Effects of *p*-Nitrotoluene on Cultured Mesencephalic Neural Stem Cells

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Current risk assessment methods for environmental chemicals are based on adult physiology. However, recent reports have shown an increased incidence of neurodevelopmental disorders which may result from exposure to chemical *in utero* and during the early postnatal period. We previously showed that exposure of neonates to the environmental chemical *p*-nitrotoluene caused hyperactivity, accompanied by changes in the expression of the mesencephalic dopamine transporter gene. In this study, we have examined the effects of *p*-nitrotoluene on cultured neural stem cells isolated from the rat mesencephalon. At embryonic day 15 (E15), these cells stained positive with antibodies against nestin, microtubule-associated proteins (MAPs), and glial fibrillary acidic proteins (GFAPs). The treatment of cultured neurospheres with *p*-nitrotoluene (1 µM; 72 hr) facilitated differentiation with two distinct morphologies outside the sphere, being neural and glial lineages. Neurospheres could therefore be used as a very simple primary assay for screening environmental chemicals for disruption of developmental programming.

Key words —— *p*-nitrotoluene, neural stem cell, neurosphere assay

INTRODUCTION

The very large number of environmental chemicals that now exist have made assessing their neural risks challenging.1) Recently, environmental estrogens, known as endocrine disruptors or endocrine-disrupting chemicals, have been identified as a diverse group of synthetic and naturally occurring compounds that mimic the action of steroidal estrogens.2) Their reproductive effects have been largely described.3) However, the nonlinear relationship between their effects and concentration has hampered their risk assessment.

Recent evidence points to an important effect of exposure to environmental neurotoxicant chemicals on the marked increase seen in neurodevelopmental disorders. We have demonstrated that intracisternal administration of some endocrine-disrupting chemicals, such as bisphenol A,4) octylphenol,5) nonylphenol,6) dibutylphthalate,6) diethylhexylphthalate,6) cyclohexylphthalate,7) and tributyltin,8) caused hyperactivity in male Wistar rats, using the method of Shaywitz *et al.* (1976), who reported that 5-day-old rat pups treated with 6-hydroxydopamine (6-OHDA) showed increased motor activity and at 2–4 weeks, showed cognitive difficulties in shuttle-box learning tests.9) We have further studied environmental evaluation of these disorders,10) and concluded that endocrine-disrupting chemicals seem to be neurotoxic to the developing rat brain, but it is still unclear whether their effects on the developing brain result from their endocrine disrupting activity or some other as yet uncharacterized process.

Many studies have investigated the carcinogenetic properties of *p*-nitrotoluene, which is used to synthesize agricultural and rubber chemicals.11, 12) Recent reports on *p*-nitrotoluene showed it had no estrogenic or androgenic effects in uterotrophic or Hershberger assays13) or in recombinant yeast screens.14) A two-generation reproductive toxicity study also failed to find any effects on endocrine or reproductive organs.15) However, we have shown that intracisternal administration of *p*-nitrotoluene in 5-day-old rats caused hyperactivity, suggesting a deficit in the developing rat brain.16)

Sensitivity to environmental chemicals is dependent on age and may be greatest *in utero* and in the early postnatal period.17) Every year a large number of new chemicals are released into the environment, so the establishment of a simple test for developmental neurotoxicity is urgent. A likely rationale may be to use neural stem cells, as they are most abundant at early developmental stages and can differentiate into neurons, glia, and oligodendrocytes. We have examined the effects of *p*-nitrotoluene on cultured rat neural stem cells to investigate their use *in vitro* to test the developmental effects of environmental chemicals.

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MATERIALS AND METHODS

Chemicals —— *p*-Nitrotoluene was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and olive oil from nacalai tesque (Kyoto, Japan).

