Exploring interactions between xenobiotics, microbiota, and neurotoxicity in zebrafish

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Abstract

Susceptibility to xenobiotic exposures is variable. One factor that might account for this is the microbiome, which encompasses all microorganisms, their encoded genes, and associated functions that colonize a host organism. Microbiota harbor the capacity to affect the toxicokinetics and toxicodynamics of xenobiotic exposures. The neurotoxicological effects of environmental chemicals may be modified by intestinal microbes via the microbiota-gut-brain axis. This is a complex, bi-directional signaling pathway between intestinal microbes and the host nervous system. As a model organism, zebrafish are extremely well-placed to illuminate mechanisms by which microbiota modify the developmental neurotoxicity of environmental chemicals. The goal of this review article is to examine the microbiota-gut-brain axis in a toxicological context, specifically focusing on the strengths and weaknesses of the zebrafish model for the investigation of interactions between xenobiotic agents and host-associated microbes. Previous studies describing the relationship between intestinal microbes and host neurodevelopment will be discussed. From a neurotoxicological perspective, studies utilizing zebrafish to assess links between neurotoxicological outcomes and the microbiome are emphasized. Overall, there are major gaps in our understanding the mechanisms by which microbiota interact with xenobiotics to cause or modify host neurotoxicity. In this review, we demonstrate that zebrafish are an ideal model system for studying the complex relationship between chemical exposures, microorganisms, and host neurotoxicological outcomes.

Zebrafish as an established model for neurotoxicology studies

Zebrafish is a non-mammalian vertebrate model that is well established as an alternative model for neurotoxicological studies (Peterson *et al.*, 2008; Horzmann & Freeman, 2016). Zebrafish contain a fully sequenced genome, and approximately 70-80% of their genes are homologous with human counterparts (Howe *et al.*, 2013). As opposed to commonly used animal models like mice and rats, zebrafish embryos develop external to the mother, such that the developing embryo can be directly exposed to xenobiotic agents (Peterson *et al.*, 2008; Horzmann & Freeman, 2016). Zebrafish organogenesis is complete by 72 hours post fertilization (hpf) (Peterson *et al.*, 2008; Horzmann & Freeman, 2016) and both embryos and larvae can be toxicologically assessed in 96-well plates or, for early developmental analyses, 384-well plates, where chemical exposures can be easily performed (Peterson *et al.*, 2008; Horzmann & Freeman, 2016).

Zebrafish neurodevelopment is generally conserved compared to humans (Lee & Freeman, 2014; Nishimura *et al.*, 2015, Schmidt *et al.*, 2012). At 6 hpf, during gastrulation, ectoderm differentiation begins (Lee & Freeman, 2014; Nishimura *et al.*, 2015). Analogous to other mammalian species, zebrafish neuroectoderm develops into a neural plate and undergoes neurulation (i.e. the process of folding the neural plate into the neural tube) (Nishimura *et al.*, 2015; Schmidt *et al.*, 2012). However, neurulation occurs via a different process in zebrafish relative to most vertebrates (Papan & Campos-Ortega, 1994; Buckley *et al.*, 2013). In zebrafish, at roughly 12 hpf, the neural plate forms a neural keel, leading to the formation of the neural rod followed by the neural tube (Papan & Campos-Ortega, 1994; Buckley *et al.*, 2013). The forebrain (diencephalon), midbrain (telencephalon), hindbrain (cerebellum), and spinal cord are apparent at 16 hpf (Kozol *et al.*, 2016). By 2-3 days post fertilization (dpf), neuronal subtypes, including GABAergic, catecholaminergic, serotonergic, and noradrenergic neurons, start to differentiate (Lee & Freeman, 2014; Nishimura *et al.*, 2015). Similar to mammas, zebrafish also contain astrocytes, microglia, oligodendrocytes, cerebellar Purkinje cells, myelin, and motor neurons and zebrafish develop a functional blood brain barrier (BBB) by 3 dpf (Fleming *et al.*, 2013). Neurotoxicological phenotypes include loss or expansion of brain ventricles, truncation of the telencephalon, and neuronal necrosis (Peterson *et al.*, 2000).

Automated behavioral tests in embryonic and larval zebrafish are widely used as a functional readout of neurodevelopment in animals exposed to environmental chemicals (Figure 1) (Fraser *et al.*, 2017; Glazer *et al.*, 2017; Dishaw *et al.*, 2014; Chen *et al.*, 2012; Bailey *et al.*, 2016; Massarsky *et al.*, 2018; Cassar *et al.*, 2018; Wolman *et al.*, 2015). Neurotoxicological effects of xenobiotics are also commonly assessed in older life stage zebrafish (Lutte *et al.*, 2018; Anichtchik *et al.*, 2004; Pereira *et al.*, 2012; Bencan & Levin, 2008; Massarsky *et al.*, 2018; Pereira *et al.*, 2012; Xu *et al.*, 2016). Embryos can be assessed for the effect of xenobiotic exposures on spontaneous movement (i.e. head and tail coilings), which begin as early as 17 hpf, depending on rearing temperature (Kokel al., 2010). Larval zebrafish behavior can be analyzed in relation to distance travelled, time spent active, or pattern of behavioral responses to stimuli like light changes (Fraser *et al.*, 2017; Glazer *et al.*, 2017; Dishaw *et al.*, 2014; Chen *et al.*, 2012; Bailey *et al.*, 2016; Massarsky *et al.*, 2018), or acoustic startle (Cassar *et al.*, 2018; Wolman *et al.*, 2015). Furthermore, larval behavior such as threat avoidance (Richendrfer & Creton, 2015; Gonzalez *et al.*, 2016), anxiety-like behavior measured through thigmotaxis (i.e. place of preference in the well) (Gonzalez *et al.*, 2016), and optomotor response (Cassar *et al.*, 2018) can also be assessed. Habituation, the most

primitive form of learning, can also be tested in larval zebrafish using an automated tracking system and repeated acoustic startles (Roberts *et al.,* 2011; Wolman *et al.,* 2015). Adult neurobehavior is also commonly assessed to investigate baseline locomotor activity (Lutte *et al.,* 2018; Anichtchik *et al.,* 2004; Pereira *et al.,* 2012), anxiety-like behavior (Bencan & Levin, 2008; Anichtchik *et al.,* 2004; Massarsky *et al.,* 2018), habituation and memory (Lutte *et al.,* 2018; Massarsky *et al.,* 2018; Pereira *et al.,* 2012), peer recognition (Massarsky *et al.,* 2018; Fernandes *et al.,* 2015), and aversive stimulus recognition (Xu *et al.,* 2016; Massarsky *et al.,* 2018). For all embryonic, larval, and adult behavioral tests, there is a lack of standardized testing procedures.

