Intracisternal Administration of *p*-n-Octylphenol into Neonatal Rats Causes Hyperactivity Concomitantly with the Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labelling (TUNEL)-Positive Cells in the Mesencephalon where Immunoreactivity for Tyrosine Hydroxylase is Reduced by the Chemical

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It is unknown which endocrine disruptors exert their effects on neuronal functions, particularly leading to behavioral alterations. To address this, we examined the effects of *p*-n-octylphenol, an endocrine disruptor, on rat behavior and cellular responses. Single intracisternal administration of *p*-n-octylphenol (87 nmol) into 5-day-old male Wistar rats caused significant hyperactivity at 4–5 weeks of age. The treated rats were about 1.5-fold more active in the nocturnal phase after administration of *p*-n-octylphenol than control rats. Immunohistochemical analyses revealed that *p*-n-octylphenol abolished immunoreactivity for tyrosine hydroxylase in the midbrain of 8 week-old rats, where terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL)-positive cells were also seen. Thus, this is the first demonstration that *p*-n-octylphenol certainly affected the developing brain, resulting in hyperactivity in the rat, most likely due to degeneration of mesencephalic tyrosine hydroxylase.

Key words —— *p*-n-octylphenol, hyperactivity, tyrosine hydroxylase, endocrine disruptor

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INTRODUCTION

There is considerable public concern about the possibility that endocrine disruptors might exert effects on neuronal functions because of the following observations. First, polychlorinated biphenyls (PCBs) distributed in the environment cross the placenta, causing *in utero* injury to the developing brain, which is correlated with a decrease in intelligence quotient.[1,2] Second, many chemicals such as dioxins, PCBs, bisphenol A, and heavy metals have been detected in human umbilical cord and cord serum, suggesting that they transfer transplacentally from mother to fetus.[3]

An emerging body of evidence is accumulating that endocrine disruptors, such as bisphenol A, transfer from the maternal rat to the fetus and that the chemicals affect the developing brain, leading to behavioral alterations.[4] Therefore, we hypothesize that endocrine disruptors might also contribute to the incidence of neurodevelopmental disorders such as attention-deficit hyperactivity disorder (ADHD) and autism,[5,6] although it is still unknown if the chemicals exert their neuronal effects on the developing brain through an endocrine-disrupting action or through yet uncharacterized processes.

The symptoms of ADHD and autism are inattention, excess impulsivity, and uncontrolled hyperactivity.[5,6] The animal model for hyperactivity was produced by Shaywitz *et al*. who demonstrated that rat pups treated with 6-hydroxydopamine (6-OHDA) by intracisternal administration at 5 days of age exhibit increased motor activity, leading to cognitive difficulties in shuttle-box learning between 2 and 4 weeks of age.[7] These observations were strikingly similar to the clinical syndrome of minimal brain dysfunction, called ADHD, found in children.

As the first step for testing our hypothesis, we employed the Supermex system to screen endocrine disruptors that cause hyperactivity in the rat. Dur-

ing the course of primary screening of several endocrine-disrupting chemicals at fixed concentrations (87 nmol), which were equivalent to the amounts of tributyltin, a neurotoxin, sufficient to alter the behavior of rats,[8] we found that *p*-n-octylphenol was positive in the system.

MATERIALS AND METHODS

Chemicals —— *p*-n-Octylphenol was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Olive oil was obtained from nacalai tesque (Kyoto, Japan).

Animals and Treatments with Chemicals —— Pregnant Wistar rats were obtained from Clea Japan (Tokyo, Japan). They were maintained in home cages and fed a standard laboratory chow (MF diet, Oriental Yeast Corp., Tokyo, Japan) and distilled water *ad libitum* at 22°C on a light-dark cycle (12 hr/12 hr) for at least one week. *p*-n-Octylphenol (87 nmol) was suspended in olive oil (10 μl) and administered intracisternally to 5-day-old male rats. Eighty-seven nmol of *p*-n-octylphenol is equivalent to 18 μg. Control rats were injected with vehicle (10 μl) alone. Pups were weaned at 3 weeks of age. Animals received humane care according to the National Institute for Environmental Studies guidelines.

