses and pro-cognitive effects of nicotine may be a reason why there is a greater association between SZ and tobacco smoking than SZ and alcoholism and additionally why therapeutic strategies for improving cognitive symptoms in SZ target nicotinic pathways. As *slit-3* mutants have demonstrated, increased sensitivity to the reinforcing properties of nicotine and ethanol in the CPP, and both drugs have reported anxiolytic properties, it is possible that their differential effect on behaviour is a consequence of differences in their interaction with the HPI axis.

Nicotine is well known for increasing cortisol levels after smoking, which, in the presence of a pre-existing down-regulation of cortisol, may be an additional mechanism through which nicotine improves cognitive function. However, studies of alcohol ingestion in humans have identified a potential role for high versus low doses of alcohol influencing either elevated or lowered plasma cortisol levels, respectively. However, the low levels of alcohol are more notably associated with anxiolytic responses, whilst high doses have been reported to cause anxiety-like behaviours (Blaine et al., 2016; Dai et al., 2002, 2007). The absence of anxiolytic effect seen in *slit-3* mutants, pre-treated with ethanol, may be a consequence of their basal alterations in HPI axis activity prior to ethanol exposure. Further examination of the altered concentration of ethanol pre-treatment may indicate dose-related shift in anxiolytic properties of ethanol in neuropsychiatric conditions with hypo- or hyper functionality of the HPI axis. Future studies with *slit-3* mutant fish should investigate how drug pre-treatment alters cortisol levels after acute stress, as it would be hypothesised that nicotine would diminish cortisol level differences between *slit-3* and controls, whilst at the dose given here, it would be expected that *slit-3* fish would maintain down-regulation of cortisol response.

2.6 Conclusion

This study provides the first evidence of a reliable animal model of *slit-3* induced SZ-associated molecular, cognitive and behavioural alterations. *Slit-3* mutant zebrafish recapitulate a broad spectrum of SZ-like phenotypes that are typically reported in patients diagnosed with SZ. Here we describe, cognitive impairments relating to reduced WM performance, increased stereotypies, heightened anxiety responses, attenuated HPI axis reactivity and alterations to the rewarding effects of drugs of abuse. In addition, we identify behavioural alterations in response to nicotine exposure, that may implicate several pathways in the increased comorbidity of tobacco smoking in SZ patients and possible therapeutic mechanisms for treating negative and cognitive symptoms associated with SZ. We also note that not all drugs with anxiolytic properties may have therapeutic effects, highlighting a need to better understand how basal biochemical mechanisms can influence anxiolytic properties of drugs. This work further supports the use of zebrafish as a model for investigating a broad spectrum of symptomologies associated with complex neuropsychiatric disorders, including abnormalities in cognitive processing. We also highlight a complicated and multifaceted role for slit-3 in the development of psychiatric conditions and mental health.

Chapter 3

The previous chapter describes a new zebrafish slit-3 model of SZ-like phenotypes, that demonstrated dysfunction of cognitive processes, increased stereotypic and anxiety-like behaviours and abnormalities in HPI axis activity and regulation. To further validate this model as exhibiting SZ-like behaviours we assessed transgenic zebrafish carrying a mutation in one of the most well studied SZ-risk genes, disc1. The following chapter describes the cognitive performance, anxiety responses and HPI axis activity in disc1 mutant zebrafish. The penultimate chapter of this section discusses the similarities and differences between the two transgenic lines.

Disrupted-in-Schizophrenia-1 (disc1) Alters Anxiety Behaviour, Cognition, Glucocorticoid and Dopamine Receptor Expression

3.1 Abstract

Disrupted-in-schizophrenia 1 *(DISC1*) has been identified as a high susceptibility gene for developing neuropsychiatric disorders, such as schizophrenia. Human and rodent studies have implicated *DISC1* in stress response and cognitive abnormalities. However, the underlying mechanisms are poorly understood. Additionally, only recently have zebrafish been utilised for understanding psychiatric disorders, such as schizophrenia and information regarding conservation of *disc1* associated changes, particularly cognitive impairment, have not been fully investigated. Here, we demonstrate that juvenile zebrafish carrying the *disc1*^{Y472} nonsense, point mutation exhibit prolonged anxiety responses, and show severe cognitive deficits, including WM impairment and the loss of strategized exploration. Quantitative RT-PCR revealed up-regulation of *gr* mRNA expression and down-regulation of *drd5* mRNA expression in the brain. Our study highlights severe cognitive deficits in *disc1* mutants that can be reliably modelled in zebrafish during juvenile stages of development. Additionally, we have identified a novel role for altered *gr* and *drd5* expression underlying abnormal stress response and cognitive function. Here, we provide further evidence of the use of zebrafish for modelling neuropsychiatric disorders and cognitive impairment.

3.2 Introduction

The *DISC1* variant in humans has been identified as one of the most important susceptibility genes for schizophrenia (SZ), but also affects a broad spectrum of other associated psychiatric illnesses (Ayhan et al., 2016; Twyman & Amin, 2009). *DISC1* variants have been repeatedly shown to cause alterations in behavioural responses and cognitive function in humans (Callicott et al., 2005; Carless et al., 2011), non-

human primates (Simen et al., 2009) and rodents (Ayhan et al., 2016; Johnstone et al., 2011; Lipina & Roder, 2014; Papaleo et al., 2012), with altered anxiety responses and deficits in WM regularly being reported in subjects carrying *DISC1* variants (Carless et al., 2011; Gamo et al., 2013; Johnstone et al., 2011; Wang et al., 2019).

Early-onset SZ manifests in childhood or adolescence and, although similar to the adult-onset form of the disorder, it represents a more severe variant (Frangou, 2009; Röpcke & Eggers, 2005; Vyas et al., 2010). Adolescence is a sensitive neurodevelopmental period with increased susceptibility to stress and the onset of stress-related psychiatric disorders, such as SZ (Blakemore, 2008; Rapoport & Gogtay, 2011; Van Os et al., 2010). Models of schizophrenia posit that this disorder is the result of environmental and genetic factors that converge with aberrant neurodevelopment that begins long before the onset of clinical symptoms (Rapoport et al., 2005; Rapoport & Gogtay, 2011) and greatly influences adult behaviour (Niwa et al., 2013). Thus, unique insights could be gained from studying early-onset forms of the disorder that could provide developmental trajectories and treatment opportunities for both early-onset and adult-onset forms of SZ (Rapoport & Gogtay, 2011).