In addition to morphological and behavioral endpoints, molecular biology approaches are also widely deployed to examine neurotoxicological outcomes in zebrafish. Targeted gene expression (Massarsky *et al.,* 2018; Pereira *et al.,* 2012) or microarrays (Liu *et al.,* 2015) have been usurped by unbiased RNA sequencing (Xu *et al.,* 2015; Chen *et al.,* 2016; Zhang *et al.,* 2017). Unless sequencing approaches are applied in specific cell types isolated from transgenic lines (Hernández *et al.,* 2018, Cao *et al.,* 2016), these approaches generally lack spatial information. *In situ hybridization* has long been used in zebrafish to illuminate spatiotemporal gene expression in the developing nervous system (Stehr *et al.,* 2006; Wen *et al.,* 2008; Hill *et al.,* 2003; Kanungo *et al.,* 2013). From a neurotoxicological perspective, xenobiotic-induced changes in gene expression are best used for hypothesis generation that can be empirically tested by gain- or loss-of-function experimentation. Historically, antisense oligonucleotide morpholinos were nearly universally utilized to study neurotoxicological mechanisms of action (Bertotto *et al.,* 2019; Chlebowski *et al.,* 2017; Tal *et al.,* 2012). However, because of concerns about off-target effects (Eisen & Smith, 2008), the emerging gold standard for mechanism delineation in a toxicological context is gene editing, often via clustered regularly interspaced short palindromic repeats (CRISPR) technique (Zabinyakov *et al.,* 2017; Farrar *et al.,* 2018), although this method also introduces off-target mutations (Tsai & Joung, 2016). Lastly, transgenic lines that allow for real-time visualization and quantitation of electrical activity in live zebrafish have been developed and used to evaluate neurotoxicity of xenobiotics (Hayashi *et al.,* 2015; Wen *et al.,* 2008; Hill *et al.,* 2003; Kanungo *et al.,* 2013). Overall, there are a wealth of tools and approaches that allow for both hazard identification and mechanistic research to examine the neurotoxicological effects of xenobiotic exposures.

Zebrafish as an emerging model for microbiota-gut-brain-axis studies

The microbiota-gut-brain axis describes the complex bidirectional communication between microorganisms that reside in the gastrointestinal (GI) tract and the host central nervous system (CNS) (Figure 2). This axis monitors and integrates intestinal functions that link emotional and cognitive centers in the brain to intestinal permeability, enteric reflex, entero-endocrine signaling, and immune activation (Carabotti et al, 2015). Bidirectional communication occurs via vagus nerve neurons that form synapses with intestinal epithelial cells and through the generation of microbial products or microbial stimulation of host cytokines and chemokines that penetrate the BBB and exert direct effects on the host nervous system (Figure 2) (Bravo *et al*, 2011; Tsavkelova *et al.*, 2000; Stephenson *et al.*, 1947; De Vadder *et al*., 2014; Wikoff *et al*., 2009). The host hypothalamus-pituitary-adrenal axis (HPA), which coordinates adaptive responses to stressors like environmental chemicals and elevates systemic proinflammatory cytokines (Breit *et al.,* 2018), also plays an important role in the microbiota-gut-brain axis (Figure 2). Communication from both

vagal and HPA pathways can regulate the activity of intestinal effector cells, such as epithelial, smooth muscle, interstitial, and enterochromaffin cells, as well as enteric neurons, which can also be influenced by the gut microbiome. These cells are responsive to an array of molecules synthesized and/or metabolized by intestinal microbiota including catecholamines, GABA, bile acids, and short chain fatty acids (SCFA) (Tsavkelova *et al.*, 2000; Stephenson *et al.*, 1947; Bravo *et al*., 2011; De Vadder *et al*., 2014). One well known host-microbiome interaction relates to the neurotransmitter serotonin, of which more than 90% is produced in the gut via microbial-dependent synthesis in enterochromaffin cells (Yano *et al.*, 2015).

Animal models such as zebrafish (Rawls *et al.*, 2004), mice (Neufeld *et al.*, 2011), rats (Mao *et al*., 2019), flies (Broderick *et al.*, 2014), and nematodes (Nguyen *et al.*, 2012) are commonly used to study the microbiota-gut-brain axis (Figure 3), often by comparing hosts that lack microbes (i.e. axenic) to colonized or conventionalized (i.e. axenic hosts subsequently colonized with microbes) animals. In this review, the term "axenic" was selected to accurately and specifically describe animals devoid of microbes rather than the more commonly used terms "germ-free" or "gnotobiotic," as not all microbes are pathogenic and animals conventionalized with known strains of microbes are also considered to be gnotobiotic.

Relative to mouse and rat models that prohibitively require the maintenance of axenic breeding colonies or lower order animal models such as flies and nematodes that are colonized by less complex microbiota comprised of 1-30 microorganisms, zebrafish represents a powerful intermediate model system where axenic offspring can be easily derived and compared to colonized animals with moderate microbiota complexity (~100-200 species) (Figure 3). In addition, the zebrafish GI tract is homologous with higher order vertebrates, containing a liver, pancreas, gall bladder, absorptive enterocytes, goblet and enteroendocrine cells, a linearly segmented intestinal tract with absorptive and secretory functions, and tight junctions and microvilli in the intestinal epithelium (Goldsmith & Jobin, 2012). However, some important differences exist (Figure 3). For example, zebrafish lack a stomach, lymph nodes, Peyer's patches, and splenic germinal centers (Danilova & Steiner, 2002). Diet and environment are also quite different between zebrafish and humans, where intestinal microbes associated with laboratory zebrafish exist at ~26-28º C as compared to human-relevant microorganisms that thrive at ~37º C (Meeker & Trede, 2008). Differences in temperature, diet, and salinity all influence the complex community structure of host-associated microbes that colonize zebrafish, which includes gram-positive and gram-negative bacteria, protozoa, fungi, and viruses (Goldsmith & Jobin, 2012).

