(AAV-8) is of particular interest as a highly efficient gene transfer vehicle for many organs and the ability to transduce glial cells in the murine brain [4]. AAV-8 vector may be useful not only for enhancing the efficacy of therapies for dysmylinating or demylinating diseases of the brain, but also in studying development of oligodendrocytes and the myelination process.

Taking advantage of the efficient and non-toxic transduction of neurons, AAV vectors have already been successfully implemented to create novel animal models of neurological disorders by expressing pathogenic genes instead of therapeutic genes. Techniques for stereotaxic injection into the rodent brain have been established and modern magnetic resonance imaging navigation systems allow precise pin-pointing of infusion sites, even in non-human primates, offering an invaluable tool for studying regions of the brain. In addition, positron emission tomography with various tracers can be used to monitor the effects of transgene expression *in vivo*. Overproduction of α-synuclein in the substantia nigra or β-amyloid in the hippocampus recapitulates the neuronal degeneration in Parkinson's disease or Alzheimer disease, respectively. Viral vector-mediated expression of an α-synuclein model is valuable not only in providing better understanding of involvement in the disease process, but also to offer a closer semblance of the neurodegeneration found in Parkinson's disease than seen with the classical neurotoxin-induced model. Monkeys injected with AAV vectors expressing human mutant α-synuclein show gradual loss of nigral dopaminergic neurons, ubiquitin containing aggregates and motor impairments [5]. Roles of specific *amyloid-β peptide* (Aβ) can be studied in AAV vector-treated animals. Infusion of AAV vectors encoding fusion of *BRI*, a causative gene for familial British dementia, and Aβ42 cDNAs resulted in elevated levels of detergent-insoluble Aβ in the hippocampus [6]. Notably, *in vivo* transduction efficiency would vary due to vector batch differences. Despite this inherent weakness, viral vector-mediated overexpression offers a valuable tool for modeling aspects of neurodegeneration.

Use of AAV vectors is superseding conventional methods for *in vivo* delivery of molecular switches in the brain. Under conventional methods, conditional knock-out mice are made using bacteriophage Pl Cre recombinase (Cre) to shut down gene expression. Transgenesis is achieved by mating mice expressing Cre with transgenic mice expressing a target sequence flanked by loxP sequences, but because phenotypic changes may occur, interpreting the effects of molecular switches on adult brain function is difficult. In contrast, delivery of Cre using AAV vector allows specific inactivation of target genes in adult mice [7].

More recently, we have developed an AAV vector that expresses Cre only after induction by tamoxifen, a synthetic estrogen [8]. This vector was originally generated to increase the safety of gene therapy for neurodegenerative diseases. We have previously demonstrated that AAV vector-mediated gene transfer of 3 enzymes for synthesizing dopamine (DA), namely tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), and GTP cyclohydrolase I (GCH), results in behavioral recovery in animal models of Parkinson's disease. We also showed that gene delivery of glial cell line-derived neurotrophic factor (GDNF) in skeletal muscles slows down the degeneration of motoneurons in a mouse model of amyotrophic lateral sclerosis (ALS). To regulate gene expression and avoid overproduction of DA or GDNF, we generated a recombinant AAV vector that expresses an improved variant of tamoxifen-inducible Cre recombinase (CreERT2), where recombinase is

fused to the mutated ligand-binding domain of the human estrogen receptor. AAV vectors that express TH or GDNF were modified by adding flanking loxP sites to coding sequence of TH or GDNF (AAV-floxed TH or AAV-floxed GDNF). Hemiparkinsonian rats received a mixture of AAV-CreERT2, AAV-floxed TH, AAV-AADC and AAV-GCH, and half were further treated with intraperitoneal 4-OH tamoxifen (4 mg/kg, 5 days). Control rats were injected with AAV-LacZ alone, or AAV-Cre that expressed Cre recombinase with AAV-floxed TH/-AADC/-GCH. Behavioral recovery was observed in rats that received AAV vectors expressing DA-synthesizing enzymes, except for those that received AAV-Cre simultaneously. After tamoxifen treatment, AAV-CreERT2-injected rats again showed behavioral impairment. DA level in lesioned striatum was significantly lower and fewer TH-immunoreactive cells were seen in the tamoxifen-treated AAV-CreERT2 group compared with the tamoxifen-untreated group. AADC activity was unchanged after tamoxifen treatment, suggesting selective removal of the TH gene. Tamoxifen-induced reductions in GDNF expression were also observed in the muscles of mice that received both AAV-floxed GDNF and AAV-CreERT2. Inducible reduction of transgene using AAV-CreERT2 should represent not only a molecular switch for increasing the safety of gene therapy, but also a powerful experimental tool for neuroscience.

Induction of RNA interference (RNAi), a gene-silencing mechanism, by expressing target-complementary short hairpin RNA (shRNA) from viral vectors is another promising technique for investigating gene function *in vivo*. Persistent and functional shRNA expressions have been reported in therapeutic experiments including a mouse model of spinocerebellar ataxia type 1 [9]. Further improvements may be achieved by engineering upstream primary microRNAs with more appropriate controlled expression vectors.

With continued advances, AAV vectors will become more valuable tools for use in neuroscience.

REFERENCES

[1] Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther.* *14*(3):316-27, 2006.

[2] Muramatsu S, Tsukada H, Nakano I, Ozawa K. Gene therapy for Parkinson's disease using recombinant adeno-associated viral vectors. *Exp Opin Biol Ther.* *5*(5):663-71, 2005.

[3] Kaplitt MG, Feigin A, Tang C, Fitzsimons HL, Mattis P, Lawlor PA, Bland RJ, Young D, Strybing K, Eidelberg D, During MJ. Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. *Lancet.* *369*(9579):2097-105, 2007.

[4] Nakai H, Fuess S, Storm TA, Muramatsu S, Nara Y, Kay MA. Unrestricted hepatocyte transduction with adeno-associated virus serotype 8 vectors in mice. *J Virol.* *79*(1):214-24, 2005.

[5] Eslamboli A, Romero-Ramos M, Burger C, Bjorklund T, Muzyczka N, Mandel RJ, Baker H, Ridley RM, Kirik D. Long-term consequences of human alpha-synuclein overexpression in the primate ventral midbrain. *Brain.* *130*(Pt 3):799-815, 2007.

[6] Lawlor PA, Bland RJ, Das P, Price RW, Holloway V, Smithson L, Dicker BL, During MJ, Young D, Golde TE. Novel rat Alzheimer's disease models based on AAV-mediated gene transfer to selectively increase hippocampal Abeta levels. *Mol Neurodegener.* *2*:11, 2007.

[7] Kaspar BK, Vissel B, Bengoechea T, Crone S, Randolph-Moore L, Muller R, Brandon EP, Schaffer D, Verma IM, Lee KF, Heinemann SF, Gage FH. Adeno-associated virus effectively mediates conditional gene modification in the brain. *Proc Natl Acad Sci U S A.* *99*(4):2320-5, 2002.

[8] Li XG, Okada T, Kodera M, Nara Y, Takino N, Muramatsu C, Ikeguchi K, Urano F, Ichinose H, Metzger D, Chambon P, Nakano I, Ozawa K, Muramatsu S. Viral-mediated temporally controlled dopamine production in a rat model of Parkinson disease. *Mol Ther.* *13*(1):160-6, 2006.

[9] Xia H, Mao Q, Eliason SL, Harper SQ, Martins IH, Orr HT, Paulson HL, Yang L, Kotin RM, Davidson BL. RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. *Nat Med.* *10*(8):816-20, 2004.

