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Zebrafish models for translational neuroscience research: from tank to bedside

Adam Michael Stewart[1,2], Oliver Braubach[3], Jan Spitsbergen[4],

Robert Gerlai[5] and Allan V. Kalueff[1*]

[1]ZENEREI Institute and International Zebrafish Neuroscience Research Consortium (ZNRC), 309 Palmer Court, Slidell, LA 70458, USA

[2]Department of Neuroscience, University of Pittsburgh, A210 Langley Hall, Pittsburgh, PA 15260, USA

[3]Center for Functional Connectomics, Korea Institute of Science and Technology, Hwarangno 14-gil 5, Seoul, 136791, Republic of Korea

[4]Department of Microbiology, Oregon State University, Nash Hall 220 Corvallis, OR 97331, USA

[5]Department of Psychology, University of Toronto at Mississauga, 3359 Mississauga Rd N Mississauga, Ontario L5L1C6, Canada

***Corresponding Author:**

Allan V. Kalueff, PhD

ZENEREI Institute, 309 Palmer Court, Slidell,

LA 70458, USA

Tel/Fax.: +1-240-328-2275

Email: avkalueff@gmail.com

Abstract

The zebrafish (*Danio rerio*) is emerging as a new important species for studying brain mechanisms and its deficits. Focusing on selected CNS disorders (brain cancer, epilepsy and anxiety) and using them as examples, we discuss the value of zebrafish models in translational neuroscience, and their contribution 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 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 strengths and limitations of zebrafish models in biomedical research and their relevance to neuroscience have been provided in recent literature[7-12], and are briefly summarized in Table 1[13].

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 experience with zebrafish and their phenotypes among various non-fish laboratories hinders the wider application of these aquatic models in brain research (see Supplementary Table 1S and [13] for details). Recognizing the potential of the zebrafish for translational neuroscience[9, 14, 17-20], here we discuss the growing role of this organism in studying brain pathogenesis and its experimental modulation.

Because of the general scope of this review, we highlight several 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, and ranging from neoplastic to neurological and neuropsychiatric illnesses (Figure 3A). However, it is important to understand that zebrafish brain studies are much broader, and cover a wide spectrum of CNS disorders and conditions that can be targeted using zebrafish (Supplementary Table 2S)[7, 13]. For example, a 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, this indicates

that zebrafish is rapidly becoming one of the main organisms in translational neuroscience and biopsychiatry research, complementing both rodent and clinical models[7, 9, 30].

Zebrafish CNS imaging

Developing novel biomarkers of brain disorders benefits from neuroimaging and neuromorphological analyses using animal models. Thus, transparency of larval zebrafish and their brain is an important advantage of this model organism[8] (Table 1). In addition, although the zebrafish brain is relatively small, it shares many organizational features with its mammalian counterpart[7], and can be used for CNS imaging. Various genetic manipulations (Glossary) have also facilitated a rapid development of transgenic strains that enable visualization of neuronal structure and function. Collectively, these features make the zebrafish an attractive model for *in vivo* and *ex vivo* whole-brain mapping of neuronal connectivity in normal and diseased animals (Figure 2). For example, because of its small size, the zebrafish brain is ideal for three-dimensional (3D) reconstruction of individual brain regions and/or whole brains using magnetic resonance imaging (MRI; Figure 2A). These methods also have necessary resolution, as the 3D reconstruction can easily generate 10 μm^3 in an ex-vivo zebrafish brain using a standard 16.4T MRI scanner, clearly sufficient to detect and monitor CNS tumors (Ullmann, personal communication, 2014). Thus, such imaging tools can can be an important advantage for mapping the progression of brain tumors or other gross structural abnormalities that occur as a result of neurological diseases.

More detailed analyses of brain structure can subsequently be conducted with simple whole-mount histochemistry and widely available laser scanning microscopy. This approach enables the creation of in-depth anatomical maps of intact brain regions 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 olfactory bulbs (green) are imaged and overlaid with information on the locations of axonal terminals (red). The small brain size enables staining and mounting the whole intact tissue sample while also producing

unprecedentedly detailed imaging of this brain region (see [31] for details). As shown in Figure 2B, this approach permits accurate imaging and reconstruction of connectivity between brain regions. For instance, the small connection shown between the olfactory bulb (OB) and telencephalon (TEL) is readily visible in a whole-mounted brain. In contrast, if the same tissue would be sectioned, this connection may not be immediately apparent, and would have to await laborious manual and digital reconstruction of the sectioned material to be revealed. Furthermore, histochemical markers for labeling neurotransmitters involved in affective and/or neurological disorders (i.e., serotonin and dopamine) have been tested in zebrafish[32], and are commercially available. This enables systems level connectivity analysis of neurotransmitter systems, with the exciting possibility of mapping normal and abnormal connectivity phenotypes rapidly, economically and relatively easily in zebrafish.

The increasing availability of sophisticated optical imaging systems[33] and new methods for clearing brain tissue (e.g., seeDB[34] and CLARITY[35]) also foster zebrafish neuroimaging applications. While originally developed in rodents (e.g., [35]), such methodological approaches become particularly useful in zebrafish, enabling mapping the substructure of certain fish brain regions without sectioning the tissue. For example, Figure 2C shows an optical cross-section of the zebrafish optic tectum, the region that receives retinal input (green). 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 anatomical images, which clearly detail the anatomical relationships between these two neuronal systems in the intact brain (which can be evaluated at multiple time points, e.g., during disease progression or aging). In addition to these anatomical analyses, zebrafish offer a unique ability to map neuronal function at the single-cell and network levels. Such experiments have mainly been conducted in larval zebrafish, which are transparent and allow brain visualization *in vivo*. Exploiting these advantages helps delineate functional aspects in multiple brain regions in live animals[36, 37]. Other important recent developments include whole-brain/single-cell functional imaging techniques (which

enable monitoring of neuronal activity in hundreds of neurons at once[38, 39]), as well as optogenetics approaches[40-43] and tracking of single pre- and post-synaptic structures (e.g., using genetically encoded calcium indicators, GECIs)[44, 45], all representing powerful tools for 'systems neuroscience' studies using zebrafish.

Finally, traditional Golgi impregnation is one of the first methods developed to visualize nervous tissue, and has been serving neuroscience research for more than a century. This approach enables high-quality microscopic visualization of the soma and dendritic arbor (where most of the neuronal volume is located)[46]. Applied to zebrafish recently, Golgi staining shows that fish neuronal morphology is very similar to that of rodents[7]. Because dendritic spines represent the loci of 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[46] (which can be used as neuromorphological biomarkers in zebrafish[7], to accompany in-depth behavioral and physiological analyses). Thus, zebrafish have become an attractive model for neuroimaging, as well as for in-depth anatomical and functional mapping of neuronal network connectivity and single-cell function[47, 48], helping to determine the neuronal bases of various brain diseases.

Automated behavioral analyses

Behavioral phenotypes are the most complex product of CNS activity, and the availability of sophisticated video-tracking techniques markedly empowers neurobehavioral analyses in zebrafish[49, 50]. 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 having modular structure and coupled with thoughtfully designed hardware, are standardized, user-friendly, but not

inexpensive. However, they are 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 allernative, the custom-made alternative systems are also available from different laboratories worldwide (e.g., [51]), and can be useful for various specific zebrafish neurophenotyping tasks and experimental set-ups.

Applications of these automated tools to anxiety research in larval and adult zebrafish are illustrated in Figure 4, showing robust anxiogenic- or anxiolytic-like responses detected and quantified by the software. For larval models, the most common endpoints are locomotion (e.g., distance traveled vs. immobility/freezing, Figure 4A)[12]. Adult zebrafish behavior (Figure 4B) is more complex, and includes multiple parameters, often with complex spatial components (see Table 2 and [6, 19, 52, 53] for details).

For some other brain disorders, such as autism (a severely debilitating developmental disorder affecting 1-2% of the global population[54, 55]), the use of zebrafish to model social deficits is based on their rich social behaviors[7]. For example, zebrafish are highly social animals and swim in shoals (Figure 4C), the disruption of which by various environmental, pharmacological or genetic factor can be easily assessed[14, 17, 56]. Fully automated video-tracking tools can 'extract' zebrafish social phenotypic data by monitoring several fish in parallel[50], and calculating the proximity of 3 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[57, 58]), the inclusion of zebrafish in such translational studies can be particularly interesting.