In both zebrafish and mammals, microbial communities become more established and diverse as the organisms mature. At the phylum level, zebrafish microbiota are variable based on laboratory environment and in general are dominated by Proteobacteria, Firmicutes, and Fusobacteria (Rawls *et al.*, 2006; Stephens *et al.*, 2016). In contrast, mice and human microbiota are dominated by Firmicutes and Bacteroidetes. Humans also contain a proportionally similar number of bacteria that belong to the phylum Actinobacteria (Kostic *et al.*, 2013). While some differences in microbiota composition between species can be attributed to environmental factors such as temperature, diet, or salinity, host factors also strongly impact the development and maturation of microbial communities within each species. For example, some studies in zebrafish have shown that microbial composition is influenced by host-specific selective pressures in the gut and are altered even when extrinsic factors such as diet and environment remain constant (Bevins *et al*.,

2011; Wong *et al*., 2012; Stephens *et al*., 2016). Additionally, axenic adult zebrafish transplanted with mouse intestinal microbiota (primarily Firmicutes) develop microbiota that resemble conventional zebrafish guts (primarily Proteobacteria) rather than conventional mouse guts (Rawls *et al*., 2006). A similar phenomenon occurs when zebrafish microbiota are transplanted into axenic mice (Rawls *et al*., 2006), suggesting that comparable host selection factors and signaling mechanisms are present in both zebrafish and mammalian models. Importantly, the moderately diverse microbiota (~100-200 species, Figure 3) within zebrafish contain functionally similar enzymes and biochemical pathways compared to mammals (e.g. rodents) (Milligan-Myhre *et al*. 2011).

One major advantage of the zebrafish system relative to higher order vertebrates is that methods to derive axenic embryonic zebrafish are well established (Rawls *et al.,* 2004; Rawls *et al.,* 2006; Phelps *et al.,* 2017; Melancon *et al.,* 2017). Axenic status can be easily assessed by culturing media samples, qRT-PCR, or metagenomic sequencing. Once axenic zebrafish are generated, they can be conventionalized via simple immersion using fish facility water or specific microbial cultures (Davis *et al.,* 2016a; Davis *et al.,* 2016b; Phelps *et al.,* 2017), or by injection (Herbomel *et al.,* 1999; Vergunst *et al.,* 2010). It is important to note that colonization with fish facility water is variable over time (Catron *et al.,* 2019a), which can result in significant differences in community structure between conventionally colonized and conventionalized control animals (Catron *et al.,* 2019b; Weitekamp *et al.,* 2019). Depending on rearing temperature and institutional animal use rules, methods for rearing up zebrafish are possible without the introduction of sterile food for up to ~6 dpf, as larvae rely on the yolk sac for nutrition (Dabrowski & Miller, 2018). Past ~6 dpf, axenic zebrafish have been successfully reared until 10 dpf using gamma irradiated powder diet (Phelps *et al.,* 2017) and 30 dpf with the addition of microbe-free live food cultures, a labor-intensive method (Melancon *et al.,* 2017).

Regarding study design, one of the strengths of alternative models for microbiota-oriented research, such as the fruit fly, the nematode worm, and the zebrafish, is the ability to easily generate and maintain multiple colonization statuses of these organisms including conventionally colonized, axenic, and conventionalized (Figure 3). Relative to less complex systems like *Drosophila* and *C. elegans*, zebrafish is particularly advantageous because it has greater genetic similarity to human/mammalian genomes, increased microbial diversity (~100-200 species) (Figure 3), and more complex assays exist to investigate microbiota-gut-brain interactions. Zebrafish therefore represents an ideal intermediate model system with sufficient microbiome complexity, yet also allows for relatively simple modification of microbial colonization status. However, the above-mentioned important disadvantages of the zebrafish model relative to mice and humans should not be overlooked (e.g. GI tract structure and microbiota composition), as key differences between species can likely influence toxicokinetic and toxicodynamic interactions with xenobiotic agents. Overall, the ability to easily manipulate larval colonization status is a key technical advantage that can be used to determine whether microbial colonization status influences the developmental neurotoxicity of exposure to environmental chemicals (Figure 1). More work is needed to understand how structural and potential functional differences in host-associated microbes impacts microbiota interactions with the brain, particularly in the context of xenobiotics exposures.

Key zebrafish microbiota-gut-brain-axis behavior studies

As mentioned previously, zebrafish behavior is commonly used as a functional readout of neurodevelopment. The same logic can be applied to evaluate the microbiota-gut-brain axis in zebrafish. In zebrafish larvae, microbiota can modulate locomotion and anxiety-related behaviors (Davis *et al.,* 2016a; Davis *et al.,* 2016b; Phelps *et al.,* 2017; Catron *et al.,* 2019b). Davis *et al.* (2016a) first reported that axenic zebrafish are hyperactive relative to colonized controls, a phenotype that our laboratory has replicated (Phelps *et al.,* 2017) and has also been observed in both mammalian studies (Diaz Heijtz *et al.,* 2011; Neufeld *et al.,* 2011) and, more recently, in *Drosophila* (Schretter *et al.,* 2018). Thigmotaxis, the demonstrated preference for the edge of a multiwall plate, is used as a functional measure of anxiety-like behavior in zebrafish (Kalueff & Stewart, 2012). Axenic zebrafish exhibit reduced anxiety-like behavior in the thigmotaxis assay (Davis *et al.*, 2016a), although this finding was not replicated in a later study (Phelps *et al.*, 2017). The lack of a standardized method for assessing thigmotaxis in zebrafish likely explains the discordant data. From a developmental perspective, Phelps *et al.* (2017) reported that conventionalization of axenic zebrafish by 6 dpf was sufficient to block hyperactivity at 10 dpf. This supports the concept that there are critical windows of nervous system development that require microbial colonization to enable control-like development in zebrafish and that conventionalization after these temporally distinct windows close is likely insufficient to recover control-like behavior (Phelps *et al.,* 2017). This is supported by mammalian data showing that colonization of axenic mice post-weaning failed to replenish reduced serotonin levels in the CNS (Clarke et al., 2013). In other words, in mice, there is a strict developmental window that requires microbial colonization for control-like establishment of serotonergic signaling (Clarke *et al.,* 2013). Exciting recent work has identified the bacterial enzyme xylose isomerase as critical modulator of sugar metabolism in flies and that subsequent activation of host octopaminergic neurons was sufficient to block axenic hyperactivity (Schretter *et al.,* 2018). Despite these advancements, the mechanism(s) by which microbial colonization influences the development and function of circuits that control stereotypic behaviors in zebrafish (i.e. larval swimming responses and thigmotaxis), and how these microbiome-host interactions are affected by xenobiotic exposure, are unknown.

