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Chapter 12

THE EMBRYONIC ZEBRAFISH AS A MODEL SYSTEM TO STUDY THE EFFECTS OF ENVIRONMENTAL TOXICANTS ON BEHAVIOR

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ABSTRACT

This chapter highlights current research using the embryonic zebrafish as a model system to study the effects of environmental toxicants on behavior. Zebrafish are ideally suited for high-throughput analyses of behavior. Hundreds of fertilized eggs can be collected daily from a single tank and the synchronously developing embryos can be exposed to environmental toxicants in a culture dish or multiwell plate. The developing motor network of the zebrafish embryo has been impressively characterized at multiple levels, from behavior to circuitry to genes, thus establishing a solid foundation for investigating mechanisms of neurobehavioral toxicity. Three assays are reviewed and their usage to screen for toxicant-induced behavioral defects in embryonic zebrafish is critically evaluated. The mechanisms of neurodevelopment are well conserved in vertebrate species in that similar genes, neurotransmitters, and hormones control early brain development and behavior in fish, mice, and humans. Consequently, behavioral assays in embryonic zebrafish may be used to screen for environmental toxicants that influence human brain development and behavior. Several recommendations are made to strengthen current approaches to accomplishing this important goal.

Keywords: Behavior assays, embryonic, stimulus-induced swimming, tail coiling, toxicant exposure

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INTRODUCTION

Anthropogenic pollution poses a clear and present danger to the healthy development of both human and nonhuman animals. Industrial and household waste products, pesticides, and other chemical pollutants have found their way, historically, accidentally, and illegally, into the earth's atmosphere, soil, and ground and surface waters including oceans at an alarming rate since the Industrial Revolution in the 1900s. Many of these contaminants enter the food chain and some bioaccumulate resulting in novel mixtures and concentrations higher than those found naturally in the environment.

Developing organisms comprise one of the groups most vulnerable to the potentially hazardous consequences of toxicant exposure (Rodier 1994). Their greater risk has been attributed to a combination of behavioral, morphological, physiological and biochemical factors that can lead to increased exposure or uptake of toxicants from the environment, greater susceptibility for organ and system damage by the toxicant, and reduced capacity for detoxification and toxicant excretion. The neurobehavioral defects induced by toxicant exposure have been attributed in large part to altered neuronal connectivity in the developing brain. Given that the mechanisms of neurodevelopment are well conserved in vertebrate species (Tropepe & Sive 2003), zebrafish behavioral screens for toxicant-induced defects are likely to reveal environmental contaminants and mixtures that would influence human brain development and behavior.

The focus of this chapter is on current usage of embryonic zebrafish behavioral assays to screen for toxicant exposure effects. The chapter begins with an overview of the merits of the developing zebrafish as a model system for environmental toxicology and brief descriptions of the main toxicants that have been used to induce behavioral defects in the zebrafish embryo. The behavioral milestones that characterize normal early embryonic development and the assays that incorporate these behaviors are summarized. The use of these behavioral assays to screen the effects of toxicant exposure on development is critically evaluated. This review is organized around three categories; spontaneous behavior assays, stimulus-elicited behavior assays, and experience-based behavior assays. Recommendations are made to strengthen future assessments of toxicant exposure effects on embryonic behavior using these assays.

THE DEVELOPING ZEBRAFISH AS A MODEL SYSTEM FOR ENVIRONMENTAL TOXICOLOGY

The zebrafish (*Danio rerio*) is emerging as a model system in the field of environmental toxicology for many of the same reasons it has gained traction as a model system in genetic and pharmacological research (Barros et al. 2008; Gerlai et al. 2000; Langheinrich 2003; Lieschke & Currie 2007; Norton & Bally-Cuif 2010). The embryos develop externally, making them easily accessible to genetic, chemical, and experimental manipulations. They can be collected in large numbers on a daily basis from even a modest colony and immediately treated with toxicants in a culture dish or multi-well plate from the one cell stage of development without affecting the parental generations. Development is extremely rapid;

within 24 hours, a fertilized egg has developed into a larva with eyes, a beating heart, and tail movements (Kimmel et al. 1995; Westerfield 2007). Figure 1 shows three different views of a zebrafish embryo in its chorion at 24 hours post-fertilization (hpf). In contrast, a day old human embryo has only just entered the two-cell stage. Zebrafish embryos are also transparent, making them easy to observe and image for morphological deformities during toxicant exposure (Beis & Stainier 2006; Fetcho & Liu 1998). In addition to these practical considerations, behavioral assays have been developed to assess sensory, motor, cognitive, and social development in the first week following fertilization.