Measurements of Spontaneous Motor Activity —— Spontaneous motor activity of the rats at 4–5 weeks of age was individually measured in a home cage with a Supermex system (Muromachi Kikai, Tokyo, Japan), as described previously.[8–11] In this system a sensor detects and measures the radiated body heat of an animal. A Supermex sensor head consists of paired infrared pyroelectric detectors. This system detects any object with a temperature at least 5°C higher than background within a cone-shaped area with a 6 m diameter and a 110° vertex. The sensor monitors motion in multiple zones of the cage through an array of Fresnel lenses placed above the cage and movement of the animal in the *X*, *Y*, and *Z* axis can be determined. Activity was measured in 15 min increments for 22–24 hr and maintained on a 12 hr light : dark cycle. Food and water were provided *ad libitum* at the beginning of counting and the rats were never disturbed in any way. Ten rats were recorded concurrently.

Immunohistochemistry —— Immunostaining was carried out as described previously[12] using the avidin-biotin alkaline phosphatase technique.[13] Rats were killed by decapitation at 8 weeks of age. The whole brain was fixed in 10% (vol/vol) phosphate-buffed formalin (pH 7.2) and embedded in paraffin. Sagittal sections (5 μm) were mounted on slides coated with BioBond (British BioCell International Ltd., Cardiff, U.K.), deparaffinized, and hydrated. The section was then permeabilized with 0.5 g/l Triton X-100 in phosphate-buffered saline (PBS), and blocked with 10% normal horse serum plus 40 g/l bovine serum albumin for 30 min at 4°C. The sample was incubated with primary monoclonal anti-tyrosine hydroxylase antibody (1 : 100; Sigma-Aldrich, St. Louis, MO, U.S.A.) in the presence of 40 g/l bovine serum albumin and 0.05 g/l Triton X-100. After three washes with PBS containing 1 g/l Tween 20, the sample was incubated with biotinylated secondary goat anti-mouse IgG (Lab Vision Corp., CA, U.S.A.). The biotinylated molecules were further incubated with streptavidin-conjugated alkaline phosphatase for 10 min at room temperature. The specimen was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Lab Vision Corp.) and counterstained with 10 g/l methylgreen.

***In situ* Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labelling (TUNEL) Labeling** —— Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL)-positive cells were identified by the TUNEL method as described previously.[14] The brain was fixed in 10% phosphate buffered formalin (pH 7.2) and embedded in paraffin. Following deparaffinization in xylene and rehydration through a series of graded ethanols, the sample was treated with 20 μg/ml proteinase K for 15 min at room temperature. After three washes in PBS, DNA 3′-end labelling was performed with 10 U of terminal deoxynucleotidyl transferase, 5 mM fluorescein-dUTP, and 45 mM dATP in 50 mM sodium phosphate, pH 6.6, containing 5 mM $CoCl_2$ and 200 mM potassium cacodylate at 37°C for 1 hr. After three washes in PBS buffer, the cover slips were mounted in a 50% glycerol solution onto a slide and then directly surveyed under a fluorescence microscope (IX70; Olympus, Tokyo, Japan). Images were captured using Viewfinder Lite ver. 1.0 camera software through a DP-50 digital camera (Olympus).

Statistics —— Statistical analyses were carried out with repeated measure analysis of variance (ANOVA) or Student's *t*-test using the StatView Ver. 5.0 software (SAS Institute, Cary, NC, U.S.A.).

