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Developmental Exposure to an Organophosphate Flame Retardant Alters Later Behavioral Responses to Dopamine Antagonism in Zebrafish Larvae

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Abstract

Human exposure to organophosphate flame retardants (OPFRs) is widespread, including pregnant women and young children with whom developmental neurotoxic risk is a concern. Given similarities of OPFRs to organophosphate (OP) pesticides, research into the possible neurotoxic impacts of developmental OPFR exposure has been growing. Building upon research implicating exposure to OP pesticides in dopaminergic (DA) dysfunction, we exposed developing zebrafish to the OPFR tris(1,3-dichloroisopropyl) phosphate (TDCIPP), during the first 5 days following fertilization. On day 6, larvae were challenged with acute administration of dopamine D_1 and D_2 receptor antagonists and then tested in a light-dark locomotor assay. We found that both developmental TDCIPP exposure and acute dopamine D_1 and D_2 antagonism decreased locomotor activity separately. The OPFR and DA effects were not additive; rather, TDCIPP blunted further D_1 and D_2 antagonist-induced decreases in activity. Our results suggest that TDCIPP exposure may be disrupting dopamine signaling. These findings support further research on the effects of OPFR exposure on the normal neurodevelopment of DA systems, whether these results might persist into adulthood, and whether they interact with OPFR effects on other neurotransmitter systems in producing the developmental neurobehavioral toxicity.

Keywords

Organophosphate; flame retardant; behavior; dopamine; zebrafish; development

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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 compounds. Among these replacements are organophosphate flame retardants, or OPFRs. It has become apparent, since then, that exposure to OPFRs is widespread. The common OPFR tris(1,3-dichloroisopropyl) phosphate (TDCIPP) has been found in over 96% of samples of dust and furniture foam, with levels as high as 1.8 mg/g (Stapleton et al., 2009; Meeker and Stapleton, 2010; Canbaz et al., 2016), in elementary schools at levels as high as 0.27 mg/g (Mizouchi et al., 2015), and in daycare centers at levels up to 0.33 mg/g (Wu et al., 2016). TDCIPP has been found in foam inside of infant products (Stapleton et al., 2011) and in hand wipe samples of children (Hoffman et al., 2015; Stapleton et al., 2014). Correspondingly, metabolites of TDCIPP and other OPFRs can be found in the urine of adults (Carignan et al., 2013; Meeker et al., 2013) and pregnant women and paired mothers and children (Butt et al., 2014; Hoffman et al., 2015), as well as in human hair and fingernails (Liu et al., 2016) and placentas (Ding et al., 2016).

It is clear from these studies that pregnant mothers, infants, and children all likely receive significant exposure to organophosphate pesticides. An emerging concern, then, is whether these organophosphate flame retardants pose a developmental neurotoxic risk on par with other organophosphate compounds, such as organophosphate pesticides. A variety of epidemiological studies have linked these pesticides to abnormal neurobehavioral development in human populations. Prenatal exposures have been linked to impairments in the development of normal reflexes (Engel et al., 2007) and social functions (Furlong et al., 2014), and in lower IQ scores (Rauh et al., 2011; Bouchard et al., 2011). Children exposed to OP pesticides prenatally also score lower on other indices of normal neurobehavioral development (Rauh et al., 2006), including indicators for attention deficit disorders (Marks et al., 2010). Studies in rodent models have replicated this epidemiological evidence. Rat pups exposed to chlorpyrifos, one of the most widely used OP pesticides, during gestation display abnormal exploratory behavior and neuromuscular development (Chanda and Pope, 1996), behavioral effects generally replicated if the exposure is moved to the early postnatal period (Dam et al., 2000). Effects arising from exposures during multiple exposure periods, including exposures spanning gestation and early postnatal periods, have been shown to persist into adulthood in rodents, and expand into multiple behavioral domains including measures of cognitive performance and memory (Levin et al., 2001; Levin et al., 2002) and anxiety and reward-seeking (Aldridge et al., 2005a; Ricceri et al., 2006; Braquenier et al., 2010; Carr et al., 2015).

Recently, a growing number of studies investigating the behavioral effects of developmental OP pesticide exposure have been conducted in the zebrafish model. Developmental exposure to the OP pesticide chlorpyrifos has been shown to cause long-term effects on novel tank exploratory behavior (Sledge et al., 2011), spatial learning (Levin et al., 2003; Sledge et al., 2011) and the response to a startling stimulus (Eddins et al., 2010) in adult zebrafish after embryonic exposure. Behavioral effects of OP exposure have also been observed in larval motility shortly following the end of a developmental exposure (Levin et al., 2004; Richendrfer et al., 2012; Dishaw et al., 2014). As seen in rodents, the magnitude of some behavioral effects is sensitive to the developmental window during which the zebrafish are exposed (Sledge et al., 2011).