Zebrafish embryos and larvae are small. Even at 7 days post-fertilization (dpf), the larvae are still only a few millimeters long and can be monitored in multiwell plates using automated imaging systems, which allows for systematic high throughput screening of the genes, pharmaceuticals, and environmental toxicants that influence behavior. Genes important for zebrafish brain development have been identified in large scale mutagenesis screens (Driever et al. 1996; Granato et al. 1996; Haffter & Nusslein-Volhard 1996; Schier et al. 1996) and can be knocked down by injecting antisense morpholinos into the developing embryo (Heasman 2002). Optogenetic systems can be used to visualize not only anatomy but also neuronal activity during development and behavior in transgenic zebrafish that have been designed to express a genetically encoded calcium sensor in their cells that fluoresces whenever the cells become active (Arrenberg, Del Bene & Baier 2009; Del Bene & Wyart 2012; Higashijima et al. 2003). Finally, any delayed deleterious effects of early life stage toxicant exposure on reproduction, sexual and social behavior, motivation, and cognition can be examined in the adult zebrafish 3-4 months post-fertilization.

In recent years, there has been considerable progress in the development and sophistication of behavioral assays for zebrafish larvae and adults (reviewed in Colwill & Creton 2011; Fero, Yokogawa & Burgess 2011; Gerlai 2011; Stewart & Kalueff 2012; Wolman & Granato 2012). In contrast, behavioral assays for zebrafish embryos have received comparatively less attention (Ahmad et al. 2012). However, the motor network of the developing zebrafish has been impressively characterized at multiple levels from behavior to circuitry to genes (Brustein et al. 2003; Drapeau et al. 2002; Granato et al. 1996; Grillner 2003; Kullander 2005; Saint-Amant & Drapeau, 1998), thus providing a solid foundation for understanding mechanisms of neurobehavioral toxicity. The goal of this chapter is to highlight the perhaps unappreciated potential of the developing zebrafish embryo for revealing neurobehavioral effects of toxicant exposure.



Figure 1. Tail coiling in a 24 hour-old zebrafish embryo. Images were collected on a Zeiss Axiovert 200M microscope with a 5x objective approximately 1 minute apart to show different orientations of the embryo during tail coiling. Scale bar = 500 μ m.

Table 1. Selected effects of toxicants on the frequency and duration of zebrafish embryo behaviors

Toxicant	Lowest ED (mg/L)	Exp (hpf)	Test (hpf)	Behavior as described in study	Effect	Source
<i>Spontaneous behavior assay</i>						
Bifenthrin	0.05	3	24	Tail movement	↑	Jin et al. (2009)
1R-enantiomer	0.1	3	18-25	Tail movement	↑ ^a	Jin et al. (2010)
1S-enantiomer	0.1	3	18, 20-24	Tail movement	↓ ^a	Jin et al. (2010)
Chlorpyrifos	0.625	2	24-26	Tail coiling Tail coiling (dur)	↑ ↑	Selderslaghs et al. (2010)
Difenoconazole	1.5 2.5	1 1	24 24	Tail lashing Tail lashing	↑ ↓	Mu et al. (2013)
Malathion	2	1-2	24	Tail lashing	↑	Fraysse et al. (2006)
Acrylamide	250 500	2 2	24-26 24-26	Tail coiling Tail coiling (dur)	↑ ↑	Selderslaghs et al. (2013)
Bisphenol A		2	24-26	Tail coiling	—	Selderslaghs et al. (2013)
IDPN	2500	2	24-26	Tail coiling Tail coiling (dur)	↑ ↑	Selderslaghs et al. (2013)
PFOS	4 4	6 6	25 19	Tail bend Tail bend (peak) ^b	↑ ↓	Huang et al. (2010)
PBDEs						Usenko et al. (2011)
BDE 28	2.5 ^c	6	24	Body flexes	↑	
BDE 47	4 ^c	6	24	Body flexes	↑	
BDE 99	13	6	24	Body flexes	↑	
BDE 100	16.8	6	24	Body flexes	↑	
BDE 153	11.7	6	24	Body flexes	↑	
BDE 183		6	24	Body flexes	—	
TMT	0.4 2 1 1	8 8 8 8	19 20 25 27	Tail bend (peak) ^d Tail bend	↑ ↓ ↓ ↓	Chen et al. (2011)
Cadmium		1-2	24	Tail lashing	—	Fraysse et al. (2006)
Methylmercury	0.028	2	24-26	Tail coiling	↓	Selderslaghs et al. (2013)
<i>Touch-induced motor response assay</i>						
Fipronil	0.333	1-2	30	Swimming	↓ ^e	Stehr et al. (2006)
TMT	1 1	8 8	27 36	Touch response Touch response	↓ ^f ↓ ^f	Chen et al. (2011)

Key to abbreviations: ED = Effective dose; Exp = age exposure began; Test = age behavior assessed; Arrows indicate direction of change (increase or decrease) in frequency or duration (dur) of behavior and — indicates no effect at any of the exposure concentrations used in the study. IDPN = Iminodipropionitrile; PFOS = Perfluorooctanesulphonicacid; PBDEs = Polybrominated diphenyl ethers; TNT = Trimethyltin chloride; ^aNo statistics were reported for these differences; ^bPeak

frequency was lower and delayed relative to control; ^cValues estimated from graph; ^dPeak frequency advanced by one hour relative to controls; ^eData not shown for 30 hpf; ^fPercent of embryos showing touch response.

