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Maternal exposure to hexachlorophene targets intermediate-stage progenitor cells of the hippocampal neurogenesis in rat offspring via dysfunction of cholinergic inputs by myelin vacuolation

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ABSTRACT

Hexachlorophene (HCP) is known to induce myelin vacuolation corresponding to intramyelinic edema of nerve fibers in the central and peripheral nervous system in animals. This study investigated the effect of maternal exposure to HCP on hippocampal neurogenesis in rat offspring using pregnant rats supplemented with 0 (controls), 100, or 300 ppm HCP in the diet from gestational day 6 to day 21 after delivery. On postnatal day (PND) 21, the numbers of T box brain 2$^+$ progenitor cells and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling$^+$ apoptotic cells in the hippocampal subgranular zone (SGZ) decreased in female offspring at 300 ppm, which was accompanied by myelin vacuolation and punctate tubulin beta-3 chain staining of nerve fibers in the hippocampal fimbria. In addition, transcript levels of the cholinergic receptor, nicotinic beta 2 (*Chrnb2*) and B-cell CLL/lymphoma 2 (*Bcl2*) decreased in the dentate gyrus. HCP-exposure did not alter the numbers of SGZ proliferating cells and reelin- or calcium-binding protein-expressing γ-aminobutyric acid (GABA)-ergic interneuron subpopulations in the dentate hilus on PND 21 and PND 77. Although some myelin vacuolation remained, all other changes observed in HCP-exposed offspring on PND 21 disappeared on PND 77. These results suggest that maternal HCP exposure reversibly decreases type-2b intermediate-stage progenitor cells via the mitochondrial apoptotic pathway in offspring hippocampal neurogenesis at 300 ppm HCP. Neurogenesis may be affected by dysfunction of cholinergic inputs into granule cell lineages and/or GABAergic interneurons as indicated by decreased transcript levels of *Chrnb2* and numbers of Chrnb2$^+$ interneurons caused by myelin vacuolation in the septal–hippocampal pathway.

1. Introduction

The hippocampal formation comprises of a group of cortical regions including the dentate gyrus and cornu ammonis (CA), and plays an important role in learning and memory. In mammals, neurogenesis continues throughout life in the hippocampal dentate gyrus (Altman and Das, 1965). Adult neurogenesis involves a multistep process (proliferation, differentiation, migration, targeting and synaptic integration) that ends with the formation of postmitotic, functionally integrated new neurons (Kempermann et al., 2004). Stem cells (type-1 cells) exist in the subgranular zone (SGZ) of the dentate gyrus and divide slowly to produce intermediate progenitor cells, a type of transient amplifying cell (Kempermann et al., 2004). Undifferentiated intermediate progenitor cells (type-2a and type-2b) divide rapidly to produce neuronally committed intermediate progenitor cells (type-3) and react to stimuli that influence neuronal generation in the

Abbreviations: Blbp, brain lipid binding protein; CA, cornu ammonis; Calb1, calbindin-D-28 K; Calb2, calbindin-D-29 K; Chrna7, cholinergic receptor, nicotinic, alpha polypeptide 7; Chrnb2, cholinergic receptor, nicotinic, beta polypeptide 2; C_T, threshold cycle; Dcx, doublecortin; GABA, γ-aminobutyric acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GD, gestational day; HCP, hexachlorophene; Hprt1, hypoxanthine-guanine phosphoribosyltransferase 1; MBP, myelin basic protein; NeuN, neuron-specific nuclear protein; Olig2, oligodendrocyte lineage transcription factor 2; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde; PND, postnatal day; Pvalb, parvalbumin; RT-PCR, reverse-transcription polymerase chain reaction; SGZ, subgranular zone; Sox2, SRY (sex determining region Y)-box 2; Tbr2, T box brain 2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling.

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hippocampal dentate gyrus (Kempermann et al., 2004). Type-3 intermediate progenitor cells produce immature neurons, which progress through a regulatory period and integrate into the granule cell layer as granule cells (Kempermann et al., 2004).

In the hilar region of the dentate gyrus, γ-aminobutyric acid (GABA)-ergic interneurons are known to make synapses with adult-born dentate granule cells and play a functional role in adult neurogenesis (Toni et al., 2008). Interneurons in the dentate gyrus are divided into specific populations based on the expression of cellular proteins such as parvalbumin, calretinin, calbindin and reelin (Freund and Buzsáki, 1996). Interneurons innervate progenitor cells in the SGZ resulting in the differentiation of progenitor cells (Freund and Buzsáki, 1996; Tozuka et al., 2005). Various neurons outside the hippocampus also make synaptic connections with neurons in the dentate gyrus. For example, cholinergic neurons originating from the septal nucleus and nucleus of the diagonal band of Broca innervate neurons in the dentate hilus. Noradrenergic neurons in the locus ceruleus innervate neurons in the SGZ (Conrad, 1974).