In adult zebrafish, two separate studies have similarly used behavior to observe how probiotic microbes influence the host nervous system. In Davis *et al*. (2016b), one-month long supplementation of adult zebrafish with *L. plantarum* (strain not specified), was shown to subtly alter intestinal microbiota and cause a small but significant reduction in anxiety-like behavior in the novel tank test. The same study also applied a five-day chronic unpredictable stress protocol that massively restructured microbial community structure (Davis *et al.,* 2016b). Restructuring was significantly ablated via supplementation with *L. plantarum*, although supplementation had no effect on serum cortisol levels (Davis *et al.,* 2016b). In a similar study, Borrelli *et al.* (2016) administered the probiotic *L. rhamnosus* IMC 501 for 28 days to adult zebrafish, resulting in small effects on shoaling behavior and altered expression of brain derived neurotrophic factor (*bdnf)* and genes related to serotonin metabolism and signaling such as paralogs of the tryptophan hydroxylase gene (*tph1a, tph1b* and *tph2*). While tantalizing, these adult studies raise two important questions. First, what are the mechanisms by which probiotic administration modifies behavior? Second, do relatively small changes in behavior functionally compromise the organism? Overall, although zebrafish has proven to be an essential animal model for studying host-microbe

interactions, more work is needed to establish causal links between intestinal microbes and host swimming behaviors.

A framework for microbiota-xenobiotic interactions

There is growing interest in understanding the mechanisms by which microbiota interact with xenobiotic agents to influence host toxicity (e.g. neurodevelopmental toxicity). This can theoretically occur via toxicodynamic interactions where chemical exposures cause dysbiosis, characterized by alterations in the community structure of host-associated microbiota that subsequently affects the host (Figure 4). Chemical-selected microbiota can also perform chemical activation or detoxification reactions (i.e. Toxicokinetic interactions) (Figure 4). Many studies in larval or adult zebrafish demonstrate the utility of the model system to describe dysbiosis following exposure to drugs or environmental chemicals. Recent evidence obtained in the zebrafish model also demonstrates toxicokinetic interactions between chemicals and microbiota in which intestinal microorganisms bioactivate or detoxify xenobiotics.

Xenobiotic exposure alters community structure of host-associated microbes (i.e. Dysbiosis)

The identification of chemical-induced dysbiosis in zebrafish is the subject of a recent review (Catron *et al.* In Press) and will not be discussed in detail here. Briefly, in zebrafish, exposure to a wide array of xenobiotic agents has been shown to disrupt the community structure of host-associated microbes including pesticides (Wang *et al.,* 2019; Zhang *et al.,* 2018; Jin *et al.,* 2017; Oliveira *et al.,* 2017), metals (Dahan *et al.,* 2018, Xia *et al.,* 2018), microplastics (Qiao *at el.,* 2019; Wan *et al.,* 2019), and antibiotics (Nadal *et al.,* 2018; Pindling *et al.,* 2018). Rather than list qualitative changes in specific taxa following various xenobiotic exposures, a key theory and examples of concordant and discordant structural data will be discussed. First, a recent study comparing the effect of six concentrations of the plasticizer Bisphenol A (BPA) or four replacement chemicals (BPAF, BPB, BPF, or BPS) on community structure and developmental toxicity in zebrafish was recently reported (Catron *et al.,* 2019a). The highest concentration evaluated using metagenomic sequencing was the No Observed Effect Concentration (NOEC) for developmental toxicity. Not all compounds tested affected microbial structure (e.g. BPAF or BPB) (Catron *et al.,* 2019a). Interestingly, the ability of xenobiotics to restructure microbiota was inversely related to their potency for developmental toxicity (Catron *et al.,* 2019a). This illustrates the principal that compounds may fail to cause dysbiosis simply because the concentrations necessary to perturb community structure cannot be tolerated by the host (Catron *et al.,* 2019a). Conversely, compounds that are well tolerated by the host may be more likely to cause structural dysbiosis. Because the zebrafish developmental toxicity assay is widely used for hazard identification and chemical prioritization, these data suggest that chemicals with lower host toxicity profiles might be more likely to simultaneously cause dysbiosis of host-associated microbes.

Discordant structural data following exposure to the same chemical agent are likely because microbiota are significantly affected by extrinsic factors including diet, genetics, age, or water quality parameters that often vary substantially across laboratories. For example, a recent publication showed that exogenous 17β-estradiol exposure (0.34–3.5 μM) failed to alter microbial community structure in larval zebrafish (Catron *et al*., 2019b), which is in line with the theory that

