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Zebrafish Models in NeuroPsychopharmacology and CNS Drug Discovery

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Abstract

Despite high prevalence of neuropsychiatric disorders, their etiology and molecular mechanisms remain poorly understood. The zebrafish (*Danio rerio*) is increasingly utilized as a powerful animal model in neuropharmacology research and in-vivo drug screening. Collectively, this makes zebrafish a useful tool for drug discovery and the identification of disordered molecular pathways. Here, we discuss zebrafish models of selected human neuropsychiatric disorders and drug-induced phenotypes. Covering a broad range of brain disorders (from anxiety and psychoses to neurodegeneration), we also summarize recent developments in zebrafish genetics and small molecule screening, which markedly enhance the disease modeling and the discovery of novel drug targets.

Keywords: Zebrafish; Behavioral models; Toxicology models; Genetic Models; Preclinical study; Model organism

TARGETS	
Other protein targets[a]	**Transporters**[c]
NAC	Vmat2
PSEN1	**Enzymes**[d]
PSEN2	AChE
Nuclear hormone receptors[b]	COX-2
GR	

LIGANDS	
ACTH	APP
CRH	GABA
KA	MB
NMDA	

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

Abbreviations:

AChE = acetylcholinesterase;
ACTH = adrenocorticotropic hormone;
ALS = amyotrophic lateral sclerosis;
AD = Alzheimer's disease;
ApoE4 = apolipoprotein E ε4;
APP = Amyloid beta A4 precursor protein;
BMAA = beta-mythylamino-alanin;
CRH = corticotrophin-releasing hormone;
COX-2 = cyclooxygenase-2;
CPP = conditioned place preference;
dpf = days post fertilization;
GABA = gamma aminobutyric acid;
GR = glucocorticoid receptors;
HPA = hypothalamus-pituitary-adrenal;
HPI = hypothalamus-pituitary-interrenal;
HSR = heat shock stress response;
microCT = micro-computer tomography;
KA = kainic acid;
MB = methylene blue;
MO = morpholino-modified antisense oligonucleotide;
NAC = N-acetylcysteine;
NFT = neurofibrillary tangles;
NMDA = N-methyl-D-aspartate;
NMJ = neuromuscular junction;
OCT = optical coherence tomography;
PPI = pre-pulse inhibition;
PSEN1 = PRESENLIN1;
PSEN2 = PRESENLININ2;
PTSD = post-traumatic stress disorder;
PTZ = pentyleneterazole;
RNAi = RNA interference;
SEA = Similarity Ensemble Approach;
SNRI = selective norepinephrine reuptake inhibitors;
SSRI = selective serotonin reuptake inhibitor;
UCMS = unpredictable chronic mild stressors;
Vmat2 = vesicular monoamine transporter 2;
WGD = whole-genome duplication

1. Introduction: Zebrafish as an emerging animal model

Widespread and debilitating, neuropsychiatric disorders have poorly understood mechanisms and often lack efficient therapies (Garakani, Mathew & Charney, 2006; Griebel & Holmes, 2013b). Identifying clinically relevant biomarkers, the underlying neurobiological mechanisms, as well as genetic and environmental factors of psychopathology, are critical steps in discovering efficacious treatments (Caspi & Moffitt, 2006; Nestler, 2013). While rodent models of human brain disorders have long been employed in this effort, they are often impeded by high-cost and experimental inefficiency (Cryan & Holmes, 2005).

The zebrafish (*Danio rerio*) has recently garnered attention as a powerful animal model for a wide range of human brain disorders (Kalueff, Echevarria & Stewart, 2014b; Kalueff, Stewart & Gerlai, 2014; Stewart et al., 2015b). Zebrafish is a small, low-cost and genetically tractable aquatic vertebrate species with a high degree of morphological, physiological and genetic homology to humans (Kalueff, Echevarria & Stewart, 2014a; Kalueff, Stewart & Gerlai, 2014). The zebrafish genome, fully sequenced, shows orthologues corresponding to ~82% of disease-related genes in humans (Howe et al., 2013). Gene expression databases (e.g., http://zfin.org/) and atlases of zebrafish brain are also available to explore the genomics and neuroanatomy of brain areas associated with neuropsychiatric disorders (Mueller & Wullimann, 2015; Ullmann, Cowin, Kurniawan & Collin, 2010; Wulliman, Rupp & Reichert, 2012).

Modeling human conditions in zebrafish empowers the discovery of potential therapeutic targets and their underlying molecular interactions (Table 1). For example, analyzing the efficacy of various compounds to ameliorate the amyotrophic lateral sclerosis (ALS)-like phenotype, a recent study found therapeutic potential of methylene blue (MB) in a mutant mTDP-43 zebrafish (Vaccaro et al., 2012). Likewise, the mTDP-43 mutant zebrafish presents with short, abnormally branched motor axons, increased oxidative stress and

aberrant escape response (Vaccaro et al., 2012). Administration of MB, a neuroprotective agent, corrects swimming and axonal phenotypes while reducing the endoplasmic reticulum (ER) stress that occurs as a result of an accumulation of unfolded mutant proteins (Vaccaro et al., 2013; Vaccaro et al., 2012). The identification of ER stress as a potential target for ALS drug treatment prompted further testing the efficacy of several related agents in a G93A mtSOD1 transgenic mouse model, which led to the identification and repositioning of guanabenz, an approved drug for hypertension, as a potential new treatment for ALS (Vaccaro et al., 2013). Clearly, the zebrafish mutant model played a critical role in the identification of new ALS treatment options.

Another example of bringing laboratory findings to the bedside includes two modulators of hematopoietic stem cells (HSC) recently discovered in zebrafish (Zon, 2014) and then becoming therapies in patients (North et al., 2007). Original screening of nearly 2500 small molecules in zebrafish identified 35 'leads' that up-regulate vital HSC genes, *runx1* and *c-myb*, ten of which modulate the prostaglandin pathway, implicating it in HSC regulation. One of these potent candidates, 16,16-dimethyl prostaglandin E2 (dmPGE2), was next tested in a mouse model, increasing the number of HSC grafted (North et al., 2007; Zon, 2014). Subsequent preclinical testing using primate blood model yielded successful results, allowing the drug to move to the approved Phase I clinical trial (Goessling et al., 2011). These studies have recently yielded positive results in leukemia patients and demonstrated the safety of the treatment, allowing it to move to Phase II testing (Cutler et al., 2013). Thus, the translatability of original zebrafish results was critical for the application of this drug in mice, and in humans (see other examples of translational approaches in Table 1).

Both larval and adult zebrafish are useful pre-clinical *in-vivo* models highly amenable to experimental, pharmacological and genetic manipulations (Barros, Alderton, Reynolds, Roach & Berghmans, 2008; Brennan, 2011; Bruni et al., 2016). Due to their transparency and

small size, larval zebrafish are particularly useful for optical manipulation and imaging of neural activity, as well as for large-scale high-throughput screens of molecular drug targets and candidate genes (Brennan, 2011; Stewart, Gerlai & Kalueff, 2015; Wyart & Del Bene, 2011). Together with recent developments in genome editing techniques (e.g., CRISPR/Cas) and automated 3D behavioral phenotyping, this makes zebrafish an ideal model to study genotype-phenotype and genotype-drug-phenotype relationships (Cachat et al., 2011b; Hwang et al., 2013; Kokel et al., 2010; Stewart et al., 2015a). Furthermore, zebrafish develop externally to the maternal organism, reach sexual maturity fast (in ~90 days), and live for ~4-5 years in the laboratory, allowing for direct and easy analyses of pathogenetic trajectories (Fonseka, Wen, Foster & Kennedy, 2016; Kalueff, Stewart & Gerlai, 2014). Complementing larval models, adult zebrafish exhibit complex behaviors (Kalueff et al., 2013a) relevant to cognition (Blaser & Vira, 2014; Gerlai, 2016), reward (Collier, Khan, Caramillo, Mohn & Echevarria, 2014; von Trotha, Vernier & Bally-Cuif, 2014), social behavior (Gerlai, 2014; Qin, Wong, Seguin & Gerlai, 2014) and affect (Gerlai, 2013; Jesuthasan, 2012; Wang et al., 2016a). Numerous experimental paradigms have been converted for aquatic models to investigate major behavioral phenotypes which are well-conserved in zebrafish and mammals (Renier et al., 2007; Stewart, Braubach, Spitsbergen, Gerlai & Kalueff, 2014a).

Rats and mice are currently the most commonly employed animals to study normal and abnormal brain functioning, with nearly 1/3 of all published neuroscience papers in 2015 utilizing rodent models, and <11% using other animal models, including zebrafish (Keifer & Summers, 2016). However, the rate of zebrafish publications is growing faster than any other model organisms, and experimental tools and resources for this organism are becoming increasingly available (Kalueff, Echevarria & Stewart, 2014b; Wyatt, Bartoszek & Yaksi, 2015). A new animal model that still requires validation across multiple domains, the zebrafish has a growing utility in high-throughput phenotyping, gene and drug screening, thus

becoming increasingly useful in neuropsychopharmacology and drug discovery research. Here, we highlight recent successes and challenges in this rapidly expanding field.

