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Using zebrafish to assess the impact of drugs on neural development and function

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Abstract

Background—Zebrafish is becoming an increasingly attractive model organism for understanding biology and developing therapeutics, because as a vertebrate, it shares considerable similarity with mammals in both genetic compositions and tissue/organ structures, and yet remains accessible to high throughput phenotype-based genetic and small molecule compound screening.

Objective/method—The focus of this review is on the nervous system, which is arguably the most complex organ and known to be afflicted by more than six hundred disorders in humans. I discuss the past, present, and future of using zebrafish to assess the impact of small molecule drugs on neural development and function, in light of understanding and treating neurodevelopmental disorders such as autism, neurodegenerative disorders including Alzheimer's, Parkinson's, and Huntington's disease, and neural system dysfunctions such as anxiety/depression and addiction.

Conclusion—These studies hold promise to reveal fundamental mechanisms governing nervous system development and function, and to facilitate small molecule drug discovery for the many types of neurological disorders.

Keywords

zebrafish; neural development; neurodegeneration; function; small molecule drug discovery; neurological disorders; autism; Parkinson's disease; Alzheimer's disease; Huntington's disease; anxiety/depression; addiction

1. Introduction

Structural similarity of the nervous system emerges at the level of vertebrates, with the central nervous system (CNS) derived from a sheet of dorsally located neuroectoderm that is regionally patterned into the forebrain, midbrain, hindbrain, and spinal cord, while the peripheral nervous system (PNS) is derived from the migratory neural crest cells. During its formation and maturation, the nervous system forms intricate connections within itself and to most if not all tissues and organs in the body, and functions to regulate complex behaviors and vital activities¹. Given such complexity and importance, it is not surprising that more than 600 human disorders afflict the nervous system (sources from National Institute of Health, http://www.ninds.nih.gov/about_ninds/ninds_overview.html). They range from those that affect neural development (e.g. mental retardation, autism, schizophrenia), to those that disrupt neural maintenance (e.g. Alzheimer's disease, Parkinson's disease) and neural function (e.g. anxiety/depression, and addictive disorders). Neurological disorders strike an estimated 50 million Americans each year, placing immense burdens to the individuals and family involved, with an annual economic cost of hundreds of billions of dollars in medical expenses and lost productivity (sources from National Institute of Health, http://www.ninds.nih.gov/about_ninds/ninds_overview.html).

Treating neurological disorders is a tremendous challenge, because the CNS is largely inaccessible to intravenously delivered cell- or protein-based therapeutic agents. Small molecule drugs that can penetrate the blood-brain barrier are therefore particularly attractive candidates for treating CNS disorders. Most of the currently available small molecule drugs that alleviate neurological symptoms are discovered in serendipitous ways, and many of them do not represent ideal treatments, either due to inadequate efficacy or troubling side effects. Furthermore, for many neurological disorders, treatments are simply not available. These situations highlight the urgent need to find novel and systematic approaches to facilitate CNS drug discovery.

This review aims to evaluate the feasibility of zebrafish as a system for discovering and studying the impact of drugs on the nervous system and related neurological disorders. First, the use of zebrafish for chemical genetics/genomics and small molecule discovery is briefly reviewed. Second, our current knowledge of the zebrafish nervous system is examined in comparison to that of mammals, at both developmental and adult stages, and in terms of its response to known drugs. Third, neurological disorders, including autism, Parkinson's disease, Alzheimer's disease, Huntington's disease, anxiety/depression, and addictive disorders, and overall strategies to model them in zebrafish, are presented. Finally, how these models can be used in small molecule drug discovery is discussed.

2. Use of zebrafish in chemical genetics/genomics and small molecule drug discovery

Chemical genetic approaches have been successfully employed in transparent embryonic and larval zebrafish to probe interesting biological processes as well as to catalyze the discovery of potential therapeutic compounds. Thus far, most studies have been conducted outside of the nervous system, and have been reviewed in detail elsewhere²⁻⁴. Here we take an overview of a few examples and issues generally related to screening and chemical compounds. For instance, small molecules have been identified that suppress mutation-caused cardiovascular defect⁵ or cell cycle arrest⁶, and those that modulate the embryonic heart rate in wild type⁷. In addition, many drugs with known effects in humans have been shown to cause similar effects in zebrafish^{8,9}. Moreover, a recent study has identified a potent small molecule, prostaglandin E₂, through zebrafish-based small molecule screening for chemical regulators of haematopoietic stem cell (HSC) homeostasis. Remarkably, this small molecule also performs similar action in mammalian HSCs, thus validating that zebrafish-based drug discovery can potentially lead to therapeutic compounds for human conditions¹⁰.

Because of the small size of embryonic and larval zebrafish, small molecule compound screens can often be carried out in a high throughput way using 96-well plates. Many small molecule libraries are available for screening. Some contain a collected set of compounds (both natural and synthetic), such as those available from Microsource Discovery systems, whereas others contain compounds synthesized through combinatorial chemistry, such as those available from ChemDiv and Chembridge².