Zebrafish studies examining the developmental neurotoxicity of OPFRs have found that exposures to various OPFRS produce morphological abnormalities and behavioral abnormalities in a similar range of concentrations in which chlorpyrifos has effects on the same endpoints (Dishaw et al., 2014; Noyes et al., 2015; Oliveri et al., 2015; Sun et al., 2016). Additionally, exposing adult fish to TDCIPP produces behavioral abnormalities in the larval offspring, implicating OPFRs in trans-generations developmental neurotoxicity (Wang et al., 2015a). It is worth noting that several studies in zebrafish have shown that multiple OPFRs have no acetylcholinesterase inhibitory activity (Wang et al., 2015b; Sun et al., 2016), suggesting that if these compounds do share that neurotoxic mechanism with organophosphate pesticides, it is likely other mechanisms are involved. One investigation into neurochemical effects found no changes in levels of dopamine in larval zebrafish following a short developmental exposure to TDCIPP, but following a chronic exposure paradigm from the embryonic stage through adulthood did lead to reduced dopamine levels in female adult fish (Wang et al., 2015b). Another study, in the PC12 cell model, found OPFRs to be at least as potent as chlorpyrifos in promoting preferential neurodifferentiation into a dopaminergic phenotype (Dishaw et al., 2011).

The possible interaction of OPFRs such as TDCIPP with the development and functioning of dopaminergic systems mirrors effects seen following developmental exposure to organophosphate pesticides. Several studies in rodents have shown that developmental exposures to chlorpyrifos can alter dopamine content in a brain region- and age-specific fashion (Slotkin et al., 2002; Aldridge et al., 2005b; Chen et al., 2011). Similarly, several studies have identified increased synaptic release of dopamine following developmental organophosphate exposure, measured as an increase in the ratio of the dopamine metabolite DOPAC to dopamine itself, as dopamine is only metabolized into DOPAC following release (Dam et al., 1999; Slotkin et al., 2002; Aldridge et al., 2005b; Slotkin and Seidler, 2007b; Eells and Brown, 2009; Slotkin et al., 2009). The adenylyl cyclase signaling cascade, which is involved in signaling downstream from dopamine receptors, has also shown to be altered following developmental organophosphate exposure (Song et al., 1997; Aldridge et al., 2003; Meyer et al., 2003; Aldridge et al., 2004; Meyer et al., 2004; Adigun et al., 2010), linking developmental exposures to both pre- and postsynaptic elements of dopamine neurotransmission.

Given the evidence that developmental exposure to OP pesticides cause short and long-term disruption of dopamine systems, we hypothesized that the OP flame retardant TDCIPP would also have neurobehavioral effects mediated via dopamine systems. Dopamine antagonists were used as probes because we hypothesized that the TDCIPP effects on dopamine systems would be subtle and would be best detected with increased vulnerability to the behavioral effects of dopamine antagonist challenge.

Zebrafish larvae exposed to TDCIPP for the first 5 days post-fertilization, at concentration shown previously to generate hypoactivity (Dishaw et al., 2014), were then challenged with either the D_1-receptor antagonist SCH-23390 or the D_2-receptor antagonist haloperidol immediately preceding a light-dark locomotor assay.

Methods

Animal Care 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 approximately 2:1. Eggs were collected via in-tank inserts approximately 1–2 hours after the lights-on phase of a 14:10 hour light:dark cycle. Eggs from approximately six such tanks were combined and rinsed with 10,000× diluted solution of bleach for one minute, followed by three likewise rinses in fresh aquarium water. Eggs were inspected under a dissection microscope and unfertilized or otherwise abnormal eggs were discarded. Approximately five-h post fertilization, eggs were randomly distributed into glass Petri dishes corresponding to differing exposures, and placed in an incubator held at 28°C and illuminated with an identical 14:10 light cycle (lights on at 8:00 AM) until 6 days post fertilization. All behavioral testing was run between 1:00PM and 5:00PM.

Chemical Exposures

At 5 hours post fertilization, zebrafish eggs were placed in separate glass Petri dishes in 40-ml of solutions of tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) (Sigma-Aldrich, St. Louis, MO, USA, purity > 98%) at either 3 or 6 μM. DMSO 0.03% in aquarium water served as a vehicle control. These solutions were renewed every 24 hours, 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 such as spinal deformity were discarded. The larval morphology assessment included spinal deformity, reduction in size for age and viability. The degree of deformity was not quantitated. The concentration range of TDCIPP tested was below the threshold for increases in lethality and dysmorphogenesis.