Another important aspect of computer-based behavioral analyses in zebrafish is their locomotion in 3D (swimming in XYZ coordinates)[52]. This situation differs markedly from typical rodent tests, which are based on animal activity in 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 2-camera experimental setup (Figure 4B) allows automated 3D neurophenotyping of zebrafish locomotion in XYZ coordinates by taking images from top and side view, 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 [52] 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, 52], but also extend the range of measurable indices (e.g., loops, tight circles, 'slide-and-fall' or 'figure-8's patterns), many of which can be sensitive to specific drug classes or behavioral profiles (e.g., withdrawal anxiety states, psychological anxiety states, fear/panic-like states or neurological/motor deficits)[52]. Furthermore, whereas 3D-based analysis are currently applied to adult zebrafish[52, 59], its high potential for drug discovery may eventually lead to 3D analyses of larval behaviors, markedly enhancing their traditional 2D screens (Figure 4A).

Behavioral phenomics

Recent progress in bioinformatics has led to a new field of neuroscience (behavioral phenomics) that links complex behavioral phenotypes of an organism to various genetic mutations and environmental manipulations[60] (Glossary). Zebrafish-based phenotyping of various mutants and transgenic fish[61-63] becomes important for neurogenetics and pharmacology, and zebrafish 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, 32], 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 utility of

zebrafish in modeling complex drug-evoked brain disorders, suggesting the potential of this species for developing new effective therapies for major groups of brain disorders. With the potential to screen hundreds of compounds per day, zebrafish HTS become 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 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). Currently, typical HTS in zebrafish 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, 61, 64]. Thus, the breadth of testing in HTS is typically preferred over the depth of phenotypic analyses. Like with 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. We argue that the use of automated 3D-based behavioral analyses in zebrafish[52], combined with IT-based 'behavior recognition' of individual patterns (e.g., circling, loops, social heading), enables zebrafish HTS to maintain their extensive nature, yet analyzing in-depth multiple novel endpoints and motor patterns.

Furthermore, despite a 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[65-67]. Most agree that a single behavioral test is not enough to characterize animal phenotype, and therefore test batteries must be used[65, 68]. One way to organize a test battery is to move from least stressful/invasive tests to most invasive, thereby reducing the chance that behavioral responses are affected by prior test history[68, 69]. Test batteries can also be organized in a "bottom up" manner, i.e., 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 the tests[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. Therefore, a compromise can be the best strategy: standardized HTS followed up by secondary tests which are custom-tailored to the functional abnormality detected. Although the principles of behavioral phenotyping have been discussed *in-depth* in rodent literature[65, 66, 71], 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 developed for the zebrafish (Glossary), 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 circuits

Importantly, mental disorders are increasingly recognized as 'circuit disorders' with abnormal connectivity between different brain areas[72] (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. For example, region-specific analyses of proto-oncogenes expression can enable mapping brain activity to specific circuits implicated in specific zebrafish behavior (Glossary). Using this *in-situ* hybridization, *c-fos* expression assessed in the light-dark paradigm links zebrafish anxiety-related behavior to habenula-related circuitry, including dorsal and ventral telencephalic areas (the zebrafish homologs of mammalian amygdala and striatum, critical for rodent anxiety behavior)[73]. Consistent with this, anxiogenic stimuli (e.g., alarm pheromone or overhead moving shadow) evoke heightened anxiety responses in transgenic zebrafish with medial habenular silenced by tetanus toxin[74]. 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)[75]. In this model, genetic inactivation of the lateral sub-nucleus of dorsal habenula evokes freezing (vs. the normal flight response), suggesting the zebrafish 'anxiety' circuit involving the lateral habenula and dorsal inter-peduncular nucleus[75]. While 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[76] and the 'circuitry nature' of various mental disorders[72], such studies can be particularly important for translational neuroscience and biological psychiatry.

Selected applications of zebrafish models

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], and illustrate 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[8, 77, 78], including modeling aggressive 'killer-cancers', such as glioblastoma, neuroblastoma and melanoma (Figure 2D)[79-81]. Brain cancers affect a significant portion of global population, with over 23000 new cases and 14000 deaths in 2013 in USA alone[82]. 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 tissue 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. While 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 eye, skull and other sites. Certain mutant lines of zebrafish develop neurogenic neoplasia quite 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 occur rarely in other species)[79]. Finally, given robust CNS cancer phenotypes in zebrafish models, they may not only increase our understanding of tumor pathways, progression and metastasis, but can also be useful for developing new anti-cancer 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 (such as epilepsy, selected here as an example) are characterized by both brain tassel 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, 61, 83]. Electroencephalographic (EEG) responses, a hallmark of epilepsy, can be recorded in both larval and adult zebrafish[61], 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, 83]. Increased brain expression of early proto-oncogenes, such as *c-fos*, is another marker of neuronal activation, and is typically elevated during seizures in both rodent models and zebrafish[3, 19, 83] (Figure 3C and Glossary).

Moreover, zebrafish are sensitive to drugs and genetic mutations known to cause epilepsy in humans and rodents (e.g., [3, 61]), 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 [62, 63]. 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[63]. Another example is zebrafish Nav1.1 mutants with genetically disrupted *scn1Lab* gene (which normally encodes a voltage-gated sodium channel)[61]. In humans, mutations in *SCN1A* cause characteristic Dravet syndrome with severe intellectual disability, impaired social development and drug-resistant seizures[61]. Paralleling clinical findings, *scn1Lab* mutant zebrafish display a similar neurological phenotype, including spontaneous seizure-like EEG activity, hyperactivity and convulsions[61]. Supporting the use of zebrafish for modelling 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)[61].

Finally, zebrafish represent powerful HTS for testing various pro- and anti-epileptic drugs[3, 61], which becomes particularly evident for screening novel anticonvulsant drugs. This typically includes exposure of many groups of experimental and control animals to a standard pro-epileptic 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 pre-exposed to different putative therapeutic agents) in the aquatic system with running convulsant-containing water offers unparallelled time/space and cost efficiency, compared to any rodent studies of a similar design[3]. Recently developed sophisticated devices for automated drug delivery and medium change in multi-well screening panels (e.g., www.noldus.nl) help develop even more powerful epilepsy-related HTS for 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 more complex neuropsychiatric disorders. For example, anxiety disorders (including generalized anxiety and other spectrum disorders, Supplementary Table 3S [84]) are among the most common human neuropsychiatric illnesses[85-87]. The increasing prevalence and emerging clinical complexity of anxiety necessitates novel therapeutic approaches[85-87] and new experimental models to develop such treatments[88]. One key strategy is to increase the range of model species to enable cross-species analyses of 'conserved' affective phenotypes[71, 88]. Decades ago, most scientists would be surprised if one suggested seemingly 'primitive' fish as a model to study complex affective disorders. However, the situation has changed dramatically 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 anatomic homology with humans and rodents in relation to anxiety traits. For instance, adult and larval zebrafish express well-

developed endocrine 'stress' axis[89, 90] (Figure 1C and Glossary) and robust anxiety-like responses (Table 1 and Figure 4B). Furthermore, zebrafish are bi-directionally sensitive to a wide range of anxiogenic and anxiolytic drugs, generally paralleling rodent and clinical data for these agents[7].

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)[91]. 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 (such as predator exposure or acute restraint), are also used in both rodent and zebrafish models of anxiety spectrum disorders (Table 1).

Finally, genetic models are useful for anxiety research in zebrafish. For example, several zebrafish outbred strains display higher baseline anxiety phenotype (as compared to low-anxiety strains[7]), and may therefore be relevant to generalized anxiety disorder and dissecting its neural underpinnings (Table 1), similar to the use of high- vs. low-anxiety mouse strains[91]. 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 1) 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[92]. The latter two strains can be important for bi-directional 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 and 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 is becoming widely available in zebrafish, gradually approaching the knowledge base for other well-studied laboratory animals. Although multiple other biological questions remain to be addressed (Outstanding Questions), the availability of both a sensitive model organism and a growing set of experimental tools (e.g., Figure 4), are particularly timely. There are also several excellent public-access biomedical databases available to zebrafish investigators working with neurogenetics, pharmacology and brain imaging (see Outstanding Questions for details).

While there is a relatively small group of highly trained zebrafish neuroscientists and pathologists, the field is expanding rapidly. 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 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.

Supplementary data: Refer to Web version on PubMed Central for supplementary material.