2. Zebrafish CNS

The overall architecture, neuroanatomical features and cellular morphology of the zebrafish CNS are generally similar to those of mammals (Kalueff, Stewart & Gerlai, 2014). For example, the medial teleost pallium contains homologous structures to the mammalian amygdala (Martín, Gómez, Salas, Puerto & Rodríguez, 2011; Mueller, Dong, Berberoglu & Guo, 2011; Portavella, Torres & Salas, 2004; von Trotha, Vernier & Bally-Cuif, 2014) – the brain structure key for affective processing and emotionality in humans. The amygdala is pathologically hyperactivated in clinical anxiety (Rauch et al., 2000; Shin, Rauch & Pitman, 2006), social anxiety disorder (Furmark et al., 2004; Stein, Goldin, Sareen, Zorrilla & Brown, 2002), and drug abuse (Buffalari & See, 2010; Mead, Vasilaki, Spyraki, Duka & Stephens, 1999). The zebrafish medial pallium shows increased Fos protein expression, a measure of neuronal activation, following both acute administration of D-amphetamine and during drug-seeking behavior in a conditioned place preference (CPP) assay (von Trotha, Vernier & Bally-Cuif, 2014), collectively supporting the role of zebrafish medial pallium as a homologous structure to the mammalian amygdala with evolutionarily conserved functions in modulating key behaviors.

Visualizing CNS activity through imaging methods is an important step to discern how the brain contributes to normal and abnormal behavior. The small size, and optical transparency of larval zebrafish allows for high resolution *in-vivo* imaging and manipulation of neural activity in behaviorally active animals (Orger & Portugues, 2016). For example, imaging neuronal activity of larval zebrafish behavior has been achieved by expressing a genetically encoded calcium indicator and recording whole-brain activity using light-sheet microscopy (Ahrens, Orger, Robson, Li & Keller, 2013). Optogenetic neuromodulation of the

transparent and genetically accessible larval zebrafish is particularly useful to investigate the neural circuitry underlying behaviors relevant to brain disorders (Knafo & Wyart, 2015). Neuronal excitation and inhibition of targeted neuronal populations has been successfully triggered in behaving larval zebrafish by expressing optogenetic actuators, including channelrhodopsin-2 and halorhodopsin (Arrenberg, Del Bene & Baier, 2009; Douglass, Kraves, Deisseroth, Schier & Engert, 2008). To date, optogenetic studies in zebrafish have largely focused on several simpler behaviors, such as escape (Douglass, Kraves, Deisseroth, Schier & Engert, 2008) and locomotion (Arrenberg, Del Bene & Baier, 2009; Ljunggren, Haupt, Ausborn, Ampatzis & El Manira, 2014), as well as sensory processing (Kubo, Hablitzel, Dal Maschio, Driever, Baier & Arrenberg, 2014). However, optogenetic neuromodulation in zebrafish help future research to create robust models of complex human neuropsychiatric disorders (Stewart et al., 2015b; Tye & Deisseroth, 2012). Furthermore, imaging neural activity in adult zebrafish is more challenging due to their larger and opaque brains. Recently applied in adult zebrafish, contrast-enhanced X-ray micro-computer tomography (microCT) with iodine as a contrasting agent provided 3D visualization of zebrafish brain anatomy in intact animals (Babaei, Hong, Yeung, Cheng & Lam, 2016). Optical coherence tomography (OCT) has also recently been used *in-vivo* in adult zebrafish to non-invasively generate real-time cross-sectional images at high resolution that and then reconstructed in 3D (Rao, Alex, Verma, Thampi & Gupta, 2009; Zhang & Yuan, 2015).

Neurochemistry is generally conserved across vertebrate species, as they share major neurotransmitters, receptors and transporters (Alsop & Vijayan, 2008; Panula et al., 2010; Panula et al., 2006). Thus, zebrafish are sensitive to major classes of pharmacological agents, such as psychostimulants (Ninkovic & Bally-Cuif, 2006), opiates (Lau, Bretaud, Huang, Lin & Guo, 2006), ethanol (Tran, Chatterjee & Gerlai, 2015), hallucinogens (Stewart, Cachat, Gaikwad, Robinson, Gebhardt & Kalueff, 2013), anxiolytics (Bencan, Sledge & Levin,

2009), antidepressants (Stewart et al., 2014) and antiopsychotics (Bruni et al., 2016). The spatial and temporal distribution of major neurotransmitter systems in zebrafish is also similar to that of mammals, and has been well described in zebrafish for glutamate, gamma aminobutyric acid (GABA), acetylcholine, dopamine, serotonin, norepinephrine and histamine (Stewart et al., 2015b). For instance, the major pathways and receptor subtypes of the dopamine system are all present in zebrafish, with the exception of the D_5 receptor (Maximino & Herculano, 2010; Panula et al., 2010; Panula et al., 2006). The amino acid sequence recently compared between zebrafish and humans for D_1-D_4 receptors shows 100% amino acid homology in the binding site for D_1 and D_3, and 85-95% for D_2 and D_4 receptors (Ek et al., 2016). Consequently, pharmacological agents that act on the dopamine system produce similar phenotypes, as dopamine antagonists or depletors impair locomotion (Giacomini, Rose, Kobayashi & Guo, 2006; Kyzar et al., 2014) and agonists predictably increase zebrafish locomotion (Irons, Kelly, Hunter, Macphail & Padilla, 2013), paralleling similar effects in rodents (Akhisaroglu, Kurtuncu, Manev & Uz, 2005; Mobini, Chiang, Ho, Bradshaw & Szabadi, 2000). The dopamine agonist apomorphine produces a U-shaped dose-response relationship for distance traveled in larval zebrafish, with low-doses increasing time in center (anxiolytic effect) and high-doses increasing thigmotaxis (anxiogenic effect) (Ek et al., 2016). Strikingly paralleling similar drug action in rats (Ek et al., 2016), these findings further support the translational value of neuropharmacological studies in zebrafish.

Mounting evidence implicates alterations of the neuroendocrine system in various brain disorders, including depression (Herbert, 2013; Holsboer, 2001), anxiety (Hek et al., 2013; Korte, 2001), addiction (Keedwell, Poon, Papadopoulos, Marshall & Checkley, 2001; Lovallo, 2006) and Alzheimer's disease (AD) (Belanoff, Gross, Yager & Schatzberg, 2001; Wahbeh, Kishiyama, Zajdel & Oken, 2008). Activation of the neuroendocrine hypothalamus-pituitary-interrenal (HPI) axis of zebrafish releases cortisol that acts on glucocorticoid

receptors, similar to the hypothalamus-pituitary-adrenal (HPA) axis in humans (Alsop & Vijayan, 2009; Griffiths, Schoonheim, Ziv, Voelker, Baier & Gahtan, 2012b; Pavlidis, Theodoridi & Tsalafouta, 2015). The zebrafish neuroendocrine system can be easily modulated by experimental, pharmacological and genetic manipulations, and fish cortisol can be sampled using various invasive and non-invasive methods (Canavello et al., 2011; Félix, Faustino, Cabral & Oliveira, 2013; Pavlidis, Sundvik, Chen & Panula, 2011). For example, genetic mutation of the glucocorticoid receptors (GR) gene in adult gr^{s357} mutant zebrafish disrupts negative feedback and cortisol signaling by abolishing the transcriptional activity of GR upon cortisol binding (Ziv et al., 2013). This elevates blood cortisol levels and evokes aberrant phenotypes, such as freezing, reduced exploration, impaired habituation and potentiated startle, most of which can be rescued in gr^{s357} mutants by a selective serotonin reuptake inhibitor (SSRI) fluoxetine. This also emphasizes the high degree of evolutionarily conservation between the neuroendocrine system and its modulation between zebrafish and humans (Griffiths, Schoonheim, Ziv, Voelker, Baier & Gahtan, 2012b; Ziv et al., 2013).

3. Zebrafish models of major CNS disorders

A clear advantage of non-human animals (like zebrafish) for modeling brain disorders is, as already mentioned, their amenability to experimental, genetic and pharmacological manipulations. Furthermore, the behavioral phenotypes, genetic factors and pharmacological sensitivity of zebrafish often show a high degree of similarity to those reported in rodent models of brain disorders and in human clinical populations (see further).

3.1. Depression and anxiety

Stress is a common risk factor for developing affective disorders, including Major Depressive Disorder (Lucassen, Oomen, Schouten, Encinas & Fitzsimons, 2016; Strüber, Strüber & Roth, 2014) and anxiety (Bystritsky, 2006). In mammals, stress response is mainly mediated by the interplay of the hypothalamus, the pituitary and the adrenal glands,

collectively forming the HPA axis (Smith & Vale, 2006). Prolonged stress and hyperactivation of the HPA axis have the potential to lower GR expression, ultimately reducing the ability to adapt and cope with stress-events (Howell, Kutiyanawalla & Pillai, 2011) and thereby triggering depression (Zhou, Zhu, Wu, Luo, Chang & Zhu, 2011).