Neurogenesis in the dentate gyrus changes with various physiological or pathological situations. Hippocampal neurogenesis is widely believed to play a vital role in the formation of hippocampal-dependent memories and the maintenance of mood function, while abnormal neurogenesis that ensues after injury contributes to aberrant synaptic reorganization in the hippocampus, and memory and mood dysfunction. We have recently shown that maternal exposure to acrylamide and glycidol, both of which target axon terminals, impairs late-stage differentiation during neurogenesis in rat offspring after developmental exposure (Akane et al., 2013b; Ogawa et al., 2012). In contrast, developmental hypothyroidism that targets a broad range of neuronal stem/progenitor cells decreases the numbers of these cells in rat offspring (Shiraki et al., 2012). In addition, maternal transient exposure to methylnitrosourea, an anti-proliferating alkylating agent, mainly targets transient populations of highly proliferative progenitor cells and does not affect the ability of stem cells to produce progenitor cells in rat offspring (Itahashi et al., 2014). These results suggest that neurogenesis can be affected by neurotoxins by targeting different cellular populations of neuronal cell lineage.

Hexachlorophene (HCP), used as an antimicrobial agent in soaps, liquid detergents and cosmetics during the 1960s, has been widely used in agriculture as a plant fungicide and pesticide (Kennedy et al., 1976). HCP is a typical neurotoxin and induces myelin vacuolation corresponding to the splitting of the intraperiod line of the myelin sheath in the cerebral white matter in animals (Lampert et al., 1973). In chronically exposed animals, segmental demyelination and remyelination develop and a few fibers undergo axonal degeneration (Maxwell and Le Quesne, 1979).

The present study was performed to examine the effect of maternal exposure to HCP on hippocampal neurogenesis of rat offspring in later life. For this purpose, we administered HCP to pregnant rats during gestation and lactation periods, and examined the dose–effect relationship on the distribution, proliferation and apoptosis of granule cell lineages in the SGZ and on the distribution of interneuron subpopulations in the dentate hilus at weaning and also at the adult stage.

2. Materials and methods

2.1. Chemicals and animals

HCP (purity: >99%) was purchased from MP Biomedicals LLC. (Santa Ana CA, USA). HCP was administered through the diet. Pregnant Crl:CD(SD) rats purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan) at gestational day (GD) 1 (the appearance of vaginal plugs was designated as GD 0) were housed individually with their offspring in plastic cages with paper bedding until day 21 after delivery. Animals were kept in an air-conditioned animal room (temperature: 23 ± 2 °C, relative humidity: 55 ± 15%) with a 12-h light/dark cycle. Pregnant rats were provided ad libitum tap water during the experimental period and with a pelleted basal diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) until the start of exposure to HCP. From postnatal day (PND) 21 (where PND 0 is the day of delivery) onwards, offspring were reared with three or four animals per cage and provided the MF diet and tap water ad libitum.

2.2. Experimental design

Pregnant rats were randomly divided into three groups of 12 animals per group. Food was supplemented with 0, 100 or 300 ppm of HCP, and dams were treated from GD 6 to PND 21. Based on a previous study (Gaines et al., 1973), the preliminary experiments initially had three groups consisting of 0, 100 and 150 ppm HCP in the diet. Myelin vacuolation was shown to occur in the white matter of adult rats and their offspring at 100 ppm; however, we did not observe any histopathological change in the brain of dams and offspring by HCP on PND 21. Because 500 ppm in the diet has been shown to induce death in dams and offspring (Rose et al., 1975), in this study, the high dose level was set at 300 ppm and the low dose level was set at 100 ppm. Dams were subjected to measurements of body weight, water consumption and food consumption daily during GD 6 and GD 21 and at 3- or 4-day intervals during the lactation period. On PND 4 after delivery, the litters were culled randomly, leaving eight female offspring per dam. If dams gave birth to fewer than eight female pups, more male pups were added to maintain a total of eight pups per litter. The male and female offspring were weighed every 3 or 4 days until PND 21. On PND 21, 10–12 female offspring per group (1 female offspring per dam) and all dams were subjected to perfusion fixation for brain immunohistochemistry through the left cardiac ventricle with cold 4% (w/v) paraformaldehyde (PFA) at a flow rate of 10 mL (offspring) and 35 mL (dams)/min after deep anesthetization with CO_2/O_2. For mRNA expression analysis, five female offspring per group (1 female per dam) were euthanized by exsanguination from the abdominal aorta under CO_2/O_2 anesthesia and subjected to necropsy. For other purposes of analysis, brain samples from 16 to 21 female and 12 to 13 male offspring per group (from 0 to 4 females and 1 to 2 males per dam) were prepared.

The remaining male and female offspring were maintained until PND 77, and body weight and food consumption were measured once a week. On PND 77, 10–12 female offspring per group (1 female offspring per dam) were perfused for immunohistochemistry at a flow rate of 35 mL/min. For mRNA expression analysis, five female offspring per group (1 female per dam) were euthanized by exsanguination from the abdominal aorta under CO_2/O_2 anesthesia and subjected to necropsy. For other purposes of analysis, brain samples from 23 to 28 female offspring per group (from 1 to 4 females per dam) and 13 male offspring per group (from 1 to 2 males per dam) were prepared.

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

2.3. Immunohistochemistry and apoptotic cell detection

Brain sections collected from dams and female offspring on PND 21 and PND 77 were subjected to immunohistochemistry. One female offspring per dam was analyzed in each group. Removed