the holobiont system evolved to tolerate fluctuations in endogenous hormones. However, a separate study in adult zebrafish exposed to approximately 0.07 μM 17β-estradiol reported a qualitative perturbation of global microbiota (Chen *et al.,* 2018b). Perhaps more attention should be given to concordant data across laboratories using the same chemical to perturb microbiota. For example, exposure to the antimicrobial agent triclosan was shown to select for the gram-negative *Pseudomonas* in zebrafish larvae (Weitekamp *et al.,* 2019) and adults (Gaulke *et al.,* 2016) indicating that certain xenobiotic-dependent alterations in community structure are conserved at multiple life stages. In addition, concordant changes in taxa following exposure to different chemical stressors might also be particularly relevant. For example, >70% sequencing reads in zebrafish larvae developmentally exposed to triclosan were associated with a single gram-negative bacteria, *Rheinheimera* (Weitekamp *et al.,* 2019). Interestingly, BPA exposure also selected for both *Rheinheimera* and *Pseudomonas* in larval zebrafish (Catron *et al.,* 2019a). This suggests that certain taxa (e.g. *Rheinheimera* and *Pseudomonas*) that are either broadly resistant to xenobiotic agents or are sensitive to chemical exposures yet exhibit swifter repopulation kinetics may serve general markers of dysbiosis in zebrafish. Overall, while these studies report chemical-dependent microbiota dysbiosis, most fail to connect changes in community structure to adverse physiological outcomes in the host (i.e. Toxicodynamic interactions) and this uncertainty is a key limitation of reported xenobiotic-induced structural dysbiosis in zebrafish and other model systems.

Chemical biotransformation of xenobiotics by intestinal microbiota in zebrafish (i.e. Toxicokinetic interactions)

Gut microbiota carry out a multiplicity of reactions to efficiently harvest nutrients from their environment. Microbial enzymes can also biotransform xenobiotics. While there are increasingly more studies showing toxicodynamic effects of chemicals on the zebrafish microbiome, toxicokinetic data in this model are rather limited. This topic was recently reviewed in the zebrafish literature (Catron *et al.,* In Press) and will therefore not be extensively explored in this review. Briefly, intestinal microbes can perform xenobiotic reductions, hydrolysis, glucuronidation, lyase reactions and nucleophilic substitutions (Rafii *et al.,* 1997; Lee and Renwick, 1995; Laue *et al.,* 2001; Peppercorn and Goldman, 1972; Takeno and Sakai, 1991; Wallace *et al.,* 2015; Cantarel *et al.,* 2012; Sutherland, 1995; Kumano *et al.,* 2016; Catron *et al.,* 2019b; Weitekamp *et al.,* 2019). Catron *et al*. demonstrated levels of estradiol and some direct estradiol metabolites were ~3x higher in axenic relative to colonized zebrafish at 10 dpf, suggesting that the microbes in this system influence estradiol metabolism and exhibit toxicokinetic interactions with the host (Catron *et al.* 2019b). Another recent paper demonstrated that colonized zebrafish contained 2.5-3x higher concentrations of parent triclosan compared to axenic zebrafish (Weitekamp *et al.,* 2019). Elevations in triclosan sulfate were also generated by triclosan-selected microbiota and triclosan-selected microorganisms were enriched for the ability to perform sulfonation reactions, which is the first step in the biochemical pathway necessary to produce triclosan sulfate (Weitekamp *et al.,* 2019). Together, these studies demonstrate that colonization status influences chemical metabolism in zebrafish and that xenobiotic biotransformation profiles are likely to be chemical-specific, given that consistent changes in parent concentrations were not observed in either axenic or colonized zebrafish across studies (Catron *et al.* 2019b; Weitekamp *et al.,* 2019). A key consideration when interpreting toxicokinetic data derived from axenic, conventionally colonized, and conventionalized zebrafish is that axenic animals may be compromised in their ability to detoxify or metabolize chemicals. This uncertainty makes it difficult to clearly delineate host *vs.*

362 microbial effects on chemical biotransformation. Nevertheless, the comparison of xenobiotic
363 biotranformation events across zebrafish with varying colonization statuses represent a powerful
364 strategy to uncover mechanisms by which host-associated microbiota influence the kinetics of
365 xenobiotic exposures.
366
367 ***Using zebrafish to investigate whether microbiota modify the developmental neurotoxicity of***
368 ***environmental chemicals***
369
370 Zebrafish are an excellent model for assessing microbiota as a modifying factor for the
371 developmental neurotoxicity of environmental chemicals (Figure 3). This is in large part due to
372 three key factors. One, because zebrafish develop external to the mother, researchers can directly
373 expose the developing embryos to xenobiotic agents. Two, because the organism initially develops
374 within an acellular chorion, it is relatively simple to generate axenic zebrafish. Three, axenic and
375 colonized zebrafish exposed to chemicals can be assessed for developmental neurotoxicity using
376 a wide array of automated behavioral assays (Figure 1).
377
378 Antibiotic treatment is commonly used to perturb microbiota. In support of a microbial link to
379 neurobehavioral development in zebrafish, conventionally reared zebrafish treated with broad
380 spectrum antibiotics exhibit hyperactivity (Phelps et al., 2017). This result is in line with
381 mammalian data reporting hyperactivity (Desbonnet et al., 2010) and increased anxiety-like
382 behavior (Bercik et al., 2011) in mice exposed to antibiotics. Interestingly, antibiotic-induced
383 hyperactivity in zebrafish phenocopies the degree of locomotor activity observed in axenic
384 zebrafish (Phelps *et al.,* 2017). From a neurotoxicology perspective, there is a single published
385 paper in zebrafish that shows a microbial-dependent behavioral effect elicited by a xenobiotic
386 exposure (Catron *et al.* 2019b). Light-phase dependent hypoactivity was reported in
387 conventionally colonized and conventionalized zebrafish, but not axenic animals, developmentally
388 exposed multiple concentrations of exogenous 17β-estradiol (Catron *et al.* 2019b). This suggests
389 that chemical-dependent hypoactivity relied on the presence of microorganisms. To our
390 knowledge, this is the first reported example showing that host-associated microbes are required
391 for the developmental neurotoxicity of a xenobiotic agent. However, despite examining microbial
392 community structure and chemical biotransformation profiles, the mechanism underlying this
393 interaction is unknown (Catron *et al.* 2019b). In contrast to 17β-estradiol, colonization status failed
394 to modify the effect of triclosan exposure on locomotor activity in zebrafish (Weitekamp *et al.,*
395 2019). Collectively, the use of the zebrafish three colonization status system coupled with targeted
396 and non-targeted analytical chemistry represents a powerful approach that can reveal toxicokinetic
397 contributions of host-associated microbes to chemical toxicity.
398
399 In addition to the three colonization cohort system, two different studies investigated the ability of
400 microorganisms to reinstate control-like behavior in xenobiotic-exposed zebrafish. In a recent
401 study, adult zebrafish were exposed to triclosan and/or the probiotic *L. plantarum* ST-III for 90
402 days (Zang *et al.,* 2019). Triclosan exposed fish exhibited increased speed and distance travelled,
403 and reduced time spent on the non-stimulus side of a T-maze assay (Zang *et al.,* 2019).
404 Supplementation with *L. plantarum* ST-III partially restored the control-like preference for the
405 non-stimulus side of the T-maze as compared to the triclosan exposed group that did not receive
406 probiotic supplementation (Zang *et al.,* 2019). In a separate study, adult wild-type zebrafish
407 exposed to ethanol for two weeks exhibited reduced anxiety-like behavior, demonstrated by