Depression has been extensively modeled in rodents (Deussing, 2006; Krishnan & Nestler, 2011) utilizing early-life (Fumagalli, Molteni, Racagni & Riva, 2007) and adulthood stresses (Seligman, Rosellini & Kozak, 1975) as well as pharmacological interventions (Barr & Markou, 2005), selective breeding or genetic engineering (Deussing, 2006). Several hallmark depression symptoms (e.g., low self-esteem, and depressed mood) are difficult to evaluate in animals, as they do not clearly display a sense of self (Deussing, 2006). In contrast, evaluation of other phenotypes, including anhedonia, comorbid anxiety or sleep- and neuroendocrine disturbances, can be easily modeled in animals (Porsolt, 2000; Seligman & Beagley, 1975). In zebrafish, depressive-like states can be evoked by a battery of unpredictable chronic mild stressors (UCMS) applied for an extended period of time (Fulcher, Tran, Shams, Chatterjee & Gerlai, 2016; Marcon et al., 2016; Piato et al., 2011). Adult fish exposed to 7-14-day UCMS exhibit reduced locomotion, altered shoaling behavior and body coloration (Gerlai, Lahav, Guo & Rosenthal, 2000). Applied to zebrafish raised in social isolation for 5 months, UCMS increases anxiety-like behaviors in the novel tank test, and reduces body weight and whole-brain dopamine and serotonin metabolite 5-HIAA levels, compared to zebrafish raised in groups (Fulcher, Tran, Shams, Chatterjee & Gerlai, 2016). The effects of UCMS on exploratory and group/shoaling behaviors are reversed by fluoxetine (an SSRI) and bromazepam, a benzodiazepine anxiolytic (Marcon et al., 2016). In addition, several key pro-inflammatory molecules, such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2), are differentially regulated in the zebrafish following 7 days of UCMS (Marcon et al., 2016). COX-2 transcription is greater in

individuals with recurrent depressive disorder, and is hypothesized to negatively affect cognitive functioning, emotionality and synaptic homeostasis (Galecki, Talarowska, Bobińska & Szemraj, 2014). Treatment with psychotropic drugs (fluoxetine, bromazepam and nortriptyline) reduces the expression of IL-6 and TNF-α (Marcon et al., 2016), highlighting the sensitivity of this model to established, clinically active antidepressants. Other pharmacological interventions, such as the administration of reserpine, produce depressive-like responses in zebrafish, including social withdrawal, motor retardation and elevated cortisol, that parallel clinical symptoms of depression (Nguyen, Stewart & Kalueff, 2014). Finally, several genetic models empower studying depression in the zebrafish. For instance, larval zebrafish with mutant glucocorticoid receptors (gr/s357) display heightened physiological responses (e.g., higher whole body cortisol levels) and dysfunctional HPI axis (Griffiths, Schoonheim, Ziv, Voelker, Baier & Gahtan, 2012a), similar to the effect observed in humans.

Anxiety disorders are debilitating psychiatric diseases with a lifetime prevalence of ~30%, higher than any other mental disorder (Kessler, 2007; Kessler, Chiu, Demler, Merikangas & Walters, 2005). There are several types of anxiety disorders, including panic disorder, post-traumatic stress disorder (PTSD), generalized anxiety disorder and specific phobias (American Psychiatric Association, 2013). The hallmark symptom of anxiety disorders is an overwhelming and exaggerated sense of worry in response to perceived threats (American Psychiatric Association, 2013), dramatically lowering patients' quality of life and work productivity (ADAA, 2016). The first line of treatment for anxiety disorders is typically a regimen of SSRIs or cognitive behavioral therapy (Bystritsky, 2006). Patients who do not respond to these treatments are then given selective norepinephrine reuptake inhibitors (SNRIs) or tricyclic antidepressants (Bystritsky, 2006). However, SNRIs or tricyclics increase the risk for tolerance and dependence (Otto, Pollack, Sachs, Reiter, Meltzer-Brody &

Rosenbaum, 1993), thereby limiting their use. Furthermore, although many treatments for anxiety disorders exist, approximately 30% of patients show no improvement (Brown, Schulberg, Madonia, Shear & Houck, 1996). This necessitates the identification and development of treatments that are devoid of the limitations in efficacy and tolerance (Griebel & Holmes, 2013a).

One of the problems with developing new treatments has been the identification of biochemical targets, genetic variants or mechanism of action for the onset of the disorder (Bystritsky, 2006; Griebel & Holmes, 2013a; Insel et al., 2011), beckoning the need for animal models. The zebrafish model is particularly amenable to high-throughput anxiolytic drug screens (Lundegaard et al., 2015). The larval zebrafish hatches from its chorion within 3 days post fertilization (dpf), and are able to inflate their swim bladder by 5 dpf and produce a broad range of behaviors (Richendrfer, Pelkowski, Colwill & Creton, 2012), see Fig. 1. For instance, staying near the periphery of the arena (thigmotaxis) reflects anxiety-like behavior, and is heightened following exposure to anxiogenic stimuli or drugs (Stewart, Gaikwad, Kyzar, Green, Roth & Kalueff, 2012). In adult fish, measures of anxiety include a latency to explore the top, or higher tendency to remain in the bottom (Stewart, Gaikwad, Kyzar, Green, Roth & Kalueff, 2012) in the novel tank test (Fig 2). In the light-dark test, allowed to freely explore brightly light and dark arenas, zebrafish spend more time in the dark (scototaxis) - an anxiety-like response which can be bidirectionally influenced by anxiolytic or anxiogenic treatments (Kalueff et al., 2013b). Genetic models of anxiety in zebrafish are also available, including the knockdown of vesicular monoamine transporter 2 (*Vmat2*) which produces an anxiety-like profile with social withdrawal and reduced exploration (Wang et al., 2016b).

In the effort to identify new treatments for anxiety and related disorders, there has also been a call to repurpose available drugs for novel applications (Lundegaard et al., 2015). This method of drug discovery has the advantage of reducing uncertainty regarding

pharmacokinetic issues or safety of the drug (Ashburn & Thor, 2004; Insel et al., 2011), thereby allowing for a more rapid drug screen and testing. For instance, N-acetylcysteine (NAC), a common mucolytic agent and antidote for paracetamol overdose, has shown promise in the treatment of several neuropsychiatric disorders (Berk, Malhi, Gray & Dean, 2013). NAC plays a role in maintaining oxidative balance germane to anxiety, and has been shown to modulate central glutamatergic pathways (Dean, Giorlando & Berk, 2011). While a growing body of evidence supports the role of glutamate in the anxiety response, there is a clear deficit of approved glutamatergic anxiolytics (Cortese & Phan, 2005). NAC administration to adult zebrafish prevents stress-induced anxiety (Mocelin et al., 2015), which is in line with previous reports of its clinical efficacy in depressed patients (Berk, Malhi, Gray & Dean, 2013). In another example of drug repositioning, potential anxiolytic targets were identified using traditional cancer treatments, as cAMP mediated anxiety in the zebrafish via crosstalk of the RAS-MAPK pathway (Lundegaard et al., 2015). The heightened anxiety-like response is attenuated by exposure to MEK inhibitors, anti-cancer treatment (Lundegaard et al., 2015), suggesting the MEK crosstalk as potential alternative target for treatments of anxiety as well.

3.2. Epilepsy

Epilepsy, which affects approximately 50 million people worldwide (WHO, 2016), is characterized by recurrent convulsions/seizures, behavioral impairments, pathological neural activity and endocrine dysfunction (Andrea Galimberti et al., 2005; Engel, 2013; Green et al., 2012; Zhang & Liu, 2008). Epilepsy can be modeled in larval and adult zebrafish (primarily by administration of convulsant drugs and genetic modifications) and evaluated by various behavioral and physiological endpoints (Cunliffe, 2015; Desmond et al., 2012; Wong et al., 2010). Characteristic behaviors for epilepsy-like states in adult zebrafish are hyperactivity, erratic swimming, loss of body posture, spasm-like corkscrew swimming (Desmond et al.,

2012) and electrical discharges in the CNS (Baraban, Taylor, Castro & Baier, 2005; Zdebik, Mahmood, Stanescu, Kleta, Bockenhauer & Russell, 2013). Experimental seizures in zebrafish can be induced by acute caffeine (250 mg/L), pentylenetetrazole (PTZ, 2.5 g/L) and picrotoxin (100 mg/L), causing hyperactivity, circular/corkscrew swimming, spasms and elevated whole-body cortisol levels (Wong et al., 2010). These symptoms are suppressed by antiepileptic drugs in both larval and adult zebrafish (Green et al., 2012), enabling the discovery of more efficacious treatments for epilepsy (Alfaro, Ripoll- Gómez & Burgos, 2011). For instance, PTZ administration not only evokes characteristic seizures, but is also accompanied by the rapid transcription of *c-fos* and *npas4* (Cunliffe et al., 2015), paralleling responses observed in seizure onset in mammals (Cunliffe et al., 2015; Loebrich & Nedivi, 2009). Finally, various genetic techniques enable the greater exploration of function for specific candidate genes (Cunliffe, 2015; Mahmood, Fu, Cooke, Wilson, Cooper & Russell, 2013; Teng et al., 2010) or anti-epileptic treatments using high-throughput and rapid screening in zebrafish (Baraban, Dinday & Hortopan, 2013; Baxendale et al., 2012; Cunliffe, 2015).