The dopaminergic system has been significantly implicated in the pathology of SZ (Brisch et al., 2014; Kokkinou et al., 2020; Peleg-Raibstein et al., 2009; Rao et al., 2019). Abnormalities in DA synthesis, availability or receptor activation have been linked to all aspects of SZ (Brisch et al., 2014). Abnormalities in DRD2 neurotransmission are associated with the positive, psychotic symptoms (Alfimova et al., 2013; Noble, 2003; Ripke et al., 2014), whilst DRD1 is implicated in negative symptoms and cognitive deficits (Rao et al., 2019). In addition to DRD1, aberrant functioning of the HPA axis has also been correlated with cognitive impairment (Jameison & Dinan, 2001) and in turn the HPA axis is regulated by DA (Sullivan & Dufresne, 2006). Thus activation and negative feedback are under the control of DA and GR for the maintenance of homeostasis in the neuroendocrine system and normal behaviour and cognition (van Looveren et al., 2019). Cognitive impairment and aberrant stress responses are hallmarks of SZ (Howes et al., 2017) and therefore, requires further investigation to unravel the complex interactions of

psychiatric susceptibility genes and dysregulation of the HPA axis and dopaminergic system, to better understand the underlying biology and potentially improve therapeutic strategies.

Animals have been crucial in providing preclinical models of neuropsychiatric disorders to fully characterise genetic and neural mechanisms underlying psychiatric pathology (Baker et al., 2020). Although rodents have been particularly useful in elucidating gene x environment interactions involved in illness onset, other animal models are moving to the forefront and providing an alternative system for investigating these complex mechanisms (Fontana et al., 2018; Maximino et al., 2015). The zebrafish is being increasingly utilized for the study of complex brain disorders, including SZ (Gawel et al., 2019; Stewart et al., 2015). Thus far, studies with zebrafish have identified similar mechanisms to those found in SZ patients and rodent models of SZ (De Rienzo et al., 2011; Demin et al., 2019; Eachus et al., 2017; Gawel et al., 2019; Thyme et al., 2019). Using several targeted *disc1* mutations, zebrafish models have found a conserved role for *disc1* in aberrant behaviour, particularly anxiety responses (Eachus et al., 2017; Tang et al., 2020), but as yet have not tested differences in cognitive abilities. Here we investigate functioning of the hypothalamus-pituitary-interrenal (HPI) axis, the zebrafish equivalent of the human HPA axis, by analysing anxiety responses, post-stress cortisol levels and *gr* mRNA expression in the brain of *disc1* mutants. Additionally, we have conducted the first analysis of cognitive function in a zebrafish model of SZ and associated dopamine receptor expression in juvenile zebrafish, revealing altered behavioural, cognitive and molecular mechanisms and a novel role for altered *drd5* expression.

3.3 Materials and methods

Animals and housing

Homozygous *disc1*^{Y472} embryos and control embryos were kindly donated by Dr Caroline Brennan (Queen Mary University, London). All animals were maintained on a 14/10-hour light/dark cycle at ~25-27°C, pH 8.4 (±0.4) on a re-circulating system (Aquaneering Inc., San Diego, CA, USA), at the University of Portsmouth in accordance to standard husbandry protocols (Nusslein-Volhard & Dahm, 2002). The

disc1 mutant line was identified from an ENU mutagenesis-based screening programme, this variant is also referred to as *disc1*^{fh292}and has been described elsewhere (De Rienzo et al., 2011). Throughout chapter this line is referred to as *disc1*^{Y472}.

Ethical statement

Experiments carried out as part of this study were under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: P9D87106F] and with approval from and in accordance with the University of Portsmouth Animal Welfare and Ethical Review Board guidelines.

Behavioural analysis

Juvenile zebrafish, at ~2 months old, were used throughout this study. Fish were transferred into the behavioural room for 30 min prior to analysis to allow for acclimatization. A total of N=24 fish (12 per group) were used for all behavioural procedures. On the first day fish were analysed in the novel tank diving test, on the second day fish were run in the FMP Y-maze. All behaviour was carried out in the Zantiks adult behavioural unit (AD unit) (Zantiks Ltd, Cambridge, UK), which tracked fish continuously whilst in the test arena. Units were web-enabled allowing live streaming video feed of zebrafish during testing, to a laptop or mobile device. Starting and finishing trials was done remotely. Pre-determined protocols were used that depicted specific zones and data was automatically logged and output into an excel file. Experimenter visibility and handling were kept to a minimum to reduce stress and distraction whilst animals were performing behavioural tasks. Ambient light was a maximum of 2 lux in testing units.

Novel tank diving apparatus

All fish were run individually in a clear rectangular tank, 20 cm length x 5 cm width x 17.5 cm depth and filled with 1 L of aquarium water. Fish were permitted free swimming for 6 min, starting from first entry into the tank. The test arena was divided into three equal horizontal zones (bottom, middle, top) to

allow evaluation of vertical activity. Anxiety behaviour was analysed using time spent in each zone and number of entries into the top zone as behavioural endpoints and distance travelled was used to assess locomotor activity.

FMP Y-maze apparatus

All fish were run individually in a white acrylic Y-maze with clear base and mesh top (to prevent escape), maze dimensions are: 5 cm length x 2 cm width x 14 cm depth, and filled with 3 L of aquarium water to allow sufficient swimming depth. Two mazes were fitted into a water-tight tank and run in the Zantiks and logging of arm entries was written into the program script, recorded automatically and output in an excel spreadsheet. Arm entries and exits were continuously logged throughout the trial and converted into a series of left and right turns. Search strategy was based on overlapping sequences of four turn choices, giving rise to 16 possible tetragram configurations. Protocols for the FMP Y-maze have been described in detail elsewhere (Cleal, Fontana, et al., 2020).

Whole-body cortisol

For determination of cortisol levels following an acute stress zebrafish were grouped 2.8L housing tanks (n=4 fish per tank). Acute stress was delivered net chasing for 5 min, then all fish were left to recover for 10 min, as previous research has shown peak cortisol responses within 3-15 min of receiving a stressor in zebrafish (Ramsay et al., 2009). Following the rest period, zebrafish were immediately culled by immersion in ice-water and snap-frozen using liquid nitrogen. Whole-body cortisol levels were detected using the Salimetrics Salivary Cortisol ELISA kit for human samples (Stratech, Cambridge, UK), which has been found to reliably detect cortisol in zebrafish using the method previously described by (Cachat et al., 2010; Parker et al., 2012a). Briefly, samples were weighed and then homogenised in 1mL of chilled PBS. 5 mL of diethyl ether was mixed with the homogenised samples and centrifuged at 7000 x g for 15 min. The top, organic layer was removed, and the process was repeated until all the cortisol-containing layer was removed, and then subsequent evaporation of the diethyl ether was continued

overnight at room temperature. The resulting cortisol was reconstituted using 1 mL of chilled PBS. The ELISA was carried out in a 96-well plate following the manufacturer's instructions. Standards were used to determine the final cortisol concentration using OD readings. Weights were used to calculate cortisol concentration per gram (ng/g^{-1}). Inter- and intra-assay coefficients of variation were determined. Data are represented as mean ± SD (n = 4 per group; assayed in duplicate).

