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Reversible effect of developmental exposure to chlorpyrifos on late-stage neurogenesis in the hippocampal dentate gyrus in mouse offspring

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ABSTRACT

The effect of developmental exposure to chlorpyrifos (CPF) on hippocampal neurogenesis was examined in male mice after maternal dietary exposure to CPF at 0, 4, 20, or 100 ppm from gestation day 10 to postnatal day (PND) 21. Cholinesterase activity was dose-dependently decreased in red blood cells at ≥4 ppm and in the brain at 100 ppm both in dams and offspring on PND 21. Immunohistochemically, doublecortin$^+$ cells were decreased at ≥20 ppm in the subgranular zone (SGZ) of the dentate gyrus, and NeuN$^+$-expressing mature neurons were decreased at 100 ppm in the hilus on PND 21. There were no differences in the numbers of progenitor populations expressing Tbr2 or M1 muscarinic acetylcholine receptors. Transcript levels of *Dcx* also decreased at ≥20 ppm, and those of *Pcna*, *Casp3*, *Bax*, *Bcl2*, *Pax6* and *Tbr2* were unchanged in the dentate gyrus by real-time RT-PCR. At PND 77, hippocampal neurogenesis was unchanged. These results suggest that developmental CPF exposure directly but transiently suppresses maturation of late-stage granule cell lineages in the SGZ and affects interneuron populations in the hilus.

1. Introduction

Chlorpyrifos (CPF) is one of the most widely used organophosphate insecticides in the world [1]. Its toxicity is related to the inhibition of cholinesterase (ChE) activity, disrupting cholinergic function in the nervous system [2]. In the most recent decade, increased concern has been raised about health risks associated with subtoxic dose levels of CPF [3]. However, despite the restriction of some of its domestic and agricultural uses by the United State Environmental Protection Agency [4] in 2000, CPF still remains one of the most widely used organophosphate insecticides in the world [5]. Especially in Europe, CPF is the top selling insecticide [6] with no restrictions imposed on use sites or application rates [7]. Because of its widespread agricultural and domestic uses, the risk of unsuspected CPF exposure from environmental contamination is high. The estimated national average consumption of CPF through food in Japan is calculated as 0.72 μg/kg-day. In pregnant women this is 0.66 μg/kg-day and in infants of 1–6 years of age it is calculated as 1.34 μg/kg-day [8]. Therefore, children, who are still in the developmental stages of life, have a higher risk of CPF exposure than adults.

Studies on CPF neurotoxicity mainly focus on its impact on acetylcholine systems and related behaviors [9,10]. The mechanism underlying the systemic effects of organophosphates is the irreversible inhibition of ChE by active oxon metabolites [11,12]. However, work over the past two decades has conclusively shown that native compounds such as CPF are themselves developmental neurotoxicants at low, nonsymptomatic exposure levels and that, consequently, the ChE biomarker is inadequate for monitoring safety [11,12]. Indeed, a number of studies on the ChE-unrelated effects underlying the developmental neurotoxicity of CPF have indicated a combination of antimitotic and pro-apoptotic mechanisms, leading to deficits in the numbers of neurons and/or glial cells [12,13].

Within the hippocampal formation of the mammalian brain, the dentate gyrus is a unique structure that can continue neurogenesis at the subgranular zone (SGZ) throughout postnatal life [14]. It forms crucial neuronal networks responsible for cognitive, emotional and memory function [15]. This postnatal neurogenesis (so-called "adult neurogenesis") occurs in the SGZ from type-1

Abbreviations: ChE, cholinesterase; Chrm1, cholinergic receptor muscarinic 1; CPF, chlorpyrifos; DCX, doublecortin; GABA, γ-aminobutyric acid; GAD67, glutamic acid decarboxylase 67; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; GD, gestation day; GFAP, glial fibrillary acidic protein; *Hprt*, hypoxanthine-guanine phosphoribosyltransferase; NeuN, neuron-specific nuclear protein; Pax6, paired box gene 6; PCNA, proliferating cell nuclear antigen; PND, postnatal day; SGZ, subgranular zone; Tbr2, T box brain 2; TSH, thyroid-stimulating hormone; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; T_3, triiodothyronine; T_4, thyroxine.

