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Bifenthrin causes transcriptomic alterations in mTOR and ryanodine receptor-dependent signaling and delayed hyperactivity in developing zebrafish (*Danio rerio*)

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ABSTRACT

Over the last few decades, the pyrethroid insecticide bifenthrin has been increasingly employed for pest control in urban and agricultural areas, putting humans and wildlife at increased risk of exposure. Exposures to nanomolar (nM) concentrations of bifenthrin have recently been reported to alter calcium oscillations in rodent neurons. Neuronal calcium oscillations are influenced by ryanodine receptor (RyR) activity, which modulates calcium-dependent signaling cascades, including the mechanistic target of rapamycin (mTOR) signaling pathway. RyR activity and mTOR signaling play critical roles in regulating neurodevelopmental processes. However, whether environmentally relevant levels of bifenthrin alter RyR or mTOR signaling pathways to influence neurodevelopment has not been addressed. Therefore, our main objectives in this study were to examine the transcriptomic responses of genes involved in RyR and mTOR signaling pathways in zebrafish (*Danio rerio*) exposed to low (ng/L) concentrations of bifenthrin, and to assess the potential functional consequences by measuring locomotor responses to external stimuli. Wildtype zebrafish were exposed for 1, 3 and 5 days to 1, 10 and 50 ng/L bifenthrin, followed by a 14 d recovery period. Bifenthrin elicited significant concentration-dependent transcriptional responses in the majority of genes examined in both signaling cascades, and at all time points examined during the acute exposure period (1, 3, and 5 days post fertilization; dpf), and at the post recovery assessment time point (19 dpf). Changes in locomotor behavior were not evident during the acute exposure period, but were observed at 19 dpf, with main effects (increased locomotor behavior) detected in fish exposed developmentally to bifenthrin at 1 or 10 ng/L, but not 50 ng/L. These findings illustrate significant influences of developmental exposures to low (ng/L) concentrations of bifenthrin on neurodevelopmental processes in zebrafish.

1. Introduction

Since the production of photostable pyrethroids in the 1970s, pyrethroid insecticides have been increasingly employed for insect control over the last four decades (Schleier and Peterson, 2011), and they now represent the fourth largest group of insecticides used worldwide (Kuivila et al., 2012; Brander et al., 2016a). Moreover, in the past decade, bifenthrin, a broadly used pyrethroid, has been increasingly employed in urban areas (Jiang et al., 2012; Weston and Lydy, 2012; Saillenfait et al., 2015). As a result, there is the potential for high influx of bifenthrin into surface waters, particularly following heavy rains. Bifenthrin is commonly detected in surface waters in the low ng/L range (Weston and Lydy, 2012), although concentrations as high as 106 ng/L have been measured. Additionally, bifenthrin is the most frequently detected pyrethroid in river sediments in North America and Australia (Nowell et al., 2013; Allinson et al., 2015), and it has the potential to bioaccumulate in fish tissues (Munaretto et al., 2013). The latter is of particular concern because fish are considered more

Abbreviations: dpf, days post-fertilization; mTOR, mechanistic target of rapamycin; PCB, polychlorinated biphenyl; RyR, ryanodine receptor

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vulnerable to pyrethroid toxicity than mammals (Glickman and Lech, 1982).

Pyrethroids are classified as type I and type II, as defined by the absence or presence of an α-cyano-3-phenoxybenzyl moiety, respectively (Soderlund, 2012). This difference in chemical structure results in distinctive toxicologic effects: type I pyrethroids cause tremors or convulsions, while exposure to type II pyrethroids predominantly causes choreoathetosis (Casida and Durkin, 2013). Bifenthrin is a type I pyrethroid that binds to the voltage-gated sodium channels in insects to prolong the action potential in nerves; it also interacts with voltage-gated sodium channels of vertebrates (Mukherjee et al., 2010; Soderlund, 2012). More recently, pyrethroids have also been shown to affect calcium signaling via interactions with voltage-gated calcium ion channels (Soderlund, 2012), and exposure to nanomolar concentrations of bifenthrin has been shown to dysregulate Ca^{2+} homeostasis in neurons cultured from developing rodent brain (Cao et al., 2014). Calcium signaling regulates diverse neurodevelopmental processes (Berridge et al., 2000; Lohmann, 2009), and perturbation of calcium signaling in the developing brain has been linked to deficits in neurobehavior (Gargus, 2009; Stamou et al., 2013).

Whilst there is a rich literature addressing the role of intracellular calcium signaling in fish models, particularly in goldfish, *Carassius gibelio* (Johnson and Chang, 2002; Sawisky and Chang, 2005), the effects of bifenthrin on calcium signaling in fish has not been previously addressed. Therefore, a main objective of this study was to assess the effects of bifenthrin on the transcriptional profile of calcium-dependent signaling molecules, in particular, ryanodine receptor (RyR) and mTOR-dependent signaling molecules, in developing fish. RyRs are calcium-dependent calcium release channels embedded in the endoplasmic reticulum that regulate calcium-dependent signaling in neurons, and their function is critical to normal neurodevelopment (Pessah et al., 2010). The mTOR signaling pathway is also critical for normal neurodevelopment (Kumar et al., 2005; Lee et al., 2011; Bowling et al., 2014; Tang et al., 2014), and it is activated by increases in intracellular calcium (Zhang et al., 2012). Both RyR (Mackrill, 2012) and mTOR signaling molecules (Hall, 2008) are conserved throughout the eukaryotic kingdom. We recently reported transcriptional alterations in key genes of both the RyR and mTOR signaling pathways in developing zebrafish exposed to μM concentrations of polychlorinated biphenyl (PCB) 95 (Frank et al., 2017). PCB 95 is an environmental contaminant known to interfere with neurodevelopment in mammalian systems by modulating calcium-dependent signaling via RyR-dependent mechanisms (Wayman et al., 2012a; Wayman et al., 2012b). Whether classes of environmental contaminants other than PCBs that also alter calcium influx, such as bifenthrin, similarly alter the transcriptional profile of RyR and mTOR signaling molecules, and whether such transcriptional changes are associated with altered phenotypes in fish is not known.

