Using Zebrafish to Unravel the Genetics of Complex Brain Disorders

Robert Gerlai

Abstract The zebrafish has been prominently utilized in developmental biology for the past three decades and numerous genetic tools have been developed for it. Due to the accumulated genetic knowledge the zebrafish has now been considered an excellent research tool in other disciplines of biology too, including behavioral neuroscience and behavior genetics. Given the complexity of the vertebrate brain in general and the large number of human brain disorders whose mechanisms remain mainly unmapped in particular, there is a substantial need for appropriate laboratory research organisms that may be utilized to model such diseases and facilitate the analysis of their mechanisms. The zebrafish may have a bright future in this research field. It offers a compromise between system complexity (it is a vertebrate similar in many ways to our own species) and practical simplicity (it is small, easy to keep, and it is prolific). These features have made zebrafish an excellent choice, for example, for large scale mutation and drug screening. Such approaches may have a chance to tackle the potentially large number of molecular targets and mechanisms involved in complex brain disorders. However, although promising, the zebrafish is admittedly a novel research tool and only few empirical examples exist to support this claim. In this chapter, first I briefly review some of the rapidly evolving genetic methods available for zebrafish. Second, I discuss some promising examples for how zebrafish have been used to model and analyze molecular mechanisms of complex brain disorders. Last, I present some recently developed zebrafish behavioral paradigms that may have relevance for a spectrum of complex human brain disorders including those associated with abnormalities of learning and memory, fear and anxiety, and social behavior. Although at this point co-application

R. Gerlai (✉)
Department of Psychology, University of Toronto Mississauga,
3359 Mississauga Road North, Rm 3035,
Mississauga, ON L5L 1C6, Canada
e-mail: robert_gerlai@yahoo.com

Curr Topics Behav Neurosci (2012) 12: 3–24
DOI: 10.1007/7854_2011_180

Published Online: 17 January 2012

of the genetics and behavioral approaches is rare with zebrafish, I argue that the rapid accumulation of knowledge in both of these disciplines will make zebrafish a prominent research tool for the genetic analysis of complex brain disorders.

Keywords Zebrafish · High throughput behavioral screening · Fetal alcohol syndrome · Alcoholism · Learning and memory · Fear and anxiety

Contents

1 Zebrafish, a Novel Research Tool with Some Advantages

Numerous laboratory organisms are available for one to employ in the analysis of how genes influence behavior or how certain biochemical processes affect the functioning of the brain. For example, rats have been traditionally utilized to screen libraries of compounds to identify drugs that may be beneficial in a range of human brain and behavioral disorders both in academic and biopharmaceutical preclinical research. The house mouse has been employed in a range of behavior genetic applications including those that probe the effects of specific genes (reverse genetics) and their roles in behavior and brain function [e.g. Gerlai et al. (1995), Pekhletski et al. (1996)]. But other, simpler, laboratory organisms including the flat worm (Giles and Rankin 2009), the sea slug (Bailey and Kandel 2008), or the fruit fly (Sokolowski 2001) have also been successfully utilized to study the biological bases of brain function and behavior. Compared to these laboratory model organisms the zebrafish is quite novel. I use the term "model" here in a very loose sense and only mean that the reason for the use of animal species in the laboratory is to isolate and mimic some aspects of complex biological phenomena, a reductionist approach that may yield results faster than in human due to the simpler features of

the studied organism and to the precisely controlled laboratory conditions. In this sense, zebrafish may be an ideal model organism.

The zebrafish strikes an optimal compromise between system complexity and practical simplicity. It is a vertebrate species with a physiology (Alsop and Vijayan 2008), brain anatomy (Tropepe and Sive 2003), and neurochemistry (Chatterjee and Gerlai 2009) characteristic of the prototypical vertebrate, and thus translationally relevant to our own species. Most importantly, the nucleotide sequence of zebrafish genes is often found highly similar (70–80% homology) to the mammalian (and human) counterparts, and the amino acid sequence of functionally relevant domains of its proteins has been found to be even more evolutionarily conserved, i.e. similar to mammalian sequences (Reimers et al. 2004; Renier et al. 2007). It is thus quite probable that a gene identified in zebrafish as involved in particular functions/dysfunctions of its brain, will have a human homolog serving similar functions and vica versa. Briefly, the translational relevance of zebrafish research is expected to be high.

The number of animals one can screen is a crucial factor in forward genetics where one does not know which and how many genes may influence the phenotypical function in question. In case of brain disorders or behavioral function, the number of such genes may be quite large and thus one may have to analyze thousands of mutants to tackle this complexity and identify appropriate mutations and thus the genes involved. One can, of course, generate the same number of zebrafish and mice for screening purposes but there are several reasons why zebrafish may be preferred. First, a single female zebrafish can produce 200 offspring at every spawning and can spawn multiple times a week. Second, zebrafish is small (4 cm long) and is highly social and thus a large number of subjects may be housed cheaply in a small animal holding room. For example, a standard zebrafish stand-alone high density rack system (e.g. Aquatic Ecosystems Inc, FL, or Aquaneering Inc. CA) with six shelves and about 23 liter tanks per shelf, can house about 2,000 zebrafish, and a 40 m^2 standard vivarium room may be fitted up with up to 10 such racks. Briefly, the same room that may house a couple of hundred mice can have about 20,000 zebrafish in it. Therefore, when it comes to large scale screening, the zebrafish has a definite cost advantage. Given the relative simplicity of this vertebrate species and the fact that it is a phylogenetically older "design" compared to mammals, one may also argue that it may allow the analysis of fundamental core mechanisms of the chosen brain function. Last, adding zebrafish to the list of already well studied vertebrates (e.g. the mouse and the rat) should facilitate cross species comparison and finding common characteristics and mechanisms, which should also enhance our ability to translate the findings to human.

2 Zebrafish, the Favorite of Geneticists

Genetics is one of the strengths of zebrafish and excellent reviews have been published on numerous genetic techniques developed for this species (Amsterdam and Hopkins 2006; Chen and Ekker 2004; Lekven et al. 2000; Patton and Zon 2001).

Here I will discuss these techniques only briefly. In addition to sophisticated gene expression analyses including quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR) and DNA microarrays (gene chip), both reverse genetic and forward genetic methods are available with zebrafish, although the former, the forward genetic approaches, have been more prevalent in zebrafish research. Reverse genetic analysis allows one to study the phenotypical effects of targeted manipulation of known genes. The main goal of forward genetic studies, on the other hand, is to discover novel genes by the introduction of random mutations.

3 Reverse Genetic Tools

Among the reverse genetic tools, TILLING has been employed successfully in zebrafish (Moens et al. 2008). Targeting induced local lesions in genomes (TILLING) allows the identification of mutations in specific genes of interest in chemically mutagenized zebrafish populations. The method was first described for mutation detection in Arabidopsis about a decade ago but since then it has successfully been adopted for zebrafish too (Moens et al. 2008). The essence of the TILLING method is the screening of chemically mutagenized populations of zebrafish using the polymerase chain reaction (PCR) for mutations in the gene of interest. Unlike in gene targeting with the use of homologous recombination in embryonic stem cells employed in the mouse (Capecchi 1989), and most recently in the rat (Tong et al. 2010), the actual mutagenesis conducted in TILLING is random, i.e. not targeted. The "trick" of TILLING is then the identification of the mutation(s) in the target gene. The identification of the mutation may be achieved using two different approaches. One is the resequencing of every single mutagenized genome (the gene of interest and its sequence is known and thus alterations in the sequence can be detected), a brute force approach that is becoming increasingly feasible with the ever improving speed of sequencing methods. The other method is based upon the use of cell, a plant-specific extracellular glycoprotein that cleaves heteroduplex DNA at single nucleotide mismatches (resulting from the introduced point mutation). Using fluorescently labeled primers to detect cell cleavage products on a LiCor acrylamide slab gel, cell can identify a heterozygous single nucleotide mismatch [for further details of the TILLING methodology and its use, see Moens et al. (2008)].

