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Developmental exposure to organophosphate flame retardants causes behavioral effects in larval and adult zebrafish

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ABSTRACT

Background: Organophosphate flame retardants (OPFRs) have grown in usage since concerns about the health effects of the previously used polybrominated flame retardants led to their being phased out. The potential for OPFRs to cause adverse health effects of their own is still unexamined. Because of their structural similarities to organophosphate pesticides, which have themselves been heavily researched and shown to be neurobehavioral teratogens, we investigated the possibility that developmental exposure to two OPFRs, triphenyl phosphate (TPHP), and tris(1,3-dichloroisopropyl)phosphate (TDCIPP) might lead to behavioral impairment across the lifespan, as has been observed with the organophosphate pesticide chlorpyrifos.
Methods: Zebrafish were exposed to 0.03 or 0.3 μM of TPHP, TDCIPP, or chlorpyrifos from 0 to 5 days post fertilization. Vehicle control consisted of 0.03% solution of DMSO. At 6 days post fertilization, larvae were tested on a locomotor assay. Separate cohorts of 6 day old larvae that were not tested on the larval assay were allowed to grow to adulthood. At 12 weeks post fertilization, these adult zebrafish were tested on a battery of behavioral assays that included tests of novel environment exploration, startle habituation, social affiliation, and predator escape.
Results: Developmental exposure altered zebrafish behavior across the lifespan. Larval zebrafish exposed to the 0.03 μM doses of chlorpyrifos or TDCIPP exhibited significant ($p < 0.05$) hyperactivity in the locomotor assay. Organophosphate exposure significantly ($p < 0.05$) altered the time course of adult zebrafish behavior in the novel environment, startle habituation, and social affiliation assays. Predator escape behavior was significantly ($p < 0.05$) reduced in fish exposed to the 0.3 μM dose of TDCIPP. Exposure also caused hyperactivity in adult fish, with fish exposed to the 0.3 μM dose of TDCIPP exhibiting significantly ($p < 0.05$) elevated locomotor behavior in the novel environment assay.
Discussion: Early developmental exposure to OPFRs produced behavioral impairment that persisted into adulthood. These findings support broader research investigating the role of organophosphate compounds, including the OPFRs used here, in developmental neurotoxicity.

1. Introduction

Recently, growing concern regarding the safety of common flame retardants has led to phase outs of the older polybrominated flame retardants and replacement with several new categories of flame retardants. Among these replacements are organophosphate flame retardants, or OPFRs. It has become apparent, since then, that exposure to OPFRs is widespread. Two common OPFRs, tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) and triphenylphosphate (TPHP), have been found in over 96% of samples of house dust and furniture foam, with levels as high as 1.8 mg/g (Stapleton et al., 2009; Meeker and Stapleton, 2010), and in elementary schools at levels as high as 0.27 mg/g (Mizouchi et al., 2015). TDCIPP has similarly been found in foam inside of infant products (Stapleton et al., 2011) and in handwipe samples of children (Hoffman et al., 2015; Stapleton et al., 2014). Correspondingly, metabolites of TPHP, TDCIPP, and other OPFRs can be found in the urine of adults (Carignan et al., 2013; Meeker et al., 2013), including pregnant women and paired mothers and children (Butt et al., 2014; Hoffman et al., 2014).

Despite the growing consensus regarding widespread OPFR exposure, there has been little research concerning whether or not these compounds are actually safer than their predecessors. Specifically, the structural similarities of OPFRs to organophosphate (OP) pesticides, which have been extensively researched and shown to cause persisting toxicity, might point to a reason to be concerned regarding possible toxic effects of OPFRs. OP pesticides such as chlorpyrifos have been revealed to be especially risky with early-life exposures causing persisting neurobehavioral impairments (see Eaton et al., 2008 for a review).

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Rodents exposed developmentally to OP insecticides display abnormal sensorimotor response emotional dysfunction, and cognition as adults (Aldridge et al., 2005, Roegge et al., 2008, Timofeeva et al., 2008a; Timofeeva et al., 2008b). In zebrafish, a rapidly growing model for studies of neurobehavioral toxicities (Bailey et al., 2013, Bailey et al., 2015), early-life exposures to chlorpyrifos causes deficits in numerous domains of sensorimotor behavior, emotional function, and cognition (Levin et al., 2003; Levin et al., 2004; Eddins et al., 2010; Sledge et al., 2011), mirroring the findings found in rodents.

With the evidence for the neurobehavioral teratology of organophosphates spanning species and compounds, several of the existing studies of OPFR toxicity have started to investigate similar endpoints. Zebrafish larvae exposed developmentally to doses of OPFRs such as TDCIPP and TPHP below those that cause overt physiological toxicities display abnormal locomotor behavior in light and dark environments (Dishaw et al., 2014; Noyes et al., 2015). One study found that, although developmental exposure to TDCIPP did not cause behavioral effects or alter monoamine levels in larval zebrafish, females exposed through to adulthood showed depressed levels of both dopamine and serotonin later in life (Wang et al., 2015). Correspondingly, in vivo work has shown that TDCIPP can affect cellular function in the PC12 cell line and increase its differentiation into both cholinergic and dopaminergic cell types (Dishaw et al., 2011).