3. The zebrafish nervous system: similarities and differences to that of mammals

In order to understand or discover the effects of drugs on the nervous system using zebrafish, it is important to know the organization and overall similarity between zebrafish and mammalian nervous systems. Our understanding of the zebrafish nervous system is still rudimentary, with most of our knowledge gathered on the first 48 hours of the organism's life. In this section, I provide an overview of the zebrafish nervous system, by discussing the emergence of brain subdivisions and differentiated neural subtypes, the connectivity and

systems-level organization in the adult brain, molecular architecture of receptors and signaling molecules expressed in the nervous system, and finally, the effects of known neuro-active drugs on zebrafish. Both the similarities and the differences between zebrafish and mammals are highlighted.

3.1 The emergence of brain subdivisions and differentiated neural subtypes during development

Zebrafish embryos are transparent and develop externally, thereby allowing the entire embryogenesis to be observed with ease (Figure 1). By the end of gastrulation (~10 hours post fertilization, -hpf), the neural plate becomes distinguishable from the rest of the ectoderm, and subsequently forms the neural tube. By 1 day post fertilization (dpf), major brain subdivisions that include the forebrain, midbrain, hindbrain, and spinal cord have formed^{11,12}. Concomitant with the emergence of brain subdivisions, the earliest clusters of neurons, namely primary neurons, appear in the center of each subdivision, and extend axonal tracts in a discrete and stereotyped pattern¹¹. These early axonal tracts may serve as a simple scaffold for the axogenesis of later born neurons. By 2–3 dpf, many different neurotransmitter-expressing neurons can be identified. These include the GABAergic^{13–15}, glutamatergic¹³, monoaminergic (dopamine, noradrenaline, serotonin, histamine)^{16–20}, cholinergic²¹, and peptidergic^{22,23} neurons. Glial subtypes (oligodendrocytes, schwann cells, and astrocytes) are detected beginning at ~4 dpf^{24,25}.

The gross anatomical organization of the CNS and PNS is relatively conserved between zebrafish and mammals. For instance, the counterparts of many brain subdivisions found in the developing mammalian brain are morphologically identifiable in the teleost (zebrafish) brain^{26,27}. Analyses of gene expression further reveal that many developmentally expressed genes, ranging from pre-pattern to proneural regulators, are orchestrated in a conserved pattern in these brain subdivisions^{28–30}. Despite these similarities, a clear difference has been observed between the development of mammalian and zebrafish telencephalic hemispheres, with evagination happening in mammals versus eversion occurring in teleosts including zebrafish^{31,32}. Such difference in the process of telencephalic formation has hampered the recognition and comparative interpretation of zebrafish telencephalic organization. For example, although recent comparative neuroanatomical analyses have postulated the homologous structures in zebrafish to the mammalian cortex, hippocampus, amygdaloid complex, and basal ganglia¹⁵, It remains to be elucidated their neuroanatomical delineations, molecular architecture, functional correlates, and developmental origins.

With regard to differentiated neural subtypes, an astonishing degree of correspondences between GABA cell populations in zebrafish and mice is observed during a critical developmental time window (2–3 dpf in zebrafish versus E12.5–13.5 in mice)^{14,15}. The development of monoaminergic and peptidergic neurons also follows similar spatial and temporal programs in zebrafish and in mammals. The dopaminergic neurons are found in the retina, olfactory bulb, ventral forebrain, and ventral midbrain in mammals, whereas in zebrafish, they are detected in the retina, olfactory bulb, and ventral forebrain only^{16,33}. Interestingly, despite the conspicuous absence of dopaminergic neurons in the zebrafish ventral midbrain, groups of dopaminergic neurons located in the diencephalic posterior tubercular area are found to project into the telencephalon, resembling the mammalian mesostriatal and mesolimbic pathways³⁴. In contrast to the conserved features observed in most parts of the brain including the ventral telencephalon, the development of the teleostean dorsal telencephalon (pallium) appears to be quite different with regard to the region homologous to the mammalian cerebral cortex, which has a highly organized, laminated pattern³⁰. Although the layered organization of the cortex is known to be a mammalian adaptation³⁵, it remains

to be determined whether major cortical neuronal subtypes identified in the mammalian cortex are present in zebrafish or not.

3.2. Connectivity and circuitry in the adult nervous system

Because of the scarcity of connectivity information in zebrafish, data from the closely related goldfish is used as a reference³⁶. Hodological studies generally have demonstrated great similarities of teleostean sensory, motor, and integrative central neural circuits in comparison with other vertebrates including mammals. The chain of synaptic relay of teleostean sensory systems ascending to the telencephalon is largely comparable to that in other vertebrates, albeit some differences do exist. For instance, there are two main diencephalic sources of sensory inputs to the teleostean telencephalon: the (periventricular) dorsal thalamus and the preglomerular region. Whereas the dorsal thalamus is the major source for sensory input to the pallial telencephalon in mammals, the preglomerular region predominantly subserves this role in teleosts, and the presence of both sources in teleosts may represent the ancestral vertebrate pattern³⁷.

