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Alterations in nitric oxide synthase-expressing neurons in the forebrain regions of rats after developmental exposure to organophosphates

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ABSTRACT

Several mechanisms have been addressed as contributors to the long lasting behavioral deficits after developmental exposure to organophosphate (OP) compounds. Here, the effects of developmental exposure to two common OP insecticides, chlorpyrifos (CPF) and diazinon (DZN), on nitric oxide synthase (NOS)-expressing neurons in the rat forebrain are reported. A daily dose of 1 mg/kg of either CPF or DZN was administered to rats during gestational days 15–18 or postnatal days (PND) 1–4. We then assessed NADPH-diaphorase and neuronal NOS (nNOS) immunohistochemistry in forebrain sections on different postnatal days. Prenatal exposure to CPF and DZN induced a transient reduction of NADPH-d$^+$/nNOS-immunoreactive (IR) neurons in most cortical regions on PND 4 but exceptionally increased them in the entorhinal/piriform cortex. On PND 15, NADPH-d$^+$/nNOS-IR neurons showed morphological abnormalities within entorhinal/piriform cortex of the rats that gestationally exposed to CPF. Postnatal exposure to CPF and DZN did not induce widespread effects on the number of NADPH-d$^+$/nNOS-IR neurons on PNDs 7 and 15 but significantly reduced them in most cortical regions and hippocampal subfields on PND 60. The OPs affected NADPH-d$^+$/nNOS-IR neurons in a sex independent manner and apparently spared them in the striatum. While the NADPH-d reactivity of microvessels was normally diminished by age, OP treated rats evidently preserved the NADPH-d reactivity of microvessels in the cerebral cortex and hippocampus. The effects of OPs on NADPH-d$^+$/nNOS-IR neurons may contribute to the long-lasting behavioral outcomes and expand the neurotransmitter system that need to be considered in OP neurotoxicity evaluations.

1. Introduction

Organophosphate (OP) compounds have the potential to elicit developmental neurotoxicity at doses below the threshold for inhibition of acetylcholinesterase (AChE), their common mechanism of acute toxicity. Accordingly, mechanisms other than AChE inhibition contribute to disruption of brain development after chronic exposure to apparently subtoxic levels of OPs. Several studies have shown that OPs disrupt basic processes involved in neuronal replication and differentiation, axonogenesis, synaptogenesis and assembly of neuronal circuits (Qiao et al., 2003; Slotkin et al., 2006, 2007, 2008; Slotkin and Seidler, 2007a, 2007b). In the developing brain, OPs differentially alter neurotransmitter systems that have been supposed as responsible for disparities in the behavioral consequences. Comparative studies have shown that developmental exposure to CPF and DZN especially induce immediate and lasting effects on cholinergic, serotonergic and catecholaminergic systems (Aldridge et al., 2004, 2005; Slotkin et al., 2001, 2008).

Nitric oxide (NO) is a short life gaseous messenger which is produced by three isoforms of nitric oxide synthases (NOS), namely neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). In the nervous system NO acts as an atypical neurotransmitter and neuromodulator and is involved in numerous physiological and pathophysiological events including synaptogenesis, synaptic plasticity, neuronal excitability, cell proliferation, cortical lamination, aging, inflammation and neuronal death (Bohme et al., 1993; Cramer et al., 1996; Dawson et al., 1994; Prast and Philippu, 2001). NO may also affect neuronal function indirectly through its major role in the regulation of blood flow (Rockland and Nayyar, 2012; Zhou and Zhu, 2009). There are reports that the heterochronous appearance and varying distribution of NOS expressing neurons in some regions of developing brain correlates with developmental processes (Bravo et al., 1997; Chung et al., 2004; Samama et al., 1995; Santacana et al., 1998) and NO production may contribute to the establishment and refinement of afferent connections (Cramer et al., 1996; Williams et al., 1994). These neurons are detected by immunohistochemistry using specific antibodies against nNOS or indirectly by nicotinamide adenosine dinucleotide phosphate-diaphorase (NADPH-d) method, which has been introduced as a histological

Abbreviations: AChE, acetylcholinesterase; CPF, chlorpyrifos; DMSO, dimethylsulfoxide; DZN, diazinon; GD, gestational day; NADPH-d, nicotinamide adenine dinucleotide phosphate-diaphorase; NO, nitric oxide; NOS, nitric oxide synthase; nNOS-IR, neuronal NOS-immunoreactive; OP, organophosphate; PND, postnatal day.

