Published in final edited form as:
Trends Neurosci. 2014 May ; 37(5): 264–278. doi:10.1016/j.tins.2014.02.011.

Zebrafish models for translational neuroscience research: from tank to bedside

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Abstract

The zebrafish (*Danio rerio*) is emerging as a new important species for studying mechanisms of brain function and dysfunction. Focusing on selected central nervous system (CNS) disorders (brain cancer, epilepsy, and anxiety) and using them as examples, we discuss the value of zebrafish models in translational neuroscience. We further evaluate the contribution of zebrafish to neuroimaging, circuit level, and drug discovery research. Outlining the role of zebrafish in modeling a wide range of human brain disorders, we also summarize recent applications and existing challenges in this field. Finally, we emphasize the potential of zebrafish models in behavioral phenomics and high-throughput genetic/small molecule screening, which is critical for CNS drug discovery and identifying novel candidate genes.

Keywords

brain disorders; anxiety; epilepsy; cancer; zebrafish; biomarkers

The developing utility of zebrafish in neuroscience research

Native to Southeast Asia, the zebrafish (*Danio rerio*) has become a popular model organism in biomedical research (Figure 1). Multiple advantages of using this species in biomedicine include high physiological and genetic homology to mammals, external fertilization, rapid

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tins.2014.02.011.

development, transparency of embryos and larvae, ease of genetic and other experimental manipulations, as well as cost- and space-effectiveness [1–6]. Detailed analyses of the strengths and limitations of zebrafish models in biomedical research and their relevance to neuroscience have been provided in recent literature [7–13], and are briefly summarized in Table 1 (also see Glossary).

Together with mammals and popular invertebrate model species (Figure 1), both larval and adult zebrafish are extensively used in central nervous system (CNS) research [14–16] and targeting various brain disorders (Figures 2–4, Table 2). However, the lack of prior experience with zebrafish models and phenotypes among various non-fish neuroscience laboratories hinders the wider application of this aquatic species in brain research (see Table S1 in the supplementary material online and [13] for details). Recognizing the potential of the zebrafish for translational neuroscience [7,9,14,17–20], we discuss the growing role of this organism in studying brain pathogenesis and its experimental modulation.

Because of the general scope of this review, here we highlight only certain areas and applications of zebrafish models, using them as representative examples. Specifically, we emphasize methodological benefits of zebrafish for brain imaging, behavioral phenomics and high-throughput screening (HTS), critical for CNS drug discovery and identifying novel candidate genes for brain disorders [7]. We also focus on selected brain disorders, chosen based on their pathogenetic nature, ranging from neoplastic to neurological and neuropsychiatric illnesses (Figure 3A). However, it is important to understand that zebrafish brain studies are much broader, covering a wide spectrum of CNS disorders and conditions that can be targeted using zebrafish (Table S2 in the supplementary material online) [7,13]. For example, marked progress has recently been made developing zebrafish models relevant to autism, sleep disorders, cognitive deficits, depression, psychoses, and addiction [5,9,21–29]. Taken together, mounting evidence indicates that the zebrafish is rapidly becoming one of the main organisms in translational neuroscience and biopsychiatry research, successfully complementing both rodent and clinical models of almost every major brain disorder [7,9,13,30].

Zebrafish CNS imaging

Developing novel biomarkers of brain disorders benefits from neuromorphological studies using animal models, including zebrafish. Zebrafish offer several important advantages in this regard (Table 1). For example, larval zebrafish are highly transparent [8] and permit an unprecedented optical access to their CNS. This feature is valuable for *in vivo* neuronal network analysis and recently developed whole brain/single cell functional imaging techniques that enable monitoring of neuronal activity in hundreds of neurons at once [31,32]. The adult zebrafish brain is relatively small, but shares many organizational features with its mammalian counterpart [7]. Although adult zebrafish are not transparent (but see [33,34]), they are also useful to study normal brain and identify anomalies associated with its pathology.

A series of experimental techniques described here can be used for economical, rapid, and high-resolution neuromorphological analyses of the zebrafish brain (Figure 2). For example,

the small zebrafish brain is ideal for 3D reconstruction of individual regions or whole brains using conventional magnetic resonance imaging (MRI; Figure 2A) [35]. This method has an isotropic resolution of 10 mm in an *ex vivo* zebrafish brain, representing the highest resolution achieved in a vertebrate brain, also comparable in slice thickness to conventional histology [35]. This allows fine details, such as fiber tracts (e.g., fasciculus retroflexus) or even some cellular layers (e.g., stratum marginale in the tectum), to be revealed [35]. Thus, this resolution is sufficient for imaging regions and their substructures in the zebrafish brain, and can be used to assess both the CNS morphological abnormalities (e.g., occurring as a result of a disease) and the gross morphology of various brain regions and their connectivity.

More detailed analyses of zebrafish brain structures can be conducted using simple whole mount histochemistry and widely available laser scanning microscopy. This approach enables an in-depth anatomical mapping of intact brain regions [e.g., olfactory bulb (OB) and telencephalon (TEL) in Figure 2B] and the pathways that connect them, without having to section and digitally reconstruct tissue samples. Figure 2B shows an example of a whole mounted zebrafish brain, where the stained inputs and partial outputs of the OB (green) are imaged and overlaid with information on the locations of axonal terminals (red). The small brain size in zebrafish enables staining and mounting the whole intact tissue sample while also permitting adequate staining and imaging of the regions that comprise this tissue (see [36] for details). As illustrated in Figure 2B, this approach permits accurate imaging and reconstruction of connectivity between brain regions (see, e.g., a small axon bundle that connects OB and TEL, readily visible in a whole mounted brain). By contrast, if the same tissue was to be sectioned, this connection may not be immediately apparent, and would have to await laborious manual and digital reconstruction of the sectioned material in order to be revealed.

Extension of this analysis may also include the use of histochemical markers for certain neurotransmitters (i.e., serotonin and dopamine, known to be involved in affective or neurological disorders). Such markers have already been tested in zebrafish [37], are commercially available, and enable systems level neurotransmitter analyses with mapping normal and abnormal connectivity phenotypes in fish.

In addition to assessing regional (Figure 2A) and interregional (Figure 2B) CNS connectivity, it is also possible to use the intact zebrafish brain for substructural imaging, again without requiring to section the tissue. Consider, for example, an optical cross-section of the zebrafish optic tectum, the region that receives retinal input (green; Figure 2C). To examine cell types involved in modulating this input, counterstaining can be performed with an antibody that detects specific (e.g., cholinergic) neurons (red; Figure 2C). Imaging this brain with a confocal microscope can generate high-resolution images, clearly revealing the anatomical relationships between these two neuronal systems in the intact brain.

Furthermore, neuromorphological analyses in zebrafish can also utilize traditional Golgi impregnation techniques. Serving neuroscience research for more than a century, this method enables high-resolution microscopic visualization of neuronal somata and dendritic arbors (where most of the neuronal volume is located) [38]. Applied to zebrafish recently, Golgi staining shows that fish neuronal ultrastructure is indeed very similar to that of

rodents [7]. Because dendritic spines receive the vast majority of excitatory input to neurons, the assessment of dendritic branching and spines reflects the health of the neurons and the integrity of the circuitry in a specific brain region [38].

Finally, the increasing availability of the newest sophisticated optical imaging systems [39] markedly advances zebrafish CNS research. Although *in vivo* optophysiological analyses of neuronal network function [40–43] have mainly been established in larval zebrafish, they can also be applied to adult fish (e.g., in the *casper* zebrafish strain [33,34], which retains high transparency into adulthood, thus permitting optical access to the CNS even in adults). Recently developed methods for clearing brain tissue (e.g., SeeDB [44] and CLARITY [45]) may further enhance zebrafish neuroimaging. Although originally developed in rodents (e.g., [45]), such methods can be particularly useful for zebrafish CNS analyses. Other important recent developments include tracking of single presynaptic and postsynaptic structures (e.g., using genetically encoded calcium indicators, GECIs) in zebrafish [46,47], representing powerful tools for ‘systems neuroscience’ studies using this organism. Collectively, these experimental approaches can produce comprehensive and powerful neuromorphological datasets to help identify disease biomarkers in the zebrafish, complementing its in-depth behavioral and physiological analyses.

Automated behavioral analyses

Behavioral phenotypes are the most complex product of CNS activity, and the availability of reliable video tracking techniques markedly empowers neurobehavioral analyses in zebrafish [27,48]. For example, both commercial and custom-made video tracking systems are used to assess larval and adult zebrafish behavior. Such automated observations are particularly suitable for measuring locomotor responses (e.g., distance traveled or speed/velocity, turning, etc.) that human observers cannot quantify [12]. Examples of commercially available software packages for zebrafish research include Ethovision developed by Noldus IT (Netherlands), LocoScan created by CleverSys, Inc. (USA), or ZebraLab produced by ViewPoint (France). Such software systems often have modular structure and are standardized, user-friendly, and coupled with thoughtfully designed hardware. Although not inexpensive, these packages are also validated by multiple international users, and typically come with regular upgrades and technical support, which becomes especially useful from a practical point of view. Offering a free alternative, the custom-made tracking systems are also available from different laboratories worldwide (e.g., [49]) and can be useful for various specific neurophenotyping tasks and experimental set-ups in zebrafish.

