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The Developing Utility of Zebrafish in Modeling Neurobehavioral Disorders

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The zebrafish (*Danio rerio*) is becoming increasingly popular in the field of neurobehavioral research, including experimental, genetic, and pharmacological models of human brain disorders. While zebrafish research is rapidly expanding, its application as a translational neurobehavioral model is still in its relative infancy. Therefore, further investigation of new models is needed for targeting more domains and new, more complex brain disorders. The main aim of this paper is to discuss recent developments in the field of zebrafish neurobehavioral research, and to outline important emerging topics for further studies.

The zebrafish (*Danio rerio*) is a promising model organism in neurobehavioral and biological psychiatry research. The robustness of zebrafish phenotypes makes this species an excellent animal for studying experimental, genetic, and pharmacological models of neurobehavioral disorders. As fish represent perhaps the dawn of the evolution of vertebrates' emotional behavior, the main aim of this paper is to outline recent developments in the field of zebrafish neurobehavioral research, and to summarize the emerging important new topics for further studies in this field. Another aim of this paper is to discuss what can be done to further improve and promote zebrafish neurobehavioral research.

Exploratory-Based Models

Zebrafish behavioral assays are currently used for high-throughput phenotyping and testing of various psychotropic drugs (Blaser & Gerlai, 2006; Levin, Bencan, & Cerutti, 2007; Lopez-Patino, Yu, Cabral, & Zhdanova, 2008), Fig. 1. A popular method of behavioral analysis in zebrafish research is the *novel tank test* (Fig. 2a), conceptually similar to the open field test used for rodents, which exhibit anxiety-like behavior by staying close to the walls (thigmotaxis), but increase exploration as they become acclimated to the new environment (Choleris, Thomasb, Kavaliersa, & Prat, 2001). Similarly, exposure to a novel environment evokes a robust anxiety response in zebrasfish (Blaser & Gerlai, 2006), as they dive to the bottom (geotaxis) until they feel safe to swim in the upper regions of

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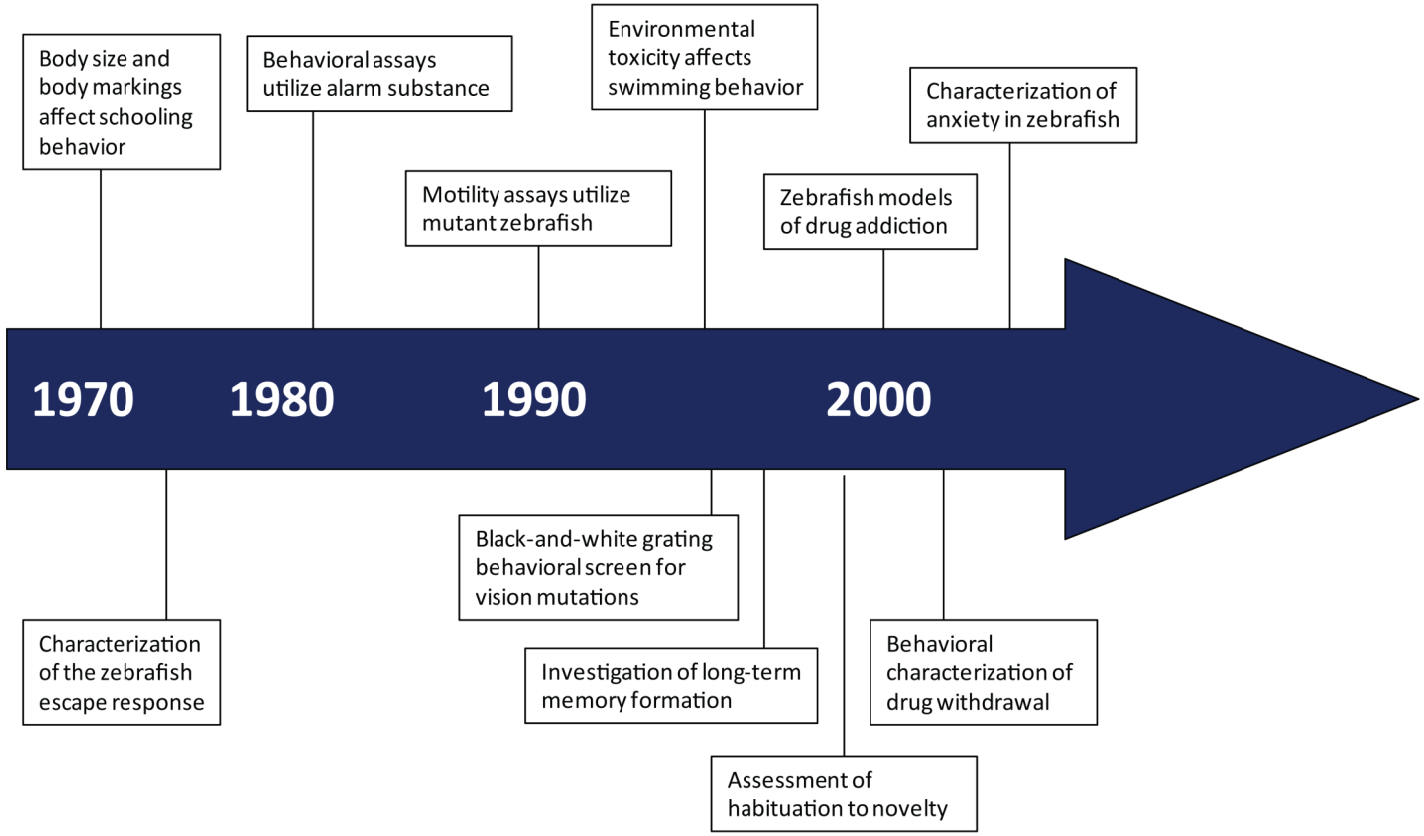
the tank. Typical endpoints in this test include the latency to enter the top, the number of transitions to the top, time spent in top, top:bottom time ratio, the number of fear/escape-like erratic movements, as well as freezing frequency and duration (Cachat et al., 2009; Levin et al., 2007; Wong et al., 2009).