RNA extraction and gDNA removal

Fish were culled using ice-water immersion, followed by severing of spinal cord. Whole brains were removed and snap frozen using liquid nitrogen. Samples were homogenised and total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's instruction. After extracting and purifying RNA, samples were treated using the RapidOut DNA Removal Kit (Thermo Scientific), following manufacturer's instructions to remove contaminating gDNA from RNA samples prior to conversion into cDNA.

cDNA synthesis

Following removal of gDNA from RNA, sample concentrations were measured using the ND 1000 spectrophotometer. To ensure the same amount of cDNA was synthesised for each sample a maximum of 1 µg of total RNA was used per reaction (the same total concentration of RNA was loaded into each reaction). Reverse transcription of mRNA to cDNA was done using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) following the manufacturer's protocol. After conversion into cDNA, samples were diluted with ddH_2O to a final concentration of 5 ng/µL. Samples were stored at -20°C until use.

Quantitative Real-Time PCR (RT-qPCR)

Quantitative real-time PCR (RT-qPCR) assays were used to validate relative gene expression based on SYBR green detection. *Gr* and *drd5* primers were predesigned by qPrimerDB (Lu et al., 2018), *eef1a* primers were based on previous studies (Parker et al., 2016; Tang et al., 2007). Primers were synthesised by Invitrogen and are listed in **Table 1**. The Thermo Scientific PowerTrack SYBR Green PCR Master Mix and the LightCycler® 96 (Roche Life Science) were used to amplify and detect the transcripts of interest. Thermal cycling conditions were as follows: 95°C for 120s (recommended by manufacturer), 35 cycles of 95°C for 15s and 60°C for 25s, in a two-step protocol. Negative reverse transcription (RT) controls and negative template controls were used to determine specificity and to check for genomic DNA contamination. Elongation factor 1 alpha (*eelf1a*) was used as a housekeeping gene (Tang et al., 2007). Gene-expression levels were calculated using delta-delta CT method and normalised to *eelf1a*. Changes in expression were relative to controls to demonstrate fold changes in *disc1* mutants. Data are presented as means ± SD (n = 6 per group; assayed in duplicate).

Table 1. Primer sets used for qPCR

Gene name	Gene	Primer sequence (Forward)	Primer sequence (Reverse)	Amplicon size
Eukaryotic translation elongation factor 1 alpha	*eef1a*	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGCATTAC	87
Glucocorticoid receptor	*gr*	ACGGTTCTATCAGCTCACTAAG	AAACTCCACGCTCAGAGATTTA	109
Dopamine receptor D5	*drd5*	CGAGAGAAAGATGAACCGTAGA	GTCCAGTTGCTTGTGTTAACC	138

Statistical analysis

Data were analysed in IBM SPSS 25.0 and GraphPad Prism 8.4.2. For data from the FMP Y-maze studentized residuals were generated from statistical models and screened for outliers. Any data point identified as an extreme value (>3*IQR) was removed from further analysis. Residuals were analysed for normality using histograms and Shapiro-Wilk normality test. Residuals for both conditions were significantly skewed, therefor all data were log transformed and a linear mixed model (LMM) was employed to assess alternations and repetitions, which, from previous work from our lab, have been identified as strategies susceptible to change with changing cognitive performance (Cleal, Fontana, et al., 2020; Cleal & Parker, 2018; Fontana, Cleal, Clay, et al., 2019). LMM analysis was carried with "total turns" as the dependent variable, "condition (wild type v *disc1*^{Y472})" and "time" as the within-subjects factor and "ID" as a random effect. Analysis was followed by Bonferroni-corrected *post-hoc* test. Locomotion was analysed using total turns completed during 1 h of exploration in the FMP Y-maze. All other data were analysed for normality using the Shapiro-Wilk test and screened for outliers using the ROUT outlier analysis (Q = 1%) and boxplot with Tukey whiskers. Outliers were removed from further analysis. Zone data from the novel tank diving test were analysed using two-way ANOVA followed Sidaks multiple comparison test, distance travelled in the novel tank, total turns in the FMP Y-maze, ELISA and qPCR data were analysed using *t*-test for normally distributed data and Mann-Whitney U test for non-normally distributed data. Results were considered significant when $p \leq 0.05$.

Due to a tracking issue with the software used, several fish were missing data from the behavioural tests. No data points were removed from ELISA or qPCR analysis due to small sample size.

3.4 Results

Juvenile *disc1* mutants maintain anxiety response

The *disc1*^{Y472} homozygous, point mutation results in the introduction of a premature stop codon located within the *N*-terminal domain of the *disc1* gene (**Fig. 1A**). To understand the impact of this mutation on juvenile behaviour, we first analysed baseline anxiety responses using the novel tank diving test. The

juvenile *disc1*^{Y472} mutants showed indistinguishable behavioural response to wild type fish for the time spent in each zone (Two-way ANOVA, $F_{(1,54)} = 0.122$, $p = 0.72$) (**Fig. 1B**) and distance travelled (Mann-Whitney U = 40, $n_1 = n_2 = 9$, $p = 0.983$, two-tailed) (**Fig. 1C**). However, there was a significant difference in the number of entries into the top of the tank, with wild type fish making nearly 8 times as many entries into the top zone compared to *disc1*^{Y472} mutants (Mann-Whitney U = 18.50, $n_1 = n_2 = 9$, $p = 0.0490$, two-tailed) (Fig. 1D). Thus, demonstrating a maintained anxiety response that in wild type fish, reduces over the course of the trial, permitting increased entries into the top zone. However, in *disc*^{Y472}mutants the anxiety of a novel environment is not alleviated with time. However, there were no evident differences in the time spent in each zone in WT compared to *disc*^{Y472} mutants.

Figure 1.