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marker that reliably stains NOS-containing neurons (Dawson et al., 1991; Hope et al., 1991; Valtschanoff et al., 1993). According to literature, NADPH-d positive (NADPH-d$^+$)/nNOS-immunoreactive (nNOS-IR) neurons first appear in the striatum and cerebral cortex on gestational day (GD) 19 but they appear in hippocampus after birth (Chung et al., 2004; Samama et al., 1995). The appearance and transient elevation of NOS expressing neurons has been proposed to be correlated to the end of neurogenesis and neural migration and this is in accord with the later development of hippocampus (Altman and Bayer, 1990; Peunova et al., 1996). Interestingly many factors that interfere with brain development also alter the neuronal count and distribution of NOS expressing neurons (Hamani et al., 1999; Rodella et al., 2006; Yan et al., 2012). Given the considerable overlap between processes that NO is involved in and those that are affected by OPs and the fact that NO can modulate the activity of different neurotransmitter systems (Prast and Philippu, 2001), it is likely that the nitrergic system takes part in the developmental toxicity of OPs.

We have recently reported the effects of developmental exposure to CPF and DZN on passive avoidance performance and nNOS-expressing neurons in the basolateral complex of amygdala (BLC). In the current study, we evaluated the effect of developmental exposure to CPF or DZN on NOS expressing neurons in three main structures of rat forebrain; cerebral cortex, hippocampus and striatum by using nNOS immunohistochemistry and NADPH-d histochemistry. We chose two windows for OP treatment: GDs 15–18, which is the time of cortical plate formation and just before appearance of NOS-containing neurons in most forebrain structures; and PNDs 1–4, which is the time that the studied structures have a large number of nNOS expressing neurons (Samama et al., 1995). CPF and DZN were administered at a dose spanning the threshold for barely-detectable, nonsymptomatic inhibition of brain AChE immediately after the end of treatment (1 mg/kg/day) (Qiao et al., 2003; Ricceri et al., 2003; Slotkin et al., 2006, 2008).

2. Materials and methods

2.1. Animals and treatments

All experiments were carried out humanely and with regard for alleviation of suffering, with protocols approved by the local animal ethics committee and in accordance with declaration of Helsinki guidelines. Male and female Wistar rats, weighting 220–260 g were purchased from Razi institute and kept in a temperature-controlled room at 22 ± 2 °C with a 12 h light–dark cycle and free access to food and water. After two weeks of acclimatization, breeding pairs were placed in a cage and females were tested each day for a sperm-positive vaginal smear indicating a mating event. Females with positive vaginal smears were considered pregnant (GD 0) and were housed separately in standard condition.