Until recently, quantification of zebrafish behavior was primarily performed manually, making it vulnerable to human error and incorrect data interpretation. However, automated video-tracking technologies are becoming widely used to analyze animal behavior, providing a standardized unbiased observation of behavioral endpoints (Egan et al., 2009a). Another advantage of using the video-tracking approach is the ability to store, replay, and reanalyze videos. Finally, video-tracking tools can calculate additional behavioral endpoints that are not available through manual observation, such as distance traveled in top/bottom, velocity, meandering and angular velocity. Comparisons of data produced by the video-tracking system with that recorded manually show a high correlation between the two (Egan et al., 2009a; Gerlai, 2005), confirming the video-tracking approach as a reliable method of analysis in zebrafish neurobehavioral research.

The light/dark box is traditionally used in rodent behavioral neuroscience, and is based on the innate aversion to open illuminated areas (scotophilia, scototaxis) (Bourin & Hascoet, 2003). Previous research has shown that while anxiolytic compounds can facilitate exploratory activity (i.e. increased entries and duration in the light part), anxiogenic drugs cause the opposite effect (Bourin & Hascoet, 2003). Importantly, this test is now being applied to zebrafish, in which they exhibit a natural preference for the dark side (Serra, Medalha, & Mattioli, 1999) (Fig. 2b). Several different modifications exist for the fish light-dark box test (e.g., Blaser, Chadwick, & McGinnis, 2010; Serra et al., 1999), consistently demonstrating the utility of light-dark situation to model zebrafish anxiety. Our own observations also support this notion, showing that "more aversive" light behaviors in zebrafish may be modulated by anxiogenic and anxiolytic drugs, strikingly paralleling the mouse light-dark behaviors.

The open field test, another apparatus traditionally used in experimental biopsychology in rodents (Carola, D'Olimpio, Brunamonti, Mangia, & Renzi, 2002; Choleris et al., 2001; Koplik, Salieva, & Gorbunova, 1995; Walsh & Cummins, 1976), also offers a promising new area of research in zebrafish. For example, some studies have applied the open field test to larval models (Lockwood, Bjerke, Kobayashi, & Guo, 2004). The utility of the open field test for adult zebrafish research also seems very logical. As in mice, zebrafish exhibit a natural tendency to stay close to walls of the apparatus, especially the corners. As they habituate to the novel arena, zebrafish predictably stray into the open central area, showing increased exploration (Fig. 2c).

Overall, this brief summary of zebrafish exploration-based paradigms leads to several important observations. First, zebrafish exploration appears to be driven by the same, evolutionarily conserved factors as rodent behavior, which is much better studied and understood. These factors include the balance between exploration (novelty-seeking, curiosity) and avoidance of aversive stimuli


Body size and body markings affect schooling behavior
Behavioral assays utilize alarm substance
Environmental toxicity affects swimming behavior
Characterization of anxiety in zebrafish
Motility assays utilize mutant zebrafish
Zebrafish models of drug addiction
1970
1980
1990
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Characterization of the zebrafish escape response
Black-and-white grating behavioral screen for vision mutations
Investigation of long-term memory formation
Assessment of habituation to novelty
Behavioral characterization of drug withdrawal