Anthropogenic Environmental Toxicants

Anthropogenic environmental toxicants include pesticides, industrial chemicals, and metals. Their presence in the environment is a direct result of human activity and their individual harmful effects on human and animal health in large quantities are well established. However, relatively less is known about their potency for disrupting normal development in low concentrations and in commonly occurring mixtures. Automated high throughput screening in animal models is an essential tool for identifying the potential risks for human development of acute and chronic exposures to these chemical cocktails. Because the zebrafish is only recently becoming more widely used as a model in behavioral studies with environmental toxicants, the paucity of studies using multiple chemicals in compounds to determine potential synergistic or additive effects remains a notable void in the literature. In this section, we briefly review the toxicants listed in Table 1 that have been the focus of recent embryonic zebrafish exposure studies. All of these studies examined the sub-chronic effects of individual toxicants, mainly pesticides and industrial chemicals, on morphological and behavioral development and all but one tested a range of concentrations.

1. Pesticides

Chlorpyrifos and malathion are organophosphate pesticides (OPs) that are widely used in agriculture in the United States and internationally. Over 60 million pounds of OPs are sprayed on crop land in the United States every year (EPA 2011) and they have been detected in air, dust, and a large portion of the foods eaten on a daily basis (Morgan et al. 2004). The adverse effects of OPs have been attributed to actions at several target sites including their inhibitory action on a key enzyme, acetylcholinesterase (AChE), which degrades the neurotransmitter acetylcholine (ACh) into choline and acetic acid. OPs inactivate AChE by phosphorylating the serine hydroxyl group located at the active site of AChE. Inactivation of AChE results in the accumulation of ACh throughout the nervous system, leading to overstimulation of muscarinic and nicotinic receptors with clinical effects. Bifenthrin is a synthetic pyrethroid (SP) used in agriculture and for indoor pest control. SPs are replacing OPs due to their low avian and mammalian toxicity but they are highly toxic to most fish. SPs neurotoxic effects have been attributed to their interaction with voltage-sensitive sodium channels (Jin et al. 2009). Fipronil is a phenylpyrazole insecticide specifically engineered to control pests by inhibiting insect gamma-aminobutyric acid (GABA) receptors. Although its affinity for mammalian GABA receptors is relatively low, evidence suggests that it is neurotoxic to developing fish, a problem given its contamination of aquatic ecosystems where fish spawn. Evidence suggests that the adverse effects of fipronil result from its action as a glycine receptor (GlyR) antagonist (Stehr et al. 2006). Difenoconazole, a broad spectrum triazole fungicide used extensively to protect rice and fruit crops, is also considered toxic to

aquatic organisms (Hinfray, Porcher & Brion 2006). However, adequate data are unavailable to determine its potential for developmental neurotoxicity (FAO/WHO 2007).