Wiley InterScience — Discover something great
Journal of Neuroscience Research 85:1752–1761 (2007)

Activated Microglia Affect the Nigro-Striatal Dopamine Neurons Differently in Neonatal and Aged Mice Treated with 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine

Hirohide Sawada,[1] Ryohei Hishida,[2] Yoko Hirata,[3] Kenji Ono,[4] Hiromi Suzuki,[4] Shin-ichi Muramatsu,[2] Imaharu Nakano,[2] Toshiharu Nagatsu,[1,4] and Makoto Sawada[4]*

[1]School of Medicine, Fujita Health University, Toyoake, Japan
[2]Division of Neurology, Department of Medicine, Jichi Medical University, Shimotsuke, Japan
[3]Department of Biomolecular Science, Faculty of Engineering, Gifu University, Gifu, Japan
[4]Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan

Microglia play an important role in the inflammatory process that occurs in Parkinson's disease (PD). Activated microglia produce cytokines and neurotrophins and may have neurotoxic or neurotrophic effects. Because microglia are most proliferative and easily activated during the neonatal period, we examined the effects of neonatal microglia activated with lipopolysaccharide (LPS) on the nigro-striatal dopamine neurons in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in comparison with activated microglia from the aged mice. By MPTP administration to neonatal mice, the number of dopamine neurons in the substantia nigra (SN) was decreased significantly, whereas that in the mice treated with LPS and MPTP was recovered to normal, along with significant microglial activation. Tyrosine hydroxylase (TH) activity, the levels of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC), and the levels of pro-inflammatory cytokines IL-1β and IL-6 in the midbrain were elevated in the neonates treated with LPS and MPTP. On the contrary, although the number of dopamine neurons in the 60-week-old mice treated with MPTP was also decreased significantly, the microglial activation by LPS treatment caused a further decrease in their number. These results suggest that the activated microglia in neonatal mice are different from those in aged mice, with the former having neurotrophic potential toward the dopamine neurons in the SN, in contrast to the neurotoxic effect of the latter. © 2007 Wiley-Liss, Inc.

Key words: microglia; dopamine neurons; neonatal mice; MPTP; cytokines

Microglia play important roles in the development, differentiation, and maintenance of neural cells in the brain. They also have immunologic functions and serve to remove dead cells by phagocytic activity after brain injury or neurodegeneration. Activated microglia may play neurotoxic roles by producing pro-inflammatory cytokines, nitric oxide (NO), and reactive oxygen species (ROS; Chao et al., 1992; Hunot et al., 1996; Cassarino et al., 1997; Liu et al., 1998; Kim et al., 2000; McGuire et al., 2001; Koutsilieri et al., 2002). Activated microglia may also play neuroprotective roles by producing neurotrophic components such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β), plasminogen, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF; Nagata et al., 1993b; Suzumura et al., 1993; Sawada et al., 1995, 1999; Elkabes et al., 1996; Miwa et al., 1997; Batchelor et al., 1999; Nakajima et al., 2001). Other cytokines produced from activated microglia, such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6, are pleiotropic, and produce either neurotoxic or neuroprotective effects (Barger et al., 1995; Liu et al., 1998; Fisher et al., 2001; Mason et al., 2001; McGuire et al., 2001; Bolin et al., 2002; Arai et al., 2004). Neurotrophic effects of microglial

Contract grant sponsor: Ministry of Health, Labor, and Welfare of Japan; Contract grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan; Contract grant sponsor: Japan Health Sciences Foundation.

*Correspondence to: Dr. Makoto Sawada, PhD, Department of Brain Life Science, Research Institute of Environmental Medicine, Nagoya University, Chikusa, Nagoya, Aichi 464-8601, Japan.
E-mail: msawada@riem.nagoya-u.ac.jp

Received 15 October 2006; Revised 15 December 2006; Accepted 18 December 2006

Published online 27 April 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21241

© 2007 Wiley-Liss, Inc.

activation were found in cell-culture studies (Nagata et al., 1993a; Elkabes et al., 1996; Miwa et al., 1997; Nakajima et al., 2001), and animal models of neurodegeneration (Rabchevsky et al., 1997; Suzuki et al., 2001; Hashimoto et al., 2005).

Parkinson's disease (PD) is a progressive neurodegenerative disorder of dopamine (DA) neurons in the substantia nigra (SN). One of the neurodegenerative mechanisms of PD is the neuroinflammatory process, by which increased levels of cytokines such as TNF-α (Mogi et al., 1994a), IL-1β, IL-6, epidermal growth factor, and TGF-α (Mogi et al., 1994b) are found in the nigro-striatal region. Microglial activation was reported to be neurotoxic in experimental PD models produced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Wu et al., 2002, 2003; Furuya et al., 2004). The source of increased levels of cytokines in the PD brain and cerebrospinal fluid (CSF) is most probably activated microglia (Nagatsu and Sawada, 2005). Imamura et al. (2003) reported that MHC class II-positive microglia produced TNF-α and IL-6, and were associated actively with damaged neurons and neurites in the SN of PD patients, suggesting that activated microglia might act for neuroprotection. They also showed that in PD patients activated microglia were observed not only in the SN, where DA cell death occurs but also in the hippocampus, where there is no cell death. Imamura et al. (2005) further reported that in patients with dementia with Lewy bodies (DLB), the levels of BDNF mRNA and immunochemically detected protein were decreased significantly in the hippocampus, where cell death occurs, but that they were not decreased in the PD hippocampus. These results suggest that activated microglia in the hippocampus in PD may be neuroprotective in contrast to their neurotoxic effect in DLB patients. Very recently, Sawada et al. (2006) proved the presence of neurotrophic and neurotoxic groups of microglia in the mouse brain.

In the present study, to explore possible age differences we investigated the neuroprotective or neurotoxic effects of activated microglia on DA neurons in the SN in vivo in neonatal mice in comparison to those of the cells in aged mice. Neonatal microglia are activated M-CSF-dependently from late gestation to 2 weeks, and are most proliferative and easily activated under normal circumstances (Sawada et al., 1990; Thery et al., 1990). Microglia are activated by lipopolysaccharide (LPS), and are the major LPS-responsive cells in the brain (Lehnardt et al., 2003).

MATERIALS AND METHODS

Animals

All experiments were carried out using neonatal and aged 60-week-old male C57BL/6 mice (Charles River Laboratories, Tokyo, Japan). Neonatal mice were obtained from purchased pregnant female mice. The animals were housed in a room with a 12-hr light/12-hr dark cycle with free access to food and water. All animal procedures were in accordance with the Jichi Medical University guidelines for animal care.

MPTP Administration

MPTP-HCl and LPS were purchased from Sigma (St. Louis, MO) and dissolved in saline. Neonatal mice were pretreated with intraperitoneal (i.p.) injections of saline or LPS (1.0 mg/kg) daily for 5 days from postnatal day 3 (P3) to P7, and then injected with MPTP (20 mg/kg i.p.) daily for 5 days. Male 60-week-old mice received 5 consecutive days of saline or LPS injections, but only a single injection with MPTP (20 mg/kg) was carried out on the last day of LPS treatment; for repeated injection of LPS and MPTP was lethal in the aged mice. Control mice received only saline injections according to the same schedule. All mice were sacrificed 24 hr after the last MPTP injection.

Preparation of Brain Tissue

For histologic analysis, mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused intracardially with 2% paraformaldehyde in 0.1 M phosphate-buffer (PB). The brains were removed and postfixed in the same fixative for 6 hr at 4°C. They were washed with 10–20% sucrose/0.1 M PB for 24 hr at 4°C and thereafter quickly frozen in Tissue-Tek OCT compound embedding matrix (Sakura Finetek, Tokyo, Japan) and cut as 8-μm-thick coronal sections with a cryostat. For biochemical analysis, mice were anesthetized and perfused with 0.1 M PB, after which their brains were dissected out, frozen quickly in liquid nitrogen, and kept at −80°C.