3.3. Molecular architecture of the zebrafish nervous system

In addition to neuroanatomical similarities, the zebrafish nervous system also expresses many signaling molecules that show similarities to that of mammals. These include major secreted proteins and their signal transduction pathways such as Sonic Hedgehog, WNTs, BMPs, and FGFs, the neurotransmitter/neuropeptide receptors and transporters including those of GABA, dopamine, glutamate, serotonin, NPY, and opioids^{38–42}.

3.4. Effects of known drugs on neural development and function in zebrafish

Zebrafish, particularly larval zebrafish, have been used in toxicological studies to analyze the effects of neurotoxins and neuroprotectants on the developing nervous system^{43–46}. For example, 7 compounds that are well-characterized for their action in mammals have been tested on embryonic and larval zebrafish to examine several parameters of neurotoxicity during development, including teratogenicity, cell death, and selected neuronal subtypes⁴⁷. This study finds that Atrazine, dichlorodiphenyltrichloroethane (DDT), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are primarily teratogenic and not specifically neurotoxic. 2,4-dichlorophenoxyacetic acid (2,4-D), dieldrin, and nonylphenol show specific neurotoxicity; dieldrin and nonylphenol are specifically toxic to catecholaminergic neurons. Malathion, although not teratogenic, shows some nonspecific toxicity. Thus, results from zebrafish show a strong correlation with mammalian data.

Effects of a number of psychoactive compounds have been evaluated in zebrafish. At physiologically relevant concentrations, ethanol has been found to affect locomotor activity, very much like its effect in mammals^{48,49}. Cocaine^{50,51}, amphetamine⁵², morphine^{53,54}, and nicotine⁵⁵ have been found to produce reward- or anxiety-related effects in zebrafish. The known teratogenic effects of some of these compounds also prompt evaluation of their effects on embryonic development. Indeed, both ethanol⁵⁶ and nicotine⁵⁷ have been shown to affect nervous system development.

Pharmacological agents that target various neurotransmitter/neuropeptide receptors or transporters have been used to treat either larval or adult zebrafish. Many have predicted effects based on their action on the mammalian nervous system. These include agents that affect the receptors for GABA, glutamate, dopamine, serotonin, acetylcholine, and opioid systems (Table 1).

In summary, our current knowledge of the teleost fish nervous system suggests that although some differences exist, remarkable similarities with that of mammals have been detected,

making it convenient to extrapolate the findings made in zebrafish to mammals. From the standpoint of drug discovery, small molecules that are bioactive in targeting zebrafish gene products are likely to have a similar action on the mammalian orthologues. Given the feasibility of zebrafish for high throughput screening that is much more difficult to carry out in mammalian organisms, it is fair to say that zebrafish offers an excellent opportunity for CNS drug discovery efforts. However, in order to seize upon this opportunity, it is critical to establish relevant models and screening assays in this organism.

4. Strategies for modeling human neurological disorders and identifying neuro-active compounds in zebrafish

In general, several alternative strategies can be employed in animal systems to model human diseases: for the disorders with defined molecular lesions, similar molecular lesions can be created in animal models; for those with defined cellular lesions, similar cellular lesions might be introduced in animal models. The overall similarity of the zebrafish nervous system to that of mammals and the particular amenability of zebrafish for candidate drug evaluation and high throughput drug screening suggests that it is a potentially fruitful system for modeling neurological disorders and moreover for subsequent drug discovery. The available molecular genetic technologies permit inactivation^{58–62} or overexpression^{63–65} of genes implicated in the human disorders. Tools to introduce defined cellular lesions in specific cell or tissue types^{66,67} have also been established in this organism. Because of the scope of this review, the readers are referred to the reviews cited above for details regarding these technologies.

One should also keep in mind, however, while determining the validity of any animal models, that it is impossible to recapitulate all the phenotypes of human disorders in any single given animal model. In this section, I highlight several human neurological disorders including autism spectrum disorders, neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, and neuropsychiatric disorders such as anxiety/depression and addiction, and discuss current efforts toward creating zebrafish models for these disorders.

4.1 Autism Spectrum Disorders

Among the different kinds of neurodevelopmental disorders, here we focus on autism spectrum disorders (ASDs). A meta-analysis of ASD prevalence rates suggests that approximately 37 in 10,000 individuals are affected, and currently 1 in 150 children is said to be diagnosed with autism⁶⁸. Autism is characterized by three distinctive behaviors: difficulties with social interaction, problems with verbal and nonverbal communication, and repetitive behaviors or narrow, obsessive interests. These behaviors can range in impact from mild to disabling. It is unclear what causes autism, and there is no clear and consistent pathology that has emerged for autism, although multiple regions of the brain including the cerebral cortex, amygdala, brainstem, and cerebellum are involved, and developmental processes including progenitor proliferation, programmed cell death, neuronal migration, axodendritic growth and pruning, synaptogenesis and synaptic transmission may be altered in ASD patients.^{69,70} Due to their less ambiguous association with ASDs and interesting biological properties, several types of genetic lesions are worth particular mentioning. 1) The gene encoding methyl-CpG-binding protein 2 (MeCP2), a transcriptional regulator widely expressed throughout the brain, is disrupted in the Rett Syndrome (RTT), a devastating X-linked ASD disorder that affects 1 in 10,000 females⁷¹. Patients with classic RTT suffer from a broad array of phenotypes that affect almost every part of the central and autonomic nervous systems, including impaired social behavior and communication skills, motor abnormalities, and the development of stereotyped movements. Another transcription regulator, Engrailed 2 (EN2), is also associated with ASDs^{72,73}. 2) Mutations in the fragile X mental retardation (*FMR1*) gene, which encodes

