2. Materials and methods

2.1. Materials

Synthetic human Aβ1-42 and Aβ25-35 were obtained from Peptide Institute Inc. Aβ25-35 was dissolved in H_2O and Aβ1-42 was dissolved in 0.1% NH_3 according to the manufacturer's instructions. Anti-MMP3 monoclonal antibody was from R&D Systems, Inc. and anti-MMP12 polyclonal antibody was from Santa Cruz Biotechnology, Inc. Anti-phospho-Akt (Serine 473), anti-Akt, antibodies were from Cell Signaling. Wortmannin and PD98059 were from Calbiochem. SB203580 was from Promega. All inhibitors were resolved in DMSO. Mouse recombinant granulocyte-macrophage colony-stimulating factor (mrGM-CSF) was from BD Bioscience Pharmingen.

2.2. Cell culture

The microglial cell line Ra2 was cultured in MGI medium [Eagle's MEM supplemented with 0.2% glucose, 5 μg/ml bovine Insulin (Sigma–Aldrich), 10% fetal bovine serum (FBS, Invitrogen)] and 0.8 ng/ml mrGM-CSF (Ito et al., 2005). Before Aβ-treatment, the Ra2 cells were cultured in an MGI medium without mrGM-CSF for 16 h. Primary microglia and primary astrocytes were prepared using newborn C57BL/6 mice and cultured as described previously (Ito et al., 2005), and cultured in MGI medium containing 0.8 ng/ml mrGM-CSF. Primary neurons were obtained from the cortex of 14-day-old C57BL/6 mouse embryos as described previously (Ito et al., 2005).

2.3. RT-PCR and real-time quantitative RT-PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Invitrogen). Conventional RT-PCR was performed at the condition of 30 cycles for MMPs or 23 cycles for β-actin at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Quantitative real-time PCR was performed on Mx3000P (Promega) using the following program: 10 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, 20 s at 60 °C, and a dissociation reaction. The reactions were carried out using 0.5 μl cDNA with SYBR Premix EX Taq (Takara). Specificity of the PCR product was confirmed by examination of dissociation reaction plots. The values were expressed as the relative expression was normalized to β-actin mRNA. For RT-PCR and real-time quantitative PCR, the primers for mouse MMPs and β-actin genes were listed in Table 1.

Table 1
RT-PCR primers

Target (product size)		Sequence (5′-3′)
MMP2 (203 bp)	Sense	CACACCAGGTGAAGGATGTG
	Antisense	AGGGCTGCATTGCAAATATC
MMP3 (173 bp)	Sense	CAGACTTGTCCCGTTTCCAT
	Antisense	GGTGCTGACTGCATCAAAGA
MMP8 (250 bp)	Sense	CCTATTTCTCGTGGCTGCTC
	Antisense	CCCACGGAGTGTGGTAGTAG
MMP9 (228 bp)	Sense	GAAGGCAAACCCTGTGTGTT
	Antisense	AGAGTACTGCTTGCCCAGGA
MMP12 (184 bp)	Sense	CCAAGCATCCCATCTGCTAT
	Antisense	GGTCAAAGACAGCTGCATCA
MMP13 (286 bp)	Sense	TGATGAAACCTGGACAAGCA
	Antisense	TCCTCGGAGACTGGTAATGG
MMP20 (270 bp)	Sense	CTCGTCCTTTGATGCAGTGA
	Antisense	CTTGGGAACCCGAAGTCATA

2.4. Immunoblotting

The cells were lysed in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 5% bromo phenol blue). Next, 50 μg of total protein were resolved by SDS–PAGE and then were transferred to PVDF membranes (Millipore). Cell-conditioned medium was centrifuged at 2000 rpm to remove dead cells and debris, and concentrated 5 times using Biomax-10 (Millipore). Immunoblotting was performed with the appropriate antibody using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia).