Applications of automated video tracking tools to behavioral research in larval and adult zebrafish are illustrated in Figure 4, showing robust anxiety-like or anxiolytic-like responses detected and quantified by the software (see in-depth discussion further). For larval models, the most common endpoints are locomotory, such as distance traveled and immobility/freezing (Figure 4A), which may often decrease or increase with anxiety, respectively [12,19]. Adult zebrafish behavior (Figure 4B) is much more complex and includes multiple parameters, often with complex spatial components (see Table 2 and [6,19,50,51] for details). Some other brain disorders, such as autism (a severely debilitating developmental

disorder affecting 1–2% of the global population [52,53]), can also be modeled using zebrafish [7]. The use of zebrafish to target social deficits is based on rich social behaviors in this species [7]. For example, zebrafish prefer to swim in shoals (Figure 4C), and the disruption of this group-forming behavior by various environmental, pharmacological, or genetic factors can be easily assessed [14,17,54]. Fully automated video tracking tools can 'extract' zebrafish social phenotypic data by monitoring several fish in parallel [48] and analyzing the proximity of three pairs of their body points relative to each other (see Figure 4D for details). With the growing application of cross-species behavioral analyses (e.g., using behavioral activity monitors to directly compare aberrant behaviors in human patients and rodent tests [55,56]), the inclusion of zebrafish in such translational neuropsychiatric studies can be particularly useful.

Another important aspect of computer-based behavioral analysis in zebrafish is their locomotion in 3D (swimming in XYZ coordinates) [50]. This situation markedly differs from typical rodent tests, which are mainly based on animal activity in a horizontal 2D plane (Figure 4B). The value of an additional (vertical) dimension is particularly important for zebrafish models, as their robust diving response to anxiogenic stimuli (Figure 4B) represents a natural zebrafish 'survival' behavior in the wild, and remains highly sensitive in laboratory zebrafish. A two-camera experimental set-up (e.g., as in Figure 4B) allows automated 3D neurophenotyping of zebrafish locomotion in XYZ coordinates by taking images from top and side views, and integrating them using various software packages into 3D signals. The ability to visualize and quantify behaviors in 3D enhances neurophenotyping using zebrafish and reveals high sensitivity of their 3D track reconstructions to various experimental manipulations (Figure 4B, see [50] for conceptual rationale and validation). For instance, 3D reconstructions of zebrafish swim patterns not only show high sensitivity to a wide array of pharmacological manipulations [5,6,50] but also extend the range of measurable indices (e.g., loops, tight circles, 'slide-and-fall' or 'figure-8' patterns). Many of them can be sensitive to specific drug classes (e.g., serotonergic vs glutamatergic agents) or behavioral profiles (e.g., withdrawal anxiety states, psychological anxiety states, fear/panic-like states, or neurological/motor deficits) [50]. Furthermore, whereas 3D-based analysis is currently applied to adult zebrafish [50,57], its high potential for drug discovery may eventually lead to 3D analysis of larval behavior, markedly enhancing their traditional '2D' HTS (Figure 4A).

Behavioral phenomics

Recent progress in bioinformatics has led to a new field of neuroscience – behavioral phenomics, that links complex behavioral phenotypes to various genetic mutations and environmental manipulations [58]. Phenotyping of various mutant and transgenic zebrafish [59–61] becomes important for neurogenetics and pharmacology, and such HTS are widely used for screening genetic mutations and small molecules, as part of behavioral phenomics approaches. Because zebrafish possess 'evolutionarily conserved' neuromediator systems with high homology to rodents and humans [7,37], the translational value of these screens is clear. Moreover, zebrafish are sensitive to all major classes of neurotropic drugs (including antipsychotics, mood stabilizers, anxiolytics, antidepressants, ethanol, sedatives, stimulants, hallucinogens, antiepileptics, anesthetic/analgesics, and cognitive enhancers) [7]. Taken

together, this demonstrates the value of zebrafish for modeling complex drug-evoked phenomena, eventually leading to new effective therapies for major groups of brain disorders [7]. Given the potential to screen hundreds of compounds per day, zebrafish HTS are critical for rapidly identifying active compounds or candidate genes to address these needs [10,12].

The possibility to develop 3D-based HTS for larval and adult zebrafish is important both practically and conceptually, because it can take zebrafish behavioral phenomics in a new direction. For example, *in vivo* HTS are based on the balance between the number of compounds to test and the number of endpoints to assess (Figure 4A). As already mentioned, typical HTS in zebrafish currently test multiple compounds in sensitive/fast 2D assays and focus on selected well-established, but relatively simple, behavioral endpoints (e.g., time spent moving, velocity, distance traveled, or heading) [10,59,62]. Thus, the breadth of testing in such HTS is typically preferred over the depth of phenotypic analyses. Similar to other species, zebrafish HTS are extensive, but limited in the ability to 'dig deeper' and extract rich behavioral information from a larger number of endpoints and their patterns. Therefore, we argue that the use of automated 3D-based behavioral analyses in zebrafish [50], combined with IT-based 'behavior recognition' of individual movement patterns (e.g., circling, loops, social heading), may enable zebrafish HTS to maintain their extensive nature, yet analyzing in-depth multiple novel endpoints and motor patterns.

Furthermore, despite general agreement among zebrafish scientists that a key advantage of zebrafish is the cost-effective HTS, how to perform such screens for behavioral phenotypes is less clear. Because behavioral phenotyping is a complex task, even for the mouse (the most frequently employed model organism of biomedical research), the organization of phenotyping screens is actively debated in the field [63–65]. Most agree that a single behavioral test is not enough to characterize an animal phenotype, and therefore test batteries must be used [63,66]. One way to organize a test battery is to move from least stressful/invasive tests to most invasive tests, thereby reducing the chance that behavioral responses are affected by prior test history [66,67]. Test batteries can also be organized in a 'bottom up' manner, that is, with the simpler behavioral functions tested first, and then gradually increasing the complexity of behavioral screening. The advantage of this strategy is in its systematic and logical, step-by-step analysis of a large number of behaviors. An alternative 'top down' strategy can also be efficient because starting analyses with the most complex behavior (e.g., memory) enables detecting all alterations that influence performance in the task designed to quantify the behavior (i.e., some associated with memory, others with simpler motor characteristics). This approach allows investigators to quickly identify mutants or drugs that did not alter the phenotype of interest and perform follow-up analyses on those animals that did show phenotypical modifications.

Another related, actively debated problem is standardization of neurophenotyping tests [68–70]. For example, although standardization is critical for consistency and cross-laboratory comparability of HTS, the new mutations or drugs may have unique effects on brain function and behavior, thereby necessitating custom-tailored paradigms and analyses. Thus, the best strategy can be a compromise – to use standardized HTS, followed up by secondary tests (custom-tailored to the functional abnormality detected). Although discussed in depth

in rodent literature ([63,64,71], Box 1) little is known about behavioral test batteries for zebrafish. Nevertheless, with the increasing number of zebrafish behavioral studies utilizing newly developed test paradigms (Table 2) and the technical advancements in movement monitoring, quantification, and analyses (see above), it is likely that neurophenotype-based HTS will be routinely employed in zebrafish. Coupled with sophisticated forward- and reverse-genetic methods (Glossary) developed for the zebrafish, these behavioral screens may provide an unprecedented coverage of critical genetic and biological mechanisms, underlying even the most complex behavioral functions or disorders of the vertebrate brain.

Dissecting zebrafish neural circuits

Neural circuits potently modulate human and animal behaviors [40,44,72], and various mental disorders are increasingly recognized as ‘circuit disorders’ due to abnormal brain connectivity [73] (Figure 3A). Can zebrafish be used to address this important aspect of CNS pathogenesis, and to dissect brain circuits and their contribution to complex behaviors? Recent experimental evidence strongly supports this notion, as region-specific analyses of the expression of proto-oncogenes can enable mapping brain activity to distinct circuits implicated in specific zebrafish behavior. For example, using *in situ* hybridization, *c-fos* expression links zebrafish anxiety-like behavior to habenula- related circuitry, including dorsal and ventral telencephalic areas (the zebrafish homologs of mammalian amygdala and striatum, critical for rodent anxiety behavior) [74]. Consistent with this, anxiogenic stimuli (e.g., alarm pheromone or overhead moving shadow) evoke heightened anxiety responses in transgenic zebrafish with their medial habenular silenced by tetanus toxin [75].

Likewise, learned aversive behavior can be assessed in zebrafish by conditioning their behaviors (e.g., using a red light) and measuring them following an aversive unconditioned stimulus (e.g., electric shock) [76]. In this model, genetic inactivation of the lateral subnucleus of dorsal habenula evokes freezing (versus the normal flight response), suggesting the zebrafish ‘anxiety’ circuit involving the lateral habenula and dorsal interpeduncular nucleus [76]. Although future studies will further dissect zebrafish neural circuits, these examples show how different approaches can already be successfully applied to develop valid zebrafish circuitry-oriented models of complex affective behaviors. Given the evolutionarily conserved nature of anxiety circuits [72] and the ‘circuitry nature’ of various mental disorders [73], such studies can be particularly important for translational neuroscience and biological psychiatry.

Selected applications of zebrafish models to disease modeling

As already mentioned, we specifically focus on several brain disorders, selected among common neoplastic, neurological, and neuropsychiatric illnesses (Figure 3A). Although brain cancer, epilepsy, and anxiety are only some of many examples of using zebrafish to study CNS disorders, they emphasize the translational value of this model organism for neuroscience research [7], also illustrating the breadth of spectrum of brain disorders which can be modeled using zebrafish.

