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Chlorpyrifos and malathion have opposite effects on behaviors and brain size that are not correlated to changes in AChE activity

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ABSTRACT

Organophosphates, a type of neurotoxicant pesticide, are used globally for the treatment of pests on croplands and are therefore found in a large number of conventional foods. These pesticides are harmful and potentially deadly if ingested or inhaled in large quantities by causing a significant reduction in acetylcholinesterase (AChE) activity in the central and peripheral nervous system. However, much less is known about the effects of exposure to small quantities of the pesticides on neural systems and behavior during development. In the current study we used zebrafish larvae in order to determine the effects of two of the most widely used organophosphates, chlorpyrifos and malathion, on zebrafish behavior and AChE activity. Embryos and larvae were exposed to the organophosphates during different time points in development and then tested at 5 days post-fertilization for behavioral, neurodevelopmental and AChE abnormalities. The results of the study indicate that chlorpyrifos and malathion cause opposing behaviors in the larvae such as swim speed (hypoactivity vs. hyperactivity) and rest. Additionally, the pesticides affect only certain behaviors, such as thigmotaxis, during specific time points in development that are unrelated to changes in AChE activity. Larvae treated with malathion but not chlorpyrifos also had significantly smaller forebrain and hindbrain regions compared to controls by 5 days post-fertilization. We conclude that exposure to very low concentrations of organophosphate pesticides during development cause abnormalities in behavior and brain size.

1. Introduction

Organophosphates are a class of neurotoxicant pesticides that are widely used on croplands worldwide. In the United States, 60 million pounds of organophosphates are used on about 60 million acres of land every year (EPA, 2011). Two of the most widely used organophosphates are chlorpyrifos and malathion (NASS, 2011). At high levels these pesticides work by inhibiting the activity of acetylcholinesterase (AChE) thereby leading to an abundance of acetylcholine in the synapses of neurons. This can lead to paralysis, trouble breathing, and even death (CDC, 2011). These acute effects have been observed in crop workers that have been exposed to these pesticides without proper protection. Lesser information is known about the effects of small quantities of these pesticides over longer durations, especially during development. Some of the developmental effects of organophosphates include delayed motor and digestive tract development, spinal deformities, edema, decreases in body weight and brain volume, reproductive dysfunction and sex dependent abnormalities in responses to social cues (Condette et al., 2015; De Felice et al., 2015, 2014; Jin et al., 2015; Mullins et al., 2015; Yu et al., 2013).

Exposure to pesticides can occur through several different routes. These include ingestion by mouth, dermal contact, or through inhalation of air and dust particles. Organophosphate pesticides are found on a large number of conventional non-organic fruits, vegetables, and grains (PAN, 2011). They have also been detected in air and dust samples, including in homes and day cares (Morgan et al., 2004). The potential exposure during childhood is concerning because children have more susceptible immune systems and are still physically developing (PAN, 2011). There have already been documented cases of neurodevelopmental and neuropsychological disorders such as attention deficit hyperactivity disorder (ADHD), autism spectrum disorders (ASD), anxiety, and depression in correlation to relatively high levels of pesticide exposure in children (Bouchard et al., 2010; Chen et al., 2011; Rauh et al., 2006).

The zebrafish is an excellent model to study the developmental effects of low levels of pesticides or other toxicants in order to determine behavioral and morphological changes after exposure

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(Eddins et al., 2010; Levin et al., 2011; Richendrfer and Creton, 2013; Richendrfer et al., 2014, 2012b). Zebrafish embryos develop outside of the mother and can be collected daily in very large numbers and the embryos can be treated directly in a petri dish with various toxicants, drugs, or pesticides. Since zebrafish larvae are transparent, they are frequently used for whole specimen imaging. The large number of transgenic zebrafish available also makes it convenient to visualize gene expression and protein localization using various fluorescent proteins (Park et al., 2000). Zebrafish embryos develop rapidly and exhibit swimming behavior, hunting, avoidance, and escape behaviors within the first week of development (Colwill and Creton, 2011a,b) making them useful for behavioral analysis. A unique behavioral assay has been created in our lab which is used to assess behavioral changes in zebrafish larvae after exposure to toxicants (Pelkowski et al., 2011; Richendrfer and Creton, 2013; Richendrfer et al., 2012a). The assay can detect very subtle differences in behavior such as swim speed, amount of rest, avoidance behavior and thigmotaxis (Pelkowski et al., 2011; Richendrfer and Creton, 2013; Richendrfer et al., 2012a).