Immunohistochemistry

Fixed brain tissues were immunostained by the double immunofluorescence method for light microscopy. Tissue sections were fully dried, then re-fixed in cold acetone for 10 min at room temperature, and washed in phosphate-buffer saline (PBS). Non-specific reactions were blocked by incubation for 1 hr at room temperature in blocking solution containing 10% normal goat serum and 1% bovine serum albumin in PBS. The sections were first incubated for 12 hr at 4°C with a blocking solution-diluted primary antibody specific for a microglial marker. They were washed with PBS and incubated with the appropriate fluorescent secondary antibody for 1 hr at room temperature. After having been washed in PBS, the sections were reacted with a second set of primary antibody against another marker and the appropriate secondary fluorescent antibody under the same conditions. For immunofluorescence staining of microglia, two monoclonal antibodies were used as primary antibodies, rat anti-CD11b (M1/70.15.11.5.2, Cell Hybridoma Bank) or rat anti-F4/80 (HB-198, Cell Hybridoma Bank). Other primary antibodies used were rabbit polyclonal anti-tyrosine hydroxylase (TH, diluted 1:5,000) (Nagatsu et al., 1977) for DA neurons, and rabbit polyclonal anti-caspase-3 (cleaved type) antibody (diluted 1:400; Cell Signaling, Beverly, MA) for apoptosis detection. The fluorophore-conjugated secondary antibodies used were goat Cy3 anti-rat IgG (diluted 1:400; Rockland, Gilbertsville, PA) and goat Alexa Fluor 488 anti-rabbit IgG (diluted 1:400; Molecular Probes, Eugene, OR). The nuclei in these sections were stained with 1 mg/ml of 4′,6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan) for 10 min, after which

the sections were mounted with aqueous DAKO fluorescence mounting medium.

Fluoro-Jade B Staining

Degenerating neurons were detected by Fluoro-Jade B (FJB) fluorescence staining (Schmued and Hopkins, 2000). After sections had been immersed in a solution containing 1% sodium hydroxide in ethanol and washed in distilled water, they were transferred to a solution of 0.06% potassium permanganate for 10 min. Then the sections were washed and stained with 4×10^{-4}% FJB solution including 0.1% acetic acid for 20 min. They were washed with distilled water, fully dried, and mounted with non-aqueous mounting medium (Entellan neu, Merck, Whitehouse, NJ).

Quantitative Morphologic Analysis

Quantitative analysis of DA neurons and microglia in the SN were carried out by double-immunostaining for TH and CD11b or F4/80, respectively. Immunofluorescence images were captured with an imaging system (Sensys, CCD Camera; Photometrics, Tokyo, Japan) connected to a computer with an image program (IP Lab software; Signal Analytics, Palo Alto, CA). Numbers of cells were counted in every fifth 8-μm section throughout the entire SN. For counting DA (A9) neurons in the SN pars compacta (SNc), the total number of these neurons were calculated from the total number of TH positive cells throughout the entire SNc (Aguirre et al., 1999), which corresponded to the representative levels from Bregma −2.92 (Franklin and Paxinos, 1997) to Bregma −3.64 (Franklin and Paxinos, 1997). The number of microglia per section of the SNc was also counted. The total number of sections per mouse was from 8–10. Cell counting was carried out at low-power magnification (100×). The data were expressed as the mean ± SD and examined for statistical differences by using the unpaired Student's *t*-test (StatView, Cary, NC).

Assay of TH Activity and Contents of Dopamine and DOPAC

TH activity was analyzed by measuring enzymatically formed L-3,4-dihydroxyphenylalanine (DOPA; Hirata et al., 2001). The incubation mixture consisted of 0.2 M Tris-acetate (pH 6.0), 20 μg catalase, 1 mM 6-methyl-5,6,7,8-tetrahydropterin, 0.1 M 2-mercaptoethanol, 0.2 mM tyrosine, and 40 μL of homogenate as enzyme. Incubation was carried out at 37°C for 10 min in a total volume of 200 μL. The contents of DOPA, dopamine, and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), were determined by high-performance liquid chromatography (HPLC) with electrochemical detection (EICOM, Kyoto, Japan).

Cytokine Analysis

Analyses of cytokines were carried out by using mouse enzyme-linked immunoabsorbent assay (ELISA) systems for TNFα, IL-1β, and IL-6 (Quantikine, R&D Systems, Minneapolis, MN). This assay is based on the quantitative sandwich enzyme immunoassay with a purified antibody specific for each cytokine. Brain tissues were weighed and homogenized in 9 vol of 50 mM Tris-HCl buffer containing 5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, and 5 μg/mL leupeptin. The homogenates were then centrifuged at 15,000 × *g* for 10 min, and supernatants were used for cytokine analysis. Briefly, a 50-μL sample or standard was added to each microplate well-coated with a primary antibody. After a wash with buffer, the identical antibody conjugated to horseradish peroxidase was added, followed by tetramethylbenzidine substrate solution as chromogen. Protein concentrations were measured with a Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) using bicinchoninic acid for the detection of Cu^{+} formed from Cu^{2+} by protein.

RNA Preparation and RT-PCR

Total RNA extracted from frozen tissue samples of midbrain using a modified acid phenol-guanidine method was used as a template for first-strand cDNA synthesis as following method. A random primer (0.1 μg) was incubated at 95°C for 10 min with the RNA (1 μg) in a volume of 30 μl, and then placed on ice for 5 min. Next, this mixture was incubated at 37°C for 90 min with a mixture of 100 U M-MLV reverse transcriptase (Gibco BRL, Grand Island, MI), 1× reverse transcription buffer, 10 mM dithiothreitol, 40 U RNase inhibitor, and 0.56 mM each of dATP, dGTP, dCTP, and dTTP in a volume of 50 μl, then heated at 95°C for 10 min. The cDNA was amplified with Taq DNA polymerase (Takara, Otsu, Japan) using primer pairs specific to NGFβ (sense primer: AGTTTTACCAAGGGAGCA, antisense primer: GGCAGTGTCAAGGGAATG), BDNF (sense primer: AAGAAAGCCCTAACCAGT, antisense primer: CGAAAGTGTCAGCCAATG), neurotrophin (NT)-3 (sense primer: GCTTATCTCCGTGGCATC, antisense primer: TGTTGTCGCAGCAGTTCG), GDNF (sense primer: GCCAGAGGATTAT-CCTGA, antisense primer: CCCAGACCCAAGTCAGTG), or NT-4/5 (sense primer: GCTGTGGACTTGCGTGG, antisense primer: GCCCGCACATAGGACTG) for 35 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min), and GAPDH (sense primer: GAAGGTGAAGGTCGGAGTC, antisense primer: GAAGATGGTGATGGGATTTC) for 30 cycles. The 195-bp (NGFβ), 260-bp (BDNF), 257-bp (NT-3), 240-bp (GDNF), 209-bp (NT-4/5), and 228-bp (GAPDH) PCR products were resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide, and photographed.

RESULTS

Morphological Alterations of Microglia in Neonatal and Aged Mice Administered MPTP

Immunohistochemical study of the DA (A9) neurons in the SNc was carried out by using antibody against TH, and activated microglia were stained with antibody against CD11b. The number of TH-positive DA (A9) neurons in the SN was decreased in MPTP-treated (MPTP) group mice, as compared with saline-treated control (saline) group mice. However, in mice treated with LPS and MPTP (LPS-MPTP) group, the number of DA (A9) neurons was recovered from MPTP group mice (Fig. 1A). In the neonatal mice, the majority