Throughout

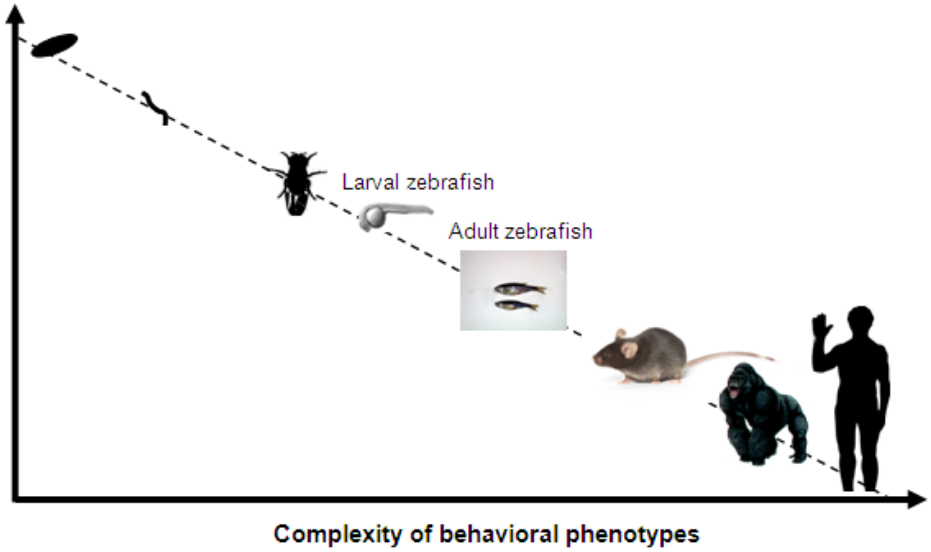

Figure 1. The developing utility of zebrafish models in behavioral neuroscience research (other models popular in biomedical research include *E. coli*, *C. Elegans*, *Drosophila*, rodents, primates and humans).

(thigmotaxis, scototaxis), thereby reconfirming the use of zebrafish in experimental and comparative biopsychology research. Finally, unlike rodent models, zebrafish behavior is 3-dimensional, and includes an additional *vertical* dimension (geotaxic top-bottom behavior), thereby introducing a novel aspect to their exploration-based phenotypes.

Zebrafish habituation behavior

Relevant to both exploration and emotionality, habituation is the simplest form of learning, and has long been used to examine animal cognitive phenotypes (Bolivar, 2009; Salomons, van Luijk, Reinders, Kirchhof, Arndt, & Ohl 2009). Habituation to novelty represents attenuation of innate behaviors, as subjects become accustomed to the environment (Leussis & Bolivar, 2006; Thompson & Spencer, 1966). Intra-session habituation reflects spatial working memory, whereas inter-session habituation is commonly used to assess middle- and long-term spatial memory (Muller et al., 1994). Despite being widely studied in various rodents (Bolivar, 2009; File & Mabbutt, 1990; Leussis & Bolivar, 2006; Ohl, Roedel, Storch, Holsboer, & Landgraf, 2002; Platel & Porsolt, 1982; Thompson & Spencer, 1966), habituation has not been extensively evaluated in zebrafish until recently (Best et al., 2008; Egan et al., 2009b; Gerlai, 2003; Goldsmith, 2004; Leimer et al., 1999; Shin & Fishman, 2002; Zon & Peterson, 2005).