Because of the poor water solubility, CPF and DZN (both from Sigma) were dissolved in dimethylsulfoxide (DMSO) to provide rapid and complete absorption (Whitney et al., 1995) and were injected subcutaneously in a volume of 1 ml/kg body weight. For prenatal exposure, dams were injected daily with CPF or DZN at dose of 1 mg/kg of body weight once daily on GDs 15–18. Control animals received equivalent injections of DMSO, which does not itself produce developmental neurotoxicity (Whitney et al., 1995). Litters were culled to eight on the day after birth (PND1) with equal number of males and females, whenever possible. For postnatal treatments CPF and DZN were injected subcutaneously at 1 mg/kg daily on PNDs 1–4. This dose has been shown previously to alter neural function without eliciting overt systemic toxicity (Slotkin et al., 2006; Whitney et al., 1995). Control animals received equivalent injections of the vehicle on the same schedule. For animals that were treated during the prenatal period, NADPH-d histochemistry and nNOS immunohistochemistry studies were performed on PNDs 4, 15 and 60 and for those that were treated during postnatal period, on PNDs 7, 15 and 60. Animals from prenatal and postnatal treated litters were weaned on PND 23, separated by sex and maintained four per cage until the day of experiment. During preweaning period pups were daily examined for fur appearance and the ear canal and eye opening. On each day of experiment, animals were weighted and one male and one female from each litter were selected and sacrificed under ether anesthesia (for PNDs 4, 7 and 15) or pentobarbital anesthesia (60 mg/kg i.p. for PND 60) by perfusion. For each day of experiment four animals of each sex were used for NADPH-d histochemistry and three for nNOS immunostaining.

2.2. Tissue preparation

Transcardial perfusion performed through the left ventricle with 0.9% saline, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, PH 7.4). The brains were removed from the skull, weighted and postfixed in the same fixative for 10–12 h at 4 °C. Blocks of forebrain were sectioned in the coronal plane every 80 μm on a vibroslice (Campden, UK). Serial sections (taken at 3 to −4.8 mm from bregma) were collected in PBS (0.1 M, pH 7.4) and every third to fourth of coronal sections were selected to follow the histochemical protocol for NADPH-d or immunohistochemistry for nNOS as described in our previous paper (Vatanparast et al., 2013) and is briefly described here.

2.3. NADPH-d histochemistry

Sections were incubated in 0.1 M PBS (pH 7.4) containing 0.5 mg/ml β-NADPH (Sigma), 0.6 mg/ml nitroblue tetrazolium (NBT, sigma) and 0.3% Triton X100 for 1 h at 37 °C in darkness. The reaction was ended by adding cold PBS to the medium and sections were serially mounted on corom-alum coated slides.

2.4. Immunohistochemistry for nNOS

Forebrain sections were incubated in PBS containing 3% H_2O_2 to quench endogenous peroxidase activity. The sections were washed twice in PBS and preincubated for 1 h in blocking buffer including 0.1% bovine serum albumin (BSA) and 0.3% triton X-100 in 0.1 M PBS to suppress non-specific binding of antibodies. Sections were incubated for 72 h at 4 °C with rabbit anti-nNOS polycolonal antibody (sigma, 1:1000) in 0.1 PBS containing 0.3% triton X-100 and 0.1% BSA. Sections were then incubated for 24 h at 4 °C in 1:500 peroxidase conjugated goat anti-rabbit IgG (Sigma). Immunoreactions were visualized by incubation in 0.05 M Tris–HCl buffer (pH 7.2) containing 0.5 mg/ml diaminobenzidine and 0.02% H_2O_2. Sections were then washed twice in PBS and transferred on corom-alum coated slides, air dried, dehydrated in the graded alcohol, cleared in xylene and cover-slipped with Entellan.

2.5. Neuronal study and cortical thickness evaluation

Sections were examined under an Olympus 4-HC light microscope to localize cells exhibiting NADPH-d or nNOS immunoreactivity. Nine sections from each brain correlating planes 11, 18, 25, 32, 39, 46, 53, 61 and 68 of Paxinos and Watson atlas (2007) were similarly selected and studied. The labeled neurons were counted bilaterally in the whole field of functionally distinct cortical areas in selected sections processed for NADPH-d and also in immunoreacted sections. The distribution of nNOS expressing neurons within layers of the cerebral cortex is changing during development (Bravo et al., 1997; Chung et al., 2004). A change in the distribution of NADPH-d$^+$/nNOS-IR neurons may cause a large bias when the neuronal count is restricted to a limited area of each functionally distinct cortical area instead of the whole field. In the rat hippocampus the border between subfield is not well marked and these regions could only be defined by estimate (Paxinos, 1995). The striatum includes a wide area of forebrain sections and shows a