Another approach that is principally a reverse genetic method, i.e. it is also aimed at the characterization of the function of known genes, is a knock down method using morpholinos (Bill et al. 2009). Morpholinos are antisense oligonucleotides (usually 25 bases long) composed of a phosphorodiamidate backbone with a morpholine ring and the same bases as DNA (Bill et al. 2009). Morpholinos are injected into the target cells and act by steric hindrance to block ribosome entry and hence prevent protein production. The antisense technology has been long employed in mammalian species but the efficiency and specificity of the oligonucleotide approach has been controversial. In zebrafish, however, the morpholino

approach has been successfully employed. For example, due to the altered backbone of the morpholinos they are not affected by nucleases and are, therefore, highly resistant to breakdown in vivo. Furthermore, because of their small size and unusual chemistry, morpholinos remain undetected for the immune system. Morpholinos are traditionally introduced into the yolk of 1–8 cell-stage embryos in which the cytoplasmic bridges connecting the embryonic cells allow rapid diffusion of the hydrophilic morpholinos leading to ubiquitous delivery. The analysis of the result of the gene expression knock down is usually limited to the embryonic stage of zebrafish. However, examples already exist suggesting that modified morpholino chemistry (the VIVO-morpholino, which allows penetration of the oligonucleotide into adult zebrafish cells) may be successfully employed in adult zebrafish as well [e.g. Kim et al. (2010)]. This is a crucial novel development considering that most complex brain disorders and higher behavioral functions can be best modeled, observed, and analyzed in the adult zebrafish.

RNA-interference, or RNAi, is yet another intriguing possibility for targeted modification of gene expression in zebrafish. RNAi is a transcriptional gene silencing mechanism-induced by short (21–23 bases long) double stranded RNA whose main mechanisms are believed to be gene expression regulation via miRNA's (endogeneous microRNA's) and defense against viral genetic material mediated by dsRNAs (double stranded RNAs), terms that represent structurally indistinguishable mRNA species. Irrespective of the physiological function, a few years ago it was realized that the cell's RNAi mechanism could be utilized for the induction of targeted knock down of gene expression by delivering double stranded short RNA sequences specific to the chosen target gene. Although zebrafish cells have been shown to possess the RNAi machinery, the functional consequences, especially the specificity of the RNAi-based manipulation, have been questioned [for review see Skromne and Prince (2008)] and thus whether this technology will lead to success in zebrafish remains to be seen. Another technology, transgenic methods, however, may offer a currently existing true and tried alternative.

In the mammalian neurobehavioral genetics field, transgenic methods have been perhaps the most fruitful reverse genetic approaches. Transgenic technologies have also been successfully employed with zebrafish [for a recent review see Skromne and Prince (2008)]. These techniques make use of a variety of methods (e.g. enzymatic approaches, transposons, and retroviruses) to enable the delivery and increase the efficiency of incorporation of foreign DNA into the genome of zebrafish thereby generating stable transgenic fish lines in which the foreign DNA is expressed. Irrespective of the mode of delivery, transgenic zebrafish may be divided into two main classes: transgenic overexpressors and dominant negative transgenics. In the former, overexpression of the transgene is achieved, for example, by delivering multiple copies of the transgene or using a strong promoter, and is used to test the functional consequences of the excess amount of translated gene product. In the latter, the expressed transgene product interferes with or blocks the function of the endogenous gene and thus allows one to test the effect of loss-of-function at the phenotypical level.

In addition to constitutive transgene expression, inducible expression systems are also available in zebrafish. Given that zebrafish tolerate a broad range of temperatures (in its natural geographical range temperatures may vary between 10 and 35°C), heatshock promoters have been successfully employed to induce transgene expression in a temporally controlled manner, and the use of focal heating has also allowed the induction of transgene expression in a spatially restricted manner, at least in superficial structures. Furthermore, the Gal4-UAS system (Scott et al. 2007), well developed for the fruit fly, the tetracycline transactivator system (Huang et al. 2005), as well as the Cre/loxP system (Langenau et al. 2005) used, for example, in the temporal and spatial control of knock out of genes in the mouse, have all been utilized in the control of transgene expression in zebrafish.

Although the classical knock out technology based upon homologous recombination in embryonic stem cells as employed in the mouse (Capecchi 1989) and most recently in the rat (Tong et al. 2010) is not yet available in the zebrafish, research in this direction is also progressing. For example, germline transmitting embryonic stem cells have been isolated from zebrafish (Fan and Collodi 2006) and methods alternative to classical gene targeting are also being explored. For example, nuclear transfer of genetically modified cultured embryonic fibroblast cells has been achieved in zebrafish, which implies that targeted genetic modification for in vivo analysis of gene function may be possible via this route (Lee et al. 2002). Furthermore, zinc finger nuclease-based knockout technology is also being developed for zebrafish (Ekker 2008). Zinc finger nucleases are genetically engineered restriction enzymes that cut the DNA sequence of interest according to their specific design. Zebrafish embryos injected with the specific custom designed zinc finger nuclease-encoding mRNA are reared to adulthood and crossed with wild type fish. As much as 25% of the resulting offspring has been shown to transmit the induced mutation, usually a frameshift allele in the germline (Ekker 2008). Last, a gene-breaking transposon-based method to generate mutations is also being developed for zebrafish (Sivasubbu et al. 2006) to mention but a few reverse genetic technologies.

The range of reverse genetic approaches discussed above is somewhat misleading, however. Although they do demonstrate how fast zebrafish genetics is evolving, many of these methodologies are not mature enough for reliable use even for embryonic or developmental biology phenotypes, the focus of most of these investigations. For complex neurobehavioral traits, virtually none of these methods have been employed.

4 Forward Genetic Tools

Unlike reverse genetic approaches, forward genetics has provided decades of consistent success with zebrafish. The first two large scale comprehensive forward genetic screens the Tubingen (Haffter et al. 1996) and Boston (Driever et al. 1996)

screens were conducted about 15 years ago and set the stage for subsequent screening studies. Since then, most forward genetic studies have utilized a chemical mutagen, ethyl-nitroso-urea (ENU), which is expected to induce single nucleotide point mutations, and when dosed appropriately, one mutation per genome on average. The advantage of ENU mutagenesis is that ENU is efficient and with it one can achieve a good coverage of the entire genome, i.e. can expect to hit a large proportion of genes as long as a large enough number of animals (thousands) are mutagenized and analyzed. The Achilles heel of ENU mutagenesis, however, is the subsequent linkage analysis, which requires several generations of crosses and cumbersome mapping. Nevertheless, due to the availability of high resolution markers developed for zebrafish, the genes carrying the induced mutations can be successfully identified using linkage analysis-based positional cloning (Knapik 2000; Patton and Zon 2001). Another concern with ENU-based forward genetic analysis is that the point mutation induced by ENU is often recessive and thus may not be observable unless bred into a homozygous form, which requires three generations of breeding to create an F3 or a backcross segregating population (Patton and Zon 2001). Although not without technical complications, an alternative that can speed up the generation of recessive homozygous mutants does exist, it is gynogenesis. For gynogenesis one may use heat shock or pressure to manipulate the cell division cycle at the earliest embryonic stage leading to haploid to diploid genome conversion and allowing the generation of homozygous fish in essentially one step without the cumbersome breeding [for review of experimental examples using gynogenesis, see Patton and Zon (2001)].