2.5. Statistical analysis

The results are expressed as the means ± SD. A statistical analysis was done using the two-tailed student's *t*-test. A *p*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Induction of MMPs by Aβ in microglia

To identify MMPs induced by Aβ stimulation in microglia, we examined mRNA expression of microglial cell line Ra2 at 16 h after 10 μM Aβ1-42 treatment by Microarray analysis (data not shown). Microarray analysis revealed several MMPs mRNA species responsive to Aβ1-42 over this time frame (Table 2). These MMPs have been shown to be secreted by inflammatory macrophages. To further analyze MMPs induced by Aβ, Ra2 was treated with 10 μM Aβ1-42 for 16 h, and the expression of MMPs was examined by quantitative real-time PCR. Among several MMPs examined, we found the expression of MMP3 (Stromelysin 1), MMP12 (Macrophage elastase) and MMP13 (Collagenase 3) mRNA to be highly increased by Aβ1-42 stimulation (Fig. 1). We examined the time course of the induction of MMPs. As shown in Fig. 2A, induction of MMP3 or MMP12 by Aβ1-42 stimulation was appeared at 6 h and lasted for 24 h. Aβ25–35 was also

Table 2
Expression of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP)

	Ratio (Aβ/control)	Control	Aβ
MMP1a	Error	−0.036	−0.001
MMP1b	1.06	0.241	0.254
MMP2	−1.04	52.682	50.516
MMP3	64.20	0.043	2.782
MMP7	1.97	0.121	0.238
MMP8	−4.76	7.893	1.657
MMP9	1.05	0.079	0.083
MMP10	12.28	0.015	0.186
MMP11	Error	0.036	−0.069
MMP12	25.96	1.140	29.612
MMP13	7.01	0.284	1.994
MMP14	1.26	0.266	0.337
MMP15	4.82	0.015	0.071
MMP16	Error	0.037	−0.030
MMP17	−1.01	0.754	0.743
MMP19	1.13	0.998	1.132
MMP20	2.23	0.258	0.577
MMP21	1.64	0.539	0.881
MMP23	1.21	0.260	0.315
MMP24	Error	−0.087	0.063
TIMP1	−2.43	0.128	0.053
TIMP2	−1.23	5.314	4.338
TIMP3	1.15	0.135	0.155
TIMP4	−19.23	0.085	0.004

found to induce these MMPs, although the strength of the induction was lower than that of Aβ1-42 stimulation (Fig. 2B). In the primary microglial cells cultured from newborn C57BL/6 mouse brain, MMP3, MMP12 and MMP13 mRNA expression were also induced by Aβ1-42 (Fig. 3). MMP3 was also expressed in primary astrocytes and neurons. The MMP3 mRNA expression in the unstimulated primary astrocytes was higher than that in the primary microglia. Aβ1-42 increased the expression of MMP3 in microglia, astrocytes and neurons. On the other hand MMP12 or MMP13 was up-regulated by Aβ1-42 only in primary microglia. MMP12 or MMP13 was induced by Aβ1-42 in neither astrocytes nor neurons (Fig. 4). We examined the protein expression of MMP3 and MMP12 by immunoblotting. Expression of MMP3 protein in Ra2 cell lysate was not detected by the stimulation of Aβ1-42. However, we could detect the expression of MMP3 in Ra2 cell-conditioned medium. And the expression of MMP3 protein was increased by the stimulation of Aβ1-42 (Fig. 5A). The expression of MMP12 was detected both in cell lysates and cell-conditioned medium. The MMP12 protein expression was increased by the stimulation of Aβ1-42 (Fig. 5B).