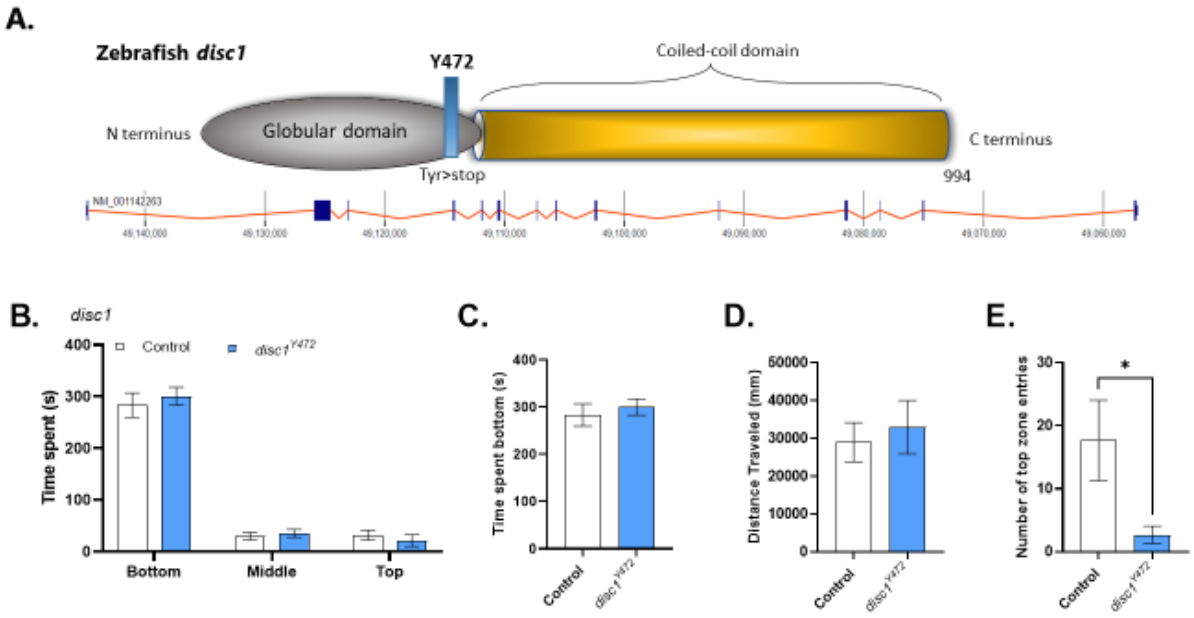

Figure 1. **A**) (upper) Schematic of *disc1* protein in zebrafish consisting of *N*-terminus, globular domain with a Tyr>Stop inserted as a point mutation at 472 amino acids (Y472) and a coiled-coil domain and C terminus, with a predicted protein length of 994 amino acids. (lower) Schematic of *disc1* intron regions (red line) and 14 exon regions (blue rectangles). (**B**) Juvenile zebrafish in the novel tank diving test, showing time spent in the bottom, middle and top zones during 6 min of free exploration of controls and *disc1*^{Y472} mutants (Two-way ANOVA). There were no differences in time spent in the bottom of the tank (**C**) or distance travelled (**D**). However, there was a significant decrease in number of entries to the top zone made by *disc1*^{Y472} mutants compared to wild type controls (*t*-test) (**E**). Bars represent mean, error bar are mean ± SEM, * $p \leq 0.05$.

Disc1 mutant juveniles demonstrate significantly altered cognitive function of WM and exploratory strategies.

To assess cognitive processing, we used the FMP Y-maze to evaluate WM and exploration strategies after 1 h of free swimming in the maze. Frequency distribution was used to plot the percentage use of each tetragram sequence throughout the duration of the trial and identify specific search patterns (**Fig. 2A**). Previous studies have highlighted two main strategies as susceptible to change related to WM processing; alternations (RLRL, LRLR) and repetitions (RRRR, LLLL) (see Part 1: Chpt 3) (Cleal, Fontana, et al., 2020). Further examination of these two strategies highlight substantial differences between *disc1*^{Y472} mutants and wild type controls, with *disc1*^{Y472} fish demonstrating significant impairment of WM, as shown by a greater than 2-fold decrease in alternation use throughout the trial (LMM; $F_{(1,72.01)} = 41.22$, $p < 0.001$) (**Fig. 2B**). Additionally, there was an increase in the use of repetitions by *disc1*^{Y472} mutants compared to wild type fish ((LMM; $F_{(1,72.01)} = 41.22$, $p < 0.000$) (**Fig. 2C**). The overall search strategy was severely impacted by cognitive impairment, as the usual alternation dominant strategy was evident in wild types, but was completely diminished in *disc1*^{Y472} fish, ultimately resulting in almost equal use of each of the 16 tetragram sequences. Thus, demonstrating random exploration, instead of application of a specific search strategy. However, as with the novel tank diving test, there was no differences observed in locomotor response between wild type and *disc1*^{Y472} mutant fish (*t*-test; $t = 0.799$, df = 12, $p = 0.440$) (**Fig. 2D**).

Figure 2.