3.3. Psychosis

Psychosis manifests as disturbances in cognition, affect, motor activity and social behavior (American Psychiatric Association, 2013), and is often accompanied by aberrant glutamatergic signaling (Merritt, McGuire & Egerton, 2013; Schobel et al., 2013). Glutamate N-methyl-D-aspartate (NMDA) receptor antagonists phencyclidine and ketamine produce psychotic symptoms in healthy volunteers, and worsen the positive, negative and cognitive symptoms of patients with schizophrenia (Merritt, McGuire & Egerton, 2013). MK-801 is a potent NMDA antagonist used to model schizophrenia in rodents, zebrafish and other animal models (Moghaddam & Jackson, 2003; Swain, Sigstad & Scalzo, 2004). Likewise, pre-pulse inhibition (PPI) is the attenuation of startle response when a weak non-startling response is

presented before the startling stimulus (Swerdlow, Geyer & Braff, 2001). Schizophrenia patients show impaired PPI (Braff, Geyer & Swerdlow, 2001) which can be rescued by antipsychotic therapy (Geyer, Krebs-Thomson, Braff & Swerdlow, 2001; Kumari, Soni & Sharma, 1999). PPI is reliably reproduced in larval zebrafish, including genetic mutants with reduced PPI currently available (Burgess & Granato, 2007). Overall, the similarity in neural pathways and startle response in the zebrafish demonstrate their utility as an unbiased platform for the discovery of regulatory genes and drugs for antipsychotic treatment.

3.4. Alzheimer's Disease

AD is a progressive neurodegenerative disease resulting in cognitive deficits, delusions, hallucinations and mood and behavior changes (Voisin & Vellas, 2009). One of the hallmark AD symptoms is the development of neurofibrillary tangles (NFT) and amyloid beta plaques (Newman, Verdile, Martins & Lardelli, 2011). There are two broad classes of AD: sporadic AD (developing at age >65), and familial AD, developing much earlier (Rossor, Iversen, Reynolds, Mountjoy & Roth, 1984). Sporadic AD accounts for more than 95% of all AD cases (Newman, Verdile, Martins & Lardelli, 2011), and is linked to the apolipoprotein E ε4 allele (ApoE4) (Selkoe, 2001). The zebrafish orthologue of this gene is *apoE* (Babin, Thisse, Durliat, Andre, Akimenko & Thisse, 1997). Early-onset familial AD (fAD) is hereditary and has been linked to mutations in the *PRESENILIN1* (*PSEN1*), *PRESENLININ2* (*PSEN2*) and *AMYLOID BETA A4 PRECURSOR PROTEIN* (*APP*) genes, orthologous to the zebrafish *psen1*, *psen2*, *appa* and *appb* genes (Newman, Verdile, Martins & Lardelli, 2011). The injection of transcription-blocking morpholinos for *psen1* disrupts notch signaling and results in aberrant somite formation (Campbell et al., 2006; Nornes, Casper, Esther, Ey & Lardelli, 2003; Nornes, Newman, Wells, Verdile, Martins & Lardelli, 2009). *Psen2* blocking produces notch-signaling defects (Campbell et al., 2006) and alters the production of spinal cord interneurons in zebrafish (Nornes, Newman, Wells, Verdile,

Martins & Lardelli, 2009), paralleling phenotypes observed in *psen1*$^{-/-}$ and *psen2*$^{-/-}$ mice (Shen, Bronson, Chen, Xia, Selkoe & Tonegawa, 1997).

Zebrafish are also valuable for studying the etiology of AD, especially the role of hypoxia as a putative risk factor (Newman, Verdile, Martins & Lardelli, 2011). Under low oxygen conditions, mitochondria may release free radicals that increase oxidative stress (Bell et al., 2007; Moussavi Nik, Croft, Mori & Lardelli, 2014). Hypoxic conditions are easily reproduced in the zebrafish by reducing water oxygen levels or via chemical mimicry of hypoxia by sodium azide (Moussavi Nik, Newman & Lardelli, 2011)). Similar to humans, hypoxic conditions in the larval and adult zebrafish upregulate several AD-related genes, including *sen1*, *psen2*, *appa*, *appb* and *bace1* (Moussavi Nik, Newman & Lardelli, 2011).

Pharmacological intervention may also help model the cognitive deficits associated with AD. For example, the cholinergic system (mediating learning and memory) is affected by AD (Fibiger, 1991), as AD patients show reduced nicotinic (nAChR) and muscarinic (mAChR) binding sites, as well as reduced acetylcholinesterase (AChE) activity (Lombardo & Maskos, 2015; Perry, Tomlinson, Blessed, Bergmann, Gibson & Perry, 1978). The muscarinic antagonist scopolamine impairs zebrafish memory without causing locomotor deficits or anxiety-like behavior (Cognato et al., 2012; Gupta, 2014; Richetti et al., 2011). Pretreatment with quercetin and rutin, two flavonoids, protects against scopolamine-induced memory impairment (Richetti et al., 2011). Flavonoids act as AChE inhibitors and can enhance learning/memory and synaptic plasticity (Ahmed & Gilani, 2009; Havsteen, 2002; Spencer, 2008). Scopolamine-induced memory impairment in zebrafish is also ameliorated by pretreatment with physostigmine, an AChE inhibitor (Kim, Lee, Kim, Jung & Lee, 2010). The ability of scopolamine to produce amnestic effects while preserving normal locomotor activity provides evidence contributing to the involvement of the cholinergic system in fish

learning and memory, and lends credence to the use of the zebrafish as a tool for drug discovery and medicines that can treat neurodegenerative diseases, including AD.

3.4. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a debilitating progressive neurodegenerative disorder affecting motor neurons in the brain and spinal cord (Rowland & Shneider, 2001). Zebrafish are a particularly attractive model for studying the function and dysfunction of spinal cord circuitry due to visual transparency at early stages of life, and because there is a high degree of functional and anatomical similarity between the zebrafish spinal cord and humans (Fetcho & O'Malley, 1995; Friedrich, Jacobson & Zhu, 2010; McGown et al., 2013). Similar to AD, there are two broad types of ALS: familial and sporadic ALS (Kiernan et al., 2011). Roughly 10% of ALS cases are inherited. The etiology of ALS is poorly understood, with a high degree of variability in genetic mutations that contribute to ALS. Nevertheless, SOD1 is the most well-understood gene to be associated with ALS (Rosen et al., 1993), and mutations in the SOD1 gene account for 20% of familial ALS cases (Valdmanis & Rouleau, 2008).

Larval zebrafish over-expressing mutant *Sod1* have abnormal neuromuscular junctions (NMJ) which worsen as the fish matures (Ramesh et al., 2010). Larval mutant fish present progressive decrease in NMJ volume (Ramesh et al., 2010), poorer performance in the forced swim test (Plaut, 2000; Ramesh et al., 2010) and reduced responsivity to repeated stimulation (Ramesh et al., 2010). Together, this indicates a defect in the neural input to the muscle, rather than defects in the intrinsic properties of the muscle (Ramesh et al., 2010). Early identification of pathogenic processes is also possible in the zebrafish through the heat-shock stress response (HSR). The HSR mechanism refolds damaged proteins in stressed cells, and is a useful tool for monitoring cellular perturbations (McGown et al., 2013). In *sod1* mutant zebrafish harboring the HSR reporter gene (*hsp70-DsRed*), fluorescence facilitates

disease mapping and spread throughout the brain (McGown et al., 2013). This method has also been used to identify neuroprotective compounds and biological targets with the potential to ameliorate early disease processes that are not yet fully understood (McGown et al., 2013).

In addition to the utility of the zebrafish in monitoring the progress of ALS symptoms, genetic mutants and pharmacological models also help identify the molecular mechanisms of this disease. For instance, the loss of function of the zebrafish orthologue *C9orf72* leads to axonal degeneration of motor neurons, and is accompanied by decreased swim speed and motility of larval zebrafish (Ciura et al., 2013). The motor deficits caused by knockdown of *C9orf72* implicate it in ALS and related neurodegenerative disorders (Ciura et al., 2013). Gene editing techniques, such as TALEN- or CRISPR, may also be used to insert point mutations in the zebrafish genome (Armstrong, Liao, You, Lissouba, Chen & Drapeau, 2016), resulting in mutant zebrafish lines recapitulating ALS. This novel methodology shows promise in the development of mutant models for other neuropsychiatric diseases as well (Armstrong, Liao, You, Lissouba, Chen & Drapeau, 2016). Pharmacological intervention with neurotoxins like beta-mythylamino-alanin (BMAA) can also be relevant to modeling ALS. Pericardiac injection of BMAA during embryonic development alters protein homeostasis and glutamate signaling, whereas fish exposed to a sublethal dose of BMAA display reduced heart rate and abnormal spinal axis formation, but can be rescued pharmacologically (e.g., by inhibiting the endocannabinoid enzyme fatty acid amide hydrolase) (Froyset, Khan & Fladmark, 2016; Purdie, Samsudin, Eddy & Codd, 2009).