a translational repressor, cause an X-linked form of mental retardation with ~15–30% prevalence of ASD⁷⁴. Mutations in Tuberous sclerosis complex (*TSC*)⁷⁵ and the *PTEN* phosphatase⁷⁶ genes, both of which are involved in translational regulation, have also been associated with ASDs, suggesting that defects in translational regulation represent one potential mechanism underlying the development of ASDs. 3) Analyses of *de novo* chromosomal deletions and duplications have hinted at the involvement of synaptic molecules including neuroligins 3 and 4 (*NLGN3* and *NLGN4*)⁷⁷, *SHANK3* (a cytoplasmic binding partner of neuroligins)⁷⁸, neurexin (*NRXN1*)⁷⁹, contactin associated protein-like 2 (*CNTNAP2*, a member of neurexin superfamily)^{80,81}, and *CACNA1C* (L-type voltage-gated calcium channel)⁸². 4) *RELN*, an extra-cellular matrix protein involved in cell adhesion and migration, is associated with ASD⁸³. While available therapies and behavioral interventions are designed to remedy specific symptoms, there is currently no cure for autism.

Given the likely developmental origins of ASDs and the suitability of zebrafish for developmental studies, surprisingly little has been done with respect to modeling ASDs in zebrafish. A review discussing the potential of the system has been published⁸⁴. This may be in part due to the complex behavioral abnormalities associated with ASDs that include deficits in language, social interaction, and breadth of interests. Deficits in language and breadth of interests are indeed difficult to model perhaps in any animal systems. However, the observed developmental and cellular defects as well as defects in social interaction can certainly be modeled in zebrafish. Indeed, zebrafish homologues of genes implicated in ASDs, including *neurexins*⁸⁵, *reelin*³⁵, *mecp2*⁸⁶, and *met*⁸⁷, have been identified. Assays that measure social interaction have been developed^{88–90}. Thus, it will be interesting to assess whether lesions in ASD-associated genes lead to developmental and cellular defects as well as defects in social interaction similar to what has been observed in mammals. Uncovering developmental and cellular defects could lead to the establishment of potential screening assays, which can be used to identify small molecule compounds that can modify such phenotypes.

4.2 Neurodegenerative disorders: Parkinson's disease, Alzheimer's disease, and Huntington's disease

Parkinson's disease—Parkinson's disease (PD) is the most common movement disorder, manifested with four primary symptoms including tremor, rigidity, bradykinesia, and postural instability. The loss of brain dopamine neurons and formation of proteinaceous aggregates known as Lewy bodies are pathological hallmarks of PD. PD usually affects people over the age of 50, but rare familial forms can strike at a much younger age. Several genes have now been definitively linked to PD. They encode α -synuclein⁹¹, Parkin⁹², DJ-1⁹³, PINK1⁹⁴, and LRRK2^{95,96}, the dysfunctions of which are thought to cause either proteasomal abnormalities or oxidative stress. At present, there is no cure for PD, but a variety of medications provide symptomatic relief. Although levodopa combined with carbidopa helps at least 75% parkinsonian cases, not all symptoms respond equally to the drug. Prolonged use of these drugs unfortunately also has troubling side effects such as the development of dyskinesia. Other drugs, such as bromocriptine, pramipexole, and ropinirole, mimic the role of dopamine in the brain, causing the neurons to react as they would to dopamine. An antiviral drug, amantadine, with possible mechanisms as a nicotinic agonist or NMDA antagonist, also appears to reduce symptoms.

Given the ease of delivering chemical compounds to zebrafish, the potential PD-inducing effects of MPTP, its metabolite MPP⁺, and the pesticides including rotenone and paraquat, have been evaluated in both larval and adult zebrafish^{97–100}. One issue relating to the validity of this MPTP neurotoxin-induced PD model is whether the loss of DA neurons represents a selective event as in humans, i.e. other groups of neurons are largely spared. Such selective effect of MPTP has indeed been observed in larval zebrafish. It will be of interest in the future

to treat larval zebrafish of a later stage and see if DA neuronal loss can be similarly observed, in order to further determine whether MPTP affects the development or the survival of DA neurons. Moreover, it will be of great interest to know whether a recovery of DA neuronal loss can be observed after the removal of MPTP. In addition to locomotor deficits, MPTP has also elicited remarkable peripheral phenotypes in zebrafish, including a difficulty in respiration and darkened skin pigmentation. It should be further validated whether the change in skin pigmentation might be related to a loss of norepinephrine contents or neuronal terminals in MPTP-treated fish. If so, this easy-to-observe phenotype might serve as a convenient indicator of MPTP toxicity on catecholamine terminals, and could be used as a simple assay for high throughput screens of chemicals that can counteract the neurotoxic effects of MPTP.