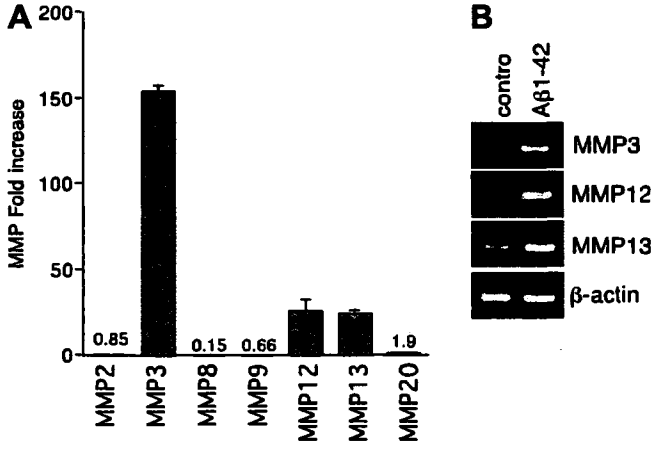

Fig. 1. Ra2 cells were treated with 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 16 h. (A) Real-time PCR of a series of MMPs mRNA were performed. The result represents the fold increase of Aβ1-42 stimulation to control. Data represent means ± SD of three separate determinations. (B) MMPs and β-actin mRNA were determined by RT-PCR.

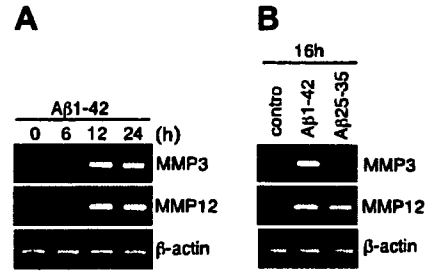

Fig. 2. Aβ stimulates MMP mRNA expressions in Ra2 cells. MMP3, MMP12 and β-actin mRNA expressions were determined by RT-PCR. (A) Time course of MMP expressions in Ra2 cells treated with 10 μM Aβ1-42. (B) Ra2 cells were treated with 10 μM Aβ1-42 or 50 μM 25–35 for 16 h.

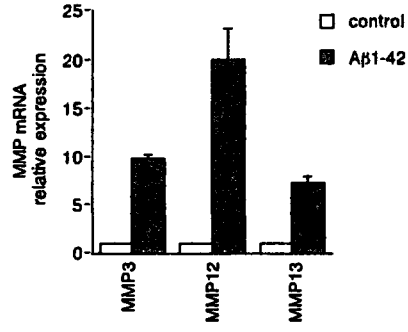

Fig. 3. Induction of MMP3, MMP12 and MMP13 mRNA by Aβ1-42 in primary microglia. Primary microglia was separated from newborn C57BL/6 mouse brain. Cultured cells were treated with 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 16 h. Extracted RNA was quantified by real-time PCR. The data represent the means ± SD of three separate determinations.

3.2. *Aβ induces the expression of MMP mRNA via the PI3-kinase signal cascade*

Next, we examined the signal cascades for Aβ-induced MMP mRNA expression by using several chemical inhibitors (Fig. 6). Wortmannin, PI3K (phosphatidylinositol 3 kinase) inhibitor, clearly inhibited the induction of MMP mRNA expression by Aβ1-42. However, PD98059, MEK inhibitor and SB203580, p38 inhibitor, did not inhibit the induction of MMP mRNA expression. We analyzed the