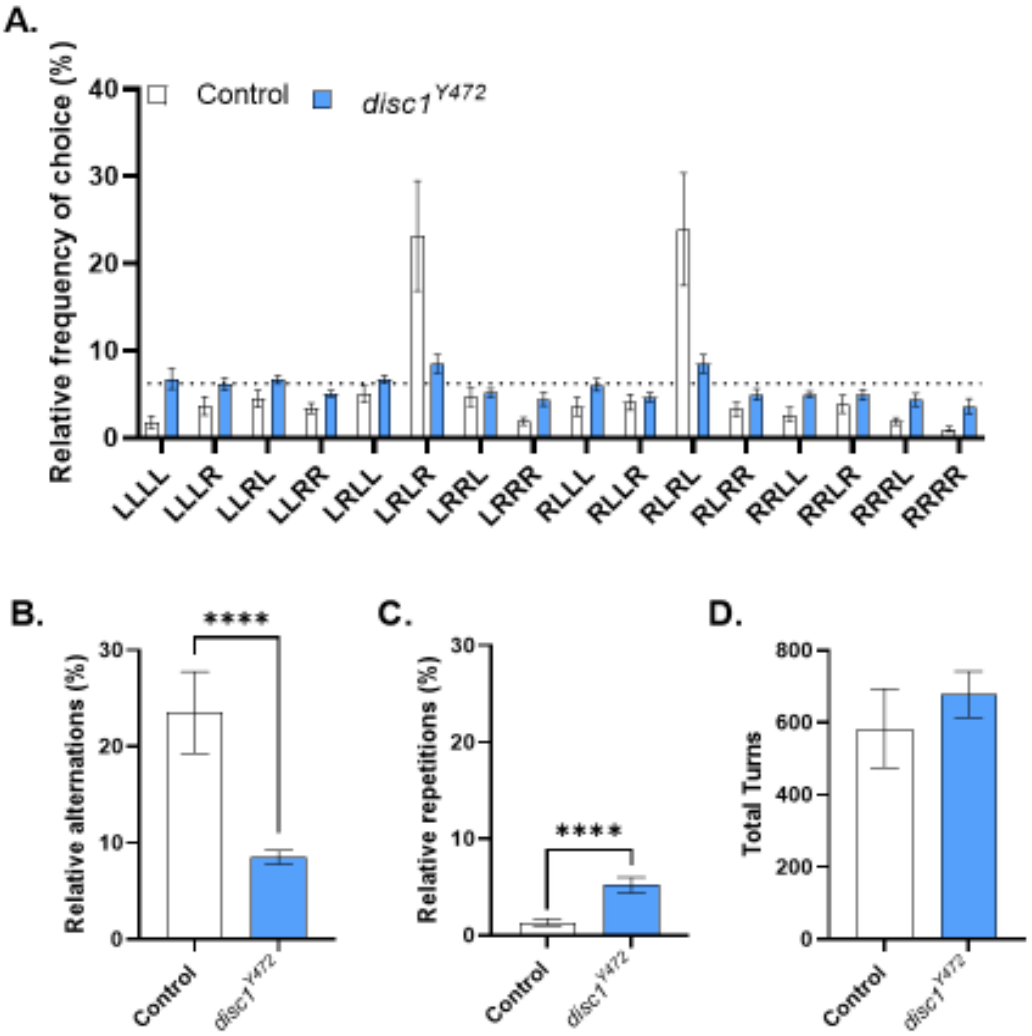

Figure 2. Search strategy after 1 h of exploration in the FMP Y-maze. (**A**) Percentage use of each tetragram sequence by 2-month old zebrafish wild type controls v *disc1*^{Y472} mutants in the FMP Y-maze (controls, n = 5; *disc1*^{Y472}, n = 10). Controls demonstrate a clear dominant use of alternations, which is not evident in *disc1*^{Y472} mutants. Percentage use of (**B**) alternations and (**C**) repetitions in controls compared to *disc1*^{Y472} mutants. (**D**) Locomotor activity was assessed using total turns. Normality was analysed using Shapiro-Wilk test. Tetragram data were log transformed and analysed using LMM followed by Bonferroni *post-hoc* test. Total turns were analysed using *t*-test. The dashed *line* denotes chance performance (approximately 6.25%). Bars are mean, error bars are mean ± SEM, **** $p \leq 0.0001$.

Abnormal expression of genes critical to the normal functioning of the HPI axis in *disc1* mutations

Brain corticosteroid receptor balance has been discovered to be involved in plasticity and cognitive performance. Specifically glucocorticoids can influence memory, contextualization and motivational responses to environmental cues and experiences (de Kloet et al., 2018). Having identified altered anxiety responses and cognitive performance, we investigated how glucocorticoid pathways may be altered in *disc1* mutants. Therefore, we analysed whole body cortisol levels following an acute stress, and expression levels of *gr* and *drd5* which directly and indirectly (respectively) modulate glucocorticoid activity. An acute stress of 5 min net chasing, followed by 10 min of rest, which has previously been shown to result in peak cortisol levels following stress, did not provoke significant differences between wild type and $disc1^{Y47f2}$ mutants (Mann-Whitney U = 8.00, $n_1 = n_2 = 4$, $p > 0.999$, two-tailed) (**Fig. 3A**). However, a significant increase in *gr* mRNA expression (Mann-Whitney U = 0.00, $n_1 = n_2 = 6$, $p = 0.002$, two-tailed) (**Fig. 3B**) and slight decrease in *drd5* mRNA expression (*t*-test; $t = 2.57$, df = 10, $p = 0.028$) (**Fig. 3C**) were evident in $disc1^{Y472}$ mutants compared to wild type controls.

Figure 3.

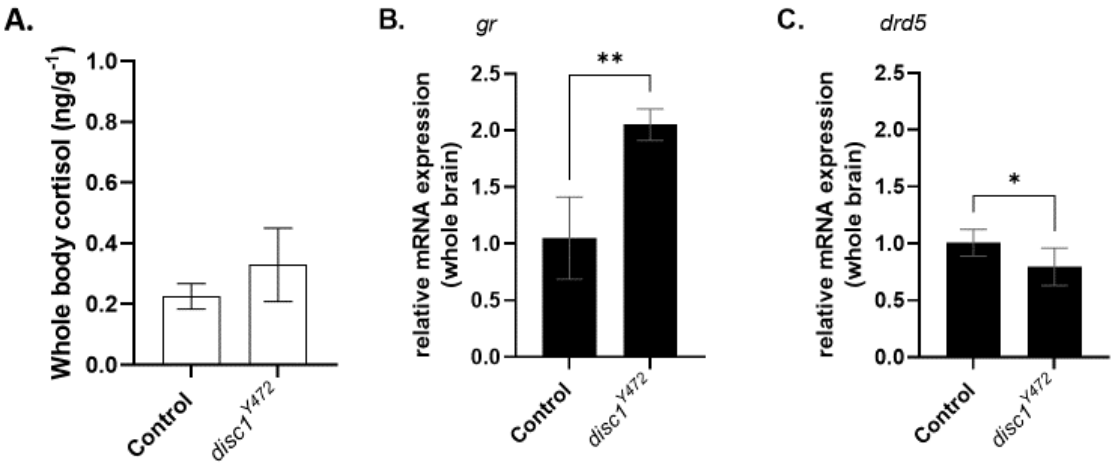

Figure 3. (**A**) Whole body cortisol following acute stress did not show any significant difference between controls and *disc1*^{Y472} mutants (n = 4 per group). Quantitative RT-PCR for *disc1*^{Y472} mutants shows a 2-fold increase in *gr* (**B**) and significant decrease in *drd5* (**C**) whole brain mRNA expression. All data were analysed for normality using Shapiro-Wilk test. Normally distributed data were analysed using *t*-test, non-normally distributed data were analysed using Mann-Whitney U test. Bars are mean and error bars are mean ± SD, **p*<0.05, ***p*<0.01.