The larvae then underwent another solution change into 40-mL solutions of SCH-23390 (Sigma-Aldrich, St. Louis, MO, USA, purity > 98%) or haloperidol (Abbott Labs, Abbott Park, Il, USA, purity > 98%) at 5.0 μM, or into aquarium water as a control for two hours prior to behavioral testing. From here, larvae in these dosing solutions were divided individually into four glass 96-well plates (two for haloperidol and two for SCH 23390) with n=29–33 per combination of TDCIPP exposure and antagonist dosing (14–16 per plate for each of the six exposure conditions in a 2 × 3 design: vehicle control, TDCIPP3 alone, TDCIPP6 alone × antagonist alone with three replicates, TDCIPPS+antagonist and TDCIPP6+antagonist). These 96-well plates were returned to the incubator for two hours until the larval motility assay. The dopamine concentrations used were determined by pilot studies to not in themselves cause increased dysmorphogenesis or leathality.

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=29–33 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 two hours 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 (5,000 lux) and 10-min at 0% illumination. An infrared camera tracked larval locomotion throughout the trial. EthoVision XT® was used to calculate the average distance moved in cm per minute for each subject.

Data Analysis

All statistical analyses were performed with Supernova/Statview (SAS, Cary, NC, USA). Type 1 error rate (α) was set at 0.05 for all tests. A mixed design analysis (ANOVA) with TDCIPP concentration, antagonist challenge, and antagonist type as between subject factors and illumination condition as the repeated measures was used for all behavioral tests. Individual fish was used as the statistical unit for larval testing. All of the treatment conditions were represented in each plate. Plate was used as a factor in the analysis. Fisher's least significant difference was used for all post-hoc analyses. A Huynh-Feldt adjustment was used to control for possible deviations from sphericity. In final analyses $p<0.05$ (two-tailed) was always set as threshold for statistical significance.

Results

Illumination condition ($F(1,365)=1071.42$, $p<0.001$), TDCIPP exposure ($F(2, 365)=4.20$, $p<0.05$), and antagonist challenge ($F(1,365)=74.09$, $p<0.001$) produced significant main effects, with larvae showing greater activity in the dark phases compared to the light phases, and with developmental TDCIPP exposure and acute antagonist challenge both reducing locomotor activity separately. The effects of TDCIPP were not significantly altered by changing illumination conditions, although those of dopamine antagonism were ($F(1,365)=15.11$, $p<0.001$), with antagonist-induced hypoactivity much more prominent in the dark phases of the assay than in the light. The two different antagonists did not significantly differ from each other in effect.

Additionally, we observed a significant interaction between TDCIPP exposure and later DA antagonist challenge ($F(2,365)=7.06$, $p<0.005$), with fish exposed developmentally to TDCIPP showing a blunted effect to an acute DA challenge (i.e., a smaller antagonist-induced decrease in activity) with either dopamine receptor antagonist (Figures 1–2). This effect did not significantly interact with either illumination condition or D_1 or D_2 antagonist type. Without DA antagonist challenge the 6 μM TDCIPP concentration caused a significant ($p<0.0005$) reduction in swimming activity. The lower 3 μM TDCIPP concentration did not produce a significant effect relative to the control group not exposed to TDCIPP.

Discussion

The current study was designed to examine the interactions between developmental OPFR exposure and acute pharmacological modulations of dopamine signaling. Developmental TDCIPP exposure altered the behavioral responses to acute dosing with the dopamine receptor antagonists SCH-23390 (D_1) and haloperidol (D_2). TDCIPP (6μM) blunted the effect dopamine antagonist induced locomotor hypoactivity in larvae. Both TDCIPP and dopamine antagonist treatment decreased activity but the effects were not additive. It is not the case that there was a floor effect because there was certainly room for further decreases in activity.

A number of possible mechanisms may be driving the reduced efficacy of the antagonist challenges in TDCIPP-exposed larvae. One possibility is that the developmental exposure produces a lasting increase in dopamine release that then counteracts the activity of the antagonists. However, exposed larvae do not exhibit the hyperactivity that would be expected from increased basal dopamine activity, or that is seen with acute dopaminergic agonism in locomotor assays of larval fish (Irons et al., 2013). Indeed, TDCIPP exposure produces hypoactivity, generating a behavioral effect in the same direction as dopamine antagonist effects. Previous research has shown in zebrafish that developmental exposure to chlorpyrifos, an organophosphate pesticide, causes decrease in dopamine levels along with an increase in dopamine turnover rates in larval fish (Eddins et al., 2011), while another study examining an exposure at the same concentration reported that larvae exhibited hypoactivity (Levin et al., 2004). Previous studies examining the effects of TDCIPP on zebrafish have corroborated a reduction in locomotor activity following developmental exposures to concentrations identical to those used here (Dishaw et al., 2014). Others have observed no change in dopamine levels in larval fish exposed to TDCIPP (Wang et al., 2015b), although dopamine turnover was not measured. The lack of hyperactivity in TDCIPP-exposed larvae suggests that other mechanisms by which synaptic dopamine might be increased and competing with the dopamine antagonists, such as decreased dopamine transporter expression or activity, may be possible.