2. Industrial Chemicals

Neural development and function can be affected by a wide variety of industrial chemicals such as acrylamide, bisphenol A (BPA), iminodipropionitrile (IDPN), perfluorooctanesulphonic acid (PFOS), polybrominated diphenyl ethers (PBDEs), and trimethyltin chloride (TMT) that are ubiquitous in consumer products. Acrylamide is a chemical used in wastewater treatment plants and in the production of dyes, paper, pesticides, and plastics. Common routes of exposure include inhalation of cigarette smoke and consumption of cooked starch-rich foods such as toast, cereal, and dried fruits. Neurotoxic effects have been observed in humans through occupational exposure and nerve terminals have been identified as a primary site of acrylamide action (Kütting et al. 2009). BPA is widely used in plastic food and beverage containers, dental sealants, cash register receipts, and polyvinyl chloride (PVC) (Biedermann, Tschudin & Grob 2010; Kang, Kondo & Katayama 2006; Welshons, Nagel & vom Saal 2006). Very low levels are consistently detected in the human population (Geens, Neels & Covaci 2012). While it can be rapidly metabolized in adults, exposures of infants and young children to BPA may pose a significant risk for developmental disorders (Groff 2010). The common mode of action of BPA is through its ability to mimic the effects of estrogen (EPA 2012a; Masuo & Ishido 2011). Nitriles, such as IDPN, are used in the synthesis of plastics, resins and synthetic fibers. Exposure to IDPN can cause neurological symptoms similar to those seen in the neuromuscular degenerative disease, amyotrophic lateral sclerosis (ALS) (Al-Deeb et al. 1994). Perfluorooctanesulfonic acid (PFOS) is a breakdown product of perfluorocarbons (PFCs) used extensively in stain repellent products, surfactants, and lubricants (Lindstrom, Strynar & Libelo 2011). PFOS has been detected in human bodily fluids and tissues including serum from blood bank donations (D'Hollander et al. 2010; Wilhelm et al. 2009) and in wildlife (Giesy & Kannan 2001). Its neurotoxicity to humans and animals is well established and PFOS contamination is of global concern (Huang et al. 2010). PBDEs are typically used in flame retardants commonly found in consumer products for the home. PBDEs persist in the environment and bioaccumulate in humans and animals (Mai et al. 2005, EPA 2012b). They are considered neurodevelopmental toxicants, targeting muscarinic cholinergic receptors in the hippocampus and disrupting thyroid hormone homeostasis (Usenko et al. 2011). Trimethyltin chloride (TMT) is used primarily in the production of PVC for consumer plastic products (Sadiki et al. 1996) and in fungicides. It has been detected in both domestic and aquatic water supplies (Borghini & Porte, 2002; Shawky & Emons 1998). TMT has been linked to neurological toxicity and profound cognitive impairments in humans and animals (Ishida et al. 1997; Nishimura et al. 2001; Stanton & Jensen 1991). Its effects typically involve damage to hippocampal pyramidal cells (Trabucco et al. 2009), deficits in the serotonergic and GABA-ergic systems, and a decrease in M1 and M2 binding sites in the hippocampus (Earley, Burke & Leonard 1992).

3. Metals

Cadmium exists naturally in the earth's crust but its occurrence as a byproduct of zinc production and its widespread use in consumer products such as batteries, solar panels, electroplating, plastic stabilizers, and pigments are what contribute to its role as an environmental toxicant. Cadmium has been detected in air, dust, soil, food and water. Neurotoxicity studies suggest that cadmium may alter the release of different neurotransmitters, thus disrupting the balance of excitation-inhibition in synaptic transmission (Minami et al. 2001). Cadmium exposure is associated with olfactory dysfunction in humans and other animals (Blechinger et al. 2007) and with neurobehavioral deficits in rodents exposed via gestation and lactation (Ali, Murphy & Chandra 1986; Desi et al. 1998).

Methylmercury, once a byproduct of several industrial manufacturing processes, is now produced largely through the burning of fossil fuels and waste containing mercury and through the methylation of mercury released into the environment (ATSDR 2012). Methylmercury bioaccumulates in aquatic food chains and poses serious health risks to aquatic wildlife in polluted ecosystems and to the developing fetus exposed through maternal consumption of contaminated fish. Methylmercury has multiple effects in the brain including disruption of muscarinic cholinergic systems, inactivation of sodium-potassium adenosine triphosphatase, and the sequestration of selenium essential to cellular and biochemical functions. Methylmercury consumption is associated with serious cognitive impairments that are especially pronounced in developing organisms.

EMBRYONIC BEHAVIOR ASSESSMENTS

Normal development of the zebrafish embryo is relatively rapid with hatching occurring between 48 and 60 hours post-fertilization in embryos incubated at 28.5°C (Kimmel et al. 1995). Embryonic assays have incorporated two behaviors, tail coiling and swimming, to investigate the neurotoxic effects of early exposure to various common environmental contaminants (see Table 1). Embryos are typically bath exposed to the toxicants in small groups in multiwell plates and behavioral assessments are usually conducted in the exposure medium. Generally, a range of toxicant concentrations is examined and, as shown in Table 1, exposure may begin as early as 1 hpf. Some studies reported excluding embryos with visible morphological malformations from behavioral assessments.

Tail coiling occurs spontaneously in embryos growing in their chorions and in dechorionated embryos. It is also well established that tail coiling can be induced by external stimulation. For example, in a dechorionated embryo, vigorous tail coiling can be triggered by gently touching the head or tail with a fine probe. As the embryo matures, mechanical stimulation of the head continues to elicit full tail coils and sometimes swimming whereas tail stimulation elicits partial tail coiling and a swim forward response (Saint-Amant & Drapeau 1998). In mature, dark-adapted embryos, exposure to an intense white light stimulus triggers whole body contractions (shaking) comprised of both tail coiling and swimming movements (Kokel & Peterson 2011; Kokel et al. 2010, 2013). These embryonic assays are useful for

distinguishing toxicant-induced changes in the timing of motor and sensory neuron development and revealing the extent of neurotoxicity. Furthermore, automated analyses of digital images make it possible to quantify these embryonic behaviors with a high level of precision, consistency and speed, factors that can enhance the suitability of these assays for high throughput screening.