ENU has been the most frequently employed mutagen for zebrafish, but other mutagenesis methods have also been successfully utilized. Perhaps the most promising among them is retrovirus mediated insertional mutagenesis (Amsterdam and Hopkins 2006). This method has the advantage over ENU because insertion of the viral genetic material into the zebrafish genome not only induces a mutation but by leaving the unique viral sequences in the genome allows fast and efficient localization and cloning of the mutated gene (Amsterdam and Hopkins 2006).

A potential concern with forward genetic approaches in zebrafish is that this species has undergone a partial genome duplication event in its evolutionary past. The concern is that the genes whose locus is on the duplicated region of the genome (approximately 20% of the genome) may be able to compensate for the induced mutation and could mask its effects if the sister gene remained active and if its function has not changed much since the gene duplication event. However, some argue that the partial "tetraploidy" may be viewed as an advantage in zebrafish for forward genetics especially when it comes to the analysis of the genetics of complex traits: the induced mutations are not expected to be lethal and may only have small quantitative effects on the phenotype, thus making functional phenotypical identification and characterization feasible. Whether these arguments turn out to be correct will have to be seen. But the few already existing examples suggest that we have some reason for optimism. Below I review such examples organized according to their disease relevance.

5 Parkinson's Disease

Parkinson's disease is the second most prevalent human neurodegenerative disease, which primarily affects the nigro-striatal dopaminergic system. Numerous genes have been identified associated with Parkinson's disease in humans three of which are believed to underlie early onset of Parkinson's Disease: PARK2 encoding Parkin, PINK1 encoding PTEN-induced putative kinase 1, and PARK7 encoding DJ-1 [for review see Bandmann and Burton (2010)]. The zebrafish orthologs of the human genes have been identified and, for example, the PARK2 gene product is known to be a 458 amino acid long protein which is 75% similar to the human protein with functionally important regions having 93% similarity. The similarities between the other two human genes with their zebrafish orthologs are also substantial. Morpholino-induced knock down of expression of these genes have led to significant dopaminergic neuron loss and/or to increased sensitivity of these neurons to toxins [although the selectivity of the effects have been questioned in the case of PARK6, reviewed by Bandmann and Burton (2010)]. Behavioral impairments resulting from the knock down have not been shown in zebrafish with one exception: Morpholino antisense knock down induced reduction of PINK1 expression led to loss of tyrosine hydroxylase staining in distinct groups of dopaminergic neurons in the zebrafish brain and when challenged with normally sub-effective concentrations of 1-methyl-4-phenyl-1,2,3,6-tetrapyradine (MPTP) the affected larval zebrafish showed reduced locomotion. Detailed swim path analysis of these larvae was not conducted, however.

6 Tauopathy and Alzheimer's Disease

Another class of neurodegenerative diseases is the tauopathies. These diseases are all defined by their underlying molecular pathology: the presence of hyperphosphorylated insoluble forms of the microtubule associated protein Tau, which is deposited in neurons in the form of observable neurofibrillary tangles. Most of these diseases are sporadic [e.g. progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and Pick's disease (PiD)], but neurofibrillary tangles have also been associated with familial early onset of Alzheimer's disease. Both transient as well as stable transgenic zebrafish lines overexpressing Tau have been generated and in both structures resembling neurofibrillary tangles have been reported [for review see Bandmann and Burton (2010)]. Behavioral analysis of the effects of these changes has not been conducted except for a rudimentary, observation-based, judgment as to whether tactile stimulation induced a normal escape response in the 48 h post-fertilization embryo expressing a mutant form of Tau (Paquet et al. 2009).

Alzheimer's disease is often mentioned under tauopathies but there are other prominent known genetic factors associated with this disease too. Alzheimer's disease is the most common form of dementia affecting over 25 million people

worldwide (Querfurth and LaFerla 2010). The histological hallmarks of the disease are neurofibrillary tangles (mentioned above) and, perhaps even more characteristically, amyloid plaques. Although the familial (heritable) form of the disease only makes up 4–5% of all cases, clearly demonstrating the importance of environmental factors, the genetic analysis of Alzheimer's disease has led to major discoveries and clarified some of the biological mechanisms core to the disease. The genes encoding amyloid-β precursor protein (APP), and presenilins PS1 and PS2 have been found to underlie familial Alzheimer's disease, and the ε4 allele of the gene encoding apolipoprotein E (ApoE) has been found to be a risk factor in sporadic (non-familial) Alzheimer's cases. The APP, PS1, PS2, and ApoE genes have all been identified in zebrafish and have been found to be highly homologous to their mammalian, and human counterparts, with certain functionally relevant regions approaching 100% identity with human (e.g. the transmembrane region of APP). Furthermore, some of the components of the γ-secretase complex mediating the processing and cleavage of APP, which may lead to the generation of the toxic Aβ (40, 42) peptide have started to be examined in zebrafish (reviewed in Xia 2010). To characterize the involvement of APP in zebrafish embryonic development, the expression of the APP gene was reduced using morpholinos, which led to significant shortening of the body length of the embryos (Joshi et al. 2009). The involvement of overexpression of APP (as in Down's patients, for example, who also develop Alzheimer's disease) or of the expression of mutant forms of APP (identified in familial Alzheimer's cases), or the role of presinilins have not been investigated in zebrafish, nor has been any studies conducted for the potential behavioral consequences of transgenic manipulations of these genes. The function of ApoE and its potential role in Alzheimer's disease related abnormalities also has not been investigated with zebrafish.

7 The Anxiety "Cluster"

Perhaps the largest cluster of human brain disorders in terms of prevalence are neuropsychiatric conditions including anxiety and stress disorders, depression, obsessive compulsive disorders, and several forms of phobias. Although both academic and pharmaceutical and biotechnology companies have been studying the potential mechanisms of these disorders and several treatment options have been developed, these diseases still represent an enormous unmet medical need. This is because the causative factors (both genetic and environmental) behind these diseases are difficult to trace and the mechanisms of the diseases are also complex. A recent review paints an optimistic picture as to the potential use of zebrafish in the analysis and modeling of neuropsychiatric conditions (Mathur and Guo 2010) and I share this optimism. For example, zebrafish possess a glucocorticoid and a mineralocorticoid receptor that have been cloned and sequenced, and has a corticoid signaling pathway highly similar to that of mammals (Amsterdam and Hopkins 2006; Denver 2009). Also, the major components

necessary for success, including the genetic tools discussed above and the novel behavioral paradigms, some of which are presented below, already exist for zebrafish. Nevertheless, one must also acknowledge that these studies with zebrafish are only starting now and thus have not produced major breakthroughs.