3.5 Discussion

In this study we report, for the first time, that zebrafish carrying a *disc1* point mutation have severely impaired WM that generated random exploration over strategized exploration, which was evident from as early as 2 months-old. Although there was no overall effect on anxiety-like behaviour, supported by similar cortisol levels in both groups following acute stress, there was evidence that initial anxiety responses (to a novel environment) perpetuated for longer in *disc1* mutants compared to wild type fish. This was shown by a significantly lowered number of transitions into the top zone by *disc1*^{Y472} mutants in the novel tank diving test. Further, we identified whole brain expression changes in two genes, *gr* and *drd5,* that play important roles in maintaining cognition and HPI axis activity, which may indicate a potential pathway responsible for aberrant behaviour in *disc1* induced neuropsychiatric disorders, including SZ.

Novelty tests are regularly used in animal models to assess anxiety responses (Harro, 2018; Lezak et al., 2017; Maximino et al., 2010). The typical response of fish when introduced to a novel environment is to dive to the bottom of the tank. Following a short period of habituation the fish gradually begins to ascend into the top area of the tank, making multiple transitions between the different zones for the remainder of the trial (Cachat et al., 2010). Using the novel tank diving test, we assessed anxiety responses in wild type fish compared to *disc1*^{Y472} mutants, using time spent in each zone and number of transitions into the top zone as behavioural endpoints. We found that, overall, there was no difference in the time spent in each zone between wild types and *disc1*^{Y472} mutants. Although, on average control fish did not spend more time at the top, they did make a significantly greater number of entries into the top zone compared to *disc1* mutants. Thus, demonstrating a reduction in anxiety-like behaviour as fish habituated to their environment. However, carriers of the *disc1*^{Y472} mutation made on average fewer than 5 transitions into the top zone compared to greater than 15 transitions made by wild type fish. The lack of transitions into the top zone is potentially reflective of a prolonged anxiety response.

Previous studies in zebrafish have reported whole body cortisol levels that correlate well with findings in the novel tank diving test (Cachat et al., 2010; Maximino et al., 2010). We, therefore,

investigated cortisol levels in wild type and *disc1* mutants following an acute stress. In line with our findings from the novel tank diving test, we found that there was no significant difference in cortisol levels between groups. Previous zebrafish studies using the *disc1*^{Y472} mutant line have investigated cortisol levels in larvae following acute stressors (alarm substance and sodium chloride) and although they reported differences between wild type stress response and baseline, they similarly do not report differences between wild type and *disc1*^{Y472} following stressor. Instead, stress reactivity differences are in comparison to basal levels of each group, not between groups (Eachus et al., 2017). These findings are in line with human studies investigating stress reactivity in patients with SZ. (Nugent et al., 2015) similarly reported no overall difference in salivary cortisol levels post-stress test, however, when dividing patients into groups of distress tolerance (completed the stress test) and intolerant (did not complete the stress test) they found prolonged cortisol reactivity that had not returned to baseline levels after 40 min post-stress test in schizophrenic patients with distress intolerance (Nugent et al., 2015). Although we did not examine cortisol levels at multiple time points following the acute stress, we hypothesis that they would be reflective of our findings in the novel tank diving test and would be maintained for a longer period than wild type controls and warrants further investigation.

Cognitive deficits are a hallmark of SZ and often present with moderate to severe impairments in patients and animal models (Ayhan et al., 2016; Keefe & Harvey, 2012). Deficits in cognitive functions, such as memory, executive function and attention have been demonstrated in early-onset SZ (development between childhood and early adulthood), which correlates with clinical severity and is comparable to adult-onset forms of the disorder, as those exhibiting early-onset cognitive deficits do not tend to show age-related improvements, as is associated with healthy peers (Frangou, 2009; Hollis, 1995; Vyas et al., 2010). One of the most commonly reported cognitive deficiencies in SZ is WM and has been demonstrated in numerous animal models (Carless et al., 2011; Gamo et al., 2013; Kvajo et al., 2008; Simen et al., 2009; Wang et al., 2019). However, as yet, no zebrafish model of SZ has examined differences in cognition. We therefore set out to assess WM abilities in mutant zebrafish carrying the *disc1*^{Y472} point mutation using the FMP Y-maze. Prior studies utilising the FMP Y-maze have reported a

specific exploration strategy whilst traversing the maze that is common to zebrafish, rodents and humans and is heavily dominated by use of alternations (RLRL+LRLR) (Cleal, Fontana, et al., 2020; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Variations in this strategy have been triggered by blocking glutamatergic, cholinergic and dopaminergic receptors prior to exploration, all reducing use of alternations, however, only high doses of D1/D5 receptor antagonist caused a simultaneous down regulation of alternations and up regulation of repetitions (Cleal, Fontana, et al., 2020). Here, we found that *disc1*^{Y472} mutants presented with severe WM impairments compared to wild type controls, demonstrating a total loss of the alternation based strategy and instead encompassing a completely random exploration pattern in which turn sequences were selected by chance. In addition to the deficit in alternation use, there was an increase in the use of repetitions (LLLL+RRRR) compared to controls. Prior studies by our group have shown increases in repetition strategies in zebrafish pre-treated with a D1/D5 receptor antagonist, but not a D2/D3 receptor antagonist (Cleal, Fontana, et al., 2020). These findings potentially suggest a role for disrupted dopaminergic signalling in altered cognitive performance in the FMP Y-maze.

To further understand the molecular mechanisms underpinning the behavioural changes seen in *disc1*^{Y472} mutants, we analysed whole brain mRNA expression of *gr* and *drd5*. Glucocorticoids and their associated receptors play a critical role in modulating stress responses through HPA axis activation in humans and HPI activation in zebrafish (Derek Alsop & Vijayan, 2009; Aponte & Petrunich-Rutherford, 2019; Ramamoorthy & Cidlowski, 2016; Wilson et al., 2013). Having already demonstrated that cortisol is not significantly upregulated following acute stress in *disc1*^{Y472} mutants, we further analysed the impact of this line on *gr* expression. We found a 2-fold increase in *gr* mRNA whole brain expression in *disc1*^{Y472} mutants compared to wild type controls. Our findings are at odds with other studies investigating *GR* regulation in SZ with many reporting a down regulation of *GR* mRNA expression in humans (Liu et al., 2020; Sinclair et al., 2011). However, rodent models of mild chronic stress have demonstrated greater than 2-fold increase in *Gr* mRNA expression in the prefrontal cortex (Wei et al., 2016). If our findings of prolonged anxiety-like behaviour in the novel tank diving test is correlated with

prolonged cortisol elevation following stress, as shown in schizophrenic patients (Nugent et al., 2015), then this may act as a potential mechanism for causing the up-regulation of *gr* mRNA and warrants further investigation.