Zebrafish models of brain cancers

Mounting evidence indicates the utility of zebrafish in cancer research, including studying metastatic potential, tumor-induced angiogenesis, extravasation, and tumorigenicity of various cancer lines under different conditions [8,77,78]. Zebrafish also appear to be useful for modeling aggressive 'killer cancers', such as glioblastoma, neuroblastoma, and melanoma (Figure 2D) [79–81]. Brain cancers affect a significant portion of the global population, with over 23 000 new cases and 14 000 deaths in 2013 in the USA alone (http://www.cancer.gov/). In children, neuroblastoma is the second most common type of brain tumor (and is also the most common extracranial solid pediatric tumor, often arising in adrenal or ganglion tissues along the spine). Adult zebrafish are a promising model for pediatric neoplasia of the brain and eye (Figure 2D) because, unlike mammals, they retain abundant embryonal neuroepithelium surrounding the ventricles of the brain, and also retain more pluripotent tissue in the eye and optic nerve. Although the CNS is the most common site for solid tumors in children, pediatric brain neoplasia is often less aggressive and can be more successfully treated than tumors in adults. Interestingly, fish species (including zebrafish) are highly resistant to spontaneous gliomas, for reasons not yet understood. However, available transgenic tumor models (Figure 2D) clearly demonstrate that zebrafish readily develop even the most aggressive forms of glioma or glioblastoma multiforme in the eye and brain, if selected genes are perturbed [80]. Early life stage exposure of zebrafish to a variety of carcinogens readily induces brain neuroblastoma, as well as nerve sheath neoplasia of the eye, skull, and other sites. Certain mutant lines of zebrafish develop neurogenic neoplasia fairly rapidly following carcinogen treatment [79]. Early life exposure of zebrafish to carcinogens and utilizing transgenic zebrafish models enables studying a wide variety of epithelial, mesenchymal, neural, and neural crest neoplasms (including some types of cancer, such as chordomas and esthesioneuroblastomas, that rarely occur in other species) [79]. Finally, given robust CNS cancer phenotypes observed in zebrafish models (e.g., Figure 2D), they may not only increase our understanding of tumor pathways, progression, and metastasis but can also be useful for developing new anticancer therapies, the need for which is currently widely recognized in the field, especially for treating aggressive brain tumors.

Zebrafish models of epilepsy

As CNS tumors represent neoplastic disorders associated with tissue damage, neurological disorders (including epilepsy, selected here as an example) are characterized by both brain tissue damage and pathological neural activity (Figure 3A). Epilepsy is a complex neurological disorder which affects ~70 million people worldwide, and has multiple underlying genetic and environmental causes with unclear pathophysiological mechanisms [3]. Commonly studied in rodents, epilepsy can also be modeled in zebrafish. For example, seizure-like behavioral and physiological responses are caused in zebrafish by various experimental manipulations [3,59,82]. Electroencephalographic (EEG) responses, a hallmark of epilepsy, can be recorded in both larval and adult zebrafish [59], to complement hyperactivity and other seizure-like behaviors, such as tremor, spasms, as well as corkscrew and circular swimming (Figure 3B,C), typically not observed in normal fish [3,19,82]. Increased brain expression of early protooncogenes, such as *c-fos*, is another marker of

neuronal activation and is typically elevated during seizures in both rodent models and zebrafish [3,19,82] (Figure 3C).

Moreover, zebrafish are sensitive to drugs and genetic mutations known to cause epilepsy in humans and rodents (e.g., [3,59]), which can help model various neurochemical and genetic aspects of epilepsy. One example of such models is the *mind-bomb* mutant zebrafish, which displays disturbed E3 ubiquitin ligase activity and *Notch* signaling, resulting in defects of brain development and in spontaneous seizures [60,61]. Analyzing agar-immobilized larvae, these studies have revealed seizure-like EEG activity and aberrant motor responses in mutant zebrafish, accompanied by altered expression of selected CNS genes [61]. Another example is zebrafish Nav1.1 mutants with the genetically disrupted *scn1Lab* gene (which normally encodes a voltage-gated sodium channel) [59]. In humans, mutations in *SCN1A* cause characteristic Dravet syndrome with severe intellectual disability, impaired social development, and drug-resistant seizures [59]. Paralleling clinical findings, *scn1Lab* mutant zebrafish display a similar neurological phenotype, including spontaneous seizure-like EEG activity, hyperactivity, and convulsions [59]. Supporting the use of zebrafish for modeling pediatric epilepsy (and monogenic epilepsy disorders in general), these mutants are also sensitive to various clinically used antiepileptic treatments (e.g., ketogenic diet, diazepam, valproate, potassium bromide, stiripentol, and clemizole) [59].

Finally, zebrafish represent powerful HTS for testing various proepileptic and antiepileptic drugs [3,59], which becomes particularly evident for screening novel anticonvulsant drugs. This typically includes exposure of many groups of experimental and control animals to a standard proepileptic agent, and identifying groups more resistant to evoked seizure-related physiological and behavioral symptoms [3]. For zebrafish, simultaneous exposure of multiple cohorts/tanks (containing hundreds of fish preexposed to different putative therapeutic agents) in the aquatic system with running convulsant-containing water offers unparalleled time/space and cost efficiency, compared with any rodent studies of a similar design [3]. Recently developed devices for automated drug delivery and medium change in multi-well screening panels (e.g., www.noldus.nl) can help develop further powerful epilepsy- related HTS using larval zebrafish.

Zebrafish models of anxiety spectrum disorders

In addition to neoplastic and neurological disorders discussed earlier, zebrafish are emerging as a promising model to study complex neuropsychiatric illnesses (Figure 4) [4,7,13]. For example, anxiety disorders (including generalized anxiety and other anxiety spectrum disorders, see Table S2 in the supplementary material online and [83]) are among the most common human neuropsychiatric conditions [84–86]. The increasing prevalence and emerging clinical complexity of anxiety disorders necessitates novel therapeutic approaches [84–86] and new experimental models to develop such treatments [87]. One key strategy is to increase the range of model species to enable cross-species analyses of ‘conserved’ affective phenotypes [71,87]. Decades ago, most scientists would be surprised if one suggested seemingly ‘primitive’ fish as a model to study complex affective disorders [13]. However, the situation has dramatically changed recently, as the field now recognizes that zebrafish ‘emotional’ behavior is complex and highly sensitive to various environmental

manipulations [19]. Zebrafish also possess significant genetic, endocrine, and anatomical homology with humans and rodents in relation to anxiety traits. For instance, adult and larval zebrafish express well-developed endocrine 'stress' axis [88,89] (Figure 1C) and robust anxiety-like responses (Table 1 and Figure 4B). In line with this, zebrafish are bidirectionally sensitive to a wide range of anxiogenic and anxiolytic drugs, generally paralleling rodent and clinical data for these agents [7].

Larval and adult zebrafish anxiety-related behaviors have already been discussed above (also see [19] for a comprehensive catalog). Table 2 summarizes currently available experimental models of zebrafish anxiety-like behavior and their striking similarity to rodent paradigms. For example, increased emotional reactivity is a core symptom of clinical anxiety and has long been utilized for modeling anxiety in rodent novelty-based paradigms (e.g., open field, light–dark, and elevated plus maze tests, based on the balance between exploration and avoidance/neophobia) [90]. Aquatic 'novelty' tests, such as the novel tank, light– dark box, and open field tests, are conceptually similar to these rodent models and have been extensively validated in zebrafish [4]. Acute stress-based models (e.g., predator exposure or acute restraint) are also used in both rodent and zebrafish models of anxiety spectrum disorders (Table 2).

Finally, genetic models are becoming increasingly useful for anxiety research in zebrafish. For example, several zebrafish strains display higher baseline anxiety phenotype than other strains [7], and may therefore be relevant to generalized anxiety disorder and dissecting its neural underpinnings (Table 1), similar to the use of high/low-anxiety mouse strains [90]. Furthermore, a promising new genetic model of affective disorders, recently developed in zebrafish [24,25], is based on disrupted negative feedback on the stress response in glucocorticoid receptor (GR) knockout zebrafish. These mutants display elevated cortisol levels and higher stress responsivity associated with anxiety-like behavior (Table 2), which can be corrected by fluoxetine [24]. In addition, mutant zebrafish with reduced anxiety have also been developed. For example, zebrafish with a mutated *fmr1* gene (encoding for the fragile X mental retardation 1 protein) display anxiolytic-like responses [91]. The latter two strains are important for bidirectional modulation of anxiety in zebrafish, potentially useful for both disease modeling and screening for novel anxiotropic drugs.

Concluding remarks: moving from tank to bedside

In summary, zebrafish represent an ideal organism for neurophenotyping, HTS, and brain imaging studies (Figures 1–4), which also facilitate *in vivo* drug discovery and genetic screening. Possessing high physiological and genetic homology to humans, zebrafish have become increasingly useful in studying a wide spectrum of human brain disorders, from brain cancer to epilepsy to complex affective disorders. Baseline data on normal and pathological behaviors or spontaneous and induced brain tissue pathology are becoming widely available in zebrafish, gradually approaching the knowledge base for other well-studied laboratory animals [7,13]. Although many biological problems remain to be addressed in zebrafish (Box 1), the availability of both a sensitive model organism and a growing set of experimental tools (e.g., Figure 4) is particularly timely. There are also

several excellent public access biomedical databases available to zebrafish investigators working with neurogenetics, pharmacology, and brain imaging (see Box 1 for details).