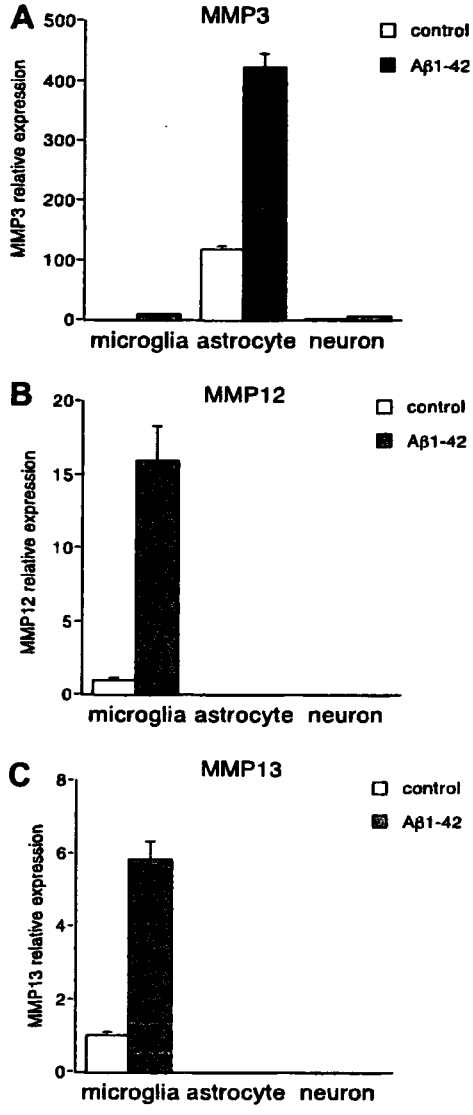

Fig. 4. Induction of MMP3, MMP12 and MMP13 mRNA by Aβ1-42 in primary microglia, primary astrocytes and primary neuron from C57BL/6 mice. Cultured cells were treated with 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 16 h. Extracted RNA was quantified by real-time PCR. The data represent the means ± SD of three separate determinations.

Akt pathways by immunoblotting. Aβ1-42 induced the phosphorylation of Akt (Fig. 7). The phosphorylation of Akt was sustained up to 2 h (data not shown).

4. Discussion

We have shown in this paper that Aβ1-42 induces the expression of MMP3, MMP12 and MMP13. MMP3 and MMP12 are the family of stromelysin and MMP13 belongs to the family of interstitial collagenases. We found that MMP12 and MMP13 were expressed only in microglia (Fig. 4). They were highly up-regulated by Aβ1-42 stimulation. On the contrary, MMP3 were expressed both in astrocytes and neurons and were weakly expressed in microglia. By the stimulation of Aβ1-42, MMP3 were highly up-regulated in astrocytes, microglia and neurons. From these results it will be suggested that increase of Aβ1-42 by aging or genetic disorder (Alzheimer's susceptible person) may gradually up-regulate the production of MMP12, and MMP13 by microglia and MMP3 in astrocytes, neurons and also microglia. By using chemical inhibitor, we have herein shown the Aβ-induced activation of MMP3, MMP12 and MMP13 to be correlated with the activation of the PI3K/Akt signaling cascades in the microglia. The expression of MMP12 in SB203580 was higher than the control without or with the stimulation of Aβ1-42 (Fig. 6). SB203580 itself may stimulate the induction of MMP12 mRNA expression. To elucidate more precise signaling pathway by Aβ1-42 stimulation in microglia needs another experiments by using dominant negative or RNAi transfection procedures.

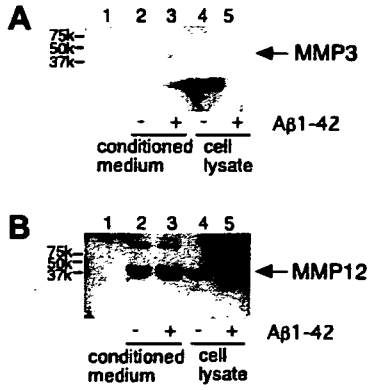

Fig. 5. MMP3 and MMP12 protein expression induced by Aβ1-42. Ra2 cells were treated with 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 16 h in serum-free medium. Cell-conditioned medium (lanes 2 and 3) and cell lysates (lanes 4 and 5) were immnoblotted using anti-MMP3 antibody (A), and anti-MMP12 antibody (B). Lane 1: serum-free medium as a negative control.