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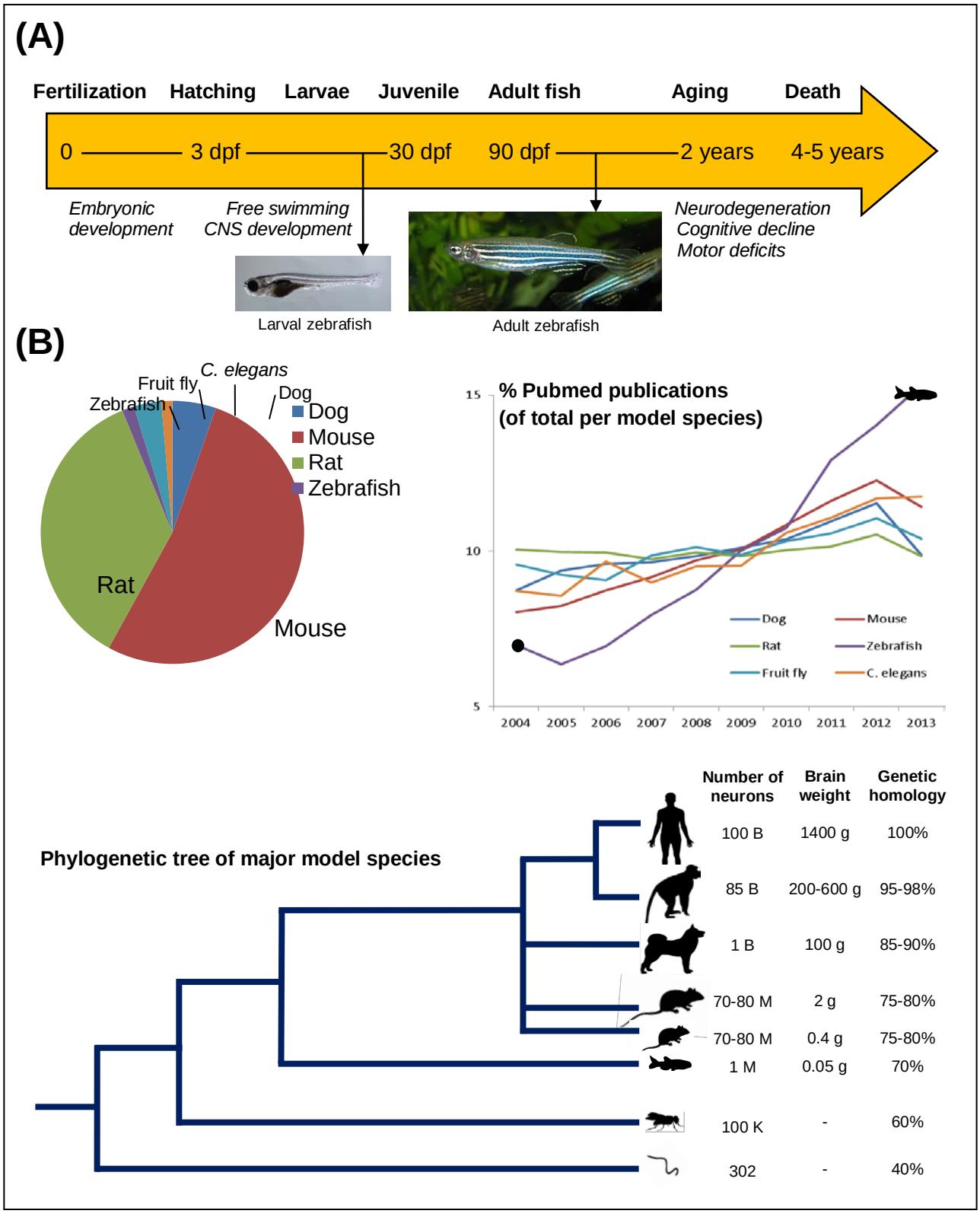
Figure 1. Adult and larval zebrafish (*Danio rerio*), and their developing utility in biomedical research (from tank to bedside).

Panel (A) outlines the lifecycle of zebrafish, from embryonic pre- (0-72 hpf) and post-hatching (72-120 hpf) stages to 'larval' (1-29 dpf), juvenile (30-89 dpf), adult (90 dpf-2 years) and aged zebrafish (>2 years)[93].

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 over 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 (*C. elegans*). Line diagram shows normalized (expressed as % of total) number of publications per respective species (zebrafish publications display the sharpest rise compared to other animal models). Bottom image: Comparative analysis of zebrafish brain vs. other model organisms. Note similar brain characteristics in zebrafish and mammals, including humans[7].

Panel (C) illustrates zebrafish neuroendocrine stress (hypothalamo-pituitary-interrenal, HPI) system (Glossary), which releases cortisol (CORT) from the inter-renal gland in response to adrenocorticotropic resealing hormone (ACTH) following the stress-evoked release of hypothalamic corticotropin-releasing hormone (CRH). The inter-renal gland in zebrafish (similar to the adrenal gland in mammals) releases cortisol (CORT) in response to adrenocorticotropic resealing hormone (ACTH) produced in the pituitary following the stress-evoked release of hypothalamic corticotropin-releasing hormone (CRH)[16, 24, 25, 94]. Acting via the negative biofeedback mechanism, CORT released to the circulation activates glucocorticoid receptors (GR) to inhibit the release of CRH and ACTH in the brain (thereby protecting zebrafish from pathological over-activation of the HPI axis). For example, acute stress evokes fast and robust CORT responses with similar time dynamics in both humans and zebrafish (bottom row); also note that zebrafish and humans both use CORT 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)* or Oscar fish *(Astronotus ocellatus*). Acute exposure of zebrafish to their natural predators (e.g., Leaf fish) induces overt anxiety-like behavior (tight shoaling and avoidance), accompanied by elevated whole-body CORT levels (**P<0.01 vs control)[6, 95]. Other stressors known to elevate CORT 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). Recent successful applications of optogenetics in zebrafish have enabled selective manipulations of the HPI axis and CORT signalling[41], further fostering stress physiology research in this model organism.


(A)
Fertilization
Hatching
Larvae
Juvenile
Adult fish
Aging
Death
0
3 dpf
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90 dpf
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Embryonic development
Free swimming CNS development
Neurodegeneration
Cognitive decline
Motor deficits
Larval zebrafish
Adult zebrafish
(B)
C. elegans
Fruit fly
Dog
Zebrafish
Dog
Mouse
Rat
Zebrafish
Rat
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15
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Dog
Mouse
Rat
Zebrafish
Fruit fly
C. elegans
Phylogenetic tree of major model species
Number of neurons
Brain weight
Genetic homology
100 B
1400 g
100%
85 B
200-600 g
95-98%
1 B
100 g
85-90%
70-80 M
2 g
75-80%
70-80 M
0.4 g
75-80%
1 M
0.05 g
70%
100 K
-
60%
302
-
40%