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stem cells and produces intermediate generations in the order of type-2a, type-2b, and type-3 cells. Type-3 cells then undergo final mitosis to differentiate into immature granule cells, and finally into mature granule cells [16]. In addition, γ-aminobutyric acid (GABA)ergic interneurons in the hilus of the dentate gyrus can control neurogenesis in the SGZ [17,18]. During the embryonic period and throughout adult life, GABAergic interneurons produce reelin, which modulates dentate granule cell progenitor migration and maintains normal granule cell integration in the neonatal and adult mammalian dentate gyrus [19]. Therefore, examination of the distribution of the neuronal progenitor cell populations and reelin-expressing interneurons may be instrumental in the detection of developmental neurotoxicity. However, the cholinergic system also regulates hippocampal adult neurogenesis, positively promoting proliferation, differentiation, integration and, potentially, survival of newborn neurons [20]. This suggests that ChE inhibitors may target neurogenesis even at the adult stage.

Despite the fact that numerous studies on neuronal and glial cell populations in the developing brain and the related behavioral effects of CPF have been conducted, little information is available concerning neurogenesis and its regulatory systems in the hippocampal dentate gyrus. To develop a rapid screening system for developmental neurotoxicants, we recently performed several studies on neurogenesis and its regulatory systems in the hippocampal dentate gyrus using histopathological parameters including immunohistochemistry in small-scale animal studies [17,21–24].

In the present study, to elucidate the effect of developmental exposure to CPF on neurogenesis, we studied the distribution, proliferation and apoptosis of granule cell lineages in the SGZ and the distribution of reelin-producing interneurons in the hilus of the hippocampal dentate gyrus in the offspring of mice exposed to CPF during pregnancy and lactation. We also evaluated the changes in ChE levels in red blood cells (RBC), blood plasma, and the forebrain, and the distribution of progenitor cells expressing cholinergic receptor muscarinic 1 (Chrm1) in the SGZ.

2. Materials and methods

2.1. Chemicals and animals

CPF (purity: 99.8%) was kindly provided by Dow Chemical Japan Ltd. (Tokyo, Japan). A total of 60 pregnant Slc:ICR mice were purchased from Japan SLC Inc. (Hamamatsu, Japan) at gestation day (GD) 1 (appearance of vaginal plugs was designated as GD 0). Animals were housed individually in polycarbonate cages with wood chip bedding maintained in an air-conditioned animal room (temperature: 24 ± 1 °C; relative humidity: 55 ± 5%) with a 12-h light/dark cycle, and allowed access to food and tap water *ad libitum*. After delivery, dams were similarly housed with their litters until postnatal day (PND) 21 (with PND 0 being the day of delivery). A regular MF basal diet (Oriental Yeast Co. Ltd., Tokyo, Japan) and water were provided *ad libitum* throughout the experimental period. All offspring consumed the regular MF basal diet and water *ad libitum* from PND 21 onwards.

2.2. Experimental design

All procedures of this study were conducted in compliance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan (June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

Dams were randomly divided into 4 groups of 15 dams each, which were treated with 0, 4, 20 or 100 ppm of CPF mixed into the powdered basal diet from GD 10 to PND 21. This exposure period was chosen because the formation of hippocampus starts on GD 11 and neurogenesis in the SGZ is active during the postnatal period from PND 3 to PND 14 in mice [25]. At PND 4, litters of dams that delivered more than 5 male and 5 female offspring per group were randomly culled, leaving 5 male and 5 female offspring. Remaining dams were killed by exsanguination under deep anesthesia and the remaining offspring were killed by rapid decapitation under anesthesia. At PND 21, which is the prepubertal stage, 10 dams and 30 male and 30 female offspring per group (3 male and 3 female offspring per dam) were subjected to necropsy as described below. The remaining male and female offspring were kept until PND 77. At PND 77, all remaining pups were subjected to adult stage necropsy as described below. The body weights and food consumption of dams and the body weights of pups were determined every three to seven days. Mortality of pups was examined daily between PND 2 and PND 77.

On PND 21 and PND 77, animals were weighed and killed by exsanguination from the abdominal aorta under deep anesthesia. Dams were examined for uterine implantation sites at necropsy on PND 21. Brain, liver and kidneys were collected at necropsy in 10 male and 10 female offspring per group. Because neurogenesis is influenced by circulating levels of steroid hormones during the estrous cycle [26], female samples taken on PND 21 and PND 77 were preserved without further analysis.