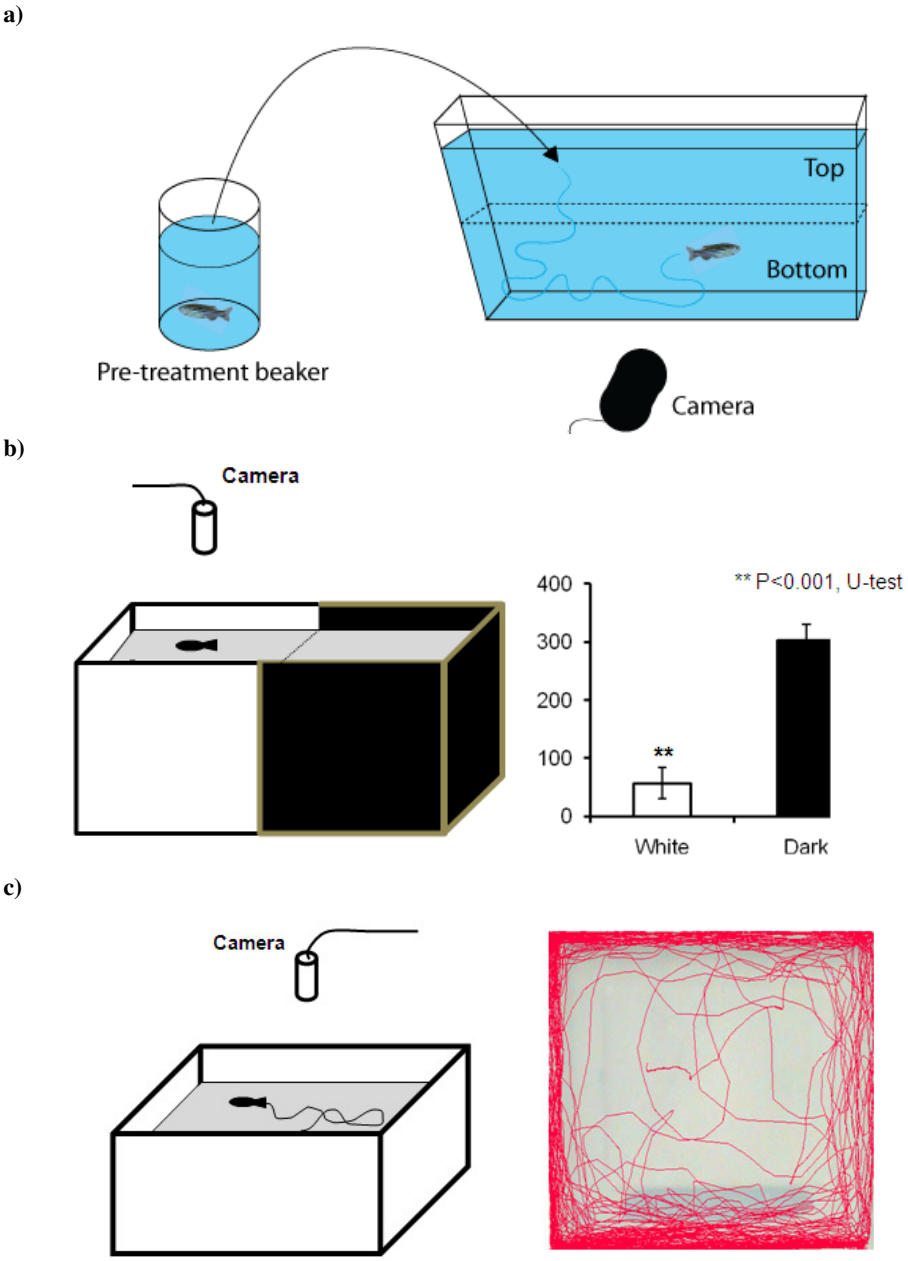

Figure 2. Experimental paradigms to study zebrafish behavior. a) The novel tank test examines novelty-evoked anxiety. When a zebrafish is exposed to a novel (potentially dangerous) environment, it initially dives to the bottom, and then gradually explores the top. Inhibited exploration, reduced speed, and increased frequency of escape-like erratic behaviors are usually associated with higher levels of anxiety elicited by different stressors. b) Normal light-dark preference in adult zebrafish (*n* = 15) tested in a 6-min light-dark box test (data obtained from manual registration of video-recorded behaviors). c) Open field thigmotaxic behavior in representative adult zebrafish tested for 30 min (top view; behavioral traces are generated using Noldus Ethovision XT7 video-tracking software).

Until recently, fish behavior was generally assumed to be instinctively-driven, with little cognitive ability (rev. in Burt de Perera, 2004). However, it is currently known that fish are capable of forming spatial memories and cognitive maps (Burt de Perera, 2004; Riedel, 1998), providing an opportunity to explore their habituation behaviors in depth. Several recent studies suggest that zebrafish can habituate to various stimuli, including conditioned place preference (Kily et al., 2008; Ninkovic & Bally-Cuif, 2006), light/dark locomotion (MacPhail et al., 2009) and startle reflex (Eddins, Cerutti, Williams, Linney, & Levin, 2009; Levin et al., 2009) testing.

Experiments undertaken in our laboratory have comprehensively characterized zebrafish habituation to novelty (Wong et al., 2009). Using short 6-min novel tank trials, we found significant increases in exploratory behavior and decreases in freezing behavior over time. We have found that during 30-min intra-session habituation trials, the zebrafish exhibited a steady increase over time in transitions to the top of the novel tank, time spent in the top, as well as a marked decrease in freezing scores, but not in erratic movements (Wong et al., 2009). Finally, by analyzing inter-session habituation (Fig. 3), with each successive day, we found significantly increased transitions to the top, a similar trend for time spent there, as well as reduced freezing behaviors. Collectively, these findings confirm robust habituation phenotypes in zebrafish (similar to that observed in various rodent models) and emphasize the utility of zebrafish to study both cognitive and emotional behaviors.

Other behavioral models

Fostering high-throughput translational paradigms in zebrafish, one area that is rapidly developing is the neurobiology of the acoustic startle reflex (ASR). In humans, ASR assesses hearing sensitivity as well as the intact activity of multiple neuronal circuits (Musiek, 2003). The startle response is evolutionarily advantageous (because it provides a form of protection from dangers in the natural habitat), and its endpoints are thought to reflect sensitization, habituation, and pre-pulse inhibition (PPI) (Koch, 1999). In humans, lowered PPI has been reported in patients with neurological damage, schizophrenia or Huntington's disease (Musiek, 2003). For example, Burgess and Granato (Burgess & Granato, 2007) have shown that PPI does modulate ASR in zebrafish just as in higher vertebrate, such as mice, also reporting that reduced PPI (modulated by dopamine agonists) can be counteracted by antipsychotic drugs (Rigdon & Weatherspoon, 1992).