Genes involved in PD have also been studied in zebrafish, including the characterization of a zebrafish orthologue of ubiquitin C-terminal hydrolase L1 (UCH-L1), which has been associated with inherited forms of PD 101. While no stable transgenic or mutational lines in PD-associated genes have been reported in zebrafish, two studies have examined the effects of transient knockdown of DJ-1 and PINK1 using morpholino antisense oligonucleotides 102-103. DJ-1 morphants are more sensitive to oxidative stress, as evidenced by a significant reduction of DA neurons in DJ-1 morphants as compared to control embryos upon hydrogen peroxide (H₂O₂) exposure. DJ-1 morphants also have increased levels of superoxide dismutase (SOD1) mRNA. While the link between DJ-1 and oxidative stress is quite intriguing, it remains unclear whether the loss of DA neurons was selective, and whether it is due to neuronal degeneration or abnormal development. PINK1 knockdown results in the severe developmental phenotype that is rescued by wildtype human PINK1 mRNA. Morphants display a moderate decrease in the numbers of central dopaminergic neurons and alterations of mitochondrial function, including increases in caspase-3 activity and reactive oxygen species (ROS) levels. When the morphants were exposed to several drugs with antioxidant properties, ROS levels were normalized and associated phenotype improved. In addition, GSK-3 β -related mechanisms can account for some of the effects of PINK1 knockdown, as morphant fish show elevated GSK3 β activity and their phenotype is partially abrogated by GSK3 β inhibitors, such as LiCl and SB216763 [3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)1H-pyrrole-2,5-dione].

In addition to understanding the mechanism of DA neuron degeneration, another potentially fruitful avenue for treating PD involves understanding the mechanisms of DA neuron development and regeneration. Work from our laboratory has employed zebrafish dopaminergic (DA) neurons to identify genetic factors and small molecule drugs that can regulate the development and maintenance of these neuronal groups¹⁶. Using a TH-GFP transgenic line (Q. Li and S. Guo, unpublished data) and/or immunofluorescent labeling to visualize DA neurons, the effect of the neurotoxin MPTP or MPP+ has been visualized (Fig. 2). A small molecule screening of ~5000 FDA-approved bioactive compounds has been carried out. With our screening assays, ~300 compounds can be easily screened per person per week. A handful of compounds have been identified that can inhibit DA neuron development (S. Chatterjee, H. Khodabakhsh, and S. Guo, unpublished data). However, no compounds that can increase the number of DA neurons have been identified so far. It is possible that the wildtype number of DA neurons represents an optimum, and it is difficult to further improve upon it by addition of a single small molecule. Therefore, screening strategies are currently being devised to create DA-deficient models in order to identify compounds that can rescue DA deficits.

Alzheimer's disease—Alzheimer's disease (AD) is an age-related, non-reversible brain disorder that develops over a period of years. Initially, people experience memory loss and confusion, gradually leading to behavioral and personality changes, a decline in cognitive abilities such as decision-making and language skills, as well as problems recognizing family and friends. It is the most common cause of dementia among people age 65 and older. Three

major hallmarks in the brain are associated with the disease processes of AD. 1) Amyloid plaques, which are composed of fragments of a protein called β -amyloid peptide. 2) Neurofibrillary tangles (NFTs), found inside neurons, are abnormal collections of the protein Tau. 3) Loss of connections between neurons responsible for memory and learning. Among the complex genetic underpinnings of AD, three genes, amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2), have been identified to play important roles in the pathogenesis of AD¹⁰⁴. Currently there are no medicines that can slow the progression of AD. Four FDA-approved medications are used to treat AD symptoms. However, they will not stop or reverse AD and appear to help individuals for only a few months to a few years. Donepezil (Aricept), rivastigmine (Exelon), and galantamine (Reminyl), all of which act as cholinesterase inhibitors, are prescribed to treat mild to moderate AD symptoms. The newest AD medication is memantine (Namenda, a NMDA receptor blocker), which is prescribed to treat moderate to severe AD symptoms.

Zebrafish orthologues of APP¹⁰⁵, PS1¹⁰⁶, and PS2¹⁰⁷ have been identified. These genes are widely expressed beginning at early embryonic stages, suggesting that they may play an important role in regulating development. Thus, creation of zebrafish models of AD will likely require conditional manipulation of the activity of these genes. On the other hand, a transient transgenic model of tauopathy has been analyzed¹⁰⁸. In this model, a FTDP-17 mutant form of human tau expressed in zebrafish neurons produces a cytoskeletal disruption that closely resembles the NFT in human disease. However, since no stable transgenic lines have been reported, it is difficult to know whether neurodegenerative and behavioral phenotypes are obtained.

As mentioned above, enhancement of cholinergic transmission has been used as a symptomatic treatment for AD. It is interesting to mention that nicotine is found to improve memory in a learning paradigm in zebrafish¹⁰⁹.