8 Autism Spectrum Disorders

The zebrafish has also been suggested for the analysis of the mechanisms of autism spectrum disorders (Tropepe and Sive 2003). The number of genetic factors underlying autism spectrum disorders is believed to be much less than in such neuropsychiatric conditions as anxiety or schizophrenia, and thus animal genetic models have been generated with much hope [for a review, see e.g. Gerlai and Gerlai (2003)]. Importantly, several of the genes implicated in the human disease have been shown to have homologs in zebrafish [for a most recent review, see Mathur and Guo (2010)]. It may therefore be possible to recapitulate some aspects of autism spectrum disorders by selectively targeting these genes and testing the effect of the genetic or pharmacological manipulations on developmental as well as behavioral characteristics in zebrafish. It is also notable that the zebrafish is a highly social species, and the novel behavioral paradigms that are being developed to induce and quantify social behavioral responses in zebrafish may also contribute to this research, for example, by allowing large scale mutaganesis screening-based identification of molecular mechanisms involved in vertebrate social behavior.

9 Schizophrenia

Schizophrenia is a neurodevelopmental disorder that often manifests first during adolescence. Unlike in the case of autism spectrum disorders the number of genes involved in schizophrenia may be extremely large (hundreds) and many of these genes may only have a minor "predisposing" effect, which have hindered the unraveling of the mechanisms of the disease. Nevertheless, some of the genes implicated in schizophrenia have been identified in zebrafish. For example, DISC1, a schizophrenia susceptibility gene, has been shown to play roles in cell migration and differentiation in the zebrafish neural crest (Drerup et al. 2009) as well as in the development of oligodendrocytes and neuronal lineages developing from olig2 expressing precursor cells. In addition to delineating the cellular and molecular roles of some schizophrenia associated genes, there is already one example for a genetic manipulation to affect zebrafish behavior. SHANK3 is a synaptic scaffolding protein whose gene was recently identified to carry mutations in some patients suffering from schizophrenia and was also found in autistic patients. Morpholino-induced knock down of the expression of the corresponding gene resulted in robust morphological abnormalities as well as impaired swimming in

response to tactile stimulation in the zebrafish larva (Gauthier et al. 2010). It may be noted, however, that the specificity of the morpholino-induced changes may be questionable given that the attempt to rescue the phenotype by injection of wild type or mutant SHANK3 mRNA sequences led only to partial success at best. Last, psychopharmacological experiments have already started to be utilized with zebrafish in the analysis and modeling of schizophrenia. One behavioral endophenotype often argued to be an important aspect of schizophrenia is reduced prepulse inhibition, or PPI. Prepulse inhibition is believed to be a measure of sensory gating. It is induced by employing a weak stimulus (the prepulse), which is expected to inhibit the reaction to a subsequent stronger startling stimulus (the pulse). Larval zebrafish exhibit PPI of the acoustic startle response similarly to what has been demonstrated in rodents [reviewed in Mathur and Guo (2010)]. PPI can be disrupted by dopamine agonists in the zebrafish larvae, an alteration that is reversed by antipsychotic drugs similarly to the mammalian situation (Braff et al. 2001). In addition to these promising psychopharmacology results, a forward genetic screen has already isolated a mutant "Ophelia", which exhibited reduced PPI (Burgess and Granato 2007). In summary, the first examples showing how zebrafish may be utilized in the investigation of the genetic mechanisms of complex brain disorders already exist. However, in most of these studies the behavioral consequences of the employed experimental manipulations were not analyzed or were studied in a rudimentary manner. This is, in general, the current weakness of the zebrafish as an experimental tool: its genetics and neurobiology have been traditionally powerful but its behavioral characteristics are largely unmapped because only a few behavioral test paradigms are available (Sison et al. 2006).

10 Expanding Our Horizon: The Need for Sophisticated Behavioral Test Paradigms in Zebrafish Research

Sophisticated behavioral paradigms may be crucial for two main reasons: first, the construct and face validity of the genetic (reverse genetics) or pharmacological models may only be fully established using behavioral tests; and two, behavioral paradigms may represent unbiased screening tools for forward genetic applications (Gerlai 2002). Arguably, behavioral analysis can efficiently probe a broad spectrum of brain functions in a large number of subjects. It is not limited to particular brain regions or neurobiological mechanisms, and it is simple and cheap to conduct (Gerlai and Clayton 1999). Arguably, high throughput behavioral screens may be able to systematically reveal mutation or drug-induced functional changes in the brain. Fortunately, for the past few years a clear upsurge of zebrafish behavioral studies is evident, indicating that behavioral neuroscience and behavior genetics has started to acknowledge the utility of this species. Below I discuss some of these recent studies focusing on the question of how behavioral analysis may be utilized for the discovery of novel genes and compounds affecting brain

dysfunction associated with complex human brain disorders. Three main behavioral focus areas will be represented below: learning and memory, which is relevant for a number of neurodegenerative diseases including Alzheimer's disease; fear and anxiety, which are important behavioral responses and behavioral states relevant for a spectrum of neuropsychiatric conditions; and social behavior, whose abnormalities may be important in the analysis of autism spectrum disorders and schizophrenia, to mention but the two most important diseases in this domain.

11 Learning and Memory

Learning and memory has been extensively studied by scholars of several scientific fields. Numerous mechanistic questions related to how learning occurs, and what memory is, have been successfully tackled. For example, by now a large number of genes and biochemical mechanisms underlying learning and memory have been identified (Sweatt 2010). Can zebrafish add anything to this wealth of knowledge? Although hundreds of genes involved in learning and memory have been identified, the mechanisms of these complex processes are far from being understood. It is likely that the number of undiscovered genes that play roles in learning and memory is large. For example, according to conservative estimates, most vertebrate genomes contain about 30,000 genes. Recent microarray studies suggest that at least 50% of all the genes of the genome are expressed in the vertebrate brain (e.g. in zebrafish), i.e. about 15,000 genes (Pan et al. 2010). Given that plasticity is perhaps the most complex aspect of brain function, it is likely that a large proportion of these genes, i.e. potentially thousands of them, are involved in some mechanisms subserving plasticity, i.e. learning and memory.

A number of laboratories have realized that the cheap and easy to breed zebrafish may offer a solution for high throughput screening which would be costly with traditional laboratory rodents. Investigators have started to characterize the cognitive capabilities of zebrafish and have already developed several test methods that can measure learning and memory efficiently and fast [for examples see Sison et al. (2006), Sison and Gerlai (2010)]. The key in these paradigms concerns automatability. Even if one needs to employ several repeated training trials, as is the case in most learning paradigms, if these trials are administered in an automated manner, and if the behavioral responses that reflect learning and memory performance are easy to measure and do not require the constant presence and attention of an experimenter, the paradigm may be run in multiple test apparati in parallel and thus become high throughput.