4. Zebrafish sensitivity to CNS drug classes

The well-documented similarity of zebrafish and mammalian neurotransmitter systems (Panula et al., 2010; Panula et al., 2006) contributes to the fact that zebrafish models display similar pharmacology and sensitivity to various CNS drugs. Using selected classes of

neuroactive drugs as examples, we will further illustrate this aspect of zebrafish models, and its relevance to the search for novel therapeutic approaches.

4.1. Antiepileptic drugs

PTZ is one of the most widely used convulsant agents in rodents and zebrafish, and produces robust seizure phenotypes suppressed to varying degrees by a wide range of known anti-epileptic drugs (Cunliffe, 2016). PTZ induction of seizures is also an effective way of medium-throughput testing for the discovery of new anti-epileptic treatments (Baxendale et al., 2012; Cunliffe, 2016). As small molecule screens may be conducted in zebrafish as early as 2 dpf, the efficacy of potential treatments is evaluated not only through behavioral testing, but through the monitoring of neural responses (e.g., *c-fos*) (Baxendale et al., 2012). Exposure to PTZ increases C-*fos* expression which is attenuated by classic anti-convulsant agents, as well as anti-inflammatory agents, natural and synthetic steroids, antioxidants, vasodilatory agents, pesticides and herbicides (Baxendale et al., 2012). However, while these drugs attenuate PTZ-induced seizures, the mechanism of their action remains unclear. In addition to PTZ, other drugs evoke seizure-like states in zebrafish (Winter, Redfern, Hayfield, Owen, Valentin & Hutchinson, 2008). Kainic acid (KA) is a common convulsant agent in rodents, and is able to produce similar effects in zebrafish (Alfaro, Ripoll- Gómez & Burgos, 2011). Glutamate receptor antagonists diminish KA-induced seizures, underscoring the utility of the zebrafish model to study glutamatergic excitatory neurotransmission. Also pertinent to the study of anti-epileptic treatment is the combination of genetic manipulation with pharmacological interventions (Cunliffe, 2015). For instance, clemizole (a histaminergic antagonist) is efficient in treating genetically evoked seizures on *scn1lab* zebrafish (Grone & Baraban, 2015), a model of Dravet syndrome (Baraban, Dinday & Hortopan, 2013) caused by SCN1A mutations with spontaneous seizures insensitive to major anti-epileptic drugs.

4.2. Antipsychotics

First generation (typical) antipsychotics are high-affinity antagonists of dopamine D2 receptors, and are the most effective treatment of psychoses (Lieberman et al., 2005). However, they produce severe side effects, including tremors, paranoia and anxiety (Miyamoto, Duncan, Marx & Lieberman, 2005). The second-generation 'atypical' antipsychotics demonstrate a lower affinity for D2 receptors and fewer side effects, relative to typical antipsychotics (Kane, Honigfeld, Singer & Meltzer, 1988). However, there remains a great need for the identification of novel treatments for psychoses, and zebrafish models can be highly useful in this endeavor. For example, administration of MK-801 induces hyper-locomotion (similar to psychomotor agitation, a characteristic symptom of schizophrenia (Seibt et al., 2010)), as well as social and cognitive deficits (Seibt, Piato, da Luz Oliveira, Capiotti, Vianna & Bonan, 2011). MK-801-induced locomotor effects are reversed by typical (haloperidol) and atypical (olanzapine and sulpiride) antipsychotics (Seibt et al., 2010). However, fish exposed to MK-801 perform poorly in an inhibitory avoidance task, and their social and cognitive deficits are restored by atypical, but not typical, antipsychotics (Seibt, Piato, da Luz Oliveira, Capiotti, Vianna & Bonan, 2011). Importantly, atypical antipsychotics have affinities for dopaminergic as well as serotonergic, glutamatergic and other neurosignaling pathways. For example, resperidone acts via D2 and serotonin 5-HT_2 receptors, and shows promise as an anxiolytic substance (Idalencio et al., 2015). Stressed fish exposed to resperidone spend more time in top of the novel tank test, have fewer transitions to the dark in the light-dark test (Magno, Fontes, Gonçalves & Gouveia, 2015) and show lower cortisol levels (Idalencio et al., 2015). The purinergeic system has been recently implicated in schizophrenia (Lara & Souza, 2000), especially since adenosine, the final product in the ectonucleotidase cascade, modulates dopamine and glutamate (Lara & Souza, 2000). In zebrafish, haloperidol reduces ATP hydrolysis and adenosine deamination, thereby

reducing synaptic adenosine levels (Seibt, da Luz Oliveira, Bogo, Senger & Bonan, 2015). The sensitivity of zebrafish ATP hydrolysis to haloperidol suggests an extracellular mechanism of action, potentially relevant to pharmacological targets (Seibt, da Luz Oliveira, Bogo, Senger & Bonan, 2015).

4.3. Drugs of abuse

Substance abuse and addiction are easily modeled in larval and adult zebrafish (Stewart et al., 2011). For example, addiction, tolerance and withdrawal can be studied using aquatic conditioned place preference (CPP) paradigms (Collier & Echevarria, 2013; Collier, Khan, Caramillo, Mohn & Echevarria, 2014; Mathur & Guo, 2010). A typical CPP setup consists of two distinct environments, which differ in their colors, visual patterns or environmental cues (Darland & Dowling, 2001). The protocol consists of three steps: initial determination of environment preference, conditioning session, and testing of final place preference. From the conditioning session, three outcomes are possible: preference for the non-preferred side, aversion of the preferred side, or no change. In zebrafish, CPP protocols generally take ~3 days (Collier, Khan, Caramillo, Mohn & Echevarria, 2014), but may also run for several weeks (Kily et al., 2008). This protocol is widely used in zebrafish, rodents and other model organisms to investigate the behavioral effects of psychoactive compounds and associative learning (Lucke-Wold, 2011) but, despite the ability to elucidate reward-seeking behavior, does not measure the drug's abuse potential (see further).

4.3.1. Alcohol and nicotine

Ethanol produces a characteristic dose-dependent effect in zebrafish. At low doses (<0.5%) ethanol increases locomotion, swim speed, and shoaling behaviors (Gerlai, Lahav, Guo & Rosenthal, 2000). A 20-min exposure to 1.00% ethanol is anxiolytic in zebrafish, whereas longer exposure to the same dose (or higher doses) impairs their locomotion and induces sedation (Gerlai, Lahav, Guo & Rosenthal, 2000; Pannia, Tran, Rampersad & Gerlai,

2014; Rosemberg et al., 2012; Tran & Gerlai, 2013). The rewarding effect of ethanol in zebrafish is seen after a single exposure to 0.25-1% (Collier, Khan, Caramillo, Mohn & Echevarria, 2014) or 1.5% (Mathur, Berberoglu & Guo, 2011), reliably changing fish CPP. A prolonged CPP paradigm (e.g., daily conditioning for 4 weeks) produces robust behavioral responses which persist following abstinence, indicating the establishment of dependence-related behavior (Kily et al., 2008). Some reports evaluating the chronic ethanol CPP treatment note the development of tolerance, as indicated by lower drug-induced hyperactivity, and decreased anxiolytic effects (Gerlai, Lee & Blaser, 2006). Drug abstinence following chronic (1 week) exposure produces robust withdrawal symptoms in adult zebrafish, including anxiety-like behavior and elevated cortisol (Cachat et al., 2011a).

Zebrafish also produce a wide range of dose-dependent responses to nicotine (Kily et al., 2008; Levin, Bencan & Cerutti, 2007). At low to moderate doses (e.g., 3 – 300 μM), nicotine evokes anxiolytic responses in the novel tank test (Levin, Bencan & Cerutti, 2007) and robust CPP that persist following a period of abstinence (Kily et al., 2008). The behavioral effects of nicotine are also susceptible to genetic variation, allowing the researchers to identify genetic candidates for human nicotine addiction (Petzold et al., 2009). Furthermore, microarray analyses of whole brain samples from nicotine-treated fish reveal an upregulation of several genes implicated in the development of drug dependence, including genes for calcineurin B and hypocretin receptor, which have both been previously linked to synaptic plasticity and neurotransmission in drug dependence (Kily et al., 2008).

4.3.2. Cocaine and amphetamines

Administration of cocaine (5, 10, and 15mg/L) to adult zebrafish produces robust arousal states, as indicated by an extension of the fins, slow circling and remaining low in the water column (Darland & Dowling, 2001). Surrounded by conspecifics, cocaine-treated zebrafish engage in aggressive behavior through dominance displays and chasing (Darland &

Dowling, 2001). Abstinence from the drug results in withdrawal symptoms within 72 h, wherein animals experience anxiety-like behavior and basal hyperlocomotion (López-Patiño, Yu, Cabral & Zhdanova, 2008). Withdrawal symptoms are counteracted by the administration of non-sedative dose of diazepam (5 µM) or cocaine (1.5 µM) (López-Patiño, Yu, Cabral & Zhdanova, 2008). Cocaine also produces dose-dependent CPP responses, with 10 mg/L causing the most robust response (Darland & Dowling, 2001). Cross-breeding wild-type females with males mutagenized through repeated exposure to N-ethyl-nitrosourea (ENA) yielded an F_1 generation, outcrossing of which results in F_2 generation tested for cocaine sensitivity in the CPP task. Low responding F_2 siblings were crossbred to yield F_3 generation which display low sensitivity to cocaine in the CPP task, demonstrating a genetic basis for the altered behavior profile (Darland & Dowling, 2001).