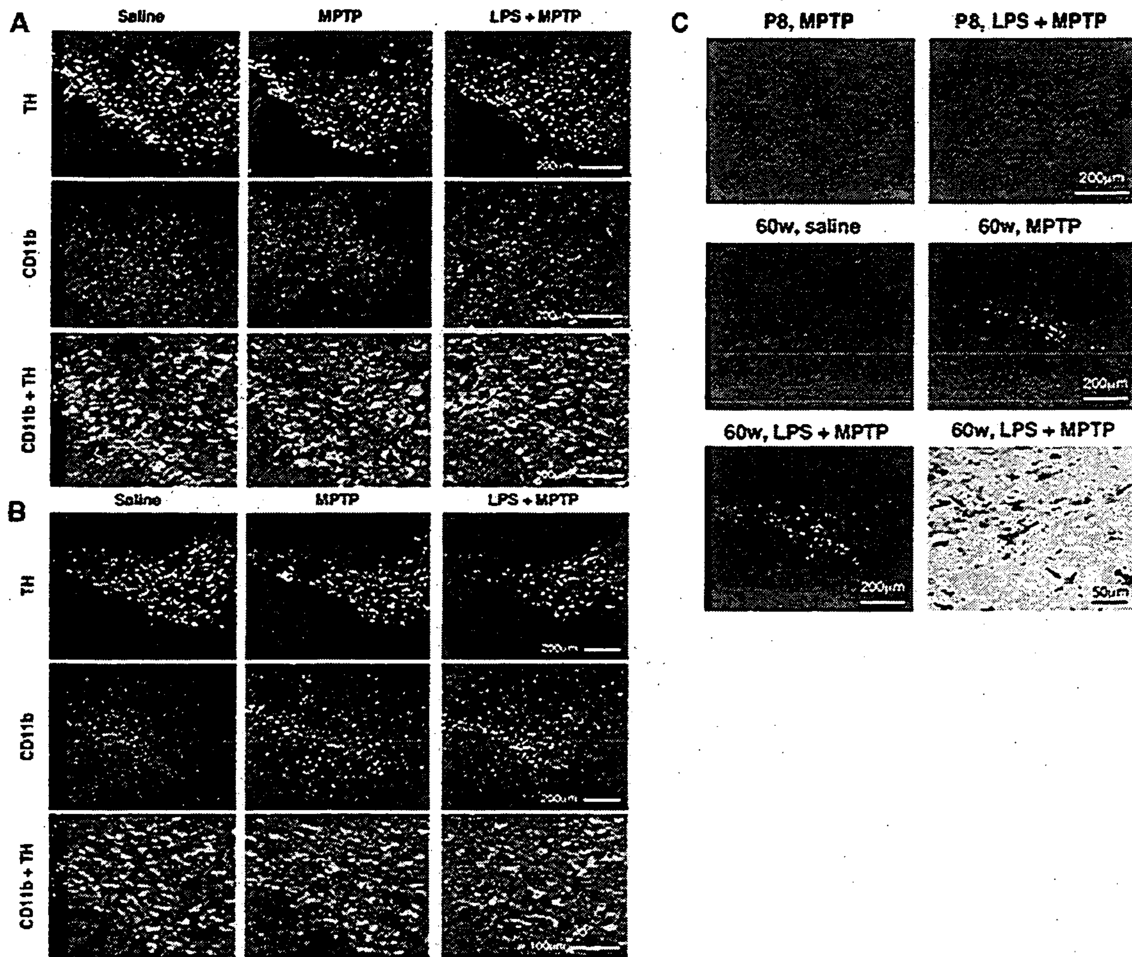

Fig. 1. Morphological changes due to MPTP administration in the SN. Immunostaining for tyrosine hydroxylase (TH)-positive dopamine (DA) (A9) neurons (merged, green) and CD 11b-positive activated microglia (merged, red) in the SN from mice treated with saline, MPTP, and LPS-MPTP are shown. **A:** In neonatal mice, DA (A9) neurons in the SN were decreased in MPTP-treated mice, whereas these neurons in the LPS-MPTP-treated mice were recovered, compared from MPTP-treated mice. The activated microglia had increased in number in the entire SN in mice treated with LPS-MPTP, as compared with saline- or MPTP-treated mice. **B:** In the aged mice, numbers of the DA (A9) neurons were decreased in the order of saline, MPTP, and LPS-MPTP treatments. In the MPTP- and LPS-MPTP-treated mice, numbers of the activated microglia were increased with their accumulation in the SNc, and the majority of the microglia showed amoeboid features. **C:** Detection of neuronal degeneration by Fluoro-Jade B (FJB) staining in MPTP-treated neonatal and aged mice. P8 refers to postnatal day 8; and 60w, to 60-week-old mice. In neonatal mice, FJB staining in the SNc of MPTP- or LPS-MPTP-treated mice were all negative. FJB staining in the SNc was negative for the aged saline-treated mice, but the MPTP- and LPS-MPTP-treated aged mice showed FJB-positive cells in their SN. Results of double staining for CD 11b-positive microglia and FJB-positive degenerative neurons in aged mice treated with LPS and MPTP are also shown. The amoeboid or ramified microglia (black) were phagocytic (arrows) or non-phagocytic (arrowheads) for FJB-positive cells (green).

of the microglia were ramified, with a small population of amoeboid ones, in the SNc or SN pars reticulata (SNr) in saline- or MPTP-treated mice. In mice treated with LPS-MPTP, activated microglia, which had thicker branched processes than resting (ramified feature) microglia, had increased in number in the entire SN (Fig. 1A). In the sections from the 60-week-old (aged) mice, the number of DA (A9) neurons was decreased in order of saline, MPTP, and LPS-MPTP groups (Fig. 1B). In the aged mice, most of the microglia were resting in the saline group, but the mice treated with MPTP and LPS-MPTP, the majority of the microglia showed amoeboid features (Fig. 1B). In both MPTP- and LPS-MPTP-treated aged mice, most of activated microglia were accumulated in the SNc, unlike the microglial distribution in the entire SN in neonatal LPS-MPTP-treated mice.

Neuronal Degeneration Due to MPTP Administration Was Observed Only in Aged Mice

DA cells in the SNc of MPTP-treated neonatal mice showed no obvious features of degeneration or cell

death as judged from the negative results of FJB staining (Fig. 1C). FJB staining of the DA (A9) neurons was negative for all of the 60-week-old of saline control mice. However, 2 of 5 MPTP-treated mice and all of the LPS-MPTP-treated mice (n = 3) were FJB-positive (Fig. 1C). The MPTP and LPS-MPTP groups of aged mice showed FJB-positive cells in their SN, and some of the activated microglia had phagocytosed degenerating FJB-positive cells (Fig. 1C). Cleaved caspase-3-positive DA (A9) neurons or microglia were not observed in the SN both in the neonatal and aged mice (data not shown).