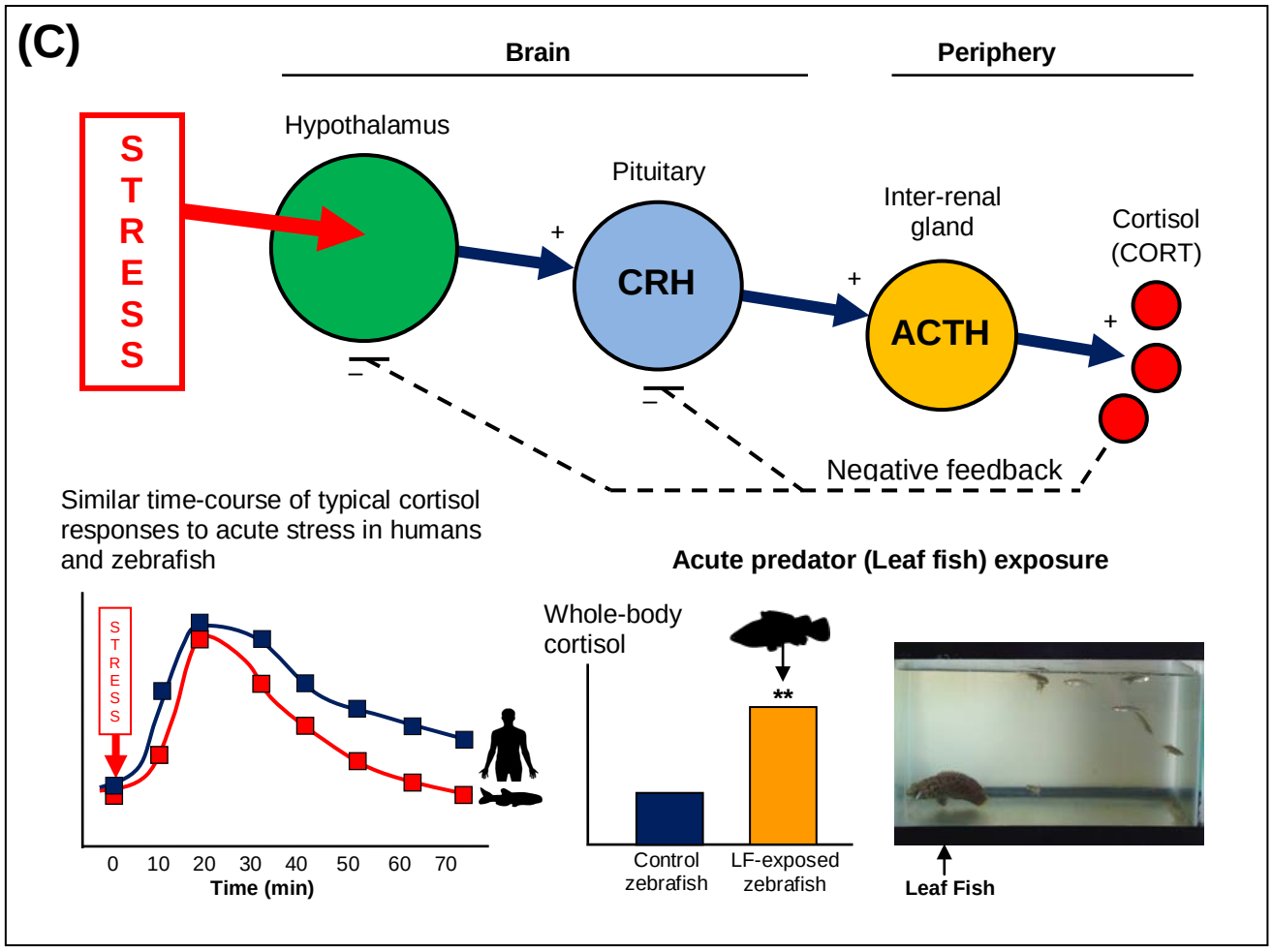

(C)
Brain
Periphery
STRESS
Hypothalamus
Pituitary
CRH
Inter-renal gland
ACTH
Cortisol (CORT)
+
+
+
–
–
Negative feedback
Similar time-course of typical cortisol responses to acute stress in humans and zebrafish
STRESS
0 10 20 30 40 50 60 70
Time (min)
Acute predator (Leaf fish) exposure
Whole-body cortisol
**
Control zebrafish
LF-exposed zebrafish
Leaf Fish

Figure 2. Visualizing zebrafish brain using various imaging techniques in adult zebrafish.

Panel (A) shows a three-dimensionally reconstructed zebrafish brain using two different types of magnetic resonance (MR), the MR histology (right) and 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[96].

Panel (B) shows a whole-mounted zebrafish brain labeled with anti-synaptic vesicle protein 2 and anti-keyhole limpet haemocyanin antibodies[31]. 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: the 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 (Braubach, 2014 Zebrafish ZB2N Case Study report).

Panel (D) shows 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): Histologic 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: Histologic appearance of melanoma of zebrafish eye (note poorly differentiated invasive melanocytes in melanoma; Spitsbergen, 2014 Zebrafish ZB2N Case Study report).

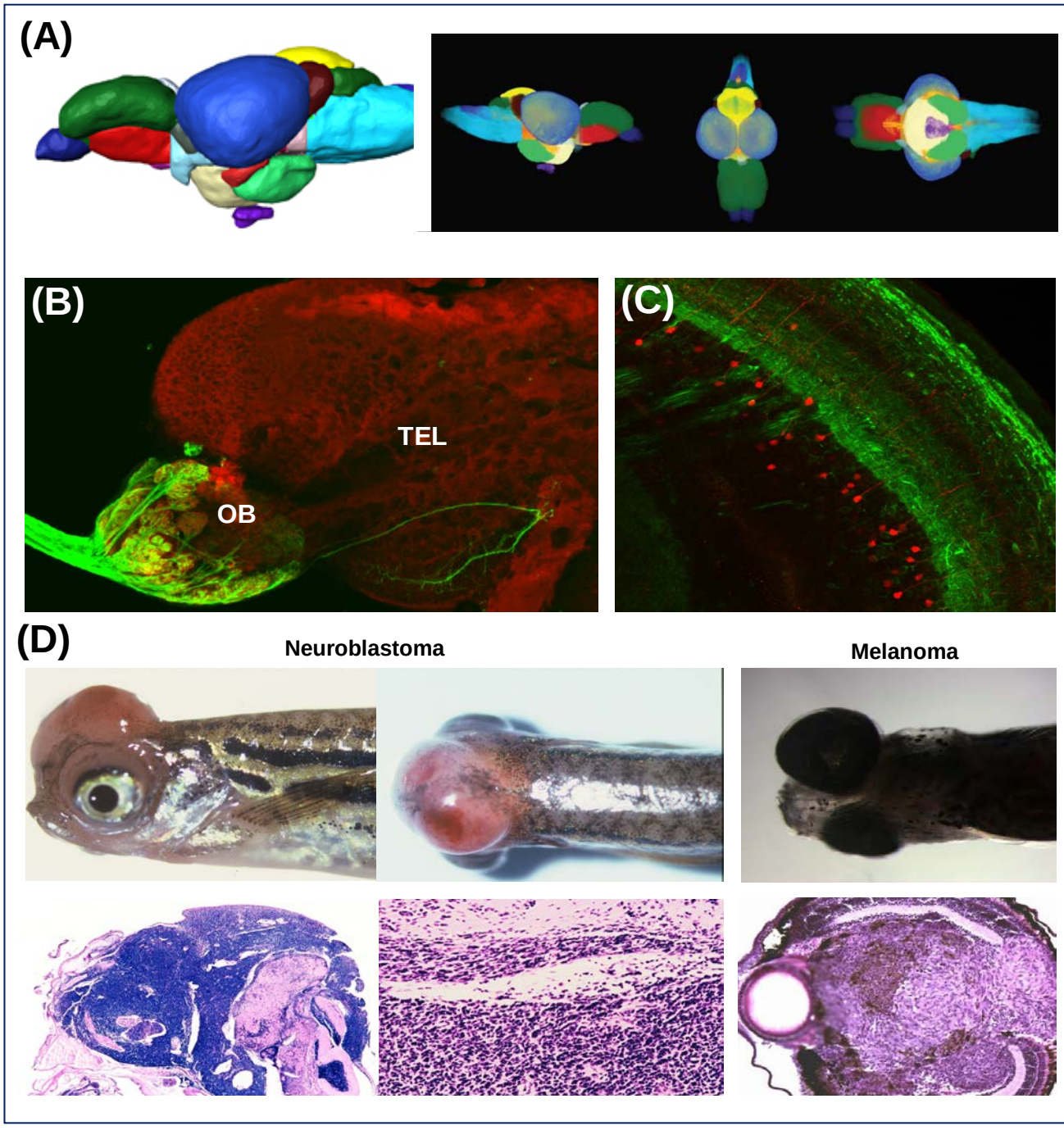

(A)
(B)
TEL
OB
(C)
(D)
Neuroblastoma
Melanoma

Figure 3. Zebrafish models and phenotypes related to epilepsy.

Panel (A) illustrates the place of epilepsy (as a neurological disorder) among other groups of CNS disorders discussed here.

Panel (B) shows a larval zebrafish embedded in agarose and paralyzed using a myorelaxant (e.g., D-tubocurarine), with electroencephalograpic (EEG) electrode inserted into the brain areas, such as tectum (invasive EEG) or placed on the scull (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 can evoked chemically (e.g., by exposure to various convulsant drugs, such as 10-15 mM pentylenetetrazole, PTZ, a blocker of the Cl-ionophore at the inhibitory gamma-aminobutyric acid GABA-A receptors). Note characteristic circling and corkscrew swimming, hyperlocomotion and elevated *c-fos* expression following pre-treatment with PTZ, *** $P < 0.001$ vs. control[3].