Although there is currently a relatively small group of highly trained zebrafish neuroscientists and pathologists, the field is expanding rapidly [13]. We also expect the growing need in their expertise, as zebrafish models become important tools in today's neuroscience research. At the same time, because zebrafish paradigms show many parallels with rodent and human phenotypes [7], it is equally important to not anthropomorphize zebrafish models and tests, and to be aware that the disorder-specific and species-specific issues may play an important role in our interpretation of zebrafish responses. Although human behavior will never be similar to fish responses (and vice versa), the evolutionarily conserved nature of complex CNS traits suggests that many human and zebrafish phenotypes share common genetic and physiological factors, representing an exciting emerging field for further translational studies in neuroscience [7,13,20,97,120].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr Jeremy Ullmann (University of Queensland, Australia), as well as Dr Lucas Noldus, Ruud Tegelenbosch and colleagues (Noldus IT, The Netherlands) for supplying images for Figures 2A and 3A, and D of this paper, and assistance in addressing reviewer's comments. The authors acknowledge the Croll and Fine laboratories (Dalhousie University, Canada) for providing the brain images for Figure 2B and C, and Mr Michael Nguyen for his assistance with this manuscript. We also thank members of the Kalueff (ZENEREI Institute, USA) and the Spitsbergen (Oregon State University, USA) laboratories for collecting samples and images used in this study. This research is supported by the National Institutes of Health/National Institute on Drug Abuse (NIH/NIDA) (A.V.K.), as well as by the NIH/National Institute on Alcohol Abuse and Alcoholism (NIAAA) and the Natural Sciences and Engineering Research Council of Canada (NSERC) (R.G.).

Glossary

Altered early proto-oncogene expression — analyses of the CNS expression of early proto-oncogenes (e.g., *c-fos*), a useful tool in zebrafish neuroscience. Activation of *c-fos* expression in the whole brain samples is frequently seen in zebrafish following pro-excitatory pharmacological modulation (e.g., by convulsant agents, Figure 3, or by selected psychostimulants and hallucinogens [27,48,111]). *c-fos* expression can also serve as a marker of neuronal activation, and is assessed more specifically in different brain regions, therefore providing functional mapping of brain activity in response to various acute or chronic challenges (see section on zebrafish circuitry for examples). Brain *c-fos* can be upregulated in zebrafish following exposure to alarm pheromone, predators, and/or novelty stress.

Behavioral phenomics — an emerging field of neuroscience that integrates multidisciplinary behavioral, physiological, and genomics research, aiming to understand the complex phenotypic consequences of genetic mutations and environmental manipulations [58] at the level of the organism (such as

zebrafish). An important goal of zebrafish behavioral phenomics is to increase the ability to measure and dissect various phenotypes (e.g., by using HTS and test batteries).

Endocrine stress responses

zebrafish possess a well-developed neuroendocrine system, generally highly homologous to that in mammals [7,112]. The zebrafish stress neuroendocrine (hypothalamo–pituitary–inter-renal, HPI) axis is similar to the human and rodent hypothalamo–pituitary–adrenal (HPA) axis, and releases cortisol following stress exposure (Figure 1C). Zebrafish cortisol responses generally correlate with behavioral indices of stress, and may be modulated experimentally (e.g., genetically or pharmacologically, Table 1) [24,25,94,107]. Methods to assess cortisol in zebrafish (including both adult [94] and larval [113] fish) include ELISA or radioligand binding assays using whole body, blood samples, and/or urine-containing water samples, where applicable.

Genetic tools

the zebrafish has been successfully utilized in ‘forward genetics’ (FG), an approach that includes the generation of random mutations (typically using chemical mutagens, such as ethylnitrosourea, ENU), with subsequent screening for phenotypical alterations [7]. FG aims to identify, based on the detected altered phenotype, novel genes whose protein products play roles in the phenotype of interest. ENU has allowed researchers to generate a large number of zebrafish mutants, and screening such mutants yielded important discoveries about the mechanisms of embryonic development, as well as larval and adult behavior and brain function. However, ENU-based FG studies require laborintensive linkage analysis-based positional cloning to identify the mutated gene. New alternative methods, including viral vector-based insertional mutagenesis or gene breaking transposon-based mutagenesis, have now been developed, enabling the identification of genes involved in neurobehavioral phenotypes more efficiently [7]. The goal of ‘reverse genetics’ (RG) is to characterize the function of known genes. Although mouse homologous recombination-based gene targeting using embryonic stem cells is a powerful RG method, it is not feasible for the zebrafish. Nevertheless, new technologies may revolutionize gene targeting as they enable more efficient targeted mutation of genes in zebrafish. One such method is the transcription activator-like effector nuclease (TALEN) technology that utilizes custom-designed artificial restriction endonuclease-like enzymes cutting at user-defined nucleotide sequences, specific to the target gene. Other RG approaches, successfully validated in zebrafish, include the morpholino knockdown and the ‘targeting induced local lesions in genomes’ (TILLING) system [7].

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Box 1. Outstanding questions

This section highlights selected outstanding questions and problems associated with the application of zebrafish models in neuroscience research (also see [7,13] for detailed discussion).

Applying optimal test batteries

Test batteries are a series of specific behavioral paradigms, clustered by category/domain (e.g., cognitive, feeding, pain, social, anxiety, depression, psychoses, reward), and are commonly used in neurobehavioral analyses, especially in rodents [64,67,114]. Test batteries depend on specific needs and research questions, but typically consist of well-characterized paradigms from the established neurobehavioral literature [67]. Combining individual tests in 'smart' ('hybrid') batteries has also been suggested to maximize the number of phenotypes assessed per trial [115]. Overall, test batteries are practical, time/cost-efficient, and valid, because they help dissociate simple performance characteristics (e.g., motor function, perception, attention, and motivation) from more complex behavioral traits, including learning, memory or anxiety [66]. In rodents, some (but not all) behavioral tests are sensitive to previous testing experience (test battery effect) [66]. Although many approaches similar to rodent models (Table 2) are used in zebrafish neurophenotyping when designing the aquatic test batteries [27], the effects of test battery on zebrafish behavior have not yet been studied and merit further scrutiny.

Exploring behavioral sex differences

Although far less studied (compared with rodents [64,67,114]), zebrafish display sex differences in behavioral and physiological responses. For example, female zebrafish display higher (than males) dopamine levels in the forebrain and lower 5-hydroxyindolacetic acid/serotonin ratios [116]. Similarly, in the cocaine withdrawal model, females exhibit earlier onset of behavioral withdrawal symptoms than male fish (who show more robust anxiogenic-like withdrawal phenotypes [22]). Given well-known sex differences in human behaviors, the effects of sex on zebrafish phenotypes in various behavioral models necessitate further experimentation (e.g., see recent data on sex and age differences in zebrafish locomotion [117]).

Understanding stress-related neuroendocrine responses

Zebrafish stress responses show striking similarity to human stress, employing cortisol as the major glucocorticoid hormone [39] (Figure 1C). Moreover, individual variation in zebrafish stress responses (similar to the coping styles in other vertebrate species) correlates strongly with basal cortisol levels and its recovery over time [118]. Given the absence of aldosterone production in zebrafish, cortisol may also function as a mineralocorticoid hormone, thereby acting physiologically at both glucocorticoid (GR) and mineralocorticoid (MR) receptors [119]. The exact contribution of these receptors in zebrafish phenotypes remains unclear [119], including their role in CNS-related responses. Although both GR and MR are implicated in medicating various zebrafish responses [16,24,25,119], their signaling pathways should be investigated further, especially in relation to stress, coping, and related neuroendocrine abnormalities.

Developing zebrafish web resources and neuro-ontologies

Currently available zebrafish online resources include multiple outstanding public access biomedical databases available to zebrafish investigators, including the Zebrafish Information Network (ZFIN, the zebrafish model organism database, www.ZFIN.org), the Zebrafish Genome (ENSEMBL) database (www.ensembl.org/index.html), the Zebrafish International Resource Center (ZIRC, www.zebrafish.org), the Zebrafish Neurophenome Project (ZNP, www.kaluefflab.com/znpindex.html), and the Zebrafish Brain Atlas (www.zebrafishbrain.org) [13,19]. Zebrafish research will markedly benefit from developing further databases dedicated to CNS phenotypes and disease models using this and related (e.g., medaka) organisms. In addition, zebrafish neurobehavioral ontology does not currently exist and also needs to be developed to be integrated into the existing animal behavioral ontologies [19].