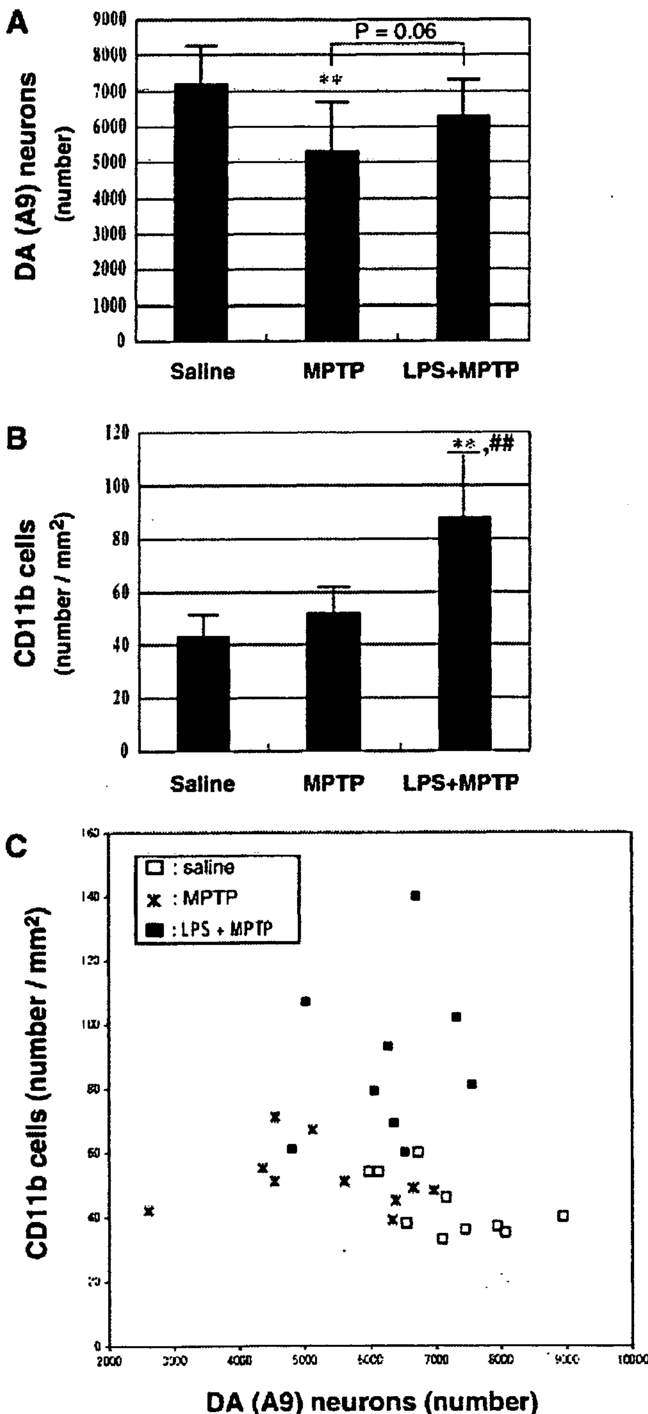

Fig. 2. Analysis of effects of LPS treatment on numbers of DA (A9) neurons and CD 11b-positive activated microglia in MPTP-treated neonatal mice. **A:** The number of DA (A9) neurons in the SN for the saline, MPTP, and LPS-MPTP groups is shown. The number of DA (A9) neurons in the MPTP group was decreased significantly, whereas that for the LPS-MPTP group was recovered. **B:** The number of CD11b-immunopositive microglial cells in the SN is shown. The LPS-MPTP group showed marked microglial activation. Values represent the mean ± SD. $^{**}P < 0.01$ vs. saline group; $^{\#\#}P < 0.01$ vs. MPTP group, by use of the unpaired Student's t-test (n = 9–10). **C:** Relationship between activated microglia and DA (A9) neurons in saline, MPTP, and LPS-MPTP groups of neonatal mice. Only slight activation of microglia and decrease in number of DA (A9) neurons were found for the MPTP group, whereas the LPS-MPTP group showed marked microglial activation and a tendency toward protection against loss of DA (A9) neurons compared to the MPTP group.

Microglial Activation by LPS Treatment Induces Neurotrophic Effects on Dopamine Cell Bodies in Neonatal Mice Administered MPTP

By MPTP administration, the number of TH-positive DA (A9) neurons in the SN of neonatal mice was significantly decreased (74% of the number for the saline group). In contrast, the number of DA (A9) neurons in the LPS-MPTP group was recovered from MPTP group (118% of the number for the MPTP group, P = 0.06) (Fig. 2A). The CD11b-positive microglia in the SN were increased markedly in number in the LPS-MPTP group (Fig. 2B). By staining with F4/80, another marker of microglia, there were no significant differences in their number among the three groups (data not shown).

The relationship between microglial activation and impairment of DA (A9) neurons in the MPTP-treated neonatal mice is shown in Figure 2C. A modest activation of microglia and a significant decrease in the number of DA (A9) neurons were observed in the MPTP group, whereas the LPS-MPTP group showed marked microglial activation and a tendency toward protection against cell toxicity, as compared with the MPTP group (Fig. 2C).

Effects of Microglial Activation on TH Activity and Levels of Dopamine and DOPAC in the Midbrain of Neonatal Mice Treated With MPTP and LPS

TH enzymatic activity and the contents of DA and its metabolite DOPAC in the three groups of neonatal mice were measured. In the LPS-MPTP group, TH activity was increased by 229% and 231% as compared to that of the saline group and the MPTP group, respectively (Fig. 3A). The contents of DA and DOPAC were

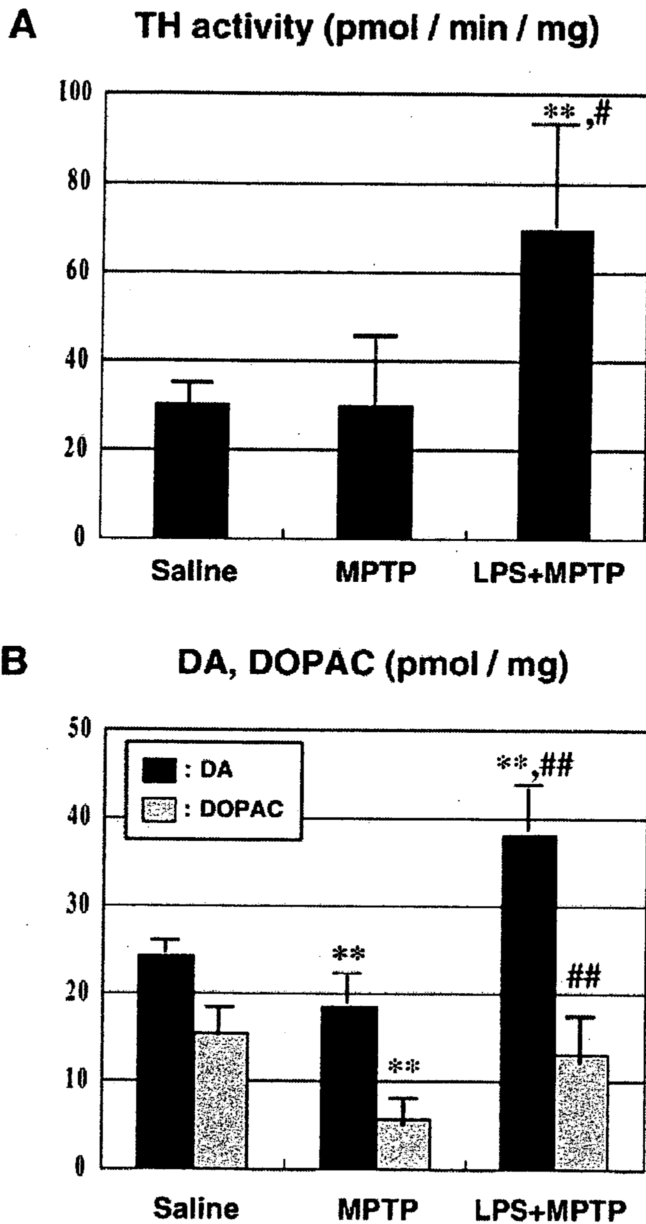

Fig. 3. Biochemical analyses of TH activity and DA and DOPAC contents in MPTP-treated neonatal mice. **A:** TH activity was measured in each of the three groups, and the LPS-MPTP group showed increased activity in the midbrain compared with the other two groups. **B:** Contents of DA and DOPAC in the midbrain after MPTP administration. Both DA and DOPAC contents were significant decreased in the MPTP group, but increased in the LPS-MPTP group. Values represent the mean ± SD. $^{**}P < 0.01$ vs. saline group, and $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs. MPTP group, by use of the unpaired Student's t-test ($n = 7$–10).

decreased in the MPTP group (DA, 76%, and DOPAC, 37% of the saline group, respectively), but these values for the LPS-MPTP group were significantly higher than those for the former group (DA; 205%, and DOPAC; 227% of the MPTP group, respectively) (Fig. 3B).