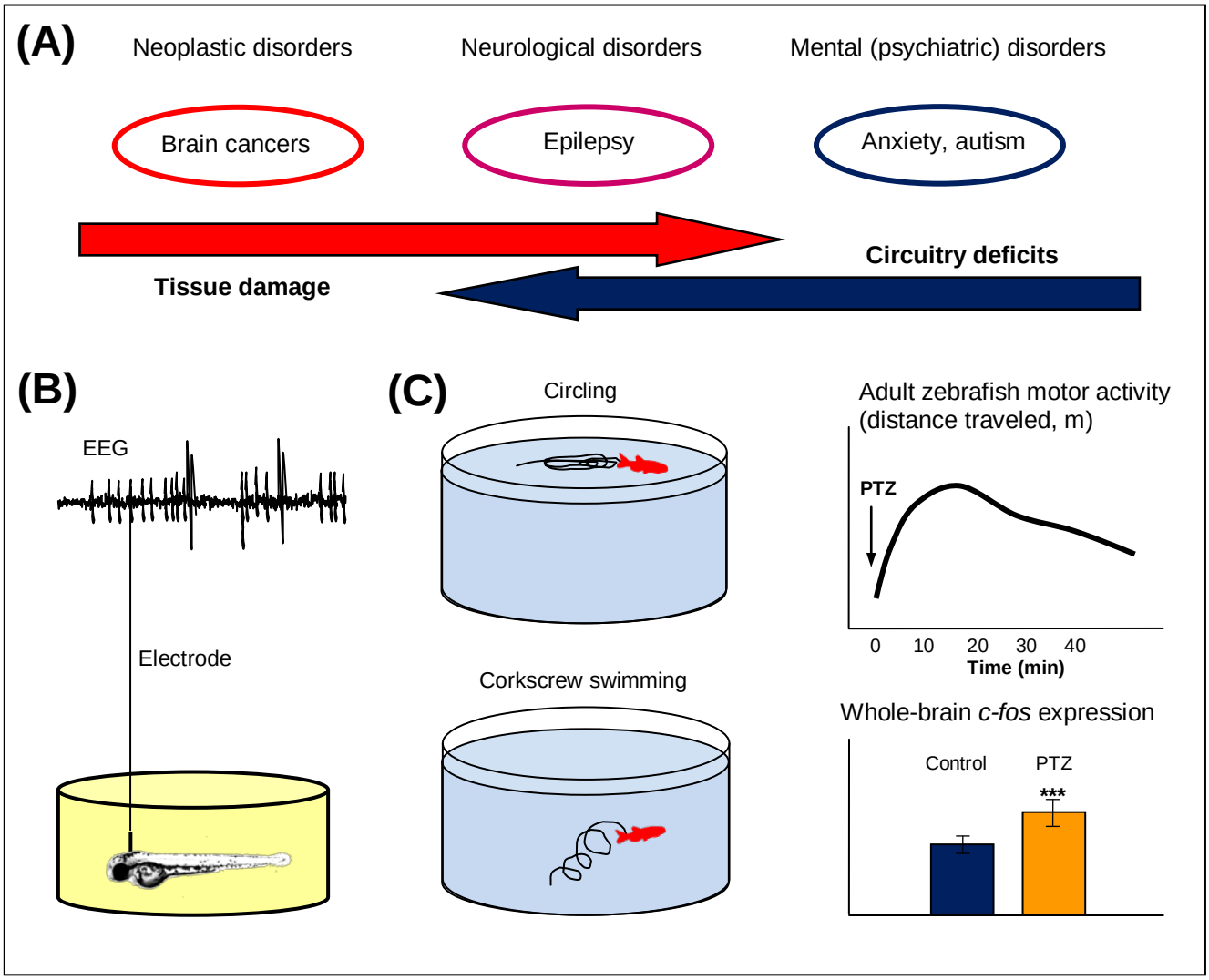

(A)
Neoplastic disorders
Neurological disorders
Mental (psychiatric) disorders
Brain cancers
Epilepsy
Anxiety, autism
Tissue damage
Circuitry deficits
(B)
EEG
Electrode
(C)
Circling
Corkscrew swimming
Adult zebrafish motor activity
(distance traveled, m)
PTZ
0
10
20
30
40
Time (min)
Whole-brain c-fos expression
Control
PTZ
***

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 vs. 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 an 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, 95]. Note distinct swimming patterns (top row), generated by a video-tracking software for untreated control (left) and experimental (right) fish treated with an 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, 95] and Table 2 for details). Consistent with this anxiolytic profile, experimental fish show significantly lower levels of cortisol [6, 95] (* $P < 0.05$ 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, 52] 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 [52] details (note that zebrafish swim in 3D (XYZ) coordinates, unlike rodent tests, where animals typically display horizontal locomotion on 2D surfaces). Right panel: a 2-camera setup which allows 3D neurophenotyping of zebrafish locomotion in XYZ coordinates (images from Noldus IT, Netherlands in collaboration with the Kalueff laboratory). The 3D neurophenotyping approach reveals robust phenotypic differences between traces in control vs. 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 a typical psychostimulant/anxiolytic action of nicotine, paralleling its profile in various other model organisms (e.g., rodents).

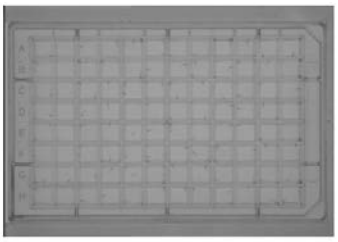
Panel (C) shows the predator exposure paradigm (see Table 2 for details) in which zebrafish tank is exposed to a nearby tank containing a predator fish (e.g., Leaf fish). As control fish (separated from the predator tank by a non-transparent plastic divider, denoted by the arrow) display low anxiety 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 zebrafish group, including bottom dwelling, unusually tight shoaling and avoidance of 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, 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 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

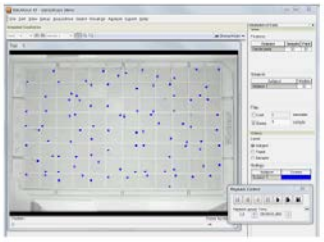
tracing 3 points – nose, center of body mass and 'tail', top view) can be used for automated decoding zebrafish social behaviors (photos by Noldus IT). Such automated tests are an invaluable tool to study zebrafish social behavior and its deficits. Typical computer-generated endpoints may include orientation (angle) towards the object, distance between selected body points and body curve patterns, which may be specific for various treatments. For example, two zebrafish (#1 and #2) that show proximity of their nose points, 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). In 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 social deficits typically observed in patients with autism (right; images: www.lovetoknow.com), paralleling zebrafish social phenotypes detected by IT-based tools.

(A)

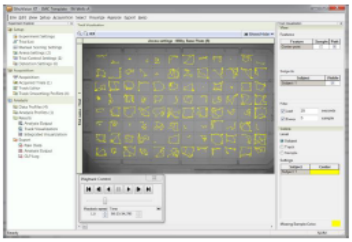
High-throughput 96-well screen for locomotor phenotyping in larval zebrafish



General top view



Object detection (dots)



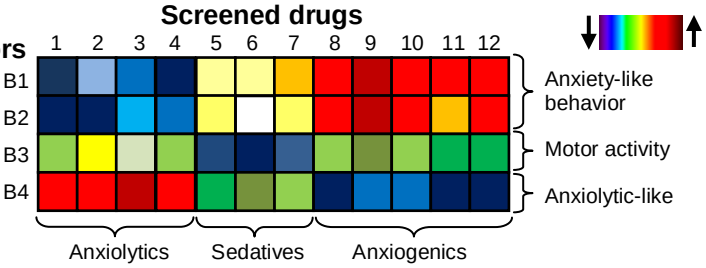
Locomotion tracking (traces)

Number of compounds tested

Number of behaviors assessed

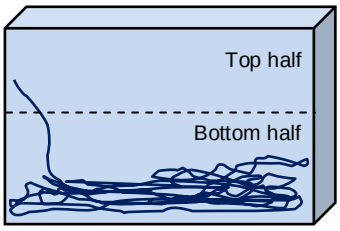
Screened drugs

Behaviors

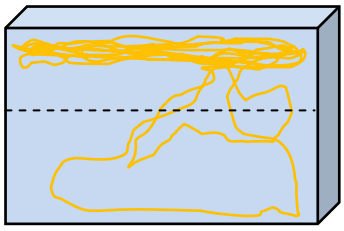


(B)

Novel tank test (traces recorded by the side-view camera)

