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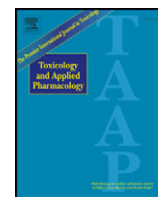

Developmental neurotoxic effects of two pesticides: Behavior and biomolecular studies on chlorpyrifos and carbaryl

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ABSTRACT

In recent times, an increased occurrence of neurodevelopmental disorders, such as neurodevelopmental delays and cognitive abnormalities has been recognized. Exposure to pesticides has been suspected to be a possible cause of these disorders, as these compounds target the nervous system of pests. Due to the similarities of brain development and composition, these pesticides may also be neurotoxic to humans. We studied two different pesticides, chlorpyrifos and carbaryl, which specifically inhibit acetylcholinesterase (AChE) in the nervous system. The aim of the study was to investigate if the pesticides can induce neurotoxic effects, when exposure occurs during a period of rapid brain growth and maturation. The results from the present study show that both compounds can affect protein levels in the developing brain and induce persistent adult behavior and cognitive impairments, in mice neonatally exposed to a single oral dose of chlorpyrifos (0.1, 1.0 or 5 mg/kg body weight) or carbaryl (0.5, 5.0 or 20.0 mg/kg body weight) on postnatal day 10. The results also indicate that the developmental neurotoxic effects induced are not related to the classical mechanism of acute cholinergic hyperstimulation, as the AChE inhibition level (8–12%) remained below the threshold for causing systemic toxicity. The neurotoxic effects are more likely caused by a disturbed neurodevelopment, as similar behavioral neurotoxic effects have been reported in studies with pesticides such as organochlorines, organophosphates, pyrethroids and POPs, when exposed during a critical window of neonatal brain development.

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Introduction

Insecticides are still widely used for their economical values as pest control worldwide, and two of the most common insecticides currently in use are organophosphate (OP) and carbamates. As these chemicals are intentionally applied, for agricultural and domestic purposes, both direct and indirect exposure to humans occur (Burns et al., 2013). This is of interest as there have been an increased incidence of neurodevelopmental disorders and dysfunctions, such as autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), developmental delays and learning disabilities associated with pesticide exposure (Bouchard et al., 2010; Shelton et al., 2014). It has been proposed that about 25% of developmental disorders resulted from direct exposure to environmental contaminants or interaction effects between environmental factors and gene susceptibility (Grandjean and Landrigan, 2006). In general, OPs and carbamates exert their acute neurotoxic effects by inhibiting the enzyme acetylcholine esterase (AChE) in the nervous system; leading to a build-up of acetylcholine in the cholinergic synapses and thereby causing cholinergic hyperstimulation (of nicotinic and muscarinic receptors). The classical symptoms of cholinergic overstimulation include salivation, lacrimation, urination and defacation; subsequently leading to muscle fasciculation, convulsions and death (Klaasen, 2001). OPs are considered to be the more adverse of the two insecticides as the AChE inhibition is considered long lasting or even irreversible (once aging has occur) compared to the carbamates, where the AChE inhibition lasts only minutes to hours. These symptoms occur when the cholinesterase inhibition surpasses ~70% (Slotkin, 2004).

In regard to the mechanism(s) behind developmental neurotoxicity (DNT), the assessment of these compounds becomes further complex. Chlorpyrifos is by far the most well-investigated OP in respect to DNT. It has been shown to elicit different behavioral aberrations in locomotor skills and cognitive performance in rats and mice, when exposed pre- or neonatally (Bjorling-Poulsen et al., 2008). Chlorpyrifos has also been connected with human neurobehavioral effects, involving delayed neurodevelopment and increased occurrence of ADHD (Rauh et al., 2006). In contrast, published DNT studies with carbamates are very sparse. This is also true for studies concerning carbaryl, a N-methyl carbamate, which is still in use today. Currently, studies on the acute effects of AChE inhibition on young and adult animals have been the basis for risk assessment (EPA, 2007). However, carbamate poisoning appears to cause similar toxic symptoms in young children as OP poisoning (Lifshitz et al., 1999); whereas the symptoms differ more between children and to adults (Lifshitz et al., 1997). Animal studies also indicate