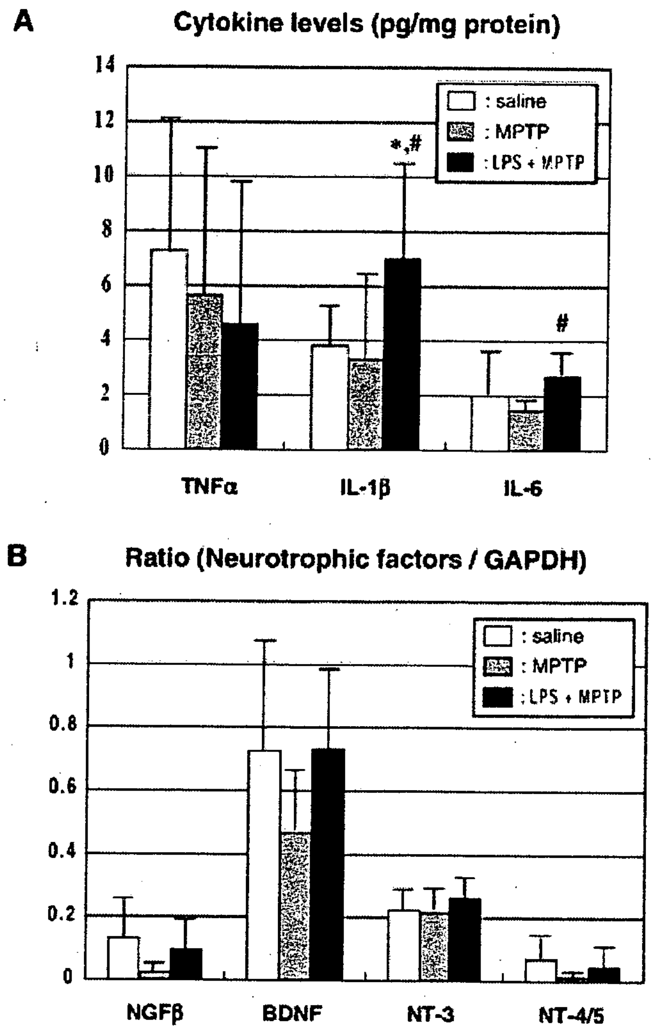

Fig. 4. Analysis of the levels of pro-inflammatory cytokines and neurotrophic factors in the midbrain of MPTP-treated neonatal mice. **A:** As to the pro-inflammatory cytokines, the TNFα level was not different among the three groups, but the IL-1β and IL-6 levels in the LPS-MPTP group were significantly higher than those in the saline and MPTP groups. **B:** The mRNA expression of neurotrophic factors, NGF-β, BDNF, NT-3, and NT-4/5 in the LPS-MPTP group were tended to higher expression than the MPTP group. Values represent the mean ± SD. $^{*}P < 0.05$ vs. saline group; $^{\#}P < 0.05$ vs. MPTP group (unpaired Student's t-test) ($n = 8$–9, pro-inflammatory cytokines; $n = 3$–8, neurotrophic factors).

Effects of Microglial Activation by LPS Treatment on Pro-Inflammatory Cytokines and Neurotrophic Factors Levels in the Midbrain of Neonatal Mice Treated With MPTP

By using the ELISA method, we analyzed the levels of pro-inflammatory cytokines, i.e., TNFα, IL-1β, and IL-6, in the brain tissues from the MPTP and LPS-MPTP groups. In the neonatal midbrain, the TNFα level was not different among the saline, MPTP, and LPS-MPTP groups. As shown in Figure 4A, IL-1β and IL-6 levels in the LPS-MPTP group were increased sig-

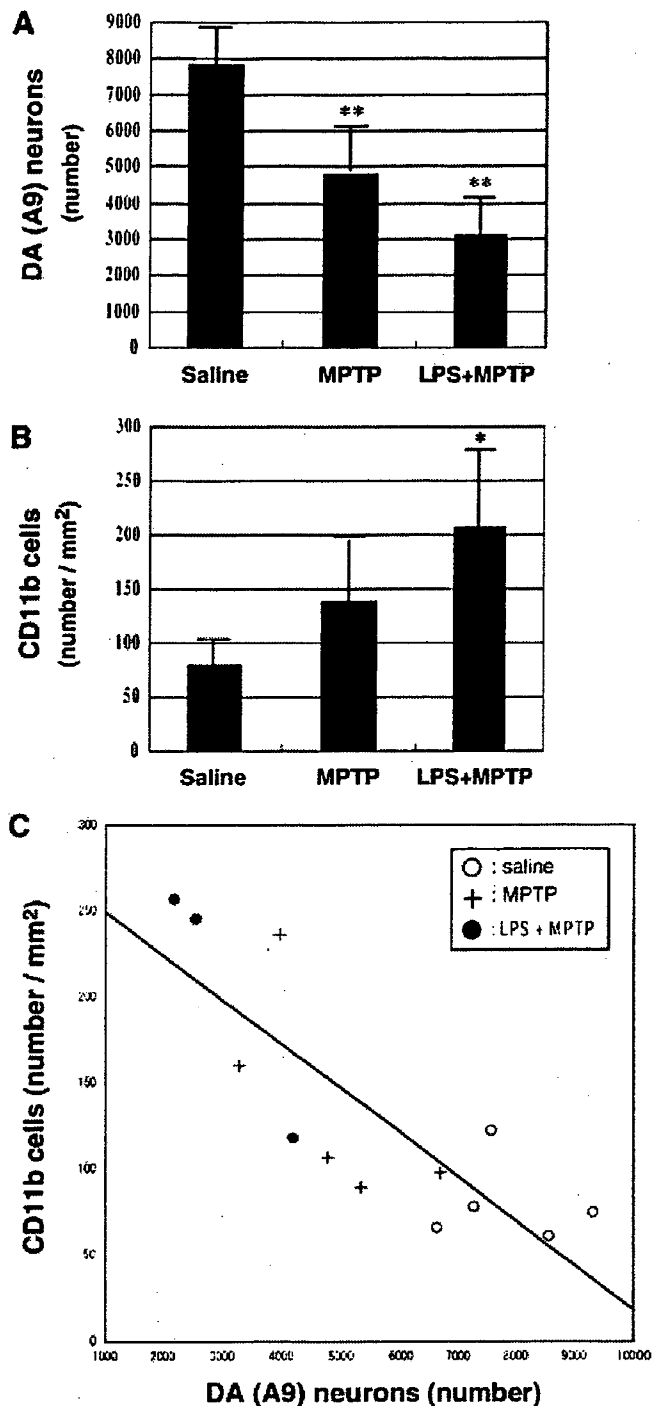

Fig. 5. Effects of microglial activation by LPS treatment on numbers of DA neurons and microglia in SN of 60-week-old (aged) mice treated with MPTP. **A:** Number of DA (A9) neurons in saline, MPTP, and LPS-MPTP groups. The number in the MPTP and LPS-MPTP groups was decreased significantly. **B:** Number of CD11b-immunopositive microglia in saline, MPTP, and LPS-MPTP groups. Severe microglial activation was observed in the LPS-MPTP group. Values represent the mean ± SD. *$P < 0.05$; **$P < 0.01$ vs. saline group (unpaired Student's t-test) ($n = 3$–5). **C:** Relationship between activated microglia and DA (A9) neurons in the SNc of MPTP-treated aged mice. An inverse correlation ($R = 0.81$) was observed between the two parameters when data for all three groups was plotted.

nificantly over those in the saline or MPTP group (IL-1β: 184% of the saline group, 207% of the MPTP group; and IL-6: 188% of the MPTP group, respectively).

The mRNA expressions of neurotrophic factors were also measured as their ratios to GAPDH by RT-PCR method. The mRNA expressions of NGF-β, BDNF, and NT-4/5 tended to decrease in the MPTP group, and these of the LPS-MPTP group were recovered to those of the saline group (Fig. 4B). As to the NT-3, the mRNA expression tended to most increase in LPS-MPTP group (Fig. 4B). However, statistical differences were not observed due to variations of the data.

Effects of Microglial Activation by LPS Treatment on the Number of Dopamine Neurons in Aged Mice Administered MPTP

Aged, 60-week-old male mice were pretreated with LPS to activate their microglia, and then given a single injection of MPTP. The number of DA (A9) neurons in the aged mice was decreased in the MPTP group (to 61% of the saline group); and that of the LPS-MPTP group was markedly decreased (to 40% of the saline group) by the single MPTP administration (Fig. 5A). The MPTP group of aged mice showed an increased number of activated microglia in their SNc; and in the LPS-MPTP group, the increase was statistically significant (Fig. 5B). The relationship between microglial activation and viability of DA (A9) neurons for the three groups of aged mice showed an inverse correlation ($R = 0.81$) (Fig. 5C).

DISCUSSION

In this study, we showed the possibility that microglia activated by treatment with LPS may have neurotrophic potential toward DA neurons in neonatal mice administered MPTP. TH activity and the levels of DA and DOPAC, as well as those of the pro-inflammatory cytokines IL-1β and IL-6, were elevated in the midbrain of LPS-MPTP-treated neonatal mice (Figs. 3,4). The cell viability of DA (A9) neurons was recovered in neonatal mice of the LPS-MPTP group compared with that for the MPTP group (Fig. 2). In contrast, the viability of these neurons in the aged mice dropped significantly by the same comparison (Fig. 5). These results may suggest that the activated microglia are different between neonatal and aged brains; i.e., the activated microglia in the neonatal brain may act for neuroprotection in MPTP-PD mice, whereas those in the same group of aged mice may be neurotoxic. Several results from cell culture systems in vitro indicate that activated microglia may act in a neuroprotective manner. The present study is the first in vivo one suggesting the neuroprotective effects of activated microglia on DA neurons in the SN of neonatal mice.