In this section, three embryonic behavioral assays are described, two of which have been used to examine the effects of environmental toxicant exposure. We provide a brief overview of their usage, consider their suitability for high throughput screening, and make recommendations for their development including suggestions for standardizing behavior-related aspects of methodology and reporting procedures to facilitate replication and comparison across studies.

1. Spontaneous Behavior

a. Tail coiling

Spontaneous tail coiling in normal embryos incubated at 28.5°C on a 14:10 light:dark cycle has been well documented at multiple levels with evidence suggesting it is mediated by a simple spinal network (Drapeau et al. 2002; Granato et al. 1996; Kimmel et al. 1995; Saint-Amant & Drapeau 1998). It is the first motor behavior to develop, appearing at 17 hpf and coinciding with the innervation of muscle by primary motoneurons. It consists initially of continuous side-to-side flexes of the tail accompanied by slow coils of the tip of the tail towards the head. Contractions usually peak at around 19 hpf and then progressively decline over the next 6-7 hours. After 24 hpf, tail contractions occur in bouts of 2 to 3 coils with up to 20 sec of inactivity between bouts (Saint-Amant & Drapeau 1998). There is negligible further change in the rate of spontaneous contractions after 26 hpf which persist through hatching (Saint-Amant & Drapeau 1998). The frequency of spontaneous tail coiling is positively correlated with the number of depolarizations in the motoneurons and the behavior is considered functionally integral to the hatching process by enabling the embryo to escape from its chorion (Kimmel, Patterson & Kimmel 1974; Saint-Amant 2006).

Spontaneous tail coiling has been used to assess the behavioral effects of embryonic exposure to acrylamide (Selderslaghs et al. 2013), bifenthrin (Jin et al. 2009, 2010), BPA (Selderslaghs et al. 2013), cadmium (Frayse, Mons & Garric 2006), chlorpyrifos (Selderslaghs et al. 2010), difenoconazole (Mu et al. 2013), IDPN (Selderslaghs et al. 2013), malathion (Frayse et al. 2006), methylmercury (Selderslaghs et al. 2013), PFOS (Huang et al. 2010), PBDEs (Usenko et al. 2011), and TMT (Chen et al. 2011). Most of these studies reported that embryos were incubated within the range 28±1°C on a 14:10 light:dark cycle but some reported using a cooler temperature, longer light period or did not specify. Behavioral assessment was always conducted in the exposure medium with all studies testing at around 24 hpf. Three studies, however, tested at additional time points: Huang et al. (2010) and Jin et al. (2010) tested at hourly intervals from 17 hpf to 25 hpf, and Chen et al. (2011) tested at hourly intervals from 19 hpf to 27 hpf. Toxicant effects were routinely measured by examining changes in the frequency of responding during one or more brief observational periods generally lasting no longer than 5 min. Details of toxicant exposures and their behavioral effects are summarized for these studies in Table 1.

Three features make this assay suitable for high throughput screening of environmental toxicants. First, there is little variability in the development of spontaneous tail coiling across untreated embryos incubated at 28.5°C. Second, the behavior can be observed in embryos in their chorions. Third, commercial and open-access software are available to automate the measurement of multiple aspects of the tail coil response including its duration and frequency. Selderslaghs et al. (2010) used automated image analysis software (EthoVision, Noldus) to derive measures of response frequency and duration from changes in the status of individual pixels between successive frames sampled at five frames per sec. They were able to set a threshold level (strong mobility) that corresponded accurately and reliably to the occurrence of a tail coil response in 24-26 hpf embryos.

Future studies of toxicant exposure effects should consider taking advantage of the detailed behavioral profile of spontaneous tail coiling developed by Saint-Amant, Drapeau and colleagues. First, standard rearing conditions should be used to optimize comparison with that profile; thus, exposed embryos should be incubated at 28.5°C on a 14:10 light:dark cycle. Cooler temperatures are known to slow embryonic development and affect behavior (Kimmel et al. 1974; Laale, 1977; Roy et al. 1999; Scheil & Kohler 2009). Even during behavior assessment, it is important to avoid cooler temperatures as the frequency of spontaneous tail coiling decreases considerably (Saint-Amant & Drapeau 1998). Second, behavior should be routinely measured at multiple time points. Hourly observations from 16 dpf through 27 dpf would be of tremendous value in determining if toxicant exposure accelerated, delayed or distorted development of spontaneous tail coiling. For example, Chen et al. (2011) found that exposure to TMT produced higher initial rates of tail bending, advanced the timing of the peak rate by 1 hour, and led to lower post-peak rates relative to controls. While this pattern suggests that TMT accelerates the development of spontaneous tail coiling, the untreated control embryos did not peak until 22 hpf, 3 hours after the peak time reported by Saint-Amant & Drapeau (1998) but matching the peak time reported by Kimmel et al. (1974) in embryos reared at 25°C. Demonstrating that TMT affects the timing of development when embryos are incubated under standard conditions is important for establishing the generality of this effect. Third, while response frequency is informative, other details such as response amplitude and duration, bout lengths, and intervals between bouts may be useful in profiling subtle toxicant effects. Image analysis software should be used to automate extraction of these components to minimize bias, inconsistency, and inefficiency associated with manual coding of behavior. Finally, because a toxicant could affect spontaneous tail coiling by acute irritation of the skin or eyes or through an acute action on the brain or nervous system, testing in the absence of the toxicant would strengthen claims that any significant difference in behavior between treated and control embryos was a direct consequence of altered neurobehavioral development.