A successful high throughput learning task design utilizes moving (animated) images of conspecifics, which are shown on a computer screen placed by each side of the experimental tank (Pather and Gerlai 2009). Previously, access to view a shoal (group) of zebrafish has been shown to represent a reward for experimental zebrafish and that this visual stimulus (the sight of a group of zebrafish) can support good learning performance (Al-Imari and Gerlai 2008). Subsequently, it has been

demonstrated that computer animated images of zebrafish can serve as a positive reinforcement (Gerlai et al. 2009a). The simple manner with which the reward could be administered allowed the development of an automated learning task (Pather and Gerlai 2009). The task is also simple. For a short period of time [20 s in Pather and Gerlai (2009)] the image of the moving shoal is shown and then it is turned off for 90 s. After this 90 s no-image period the image of the moving shoal is shown on the opposite side of the test tank for again 20 s, and the sequence repeats itself. As a result of the alternating image presentation sides, zebrafish have to make a choice during the no-image period as to whether they stay close to the side where the image was just shown, or move to the opposite side, where the image will appear. The natural tendency of zebrafish is to stay close to its conspecifics and thus initially zebrafish spend the highest amount of time near the side where the image was shown last. However, as the training proceeds, zebrafish spend increasingly longer amount of time near the side that *will* show the image. There are several important points to make about these results. First, the motivation to stay close to conspecifics does not habituate over time and thus the experimental subjects remain motivated to perform in this task, a major advantage compared to the use of food, which satiates zebrafish quickly. Second, the stimulus is a visual cue administered precisely using consumer grade (i.e. cheap) video-equipment. Third, the behavioral response (distance from stimulus screen) is easy to quantify using video-tracking systems and/or motion detectors (e.g. photocell detector arrays). Fourth, multiple trials [in Pather and Gerlai (2009), thirty trials] can be administered without the intervention by the experimenter. The fish stays in the test tank and is given the stimuli and their responses are measured repeatedly across the continuous sequence of trials. As a result of all these above features, the paradigm is fully automated and thus multiple set-ups can be run in parallel. Although the 30 trials required 3,300 s (55 min) per experimental fish (Giles and Rankin 2009), one can easily set up several such test apparati. Briefly, the throughput of the task can be dramatically increased by scaling up. In our facility a 20 m^2 test room could be easily fitted with 50 such test apparati, i.e. in an eight work hour day, one can test 400 zebrafish in a single room using this learning task, a sufficiently high throughput even for large scale mutagenesis screens (Haffter and Nüsslein-Volhard 1996).

The above paradigm is new and thus there are numerous unexplored questions one may need to address. For example, we do not know whether zebrafish can forecast the future, i.e. whether their performance improvement was due to better timing of their responses (knowing when and where the shoaling image will appear in the near future). It is possible that the performance improvement of the fish was simply due to acquisition of CS-US association: disappearance of the stimulus on one side serving as the conditioned stimulus predicted the reappearance of the unconditioned stimulus, the shoal image, on the opposite side. There are many questions that concern possible optimization of the task as well: is the 20/90 s stimulus/no-stimulus interval ratio the best? Could longer tanks (the original experiment was conducted in a 50 cm long tank, a distance that can be easily traversed by the fast zebrafish) be more appropriate allowing more sensitive detection of performance improvements/deficits? Also, how would other

stimulus/no-stimulus schedules (random versus fixed ratio, increasing stringency versus constant) affect the behavior of the fish? Last, mechanistic questions as to what neuroanatomical structures subserve the task, what drugs may influence performance in it, and how sensitive it may be to detect mutation-induced changes all will have to be explored. Clearly, there are many questions when one introduces a new paradigm. Nevertheless, the above example demonstrates how one can utilize species-specific perceptual, motor and motivational characteristics to design relatively simple and high throughput behavioral test methods that may allow addressing many of the above questions in the future.

The above learning task have an important temporal component, the delay between the stimulus presented on one versus the other side of the tank and as such may allow the analysis of a complex forms of learning in zebrafish known to be associated with the hippocampus in mammals, trace conditioning (McEchron and Disterhoft 1999) and/or acquisition of relational memory (Cohen et al. 1997). Although fish do not have a structure whose circuitry resembles that of the mammalian hippocampus, they do possess a brain region, the lateral pallium that is believed to be a structure homologous to the mammalian hippocampus (Vargas et al. 2009). Furthermore, fish without the classical mammalian hippocampal circuitry have also been found to be able to learn spatial learning paradigms, a class of tasks that is associated with hippocampal function in mammals (Salas et al. 1996). Spatial learning has also been demonstrated in zebrafish (Sison and Gerlai 2010), however, the spatial task employed (learning to find a particular location in a plus maze) was extremely time consuming. It required many repeated trials which could be administered only manually. Could one design a high throughput spatial task for zebrafish?

This question was answered in a recent study (Gómez-Laplaza and Gerlai 2010) that demonstrated good learning performance of zebrafish in a latent-learning paradigm. The paradigm consisted of two phases, a long training phase and a brief probe trial. During the training phase zebrafish were allowed to explore a complex maze which consisted of a starting chamber that was connected to a goal chamber by a left and right tunnel. Zebrafish were allowed to explore the maze in groups of ten (a shoal) for 16 consecutive days, each day once for 50 min. Allowing zebrafish to swim around the maze in ten-member shoals facilitated active exploration and reduced passive fear responses. During the exploration of the maze certain shoals were allowed to go through only one of the tunnels, i.e. there was a set of fish for which only the left tunnel was open and the right tunnel was blocked and another for which the left tunnel was blocked and only the right tunnel was open, and yet another group for which both tunnels were open, a spatial exploration task. The second part of the paradigm was a short (10 min long) probe trial, during which both tunnels of the maze were open, a shoal of stimulus fish was placed inside a transparent container and into the goal chamber of the maze, and the experimental fish were tested singly in the maze. Given the social nature of zebrafish, the experimental subject was highly motivated to get as close to the stimulus fish in the goal chamber as possible. Which route, the right versus the left tunnel, the experimental fish took was video-recorded and analyzed. The results showed that those fish that experienced the right tunnel open during the maze

exploration phase of the paradigm also used the right tunnel during the probe trial, those fish that experienced the left tunnel open used the left tunnel during the probe trial and those fish that experienced both the left and right tunnel open chose randomly. Why is this paradigm high throughput? Although the exploration phase of the paradigm took 16 days, because the fish were not monitored and their behavior was not analyzed, one could set up a large number of mazes and train a large number of fish every day. The probe trial was conducted for every fish separately, but it lasted only for 10 min per fish and the swim path of the fish could be quantified using automated video-tracking techniques (Blaser and Gerlai 2006). Thus this phase of the paradigm could also be made high throughput. Furthermore, given the spatial nature of the task, this paradigm is likely to be capable of tapping into complex forms of learning and memory.

There are again many questions about this novel paradigm. What motivates the fish to learn the maze? In other words, why fish remember the tunnel they explored before? This form of learning is termed latent learning because apparently there is no external experimenter controlled motivator (positive or negative reinforcement) presented. However, it has been argued (Gómez-Laplaza and Gerlai 2010), based on prior supporting evidence, that exploration of novelty itself is rewarding in this task and the novel aspect of the maze is what kept the fish motivated to explore and learn. The results of this study also suggested that learning in this paradigm was likely based upon acquiring and remembering external visual cues, i.e. spatial learning, a hypothesis that will need to be proven in the future. But again, despite the novel aspect of the task and the fact that there may be numerous questions one could explore with it, the paradigm does appear to be appropriate for high throughput screening of learning and mnemonic characteristics of zebrafish and mutation-induced changes in these characteristics.

There are numerous human disorders associated with memory loss and/or impairment of cognitive function, perhaps the most devastating and prevalent is Alzheimer's disease discussed above. But milder forms of memory problems, mild cognitive impairment (MCI) and age-related memory decline also affect a large percentage of the aging human population in the twenty-first century. Given the large unmet medical need associated with these diseases and the potential complexity of the genetic mechanisms underlying them (Haffter et al. 1996), the importance of appropriate screening tools with which mutation-induced changes in learning and memory processes may be identified is unquestionable.