There are many reports indicating the neurotoxic effects of activated microglia especially in aged animals. Cultures of amyloid β-peptide (Aβ)-stimulated microglia

from aged rats were reported to show more evidence of toxicity than those from middle-aged or embryonic mice (Viel et al., 2001). Furthermore, MPTP neurotoxicity was greater in aged mice than in young mice, and was accompanied by age-related microglial activation (Sugama et al., 2003). These reports agree with the present findings that activated microglia in old animals play a toxic role. LPS treatment caused neurotoxic effects on DA neurons in various cell culture systems (Kim et al., 2000; Gayle et al., 2002; Gao et al., 2003) or by direct injection into the SN (Castano et al., 2002; Arai et al., 2004; Iravani et al., 2005). The degree of neuronal injury may depend on the concentration of LPS used for treatment. The neurotoxicity of microglia was increased by the production of TNFα by the cells in response to LPS stimulation (Sawada et al., 1989, 1995).

Activated microglia may produce not only neurotoxic effects, but also neuroprotective ones depending upon their environmental situation. The present results agree with the previous results of Imamura et al. (2003, 2005) and Sawada et al. (2006), who demonstrated the existence of toxic and neuroprotective subsets of activated microglia. Vilhardt et al. (2002) discovered a toxic change in microglia, from neuroprotection to neurotoxicity, by transfecting the cells with cDNA encoding HIV-1 Nef protein, indicating the conversion from a neurotrophic to a neurotoxic subtype of microglia. During aging a similar toxic change may be induced in the microglia of the brain.

On the other hand, the neurotrophic effects of microglial activation induced by LPS have also been found in several cell culture studies (Mallat et al., 1989; Miwa et al., 1997; Elkabes et al., 1998; Nakajima et al., 2001; Kramer et al., 2002). The neurotrophic effects of LPS may be explained by the fact that LPS induces the secretion of not only pro-inflammatory cytokines but also neurotrophic compounds. LPS stimulation increases the microglial secretion of NT-3, NT-4/5, NGF, and BDNF (Miwa et al., 1997; Elkabes et al., 1998; Nakajima et al., 2001). A rat model of spinal cord injury showed improvement in locomotor function by an LPS-elicited increase in the level of neuroprotective GDNF (Hashimoto et al., 2005). Plasminogen produced by LPS-treated microglia was reported to promote the development of DA neurons (Nakajima et al., 1992; Nagata et al., 1993b). Pro-inflammatory cytokines, such as TNFα, IL-1β, and IL-6, produced from activated microglia, are pleiotropic, and act for either neuroprotection or neurotoxicity.

Neuroprotective and neurotoxic effects of microglia in neonate and adult mice are possible explanation of the present results. However, cautious interpretation is required in considering the complexity of the present experimental condition. Because in our experiment, LPS treatment was carried out by systemic injection, microglial activation may occur in the entire brain. Although different effects of microglia in neonatal and adult mice are one probable explanation of the present results, the comparison between the changes observed in the neonatal and adult mice is very difficult, because many factors can affect the final outcome. Cells that respond directly to LPS are microglia, but we induced systemic inflammation. Thus, many cells, such as astrocytes, vascular endothelial cells, and in particular, T cells, may be involved. The sensitivity of DA neurons to MPTP is different depending on the age (Jarvis and Wagner, 1985; Ali et al., 1993). It is hard to judge whether the dosage of MPTP/LPS, we employed is appropriate to induce the exactly comparable effect to the neurons and microglia of both neonatal and adult mice. The present results showing microglial activation and protection by LPS against dopaminergic damage in the SN in neonatal mice and neurotoxic effect in aged mice suggest that most probably activated microglia in neonatal mice may act for neuroprotection.

ACKNOWLEDGMENTS

We thank Dr. S. Hashimoto (Fujita Health University, School of Medicine) for a discussion about statistics and Dr. K. Tsuchida (Fujita Health University, Institute for Comprehensive Medical Science) for his support of this work. Contract grant sponsor: Ministry of Health, Labor, and Welfare of Japan (M.S.); Ministry of Education, Culture, Sports, Science, and Technology of Japan (M.S.); Japan Health Sciences Foundation (M.S.).

REFERENCES

Aguirre JA, Cintra A, Hillion J, Narváez JA, Jansson A, Antonelli T, Ferraro L, Rambert FA, Fuxe K. 1999. A stereological study on the neuroprotective actions of acute Modafinil treatment on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced nigral lesions of the male black mouse. Neurosci Lett 275:215–218.

Ali SF, David SN, Newport GD. 1993. Age-related susceptibility to MPTP-induced neurotoxicity in mice. Neurotoxicology 14:29–34.

Arai H, Furuya T, Yasuda T, Miura M, Mizuno Y, Mochizuki H. 2004. Neurotoxic effects of lipopolysaccharide on nigral dopaminergic neurons are mediated by microglial activation, interleukin-1β, and expression of caspase-11 in mice. J Biol Chem 279:51647–51653.

Barger SW, Hörster D, Furukawa K, Goodman Y, Krieglstein J, Mattson MP. 1995. Tumor necrosis factors α and β protect neurons against amyloid β-peptide toxicity: evidence for involvement of a κB-binding factor and attenuation of peroxide and Ca^{2+} accumulation. Proc Natl Acad Sci U S A 92:9328–9332.

Batchelor PE, Liberatore GT, Wong JYF, Porritt MJ, Frerichs F, Donnan GA, Howells DW. 1999. Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. J Neurosci 19:1708–1716.

Bolin LM, Strycharska-Orczyk I, Murray R, Langston JW, Monte DD. 2002. Increased vulnerability of dopaminergic neurons in MPTP-lesioned interleukin-6 deficient mice. J Neurochem 83:167–175.

Cassarino DS, Fall CP, Swerdlow RH, Smith TS, Halvorsen EM, Miller SW, Parks JP, Parker WD Jr, Bennett JP Jr. 1997. Elevated reactive oxygen species and antioxidant enzyme activities in animal and cellular models of Parkinson's disease. Biochim Biophys Acta 1362:77–86.

Castano A, Herrera AJ, Cano J, Machado A. 2002. The degenerative effect of a single intra-nigral injection of LPS on the dopaminergic system is prevented by dexamethasone, and not mimicked by rh-TNF-α, IL-1β and IFN-γ. J Neurochem 81:150–157.

Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK. 1992. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. J Immunol 149:2736–2741.

Elkabes S, DiCicco-Bloom EM, Black IB. 1996. Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. J Neurosci 16:2508–2521.

Elkabes S, Peng L, Black IB. 1998. Lipopolysaccharide differentially regulates microglial trk receptor and neurotrophin expression. J Neurosci Res 54:117–122.

Fisher J, Mizrahi T, Schori H, Yoles E, Levkovitch-Verbin H, Haggiag S, Revel M, Schwartz M. 2001. Increased post-traumatic survival of neurons in IL-6-knockout mice on a background of EAE susceptibility. J Neuroimmunol 119:1–9.

Franklin KBJ, Paxinos G. 1997. The mouse brain in stereotaxic coordinates. London: Academic Press.

Furuya T, Hayakawa H, Yamada M, Yoshimi K, Hisahara S, Miura M, Mizuno Y, Mochizuki H. 2004. Caspase-11 mediates inflammatory dopaminergic cell death in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. J Neurosci 24:1865–1872.

Gao HM, Hong JS, Zhang W, Liu B. 2003. Synergistic dopaminergic neurotoxicity of the pesticide rotenone and inflammogen lipopolysaccharide: relevance to the etiology of Parkinson's disease. J Neurosci 23:1228–1236.