2. Stimulus-Induced Behavior

a. Touch-induced motor responses

The emergence and progression of touch-induced motor activity have been well characterized at the levels of spinal cord circuitry and behavior (Saint-Amant & Drapeau 1998; Pietri et al. 2009). Sensitivity to tail touch is mediated between 24 and 60 hpf by the

primary mechanosensory Rohon-Beard cells (RBs), the only sensory neurons in the tail (Kuwada, Bernhardt & Nguyen 1990; Metcalfe et al. 1990). These RBs have been shown to develop novel connections involving glutamatergic transmission to drive motoneuron activation through a rostral loop in the spinal cord (Pietri et al. 2009). For the behavior studies, Saint-Amant and Drapeau (1998) applied a flamed microelectrode deflected by a 1-ms square voltage pulse to the head (nose) or tail (tip) of dechorionated embryos incubated at 28.5°C on a 14:10 light:dark cycle. They found that two behaviors, tail coiling and swimming, can be elicited by touch depending on the age of the embryo. The key findings for each behavior are briefly summarized.

i. Tail coiling

Starting at 21 hpf, mechanical stimulation of either the head or the tail elicited rapid tail coils, contralateral to the stimulus, that served to reorient the larvae away from the stimulus. By 24 hpf, the speed of touch-induced tail coils was determined to be approximately double that of spontaneous tail coiling. The similarity in tail coil responses evoked by head and tail stimulation was observed up to 26 hpf.

ii. Swimming

Starting at 27 hpf, differences were seen in the response to tail and head stimulation. Tail touch elicited bends or partial coils of the tail which served initially to propel the dechorionated larvae forward a distance of one body length. Over the next few hours, the swim response became stronger. By 36 hpf, almost all dechorionated larvae swam away from the stimulus when touched on the tail. In contrast, head touch continued to elicit tail coiling but swimming was observed in only about half of the larvae at 36 hpf.

Touch-induced motor response assays have been used to assess the effects of embryonic exposure to fipronil (Stehr et al., 2006) and TMT (Chen et al. 2011). Details are summarized in Table 1. The methodologies used in these two studies varied from that used by Saint-Amant and Drapeau (1998). Stehr et al. (2006) used dark-reared embryos although they were incubated at 28.5°C. At 30 hpf, dechorionated embryos were gently touched on the head with a nylon monofilament up to three times. Larvae were counted as responders if they swam away from the stimulus on its first, second or third presentation but these data are not shown. Chen et al. (2011) manually dechorionated embryos at 24 hpf and then recorded touch responses to manual stimulation of the tail by a rounded probe repeated three times first at 27 hpf and then at 36 hpf. However, the conditions for incubation and the response criteria were not reported.

Clearly, the potential of touch-induced motor assays for revealing toxicant exposure effects has yet to be realized. Two issues that might have discouraged its use and limited its suitability for high throughput screening studies have recently been addressed with some success. Mandrell et al. (2012) have developed an automated platform for dechorionating embryos in pronase at 4 hpf and a robotic system for sorting and placing them in 96-well plates at 6 hpf. At 120 hpf, the low rate (<5%) of morphological abnormalities of these larvae was comparable to that of controls which were manually dechorionated and placed in 96-well plates. A third issue, not yet resolved, concerns the manual application of touch stimulation. This is a labor intensive, repetitive procedure and not without bias and inconsistency in stimulus delivery. Although Saint-Amant and Drapeau (1998) devised a system that partially

addressed the consistency issue, it was still labor intensive. Building a robotic tool to automate application of the touch stimulus is a crucial next step not only for high throughput studies but for eliminating stimulus variability and bias in studies of toxicant exposure effects.