12 Fear and Anxiety

Fear (induced by particular negative stimuli) and anxiety (a more diffuse and prolonged behavioral state not associated with particular induction stimuli) affects a large percentage of the human population (Weisberg 2009) and despite concerted efforts by pharmaceutical research companies and academic laboratories and despite the existence of several drugs, proper treatment is still not available for a

proportion of patients. It has been argued by several researchers that zebrafish may be successfully utilized to study and model some of the mechanisms of vertebrate fear and/or anxiety (Gerlai 2010). For example, fear responses have been reliably induced in zebrafish using a chemical cue, the alarm substance (Speedie and Gerlai 2008). Alarm substances have been shown to elicit fear and panic reactions in a broad range of fish species (Speedie and Gerlai 2008). These substances, which are produced by epidermal club cells in the skin of many fish species, are released when the skin is cut or damaged. In nature, the alarm substance is believed to signal danger, perhaps the presence of an actively hunting predator (piscivore fish species or a bird of prey). In the laboratory, the alarm substance has been successfully utilized to experimentally induce fear responses. Zebrafish have also been shown to reliably respond to this chemical cue with alarm reactions that include erratic movements (zig-zagging), jumping (or leaping) and freezing (complete immobility) (Speedie and Gerlai 2008). From the perspective of mutagenesis screening, however reliable these responses may seem, the alarm substance approach suffers from a major disadvantage. This substance has to be extracted from the skin of conspecifics which entails cutting or homogenizing the skin of freshly sacrificed fish and washing, diluting the extract. Because of the variability inherent in this extraction process, the exact dose and potency of the substance can not be ascertained across multiple experiments (multiple extractions). Recently, however, zebrafish has been shown to respond to a synthetic alarm substance that shares a key chemical structural element with that of natural alarm substances from several fish species (Parra et al. 2009). Hypoxantin-3-N-Oxide, H3NO, has been found to induce alarm reactions in zebrafish similar to those elicited by the natural alarm substance (Parra et al. 2009). Thus, it is now possible to precisely control the dose of the alarm substance and reduce unwanted experimental error variation, a crucial requirement for high throughput mutagenesis screens.

Although the synthetic alarm substance, H3NO, now allows precise and replicable fear induction, this method suffers from a drawback. Olfactory cues are notoriously difficult to work with. The onset (delivery) of the cue and now also its dose can be precisely controlled, however, its offset (washout) is difficult to achieve. In most behavioral paradigms, experimenters want to introduce the subject to its test chamber (tank) and let the subject habituate, establish a stable baseline behavior before administering the cue (the alarm substance in this case). This allows pre- and post-cue delivery periods to be compared and thus is a more powerful experimental design. Ideally, after the delivery of the cue and recording the effects of this delivery, one would like to turn it off and again compare periods during and after cue delivery. But this is quite cumbersome with olfactory cues. Furthermore, even if the experimental paradigm does not require turning off the cue during the behavioral session, the cue may be difficult to remove from the tank for the next subject. Residual amounts of the alarm substance may remain in the test tank even after emptying and refilling the test tank. As even trace amounts of the alarm substance may influence the behavior of the fish, this olfactory cue is difficult to work with especially when one wants to run a large number of fish as required for mutagenesis screening.

To circumvent the above issues, cues of other modalities have been tried for the induction of fear responses. Zebrafish, being a diurnal vertebrate, has excellent vision and respond well to visual cues. Zebrafish have been demonstrated to respond differentially to the sight of live fish according to whether the fish species shown were predatory or harmless and whether they were sympatric (coinhabiting the geographical region) or allopatric with zebrafish (Bass and Gerlai 2008). The latter study also demonstrated that zebrafish uniquely responded to a sympatric predator, the Indian leaf fish (*Nandus nandus*) and that the sight (solely visual stimuli) of the predator was sufficient to induce a maximal fear response (erratic movements and jumps). Utilizing this finding, subsequently zebrafish have been found to exhibit significant antipredatory responses not only to the sight of live Indian leaf fish but also to animated (moving) computer images of this species (Gerlai et al. 2009b). In this latter paradigm, both the presentation of stimuli and the recording and analysis of the fear responses were conducted in an automated computerized manner, i.e. the test paradigm was scalable and thus potentially appropriate for high throughput screening. Although numerous parameters of this automated fear paradigm will have to be optimized (e.g. size of the test tank, size and speed of movement of the predator image, presence or absence of hiding places, level of illumination, etc.), the results demonstrate the feasibility of high throughput screening for agents (mutations or pharmaceutical compounds) that may have fear altering properties.

13 Social Behavior

The last behavioral focus area I discuss in this paper is social behavior. Social behavior is a common term for a range of complex behavioral phenomena from agonistic (aggressive) encounters to reproductive (courtship) behaviors. Here I focus on a behavior within this broad range termed affiliative behavior, social cohesion or group forming. Affiliative or group forming behaviors are characteristic of our own species. Humans tend to form groups, which in modern history led to the development of the complex society where a set of rules govern. We are particularly sensitive to social signals and tend to spontaneously follow a large number of complex social rules. Briefly, being social is an inherent human trait. There are numerous human disorders that are associated with abnormalities in social behavior, one prominent example is the autism spectrum disorders (ASD). Treatment for ASD and other forms of abnormal social behaviors is lacking for two main reasons. One, the mechanisms underlying these diseases are unclear. Two, the mechanisms underlying social behaviors in general are not understood. Laboratory model organisms have been proposed to speed up the discovery of such mechanisms [for review see Gerlai and Gerlai (2003)]. The question as to whether autism may be modeled using animals is not trivial, however. Some may be skeptical and say that in order to model autism in animals one would need to understand its mechanism first in humans, so what is the use of animal research? Nevertheless, it

is becoming clearer that even such complex phenomena as social behavior has not only face but also construct validity in animal models, i.e. not only looks similar in animals but may also be mechanistically similar to that of our own species. Briefly, it may make sense to study social behavior in vertebrates other than humans, discover the underlying mechanisms in the laboratory organism, and look for translational aspects of the work, i.e. human homologs. Zebrafish is perhaps the most social vertebrate model organism currently under study in the laboratory. Zebrafish are found swimming in groups in nature, a behavior that they maintain under the artificial confines of the laboratory (Engeszer et al. 2007). It is this swimming together response, or shoaling, that may be an excellent behavioral phenomenon to study from a translational perspective. Answering such questions as to what neurobiological mechanisms (circuits, synaptic processes, biochemical interactions) underlie group forming or social cohesion in zebrafish may help us understand human social behavior and ultimately perhaps the mechanisms of the abnormalities of human social behavior. The first step in this research could be the characterization of social behavior in zebrafish followed by the development of behavioral test paradigms that could detect mutation or drug-induced changes in brain function at the level of social behavior. Below I present some examples of recent discoveries with zebrafish that may be useful to make the first steps in this direction.