What is the biological function of MMPs in AD? Recent evidence has linked MMPs to various pathological conditions in the central nervous system, including ischemia, multiple sclerosis and AD (Maeda and Sobel, 1996). MMP9 has been shown to be synthesized in neurons of human hippocampus, which is capable of degrading the Aβ (Backstrom et al., 1996). Another aspects of MMPs are related to innate immunity. The lesions that develop, called senile plaques, are extracellular deposits principally composed of insoluble aggregates of Aβ, infiltrated by reactive microglia and astrocytes (Yankner and Mesulam, 1991; Mullan and Crawford, 1993). By using human THP-1 cells, Aβ has been shown to induce MMP9 together with TNF-α (Chong et al., 2001). We have shown that MMP3, MMP12 and MMP13 are up-regulated by Aβ1-42 stimulation. These matrix proteases are a hallmark of inflammation with MMPs considered to be important effectors of inflammatory process and also essential for leukocyte extravasation and migration (Luckow et al., 2004). We have to be in mind that MMPs produced further enhance the inflammatory processes. Recent paper has shown that apoptotic neuronal cells release MMP3, which

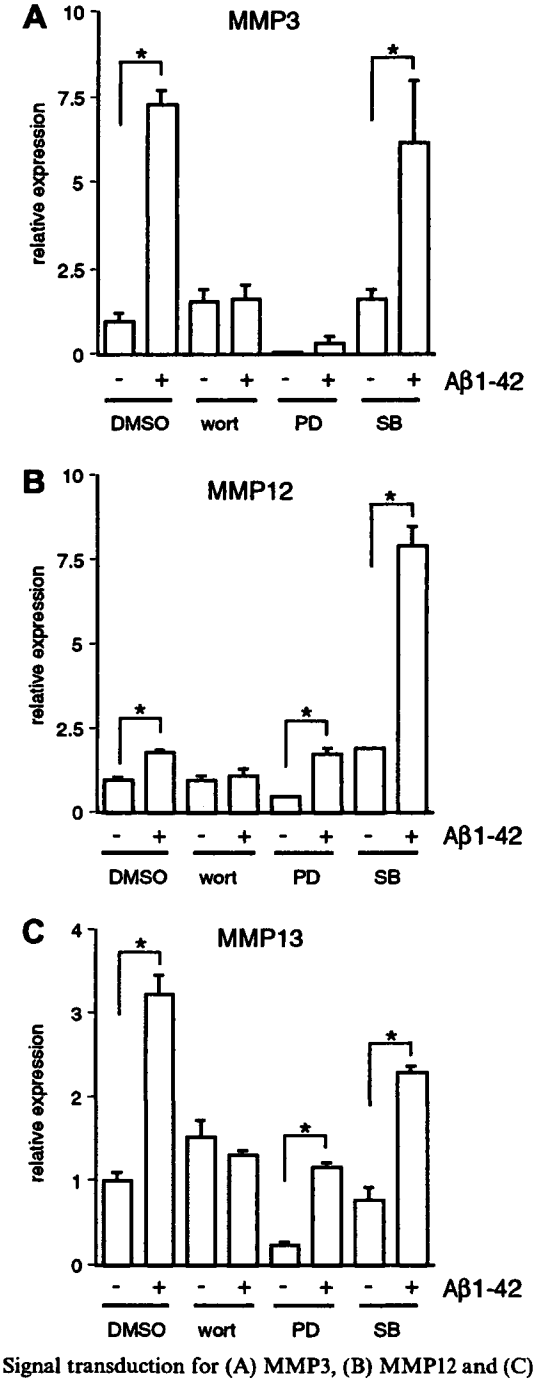

Fig. 6. Signal transduction for (A) MMP3, (B) MMP12 and (C) MMP13 mRNA expression induced by Aβ1-42. Ra2 cells were pre-incubated with 200 nM wortmannin (wort), 10 μM PD98059 (PD) or 10 μM SB203580 (SB) for 30 min before the addition of 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 6 h. Total RNA was extracted and quantified by real-time PCR. The data represent the means ± SD of three separate determinations (*$p < 0.05$).

induces microglial activation. Activated microglia produces TNFα, IL-6 and IL-1β (Kim et al., 2005). MMP12 has been shown to activate other MMPs such as MMP-2 and MMP-3, by which MMP12 exacerbates the cascade of proteolytic processes (Matsumoto et al., 1998).