Future studies of toxicant exposure effects should exploit the time-dependent changes in the pattern of responding to head and tail stimulation to profile more subtle influences on behavior. Inclusion of untreated control embryos along with solvent controls and the use of standard incubation conditions are essential for optimal comparisons with the corpus of existing information about normal embryonic development.

b. The photomotor response (PMR)

From 30 to 42 hpf, normally developing embryos incubated at 28.5°C exhibit only a low level of spontaneous motor activity (Saint-Amant & Drapeau, 1998). During this period, Kokel, Peterson and their colleagues discovered that dark-raised or dark-adapted embryos display a robust motor response (shaking) following a 1-sec pulse of intense white light (Kokel & Peterson, 2011; Kokel et al. 2010, 2013). This photomotor response (PMR) occurs with a latency of 1-2 sec and involves high-frequency body flexions and tail oscillations for 5-7 sec followed by a refractory period during which spontaneous activity is suppressed and a second light pulse is ineffective in eliciting motor activity. Only after ten minutes of dark re-adaptation between photic stimulation will animals respond to a repeated light pulse. Through a series of elegant experiments, Kokel et al. (2013) determined that the neural circuitry of the PMR involves nonvisual neurons in the hindbrain that are sensitive to light between 480nm (blue) and 560nm (green) but not to shorter or longer wavelengths.

Kokel et al. (2010) developed a quantitative method to represent motor activity throughout the different phases of the PMR in the form of a behavioral barcode. Using this method, they examined the PMR phenotypes for a large number of pharmaceutical compounds which they then sorted into clusters based on common behavioral profiles. Interestingly, phenoclusters emerged with shared pharmacological targets such as dopamine or beta-adrenergic receptor agonists. This approach may prove immensely useful for characterizing the putative cellular targets of toxicants and novel chemical compounds based on their shared similarity to these established PMR phenotype clusters, just as comparison of toxicant-induced phenotypes to genetic mutants has helped reveal potential cellular targets (Stehr et al. 2006).

The PMR assay was specifically developed for rapid screening of neuroactive small molecules and has several desirable qualities for high throughput analyses. Embryos can be tested in their chorions and both stimulus delivery and response measurement are fully automated. However, precise topographical information about the motor responses is not captured by the automated methods used to calculate motor activity from changes in average pixel intensity between consecutive digital images. To date, we have been unable to locate any studies using the PMR assay to assess the effects of toxicant exposure on neurodevelopment in zebrafish embryos. Going forward, combining this assay with the spontaneous and touch-induced motor activity assays may prove to be a fruitful strategy for disentangling the effects of toxicant exposure on the rate of development from disturbances in development.

3. Experience-Based Behavior

There should be little reason to doubt that the behavior of adult zebrafish can be modified by past experience. Studies have shown that what adult zebrafish do or where they go can be influenced by food or other rewards or by avoidance of aversive outcomes such as shock (Colwill et al. 2005; Echevarria et al. 2008; Levin & Cerutti 2009; Sison & Gerlai 2010). There is also good evidence that the behavior of zebrafish larvae can be influenced by past experience (O'Neale et al. 2013; Roberts et al. 2011; Valente et al. 2012; Wolman et al. 2012). Recently, we showed that larvae as young as 5, 6, and 7 dpf will behave differently in the presence of a moving visual stimulus depending on their recent prior experience with that stimulus (O'Neale et al. 2013).

Collectively, the experiences used in these examples to change behavior are typically thought to recruit the operation of one or more elementary learning processes (habituation, Pavlovian conditioning and instrumental learning). To the best of our knowledge, comparable studies to determine if the behavior of embryonic zebrafish can be modified by such experiences have yet to be done. However, an interesting observation reported in passing by Saint-Amant and Drapeau (1998) suggests that it might, in fact, be possible to demonstrate an experience-based alteration in stimulus-induced motor behavior in zebrafish embryos as young as 21 hpf. Elaborating on this finding, we outline a strategy for an experience-based behavior assay that could be used to reveal potential effects of toxicant exposure on embryonic learning.

Saint-Amant and Drapeau (1998) devised a 5-min protocol in which mechanical stimulation was applied to the heads or tails of different agarose-restrained dechorionated embryos first at 1 Hz and then at 2 Hz for 1 min periods, each preceded and followed by 1 min periods with no touch stimulation. Measured at hourly intervals from 21 hpf to 24 hpf, the frequency of tail contractions was found to increase with age during the stimulation periods but to decline with age during the no stimulation periods. Relevant here was an incidental and somewhat cryptic remark that accompanied the presentation of these results indicating that there 'was a decline in the responses to higher-frequency stimulation, suggesting habituation' (Saint-Amant & Drapeau 1998:626).