In the current study, we exposed zebrafish to low levels of chlorpyrifos and malathion that are relevant to levels that are found in the human diet (Lu et al., 2008, 2006). Larvae were exposed during different days of development and then analyzed for behavior at 5 days post fertilization (dpf), a timepoint chosen because larval activity does not change at this age with external feeding (Clift et al., 2014). Concurrent time points were used in order to analyze AChE activity using the Ellman assay (Ellman et al., 1961). Our results indicate that the pesticides used have opposing effects on behavior and are able to cause changes in behavior without affecting larval AChE activity. In order to determine morphological abnormalities in the brain after treatment with pesticides, measurements were made in larvae at 3, 4, and 5 dpf in the forebrain, midbrain, and hindbrain. Differences in forebrain and hindbrain size were found after treatment of larvae with malathion but not chlorpyrifos. The results presented in the current study suggest that organophosphate pesticides have diverse effects on brain development and behavior, which should be considered when setting health and food guidelines for pregnant women and children.

2. Materials and methods

2.1. Animal care and housing

Adult wild type zebrafish were obtained from Carolina Biological Supply and have been housed at Brown University over several generations in multiple 10 and 20 gallon tanks. The transgenic line Tg(HuC:Kaede) was kindly provided by Dr. Joseph Fetcho in 2011 and has been maintained at Brown University since this time. For breeding purposes, the zebrafish were kept in mixed male and female populations under a 14 h light/10 h dark cycle. The fish were fed a combination of Gemma fish food, frozen brine shrimp, and freeze-dried bloodworms. Embryos were collected from the tanks at the beginning of the light cycle (“dawn”) and were immediately transferred to deep Petri dishes (Fisher 08-752-11Z) containing ‘egg water’ (0.06 g of Instant Ocean and 0.25 mg of methylene blue per liter of deionized water). The embryos were then treated with various dilutions of chlorpyrifos (ChemService F2057) and malathion (ChemService F2118), and grown in an incubator at 28.5 °C on a 14 h light/10 h dark cycle, and at a density of 60–70 embryos per culture dish.

2.2. Toxicant exposure

Larvae were exposed to egg water (EW), dimethyl sulfoxide (DMSO) and three concentrations of chlorpyrifos (CPF) (from 0.001 to 0.1 μM) or to four concentrations of malathion (from 0.001 to 1 μM) during different time points in development for the behavioral assays. These concentrations were chosen based upon previous results indicating that concentrations of chlorpyrifos >0.1 μM affect larval morphology (Richendrfer et al., 2012b) and because these concentrations are relevant to the levels found in the human diet (Lu et al., 2008, 2006). On days larvae were not treated, all groups were housed in egg water. Behavioral analyses were performed at 5 dpf. Therefore, some experimental groups were in the pesticide treatment during behavioral testing depending upon the time point of exposure. The same treatments were given to larvae and then AChE activity was evaluated using the Ellman assay (Ellman et al., 1961). Both chlorpyrifos and malathion were dissolved in dimethyl sulfoxide (DMSO) as 1000× stocks that were stored at −20 °C. The control groups were housed in egg water containing 0.1% DMSO. To obtain the desired final concentrations, 50 μl of each 1000× stock was dissolved into 50 ml of egg water in respective culture dishes. Dead embryos were removed daily from culture dishes and all solutions were replaced daily until 5 dpf, when behavioral analyses were conducted.

2.3. Behavioral analysis

The behavioral assays were carried out in 5 lane plates, which were made by placing a specialized mold into liquid agarose and then letting the mold cool. Five larvae were placed in each lane in 5 lane plates; the solution that the larvae were housed in was used to fill up the agarose lanes. The plates were positioned on top of a laptop screen; four plates fit onto one laptop screen. The assay utilized a PowerPoint presentation shown to the larvae for 30 min (Richendrfer and Creton, 2013) (Fig. 1). The results of the behavioral assays were used to analyze thigmotaxis, avoidance behavior, rest, and swim speed.

For the first half of the behavioral assay there were no visual stimuli (Fig. 1A), in the second half a red moving bar was shown to

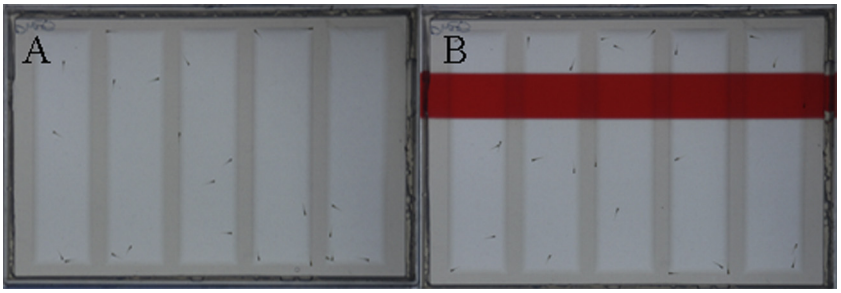

Fig. 1. Behavioral assay PowerPoint presentation. Five lane agarose plates containing five larvae per lane placed on top of a laptop screen displaying a PowerPoint presentation. Image (A) shows the blank 15 min portion of the PowerPoint presentation while image (B) shows 15 min portion of the PowerPoint presentations with the red aversive bar stimulus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)