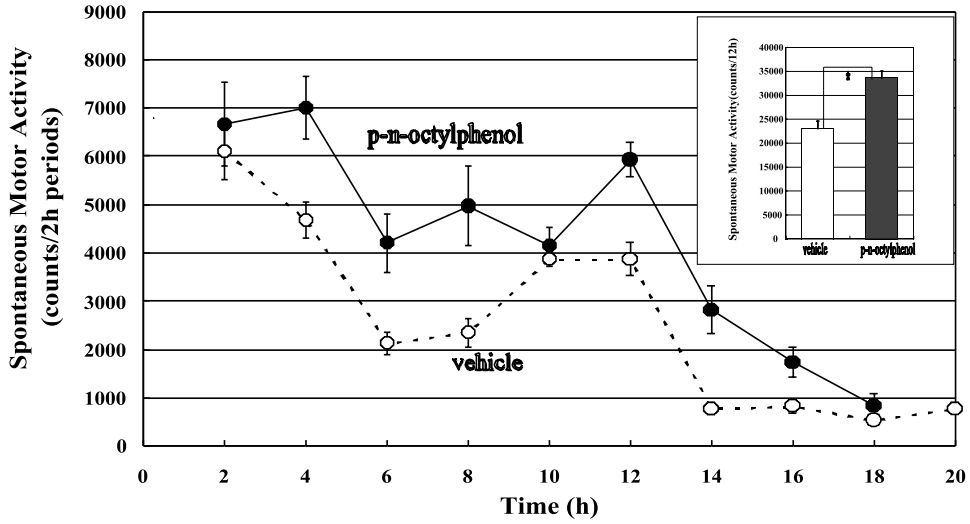

Fig. 1. Behavioral Traits

Spontaneous motor activity of 10 μl of vehicle (○)- and 87 nmol in 10 μl of *p*-n-octylphenol (●)-treated Wistar rats was measured by the Supermex system. Activity measured during 2 hr intervals is plotted. Data are indicated as mean ± S.E. ($n = 5$). (Inset) Total spontaneous motor activity at nighttime. Spontaneous motor activity at nighttime was integrated. Open: vehicle; closed: *p*-n-octylphenol treatment. Data are indicated as mean ± S.E. *Significantly different from control rats ($p < 0.001$).

RESULTS AND DISCUSSION

p-n-Octylphenol (87 nmol equivalent to 18 μg) was intracisternally administered into the 5-day-old rats and their spontaneous motor activity was measured by the Supermex system at 4–5 weeks of age (Fig. 1). The control rats were given 10 μl of olive oil alone. *p*-n-Octylphenol-treated rats were significantly more hyperactive than vehicle-treated rats in the nocturnal phase. It was notable that the kinetics of the hyperactivity of the *p*-n-octylphenol-treated rats were different from those of tributyltin-caused hyperactive rats in which was most prominent in a novel environment throughout the nighttime.[8] The total activity of the *p*-n-octylphenol-treated rats for 12 hr in the dark was 1.5 times higher than that of control rats (Fig. 1 inset; $p < 0.001$, Student's *t*-test after ANOVA).

The weight and growth characteristics of the endocrine disruptor-treated rats were indistinguishable from those of the solvent-treated rats (data not shown).

We then investigated the cellular effects of *p*-n-octylphenol by immunohistochemistry. Brain tissue at 8 weeks of age was processed for the immunoreactivity of tyrosine hydroxylase, a rate-limiting enzyme for catecholamine synthesis. The substantia nigra in the control tissue was strongly stained with the anti-tyrosine hydroxylase antibody used (Figs. 2A and 2B). In contrast, a single intracisternal injection of *p*-n-octylphenol (87 nmol) into rat pups resulted in a reduction in tyrosine hydroxylase immunoreactivity in the substantia nigra, suggesting degeneration of dopaminergic neurons (Figs. 2C and 2D). The reduction in immunoreactivity of tyrosine hydroxylase was also seen in ventral tegmental areas.

Since the large reduction in immunoreactivity for tyrosine hydroxylase in the midbrain by *p*-n-octylphenol could reflect neurodegeneration of the dopaminergic neurons, we next carried out TUNEL staining using brain sections from 8 week-old rats. No labeling was found in the control brain (Fig. 3A). In contrast, as seen in Fig. 3B, TUNEL-positive cells were detected in the *p*-n-octylphenol (87 nmol)-treated brain, demonstrating that the chemical caused cell death in the substantia nigra.

DNA macroarray analyses of both the striatum and mesencephalon from *p*-n-octylphenol-treated rat brains showed subtle alterations (data not shown). This result suggested that the robust behavioral alterations in the chemical-treated hyperactive rats may be primarily dependent on posttranscriptional mechanisms and modification of related neural circuits.

Thus, in this study we could detect behavioral alterations in rats produced by *p*-n-octylphenol, an endocrine disruptor, using the Supermex system. This indicated that *p*-n-octylphenol certainly affected the developing brain, resulting in hyperactivity that

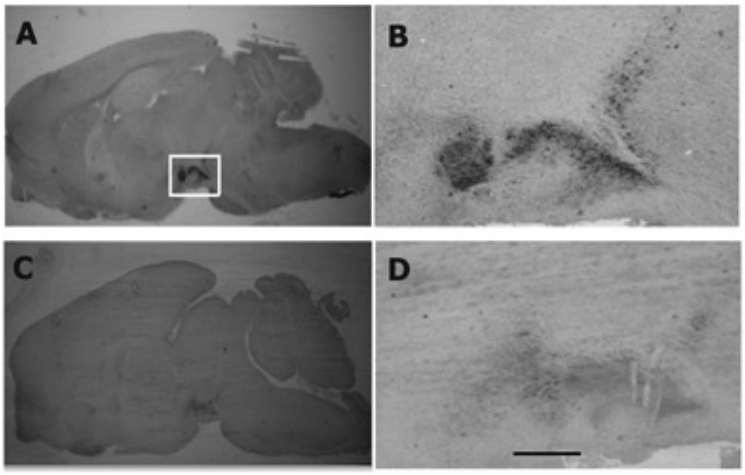

Fig. 2. Tyrosine Hydroxylase Immunohistochemistry

After intracisternal injection of vehicle alone (A and B) or *p*-n-octylphenol (87 nmol; C and D) into 5-day-old rats, brain tissues at 8 weeks of age were stained with anti-tyrosine hydroxylase antibody. The specimens were observed under a stereomicroscope (Olympus model SZX 12). B and D are magnifications of the areas indicated in panels A and C, respectively. Scale bar = 0.5 mm.