Huntington's disease—Huntington's disease (HD) is an autosomal dominantly inherited, trinucleotide repeat disorder. The mutant protein huntingtin (Htt) inhabits an expanded polyglutamine (polyQ) repeat at its N terminal region. Both gain-of-function and haploinsufficiency of the HD gene (also referred to as IT15) seem to contribute to the development of this disorder, but the precise mechanisms leading to HD are still poorly understood^{110,111}. Apart from symptomatic treatment to manage the movement abnormalities, no drugs are available that can slow down the progression of HD¹¹².

The zebrafish orthologue of HTT has been identified¹¹³. Its requirement for zebrafish development and iron utilization has been revealed through morpholino knockdown¹¹⁴. Although loss-of-function of HTT did not lead to selective neurodegeneration, mis-expression of poly-Q expanded HTT fragment does cause protein aggregation and neuronal death. Interestingly, molecular chaperones can suppress aggregate formation and neuronal death, and several classes of small molecule compounds including anti-prion compounds have been validated to inhibit poly-Q aggregate formation in zebrafish^{115,116}. These studies show that zebrafish is a promising system for modeling HD and for finding potential therapeutic treatment of HD.

4.3 Neuropsychiatric disorders: anxiety/depression and addictive disorders

Six forms of anxiety disorders have been described in humans¹¹⁷: 1) Panic disorder is characterized by unpredictable, sudden attacks of intense anxiety; 2) Generalized anxiety disorder is marked by exaggerated worry and tension, even though there is little or nothing to provoke it. 3) Social phobia, also called social anxiety disorder, involves overwhelming anxiety and excessive self-consciousness in daily social situations. 4) Specific phobias are intense fears of some specific objects that in reality pose little or no danger. 5) Obsessive-compulsive

disorder involves anxious thoughts and repetitive rituals, which, when performed, help reduce anxiety. 6) Post-traumatic stress disorder (PTSD) is characterized by persistent frightening thoughts and intrusive memories of a terrifying event. Currently two types of treatment are available for anxiety disorders¹¹⁸: they are medications (many are classical examples of serendipity in drug discovery) and specific types of psychotherapy. Although these treatments are effective in some people, many patients are left with residual symptoms or experience side effects that limit the use of these currently available medications.

Abuse and addiction to alcohol, nicotine, and illegal substances manifest as the inability to break free from these substances, despite a full awareness of their adverse effects by the addicted individuals. Although still poorly understood, addiction is believed to be a result of long-term interactions between addictive substances and multiple brain neural systems, leading to maladaptation of brain signaling pathways. Medications used to treat drug addiction include drugs that antagonize the action of addictive substances. For example, Naltrexone helps prevent relapse to alcohol and heroin abuse, although many side effects exist. In addition to drug therapy, behavioral treatments are also used but with limited success. Because of the complexity and environmental attributions to neuropsychiatric disorders, despite the strong underlying genetic basis, definitive genetic factors remain to be identified in most cases.

Several behavioral paradigms have been exploited to model fear/anxiety in zebrafish, which includes phototaxis^{48,119}, thigmotaxis⁴⁹, behavioral responses to alarm substance^{120,121}, anti-predator behavior¹²², and novelty-elicited diving response⁵⁵. While the novelty induced diving is shown to be affected by nicotine and nicotinic antagonists, implicating the involvement of both $\alpha 7$ and $\alpha 4\beta 2$ receptors¹²³, rigorous pharmacological validations of other behavioral paradigms remain to be carried out.

The psycho-stimulatory^{48,49} and reinforcing^{50,52–54} effects of alcohol and drugs of abuse have been examined in zebrafish. These studies show that zebrafish readily respond to addictive substances, and neural substrates such as dopamine, serotonin, opioid systems, and acetylcholine systems are involved.

Behavior-based approaches to neuroactive drug discovery have a long history with much serendipity. For example, many psychoactive substances such as ethanol, caffeine, and opium have been discovered due to their rapid perceptual and behavioral effects on humans and other animals. However, is it feasible to use behavior as a read out in a small molecule based drug screening? Behavioral assays are often more complex than cell based assays and subject to more variability from both genetic and environmental influences. A recent review by Kokel and Peterson argues that these problems can be overcome, and such screening represents a necessity for systematic drug discovery¹²⁴.

5. Conclusions

The zebrafish nervous system is similar to that of mammals, and is accessible to small molecule drugs. These features make zebrafish a potentially suitable organism to evaluate the impact of drugs on neuronal development and function. However, before such potential can be fully realized, a better understanding of its nervous system and further establishment of appropriate models for human conditions are needed. For the purpose of drug discovery, the small size and relative abundance of zebrafish embryos allow for 96- well format chemical screening. However, such phenotype-based screens still provide much lower throughput than *in vitro* assay-based screening. Thus, additional technological breakthroughs in animal handling, imaging, and quantification are necessary to help further automate the screening process as much as possible.

6. Expert opinion

At present, the drug discovery process is either largely serendipitous or based on *in vitro* screens. Serendipity is not a reliable and rational approach, whereas *in vitro* assay-based screening often leads to compounds that have limited *in vivo* efficacy or unforeseeable toxicity. The pre-clinical drug testing process almost exclusively uses mammalian animal models, with a low throughput and high cost. The use of zebrafish could potentially facilitate both drug screening and the processes of evaluating candidate drugs. Investment into this organism, to further understand its biology and disease modeling capability, and to facilitate technological build-ups, has the possibility of revolutionizing drug discovery for CNS disorders.

