NeuroToxicology 36 (2013) 42–48

Contents lists available at SciVerse ScienceDirect

NeuroToxicology

Short communication

Delayed hippocampal effects from a single exposure of prepubertal guinea pigs to sub-lethal dose of chlorpyrifos: A magnetic resonance imaging and spectroscopy study

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ARTICLE INFO

Article history:
Received 11 October 2012
Accepted 3 February 2013
Available online 11 February 2013

Keywords:
Chlorpyrifos
Magnetic resonance imaging
^{1}H magnetic resonance spectroscopy
Myo-inositol
Hippocampus

ABSTRACT

This study was designed to test the hypothesis that *in vivo* magnetic resonance imaging (MRI) and spectroscopy (MRS) can detect in adulthood the neurotoxic effects of a single exposure of prepubertal guinea pigs to the organophosphorus pesticide chlorpyrifos. Twelve female guinea pigs were given either a single dose of chlorpyrifos (0.6 × LD50 or 300 mg/kg, sc) or peanut oil (vehicle; 0.5 ml/kg, sc) at 35–40 days of age. One year after the exposure, the animals were tested in the Morris water maze. Three days after the end of the behavioral testing, the metabolic and structural integrity of the brain of the animals was examined by means of MRI/MRS. In the Morris water maze, the chlorpyrifos-exposed guinea pigs showed significant memory deficit. Although no significant anatomical differences were found between the chlorpyrifos-exposed guinea pigs and the control animals by *in vivo* MRI, the chlorpyrifos-exposed animals showed significant decreases in hippocampal *myo*-inositol concentration using MRS. The present results indicate that a single sub-lethal exposure of prepubertal guinea pigs to the organophosphorus pesticide chlorpyrifos can lead to long-term memory deficits that are accompanied by significant reductions in the levels of hippocampal myo-inositol.

1. Introduction

One of the drawbacks of an increasingly modernized agricultural society is the exposure of the human population to the chemical agents used to maintain it. Though banned for residential use, organophosphorus compounds such as chlorpyrifos are widely used as agricultural insecticides, and are known to have toxic effects on the developing human brain (Marks et al., 2010; Rauh et al., 2006). Chlorpyrifos acts as an irreversible inhibitor of the enzyme acetylcholinesterase (AChE), which catalyzes the hydrolysis of the neurotransmitter acetylcholine, and as such, is vital for regulating the function of the cholinergic system in the peripheral and central nervous systems. In humans, exposure to low levels of chlorpyrifos can occur *via* ingestion of contaminated food, inhalation of airborne particles, contact with household dust and residues, and living near agricultural fields treated with the compound (Fenske et al., 2002).

The presence of chlorpyrifos in the environment poses a particular danger to children. In the general US population, urine metabolites associated with chlorpyrifos are higher in children (6–11 years) than in adults (Crinnion, 2010; Lambert et al., 2005). Children exposed to chlorpyrifos while in the womb have an increased risk of delays in mental and motor development at age three and an increased occurrence of pervasive developmental disorders such as attention deficit hyperactivity disorder (ADHD) (Rauh et al., 2006). Recent studies in rodents exposed to chlorpyrifos show that altered neurogenesis and neurotransmission may occur even without overt signs of cholinergic toxicity (Aldridge et al., 2005; Betancourt et al., 2006; Howard et al., 2005; Ricceri et al., 2006; Roy et al., 2005; Slotkin et al., 2006a,b). Chlorpyrifos can also disrupt the developing brain during glial cell proliferation and differentiation which can further contribute to alterations in myelin synthesis, changes in synaptic plasticity, and

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0161-813X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neuro.2013.02.002

in general lead to abnormalities in morphology (Garcia et al., 2005). Prenatal exposure to low level organophosphorus pesticides and in particular chlorpyrifos have been shown to be associated with poor intellectual development in 7-year old children and increased frontal and parietal cortical thinning with increased exposure (Bouchard et al., 2011; Rauh et al., 2012). The evidence of increased exposure of children to organophosphorus pesticides correlating with increased incidence of neurological deficits highlights the importance of detecting abnormalities in brain metabolites that may be used as prognostic measures of the severity of the intoxication. Given the near ubiquitous presence of these pesticides in the modern environment (Crinnion, 2010), it is recognized that there is a need to improve the detection and monitoring of their subtle, yet detrimental neurological effects.

The sensitivity, precision, and non-invasive nature of MRI makes it a valuable *in vivo* method to analyze the temporal and spatial evolution of brain pathology following exposure of guinea pigs to organophosphorus compounds (Gullapalli et al., 2010). Specifically, it has been demonstrated that T_2 is significantly shortened in different brain regions of guinea pigs exposed to this nerve agent. Measurements of transverse relaxation time (T_2 or T_2^*) were proven to be very sensitive in detecting disruption of the structural integrity of the brain following a single exposure to soman. T_2^* mapping technique is a preferred magnetic resonance imaging (MRI) method used to measure iron concentrations indirectly, with higher T_2^* relaxation times being associated with lower iron levels (House et al., 2007; Langkammer et al., 2010). Abnormal iron accumulation in brain tissue is strongly linked to oxidative stress and neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Brass et al., 2006; Berg and Youdim, 2006). It is possible that neurodegeneration stemming from chlorpyrifos exposure may be detectable with this method, with affected brain areas being characterized by shortened T_2^* relaxation times.