Olfaction plays a key role in zebrafish behavioral responses. In line with this, recent studies (Braubach, Wood, Gadbois, Fine, & Croll, 2009) have characterized zebrafish olfactory behaviors and their modifications through learning. Exposing zebrafish to L-alanine and L-valine (two amino acids that induce appetitive behavior), the researchers measured zebrafish appetitive behavior by counting >90º turns in a circular flow-through tank. These experiments paired the amino acids (unconditioned stimulus) with the conditioned stimulus

(phenylethyl alcohol), thereby eliciting a conditioned response to a neutral stimulus by pairing it with an olfactory cue (Braubach et al., 2009).

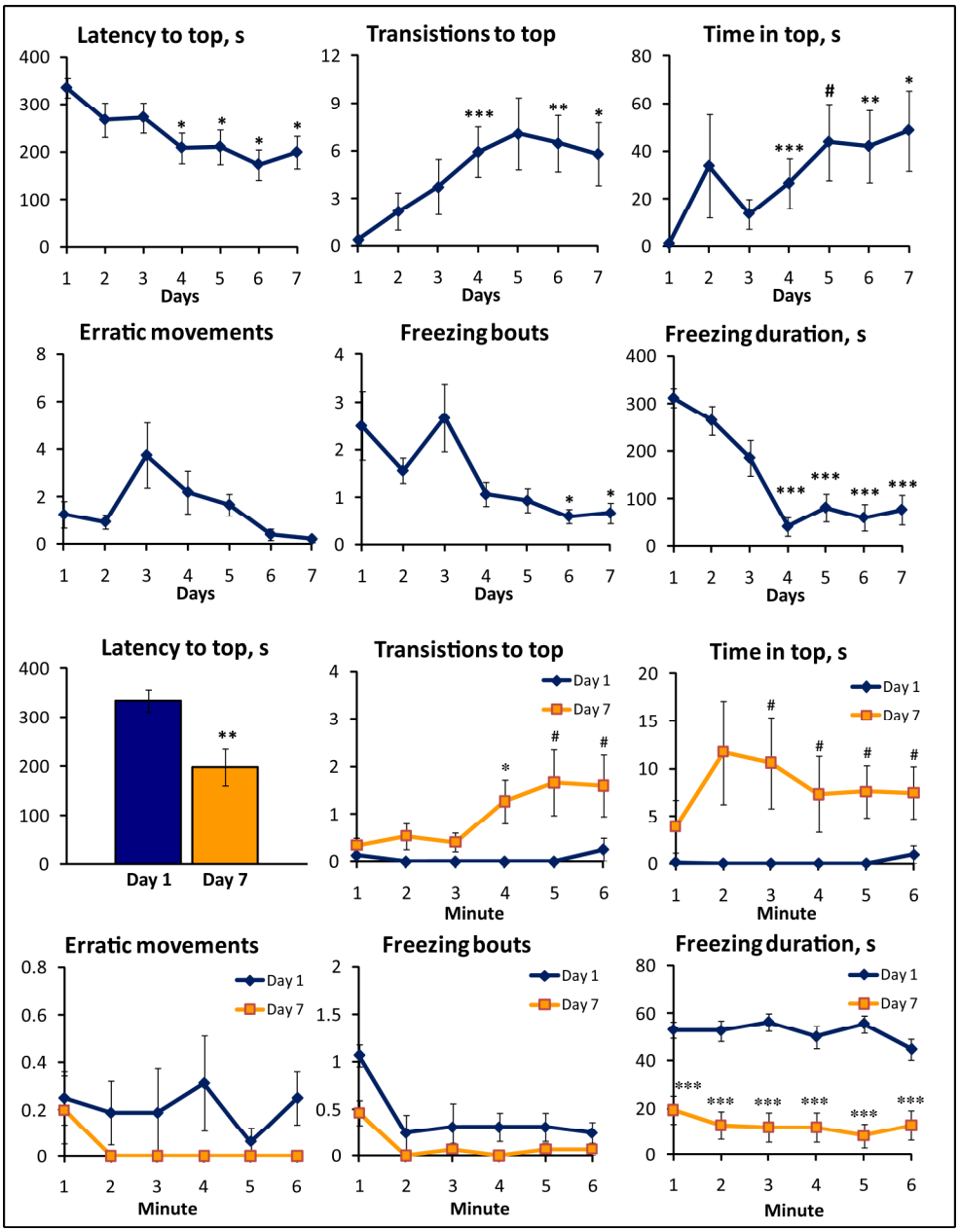

Figure 3. Habituation responses in zebrafish tested daily in the 6-min novel tank for 7 days (n =23), adapted from (Wong et al., 2009); *$p < 0.05$, **$p < 0.01$, *** $p < 0.005$, #p = 0.05-0.1, trend (U-test with Bonferroni correction, where applicable, vs. Day 1 of the test) for significant ANOVA data).