Methamphetamine is a potent psychostimulant with high addiction potential, and its abuse is comorbid with psychiatric disorders, including anxiety and depression (Akindipe, Wilson & Stein, 2014). Currently, there are no effective medications for the treatments of methamphetamine abuse. The zebrafish demonstrates sensitivity to methamphetamine and is a useful model to study effective medications, and methamphetamine-related comorbidities (Mi et al., 2016). For instance, the acute administration of methamphetamine induces avoidant behavior and increases swim speed in the open field and mirror stimulation task (Mi et al., 2016), attenuated by *I*-Scoulerine, an agent acting on dopaminergic and serotonergic systems (Mi et al., 2016). The cholinergic system may also play a role in modulating the rewarding effects of various psychoactive drugs, and genetic impairment of AChE does reduce amphetamine-induced CPP in adult zebrafish (Ninkovic et al., 2006). Finally, the pharmacological inhibition of AChE reduces addictive potential of cocaine and morphine in mice (Hikida, Kitabatake, Pastan & Nakanishi, 2003), suggesting that targeting the acetylcholine system may lead to reducing the addictive properties of drugs.

4.3.3. Hallucinogens

Hallucinogenic agents can be classified under three broad categories: (1) classic serotonergic psychedelics, (2) dissociatives, which primarily act as NMDA antagonists, and (3) deliriants, which act as anticholinergic agents (Kyzar & Kalueff, 2016). Classic serotonergic psychedelics (e.g., lysergic acid diethylamide (LSD), mescaline and psilocybin) alter zebrafish locomotion, shoaling and anxiety-like behaviors and whole body cortisol levels (Kyzar & Kalueff, 2016). Ketamine, a dissociative psychedelics, produces a dose-dependent anxiolytic effect in the zebrafish, and decreases whole body cortisol levels (De Campos, Bruni & De Martinis, 2015). The deliriant psychedelic atropine affects cholinergic neural activity in the zebrafish (Park, Lee, Kim & Lee, 2008). Although hallucinogens remain understudied in zebrafish, the available data demonstrate their sensitivity to various known drugs, and may allow for the discovery of therapeutic targets, especially given the growing recent interest in hallucinogenic agents (Kyzar & Kalueff, 2016).

4.3.4. Sedatives

Sedatives are generally prescribed for the treatment of anxiety disorders, and produce anxiety reduction, disinhibition and sedation, mainly modulating the histaminergic, GABA-ergic and adrenergic systems (Koob, 1992). Zebrafish share similarity to the mammalian GABA-A and B receptor subunits, and histamine H1 receptor (Renier et al., 2007), and are highly sensitive to a wide range of sedatives. For example, high doses of chlordiazepoxide significantly reduce swim speed (Bencan, Sledge & Levin, 2009), whereas diazepam has a biphasic effect on anxiety, with low-to-moderate doses reducing bottom dwelling, and higher doses causing sedation (Bencan, Sledge & Levin, 2009). Chronic 2-week exposure to diazepam following by abstinence produces withdrawal-like symptoms in zebrafish, including anxiety in the light dark preference task (Cachat et al., 2011a). While this highlights the utility of zebrafish as a model for sedative-related withdrawal, there is a clear

lack of studies that evaluate the rewarding or aversive effects of sedatives in zebrafish (which can easily utilize the CPP protocol to generate invaluable information on behavioral effects of these drugs).

5. Perspectives on small molecule and genetic screening in zebrafish

5.1. Understanding genetic and anatomical differences from mammalian models

A member of the teleost group, the zebrafish has arisen from a common ancestor ~340 million years ago (Amores, Catchen, Ferrara, Fontenot & Postlethwait, 2011). The ancestor had undergone an additional round of whole-genome duplication (WGD), an event that is responsible for the diversification of gene function and phenotype in zebrafish (Meyer & Schartl, 1999). Of the homologous genes, 71.4% of human genes have at least one zebrafish orthologue and, 47% of human genes have a one-to-one zebrafish orthologue (Amores, Catchen, Ferrara, Fontenot & Postlethwait, 2011). Of the genes for which zebrafish have more than one orthologue, only few have been studied and functionally characterized. Thus, the current lack of understanding of many zebrafish orthologues of human genes is a potential problem with this model. For instance, humans possess three *Period* (*Per*) genes: *Per1*, *Per2*, and *Per3* (Wang, 2008) encoding regulatory elements in the circadian clock, which are also responsible for growth, rest, and hormone production (Danilova, Krupnik, Sugden & Zhdanova, 2004; Pando & Sassone-Corsi, 2002; Vatine, Vallone, Gothilf & Foulkes, 2011). Zebrafish have two *per1* genes (*per1a* and *per1b)*, but only one *per2* and one *per3* (Wang, 2008). The *per1a* and *per1b* genes show distinct temporal and spatial expression, and their roles in the circadian clock is poorly understood (Wang, 2008). Transgenic models may help to elucidate the functions of the zebrafish per1 genes, and provide insight to their role in maintaining circadian rhythms. The serotonin transporter (*sert*) genes have also been duplicated during WGD in zebrafish, which possess two *sert* genes: *serta* and *sertb* (Wang, Takai, Yoshioka & Shirabe, 2006). These genes have high homology

to vertebrate serotonin transporter genes, suggesting a conservation of function (Wang, Takai, Yoshioka & Shirabe, 2006). Thus, despite an additional WGD, it does not render the zebrafish model unusable. Rather, the study and functional identification of genes may help better understand molecular interactions, which will further clarify the efficacy of drugs and their therapeutic targets.

Furthermore, despite significant neuroanatomical similarity discussed above, some differences between zebrafish and mammals must be critically considered. For example, while several regions in the mammalian brain do not have clear structural homologous counterparts in zebrafish, including the substantia nigra and hippocampus (Mueller, Dong, Berberoglu & Guo, 2011; Panula et al., 2010), they share functional homology with selected groups of zebrafish neurons. Thus, a small population of dopaminergic cells in the posterior tuberculum is a strong candidate for the zebrafish homologue of the substantia nigra (Kaslin & Panula, 2001), as shown by neurotoxin lesion studies (Sallinen et al., 2009). Likewise, the lateral part of the zebrafish pallium contains homologous structures to the mammalian hippocampus (von Trotha, Vernier & Bally-Cuif, 2014), thereby fostering further cognitive studies in zebrafish models. One stark difference from humans is the fact that zebrafish lack a cortex, its homologue, or even molecular markers that may be used to identify a cortex region (Mueller, Dong, Berberoglu & Guo, 2011; Northcutt, 2008). This aspect may limit translation of findings between zebrafish and humans, especially on aberrant executive functioning commonly observed in psychiatric diseases (Parker, Brock, Walton & Brennan, 2013). However, given potential limitations of the model, it is necessary to evaluate its face and construct validity. Face validity determines whether the model resembles the disease in question, while construct validity determines whether the model measures what it has set out to measure. Because the rodent models often fulfill these validity criteria, many zebrafish behavioral tasks have been modified from rodent paradigms (Levin, Bencan & Cerutti, 2007).

For instance, for modeling anxiety disorders, the zebrafish has become an adept model in the identification of stress-inducing stimuli and psychoactive agents in various novelty-based paradigms (Abreu, Giacomini, Kalueff & Barcellos, 2016; Bencan, Sledge & Levin, 2009; Levin, Bencan & Cerutti, 2007). Deficits in cognitive and behavioral flexibility, commonly reported in patients with psychiatric diagnoses, may also be modeled in zebrafish. Behavioral flexibility - the ability to adapt responses to changing environmental conditions - is often studied in rodents using a reversal of contingencies in choice-discrimination tasks (Ragozzino, Detrick & Kesner, 1999; Saus et al., 2010). These tasks rely on the ability of the animal to demonstrate a reversal of learning. Zebrafish have demonstrated the ability to adapt to changing environmental contingencies, and their capacity for reversal learning shows similar patterns to that observed in rodents (Colwill, Raymond, Ferreira & Escudero, 2005). Thus, although zebrafish may lack a proper cortex, they retain the ability to perform executive functions, such as maintaining attention and behavioral flexibility (Parker, Brock, Walton & Brennan, 2013). However, we still know relatively little about the neural circuits and how different neurotransmitter systems may functionally interact in zebrafish (Parker, Brock, Walton & Brennan, 2013). Identifying the function of neural circuits in this fish, and their reciprocity with other systems, become critical to understanding the molecular basis of behavior, and in identifying therapeutic targets for diseases.