increased vertical exploration and time spent in the top part of the tank, using the novel tank assay (Schneider *et al.,* 2016). In this case, supplementation with a different probiotic strain of *Lactobacillus* (i.e. *L. rhamnosus* GG) was not sufficient to block ethanol-dependent behavioral effects (Schneider *et al.,* 2016).

In addition to aforementioned behavioral studies, there is a single zebrafish study that correlated specific microbial taxa with several transcriptional and physiological endpoints in adult zebrafish exposed to a mixture of five brominated flame retardants (i.e. pentaPBDE mixture or DE-71) (Chen *et al.,* 2018a). Interestingly, the presence of *Chlamydia*, Thaumarchaeota, or *Mycoplasma* was inversely correlated with intestinal serotonin levels (Chen *et al.,* 2018a), an essential neurotransmitter that is often disrupted in mood disorders. It remains to be seen whether chemical-dependent alterations in serotonin synthesis and/or turnover can be causally linked to dysbiosis or selection of specific taxa or to behavioral manifestations in the host.

Taken together, these studies illustrate the utility of the zebrafish model to investigate the interaction between xenobiotics and the host nervous system. These studies also support the concept that causal linkages between chemical exposures and behavioral and/or neurologically relevant molecular endpoints are possible when using the three-colonization status zebrafish experimental system. However, to truly move this field forward, more research is needed to understand the fundamental mechanisms by which microbes influence neurodevelopment and function and whether these same pathways are sensitive to disruption following exposure to widely occurring xenobiotic agents.

Conclusion

Deconstructing the influence of intestinal microbes on the neurotoxicity of environmental chemicals is an exciting and emerging field of study. Zebrafish is an excellent model with which to unravel the complex relationship between xenobiotic agents, microbiota, and the host nervous system. In this research domain, the major strength of the model is the ability to directly expose colonized and axenic zebrafish to environmental chemicals then assess the full complexity of the developing nervous system using multifaceted automated behavioral phenotyping. However, major limitations of the model should not be overlooked. Zebrafish lack major GI tract organs such as a stomach and lymph nodes that can affect their response to xenobiotic agents, particularly in the context of the microbiota-gut-brain axis. Also critical from a toxicological perspective, zebrafish microbiota is comprised of unique genera relative to mice, rats, and humans. Much more work is needed to understand whether compositional differences in taxonomy result in xenobiotic toxicokinetic and/or toxicodynamic alterations that are significant at the level of host physiology. Overall, there are major gaps in our understanding of the interactions between environmental chemicals, microbiota, and host nervous system development, function, and disease. To address this, future work should expand this innovative experimental system to include colonization with specific strains or communities of bacteria, a more diverse repertoire of automated behavioral endpoints, and the use unbiased hypothesis-generating approaches (i.e. transcriptomics, metabolomics, and/or proteomics) to ultimately illuminate novel mechanisms by which xenobiotics and microbial-products converge to modulate host nervous system development and function.

Figures

Figure 1: Using the zebrafish multi-colonization system to test whether microbiota affect the developmental neurotoxicity of environmental chemicals. The use of conventionally colonized, axenic, conventionalized, and/or monocolonized zebrafish, coupled with behavior and molecular assays, can be used to uncover interactions between host-associated microbes and xenobiotics that provoke developmental neurotoxicity in the host organism.

Figure 2: The microbiota-gut-brain axis. This axis allows for bidirectional communication between intestinal microbiota and the host nervous system. Key elements of the pathway include the vagus nerve, hypothalamus-pituitary-adrenal axis, and microbial production of bile acids, neuroactive dietary metabolites, and neurotransmitters, and microbial stimulation of neuroactive host-derived cytokines.

Figure 3: Strengths and weaknesses of animal models commonly used for microbiome research. [1]Keane *et al.,* 2011; [2]Fritz *et al,* 2013; [3]Bedell *et al.,* 1997; [4]Kostic *et al.,* 2013; [5]Howe *et al.,* 2013; [6]Patton and Zon, 2001; [7]Goldsmith and Jobin, 2012; [8]Rawls *et al.,* 2004; [9]Trinder *et al.,* 2017; [10]Koyle *et al.,* 2016; [11]Schafer, 2005; [12]Clark and Walker, 2018.

Figure 4: A framework for microbiota-xenobiotic interactions. Axenic zebrafish (Catron *et al.,* 2019b; Davis *et al.,* 2016a; Phelps *et al.,* 2017; Weitekamp *et al.* 2019), mice (Diaz Heijtz *et al.,* 2011; Neufeld *et al.,* 2011), and flies (Schretter *et al.,* 2018) exhibit hyperactivity, as do zebrafish (Phelps et al., 2017) and mice (Desbonnet et al., 2010) exposed to antibiotics. Chemical exposures can elicit dysbiosis of host-associated microbes. Chemical-selected microbes harbor the capacity to biotranform xenobiotic agents (i.e. Toxicokinetic interaction). More work is needed to understand whether chemical-induced dysbiosis or altered xenobiotic transformations cause developmental toxicity in the host organism (i.e. Toxicodynamic interaction).