Finally, social behaviors are commonly seen in zebrafish, raising the possibility of modeling social behavior in these animals. In rodents, social defeat stress has been shown to induce anxiety and depression in the "loser" animals (Becker, Zeau, Rivat, Blugeot, Hamon, & Benoliel, 2008; Koolhaas, De Boer, De Rutter, Meerlo, & Sgoifo, 1997). While most research in social defeat has been focused on higher organisms (Bjorkqvist, 2001; Koolhaas et al., 1997), zebrafish are also capable of establishing dominant-subordinate relationships and exhibiting agonistic behavior (Larson, O'Malley, & Melloni, 2006), opening an interesting new avenue for further study. For example, it may be possible that with repeated aggression (induced through many ongoing social defeat trials), some fish would emerge as persistent "losers", leading to chronic social defeat similar to that observed in rodents.

Current Approaches and Methodological Considerations

Larval vs. adult zebrafish research

Larval zebrafish have emerged as a popular model for a number of brain pathologies. Larvae display learning, sleep, drug addiction, and other quantifiable neurobehavioral phenotypes (Best & Alderton, 2008). Another advantage of using zebrafish larva is the ability to study multiple animals simultaneously within a high-throughput battery (Best & Alderton, 2008; Best et al., 2008; Creton, 2009). However, such models have some limitations, since they do not exhibit the rich behavior of the adult animals (e.g., Creton, 2009, Fig. 1). Also, larval models have somewhat limited developmental applications, for example, lacking fully established mediatory and endocrine systems (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995), as well as some neural circuits and projections (Kastenhuber, Kratochwil, Ryu, Schweitzer, & Driever, 2010). Likewise, behavioral endpoints observed in larval animals may not be fully translated (or have good homology) to adult subjects' behavior. Thus, larval research is unable to fully replace the adult zebrafish studies. This notion is important, since zebrafish neuroscientists sometimes remind us of Montekki and Capuletti in the larval vs. adult zebrafish dilemma. Although testing multiple adult fish simultaneously may be an interim solution, a better strategy may be to accept both approaches and use them complementarily to advance zebrafish research.

Thinking outside the brain: endocrine responses to stress in zebrafish

Thinking outside of the traditional "box" is important in experimental modeling of brain disorders (Kalueff, LaPorte, Murphy, & Sufka, 2008; Kalueff, Wheaton, & Murphy, 2007). Here we argue that focusing on bodily processes, in addition to pure brain mechanisms, may be a fruitful direction of zebrafish biobehabioral research. The hypothalamic-pituitary-adrenal (HPA) axis mediates the endocrine response to stress in humans and mammals (Alsop & Vijayan, 2008). Under stress, the paraventricular nucleus of the hypothalamus produces

corticotropin releasing factor (CRF), which is delivered to the anterior pituitary gland via the hypothalamic-hypophysial portal blood vessel system (Suzuki, Kawasaki, Ohnishi, Nakamura, & Ueta, 2009). CRF stimulates the anterior pituitary gland, causing release of andrenocorticotropic hormone (ACTH) into the blood stream (Tsigos & Chrousos, 2002). When stimulated by ACTH, the adrenal cortex synthesizes glucocorticoid hormones that modulate the stress reaction (Dedovic, Duchesne, Andrews, Engert, & Pruessner, 2009; Pruessner et al., 2010).

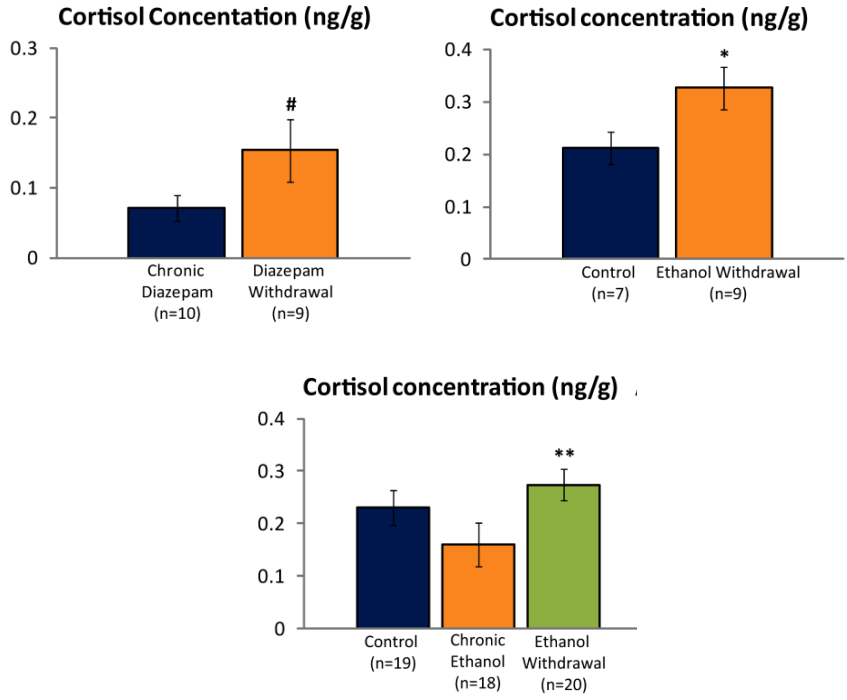

Figure 4. Zebrafish endocrine responses (whole-body cortisol, ng/g fish) to withdrawal from diazepam and ethanol. Left to right then down: 72-h withdrawal from chronic diazepam (72 µg/mL, 2 weeks); 12-h withdrawal from chronic ethanol (0.3%, 1 week); chronic ethanol exposure (0.3%, 1 week) and 12-h withdrawal from chronic ethanol (0.3%, 1 week). Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, #$p = 0.05$-0.1, trend, U-test).