It has been reported that MMP1, MMP3 and MMP9 are up-regulated in AD (Asahina et al., 2001; Leake et al., 2000; Lorenzl et al., 2003; Yoshiyama et al., 2000). By these findings genetic association between MMP and AD have been studied. The studies in Finland indicated the interaction between MMP3*5A and APOE 4 alleles increases the risk of AD (Saarela et al., 2004). However, no clear association between MMP3 and MMP9 polymorphisms and AD in Japanese (Shibata et al., 2005). From our results MMP12 and MMP13 may engage in progression of AD. Further works using clinical material are waiting.

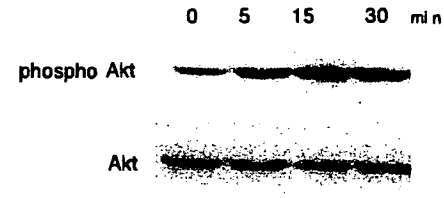

Fig. 7. Aβ1-42 induces the phosphorylation of Akt. Ra2 cells were treated with 10 μM Aβ1-42 for indicated times. Cell lysates were analyzed by immunoblotting using anti-phospho Akt (Ser 473) antibody. The same blot was reprobed with anti-Akt.

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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

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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

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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 -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 × 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

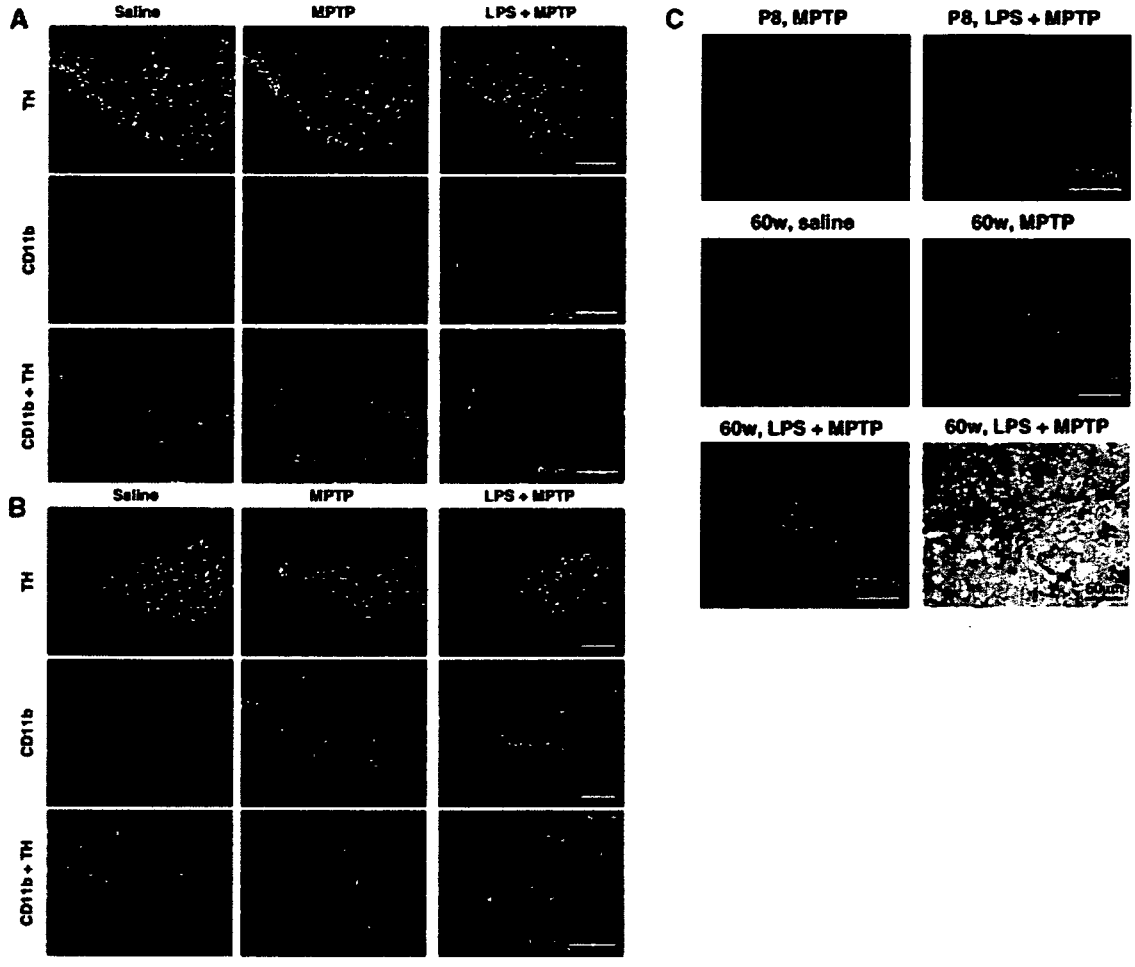


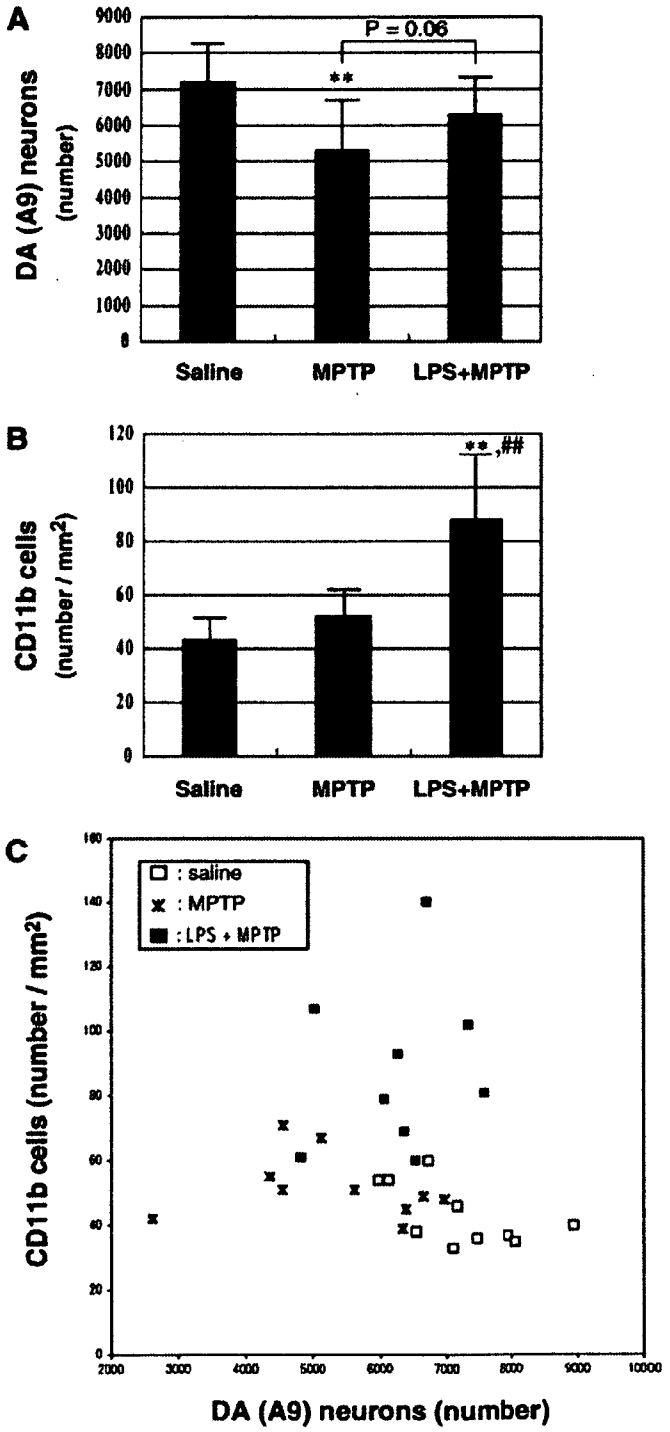
Fig. 1. Morphological changes due to MPTP administration in the SN. Immunostaining for tyrosine hydroxylase (TH)-positive dopamine (DA) (A9) neurons (merged, green) and CD 11b-positive activated microglia (merged, red) in the SN from mice treated with saline, MPTP, and LPS-MPTP are shown. **A:** In neonatal 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 (= 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).