2.3. Determination of cholinesterase (ChE) activity

ChE activity was measured in samples taken from dams on PND 21 ($n = 6$ per group) and male offspring on PND 21 and PND 77 ($n = 6$ per group for each stage).

Blood samples were collected under anesthesia from the abdominal aorta in blood collection tubes containing heparin sodium. An aliquot of blood was mixed with distilled water containing 1% Triton X-100 (Sigma–Aldrich Japan K.K., Tokyo, Japan) and hemolyzed for analysis of ChE in total blood. For the calculation of ChE activity in the RBC, the hematocrit was measured using a hematology analyzer (Advia 120, Siemens Medical Solutions Diagnostics, Tokyo, Japan). The remaining blood was centrifuged to obtain plasma.

The frontal lobe of the brain was removed, weighed, frozen in liquid nitrogen and stored at −80 °C until analysis. A brain sample was homogenized in distilled water containing 1% Triton X-100.

The ChE activities of the blood, plasma and brain samples were analyzed using a clinical chemistry automatic analyzer (TBA-120FR, Toshiba Medical Systems Corporation, Tochigi, Japan) with acetylthiocholine iodide as a substrate.

2.4. Hormone analyses

Blood samples were collected under anesthesia from the abdominal aorta of the remaining 24 male offspring per group on PND 21, and from 10 male offspring per group on PND 77. Serum was prepared and stored at −80 °C for measurement of thyroid-stimulating hormone (TSH), triiodothyronine (T_3) and thyroxine (T_4) concentrations at Mitsubishi Chemical Medience (Tokyo, Japan).

2.5. Immunohistochemistry and apoptotic cell detection

For immunohistochemical analysis, brains from male offspring killed at PND 21 and PND 77 were fixed in Bouin's solution at room temperature overnight. Samples from 10 male animals from 10 dams (one male per dam) per group were subjected to analysis at each time point. Coronal slices of 3 μm in thickness at the positions of −2.2 mm from the bregma embedded in paraffin were prepared for immunohistochemical staining.

Sections were incubated overnight at 4 °C with the antibodies listed in Table 1. To quench endogenous peroxidase, slides were incubated in 0.3% (v/v) hydrogen peroxide in absolute methanol for 30 min. Immunodetection was carried out using a Vectastain® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3′-diaminobenzidine (DAB)/hydrogen peroxide (H_2O_2) as the chromogen, as previously described [24]. The sections were then counterstained with hematoxylin and coverslipped for microscopic examination.

For evaluation of apoptosis in the SGZ of the dentate gyrus, terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) assay was applied to brain sections from the same 10 male offspring per group that were killed on PND 21. Deparaffinized sections were treated with 20 μg/mL proteinase K in phosphate buffered saline (PBS; pH 7.4) for 15 min at room temperature, and then incubated in 3.0% hydrogen peroxide in PBS for 5 min. Detection of apoptotic bodies was carried out using the Apop Tag® *in situ* apoptosis detection kit S7100 (Millipore Corporation, Billerica, MA, USA) according to the instructions provided by the manufacturer with DAB/H_2O_2 as the chromogen.

2.6. Morphometry of immunolocalized and apoptotic cells

Reelin$^+$-immunoreactive interneurons and neuron-specific nuclear protein (NeuN)$^+$ neurons, indicating postmitotic interneurons and glial fibrillary acidic protein (GFAP)-expressing astrocytes distributed in the hilus of the dentate gyrus, were bilaterally counted and normalized for the number per unit area of the hilar area (polymorphic layer) as previously described [17]. Large cornu ammonis 3 neurons distributed in this area were easily distinguished from hilar interneurons and excluded from counting as previously described [17]. Apoptotic bodies as detected by TUNEL assay, proliferating cells as detected by nuclear immunoreactivity of proliferating cell nuclear antigen (PCNA), and paired box gene 6 (Pax6)$^+$, T box brain 2 protein (Tbr2)$^+$, and doublecortin (DCX)$^+$ cells indicating granule cell lineage were bilaterally counted in the SGZ of the dentate gyrus and normalized to the length of the granule cell layer measured as previously described [17]. To examine the effects of cholinergic stimulation on the neuronal progenitors in the SGZ, Chrm1$^+$ progenitor cells were bilaterally counted in the SGZ and normalized for the length of the granule cell layer [20]. For quantitative measurements of each immunoreactive cellular component, digital photomicrographs at 200- or 400-fold magnification were taken using a BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) attached