A similar evolutionarily conserved mechanism has been found in zebrafish (To et al., 2007), whose hypothalamus-pituitary-interrenal (HPI) axis is homologous to the HPA axis. With cortisol as the main mediator of the physiological response to stress (Winberg, Nilsson, Hylland, Soderstom, & Nilsson, 1997), zebrafish may be an excellent model for endocrine research (Winberg et al., 1997). Figure 4 summarizes recent data generated by our lab from a series of experiments evoking strong anxiety in zebrafish. The consistency of increased whole-body cortisol concentrations following stressful stimuli is in line with behavioral data gathered in these and previous studies (Egan et al., 2009a).

Such ability to parallel physiological responses with behavioral phenotypes provides researchers with an important tool for investigating stress-related phenomena.

Neurodegenerative disorders

The two key endophenotypes of Alzheimer's disease (AD) include a buildup of amyloid-beta plaques in the nervous system, and a parallel production of uncoordinated meshwork of neurofibrillary tangles caused by damaged Tau protein (Best & Alderton, 2008; Paquet et al., 2009). As suggested, these neuronal damages can lead to memory impairment, and have specifically been noted in 50% of patients with dementia (Vandenberghe & Tournoy, 2005). Therefore, it is possible that learning and memory acquisition in zebrafish can be effectively modeled based on amyloid-beta plaques and tangled neuron formation.

Paradigms which use ASR, raised platform, or T-maze arenas can assess learning and memory capabilities in fish (Best & Alderton, 2008), including tauopathic zebrafish (Barut & Zon, 2000; Paquet et al., 2009) highly relevant to AD. Likewise, Parkinson's disease (PD), the most common movement disorder in humans, is also well-studied in zebrafish (Paquet, Schmid, & Haass, 2006; Shankaran, Schmid, & Kahle, 2006). In addition, various PD-inducing drugs have also been evaluated in both larval and adult zebrafish (e.g., Guo, 2009).

Addiction

Recent analyses of gene expression changes following acute or chronic exposure to drugs of abuse (Kily et al., 2008), have established the genetic correlates of addiction. For example, chronic treatment of zebrafish with ethanol and nicotine alters the expression of multiple CNS genes, some of which have been identified as components of the addiction pathways in mammals (Kily et al., 2008). Further evidence also suggests the sensitivity of zebrafish to drug withdrawal, which is the cornerstone of addictive behavior (Cachat et al., 2009).

For instance, ethanol discontinuation disrupts zebrafish shoaling behavior (Gerlai, Chatterjee, Pereira, Sawashima, & Krishnannair, 2009), while cocaine withdrawal evokes marked alterations in their locomotion (Lopez-Patino et al., 2008; Lopez Patino, Yu, Yamamoto, & Zhdanova, 2008). Our laboratory has demonstrated that withdrawal also modulates zebrafish cortisol levels (Fig. 4), implicating their cortisol abnormalities as a phenotype (Cachat et al., 2009) consistent with glucocorticoid dysregulations in human and rodent withdrawal syndrome (Borlikova, Le Merrer, & Stephens, 2006; Keedwell, Poon, Papadopoulos, Marshall, & Checkley, 2001; Lovallo, 2006; Rabbani, Hajhashemi, & Mesripour, 2009).

New Potential Applications of Zebrafish Models

Serotonin syndrome

As the clinical use of selective serotonin reuptake inhibitors (SSRIs) is rapidly increasing, their toxicity also becomes a serious biomedical problem. Serotonin syndrome (SS) is a severe adverse drug interaction characterized by altered mental status, autonomic dysfunction, and neuromuscular abnormalities. SS symptoms can include agitation, delirium, coma, mydriasis, diaphoresis, hyperthermia, tachycardia, fluctuating blood pressure, tremors, rigidity, myoclonus, and seizures. Although mild cases of the condition usually resolve within 24-72 h (Martin, 1996), SS is particularly difficult to diagnose, and may have a rapid development (Boyer & Shannon, 2005). While SS has been previously modeled in rodents (Fox, Jensen, Gallagher, & Murphy, 2007; Gingrich & Hen, 2001; Kalueff, Fox, Gallagher, & Murphy, 2007), this condition has not been assessed in zebrafish, although they also have a well-developed serotonergic system.