It is not yet understood whether early-life exposure to OPFRs can cause behavioral or cognitive abnormalities that persist into adulthood, as seen with OP pesticides. The present study seeks to examine whether developmental exposure to the OPFRs TDCIPP and TPHP leads to changes in behavior in larval and adult zebrafish. These exposures were done at doses equimolar to those of chlorpyrifos previously shown to cause lasting behavioral effects in zebrafish (Levin et al., 2003; Levin et al., 2004; Eddins et al., 2010; Sledge et al., 2011). Zebrafish exposed to two doses of chlorpyrifos, TDCIPP, or TPHP (Appendix Fig. 1) for the first 5 days post fertilization were tested on behavioral endpoints both as larvae and as adults.

2. Methods

2.1. Subject housing and husbandry

Zebrafish (AB* strain) were bred from a colony originating with progenitors obtained from the Zebrafish International Resource Center (ZIRC, Eugene, OR, USA). Breeding tanks of N = 12–15 were maintained with a male-to-female ratio of 2:1. Eggs were collected via in-tank inserts approximately 1–2 h after the lights-on phase of a 14:10 h light:dark cycle. Eggs from approximately 6 such tanks were combined and rinsed with 10,000× diluted solution of bleach for 1 min, followed by 3 likewise rinses in fresh aquarium water. Eggs were inspected under a dissection microscope and unfertilized or otherwise abnormal eggs were discarded. Approximately 5 h post fertilization, eggs (N = 60) were randomly distributed into glass Petri dishes corresponding to differing exposures and placed in an incubator held at 29 °C and illuminated with an identical 14:10 light cycle until 6 days post fertilization.

Fish aged 6 days post fertilization and older were housed in 3 L tanks on a circulating aquarium rack system (Aquatic Habitats/Pentair Aquatic Eco-Systems, Apopka, FL, USA). Aquarium water was made from a mixture of sea salt (Instant Ocean, 0.5 parts per thousand) and buffer (Seachem Neutral Regulator, 2.5-g/19 L H_2O) dissolved in de-ionized water and was maintained at 26 °C. Water chemistry, salinity, and temperature were monitored biweekly. Fish were fed twice per day: a suspension of 24-h-old brine shrimp raised in-house (origin Brine Shrimp Direct, Ogden, UT, USA) in the morning and solid food (TetraMin Tropical Flakes, Blacksburg, VA, USA) in the evening. Younger fish (until 3–4 weeks post fertilization) were supplemented with smaller-particle solid food (Brine Shrimp Direct Golden Pearl). All adult behavioral testing was conducted between 11:00 AM (2 h post lights-on) and 6:00 PM, with testing time counterbalanced across experimental groups. On days of behavioral testing, the evening feeding was withheld until testing was complete. All larval testing was run between 3:00 PM and 5:00 PM.

2.2. Chemical exposures

At 5 h post fertilization, zebrafish eggs (N = 60) were placed in separate glass Petri dishes in 40 ml of solutions of chlorpyrifos, tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), or triphenyl phosphate (TPHP) (Sigma–Aldrich, St. Louis, MO, USA), at either 0.03 or 0.3 μM. DMSO 0.03% in aquarium water served as a vehicle control. These solutions were renewed every 24 h, through 5 days post fertilization. Hatched larvae were then placed into fresh aquarium water for 24 h, at which point they were examined under a dissecting microscope. Larvae exhibiting arrested development or malformations were discarded. All other larvae were transferred to the aquarium rack system or prepared for the larval motility assay.

2.3. Larval motility assay

After 6-day-old larvae were inspected, they were placed into 96-well plates with glass well inserts each with 0.5 ml of aquarium water (n = 23–28 per exposure condition, over two exposure replicates). Exposure conditions were all represented within each plate and across multiple plates. Plates were then returned to the incubator for an hour before being placed into a DanioVision™ lightbox running EthoVision XT® tracking software (Noldus, Wageningen, The Netherlands). Locomotor activity was tracked during a paradigm in which an initial 10-min acclimation period in the dark (0% illumination) was followed by 2 cycles of 10 min at 100% illumination (5000 lx) and 10 min at 0% illumination. An infrared camera tracked larval locomotion across the 50-min trial. EthoVision XT® was used to calculate the average distance moved in cm per minute for each subject.

2.4. Adult behavioral test battery

6-day-old larvae distinct from those used in the larval motility assay were transferred to the aquarium rack systems and allowed to age normally to 12 weeks of age (n = 24–31 per exposure condition over two exposure replicates). Over approximately the following 2 weeks, the adult fish were run through a series of behavioral tests assessing various behavioral and cognitive functions.

2.5. Novel environment exploration

Adult fish were assessed for novel environment exploration in a test described previously (Bencan and Levin, 2008; Levin et al., 2007). Briefly, fish were placed into 1.5-L tanks filled 10 cm high with aquarium water. A camcorder feeding into EthoVision XT® software was used to track the position of the fish across a 5-min trial and to calculate both the average distance moved in cm per minute and the average distance from the floor of the tank in cm per min of the test.

2.6. Startle habituation

Habituation to a startling stimulus was tested in adult fish via a protocol used previously (Eddins et al., 2010; Sledge et al., 2011). Adult fish were placed into 40 ml of water in one of 8 translucent plastic cups (5.7 cm in diameter) arranged in a 4 × 2 array. Below each cup was a centrally located push solenoid that could be controlled to deliver a sudden tap to the bottom of the cup. A camcorder positioned above the cups was used to record the locomotion of the fish in all 8 cups at once. Following a 5-min acclimation period after the fish were placed in the cups, 10 taps were delivered once per minute over 10 min. EthoVision XT® software was used to control the solenoids and to calculate the average distance moved in cm during the 5 s preceding and following each of the 10 taps.