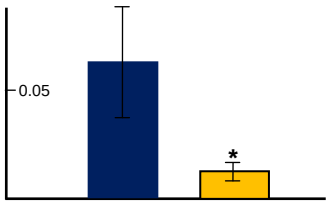


Control fish

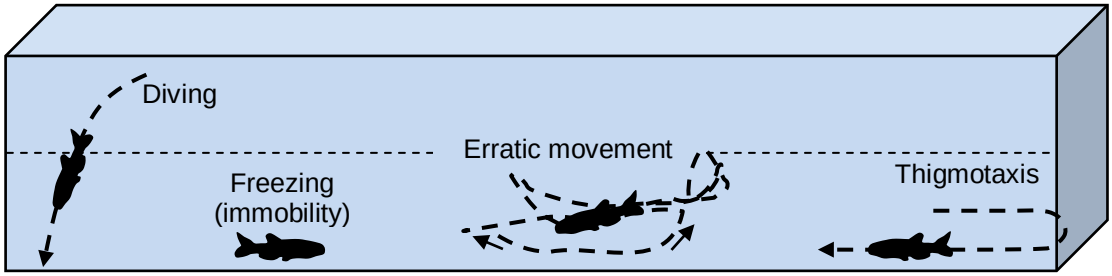


Fluoxetine-treated fish

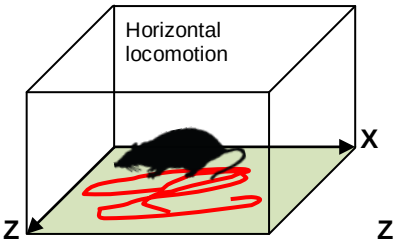
Whole-body cortisol (ng/g)



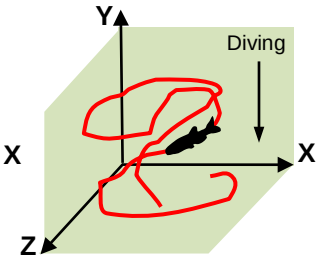
Typical zebrafish behaviors in the novel tank test

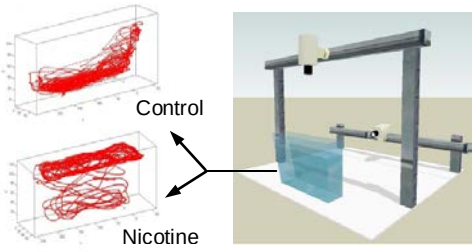


Increased dimensionality of motor neurophenotyping: from 2D in rodents to 3D in fish



Rodent (2D) 'open field' test





3D nature of zebrafish swimming and 3D-based videotracking

(C)

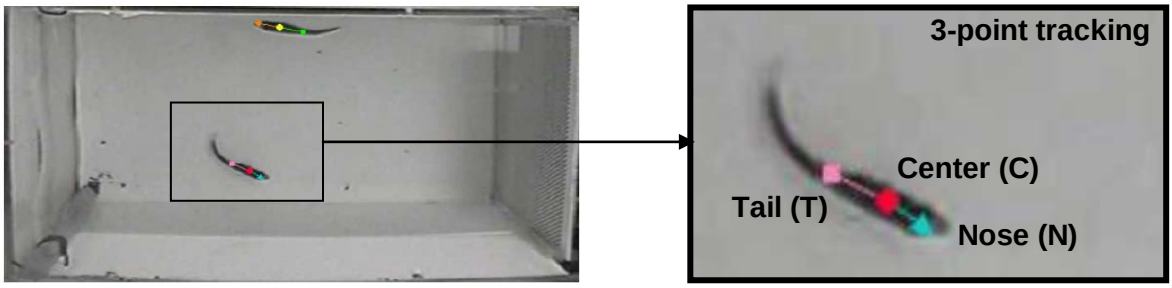
Low-stress control fish (divider present) | Predator (Leaf Fish) home tank | Stressed 'exposed' fish (divider removed)

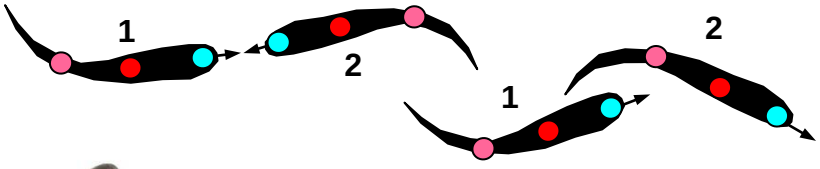
(D)

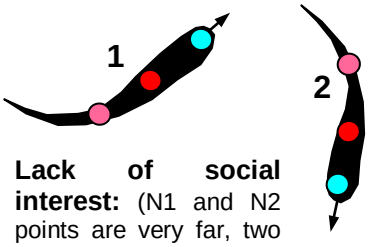
Social behaviors

Shoaling

Automated behavior recognition







Social investigation: 'approach' (N1 and N2 are very close, heading head-to-head towards each other)

Normal children

Social investigation: 'follow/chase' (N1 and T2 are very close, heading nose-to-tail in the same direction)

Lack of social interest: (N1 and N2 points are very far, two fish heading in different directions)

Autistic children

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 following pro-excitatory pharmacological modulation (e.g., by convulsant agents, Figure 3, or by selected psychostimulants and hallucinogens[27, 50, 97]). *C-fos* expression can also serve as a marker of neuronal activation, and 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 up-regulated 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[60] 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, see Glossary).

Endocrine stress responses: zebrafish possess a well-developed neuroendocrine system, generally highly homologous to that in mammals[7, 98]. The zebrafish stress neuroendocrine (hypothalamo-pituitary-interrenal, HPI) axis is similar to the human and rodent hypothalamo-pituitary-adrenal (HPA) axis, and releases cortisol (CORT) following the stress exposure (Figure 1C). Zebrafish CORT responses generally correlate with behavioral indices of stress, and may be modulated experimentally (e.g., genetically or pharmacologically, Table 1)[24, 25, 95, 99]. Methods to assess CORT in zebrafish (including both adult[95] and larval[100] fish) apply ELISA or radio-ligand binding assays using whole-body, blood samples and/or urine-containing water samples.

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 upon 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 labor intensive 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 nucleases (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].

Outstanding Questions

This section highlights selected outstanding questions and problems associated with the application of zebrafish models in neuroscience research.

Applying optimal test batteries. Test batteries are series of specific behavioral paradigms, clustered by category/domain (e.g., cognitive, feeding, pain, social, anxiety, depression, psychoses, reward), and commonly used in neurobehavioral analyses, especially in rodents[66, 69, 101]. Test batteries depend on specific needs and research questions, but typically consist of well-characterized paradigms from the established neurobehavioral literature[69]. Combining individual tests in 'smart' ('hybrid') batteries has also been suggested to maximize the number of phenotypes assessed per trial[102]. 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[68]. In rodents, some (but not all) behavioral tests are sensitive to previous testing experience (test battery effect)[68]. Although approaches similar to rodent models 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. Albeit far less studied (as compared to rodents[66, 69, 101]), 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[103]. 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.

Understanding stress-related neuroendocrine responses. Zebrafish stress responses show striking similarity to human HPA axis, employing CORT as the major glucocorticoid hormone[41] (see Glossary and Figure 1C). Moreover, individual variation in zebrafish stress responses (similar to the coping styles in other vertebrate species) correlates strongly with basal CORT levels and its recovery over time[104]. Given the absence of aldosterone production in zebrafish, CORT may also function as a mineralocorticoid hormone, thereby acting physiologically at both glucocorticoid (GR) and mineralocorticoid (MR) receptors[105]. The exact contribution of these receptors in zebrafish phenotypes remains unclear[105], including their role in CNS-related responses. Although both GR and MR are implicated in medicating various zebrafish responses[16, 24, 25, 105], 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 Zebrafish Brain Atlas (www.zebrafishbrain.org). Zebrafish research will benefit markedly from developing further databases dedicated to CNS phenotypes and disease models using this and related (e.g., medaka) organisms. In addition, zebrafish neurobehavioral ontology currently does not exist, and needs to be developed as well, to be integrated into the existing animal behavioral ontologies[19].

Table 1. Selected advantages and limitations of zebrafish models for biomedical and translational neuroscience research (adapted from [13])

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 sequences genome
Ease of genetic manipulation, availability of a wide range of genetic tools for 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 manipulate it *in-vivo* - e.g., by injecting drugs or genes).
External development (zebrafish can be exposed to various environmental factors neonatally outside of maternal organism, in more experimentally controllable environment)
High space/cost-efficiency and excellent potential for high-throughput screens (HTS)
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 (can be better assessed using newest imaging techniques), see text and Figure 2 for details (also note transparent brains in larval zebrafish)
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 is available).