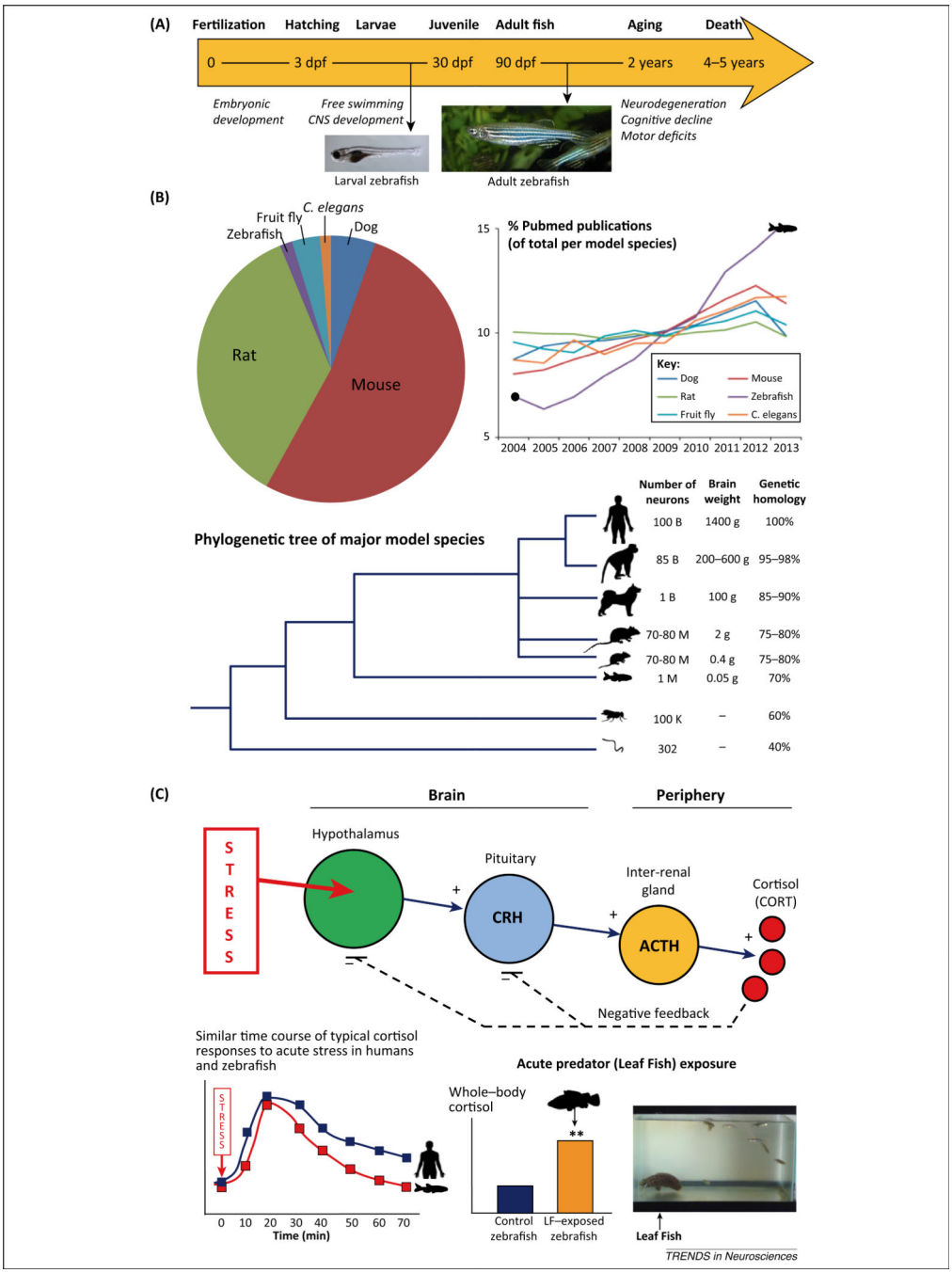


Figure 1.

Adult and larval zebrafish (*Danio rerio*), and their developing utility in biomedical research (from tank to bedside). Panel **(A)** outlines the life cycle of zebrafish, from embryonic pre-hatching (0–72 hpf) to post-hatching stages, including larval (3–29 dpf), juvenile (30–89 dpf), adult (90 dpf–2 years), and aged zebrafish (>2 years) [92]. Panel **(B)** shows the utility of zebrafish in biomedical research in 2004–2013. The number of PubMed publications (pie diagram) was assessed in December 2013 for various model organisms, yielding more than 532 000 publications for mice, 361 000 for rats, 54 000 for dogs, 34 000 for fruit flies, 15

000 for zebrafish, and 13 000 for nematodes (*Caenorhabditis elegans*). Line diagram shows normalized (expressed as % of total) number of publications per respective species (note that zebrafish publications display the sharpest increase compared with other animal models [13], shown as the phylogenetic tree; bottom left). Bottom right: comparative analyses of zebrafish brain versus other model organisms; note generally similar brain characteristics in zebrafish and mammals, including humans [7]. Panel **(C)** illustrates the zebrafish neuroendocrine stress (hypothalamo–pituitary–inter-renal, HPI) system, which releases cortisol from the inter-renal gland (similar to the adrenal gland in mammals) in response to adrenocorticotropic hormone (ACTH) following the stress-evoked release of hypothalamic corticotropin-releasing hormone (CRH) [16,24,25,93]. Acting via the negative biofeedback mechanism, cortisol released to the circulation activates glucocorticoid receptors (GRs) to inhibit the release of CRH and ACTH in the brain (thereby protecting zebrafish from pathological overactivation of the HPI axis). For example, acute stress evokes fast and robust cortisol responses with similar time dynamics in both humans and zebrafish (bottom row); also note that zebrafish and humans both use cortisol as their main stress hormone, unlike rodents (which use corticosterone) [16,24,25]. Strong behavioral and physiological stress responses can be evoked in zebrafish by acute exposure to their natural predators (e.g., Indian Leaf Fish, *Nandus nandus*) or other predator fish, such as African Leaf Fish (*Ctenopoma acutirostre*) and Oscar Fish (*Astronotus ocellatus*). Acute exposure of zebrafish to their natural predators (e.g., Leaf Fish, LF) induces overt anxiety-like behavior (tight shoaling and avoidance), accompanied by elevated whole body cortisol levels (**$P < 0.01$ vs control) [6,94]. Other stressors known to elevate cortisol in zebrafish include crowding stress, alarm pheromone exposure, acute restraint stress, novelty stress, social confrontations, drug withdrawal, or pharmacological treatments with various anxiogenic agents (Table 2). Importantly, recent successful applications of optogenetics in zebrafish have enabled selective manipulations of the HPI axis and cortisol signaling [39], further advancing stress physiology research utilizing this model organism. Abbreviations: hpf, hours post-fertilization; dpf, days post-fertilization.

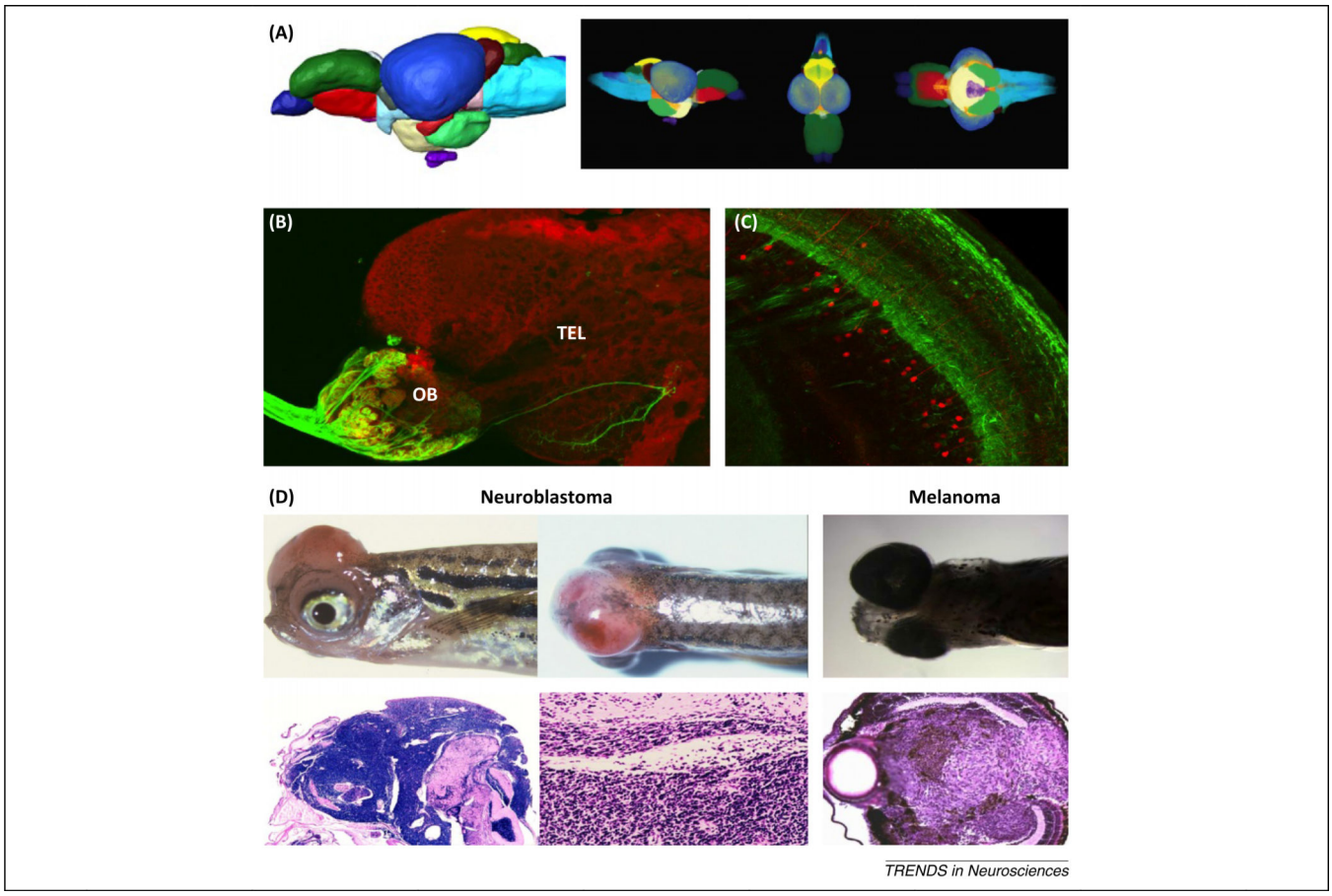


Figure 2.