Here we suggest that when attempting to model SS in zebrafish, we may need to select behavioral (e.g., anxiety, immobility) endpoints, and focus on the toxic effects of a combination of several serotornergic drugs (since a single drug may not reliably and effectively induce SS). For instance, a monoamine oxidase A inhibitor administered in conjunction with an SSRI can be expected to induce SS-like states in zebrafish, similar to the SS-like states that the two drugs would evoke in mice or humans. Agents that can be expected to achieve this result include fluoxetine, tranylcypromine, olanzapine or clomipramine. In parallel to behavioral abnormalities, endocrine and/or neurochemical (e.g., brain serotonin levels) endophenotypes can be assessed to more fully mimic fish SS-like behaviors.

Depression

Though attempts to model depression in zebrafish have so far been non-existent, some attempts have been made to model bipolar depression in mice. One such model is based on administration of one drug followed by another drug causing the opposing behavioral effects. For example, a single administration of psychostimulants such as amphetamine or methamphetamine causes hyperactivity, which is then used to test the efficacy of anti-manic treatments such as lithium and valproate. Furthermore, behavioral sensitization by repeated administration of psychostimulants such as amphetamine, methamphetamine, and cocaine has also been used as a model of bipolar disorder in mice (Kato, Kubota, & Kasahara, 2007). Since repeated exposure to cocaine can induce an oscillation or cycling in a variety of neurochemical and physiological systems (Antelman et al., 1998), we suggest that it may also be possible to evoke ”bipolar” behavior in zebrafish, for example, by using a combination of cocaine and anti-psychotic agents.

LPS response and sickness behavior

Cytokine-mediated sickness behavior is an animal syndrome that includes decreased locomotor activity, inhibited exploration of their physical and social environment, reduced food and water intake, and impaired learning and memory (Dantzer, 2001). Notably, zebrafish possess a wide array of cytokines, similar to humans and mice (Lieschke, 2001). Drawing on already established knowledge that bacterial lipopolysaccharide (LPS) is capable of inducing sickness behavior in zebrafish via the induction of pro-inflammatory cytokines (Henry et al., 2008), LPS exposure may possibly serve as a model for sickness behavior in zebrafish.

The inflammatory response is initiated by the uptake of bacteria and their products by the cells of the innate immune system, which, in turn, continues with various mechanisms, including the elevation of cytokines and/or chemokines such as TNF-α, IL-1, IL-6, and IL-8 (Decker, 2004). While affective pathogenesis is attributed to various exogenous stressors (Nemeroff, 2007; Nutt, 2000), recent studies have directly linked affective disorders with various cytokines (Asberg et al., 2009; Hoge, Brandstetter, Moshier, Pollack, Wong, & Simon, 2009; Jonsdottir, Hagg, Glise, & Ekman, 2009; Lu, Jensen, Huang, Kealey, Blair,& Whitehead, 2009). Therefore, the induction of a cytokine response via LPS in zebrafish may produce a promising model of cytokine-mediated behavioral syndromes.

Schizophrenia and autism

Traditional methods of modeling schizophrenia in animals involve dopamine agonist-mediated hyperactivity, and measuring the responses to antipsychotic dopaminergic antagonists, including the elevated levels of dopamine metabolites, dihyroxyphenylacetic acid, and homovanilic acid (found in the cerebral spinal fluid and urine of human patients). We suggest the utilization of the same antipsychotic drugs administered to rodents (such as haloperidol, clozapine, risperidone, and olanzapine), could produce similar behavioral responses in zebrafish. Clearly, more models of schizophrenia in zebrafish are needed. The only current model to date was developed through Burgess and Granato's successful use of sensory gating, in which it was demonstrated that antipsychotic drugs can suppress disruptions in zebrafish PPI induced by dopamine agonists (Burgess & Granato, 2007).

Many of behavioral abnormalities associated with autism spectrum disorders (ASDs) are inherently difficult to model in animals. However, previous experimentation suggests that it is possible to model defects in social interaction in zebrafish, as well as the developmental and cellular defects that correspond to such symptomology. For instance, zebrafish homologues of the genes implicated in ASDs (such as *neurexins*, *reelin*, *mecp2*, and *met*) have been identified, and assays that measure social interaction have been developed (Colman, Baldwin, Johnson, & Scholz, 2009). Thus, like mice, but empowered by the ease of genetic manipulations, zebrafish may lead to new experimental and genetic models relevant to ASD.

Conclusions

With an organism as promising as the zebrafish, it is imperative to develop new models and to refine and expand upon current models in order to reflect this species' full potential. In addition to establishing new models and paradigms, adding new behavioral endpoints and using novel observation methods, such as automated video-tracking systems, will bolster the utility of zebrafish in neurobehavioral research. Using biomolecular markers (such as gene expression or endocrine measures) to parallel zebrafish physiology with behavioral data is another important direction of research. Finally, expanding the area of zebrafish research by including cross-domain modeling (e.g., drug withdrawal/anxiety), new disorders (e.g., ASD, schizophrenia), and new pathways (e.g., brain genes, central and peripheral cytokines) may lead to new translational models using both larval and adult zebrafish.

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