In addition to MRI having high sensitivity to detect subtle structural brain changes induced by neurotoxins, localized *in vivo* ^{1}H MRS provides a unique opportunity for non-invasively measuring neurotoxicant-induced alterations in brain metabolism (Xu et al., 2011). A wealth of neurochemical information can be obtained from high resolution ^{1}H MRS of the brain.

N-acetylaspartate (NAA), one of the most salient metabolites in the spectrum, is an amino acid specific to neurons, and as such, is indicative of their presence and level of function (Tallan et al., 1956; Birken and Oldendorf, 1989). In a similar manner, myo-inositol (mI) is a vital component of the phosphatidylinositol second messenger system and is a reliable marker of astrocytes (Kim et al., 2005; Brand et al., 1993). Choline (glycerophosphocholine + phosphocholine, GPC + PCh) is known to be associated with cellular density and membrane turnover (Miller et al., 1996), in addition to being the precursor of acetylcholine, a neurotransmitter greatly affected by chlorpyrifos (Cohen and Wurtman, 1975). Glutamate (Glu) and glutamine (Gln) are associated with neurotransmission, with Glu being the most abundant excitatory neurotransmitter. Glu plays a key role in long-term potentiation, a cellular mechanism underlying learning and memory (Riedel et al., 2003; McEntee and Crook, 1993), though excess of Glu in the brain has also been associated with excitotoxicity. Gln is vital to cerebral function, being involved in detoxification and regulation of neurotransmitter activities. Gln is synthesized from Glu by glutamine synthetase in astrocytes (Ross, 1991). Together, these common and reliably detectable metabolites along with structural assessment can provide important information on the metabolic and functional integrity of the brain.

In the present study we examined the effect of a single sub-lethal exposure of prepubertal guinea pigs to chlorpyrifos on the structural and biochemical integrity of the brain in adulthood. Guinea pigs (*Cavia porcellus*) were selected as the ideal animal model in this study, due both to their similarities to humans in terms of relative brain development after birth, and the sensitivity of their cholinergic system to organophosphorus pesticides. Specifically, rats and mice have higher levels of circulating carboxylesterases, enzymes that hydrolyze and inactivate organophosphorus agents, making them a far less sensitive model of exposure to organophosphorus toxicants (Albuquerque et al., 2006; Fonnum et al., 1985; Ecobichon et al., 1978). Guinea pigs also give birth to young that are neuroanatomically mature and are a closer match to humans in the timing of prenatal and postnatal development (Dobbing and Sands, 1970).

2. Methods

2.1. Animal model

A total of 12 female Hartley Guinea Pigs ([Crl(HA)Br]; 35–40 days old) were obtained from Charles River Laboratories (Wilmington, MA) and acclimated for a period of seven days before treatment. Animals were kept in a light- and temperature-controlled animal care facility, with food and water provided *ad libitum*. Individual animals were randomly and equally assigned to either the chlorpyrifos treatment group or the vehicle group. The treatment group was injected subcutaneously between the shoulder blades with chlorpyrifos (ChemService, West Chester, PA) dissolved in peanut oil (300 mg/kg, from Sigma Aldrich, St. Louis, MO), and the vehicle group was injected with peanut oil (0.5 ml/kg, sc).

The oral LD50 of chlorpyrifos in guinea pigs is 504 mg/kg, and in general, the oral and subcutaneous LD50s of organophosphorus compounds are very similar (McCollister et al., 1974). Thus, the dose of chlorpyrifos used here corresponds to approximately 0.6 × LD50, which has been reported to be below the threshold for organophosphorus-induced seizures (Shih and McDonough, 1997) Animals were dosed with chlorpyrifos subcutaneously rather than orally because the soft palate of the guinea pig is continuous with the base of the tongue, and the opening through which to pass a feeding tube is very small. Despite all the care to avoid damage to the oral cavity, gavage is very stressful to guinea pigs and interferes with their normal feeding behavior.

Animals were monitored every 15 min after treatment for the first 2 h, and then every hour for the next 8 h. No animals displayed signs of overt toxicity. The Morris water maze (MWM) test and *in vivo* MRI/MRS scans were performed one year after administration. All experiments were carried out in accordance with the rules and regulations set forth by the University of Maryland School of Medicine Institutional Animal Care and Use Committee regarding the care and use of animals under a protocol approved by the committee, and complied with the principles of the '1996 Guide for the Care and Use of Laboratory Animals.

2.2. Morris water maze test

At 11 months after chlorpyrifos or vehicle administration, the MWM, a behavioral task that is dependent on the integrity of hippocampal functioning (Morris, 1984), was used to assess the cognitive performance of the animals. Animals were tested according to the protocol described in Mamczarz et al. (2011). A large circular galvanized tub (180-cm diameter, 60-cm height) was filled with tap water mixed with non-toxic black tempera paint to a depth of 40 cm. The tub was divided virtually into four quadrants. Upon placement in the water, the animals were allowed to swim to find a hidden and submerged platform (27 cm in diameter) placed in the center of one of the quadrants. Navigation to the platform