Visualizing zebrafish brain using various imaging techniques in adult zebrafish. Panel **(A)** shows a 3D reconstructed zebrafish brain using two different types of magnetic resonance (MR) imaging: the MR histology (right) and the diffusion tensor imaging (left). The resolutions are among the highest achieved in a vertebrate brain, further establishing teleost fish as an excellent model for brain imaging [35]. Panel **(B)** shows a whole mounted zebrafish brain labeled with anti-synaptic vesicle protein 2 and anti-keyhole limpet hemocyanin antibodies [36]. Marked in green are the input (axons from sensory neurons in the sensory epithelia) and partial output from the zebrafish olfactory bulb (OB), and highlighted in red are synaptic terminals formed by long-range and local pathways. The brain in this image was hemisected along its midline (TEL, telencephalon; dorsal part is up and ventral is down). Panel **(C)** shows an optical section of an intact zebrafish brain showing transgenically labeled inputs to the optic tectum (HuC:chameleon green) and immunostained cholinergic neurons (anti-Chat) likely to receive and/or modulate the incoming neural signals. This panel reveals the interconnectedness of different neuronal pathways at high spatial resolution, and was obtained from an intact zebrafish brain (O. Braubach, 2014 Zebrafish case study). Panel **(D)** shows central nervous system (CNS) cancers, such as brain neuroblastoma and eye melanoma, in adult zebrafish. Left panel (top): neuroblastoma (side and top view) in adult zebrafish of KOLN wild type strain treated by bath exposure to a mutagen, ethylnitrosourea (ENU, 2.5 mM), as a 3-week-old fry. Left panel (bottom):

histological sections of neuroblastoma in adult zebrafish (treated as embryos with the carcinogen agent methylazoxymethanol acetate). Right panel: enlarged right eye and melanoma in a 4-week-old transgenic zebrafish (top) with activated *Smoa1* expressed under control of the *krt4* promoter [80]. Bottom image: histological appearance of melanoma of zebrafish eye (note poorly differentiated invasive melanocytes in melanoma; J. Spitsbergen, 2014 Zebrafish case study).

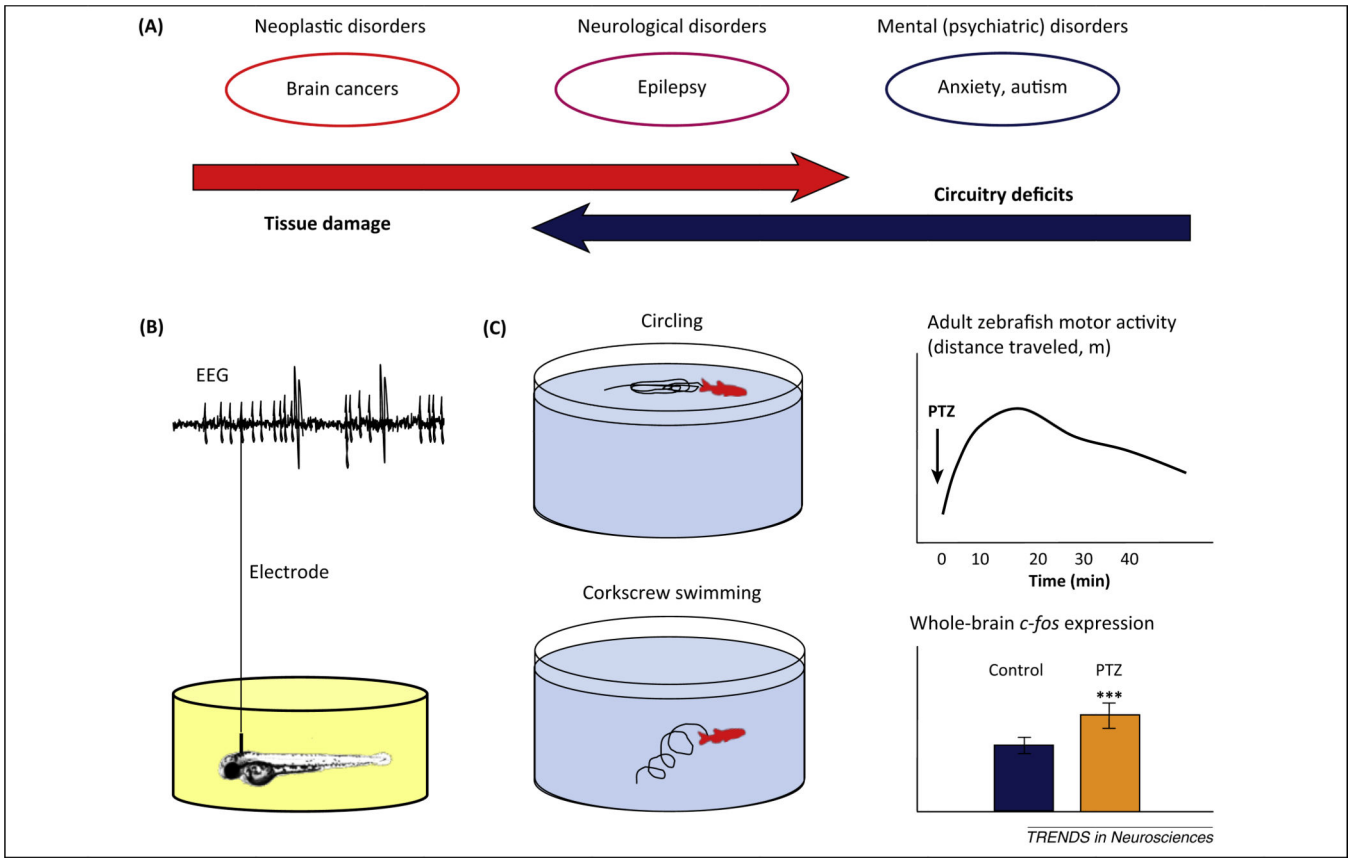
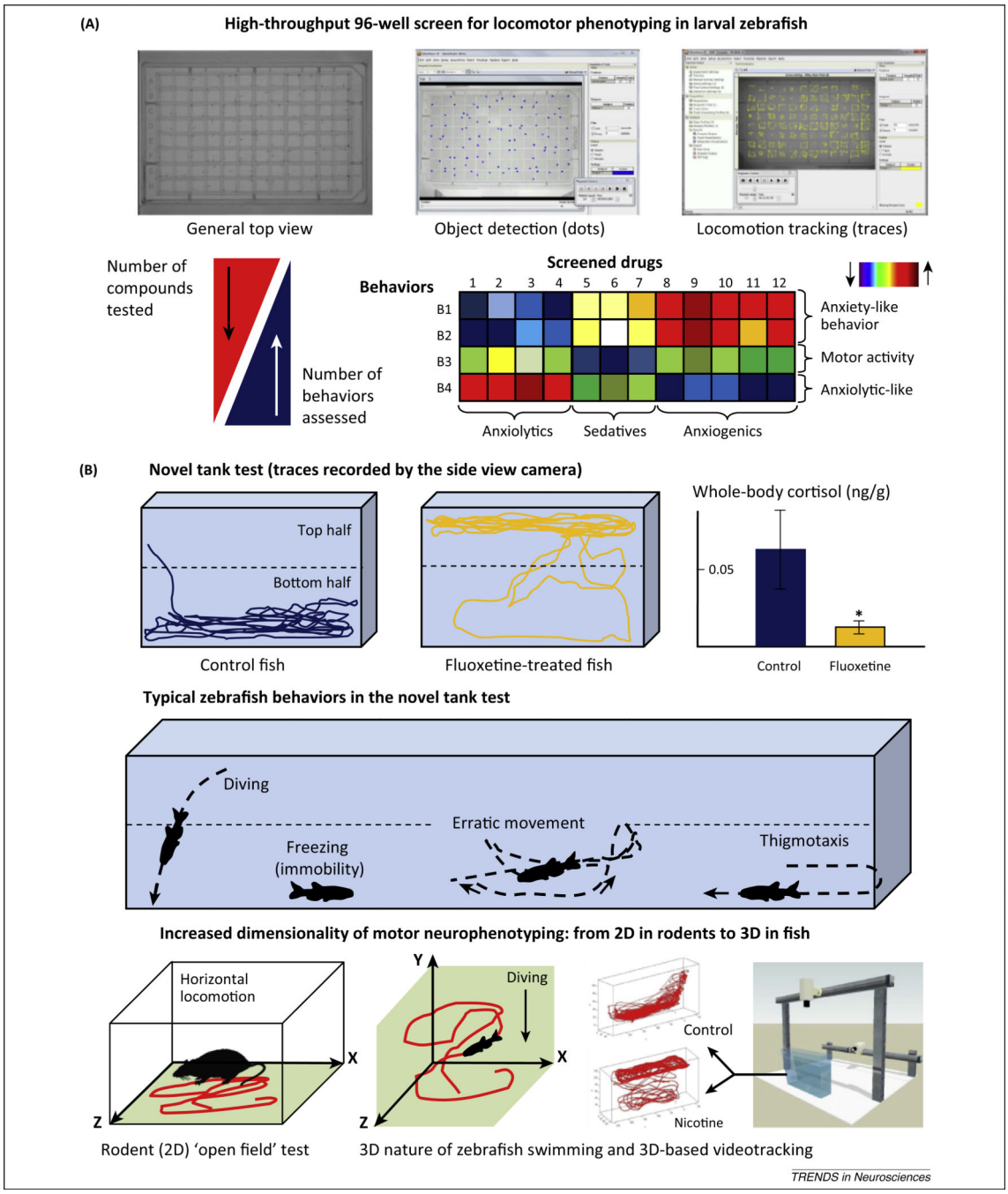


Figure 3.