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that neonates are more sensitive to OPs and carbamate exposure compared to adults (Pope et al., 1991; Pope, 1997; Moser et al., 2010). Moreover, neonates exposed to OPs have a higher systemic toxicity than do adults, although they recover more quickly from cholinesterase inhibition, which is largely due to higher synthesis rate of new cholinesterase and lower carboxylesterase activity in younger animals (Song et al., 1997; Vidair, 2004). Nevertheless, the pathways for metabolism of AChE-inhibitors seem to be compound specific as different OPs and carbamates show disparate metabolic patterns (Moser and Padilla, 2011). DNT effects have been elicited even when AChE inhibition levels were below ~70% (Ahlbom, 1995; Dam et al., 2000; Levin et al., 2001; Garcia et al., 2003), therefore effects have been suggested to be linked to alternative cholinergic mechanisms, or alternatively that a short period of cholinesterase inhibition is sufficient to cause disturbance in the brain development (Slotkin, 2004).

The mammalian central nervous system goes through several distinct developmental stages before reaching full maturation. During these stages the brain is more sensitive to toxic insults because of the dependence on the temporal and regional emergences of developmental processes. One of these critical periods of brain development is known as the "brain growth spurt (BGS)" and is characterized by rapid increase of brain size, because of biochemical processes such as synaptogenesis, neuronal proliferation and myelination (Dobbing and Sands, 1979). These processes are regulated by numerous proteins, such as calcium/calmodulin-dependent kinases II (CaMKII), growth-associated protein-43 (GAP-43), glutamate receptor 1 (GluR1), postsynaptic density protein-95 (PSD95), synaptophysin and tau (Wiedenmann and Franke, 1985; Navone et al., 1986; Benowitz and Routtenberg, 1997; Rongo and Kaplan, 1999; Ehrlich and Malinow, 2004; Wang and Liu, 2008; Traynelis et al., 2010). The sequence of neurodevelopmental processes is similar between human and murine animals; the primary difference is the timing of the processes. The human BGS is perinatal, starting around the third trimester, peaking around birth and extends up to the first years of life. The murine BGS is neonatal, starts from birth, peaks around postnatal day (PND) 10 and continues up to 3–4 weeks of life (Davison and Dobbing, 1968; Dobbing and Sands, 1979; Kolb and Whishaw, 1989; Semple et al., 2013).

Developmental neurotoxic insults by pesticides, such as organochlorines (OC), OPs and pyrethroids, occurring during the BGS have shown to result in behavioral and cognitive deficits not apparent until adulthood (Ahlbom, 1995; Levin et al., 2001; Lee et al., 2015). Previously, behavioral effects have also been observed in studies with other environmental contaminants, such as polybrominated biphenyl ethers (PBDEs), perfluorinated compounds (PFCs) and bisphenol A (Viberg et al., 2006, 2011, 2013). These compounds have also been shown to alter the levels of proteins important for normal brain development, when exposure occurs during the BGS (Viberg, 2009a; Viberg and Lee, 2012; Lee and Viberg, 2013).

The objective of the present study was to investigate whether a single oral exposure to an OP, chlorpyrifos, or a carbamate, carbaryl, during the peak of the BGS, can cause neurochemical changes in the protein levels of CaMKII, GAP-43, GluR1, PSD95, synaptophysin and tau in the mouse brain, and induce adult neurobehavioral disruptions. We also investigate if the possible developmental neurotoxic effects are linked to AChE inhibition in the brain.