A possible alternative explanation would involve a mechanism blunting post-synaptic dopaminergic processes, such as downregulation of dopamine receptors or a disruption of signaling cascades downstream of dopamine receptors. This would manifest both as a reduction in dopamine activity, explaining the hypoactivity seen independently with both TDCIPP exposure and dopamine receptor antagonism, and as a decreased sensitivity to receptor antagonism, as activity from the receptor forward would already be diminished, possibly below the level created by the antagonist on its own. While there have been no studies specifically examining developmental exposure to OPFRs or other organophosphates and effects on dopamine receptor density or activity, studies examining acute exposures to multiple organophosphate compounds in adult rats have shown it to decrease D_2 receptor binding (Naseem 1990; Choudhary et al., 2002). Additionally, multiple studies have linked exposure to organophosphate pesticides to disruptions in cell signaling components important to dopamine receptor signaling, notably including the adenylyl cyclase signaling cascade and linkage between this cascade and G-protein-coupled receptors (Song et al., 1997; Aldridge et al., 2003; Meyer et al., 2003; Aldridge et al., 2004; Meyer et al., 2004;

Adigun et al., 2010). Finally, developmental exposure to organophosphate pesticides has also been linked to changes in transcriptional profiles for the D_2 dopamine receptor and components of adenylyl cyclase signaling (Slotkin and Seidler, 2007a). None of these findings have been yet studied for organophosphate flame retardants. However. this does raise the hypothesis that these changes could underlay the observed effects that developmental TDCIPP exposure has on acute dopamine receptor antagonist response.

Of course, TDCIPP also likely has other, non-dopaminergic, mechanisms by which it can affect brain development and behavioral function. Probably, no chemical interacts with physiological processes via just one mechanism. The pattern of effects in the current study suggests non-dopaminergic effects. TDCIPP does not appear to simply act in opposition to dopaminergic agonists as an agonist. TDCIPP when given alone did not induce locomotor hyperactivity as a dopaminergic agonist would be expected to do. Likely, dopaminergic systems are just one target for TDCIPP actions on neurobehavioral development.

Further research is needed to directly link the altered behavioral response to haloperidol in TDCIPP-exposed larvae to effects of TDCIPP on dopamine systems themselves. The blunted response to the acute dopamine antagonist challenge could be a byproduct of hypoactivity caused by TDCIPP affecting other neurotransmitter systems. A non-dopaminergic effect could either inhibit responses via dopamine receptors in parallel to its effects on locomotion or lower the locomotor response to a behavioral “floor” completely independent of changes in the dopamine system. It is difficult to disentangle these possibilities without further pharmacological and neurochemical characterization of the state of dopaminergic transmission in TDCIPP-exposed fish, or without similar studies examining other neurotransmitter systems.

This study is among the first to couple developmental exposures to organophosphate flame retardants with later acute challenges to dopaminergic drugs in a behavioral paradigm. Our findings that the behavioral effects of acute dopamine receptor neurotransmission are blunted following exposure to the OPFR TDCIPP, coupled with our previous research showing lasting behavioral abnormalities after developmental exposures to lower concentrations of TDCIPP and another OPFR, TPHP (Oliveri et al., 2015) suggest that these chemicals are possibly toxicants of concern. Given the growing comparisons between the OPFRS and organophosphate pesticides (themselves well-characterized neurobehavioral teratogens), as well the ubiquity of human exposure, further work characterizing the extent of the neurotoxicology of organophosphate flame retardants such as TDCIPP is needed.

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Highlights

1. The OPFR tris(1,3-dichloroisopropyl) phosphate (TDCIPP), during 5 days post-fertilization (dpf) decreased larval motility 6 dpf.
2. Acute dopamine D_1 and D_2 antagonism with SCH-23390 and haloperidol in zebrafish larvae decreased locomotor activity 6 dpf.
3. TDCIPP blunted dopamine D_1 and D_2 antagonist-induced decreases in activity.