Isolation of Rat Neural Stem Cells —— Pregnant Wistar rats at embryonic day 14 (E14) were obtained from Clea (Tokyo, Japan), and maintained in home cages at 22°C on a 12 hr light-dark cycle, on MF diet (Oriental Yeast Corp., Tokyo, Japan) and distilled water *ad libitum*. On E15, they were killed by diethyl ether overdose. The embryos were removed and transferred to minimal essential medium (MEM; Sigma-Aldrich, Tokyo, Japan). Subsequently, the mesencephalons were dissected from the embryos and enzymatically digested with 50 U deoxyribonuclease I (Takara Corp., Kyoto, Japan) and 0.8 U papain (Sigma-Aldrich) at 32°C for 12 min. After stirring, the digestion mix was passed through a 70 µm cell strainer (BD Biosciences, Bedford, MA, U.S.A.). The run-through, containing the neural stem cells, was centrifuged at $800 \times g$ for 10 min, resuspended in Dulbecco's Modified Eagle's Medium/F12 medium (DMEM : F12, 1 : 1; Invitrogen, Tokyo, Japan), supplemented with B27 (Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Inc., Minneapolis, MN, U.S.A.) and 10 ng/ml epidermal growth factor (EGF; Roche Applied Science, Tokyo, Japan), and cultured in uncoated dishes without serum. Fresh culture medium containing EGF and bFGF was added after 3–4 days in culture.

Immunostaining —— For whole brain samples, 2-day-old rats were sacrificed by decapitation. Immunohistochemistry was carried out as described previously.[4–7] In brief, fixed 30 µm coronal sections were blocked, incubated with primary anti-nestin monoclonal antibody (1:100; Chemicon, Temecula, CA, U.S.A.) and then with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1 : 200; Sigma-Aldrich). Nuclei were counterstained with 4′6-diamino-2-phenylindole (DAPI; Invitrogen).

Cultured stem cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.5% Triton X-100 and labeled as described previously,[18, 19] using primary antibodies against nestin (1 : 100; Chemicon), microtubule-associated proteins (MAPs; 1 : 100; Sigma-Aldrich), and glial fibrillary acidic protein (GFAP; 1 : 100; Sigma-Aldrich), or cy3-conjugated GFAP (1 : 100; Sigma-Aldrich). Secondary antibodies conjugated with Alexa 405 (1 : 200; Invitrogen), FITC (1 : 200; Sigma-Aldrich), tetramethylrhodamine isothiocyanate (TRITC) (1 : 200; Sigma-Aldrich), or Alexa 488 (1 : 200; Invitrogen) were properly used. Specimens were then observed with an inverted microscope (IX-70; Olympus, Tokyo, Japan) and images were captured using Viewfinder Lite version 1.0 camera software and a DP-50 digital (Olympus) or Leica TCS SP5 confocal microscope system equipped with an AF6000 inverted microscope (Leica Microsystems, Tokyo, Japan).

RESULTS AND DISCUSSION

Since neonatal exposure to endocrine-disrupting chemicals such as bisphenol A, octylphenol, and cyclohexylphthalate are known to affect the development of mesencephalic dopaminergic neurons,[4–8, 16] we first identified neural stem cells in the neonatal midbrain. Figure 1 shows nestin, a marker protein for neural stem cells, was found around the dorsal cerebral aqueduct, suggesting that the fetal mesencephalon would be a good source of neural stem cells.

We isolated neural stem cells from E15 rat embryos (Fig. 2A), using pooled mesencephalons from 12 fetuses. After 7 d in culture, neurospheres appeared (Fig. 2B), suggesting self-renewal occurred. Neurospheres of about 200 µm in diameter consisted of about 10^3 cells.

To identify neural stem cells, we stained the neurospheres with an anti-nestin antibody, as shown in Fig. 3A. The nestin-positive cells were localized both at the edge and within the spheres. Since neural stem cells are multipotent for neural differentiation, we also immunostained the neurospheres for MAPs, which were located in cells at the edge of the spheres (Fig. 3B). Since on E15, when we isolated the neuronal stem cells, rat embryos are undergoing gliogenesis, we stained the neurospheres with anti-GFAP antibody, which mainly stained cells at the sides of the spheres (Fig. 3C). Our results suggested that heterogeneous cell populations were present in neurospheres, at late embryonic stages.