Material and methods

Animals and chemicals

Pregnant NMRI mice were purchased from Scanbur, Sollentuna, Sweden and housed individually in plastic cages in a room with an ambient temperature of 22 °C and 12/12 h cycle of light and dark. The animals had free access to standardized food pellets (Lactamin, Stockholm, Sweden) and tap water ad libitum. The day of birth was assigned PND 0; the litters were culled to 10–12 pups within 48 h after birth. At the age of 3–4 weeks male and female mice were separated and the female mice were euthanized and the male mice were kept in their respective treatment groups, together with their siblings. Each litter contained 4–7 animals. Experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), after approval from the local ethical committee (Uppsala University and Agricultural Research Council) and by the Swedish Committee for Ethical Experiments on Laboratory Animals, approval number C195/12.

Chlorpyrifos (purity > 99%, CAS number 2921-88-2 and linear formula $C_6H_{11}Cl_3NO_3PS$) and carbaryl (purity > 99%, CAS number and linear formula $C_{12}H_{11}NO_2$) were purchased from Sigma-Aldrich, Stockholm, Sweden. Chlorpyrifos and carbaryl were dissolved in an egg lecithin (Merck, Darmstadt, Germany) and peanut oil (*Oleum arachidis*) mixture (1:10) and sonicated with water to yield a 20% (w:w) fat emulsion vehicle containing 0.01, 0.1 or 0.5 mg chlorpyrifos/ml; 0.05, 0.5 or 2.0 mg carbaryl/ml, respectively. This was done to emulate the fat content of mouse milk (~14%) for a physiologically appropriate absorption and hence distribution (Keller and Yeary, 1980; Palin et al., 1982).

Treatment

The mice used for the acetylcholine esterase (AChE)-inhibition assay were administered 5 mg chlorpyrifos/kg body weight; 5.0 or 20.0 mg carbaryl/kg body weight, respectively, as a single oral dose via a metal gastric tube, on PND 10. Control mice received 10 ml/kg of the 20% fat emulsion vehicle/kg body weight. The mice were euthanized 1, 3, 6, 12, 24 or 36 h after exposure and whole brain (without cerebellum) was dissected out, snap frozen in liquid nitrogen and stored in −80 °C until analysis. Four animals for each treatment group and time point, plus 4 control animals for each treatment group and time point were used.

The mice used for protein analysis were administered 5 mg chlorpyrifos/kg body weight; 0.5, 5.0 or 20.0 mg carbaryl/kg body weight, respectively, as a single oral dose via a metal gastric tube on PND 10. Control mice received 10 ml of the 20% fat emulsion vehicle/kg body weight. The mice were euthanized 24 h or 4 months after exposure and the hippocampus and cerebral cortex brain regions were dissected out and snap frozen in liquid nitrogen and stored in −80 °C until analysis and stored as mentioned above. Five to eight animals for each treatment group and time point were used.

The mice intended for the behavioral analysis studies were administered 0.1, 1.0 or 5 mg chlorpyrifos/kg body weight; 0.5, 5.0 or 20.0 mg carbaryl/kg body weight, respectively, as a single oral dose via a metal gastric tube on PND 10. Control mice received 10 ml of the 20% fat emulsion vehicle/kg body weight. At 2 and 4 months of age, the mice were subjected to behavioral testing, and subsequently after the second behavioral performance (4 months of age) were euthanized for brain tissue sampling. Twelve animals for each treatment group and time point were tested.

The mice were monitored for any signs of toxic symptoms (e.g. muscle fasciculation and convulsions) and abnormalities during the entire experimental period (from PND 0 to adult age) without any further treatment. Body weight was measured at PND 10, PND 11 and day of separation (PND 25) and of sacrifice (4 month of age).

AChE inhibition assay

The whole brain was weighed, homogenized and sonicated in 0.1 M K-phosphate buffer [pH 8; 0.1 M KH_2PO_4 and 0.1 M K_2HPO_4] to yield a protein content of 70 mg/ml tissue, aliquotted and stored in −80 °C until use. The homogenates were further diluted to 2 mg/ml tissue, and 10 μl of homogenate was mixed with 25 μl 0.1 M acetylthiocholine iodide, 25 μl 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and 0.1 M K-phosphate buffer to yield a total reaction volume of 2.5 ml. The absorbance was measured immediately at 412 nm and the cuvettes were

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