Dopamine is a major factor in neuroendocrine function, cognition and psychosis (Lee et al., 2000). Dysregulation of the dopaminergic system has been heavily implicated in SZ (Dahoun et al., 2017; Kokkinou et al., 2020; Tost et al., 2010). Dopamine D1 (DRD1) and D2 (DRD2) receptors have been extensively studied in SZ, with many current antipsychotic medications acting as DRD2 antagonists and DRD1 implicated in the negative symptoms (Alfimova et al., 2013; McCutcheon et al., 2020; Price et al., 2014; Takahashi et al., 2012). However, relatively little has been investigated in relation to DRD5 mostly notably due to the lack of specific ligands that can differentiate between DRD1 and DRD5 (Moraga-Amaro et al., 2016). However, this receptor is worth further examination as DRD5 has been linked with regulating the HPA axis and GR expression and may provide an alternative mechanism by which neuropsychiatric disorders exert influence over cognitive domains (Perreault et al., 2013). Therefore, we have investigated whole brain mRNA expression of *drd5* in *disc1* mutant zebrafish. We found that *drd5* was significantly down-regulated compared to wild type controls. Previous studies investigating the effects of glucocorticoids on DRD5 found that high levels of circulating glucocorticoids down regulate DRD5 and disrupt the balance between DRD2 and DRD5 interaction in response to dopamine availability (Lee et al., 2000). Similar to the up-regulation of *gr* mRNA, altered cortisol levels could also cause the down-regulation of *drd5* reported here. In addition to this, previous studies utilising knockdown and knockout models of Drd5 in rodents have demonstrated impaired WM performance, demonstrating a critical role for DRD5 in cognitive function, particularly WM (Carr et al., 2017; Moraga-Amaro et al., 2016). However, it is worth noting that several studies have identified regional specific alterations in DRD5 expression (Bai et al., 2017), this combined with regional expression of GR in SZ (Sinclair et al., 2011) may implicate localized effects of dysregulation of the dopaminergic and neuroendocrine systems that perpetuate adolescent stage cognitive deficits and altered anxiety responses that are commonly reported in SZ, and other neuropsychiatric disorders associated with DISC1 mutations.

3.6 Conclusion

Here we demonstrate that *disc1* mutant zebrafish, show maintained anxiety-like behaviour that is not alleviated by acclimatization to a novel environment, indicating altered anxiety response. Examination of cognitive performance in the FMP Y-maze reveals severe cognitive impairment, particularly in WM, indicating a complete loss of strategy formation, ultimately resulting in purely random exploration of the maze. Furthermore, upregulation of *gr* and downregulation of *drd5* mRNA expression suggest possible mechanisms that may underpin anxiety and cognitive alterations in *disc1*^{Y472} mutants. Together our data demonstrate a significant impact of *disc1* mutation on normal behaviour and cognitive function in juvenile zebrafish, resembling findings in humans and rodents and implicate a role for the *drd5* pathway in maintaining normal behavioural and cognitive function.

Chapter 4

4.1 General Discussion

In the previous section we describe a new zebrafish *slit-3* mutant line that exhibits a broad spectrum of cognitive, behavioural and biochemical abnormalities. With human GWAS data implicating aberrant *SLIT-3* functioning as high risk for the development of SZ. We hypothesised that altered phenotypes in the *slit-3* zebrafish model, represents SZ-like characteristics. To validate this hypothesis, we tested a well-known SZ-mutant line, *disc1*, and assessed central features of SZ, including cognitive function, anxiety responses and markers of HPI axis activation and regulation in both zebrafish models. Below is a description of their respective findings.

Slit-3 mutants tested in the FMP Y-maze demonstrated a significant down-regulation of the use of alternations, which has been extensively described throughout this thesis as pertaining to a reduction in WM performance. The same examination with *disc1* mutants revealed a substantial inhibition of the alternation strategy, reducing their use to near chance selection, making the alternation-based strategy indistinguishable from all other tetragram strategies. This marks a significant deficit in WM performance in *disc1* mutants compared to *slit-3* mutants, and greater still compared to controls. Interestingly, such substantial reduction in the use of alternations has only previously been seen with zebrafish pre-treated with MK-801 and the D1 antagonist SCH-23390 (see Part 1: Chpt 3), potentially implicating the NMDA or D1 pathways as the underlying mechanisms in the disruption of WM performance. Additionally, both models showed up-regulation of stereotypic behaviours, as demonstrated by a significant increase in the use of repetitions compared to controls. The increase in stereotypic behaviours was greatest in *slit-3* mutants, which used this strategy at greater than chance levels, whilst *disc1* mutants maintained low overall levels, but increased use compared to controls.

Next we examined anxiety response to a novel environment, tested using the novel tank diving paradigm. *Slit-3* mutants showed increased time spent at the bottom of the tank, indicating increased anxiety. This was accompanied by novelty-induced hyperlocomotion, described as such due to the lack of differences in locomotor activity after 1 h in the FMP Y-maze during which there is substantial habituation time, which may alleviate hyperlocomotor responses. *Disc1* mutants showed no differences in time spent bottom dwelling or changes in locomotor activity. However, it is worth noting that *disc1* controls appeared to spend a substantial amount of time at the bottom, potentially resulting in ceiling effects that make it difficult to identify differences between control and mutant groups. This could potentially be a factor of age, as *disc1* mutants were tested at 2-month old, whilst *slit-3* mutants were tested as adults (6 months-old), and this could act as a confound when investigating anxiety behaviour.

Finally, we examined markers of HPI axis activation and regulation by analysing whole-body cortisol following acute stress and basal *gr* mRNA expression, respectively. *Slit-3* mutants showed a decrease in whole-body cortisol following acute stress, but showed no differences in *gr* expression between mutants and controls. *Disc1* mutants showed the complete inverse of these findings, with no differences in post stress cortisol levels compared to controls, but significant up-regulation of basal *gr* mRNA expression.

Combined these data describe two zebrafish lines that exhibit significant cognitive, behavioural and biochemical abnormalities compared to their respective controls. Similarities between cognitive deficits in *disc1* mutants compared to MK-801 suggests robust replication of the cognitive symptoms associated with SZ. However, the *slit-3* mutant line, despite showing lesser cognitive deficits, has greater dysregulation of the HPI axis, anxiety-like behaviours and stereotypies that are commonly described in SZ patients.