To address these questions, we exposed wildtype zebrafish to low (ng/L) concentrations of bifenthrin during early development. Previous studies have examined both acute (Jin et al., 2009) and developmental toxicity (Shi et al., 2011; Tu et al., 2016) of bifenthrin in zebrafish, but there has been no study examining potential neurodevelopmental effects following exposure to picomolar concentrations of pyrethroids. Since the effects of developmental exposures to neurotoxic chemicals can manifest at later life stages (Levin et al., 2003), zebrafish were assessed for transcriptional and behavioral effects immediately following the cessation of bifenthrin exposure at 5 days post-fertilization (dpf), and at 19 dpf, 14 d after exposure ended.

2. Materials and methods

2.1. Chemicals

Bifenthrin (CAS# 82657-04- 3, purity > 98%) was purchased from Chem Service (West Chester, PA, USA).

2.2. Fish husbandry and spawning

All research involving zebrafish, fish husbandry and spawning were performed in accordance with UC Davis Institutional Animal Care and Use Committee (IACUC) protocol #17645. Adult wild-type, Tropical 5D zebrafish (*Danio rerio*) were kept in 2.8 L tanks at a density of 5–7 fish per L at 28.5 ± 0.5 °C on a 14 h light:10 h dark cycle in a recirculating system (ZS560 in Light Enclosure, Aquaneering, San Diego, CA, USA). Culture water pH and conductivity were continually monitored, and pH values ranged between 7.2 and 7.8; electric conductivity between 600 and 800 μS cm^{-1}. Adult fish were fed twice a day with *Artemia nauplii* (INVE Aquaculture, Inc., Salt Lake City, UT, USA) and commercial flake (a combination of Zebrafish Select Diet, Aquaneering, San Diego, CA, USA and Golden Pearls, Artemia International LLC, Fairview, TX, USA). Embryos were obtained by naturally spawning groups of eight to twelve fish in a 1:2 female/male ratio. Spawning time was coordinated using a barrier to separate male and female fish to produce age-matched fertilized eggs. Fertilized eggs were collected within 60 min of spawning.

2.3. Bifenthrin exposures and recovery period

Bifenthrin concentrations were chosen based on similar studies that evaluated the impact of bifenthrin on endocrine disruption in fish (Brander et al., 2012; DeGroot and Brander, 2014) and were within the range of concentrations present in aquatic habitats (Weston et al., 2009; Weston and Lydy, 2012; Weston et al., 2014; Weston et al., 2015a,b). Fish from five independent spawns obtained on different days were used to obtain five biological replicates (n = 5) for each treatment. Transcriptional and behavioral responses were evaluated across four time points – 1, 3, 5, and 19 dpf – in four experimental groups: three bifenthrin exposures – 1, 10 and 50 ng/L bifenthrin pre-dissolved in methanol (MeOH) – and a vehicle (0.01% MeOH v/v) control group (ASTM, 2014). Exposures were performed from 2 hpf to 5 dpf, and were followed by a 14 day recovery period to 19 dpf. A total of 480 embryos per spawn were split into groups of 30, directly transferred into 16 different glass petri dishes (100 × 20 mm; 30 embryos/dish; 4 replicates per concentration, one for every investigated time point) containing 60 mL standardized Embryo Medium (Westerfield, 2007), and placed into an incubator at a constant temperature of 28.1 ± 0.7 °C, with a 14 h light: 10 h dark photoperiod. Embryos remained in the glass petri dishes throughout the exposure period, and were randomly sampled for transcriptomic and behavioral evaluations. All fish were examined to exclude individual fish with obvious abnormalities. Water changes (80%) were performed daily and physicochemical parameters remained constant throughout the tests: pH 7.6 ± 0.2, dissolved oxygen 8.3 ± 0.3 mg/L and specific conductance of 2195 ± 85 μS cm^{-1}.

At 5 dpf, 30 fish from each group were transferred into 1.8 L glass tanks (Mason jars, Erie, PA, USA), containing 1.6 L of culture water from the recirculating system of the adult fish and maintained for a 14 d recovery period until 19 dpf. Fish were fed twice a day with commercial flake from 5 to 19 dpf. Feeding was supplemented with live *Artemia nauplii* starting at 10 dpf, and 80% water changes were performed 60 min after the second feeding took place. To rule out the possibility that differences in transcriptome and behavioral readouts in bifenthrin-exposed vs. vehicle control fish were due to differences in food consumption during the 14 d recovery period, upon completion of the behavioral assays at 19 dpf, all fish were euthanized on ice and transferred into an aluminum dish and placed overnight in a New Brunswick incubator E24 (Eppendorf, Germany) at 70 °C. The mean dry weight of fish from each exposure group (n = 5) was then measured using a Mettler Toledo AL104 precision scale (Mettler Toledo, Columbus, OH, USA, sensitivity of 0.0001 g ± 0.0002 g). The data from this experiment is presented in Fig. S5. Physicochemical parameters remained constant throughout the recovery period: pH 7.5 ± 0.1, dissolved oxygen 7.9 ± 0.2 mg/L and specific conductance 875 ± 25 μS cm^{-1}.