Species differences in blood-brain barrier (BBB). While zebrafish develop 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 (albeit 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[a]

Test	Zebrafish phenotypes	Relevance to		References
		clinical anxiety[b]	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, 106-111
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	73, 107, 112
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	113, 114
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])	?	115

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 attenuates these responses	SP, PD (PTSD[b])	Predator image test	[116]
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	[117]
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	[118, 119]
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	[59]
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	[53]
Other drug-related anxiety	Characteristic diving behavior, increased thigmotaxis, reduced exploration in novelty tests, increased erratic behavior and freezing, as well as elevated whole-body	GAD, SMA	Withdrawal-evoked anxiety	[6, 99]

(withdrawal)	cortisol and brain *c-fos* following single or repeated withdrawal from selected drugs (alcohol, benzodiazepines, barbiturates, opioids or psychostimulants)			
Genetic models of anxiety	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 to less anxious zebrafish strains, such as AB or Tu strains)	GAD	High-anxiety mouse and rat strains	7, 120
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
Optogenetic models	Optogenetically-mediated 'hypercortisolic' zebrafish, displaying stress-like cortisol and behavioral (hyperlocomotor) responses	GAD (PTSD[b])	Elevated cortisol and stress behavior	41
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	[121]
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	[94]

[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)[84]

[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[84], 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)[73]

[d] Similar anxiety-like responses can be elicited by the predator's image presentation on computer screen[20, 114]

[e] For example, highly 'anxious' serotonin transporter (SERT) knockout mice and rats[122]

Supplementary Table 1S. Common reasons why neuroscientists are cautious to work with zebrafish models (based on [13])

Considerations	Comments
Why zebrafish?	See Table 1 for discussion of multiple advantages of zebrafish models
Conservative nature of Science: Is it 'safe' to try a new model organism?	Science constantly evolves. We shall be creative, and think outside the box
Lack of understanding of physiological complexity: Do zebrafish have brains? Do they have mitochondria?	Zebrafish show a remarkable genetic and physiological homology to humans. Many systems are highly conserved, and many genes show >95% homology.
Lack of information: Can zebrafish feel pain? Do they have affective behaviors? Can you model anxiety or depression in fish?	Yes. Zebrafish pain-related and emotional behavior is currently recognized in the field[19]
Genome duplication concerns: Zebrafish have duplicated genome. The system is just too complicated	Zebrafish genome duplication affects some, but not all, genes. In most cases, the 'duplicated' copies encode very similar proteins (e.g., receptors or transporters) with similar/overlapping pharmacology and biochemistry.
Habits: My mentor worked with rodents, and the mentor of my mentor worked with rodents	Many discoveries were made using novel model organisms. Try a new field (plus reduce competition with your mentors, so they get their grants funded too).
Lack of husbandry skills: I do not know how to raise the fish. We do not have the fish facility.	It is generally quite easy; consult your vivarium staff. To start new projects, the laboratory only need a small room (if you start a new model organism, your university may be very supportive space-wise)
Methodological concerns: How can I even give drugs to fish?	Use the same delivery methods as with other model organisms: e.g., systemic (via immersion), intraperitoneally, subcutaneously, with food, or using intracerebroventricular injections.
Concerns about animal use and care committees (IACUCs): They will raise too many questions if I work with fish	IACUCs are becoming well-aware of the rapidly growing utility of zebrafish in biomedicine. While they sometimes may have 'interesting' views on zebrafish research, educate and work with IACUCs. Using a lower vertebrate adheres to the 3R principles
Funding concerns: NIH does not like zebrafish. My study section will reject our grant if I propose to use fish	NIH is very interested in zebrafish research. Recently, they built the worlds' biggest zebrafish facility in Bethesda (MD), and also have the trans-NIH zebrafish initiative. NIH is also getting more and more zebrafish grant applications each cycle, and now tries to include zebrafish experts in their panels. NIH study sections are also 'warming up' to zebrafish
Zebrafish field is too small, and may not be worth investing in it. Will I have job in this field?	The zebrafish field is rapidly growing (Figure 1B), and may become a perfect academic investment. Also, there is a growing number of companies developing and using zebrafish *in-vivo* HTS to discover new therapies
Fiscal concerns: Zebrafish may be too expensive for my lab	Many labs we know are switching to zebrafish to *save* money (e.g., zebrafish models are 500-1000 times cheaper than similar mammalian studies).
I do not understand fish behavior! Can I decode fish behavior well enough to model complex brain disorders?	Like with any other area of science, it takes time, as more models become developed, and zebrafish responses become clearer now than a decade ago (e.g., see [19]).
Are there enough zebrafish data resources?	There are multiple outstanding public-access biomedical databases available to zebrafish investigators (see Outstanding Questions)

Supplementary Table 2S. Zebrafish models relevant to human neuropsychiatric disorders (based on 'The Diagnostic and Statistical Manual of Mental Disorders' (DSM-5) of the American Psychiatric Association (APA, 2013)[84]. + Pertinent zebrafish models available (including models targeting selected, but not all symptoms); ? unclear, or no models available yet; n/a the specific disorder is not applicable to zebrafish (e.g., with symptoms including impaired reading, writing or speaking, which are impossible in zebrafish) (adapted from [13]).

Human neuropsychiatric disorders	**Availability of relevant models in:**	
	rodents	**zebrafish**
1. Neurodevelopmental Disorders		
Intellectual Disabilities		
Intellectual Disability (Intellectual Developmental Disorder)	+	+
Global Developmental Delay	+	+
Unspecified Intellectual Disability (Intellectual Developmental Disorder)	+	?
Communication Disorders		
Language Disorder	n/a	n/a
Speech Sound Disorder (previously Phonological Disorder)	n/a	n/a
Childhood-Onset Fluency Disorder (Stuttering)	n/a	n/a
Social (Pragmatic) Communication Disorder	?	n/a
Unspecified Communication Disorder	?	n/a
Autism Spectrum Disorders	+	+
Attention-Deficit/Hyperactivity Disorder (ADHD)		
Attention-Deficit/Hyperactivity Disorder (ADHD)	+	+
Other Specified and Unspecified Attention-Deficit/Hyperactivity Disorders	?	?
Specific Learning Disorder	n/a	n/a
Motor Disorders		
Developmental Coordination Disorder	+	+
Stereotypic Movement Disorder	+	+
Tourette's Disorder	+	?
Persistent (Chronic) Motor or Vocal Tic Disorder	+	?
Provisional Tic Disorder	+	?
Other Specified and Unspecified Tic Disorders	?	?
Other Neurodevelopmental Disorders		
Other Specified and Unspecified Neurodevelopmental Disorders	?	?
2. Schizophrenia Spectrum and Other Psychotic Disorders		
Psychoses		
Schizotypal (Personality) Disorder	?	n/a
Delusional Disorder	?	n/a
Brief Psychotic Disorder	+	+
Schizophreniform Disorder	+	?
Schizophrenia	+	+
Schizoaffective Disorder	+	?
Substance/Medication-Induced Psychotic Disorder	+	+
Psychotic Disorder Due to Another Medical Condition	+	+
Other Schizophrenia Spectrum and Other Psychotic Disorders	?	?
Catatonia		
Catatonia Associated With Another Mental Disorder (Catatonia Specifier)	+	?
Catatonic Disorder Due to Another Medical Condition	+	?
Unspecified Catatonia	?	?

3. Bipolar and Related Disorders		
Bipolar Disorders		
Bipolar I and II Disorders	+	+
Cyclothymic Disorder	?	?
Substance/Medication-Induced Bipolar and Related Disorder	+	+
Bipolar and Related Disorder Due to Another Medical Condition	+	+
Other Specified and Unspecified Bipolar and Related Disorders	?	?
4. Depressive Disorders		
Depressive Disorders		
Disruptive Mood Dysregulation Disorder	+	+
Major Depressive Disorder, Single and Recurrent Episodes	+	+
Persistent Depressive Disorder (Dysthymia)	+	+
Premenstrual Dysphoric Disorder	?	n/a
Substance/Medication-Induced Depressive Disorder	+	+
Depressive Disorder Due to Another Medical Condition	+	+
Other Specified and Unspecified Depressive Disorders	?	?
5. Anxiety Disorders		
Anxiety spectrum disorders		
Separation Anxiety Disorder	+	?
Selective Mutism	n/a	n/a
Specific Phobia	+	+
Social Anxiety Disorder (Social Phobia)	+	+
Panic Disorder	+	+
Panic Attack (Specifier)	+	?
Agoraphobia	+	?
Generalized Anxiety Disorder	+	+
Substance/Medication-Induced Anxiety Disorder	+	+
Anxiety Disorder Due to Another Medical Condition	+	+
Other Specified and Unspecified Anxiety Disorders	?	?
6. Obsessive-Compulsive and Related Disorders		
Obsessive-Compulsive Disorder (OCD)	+	?
Body Dysmorphic Disorder	n/a	n/a
Hoarding Disorder	+	n/a
Trichotillomania (Hair-Pulling Disorder)	+	n/a
Excoriation (Skin-Picking) Disorder	+	n/a
Substance/Medication-Induced OCD and Related Disorder	+	?
OCD and Related Disorder Due to Another Medical Condition	+	?
Other Specified and Unspecified OCD and Related Disorders	?	?
7. Trauma- and Stressor-Related Disorders		
Reactive Attachment Disorder	?	?
Disinhibited Social Engagement Disorder	?	?
Posttraumatic Stress Disorder (PTSD)	+	+
Acute Stress Disorder	+	+
Adjustment Disorders	?	?
Other Specified and Unspecified Trauma- and Stressor-Related Disorder	?	?
8. Dissociative Disorders		
Dissociative Identity Disorder	n/a	n/a
Dissociative Amnesia	?	?
Depersonalization/Derealization Disorder	n/a	n/a
Other Specified and Unspecified Dissociative Disorders	?	?
9. Somatic Symptom and Related Disorders		
Somatic Symptom Disorder	?	?
Illness Anxiety Disorder	n/a	n/a
Conversion Disorder (Functional Neurological Symptom Disorder)	?	?
Psychological Factors Affecting Other Medical Conditions	n/a	n/a
Factitious Disorder	?	?