Zebrafish forms groups and swims in group formation but due to unavailability of appropriate behavioral quantification methods, the complexities of this behavior were not properly described in the past. By now, however, methods have been designed that allow the quantification of numerous parameters of shoaling behavior, including moment to moment changes of the distances among every possible pairs of fish within the shoal (Miller and Gerlai 2007, 2008). A periodic (cyclical) fluctuation of shoal cohesion has been discovered in zebrafish (Miller and Gerlai 2008). Analysis of shoaling is now further developed to allow high throughput automated tracking of multiple fish and thus the precise description of how the entire shoal behaves. This method may enable one to screen for mutations but would require the use of a group of fish that carry the same mutation, which would necessitate breeding an extra generation (i.e. testing not the individual mutant fish but its offspring). Perhaps a faster behavioral screening method may be to test single fish and its response to social stimuli. The disadvantage of the latter approach is that complex group dynamics may not be detected but the advantage is that the test would save the extra generation of breeding.

Testing responses of individual fish to social stimuli has been achieved with an experimental set up similar to the predator visual stimulus paradigm (Gerlai et al. 2009a). Here the computer monitor placed on the side of the experimental tank shows animated (moving) images of zebrafish (five fish in this case). Each fish on the monitor moves independently and in different randomized directions and with a speed that changes from second to second while remaining within the range of the speed of normally swimming zebrafish. This artificial “shoal” elicits a robust behavioral response. The single experimental fish placed in the test tank usually does not exhibit a preference for any sides of the tank, explores the entire tank, and thus its position when averaged over a period of time (e.g. for one minute

intervals) ends up to be in the middle of the tank, which is 25 cm away from the computer screen in case of a 50 cm long tank. However, as soon as the computer screen shows the artificial shoal, the experimental zebrafish moves closer to the computer screen and on average stays about 10 cm away from it, a distance that is similar to what has been obtained with freely moving zebrafish in a real shoal (Miller and Gerlai 2008). Given that the visual stimulus that elicits the response is computer controlled and the subject's distance from the stimulus screen is recorded using computerized video-tracking, the entire test paradigm is automated, i.e. does not require the presence of the experimenter during the behavioral recording session. The paradigm therefore is high throughput and has utility in screening for mutation or drug-induced changes in social behavior. Indeed, this paradigm has been already used to detect strain (genetic) differences between populations of zebrafish, and alcohol and dopamine receptor antagonist-induced changes in social behavior [Gerlai et al. (2009a) and unpublished results].

14 Concluding Remarks

The excellent genetic tools developed for zebrafish have already provided promising results in the analysis of complex brain disorders. Importantly, several genes implicated in a number of human brain disorders have been shown to have zebrafish homologs. The function of these genes in embryonic development, and in a few cases in behavioral responses has started to be investigated. The genetic tools are constantly refined. Increasing number of genetic markers is becoming available for linkage analysis-based gene localization in random mutagenesis. Reverse genetic tools are also rapidly developing. In addition, but also very importantly, numerous novel behavioral paradigms are being developed and our understanding of the behavioral responses and capabilities of zebrafish has been exponentially increasing over the past few years suggesting that efficient and high throughput phenotypical screening applications (Gerlai 2002) are becoming reality for zebrafish. Given the fact that behavior is the output of the brain and that vertebrates share numerous biological features, including nucleotide sequence homologies, it is likely that zebrafish neurobehavioral genetics will facilitate the identification of numerous genes and compounds leading to the understanding and better treatment of human brain disorders.

Acknowledgments Supported by NIH/NIAAA (USA) and NSERC (Canada).

References

Al-Imari L, Gerlai R (2008) Conspecifics as reward in associative learning tasks for zebrafish (*Danio rerio*). Behav Brain Res 189:216–219

Alsop D, Vijayan MM (2008) Development of the corticosteroid stress axis and receptor expression in zebrafish. Am J Physiol Regul Integr Comp Physiol 294:R711–R719

Amsterdam A, Hopkins N (2006) Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. Trends Genet 22:473–478
Bailey CH, Kandel ER (2008) Synaptic remodeling, synaptic growth and the storage of long-term memory in Aplysia. Prog Brain Res 169:179–198
Bandmann O, Burton EA (2010) Genetic zebrafish models of neurodegenerative diseases. Neurobiol Dis 40:58–65
Bass SLS, Gerlai R (2008) Zebrafish (*Danio rerio*) responds differentially to stimulus fish: the effects of sympatric and allopatric predators and harmless fish. Behav Brain Res 186:107–117
Bill BR, Petzold AM, Clark KJ, Schimmenti LA, Ekker SC (2009) A primer for morpholino use in zebrafish. Zebrafish 6:69–77
Blaser R, Gerlai R (2006) Behavioral phenotyping in zebrafish: comparison of three behavioral quantification methods. Behav Res Meth 38:456–469
Braff DL, Geyer MA, Light GA, Sprock J, Perry W, Cadenhead KS et al (2001) Impact of prepulse characteristics on the detection of sensorimotor gating deficits in schizophrenia. Schizophr Res 49:171–178
Burgess HA, Granato M (2007) Sensorimotor gating in larval zebrafish. J Neurosci 27:4984–4994
Capecchi MR (1989) Altering the genome by homologous recombination. Science 244:1288–1292
Chatterjee D, Gerlai R (2009) High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. Behav Brain Res 200:208–213
Chen E, Ekker SC (2004) Zebrafish as a genomics research model. Curr Pharm Biotechnol 5: 409–413
Cohen NJ, Poldrack RA, Eichenbaum H (1997) Memory for items and memory for relations in the procedural/declarative memory framework. Memory 5:131–178
Denver RJ (2009) Structural and functional evolution of vertebrate neuroendocrine stress systems. Ann N Y Acad Sci 1163:1–16
Drerup CM, Wiora HM, Topczewski J, Morris JA (2009) Disc1 regulates foxd3 and sox10 expression, affecting neural crest migration and differentiation. Development 136:2623–2632
Driever W, Solnica-Krezel L, Schier AF, Neuhauss, SCF, Malicki J, Stemple DL, Stainier DYR, Zwartkruis F, Abdelilah S, Rangini Z, Belak J, Boggs C (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. Development 123:37–46
Ekker SC (2008) Zinc finger-based knockout punches for zebrafish genes. Zebrafish 5:121–123
Engeszer RE, Patterson LB, Rao AA, Parichy DM (2007) Zebrafish in the wild: a review of natural history and new notes from the field. Zebrafish 4:21–40
Fan L, Collodi P (2006) Zebrafish embryonic stem cells. Methods Enzymol 418:64–77
Gauthier J, Champagne N, Lafreniere RG, Xiong L, Spiegelman D, Brustein E et al (2010) De novo mutations in the gene encoding the synaptic scaffolding protein SHANK3 in patients ascertained for schizophrenia. Proc Natl Acad Sci USA 107:7863–7868
Gerlai R (2010) Zebrafish antipredatory responses: a future for translational research? Behav Brain Res (in press)
Gerlai R (2002) Phenomics: fiction or the future? Trends Neurosci 25:506–509
Gerlai J, Gerlai R (2003) Autism: a large unmet medical need and a complex research problem. Physiol Behav 79:461–470
Gerlai R, Clayton NS (1999) Analysing hippocampal function in transgenic mice: an ethological perspective. Trends Neurosci 22:47–51
Gerlai R, Chatterjee D, Pereira T, Sawashima T, Krishnannair R (2009a) Acute and chronic alcohol dose: population differences in behavior and neurochemistry of zebrafish. Genes Brain Behav 8:586–599
Gerlai R, Fernandes Y, Pereira T (2009b) Zebrafish (*Danio rerio*) responds to the animated image of a predator: towards the development of an automated aversive task. Behav Brain Res 201:318–324
Gerlai R, Wojtowicz JM, Marks A, Roder J (1995) Over-expression of a calcium binding protein, S100ß, in astrocytes alters synaptic plasticity and impairs spatial learning in transgenic mice. Learn Mem 2:26–39