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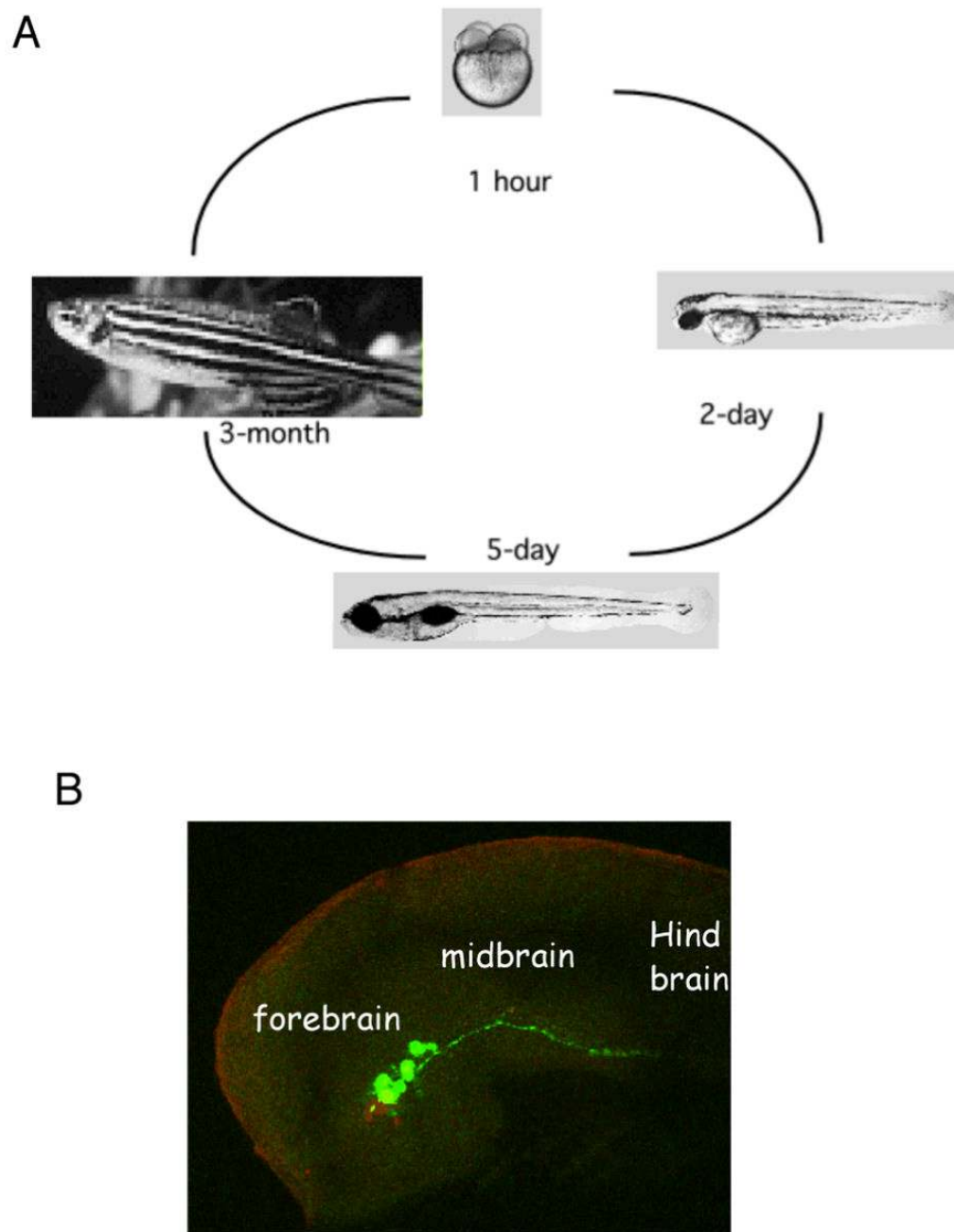
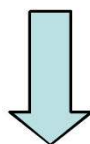
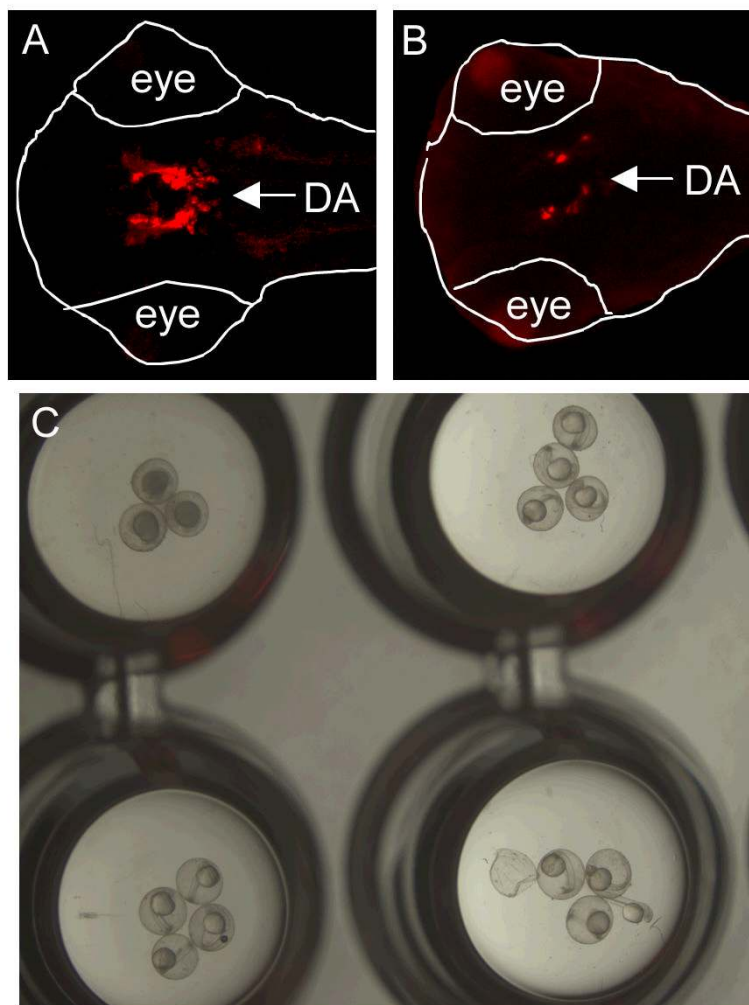