Other Specified and Unspecified Somatic Symptom and Related Disorders	?	?
10. Feeding and Eating Disorders		
Pica	+	?
Rumination Disorder	?	?
Avoidant/Restrictive Food Intake Disorder	+	+
Anorexia Nervosa	+	+
Bulimia Nervosa	+	+
Binge-Eating Disorder	+	+
Other Specified and Unspecified Feeding or Eating Disorders	?	?
11. Elimination Disorders		
Enuresis	?	?
Encopresis	?	?
Other Specified and Unspecified Elimination Disorders	?	?
12. Sleep-Wake Disorders		
Insomnia Disorder	+	+
Hypersomnolence Disorder	+	+
Narcolepsy	+	+
Obstructive Sleep Apnea, Hypopnea	?	n/a
Central Sleep Apnea	?	n/a
Sleep-Related Hypoventilation	?	?
Circadian Rhythm Sleep-Wake Disorders	?	?
Non–REM Sleep Arousal Disorders (Sleepwalking, Sleep Terrors)	n/a	n/a
Nightmare Disorder	n/a	n/a
Rapid Eye Movement Sleep Behavior Disorder	?	?
Restless Legs Syndrome	?	n/a
Substance/Medication-Induced Sleep Disorder	+	+
Other Specified and Unspecified Insomnia Disorders	+	?
Other Specified and Unspecified Hypersomnolence Disorders	?	?
Other Specified and Unspecified Sleep-Wake Disorders	?	?
13. Sexual/Gender Dysfunctions		
Delayed Ejaculation	+	n/a
Erectile Disorder	+	n/a
Female Orgasmic Disorder	?	n/a
Female Sexual Interest/Arousal Disorder	?	?
Genito-Pelvic Pain/Penetration Disorder	?	n/a
Male Hypoactive Sexual Desire Disorder	?	?
Premature Ejaculation	?	n/a
Substance/Medication-Induced Sexual Dysfunction	+	+
Other Specified and Unspecified Sexual Dysfunction	?	?
Gender Dysphoria	n/a	n/a
14. Disruptive, Impulse-Control, and Conduct Disorders		
Oppositional Defiant Disorder	n/a	n/a
Intermittent Explosive Disorder	n/a	n/a
Conduct Disorder	n/a	n/a
Antisocial Personality Disorder	?	?
Pyromania	n/a	n/a
Kleptomania	n/a	n/a
Other Disruptive, Impulse-Control and Conduct Disorders	?	?
15. Substance-Related and Addictive Disorders		
<u>Substance-Related Disorders</u>		
Alcohol Use Disorder, Intoxication, Withdrawal	+	+
Other Alcohol-Induced and Related Disorders	+	+
Caffeine Intoxication, Withdrawal, Caffeine-Induced and Related Disorders	+	+
Cannabis Use Disorder, Intoxication, Withdrawal	+	+
Other Cannabis-Induced and Related Disorders	+	+
Hallucinogen Use Disorders, Intoxication	+	+

Hallucinogen Persisting Perception Disorder	?	?
Other Hallucinogen-Induced and Related Disorder	+	+
Inhalant Use Disorder, Intoxication, Inhalant-Induced and Related Disorder	+	+
Opioid Use Disorder, Intoxication, Withdrawal,	+	+
Other Opioid-Induced and Related Disorder	+	+
Sedative, Hypnotic or Anxiolytic Use Disorder, Intoxication, Withdrawal	+	+
Other Sedative-, Hypnotic- or Anxiolytic-Induced and Related Disorders	+	+
Stimulant Use Disorder, Intoxication, Withdrawal	+	+
Other Stimulant-Induced and Related Disorders	+	+
Tobacco Use Disorder, Withdrawal	+	+
Other Tobacco-Induced and Related Disorder	+	+
Other (or Unknown) Substance Use, Intoxication, Withdrawal	+	+
Other (or Unknown) Substance–Induced and Related Disorders	+	+
Non-Substance-Related Disorders: Gambling Disorder	n/a	n/a
16. Neurocognitive Disorders		
Delirium (including Other Specified Delirium and Unspecified Delirium)	?	?
Major and Mild Neurocognitive Disorders		
Major and Mild Neurocognitive Disorders	+	+
Major or Mild Neurocognitive Disorder Due to Alzheimer's Disease	+	+
Major or Mild Frontotemporal Neurocognitive Disorder	?	?
Major or Mild Neurocognitive Disorder With Lewy Bodies	?	?
Major or Mild Vascular Neurocognitive Disorder	+	+
Major or Mild Neurocognitive Disorder Due to Traumatic Brain Injury	+	+
Substance/Medication-Induced Major or Mild Neurocognitive Disorder	+	+
Major or Mild Neurocognitive Disorder Due to HIV Infection	?	n/a
Major or Mild Neurocognitive Disorder Due to Prion Disease	?	?
Major or Mild Neurocognitive Disorder Due to Parkinson's Disease	+	+
Major or Mild Neurocognitive Disorder Due to Huntington's Disease	+	+
Major/Mild Neurocognitive Disorder Due to Another Medical Condition	+	+
Major or Mild Neurocognitive Disorder Due to Multiple Etiologies	+	+
Unspecified Neurocognitive Disorder	?	?
17. Personality Disorders		
General Personality Disorder		
Cluster A Personality Disorders: Paranoid Personality Disorder, Schizoid Personality Disorder, Schizotypal Personality Disorder	n/a	n/a
Cluster B Personality Disorders: Antisocial Personality Disorder, Borderline Personality Disorder, Histrionic and Narcissistic Personality Disorders	n/a	n/a
Cluster C Personality Disorders: Avoidant Personality, Dependent Personality and Obsessive-Compulsive Personality Disorders	n/a	n/a
Other Personality Disorders		
Personality Change Due to Another Medical Condition	n/a	n/a
Other Specified and Unspecified Personality Disorders	n/a	n/a
18. Paraphilic Disorders		
Voyeuristic Disorder	n/a	n/a
Exhibitionistic Disorder	n/a	n/a
Frotteuristic Disorder	n/a	n/a
Sexual Masochism Disorder	n/a	n/a
Sexual Sadism Disorder	n/a	n/a
Pedophilic Disorder	n/a	n/a
Fetishistic Disorder	n/a	n/a
Transvestic Disorder	n/a	n/a
Other Specified and Unspecified Paraphilic Disorders	n/a	n/a
19. Other Mental Disorders		
Other Specified Mental Disorder Due to Another Medical Condition	?	?
Unspecified Mental Disorder Due to Another Medical Condition	?	?
Other Specified Mental Disorder	?	?
Unspecified Mental Disorder	?	?

Medication-Induced Movement Disorders and Other Adverse Effects	+	+
20. Other Conditions That May Be a Focus of Clinical Attention		
Attenuated Psychosis Syndrome	+	+
Depressive Episodes With Short-Duration Hypomania	+	?
Persistent Complex Bereavement Disorder	n/a	n/a
Caffeine Use Disorder	?	?
Neurobehavioral Disorder Associated With Prenatal Alcohol Exposure	+	+
Suicidal Behavior Disorder	n/a	n/a
Non-suicidal Self-Injury	?	n/a

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