Giles AC, Rankin CH (2009) Behavioral and genetic characterization of habituation using *Caenorhabditis elegans*. Neurobiol Learn Mem 92:139–146

Gómez-Laplaza LM, Gerlai R (2010) Latent Learning in Zebrafish (*Danio rerio*). Behav Brain Res 208:509–515

Haffter P, Nüsslein-Volhard C (1996) Large scale genetics in a small vertebrate, the zebrafish. Int J Dev Biol 40:221–227

Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, Van Eeden FJM, Jiang YJ, Heisenberg CP, Kelsh RN, Furutaniseiki M, Vogelsang E, Beuchle D, Schach U, Fabian C, Nüsslein-Volhard C (1996) The identification of genes with unique and essential function in the development of the zebrafish, *Danio rerio*. Development 123:1–36

Huang CJ, Jou TS, Ho YL, Lee WH, Jeng YT, Hsieh FJ, Tsai HJ (2005) Conditional expression of a myocardium-specific transgene in zebrafish transgenic lines. Dev Dyn 233:1294–1303

Joshi P, Liang JO, Dimonte K, Sullivan J, Pimplikar SW (2009) Amyloid precursor protein is required for convergent-extension movements during zebrafish development. Dev Biol 335:1–11

Kim S, Radhakrishnan UP, Rajpurohit SK, Kulkarni V, Jagadeeswaran P (2010) Vivo-Morpholino knockdown of alpha IIb: a novel approach to inhibit thrombocyte function in adult zebrafish. Blood Cells Mol Dis 44:169–174

Knapik EW (2000) ENU mutagenesis in zebrafish—from genes to complex diseases. Mamm Genome 11:511–519

Langenau DM, Feng H, Berghmans S, Kanki JP, Kutok JL, Look AT (2005) Cre/lox-regulated transgenic zebrafish model with conditional myc-induced T cell acute lymphoblastic leukemia. Proc Natl Acad Sci USA 102:6068–6073

Lee KY, Huang H, Ju B, Yang Z, Lin S (2002) Cloned zebrafish by nuclear transfer from long-term-cultured cells. Nat Biotech 20:795–799

Lekven AC, Helde KA, Thorpe CJ, Rooke R, Moon RT (2000) Reverse genetics in zebrafish. Physiol Genomics 2:37–48

Mathur P, Guo S (2010) Use of zebrafish as a model to understand mechanisms of addiction and complex neurobehavioral phenotypes. Neurobiol Dis 40:66–72

McEchron MD, Disterhoft JF (1999) Hippocampal encoding of non-spatial trace conditioning. Hippocampus 9:385–396

Miller N, Gerlai R (2008) Oscillations in shoal cohesion in zebrafish (*Danio rerio*). Behav. Brain Res 193:148–151

Miller N, Gerlai R (2007) Quantification of shoaling behaviour in zebrafish (*Danio rerio*). Behav. Brain Res 184:157–166

Moens CB, Donn TM, Wolf-Saxon ER, Ma TP (2008) Reverse genetics in zebrafish by TILLING. Brief Funct Genomic Proteomic 7:454–459

Pan Y, Razak Z, Mo K, Westwood JT, Gerlai R (2010) Chronic alcohol exposure induced gene expression changes in the zebrafish brain. Genes Brain Behav (in press)

Parra KV, Adrian JC Jr, Gerlai R (2009) The synthetic substance hypoxanthine 3-N-oxide elicits alarm reactions in zebrafish (*Danio rerio*). Behav. Brain Res 205:336–341

Paquet D et al (2009) A zebrafish model of tauopathy allows in vivo imaging of neuronal cell death and drug evaluation. J Clin Invest 119:1382–1395

Pather S, Gerlai R (2009) Shuttle box learning in zebrafish. Behav Brain Res 196:323–327

Patton EE, Zon LI (2001) The art and design of genetic screens: zebrafish. Nat Rev Genet 2:956–966

Pekhletski R, Gerlai R, Overstreet L, Huang X-P, Agopyan N, Slater NT, Roder J, Hampson DR (1996) Impaired motor learning and short-term synaptic plasticity in mice lacking mGluR4 metabotropic glutamate receptors. J Neurosci 16:6364–6373

Querfurth HW, LaFerla FM (2010) Alzheimer's disease. N Engl J Med 362:329–344

Reimers MJ, Hahn ME, Tanguay RL (2004) Two zebrafi sh alcohol dehydrogenases share common ancestry with mammalian class I, II, IV, and V alcohol dehydrogenase genes but have distinct functional characteristics. J Biol Chem; 279:38303–38312

Renier C, Faraco JH, Bourgin P, Motley T, Bonaventure P, Rosa F, Mignot E (2007) Genomic and functional conservation of sedative-hypnotic targets in the zebrafish. Pharmacogen Genomics 17:237–253

Salas C, Rodríguez F, Vargas JP, Durán E, Torres B (1996) Spatial learning and memory deficits after telencephalic ablation in goldfish trained in place and turn maze procedures. Behav Neurosci 110:965–980

Scott EK, Mason L, Arrenberg AB, Ziv L, Gosse NJ, Xiao T, Chi NC, Asakawa K, Kawakami K, Baier H (2007) Targeting neural circuitry in zebrafish using GAL4 enhancer trapping. Nat Methods 4:323–326

Sison M, Gerlai R (2010) Associative learning in zebrafish (*Danio rerio*) in the plus maze. Behav Brain Res 207:99–104

Sison M, Cawker J, Buske C, Gerlai R (2006) Fishing for genes of vertebrate behavior: zebra fish as an upcoming model system. Lab Animal 35:33–39

Sivasubbu S, Balciunas D, Davidson AE, Pickart MA, Hermanson SB, Wangensteen KJ, Wolbrink DC, Ekker SC (2006) Gene-breaking transposon mutagenesis reveals an essential role for histone H2afza in zebrafish larval development. Mech Dev 123:513–529

Skromne I, Prince VE (2008) Current perspectives in zebrafish reverse genetics: moving forward. Dev Dyn 237:861–882

Sokolowski MB (2001) Drosophila: genetics meets behaviour. Nat Rev Genet 2:879–890

Speedie N, Gerlai R (2008) Alarm substance induced behavioral responses in zebrafish (*Danio rerio*) Behav. Brain Res 188:168–177

Sweatt JD (2010) Mechanisms of memory. 2nd edn, Elsevier, Amsterdam, p 343

Tong C, Li P, Wu NL, Yan Y, Ying QL (2010) Production of p53 gene knockout rats by homologous recombination in embryonic stem cells. Nature 467:211–213

Tropepe V, Sive HL (2003) Can zebrafish be used as a model to study the neurodevelopmental causes of autism? Genes Brain Behav 2:268–281

Vargas JP, López JC, Portavella M (2009) What are the functions of fish brain pallium? Brain Res Bull 79:436–440

Weisberg RB (2009) Overview of generalized anxiety disorder: epidemiology, presentation, and course. J Clin Psychiatry 70(Suppl 2):4–9

Xia W (2010) Exploring Alzheimer's disease in zebrafish. J Alzheimer's Dis 20:981–990