We finally examined the effects of *p*-nitrotoluene on cultured neurospheres, grown in the presence of EGF and bFGF, by exposing them to a variety of concentration of *p*-nitrotoluene (0.01–1 µM) for 72 hr at 37°C. Following by fixation and permeabilization of the treated neu-

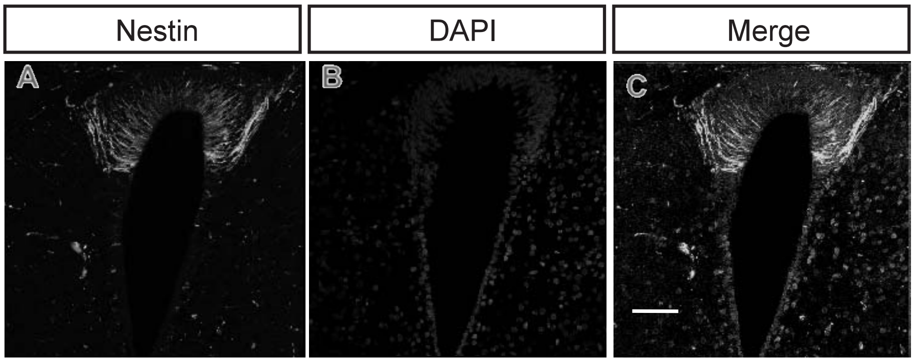

Fig. 1. Nestin-Positive Cells in the Rat Neonatal Midbrain

Coronal sections of the midbrain of 2-day-old rats were labeled with anti-nestin antibody (A) and counter-stained with DAPI (B). The merged image of A and B is shown in C. Scale bar: 50 μm.

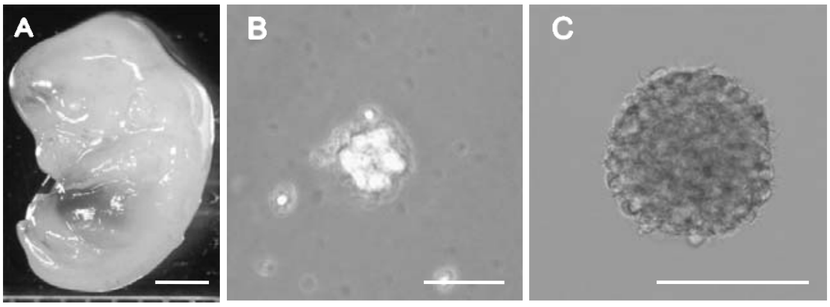

Fig. 2. (A) An E15 Rat Fetus Showing the Mesencephalon, (B, C) Primary Neurospheres Grown From E15 Fetuses, in Uncoated Dishes, in the Presence of bFGF and EGF

Small spheres were apparent after 1–2 d in culture (B) and grew to large colonies in 11 days (C). Scale bar: 2 mm (A); 25 μm (B); 100 μm (C).

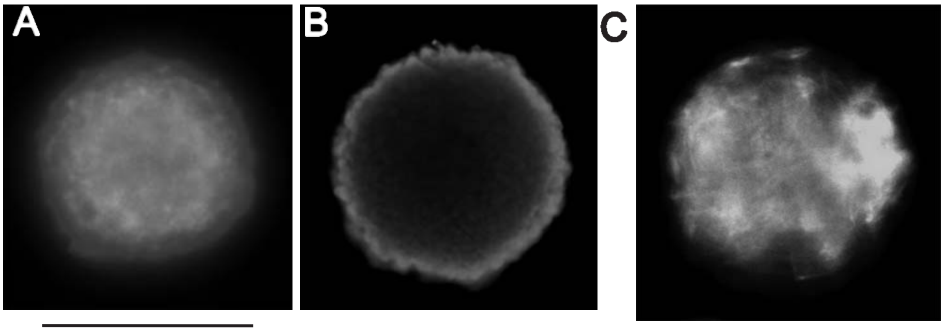

Fig. 3. Identification of Cultured Mesencephalic Neurosphere

Neurospheres were grown in the presence of bFGF and EGF, and immunostained with anti-nestin antibody (A), anti-MAPs antibody (B), or anti-GFAP antibody (C). The specimens were then observed under a fluorescence microscope. Scale bar: 100 μm.

rospheres, the specimens were immunostained with antibodies for nestin, MAPs, and GFAP. Confocal microscope analysis indicated the localization of immunoreactivities for nestin, MAPs, and GFAP. High concentration of *p*-nitrotoluene (1 μM) caused dramatic changes in morphology (Fig. 4); MAP+ cells were round, whereas GFAP+ cells were fibrillar outside the sphere. Effects of lower concentration were seen in the different patterns of immunostaining of the cell populations as compared to those of untreated sphere, probably reflecting the predominant asymmetric division by the chemical. The critical concentration of the chemical would be between 0.1–1 μM to exert morphological effects on the neurosphere.