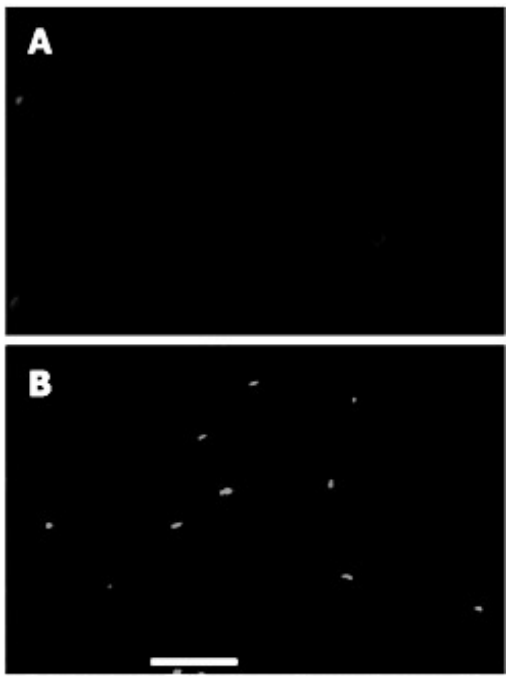

Fig. 3. *In situ* TUNEL Staining

Sections of brains from control (A) and *p*-n-octylphenol (87 nmol)-treated rats (B) at 8-weeks of age were labeled with 5 mM fluorescein dUTP in the presence of 10 U terminal deoxynucleotidyl transferase. Scale bar = 50 μm.

is also seen in cases of ADHD and autism[5,6] as well as 6-OHDA-lesioned hyperactive model rats.[11]

To our knowledge, there has been no other reports on the neurotoxicity of *p*-n-octylphenol, while numerous studies of the estrogenicity as well as carcinogenicity of the chemical have been carried out.[15–17] Our data clearly demonstrated that *p*-octylphenol causes hyperactivity, comparable to that of hyperkinetic model rats produced by 6-OHDA.[11] Eighty-seven nmol of *p*-octylphenol is equivalent to about 18 μg, which may be a possible environmental level when one considers that humans could be exposed to microgram amounts of bisphenol A from a variety of sources such as food cans and sealants used in dentistry.[18,19]

Tyrosine hydroxylase is an oxidatively labile enzyme whose level of activity is determined, in part, by redox regulation of a disulfide linkage with glutathione.[20] Catechol-quinone reduced tyrosine hydroxylase activity to an extent that is related to cysteine modifications.[21] By analogy, it is possible that the toxicity of *p*-n-octylphenol may be attributed to the degeneration of dopamine neurons, leading to hyperkinetic rats as seen in the case of 6-OHDA,[7] although we could not eliminate the possibility that the estrogenic nature of *p*-n-octylphenol or the alteration of gene expression by *p*-n-octylphenol might, at least in part, be involved.

Recently, a new rat model for ADHD, designated wig rats, was established by gene transfer from Long-Evans Cinnamon to the Wistar King-Aptekman/Hokkaido strain.[22] They are hyperactive and the behavioral abnormalities are transmitted by a single gene, which has not been identified. Although the behavioral phenotypes of wig rats and *p*-n-octylphenol-induced hyperactive rats are apparently different, the attenuation of immunoreactivity for tyrosine hydroxylase was also observed in wig rats,

supporting the crucial role of the enzyme in rat hyperactivity (manuscript in preparation).

During development of the nervous system, a large proportion of neurons die by apoptosis; about 50% of embryonic postmitotic neurons ultimately die during the period when synapses are formed between neurons and their targets.[23,24] Furthermore, apoptosis has been observed in several neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease.[25,26] It is therefore most likely that apoptotic processing might be involved in the *p*-n-octylphenol-induced hyperkinesia. Further study will be required for elucidating the molecular mechanism underlying motor hyperactivity.

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