THE LONG-TERM NEUROEFFECTS OF NEONATAL BISPHENOL A TREATMENT IN ADULT RATS

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Abstract

Bisphenol A (2,2-bis[4-hydroxyphenyl]propane) is an environmental chemical that has both neurotoxic and oestrogenic effects. Intracisternal administration of bisphenol A to neonatal rats has been reported to cause hyperactivity in juveniles. Here we examined the long-term effects of neonatal bisphenol A treatment in adult rats. Apoptotic nuclei were observed in the mesencephalons of adult rats that received bisphenol A treatment during development, and their immunoreactivity for tyrosine hydroxylase, but not glutamic-acid decarboxylase, was significantly reduced. Furthermore, immunohistochemical analyses revealed the aggregation of alpha-synuclein in the substantia nigra of the adult rats, which is a pathological feature of Parkinson disease. Our findings, together with the results of previous studies, confirm that bisphenol A alone can induce hyperactivity in juveniles, but not in adulthood when pathological features of neurodegeneration were still observed.

Introduction

Hyperactivity among children was first described by von Economo in cases of encephalic lethargica[1]. Hyperactivity, sleep disorders and antisocial personality disorder are all associated with this disease in childhood and Parkinsonism is observed in adult cases[1]. This suggests that the etiology of hyperactivity in children could involve the potentially irreversible degeneration of dopaminergic neurons.

An animal model for hyperactivity was produced by Shaywitz and colleagues, who demonstrated that 5-day-old rat pups that were treated with 6-hydroxydopamine (6-OHDA) via intracisternal administration developed increased motor activity, leading to cognitive difficulties in shuttle-box learning at 2–4 weeks of age[2]. These observations are similar to the clinical syndrome of minimal brain dysfunction in children, which is currently known as attention-deficit-hyperactivity disorder (ADHD). Brain dopamine levels were found to be depleted in 6-OHDA-treated rat pups. This neurotransmitter might therefore have a role in the pathogenesis of the disorder.

Using the methods described by Shaywitz and co-workers, we recently demonstrated that intracisternal administration of environmental chemicals, such as bisphenol A[3], *p*-n-octylphenol[4], *p*-nitrotoluene[5], and dicyclohexylphthalate[6], to 5-day-old rats caused hyperactivity at 4-5 weeks of age. However, neither the molecular mechanism of these activities nor the long-term effects of bisphenol A have been described previously.

Materials and Methods

Animals

Pregnant female and 8-week-old male Wistar rats were obtained from Clea Japan (Tokyo, Japan). Animals were housed in cages at 22°C with a 12-h light–dark cycle, and fed a standard laboratory chow (MF diet; Oriental Yeast Corp., Tokyo, Japan) and distilled water *ad libitum*. Approximately 50 male pups were born from 10 pregnant females, 5-7 of which were randomly housed and weaned at 3 weeks of age. All animals were treated humanely in accordance with the guidelines of the National Institute for Environmental Studies, Japan.

Measurements of spontaneous motor activity

Bisphenol A was suspended in 50% ethanol and made up to the required volume with olive oil. A total of 87 nmol in 10 μl olive oil per rat was intracisternally injected into 5-day-old pups. Control animals received 10 μl of vehicle alone. Spontaneous motor activity was measured at 4-5 weeks of age using the Supermex system (Muromachi Kikai, Tokyo, Japan). Activity was measured at 15-min intervals for 24 h under a 12-h light–dark cycle. Food and water were provided *ad libitum* and the rats were not disturbed during the assessment period.

Immunohistochemistry

Immunostaining was carried out as described previously[5]. Rats were sacrificed by decapitation at the ages

indicated. Whole brain samples were fixed in 10% phosphate-buffed formalin (pH 7.2) and embedded in paraffin. Sagittal and coronal sections (5 μm) were mounted on siliconized slides (Matsunami, Osaka, Japan), deparaffinized and hydrated. Each section was permealized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) and blocked with 10% normal horse serum plus 4% bovine serum albumin for 30 min at 4°C. Samples were incubated with the primary antibodies described below in the presence of 4% bovine serum albumin and 0.05% Triton X-100, tyrosine hydroxylase (1:100; Sigma-Aldrich), glutamic-acid decarboxylase 65/67 (1:100; Sigma-Aldrich), and α-synuclein (1:100; MP Biomedicals Inc.). After three washes with PBS containing 0.1% Tween 20, each sample was incubated with fluorescence-labelled secondary goat anti-mouse or rabbit immunoglobulin G (IgG; 1:2,000; Sigma-Aldrich), or biotinylated secondary goat-rabbit IgG (1:2,000; Lab Vision Corp., Fremont), for 60 min at room temperature. Each fluorescence-labelled specimen was washed twice with PBS and observed under a microscope (IX70; Olympus, Tokyo), or the biotinylated molecules were labelled with streptavidin-conjugated alkaline phosphatase and visualized with 3,3′-diaminobenzidine or 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. Images were captured using Viewfinder Lite version 1.0 camera software and a DP-50 digital camera (Olympus).

***In situ* TUNEL labelling**

In situ TUNEL labelling was performed using an Apoptotag *In Situ* Cell Death Detection kit (Intrerigene, Purchase, NY).