Zebrafish models and phenotypes related to epilepsy. Panel **(A)** illustrates the place of epilepsy (as a neurological disorder) among other groups of central nervous system (CNS) disorders discussed here. Panel **(B)** shows a larval zebrafish embedded in agarose and paralyzed using a myorelaxant (e.g., D-tubocurarine), with electroencephalographic (EEG) electrodes inserted into the brain areas, such as tectum (invasive EEG), or placed on the skull (non-invasive 'surface' EEG) to record brain activity (e.g., typical tectal field recordings shown above). Panel **(C)** shows experimental seizures in adult zebrafish that can be evoked chemically (e.g., by exposure to various convulsant drugs, such as 10–15 mM pentylenetetrazole, PTZ, a blocker of the Cl^- ionophore at inhibitory gamma-aminobutyric acid GABA-A receptors). Note characteristic circling and corkscrew swimming, hyperlocomotion, and elevated *c-fos* expression following pretreatment with PTZ, *** $P <$ 0.001 versus control [3].



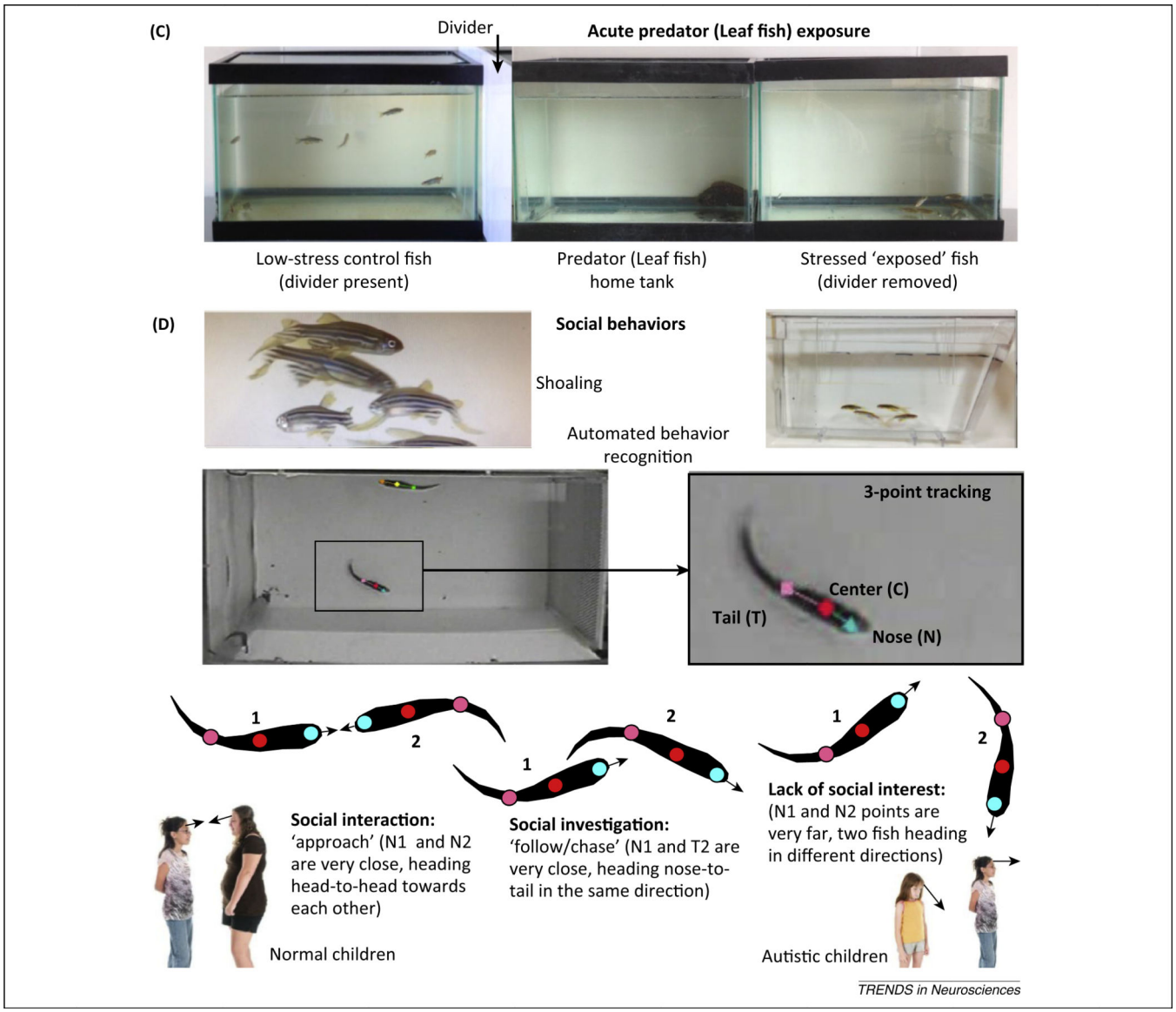


Figure 4.

Phenotyping zebrafish anxiety-related and social behavior. Panels (A) and (B) illustrate the utility of modern video tracking techniques for larval and adult zebrafish neurophenotyping. Panel **(A)** shows a 96-well high-throughput screen (HTS) for larval zebrafish, with the typical set-up (top view) and application of video tracking software to quantify zebrafish locomotor responses. Bottom row illustrates general principles of zebrafish HTS, screening a large number of chemical compounds (e.g., 1–12) from the library, and assessing various drug-induced behaviors (B1–B4) using zebrafish (color denotes decrease or increase of individual behaviors, as it moves from blue to red). Based on clustering these responses, HTS can detect psychotropic properties (e.g., anxiolytic versus sedative). Common anxiety-like behaviors in larval zebrafish HTS include increased immobility (freezing) frequency and duration, whereas anxiolytic-like behavior will often manifest in increased center dwelling [12,19]. In a simplified example, drugs evoking anxiolytic-like behaviors B1/B2

without hyperlocomotion B3 are recognized as potential anxiolytic agents; anxiogenic-like behaviors B4 and reduced B1/B2 without hyperlocomotion B3 can be interpreted as potential anxiogenic agents, whereas agents evoking anxiolytic-like behaviors B1/B2 combined with hypoactivity (reduced B3) may be interpreted as 'sedative' compounds. Panel **(B)** shows swimming patterns in the standard novel test tank, one of the most popular zebrafish behavioral assays [6,94]. Note distinct swimming patterns (top row), generated by video tracking software for untreated control (left) and experimental (right) fish treated with the classical antidepressant/anxiolytic drug fluoxetine (0.1 mg/l) for 2 weeks. The traces reveal marked differences in overall exploration and swimming activity, as control fish dwell mostly at the bottom and fluoxetine has the opposite, anxiolytic effect (see [6,94] and Table 2 for details). Consistent with this anxiolytic profile, experimental fish show significantly lower levels of cortisol [6,94] (* $P < 0.05$ vs control). Middle row: a diagram showing typical zebrafish anxiety-like responses in the novel tank test, including: (i) diving response, (ii) freezing/immobility, (iii) erratic movement, and (iv) thigmotaxis (staying close to the walls), which all increase during high-anxiety states (see [19,50] for detailed definitions of these behaviors), but can be rescued by anxiolytic treatments. Bottom row illustrates the importance of zebrafish behavioral analyses in 3D, to complement traditional 2D approaches; see [50] for details [note that zebrafish swim in 3D (XYZ) coordinates, unlike rodent tests, where animals typically display horizontal locomotion on 2D surfaces]. Right panel: a two-camera set-up which allows 3D neurophenotyping of zebrafish locomotion in XYZ coordinates (images from Noldus IT, The Netherlands in collaboration with the Kalueff laboratory). The 3D neurophenotyping approach reveals robust phenotypic differences between traces in control versus anxiolytic (5–10 mg/l nicotine-treated) zebrafish cohorts, including increased top exploration with reduced bottom dwelling and freezing. Note that nicotine-exposed fish demonstrate a consistent top dwelling, present for the entire duration of the trial. 3D reconstruction of their traces reveals anxiolytic-like 'top dwelling', largely concentrated at the water surface, yet with overt active swimming along the tank periphery. Such lack of anxiety coupled with thigmotactic behavior is consistent with typical psychostimulant/anxiolytic action of nicotine, paralleling its profile in various other model organisms (e.g., rodents). Panel **(C)** shows the predator exposure paradigm (see Table 2 for details) in which the zebrafish tank is exposed to a nearby tank containing a predator fish (e.g., Leaf Fish). As control fish (separated from the predator tank by a non-transparent plastic divider, denoted by the arrow) display low anxiety and swim in the middle and top areas of the tank in relatively 'relaxed' loose shoals, the removal of the divider results in overt 'aversive' anxiety-like behavior in the zebrafish group, including bottom dwelling, unusually tight shoaling and avoidance of the predator (by gathering in the farthest opposite corner). Panel **(D)** shows typical zebrafish 'group' (shoaling) behaviors and its potential relevance to human disorders. Zebrafish are highly social animals and spend the majority of time in social groups [e.g., staying within 1–2 body lengths (~2.5–5 cm) from each other]. In addition to reflecting anxiety-like responses (panel C), zebrafish shoaling behavior may be useful for modeling normal and pathological social behaviors, such as autism spectrum disorder [21]. For example, tracking zebrafish body shape (by simultaneous tracing three points – nose N, center of body mass C, and tail T; top view) can be used for automated decoding zebrafish social behaviors (photos by Noldus IT). Such automated tests are an invaluable tool to study zebrafish social behavior and its deficits. Typical computer-

generated endpoints may include orientation (angle) towards the object, distance between selected body points, and body curve patterns, which may be specific for various treatments. For instance, two zebrafish (#1 and #2) that show proximity of their nose points N1 and N2, tracked by the computer, are most likely to engage in social interaction (left). Heading in the same direction nose-to-tail (middle image) can be detected as 'chasing/following' behavior (see [19] for details of zebrafish ethogram). By contrast, two 'uninterested' zebrafish (right image) are detected by the software as heading in different directions without proximity of their nose points; the latter pattern is common in zebrafish with social deficits (see [21] for review). Bottom row: examples of normal human social interaction (left) and overt social deficits (right; typically observed in patients with autism; images: www.lovetoknow.com) which parallel zebrafish social phenotypes detected by IT-based tools, also see similar aberrant social behaviors in mouse models of autism [21,65,67].