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Figure 1

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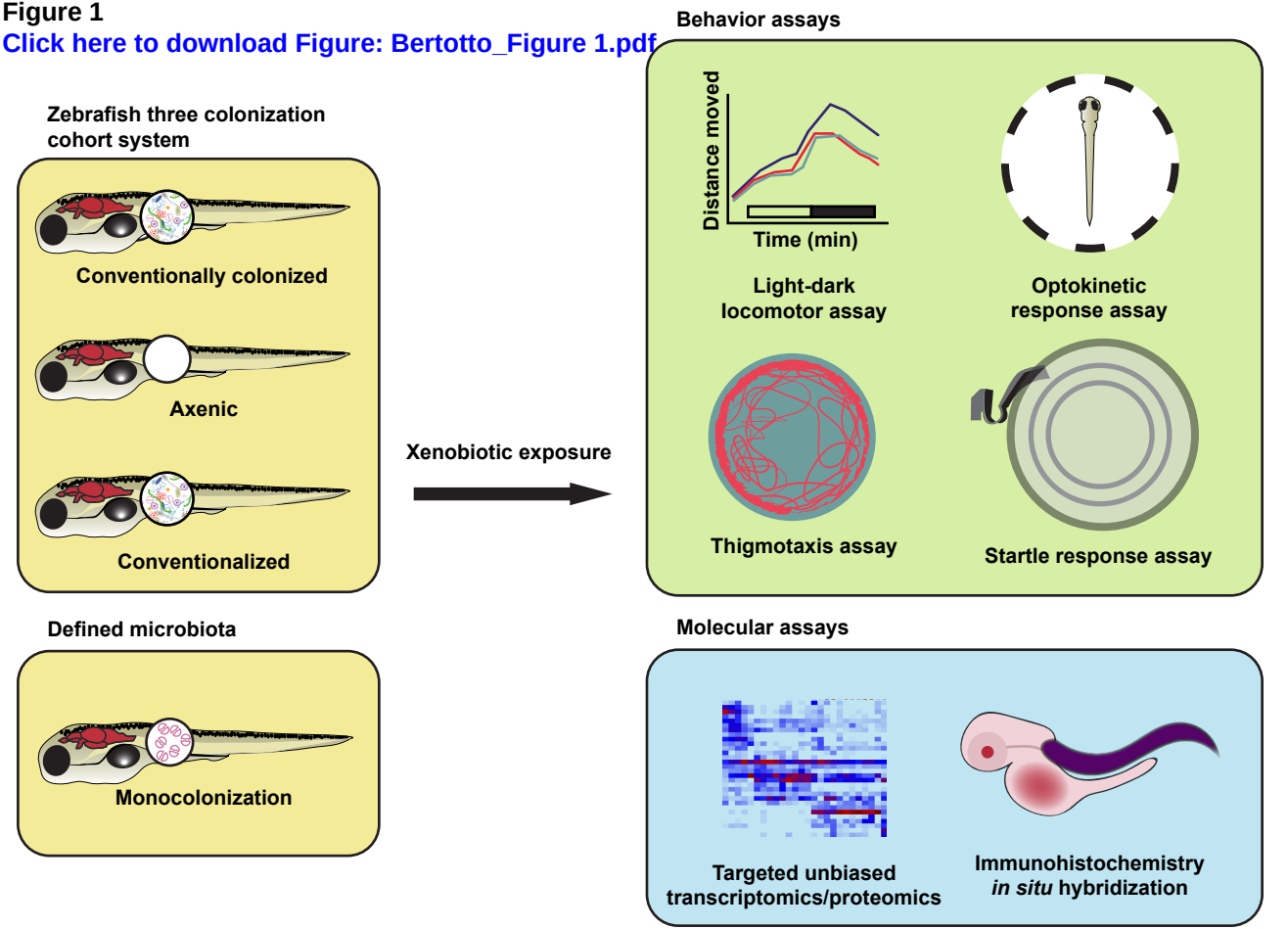

Figure 2

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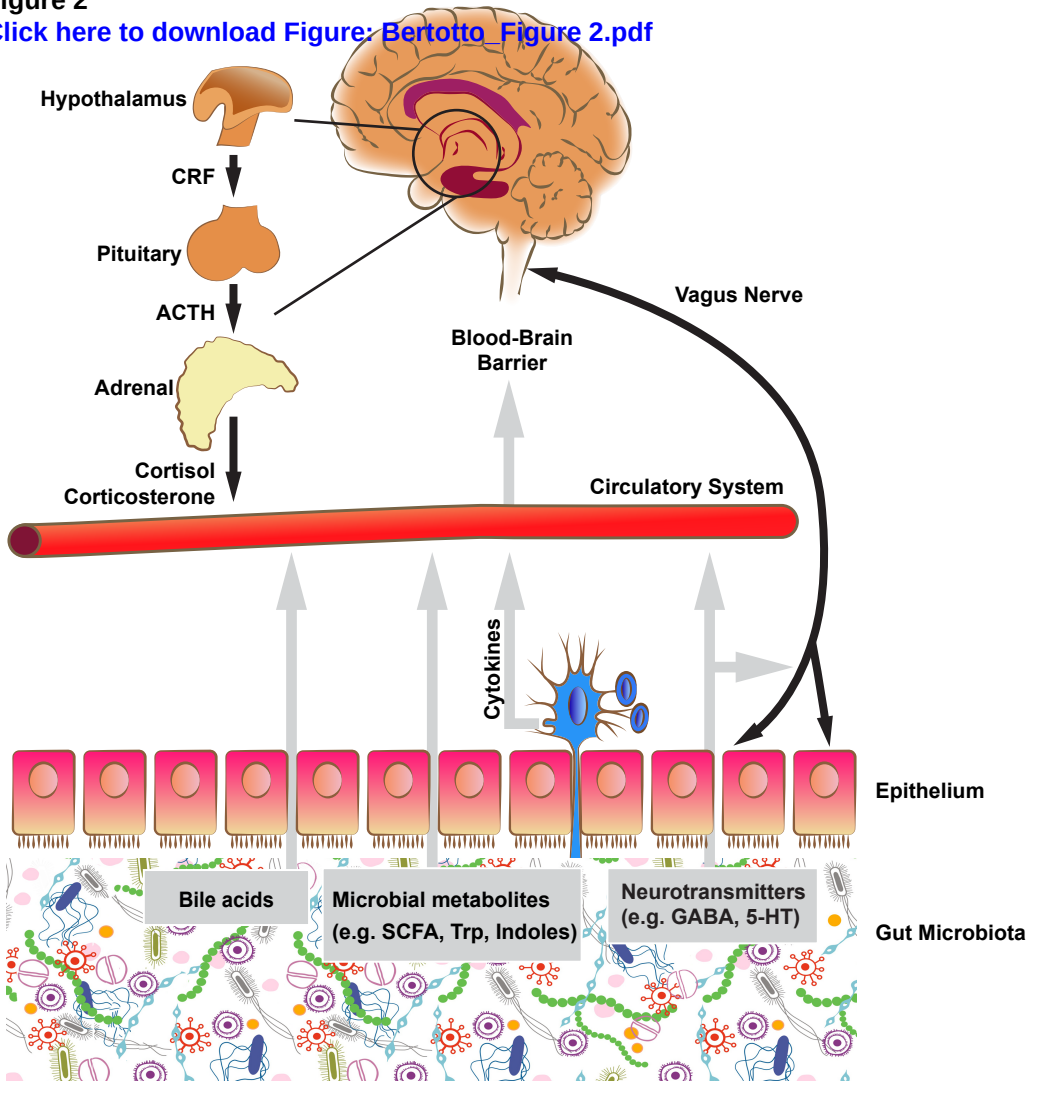