Results and Discussion

Hyperactive behaviour in the juvenile

We first investigated the effects of intracisternal administration of 20 μg of bisphenol A (equivalent to 87 nmol in 10 μl of olive oil per rat) on the developing rat brain. Control animals were given 10 μl of olive oil alone. Bisphenol A had a significant effect on motor activity ($p<0.0001$ by ANOVA): the average total spontaneous motor activity during dark phase was 1.6-times higher in the bisphenol A-treated rats than in the vehicle-treated control rats ($p<0.0001$, Student's t test). The pattern of the rhythmic curve did not differ between the control and treated groups, which indicated that the normal rhythmicity was retained.

Long-term effects of neonatal bisphenol A-treatment in adult rats

We then investigated the long-term effects of bisphenol A using immunohistochemical analyses. Brain tissues from 8-week-old rats that were hyperactive juveniles were tested for immunoreactivity to tyrosine hydroxylase, which is a rate-limiting enzyme in catecholamine synthesis. In the control rats, the substantia nigra or ventral tegmental area of the brain tissue strongly stained with the anti-tyrosine hydroxylase antibody. However, a single intracisternal injection of bisphenol A (20 μg) into rat pups resulted in a large reduction of tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta and the ventral tegmental area; this indicated the degeneration of dopaminergic neurons. Immunostaining for glutamic-acid decarboxylase, which is involved in γ-aminobutyric-acid synthesis, revealed the immunoreactive enzyme in the substantia nigra pars reticulata. This confirms that the effects of bisphenol A are specific to the dopaminergic neurons.

To examine whether the bisphenol A-induced reduction of immunoreactive tyrosine hydroxylase in the mesencephalon reflected degeneration of the dopaminergic neurons, we performed terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL). No labelled cells were observed in the control brain tissue. By contrast, TUNEL-positive cells were detected with nuclear condensation in the treated group, which indicated that bisphenol A induced apoptotic cell death in the substantia nigra.

Pathological features of neurodegenerative diseases

Bisphenol A appears to cause the selective degeneration of dopaminergic neurons, similar to that seen in Parkinson disease. We therefore performed an immunohistochemical analysis in order to examine the pathological features of Parkinson disease in the chemically lesioned rats.

α-Synuclein is reported to be a key component of the pathological process of neurogeneration. The control tissues showed faint staining, whereas a significant aggregation of α-synuclein was seen in the substantia nigra pars compacta of adult rats that were neonatally treated with bisphenol A. Furthermore, diffuse staining for α-synuclein was observed much more in the cortex of chemically treated rats compared with that in the controls.

Our results demonstrate that a single intracisternal administration of bisphenol A to neonatal rats induced the

aggregation of α-synuclein in adult animals. These traits are observed in the many neurodegenerative disorders, including Parkinson disease

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Selective nigrostriatal dopaminergic degeneration in adult rats

To more directly investigate whether bisphenol A causes nigrostriatal dopaminergic degeneration in adult rats, 20 μg of this chemical was microinjected into the unilateral substantia nigra of 9-week-old animals. Two weeks after surgery, the brain tissue was subjected to immunostaining for striatal tyrosine hydroxylase. A significant reduction in the level of immunoreactive tyrosine hydroxylase was observed in the ipsilateral striatum of bisphenol A-lesioned rats, whereas strong immunoreactivity was detected in the contralateral striatum. Similar results were seen in 6-OHDA-lesioned Parkinsonian rats. By contrast, strong immunoreactivities were detected in both sides of the striatum of control rats. These results confirm that bisphenol A stimulates the degeneration of nigrostriatal dopaminergic neurons, similar to that seen in Parkinsonian rats.

The estrogenic activity of bisphenol A has been reported. Furthermore, there is considerable public concern about the possibility that environmental chemicals such as bisphenol A might exert effects on neuronal functions. In this study, we demonstrated that a single intracisternal administration of bisphenol A to neonatal rat pups resulted in hyperactivity in nocturnal phases, and Parkinsonism was observed in adult rats.

Currently, hyperactivity was associated with neurodevelopmental disorders such as ADHD or autism whose etiology remains unknown. As Parkinson disease is caused by the selective loss of dopaminergic neurons, it is possible that irreversible damage to these cells might also be associated with hyperactivity in von Economo's encephalic lethargica. It has been demonstrated that bisphenol A was converted to bisphenol *o*-quinone *in vitro*. Tyrosine hydroxylase is an oxidatively labile enzyme whose level of activity is determined, in part, by redox regulation of disulfide linkage with glutatione. Catechol-quinone reduced tyrosine hydroxylase activity to an extent that is related to cysteine modification, therefore, it is possible that the toxicity of bisphenol A may be attributed to degeneration of dopaminergic neurones leading to hyperkinetics behaviour of the rats.

Our present results demonstrate that bisphenol A has irreversible long-term effects on the central nervous system. The rat model described here will help to elucidate the epigenetic etiology of multifactorial diseases, such as hyperkinesias, which are seen in patients with ADHD or autism, as well as that of von Economo's encephalic lethargica *per se*[1].

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