5.2. Perspectives on automated and high-throughput screening

Zebrafish models are highly amenable to behavioral, genomic and proteomic testing (Jones & Norton, 2015; Purushothaman et al., 2015), as they combine a relative neural simplicity with behavioral complexity sufficient for studying multiple behavioral processes from sleep (Purushothaman et al., 2015; Rihel et al., 2010; Zhdanova, 2006) to anxiety (Richendrfer, Pelkowski, Colwill & Creton, 2012; Stewart, Gaikwad, Kyzar, Green, Roth & Kalueff, 2012). Custom-made and commercial video-tracking software can record a wide

range of zebrafish behavioral measures, including velocity, distance traveled, place preference (e.g., top vs. bottom, light vs. dark, center vs. periphery), and specific patterns (e.g., erratic swimming, stereotypic circling) (Conklin, Lee, Schlabach & Woods, 2015; Pérez-Escudero, Vicente-Page, Hinz, Arganda & de Polavieja, 2014). The automation of zebrafish video-tracking enables several behavioral outcomes to be recorded simultaneously, removing the need to repeat the experiment and/or watch and re-watch videos manually each time a new outcome is measured (Conklin, Lee, Schlabach & Woods, 2015; Pérez-Escudero, Vicente-Page, Hinz, Arganda & de Polavieja). It is also possible to record zebrafish social groups, for example, assessing fish shoaling behaviors, presently capable of tracking multiple (e.g., 8-16) animals per arena (Noldus, 2016b) to extract rich behavioral data from average inter-fish distance to shoal polarization and cohesion (Stewart, Braubach, Spitsbergen, Gerlai & Kalueff, 2014b). Larval zebrafish allow for recording of even more (e.g., 96) animals, tracking their swim patterns simultaneously (Noldus, 2016a). An added advantage of technological advancements in this rapidly growing field of zebrafish phenomics is the automatization of drug administration, and the computerization of stimulus exposure - e.g., in drug addiction or fear conditioning paradigms (Saverino & Gerlai, 2008), which collectively improves the standardization of testing procedures, efficient data collection, as well as increased throughput and data reproducibility (Love, Pichler, Dodd, Copp & Greenwood, 2004; Stewart, Gerlai & Kalueff, 2015).

Zebrafish are further amenable to high-throughput in-vivo screening as their multiple behavioral parameters can be monitored in 3D (Stewart et al., 2015a). For example, the X, Y, and Z swim trajectories can be traced by two cameras, generating two 2D-trajectory files integrated to produce a 3D trace of the swim pattern which can help identify unique drug-induced phenotypic profiles (Stewart et al., 2015a). Advances in behavioral recognitions allow for a more detailed in vivo analysis of behavioral phenotype (Stewart, Gerlai &

Kalueff, 2015). For instance, software that can discriminate between the tail, mid-body and nose of the zebrafish are well capable of quantifying locomotion, and interpreting complex behaviors such as chasing or nipping, chasing (Kalueff et al., 2013b; Stewart, Gerlai & Kalueff, 2015). These methods are especially useful in polypharmacology studies using pharmacological agents that act on multiple targets (McCarroll, Gendelev, Keiser & Kokel, 2016). As many psychiatric disorders are linked to deficits in several neurotransmitter systems and have multigenic etiologies (Kendler, Aggen & Neale, 2013; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), this possibility in zebrafish screens becomes particularly important. Computational techniques, such as hierarchical clustering or Similarity Ensemble Approach (SEA), also help identify target hits and prediction of target interactions with psychoactive compounds. The combination of behavioral phenotyping and computation techniques are useful in the development and discovery of new medical targets, including *in-vivo* behavioral phenotyping of single target compounds in 2-D or 3-D tracking, producing their unique swim traces (thigmotaxis, scototaxis, average swim speed, etc.), identifying the compounds which produce the desired behavioral phenotype (hit compounds), and their subsequent in-depth analyses with algorithms that predict their biological target(s) to generate hypotheses of target combinations (McCarroll, Gendelev, Keiser & Kokel, 2016). Once identified, multiple hit compounds can then be tested in combination in-vivo (McCarroll, Gendelev, Keiser & Kokel, 2016), probing their ability to work in concert to achieve a desired therapeutic outcome.

5.3.Perspectves on genetic zebrafish models

As already noted, genetic manipulations are critical on animal studies to identify candidate genes associated in the etiology of a disease. Short-term genetic manipulation is achieved through injection of morpholino-modified antisense oligonucleotides (MOs) (Nasevicius & Ekker, 2000), or small interfering RNA (siRNA) (de Rienzo, Gutzman & Sive,

2012) to engage in loss-of-function studies (Kalueff, Stewart & Gerlai, 2014). MOs target specific translational inhibitors and effectively reduce gene expression (Nasevicius & Ekker, 2000). RNA interference (RNAi) is a process in which RNA molecules inhibit the translation of targeted mRNA molecules (de Rienzo, Gutzman & Sive, 2012). These methods demonstrate efficacy in targeting specific genes and in producing altered phenotypes, although the efficacy of MOs has been questioned recently (Kok et al., 2015; McCammon & Sive, 2015).

The development of mutant zebrafish provides a more stable behavioral phenotype, because rather than produce a knockdown of a given gene, it completely eliminates the target gene product (Amsterdam & Hopkins, 2006; Stewart et al., 2014). Mutants are created through retroviral insertional mutagenesis, wherein a DNA basepairs are integrated into the organism's preexisting DNA (Amsterdam & Hopkins, 2006), or through chemical mutagenesis. Chemical mutagenesis involves exposing the male zebrafish to the methylating agent ethylnitrosourea weeks before mating in order to allow the mutation to fix in the spermatogonia just before they mature to sperm (Amsterdam & Hopkins, 2006; Wienholds, van Eeden, Kosters, Mude, Plasterk & Cuppin, 2003). Mutant and morphant zebrafish are used in a wide range of studies, and provide a deeper understanding on the roles and importance of specific receptors and biological targets (Griffiths, Schoonheim, Ziv, Voelker, Baier & Gahtan, 2012a; Haesemeyer & Schier, 2015). For instance, in developing a mutant model of autism, a highly active set of genes was discovered with a large genetic target, providing a deeper look in to the functional changes associated with gene deletion and duplication (Blaker-Lee, Gupta, McCammon, De Rienzo & Sive, 2012). Furthermore, the size of the genetic target, which had previously been unknown, was elucidated allowing for targeted assays in higher vertebrates and mammals (Blaker-Lee, Gupta, McCammon, De Rienzo & Sive, 2012). Similarly, loss of function mutations for the synaptic machinery genes

stxbp1a and *stxbp1b* produce robust phenotypes (Grone et al., 2016). In humans, these genes are linked to various neurodevelopmental disorders and epilepsy (Carvill et al., 2014; Saitsu et al., 2008). Homozygous *stxbp1a* knockdown results in immobility, reduced heart rate, reduced metabolism, and early death (Grone et al., 2016). Heterozygous *stxbp1a* knockdown produces markedly fewer deleterious effects; aside from a slight reduction in behavioral response to a startle stimulus, larval zebrafish produce normal behavior (Grone et al., 2016). Homozygous *stxbp1b* mutations yield zebrafish that present with epileptic seizures, along with normal mobility, metabolism and heart rate (Grone et al., 2016). The wide range of behavioral and physiological effects of the loss of function mutations for *stxbp1a* and *stxbp1b*, coupled with the functional similarity to the mammalian genes (Saitsu et al., 2008), highlight the potential for the zebrafish model to be used in the mechanistic and epigenetic study of neurodevelopmental and neuropsychiatric diseases.

6. Conclusion

Neuropsychiatric conditions afflict human population globally, and have tremendous personal and societal costs (Garakani, Mathew & Charney, 2006; Griebel & Holmes, 2013a). Animal models have long been used in neuropsychiatric studies to better understand human disease states, and play a key role in the identification of biological and molecular targets, aiming at developing safer and more effective treatments (Keifer & Summers, 2016; Krishnan & Nestler, 2011). Zebrafish are a promising new animal model which continues to provide important insights into the etiology of CNS diseases (Kalueff, Echevarria & Stewart, 2014b; Kalueff, Stewart & Gerlai, 2014). The homology of key brain regions between zebrafish and mammals underscores the utility of zebrafish models in neurobehavioral and neuropsychiatric studies. Furthermore, the conservation of neural pathways between zebrafish and mammals allows for the bi-directional translation of findings (Renier et al., 2007; Stewart, Braubach, Spitsbergen, Gerlai & Kalueff, 2014b). Current genetic tools,

tracking techniques and statistical algorithms foster gaining a deeper understanding of molecular pathways, developing new compounds or repurposing established drugs (Stewart et al., 2015a). Together with high sensitivity of zebrafish to known anxiolytic, antipsychotic and other CNS drugs, this provides researchers with a well-rounded model organism capable of identifying molecular targets for drug treatment and empirical testing of these hypotheses (Hwang et al., 2013; Kokel et al., 2010; Stewart et al., 2015a).

Conflict of interest:

The authors declare that there are no conflicts of interest.