The developing nervous system is especially vulnerable to damage by toxic agents. Therefore, availability of neural stem cells is useful for testing

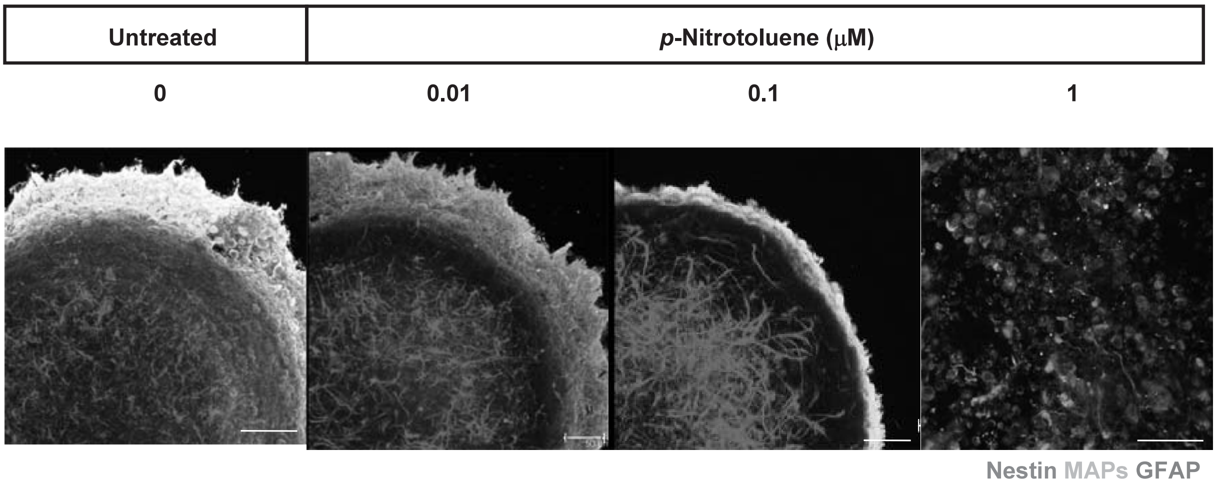

Fig. 4. Effects of *p*-Nitrotoluene on Cultured Neurospheres

The neurospheres were exposed to a variety of concentration of *p*-nitrotoluene for 72 hr at 37°C, as indicated and triple-labeled with antibodies for nestin (gray layer), MAPs (white circle), and GFAP (gray fibril). The specimens were then observed under a confocal microscope system. Scale bar: 50 μm.

the developmental effects of environmental chemicals since neural stem cells play an essential role in the development of the nervous system.

There are a few reports of neurotoxicity using neural stem cells; lead causes a significant inhibition of proliferation,[20)] whereas methylmercury[21)] and manganese[22)] induce apoptosis of neural stem cells. To our knowledge, there has been no report of the neurotoxicity of *p*-nitrotoluene, except our previous report[16)] although numerous studies of the carcinogenicity of the chemical have been carried out.[11, 12)] In this study, we used cultured mesencephalic neural stem cells to test the neurotoxicity of an environmental chemical. This idea was based on the fact that dopaminergic neurons in the neonatal midbrain are highly sensitive to some environmental chemicals.[4–8)] We identified neural stem cells in the postnatal midbrain (Fig. 1), showing the fetal mesencephalon was a good source for growing neurospheres *in vitro* (Fig. 2). Remarkably, high concentration of *p*-nitrotoluene (1 μM; 72 hr) changed the morphology of cells outside the sphere (Fig. 4), with two distinct cell types, typical of neural and glial lineages, arising. Thus, based on cell morphology, this method could function as a primary screen for environmental neurotoxins that disrupt development.

Responsiveness of cultured neural cells to *p*-nitrotoluene is dependent on cell types in culture; *p*-nitrotoluene (1 μM) facilitates neurite outgrowth of human neuroblastomas NB-1 cells, but not of SH-SY5Y cells (unpublished data). In these cultures, however, it is impossible to examine the developmental effects of the chemical, as seen in the neurosphere assay.

Although the rat mesencephalon at E15 is surgically easy to dissect (Fig. 2A), at this stage it has been committed to glial fate. For more detailed investigation of the neurodevelopmental effects of these chemicals, further studies are required, using fetal neural stem cells derived during neurogenic periods.

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