We conclude that further work is required to validate the role of *slit-3* in SZ, however, this model does provide a transgenic zebrafish line with extensive neurological perturbations that are common to a range of psychiatric disorders, including SZ, and thus provides an alternative line for investigating aspects of complex neuropsychiatric disorders.

Chapter 5

5.1 Final Discussion

The goal of this thesis was two-fold. To utilize zebrafish as an emerging model of complex disorders and to examine the cognitive symptomology associated with many neuropsychiatric conditions, as these can be life-long and significantly impact quality of life. Lack of progression in identifying clinically relevant therapeutic strategies over the last half a century has highlighted a need for a different approach and zebrafish offer such an alternative. The second aim was to design a non-invasive, robust, high throughput cognitive task that could demonstrate high translational relevance to clinical measures of disease. Part 1 of this thesis characterises and validates the FMP Y-maze as a clinically relevant cognitive task that can be used to assess cognitive function, particularly working memory and cognitive flexibility in model organisms ranging from flies, fish and rodents to humans.

Part 1 set out to validate the FMP Y-maze using pharmacological intervention to identify potential neurotransmitter systems critical to normal exploration of the maze. We identified glutamatergic and dopaminergic systems, particularly through D1-like receptors, and to a lesser extent cholinergic systems, as critical for the underlying working memory and executive functions required to strategically navigate the maze. Cross-species tests identified commonalities between vertebrate organisms in the way they naturally explore a novel, non-reinforced environment, and identified a dominant search strategy based on alternating left and right turns. Subsequent chapters set out to examine the applications and limitations of the FMP Y-maze, primarily using zebrafish, but integrating other models, such as rodents and humans to reaffirm the use of zebrafish for studying complex human disorders, but also to further validate the robustness of the FMP Y-maze. Ontogenetic evaluation of cognitive development, in tangent with examination of the developing neuroendocrine system, identified juvenile stages in zebrafish, similar to adolescent stages in humans, as a critical period of

increased vulnerability to stress-pathologies and onset of neuropsychiatric disorders. Continued examination of maturing cognitive function identified deficits in working memory and cognitive flexibility in healthy ageing, the relevance of which was confirmed by examining a healthy human cohort using the virtual FMP Y-maze and replicating our findings from zebrafish. These two studies identify zebrafish as a model organism suitable to assessing cognition from larvae to ageing and have the additional benefit that all of these stages can be modelled in as little as two years. Finally, we used a genetic mouse model of AD to provide further validation of the FMP Y-maze and demonstrate its potential use as a cognitive task used for investigating neurodegenerative disorders.

Parts 2 & 3 focus on the original aim of the thesis and use zebrafish to investigate aspects of neuropsychiatric disorders that converge on aberrant cognitive processing. Part 2 investigates the long-term effects of prenatal alcohol exposure on longitudinal cognitive deficits that could be identified in adulthood, despite only being exposed to a low concentration of drug. This highlights the sensitivity of the developing brain to environmental perturbations and long-term effects of early cognitive disruption. The negative consequences of prenatal drug exposure were in stark contrast to the next chapter which implicated chronic drug exposure regimens during adulthood with cognitive enhancing effects of nicotine and amphetamine on working memory performance in the FMP Y-maze. However, repeat exposure caused divergent effects of nicotine treated compared to amphetamine treated fish. Nicotine continued to exert pro-cognitive effects, enhancing working memory and maintaining cognitive flexibility. Amphetamine, on the other hand, demonstrated continued increases in use of alternations, but at the expensive of other strategies. Sensitizing effects of amphetamine resulted in the complete block of cognitive flexibility and potentially identified the first stages of drug-induced maladaptive behaviours. Thus, once again, demonstrating differences in the roles of the dopaminergic and cholinergic systems in influencing cognitive processes.

Finally, Part 3 analyses two transgenic lines, modelling aspects of neuropsychiatric disorders, such as aberrant cognitive function, anxiety responses and dysregulation of the HPI axis. The first chapter aims to generate and characterise a new zebrafish mutant line with schizophrenia-like

phenotypes. CRISPR/Cas9 genome editing was used to generate a *slit-3* mutant line. Behavioural and biochemical characterisation identified significant cognitive impairment in the FMP Y-maze, increased stereotypies, heighten anxiety responses and severe dysregulation of HPI axis activation in *slit-3* mutants. Previous studies had identified mutations in *slit-3* with increased sensitivity to the rewarding effects of nicotine, found in humans and zebrafish. Additionally, GWAS studies have implicated *SLIT-3* as a high susceptibility gene for the development of schizophrenia. This information combined with our data identified *slit-3* as a potential genetic link between the extremely high comorbidity of nicotine addiction in schizophrenic patients. Thus, having identified schizophrenia-like behaviours in *slit-3* mutant fish, we examined the impact of nicotine treatment on cognitive performance and anxiety responses and found that nicotine attenuated cognitive impairments and reduced anxiety, restoring fish to near control levels. To further this investigation, we tested another well-known anxiolytic drug, alcohol, to identify if responses to nicotine was due to the drugs anxiolytic effects or rewarding properties. We found that *slit-3* mutants showed greater preference for the ethanol-paired cue than controls in the CPP, but had no effect on anxiety responses. Our data further identify a role for stimulation of the cholinergic system as a mechanism for enhancing cognitive performance both in health and disease as well as a role for reducing anxiety, without negatively impacting other domains such as cognitive flexibility.

Future directions

The global pandemic has had a huge impact on this work and resulted in much research left incomplete. However, the data that has been compiled paves the way for many future studies. Covid-19 has brought about unprecedented social isolation, future uncertainty, grave health issues and death and there is little doubt of the huge impact that this event will have on the short and long-term mental health of the global population. Understanding the molecular and neurophysiological mechanisms of neuropsychiatric disorders is even more critical than ever and so is the need to find effective therapeutic strategies to combat mental illness. The potential of the FMP Y-maze to bridge the gap between findings

in animal models and the clinic is exciting and requires more work to fully appreciate its potential. As such, future plans for human studies using the FMP Y-maze to investigate cognitive changes in people suffering with mild cognitive decline, schizophrenia, autism, depression and anxiety are already in place. Generation of new and improved genetic models of these disorders will allow direct comparisons of cognitive performance and potentially, increased success in finding effective therapeutic strategies. Being able to robustly translate findings from animal models to humans is going to prove imperative to the future effort of restoring balance to the world's mental health.

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