Table 1

Selected advantages and limitations of zebrafish models for biomedical and translational neuroscience research (adapted from [13], also see [7] for discussion)

Model advantages
An *in vivo* model and a vertebrate species with common conserved cell types, organs, and physiological systems (e.g., stress endocrine axis, Figure 1C)
Sufficient physiological complexity and high physiological homology to humans
High genetic homology to humans; genetically tractable organism with fully sequenced genome
Ease of genetic manipulation and the availability of a wide range of genetic tools for the zebrafish (see Glossary)
Quick and abundant reproduction (e.g., a single female lays several hundred eggs each week)
Rapid development (hatching in <3 days and becoming mature by day 90); helpful for studying neurodevelopmental disorders. Development from 'transparent' eggs; transparent embryos (enables monitoring organ development and manipulating it *in vivo* – e.g., by injecting drugs or genes)
External development (zebrafish can be exposed to various environmental factors neonatally outside of the maternal organism, in a more experimentally controllable environment)
High space/cost-efficiency and excellent potential for high-throughput screens
Availability of various zebrafish strains, with over 1000 transgenic and mutant zebrafish strains
Adherence, as a lower vertebrate, to the 3R principles (replacement, refinement, reduction)
Smaller brains (which can be better assessed using the newest imaging techniques), see text and Figure 2 for details
Model limitations
Duplication of genome (some zebrafish genes have two copies instead of one, as in mammals)
Not as many well-characterized inbred strains as mice have (note that zebrafish, and fish in general, unlike rodents, do not tolerate inbreeding and rapidly lose fertility with inbreeding)
Drugs which are not water-soluble can be problematic to administer by water immersion (but the use of solvents as well as other routes are available)
Species differences in blood–brain barrier (BBB). Although zebrafish develop the BBB similar to that of humans, species differences exist and may affect permeability for certain drugs
Some complex behaviors develop over time (e.g., social behaviors are not prominent in larval fish)
Parental care is not known (although key for modeling some developmental disorders, such as autism, and may require alternative species to be used)
Certain brain areas are not as developed as in mammals (e.g., cortex), and some CNS structures in zebrafish are still difficult to map to their mammalian counterparts (this knowledge gap may complicate the interpretation of circuitry–behavior interplay)

Table 2

Selected experimental models of anxiety in zebrafish, and their relevance to rodent models of anxiety and human anxiety spectrum disorders (see [4,6,20,51] for details)[a]

Test	Zebrafish phenotypes	Relevance to		Refs
		Clinical anxiety	Rodent models	
Novel tank test	Characteristic diving behavior, thigmotaxis (peripheral swimming), reduction of exploration (especially in the top part of novel tanks, Figure 4B), increased erratic behavior and freezing/ immobility, elevated whole body cortisol and brain *c-fos* (these responses are highly sensitive to anxiolytic and anxiogenic agents)	GAD, AP	Novelty-based (open field, elevated plus maze) tests	[4,20,95–100]
Light–dark box	Avoidance of 'white' arenas (scototaxis), reduced exploration, and fewer visits to the white, elevated whole body cortisol and brain *c-fos* (these responses are highly sensitive to anxiolytic and anxiogenic agents)[c]	GAD, PD	Light–dark box	[74,96,101]
Predator fish exposure	Characteristic diving following acute exposure of predator fish, increased erratic behavior and freezing, elevated whole body cortisol and brain *c-fos* (Figure 1C), as well as increased escape behavior (increased distance from the stressor, Figure 4C). In group-tested fish, also induces characteristic shoal tightening (Figure 4C)[d]	SP, PD (PTSD[b])	Predator (cat, fox, snake) exposure paradigm	[102,103]
Robotic 'predator fish' exposure	Aversive responses in a preference test and in traditional anxiety/ fear-related tests (e.g., light–dark box). These responses are reduced by conventional anxiolytic drugs (e.g., ethanol)	SP, PD (PTSD[b])	?	[104]
Animated bird silhouette presentation	The model uses exposure to an animated (moving) image of a bird silhouette, decreasing the distance of the zebrafish from the bottom of the tank and increasing erratic movements. Anxiolytic treatments (e.g., ethanol) dose-dependently attenuate these responses	SP, PD (PTSD[b])	Predator image exposure test	[105]
Beaker stress	Elevated whole body cortisol and other anxiety-like behaviors following exposure to a beaker (novelty stress, social isolation stress)	GAD, PD	Social isolation stress, novelty-based tests	[13]
Alarm pheromone exposure	Characteristic diving behavior, reduced exploration, increased erratic behavior and freezing, elevated whole body cortisol, and brain *c-fos* expression. Recent studies implicate the medial habenula and interpeduncular nucleus in mediating these responses.	SP, PD	Predator (cat, fox) odor exposure paradigm	[97,106]

Test	Zebrafish phenotypes	Relevance to		Refs
		Clinical anxiety	Rodent models	
Shoaling test	Acute stress (novelty, predator, or alarm pheromone exposure) evokes overt changes in zebrafish shoals, including tightening the shoals (Figure 4C), as well as increased thigmotaxis and bottom dwelling.	GAD, SAD (PTSD[b])	Mouse social behavior test	[57]
Pharmacogenic anxiety	Characteristic diving behavior, increased thigmotaxis, reduced exploration (e.g., in the novel tank or the light–dark box), increased erratic behavior and freezing, as well as elevated whole body cortisol and brain *c-fos* following exposure to anxiogenic drugs (e.g., pentylenetetrazole or caffeine).	GAD, SMA	Pharmacogenic anxiety	[51]
Other drug-related anxiety (withdrawal)	Characteristic diving behavior, increased thigmotaxis, reduced exploration in novelty tests, increased erratic behavior and freezing, as well as elevated whole body cortisol and brain *c-fos* following single or repeated withdrawal from selected drugs (alcohol, benzodiazepines, barbiturates, opioids, or psychostimulants)	GAD, SMA	Withdrawal- evoked anxiety	[6,107]
Genetic models of anxiety: various wild type strains	The availability of several strains of zebrafish [e.g., leopard strain or wild Indian karyotype (WIK), derived from wild caught Indian zebrafish] as well as wild caught zebrafish (e.g., caught in the wild in India) which display high baseline anxiety (compared with less anxious zebrafish strains, such as AB or Tu strains)	GAD	High-anxiety mouse and rat strains	[7,108]
Mutant zebrafish models	Mutant strains (e.g., glucocorticoid receptor knockout zebrafish) showing elevated cortisol with increased anxiety-like behaviors (elevated freezing, reduced exploration) in novelty tests	GAD, comorbidity with depression	Mutant rodents with high-anxiety phenotypes[e]	[24,25]
Transgenic models	Anxiogenic stimuli (e.g., alarm pheromone or overhead moving shadow) evoke heightened anxiety responses in transgenic zebrafish with medial habenular silenced by tetanus toxin	GAD, PD (PTSD[b])	Transgenic rodents with high-anxiety phenotypes	[75]
Optogenetic models	Optogenetically mediated ‘hypercortisolic’ zebrafish, displaying stress-like cortisol and behavioral (hyperlocomotor) responses	GAD (PTSD[b])	Elevated cortisol and stress behavior	[39]

Test	Zebrafish phenotypes	Relevance to		Refs
		Clinical anxiety	Rodent models	
Startle test	The startle response is the instinctive reaction of zebrafish to novel unexpected and/or aversive stimuli (e.g., bright light, tapping/vibration, or loud sound). Anxiogenic factors typically potentiate, and anxiolytic factors reduce, startle responses in zebrafish (similar to their effects in rodents and humans)	GAD, PD (PTSD[b])	Startle test	[19]
Sleep deprivation	Sleep-deprived fish display increased anxiety-like behavior (e.g., increased preference for dark in the light–dark box)	GAD	Sleep deprivation	[23]
Acute restraint stress (ARS)	Increased anxiety-like behavior (similar to rodent responses) following acute (e.g., 90-min) ARS	PD, SP	ARS	[109]
Unpredictable chronic stress (UCS)	Increased anxiety-like behaviors and CRH expression (paralleling rodent responses) following the UCS protocol (e.g., applied for 7 or 14 days)	GAD (PTSD[b])	UCS	[93]

[a] Major anxiety spectrum disorders include separation anxiety (SA), specific phobias (SP), social anxiety disorder (SAD), panic disorder (PD), agoraphobia (AP), generalized anxiety disorder (GAD), substance/medication-induced anxiety (SMA) [83].

[b] These zebrafish models may also be relevant to post-traumatic stress disorder (PTSD), which, albeit recognized as a separate disorder from the anxiety spectrum [83], shares many symptoms and is frequently comorbid with anxiety disorders.

[c] Neuronal activity mapping using *c-fos* reveals the engagement of the medial zone of the dorsal telencephalic region and the dorsal nucleus of the ventral telencephalic area (the teleost anatomical homologs of the mammalian amygdala and striatum) [74].

[d] Similar anxiety-like responses can be elicited with an image presentation of the predator on a computer screen [20,103].

[e] For example, highly ‘anxious’ serotonin transporter (SERT) knockout mice and rats [110].