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Figure 1. The use of automated video-tracking to simultaneously assess multiple phenotypes in larval zebrafish. Panel (a) shows a 96-well holding plate to administer several compounds to larval zebrafish. Fish behaviors are recorded by an overhead camera, and images are processed through tracking software. Swim traces garnered from the tracking software allow the researcher to assess the effects of the compounds administered. Panel (b) shows an example of a swim trace in which the larval zebrafish remains towards the walls (wall-hugging behavior). Panel (c) shows an example of the opposite swim pattern in which the larval zebrafish actively explores its environment, including the center of the tank.

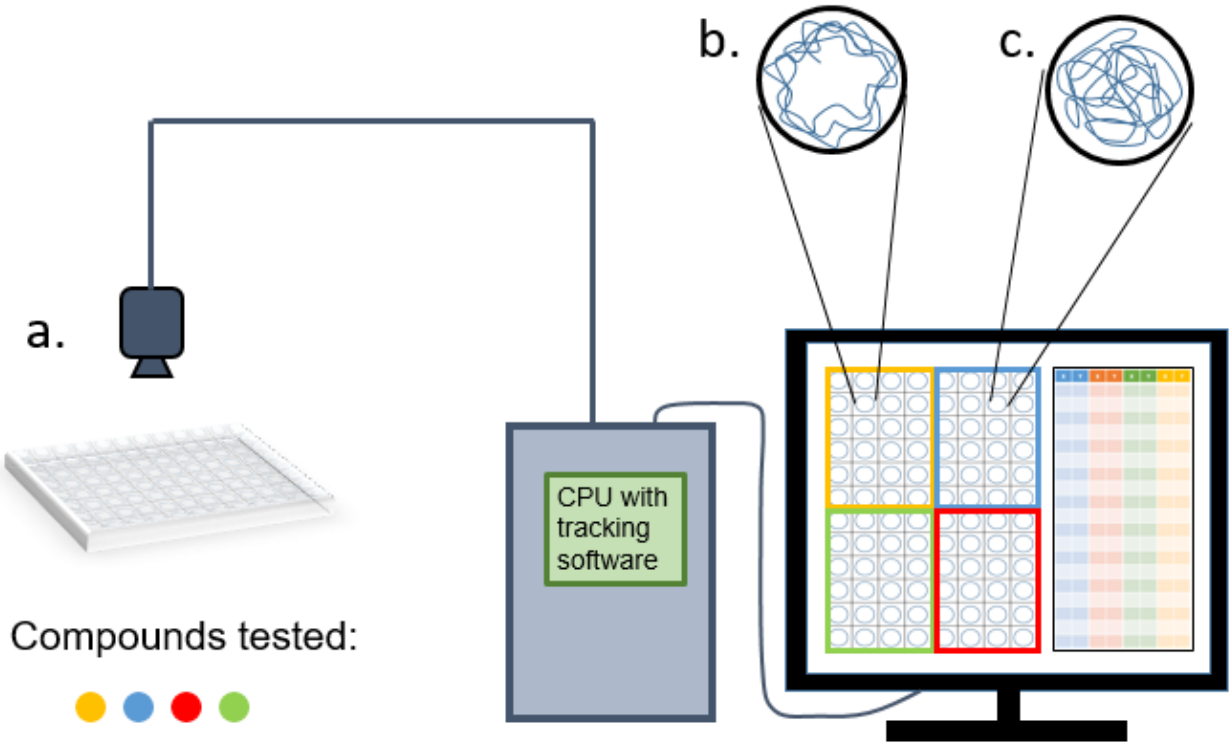

Figure 2. The use of computational techniques to identify novel therapeutic targets in adult zebrafish. Testing psychoactive compounds across various strains and transgenic lines of zebrafish in a wide range of behavioral and cognitive tasks can be used to generate a data library. Computational tools, such as hierarchical modeling and similarity ensemble approach (SEA), can help identify target hits, enabling the predictions about the effects of various drug combinations. The generated hypotheses may then be tested *in vivo* using larval or adult animals.

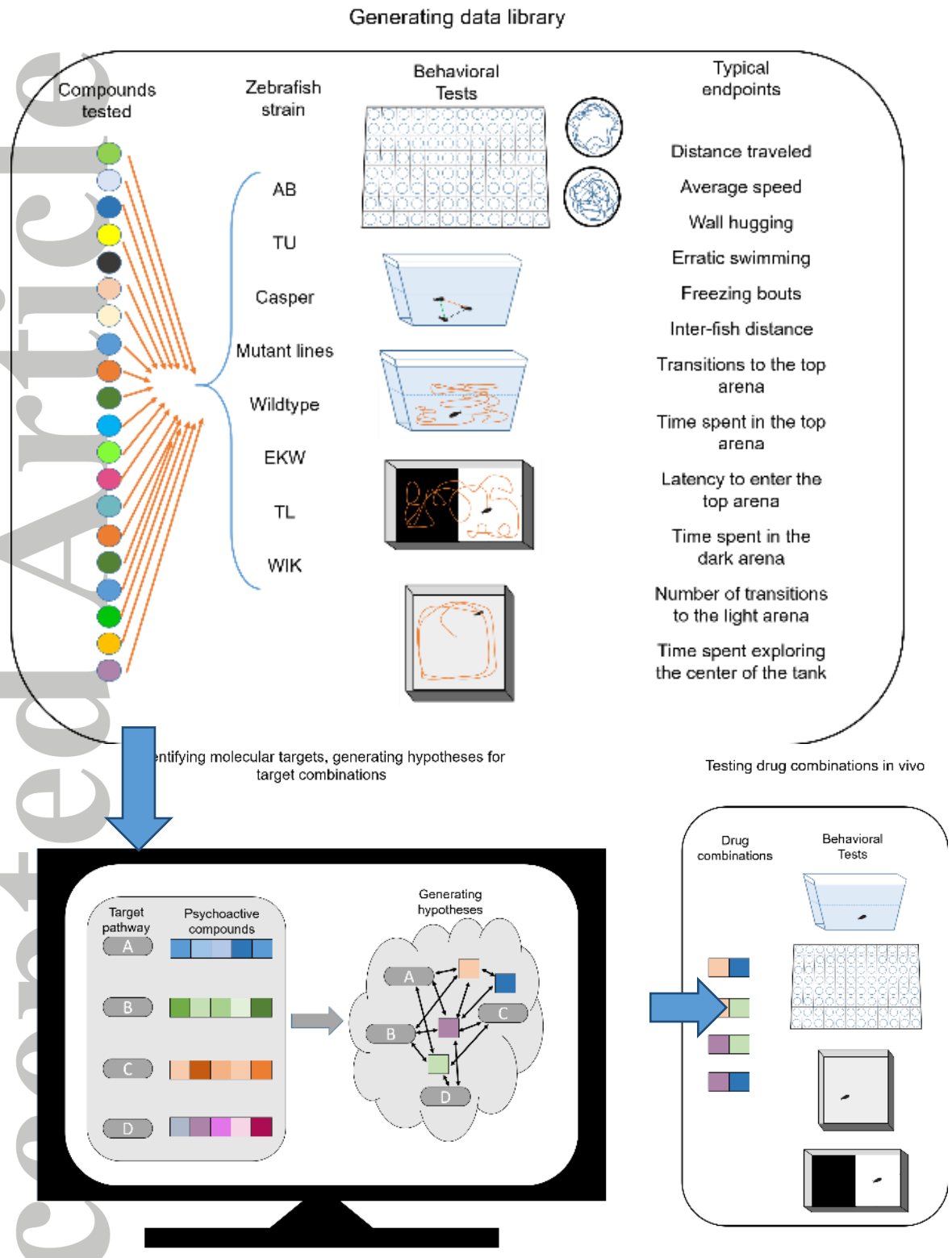

Table 1: Selected examples of translational successes using the zebrafish model for drug discovery

Human disease	Zebrafish model	Outcome	References
Pontocerebellar hyperplasia	*Tsen54* antisense morpholino	Linking a loss-of-function mutation in the *tsen54* gene to brain hypoplasia	(Kasher et al., 2011)
	R44X-loss-of-functoin mutant	Linking homozygous mutation of CLP1 (a member of the tRNA splicing endonuclease complex, TSEN) to abnormal spinal neurons, curved body, small head and eyes, and an early death in fish, helped identify this mutation as a risk factor for human condition	(Schaffer et al., 2014)
Spinal cord injury	*Heat shock transgenic lines*	Zebrafish show high capacity for axonal regeneration following spinal cord injury, especially through the activation of Fgf signaling. Increasing Fgf signaling in mammalian spinal injury sites may encourage glial cell differentiation, and lead to favorable conditions for axonal regeneration	(Goldshmit, Sztal, Jusuf, Hall, Nguyen-Chi & Currie, 2012)
Schizophrenia	Tg(*huC*:eGFP)	The *Rgs4* gene is associated with the onset and development of schizophrenia. Using the transgenic zebrafish line, *rgs4* was found to be essential for axon formation, providing the first in vivo evidence supporting the role of *rgs4* in schizophrenia	(Cheng et al., 2013)

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