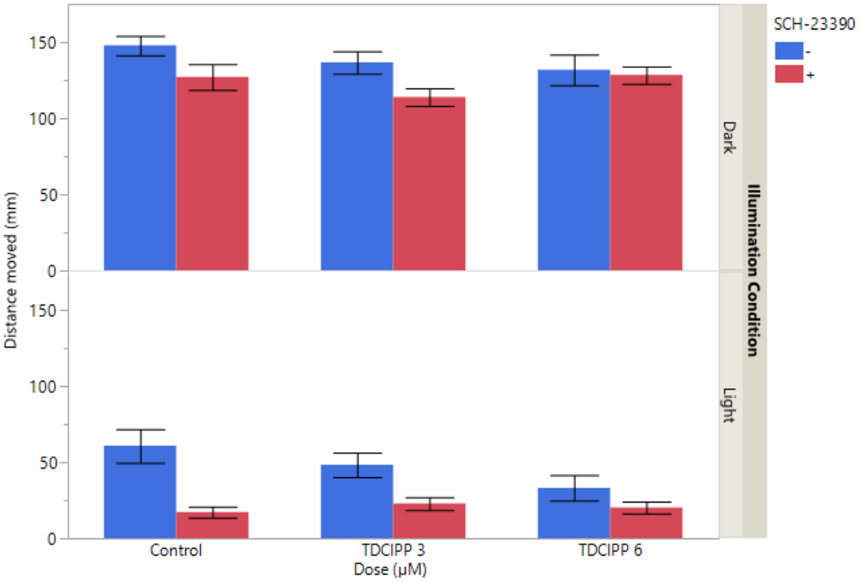

Figure 1. Developmental TDCIPP Exposure and Acute SCH-23390 Administration

TDCIPP-SCH-23390 Interactions: The mean distance moved (in mm) by larval zebrafish per minute of light and dark phases of the larval locomotor assay. Error bars represent SEM. (N=29–33 per combination of TDCIPP exposure and antagonist dosing). Significant main effects were seen for Illumination condition ($p<0.001$), TDCIPP exposure ($p<0.05$), and antagonist challenge ($p<0.001$). There was a significant interaction between TDCIPP exposure and later DA antagonist challenge ($p<0.005$), with fish exposed developmentally to TDCIPP showed a blunted effect to an acute DA challenge.

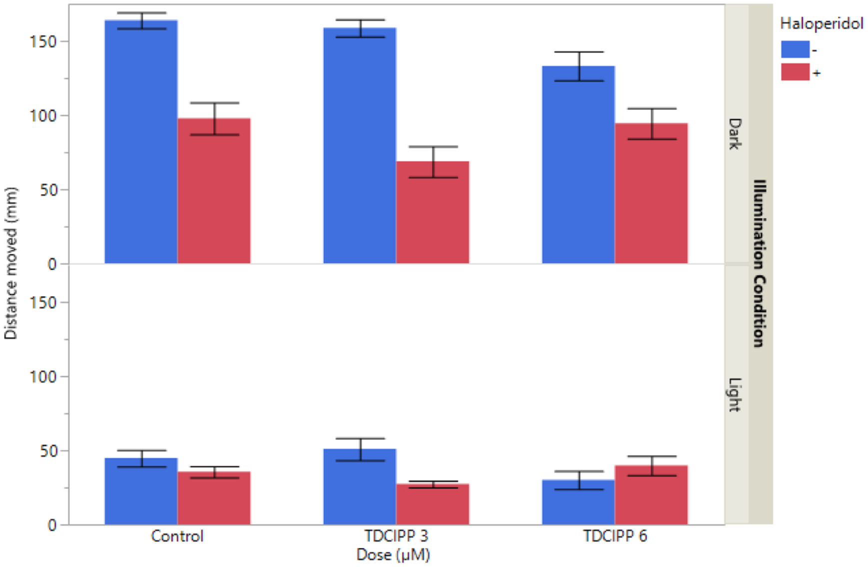

Figure 2. Developmental TDCIPP Exposure and Acute Haloperidol Administration

TDCIPP-Haloperidol Interactions: The mean distance moved (in mm) by larval zebrafish per minute of light and dark phases of the larval locomotor assay. Error bars represent SEM. (N=29–33 per combination of TDCIPP exposure and antagonist dosing). Significant main effects were seen for Illumination condition ($p<0.001$), TDCIPP exposure ($p<0.05$), and antagonist challenge ($p<0.001$). There was a significant interaction between TDCIPP exposure and later DA antagonist challenge ($p<0.005$), with fish exposed developmentally to TDCIPP showed a blunted effect to an acute DA challenge.