We have previously commented, as have others, on the not insignificant problems associated with interpreting a decline in a response to a repeatedly presented stimulus as evidence of learning, specifically habituation (Davis 1970; Davis & Wagner 1968, 1969; O'Neale et al. 2013; Rescorla 1988; Rescorla & Holland 1976). In our demonstration of stimulus learning in larval zebrafish, we used the t1-t2 framework proposed by Rescorla and Holland (1976) to avoid the problem of confounding the opportunity for learning (t1) with the assessment of that learning (t2). In one experiment, we measured the responses of two groups of larvae to a moving visual stimulus. One of the groups had been familiarized with this stimulus but the other had not. The familiar group displayed significantly less freezing in the presence of the moving visual stimulus than did the unfamiliar group. This difference in behavior of the two groups to an identical target could only be attributed to the effect of their prior differential experience with that target.

Building on Saint-Amant and Drapeau's (1998) observation, it is a fairly straightforward matter to apply the t1-t2 logic to the development and implementation of an assay for demonstrating habituation in the zebrafish embryo. Essentially, two groups of dechorionated

embryos would be tested with mechanical stimulation of the tail for a one minute period. One group would have received prior presentations of this touch stimulus whereas the other group would have been spared those presentations. A difference in behavior between the two groups during identical testing would confirm an effect of their differential prior experience, a result suggestive of simple learning.

It is important to acknowledge that prior experience may alter behavior for reasons other than learning. For example, repeated exposure to a stimulus may induce sensory adaptation, motor fatigue, or even sensitization, and recent experience with a brief stimulus event may produce pre-pulse inhibition (Burgess & Granato 2007). Methods have been devised to help distinguish among these possibilities. For instance, in the preceding example, motor fatigue may be just as reasonable an explanation for the difference between the two groups as learning. Examining spontaneous rates of contraction and the effectiveness of head stimulation in eliciting a contraction, exposing the control group at t1 to a different stimulus that elicits the same response, or using a dishabituation procedure are some of the strategies that can be deployed to distinguish between motor fatigue and habituation.

Saint-Amant and Drapeau (1998) laid the groundwork for developing a very powerful approach for studying learning in embryos. Finding a way to fully automate delivery of an effective touch stimulus or its equivalent is essential for making progress in this area and is certain to yield appreciable payoff in the long-term. Low level exposure to toxicants during development may exert subtle effects on behavior due to disruption of neuronal connectivity that may go undetected through inspection of external morphological development and gross anatomy. An embryonic assay for reliably demonstrating an effect of prior experience may serve as a valuable tool to screen for subtle neurobehavioral effects of toxicant exposure at environmentally relevant concentrations and mixtures.

CONCLUSION

Behavioral assessments of toxicant exposure have been carried out at the embryonic stage although there are currently limitations. Tail coiling is already a promising candidate behavior for high throughput analyses for several reasons. It is the first behavior to appear after fertilization, its measurement can be automated, and its occurrence can be either spontaneous or elicited by external stimulation. All of the studies reviewed here conducted assessments in the presence of the exposure medium making it difficult to determine if the observed behavioral perturbations were due to alterations that had already occurred in the developing nervous system or to a direct effect of the toxicant on behavior at the time of assessment. Stehr et al. (2006) did include a condition in which fipronil treated and solvent control embryos were tested in system water. In contrast to the deficit observed in touch-induced swimming at 30 hpf following chronic exposure, they found no effect of an 8-hr exposure pulse (.5 mg/L) prior to 24 hpf on touch-induced swimming assessed at 48 hpf. This result is interesting because it suggests that over time a developing nervous system may actually recover from some toxicant exposure effects.

Future studies of toxicant effects on development should focus on automating procedures for response measurement and stimulus delivery, conducting assessments in the absence of the toxicant at multiple time points, and perhaps expanding assessments to other behaviors

that emerge later during embryonic development such as the rhythmic movement of the pectoral fins and abrupt, periodic changes in the position of the embryo, two or three times a minute (Wielhouwer et al. 2011). Additionally, the practice of rearing and testing embryos individually should be adopted. Not only are the effects of the presence of a dying embryo eliminated but one can track the behavior of individuals over time and compare performance on a variety of assays. There may also be merit to standardizing methodology which varied widely across the studies reviewed here. At the very least, it is important to include untreated controls alongside solvent controls particularly in studies using embryos collected from fish obtained from commercial suppliers and pet stores and where there are significant deviations from the recommended conditions for embryo incubation. That said, using different strains and cooler temperatures to slow embryonic development may serendipitously reveal toxicant effects on brain and behavior that might not otherwise be detected. Such findings could have translational significance for understanding why typical risk factors such as maternal poor diet, poverty, or stress may heighten the vulnerability of a developing human fetus to the adverse effects of toxicant exposure.

Finally, we cannot overstate the importance of developing behavioral assays for assessing the effects of experience in the embryonic zebrafish. The combination of sophisticated behavioral tools, powerful optogenetic techniques, and the growing library of transgenic zebrafish lines offers a formidable arsenal for using the embryonic zebrafish to unlock the mysteries of both normal and perturbed neurobehavioral development.

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