Gayle DA, Ling Z, Tong C, Landers T, Lipton JW, Carvey PM. 2002. Lipopolysaccharide (LPS)-induced dopamine cell loss in culture: roles of tumor necrosis factor-alpha, interleukin-1beta, and nitric oxide. Brain Res Dev Brain Res 133:27–35.

Hashimoto M, Nitta A, Fukumitsu H, Nomoto H, Shen L, Furukawa S. 2005. Inflammation-induced GDNF improves locomotor function after spinal cord injury. Neuroreport 16:99–102.

Hirata Y, Kiuchi K, Nagatsu T. 2001. Manganese mimics the action of 1-methyl-4-phenylpyridinium ion, a dopaminergic neurotoxin, in rat striatal tissue slices. Neurosci Lett 311:53–56.

Hunot S, Boissiere F, Faucheux B, Brugg B, Mouatt-Prigent A, Agid Y, Hirsch EC. 1996. Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. Neuroscience 72:355–363.

Imamura K, Hishikawa N, Sawada M, Nagatsu T, Yoshida M, Hashizume Y. 2003. Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. Acta Neuropathol 106:518–526.

Imamura K, Hishikawa N, Ono K, Suzuki H, Sawada M, Nagatsu T, Yoshida M, Hashizume Y. 2005. Cytokine production of activated microglia and decrease in neurotrophic factors of neurons in the hippocampus of Lewy body disease brains. Acta Neuropathol 109:141–150.

Iravani MM, Leung CCM, Sadeghian M, Haddon CO, Rose S, Jenner P. 2005. The acute and the long-term effects of nigral lipopolysaccharide administration on dopaminergic dysfunction and glial cell activation. Eur J Neurosci 22:317–330.

Jarvis MF, Wagner GC. 1985. Age-dependent effects of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). Neuropharmacology 24:581–583.

Kramer BC, Yabut JA, Cheong J, JnoBaptiste R, Robakis T, Olanow CW, Mytilineou C. 2002. Lipopolysaccharide prevents cell death caused by glutathione depletion: possible mechanisms of protection. Neuroscience 114:361–372.

Kim WG, Mohney RP, Wilson B, Jeohn GH, Liu B, Hong JS. 2000. Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia. J Neurosci 20:6309–6316.

Koutsilieri E, Scheller C, Grünblatt E, Nara K, Li J, Riederer P. 2002. Free radicals in Parkinson's disease. J Neurol 249(Suppl):II/1–II/5.

Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T. 2003. Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. Proc Natl Acad Sci U S A 100:8514–8519.

Liu J, Marino MW, Wong G, Grail D, Dunn A, Bettadapura J, Slavin AJ, Old L, Bernard CC. 1998. TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. Nat Med 4:78–83.

Mason JL, Suzuki K, Chaplin DD, Matsushima GK. 2001. Interleukin-1β promotes repair of the CNS. J Neurosci 21:7046–7052.

Mallat M, Houlgatte R, Brachet P, Prochiantz A. 1989. Lipopolysaccharide-stimulated rat brain macrophages release NGF in vitro. Dev Biol 133:309–311.

McGuire SO, Ling ZD, Lipton JW, Sortwell CE, Collier TJ, Carvey PM. 2001. Tumor necrosis factor α is toxic to embryonic mesencephalic dopamine neurons. Exp Neurol 169:219–230.

Miwa T, Furukawa S, Nakajima K, Furukawa Y, Kohsaka S. 1997. Lipopolysaccharide enhances synthesis of brain-derived neurotrophic factor in cultured rat microglia. J Neurosci Res 50:1023–1029.

Mogi M, Harada M, Riederer P, Narabayashi H, Fujita K, Nagatsu T. 1994a. Tumor necrosis factor-alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. Neurosci Lett 165:208–210.

Mogi M, Harada M, Kondo T, Riederer P, Inagaki H, Minami M, Nagatsu T. 1994b. Interleukin-1 beta, interleukin-6, epidermal growth factor and transforming growth factor-alpha are elevated in the brain from parkinsonian patients. Neurosci Lett 180:147–150.

Nagata K, Takei N, Nakajima K, Saito H, Kohsaka S. 1993a. Microglial conditioned medium promotes survival and development of cultured mesencephalic neurons from embryonic rat brain. J Neurosci Res 34:357–363.

Nagata K, Nakajima K, Kohsaka S. 1993b. Plasminogen promotes the development of rat mesencephalic dopaminergic neurons in vitro. Brain Res Dev Brain Res 75:31–37.

Nagatsu I, Kondo Y, Inagaki S, Karasawa N, Kato T, Nagatsu T. 1977. Immunofluorescent studies on tyrosine hydroxylase: application for its axoplasmic transport. Acta Histochem Cytochem 10:494–499.

Nagatsu T, Sawada M. 2005. Inflammatory process in Parkinson's disease: role for cytokines. Curr Pharm Design 11:999–1016.

Nakajima K, Tsuzaki N, Nagata K, Takemoto N, Kohsaka S. 1992. Production and secretion of plasminogen in cultured rat brain microglia. FEBS Lett 308:179–182.

Nakajima K, Honda S, Tohyama Y, Imai Y, Kohsaka S, Kurihara T. 2001. Neurotrophin secretion from cultured microglia. J Neurosci Res 65:322–331.

Rabchevsky AG, Streit WJ. 1997. Grafting of cultured microglial cells into the lesioned spinal cord of adult rats enhances neurite outgrowth. J Neurosci Res 47:34–48.

Sawada M, Kondo N, Suzumura A, Marunouchi T. 1989. Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. Brain Res 491:394–397.

Sawada M, Suzumura A, Yamamoto H, Marunouchi T. 1990. Activation and proliferation of the isolated microglia by colony stimulating factor-1 and possible involvement of protein kinase C. Brain Res 509:119–124.

Sawada M, Suzumura A, Marunouchi T. 1995. Cytokine network in the central nervous system and its roles in growth and differentiation of glial and neuronal cells. Int J Dev Neurosci 13:253–264.

Sawada M, Suzumura A, Hosoya H, Marunouchi T, Nagatsu T. 1999. Interleukin-10 inhibits both production of cytokines and expression of cytokine receptors in microglia. J Neurochem 72:1466–1471.

Sawada M, Imamura K, Nagatsu T. 2006. Role of cytokines in inflammatory process in Parkinson's disease. J Neural Transm Suppl:373–381.

Schmued LC, Hopkins KJ. 2000. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res 874:123–130.

Sugama S, Yang L, Cho BP, DeGiorgio LA, Lorenzl S, Albers DS, Beal MF, Volpe BT, Joh TH. 2003. Age-related microglial activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration in C57BL/6 mice. Brain Res 964:288–294.

Suzuki H, Imai F, Kanno T, Sawada M. 2001. Preservation of neurotrophin expression in microglia that migrate into the gerbil's brain across the blood-brain barrier. Neurosci Lett 312:95–98.

Suzumura A, Sawada M, Yamamoto H, Marunouchi T. 1993. Transforming growth factor-β suppresses activation and proliferation of microglia in vitro. J Immunol 151:2150–2158.

Thery C, Hetier E, Evrard C, Mallat M. 1990. Expression of macrophage colony-stimulating factor gene in the mouse brain during development. J Neurosci Res 26:129–133.

Viel JJ, McManus DQ, Smith SS, Brewer GJ. 2001. Age- and concentration-dependent neuroprotection and toxicity by TNF in cortical neurons from β-amyloid. J Neurosci Res 64:454–465.

Vilhardt F, Plastre O, Sawada M, Suzuki K, Wiznerowicz M, Kiyokawa E, Trono D, Krause KH. 2002. The HIV-1 Nef protein and phagocyte NADPH oxidase activation. J Biol Chem 277:42136–42143.

Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, Choi DK, Ischiropoulos H, Przedborski S. 2002. Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. J Neurosci 22:1763–1771.

Wu DC, Teismann P, Tieu K, Vila M, Jackson-Lewis V, Ischiropoulos H, Przedborski S. 2003. NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. Proc Natl Acad Sci U S A 100:6145–6150.