Figure 3

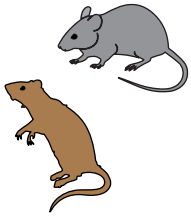
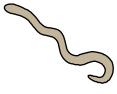
Animal Model	Advantages	Disadvantages
Mouse (*M. musculus*) and Rat (*R. novergicus*) 	• High genetic conservation with the human genome (~80-90%)[1, 2] • Large availability of genetically modified and disease-specific models[3] • Gastrointestinal (GI) tract functions, anatomical structure, and metabolism similar to humans[2] • Microbiota composition similar to humans (dominated by Firmicutes and Bacteroidetes)[4]	• Long gestational period[2] • Diet differs from humans[2] • Cage specific differences in microbiota community structure[2, 4] • **Need to maintain axenic breeding colony for developmental toxicology studies[2, 4]** • Low throughput for toxicity testing[2, 4]
Zebrafish (*D. rerio*) 	• Considerable genetic conservation with the human genome (~70%)[5] • Large availability of genetically modified models[6] • High fecundity, eggs released every one-to-two weeks[6] • Transparent during early development[6] • **Axenic animals can be derived using simple and well-established protocols[8]** • **Simple mono or conventional colonization by immersion[8]** • Diversity of automated behavioral assays to assess microbiota-gut-brain axis[6] • Medium-throughput model for toxicity testing[6]	• GI track lacks distinguishable lymph nodes, splenic germinal centers and Peyer's patches[7] • Diet and environment significantly differ from humans[2] • Microbiota composition markedly different from human (dominated by Proteobacteria and Fusobacteria)[4] • No established protocols for generating axenic
Fruit fly (*D. melanogaster*) 	• Highly fecund, eggs released every day[9] • Rapid development and generation time[9] • Axenic animals can be derived using simple and well-established protocols[9] • Mono or conventional colonization via diet[10] • High-throughput potential for toxicity testing[9]	• Low conserved homology with human genome (~50%)[9] • GI tract consists of a simple epithelium, surrounded by visceral muscles, nerves, and tracheae[9]
Nematode worm (*C. elegans*) 	• Highly fecund, eggs released every day[11] • Rapid development and generation time[12] • Transparent until adulthood allowing real-time visualization[12] • Axenic animals can be derived using simple and well-established protocols[12] • Mono or conventional colonization via bacterial diet[12] • High-throughput screening potential[12]	• Low conserved homology with human genome (~35%)[12] • Basic GI tract consisting of a tube of enterocyte cells[12] • Microbiota composition typically consists of a

Figure 4

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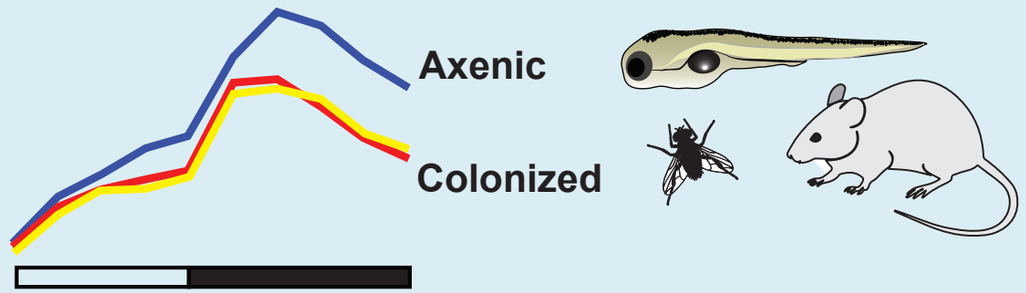
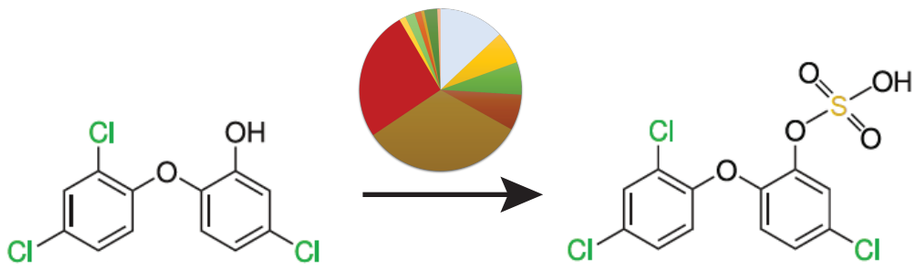
A framework for microbiota-xenobiotic interactions

I. Axenic Hyperactivity



II. Chemical exposures cause dysbiosis

III. Chemical-selected microbes perform biotransformations



Toxicodynamic interaction

Toxicokinetic interaction