Figure 1. The zebrafish life cycle (A) and the developing brain (B, lateral view of ~ 1-day old embryonic head region with the dorsal side up), with the visualization of dopaminergic (anti-TH, green) and serotonergic (anti-serotonin, red) neurons. Images in (A) are form ZFIN.org. Dopaminergic and serotonergic neurons are visualized through antibody labeling of tyrosine hydroxylase and 5HT respectively.



Small molecule compounds that regulate DA neuron development and maintenance

Figure 2. Identification of small molecule compounds that can regulate DA neuron development and maintenance. DA neurons are visualized through tyrosine hydroxylase immunostaining in normal (A) and MPTP-treated (B) embryos (ventral view with the anterior to the left). (C) Visualization of zebrafish embryos in a 96-well plate, showing the relative size of a zebrafish embryo to the well of the 96-well plate, and allowing one to appreciate the suitability of embryonic and larval zebrafish for high throughput screens. These embryos are first screened for morphological defects, followed by screening for DA neuron phenotypes.

Table 1

Agonists and antagonists of various neurotransmitter- or neuropeptide-systems that have predicted effects in zebrafish

Category	Compound name	Known action in mammals	Observed effects in zebrafish
Dopamine	Bupropion	dopamine reuptake transporter blocker	abolished spontaneous fictive swim episodes at 3 dpf ¹²⁵
	L741,626; sulpiride	D2 receptor-specific antagonists	increased the frequency of swim episodes at 3 dpf ¹²⁵ reduce morphine preference in adult zebrafish ⁵³
	Apomorphine	Dopamine receptor agonist	suppressed prepulse inhibition of startle in larval zebrafish ¹²⁶
	Haloperidol	Dopamine receptor antagonist	attenuated the apomorphine induced deficit of PPI ¹²⁶
	SCH 23390	D1 receptor-specific antagonist	reduce morphine preference in larval and adult zebrafish ^{53,54}
	SKF38393	D1 receptor-selective agonist	Decrease K ⁺ current recorded from isolated ON bipolar cells ¹²⁷
Serotonin	Fluoxetine (Prozac)	selective serotonin reuptake inhibitor	Causes a transient decrease in spontaneous swimming activity ¹²⁸
	Quipazine	5-HT receptor agonist	Increase motor output in larval zebrafish ¹²⁹
	Methysergide Ketanserin	5-HT receptor antagonist	Decrease motor output in larval zebrafish ¹²⁹
Glutamate	Ketamine AP5	NMDA receptor antagonist	Dose dependent effect on PPI ¹²⁶ Causes complex changes of individual mitral cell responses and spatio-temporal activity patterns ¹³⁰
	NBQX	AMPA/Kainate receptor antagonist	Produces diverse effects on mitral cell activity in an explant of intact brain and nose ¹³⁰
	GYKI 52466	AMPA antagonist	Prevents AMPA-induced increase of acetylcholine release at NMJ ¹³¹
	Sym 2801	KAR antagonist	Prevents KA-induced increase of acetylcholine release at NMJ ¹³¹
GABA	Baclofen	GABA _B receptor selective agonist	Attenuates Ca ²⁺ influx in ORN axon terminals during an odor response ¹³²
	CGP54626	GABA _B receptor selective antagonist	Increases Ca ²⁺ influx in ORN axon terminals during an odor response ¹³²
	Gabazine	GABA _A receptor selective antagonist	Reduces the topological reorganization and the decorrelation of MC activity patterns during an odor response ¹³²
	Diazepam	GABA receptor agonist	Reduces larval locomotor activity ¹³³
Opioids	Naloxone	Opioid receptor antagonist	Reduce morphine preference in larval and adult zebrafish ^{53,54}