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, = 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. 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. ** 0.01 vs. saline group; ## 0.01 vs. MPTP group, by use of the unpaired Student's -test (= 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.

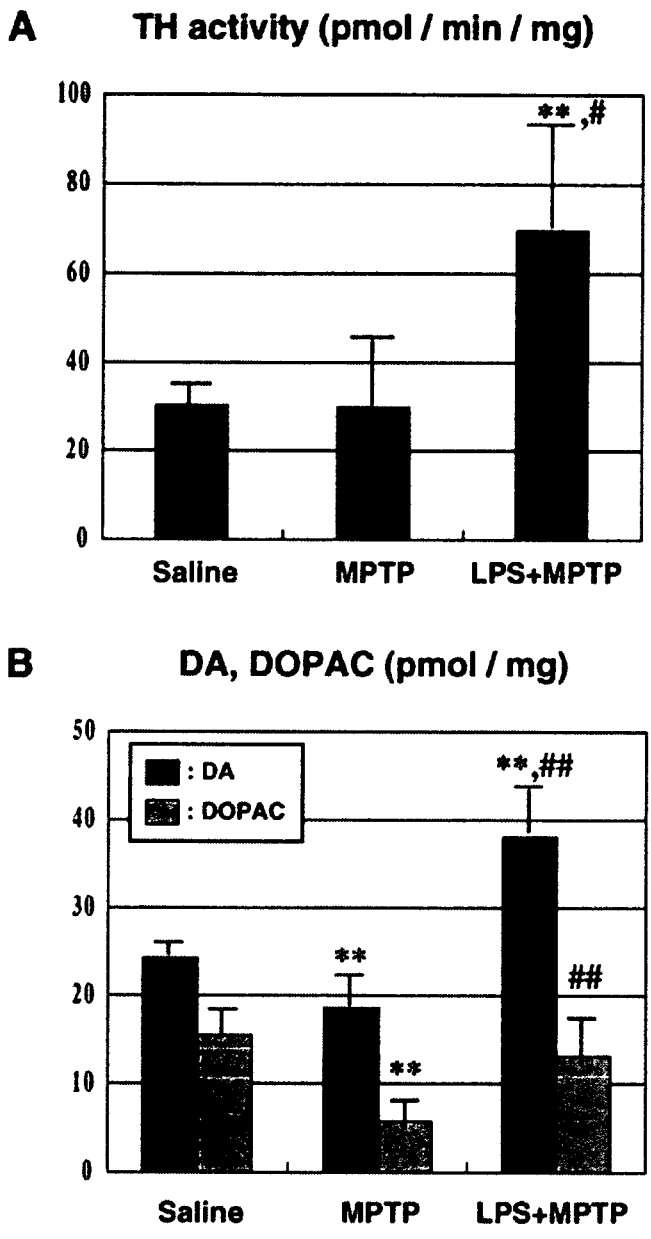


Fig. 3. Biochemical analyses of TH activity and DA and DOPAC contents in MPTP-treated neonatal mice. **A:** TH activity was measured in each of the three groups, and the LPS-MPTP group showed increased activity in the midbrain compared with the other two groups. **B:** Contents of DA and DOPAC in the 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. ** 0.01 vs. saline group, and # 0.05; ## 0.01 vs. MPTP group, by use of the unpaired Student's -test (= 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).

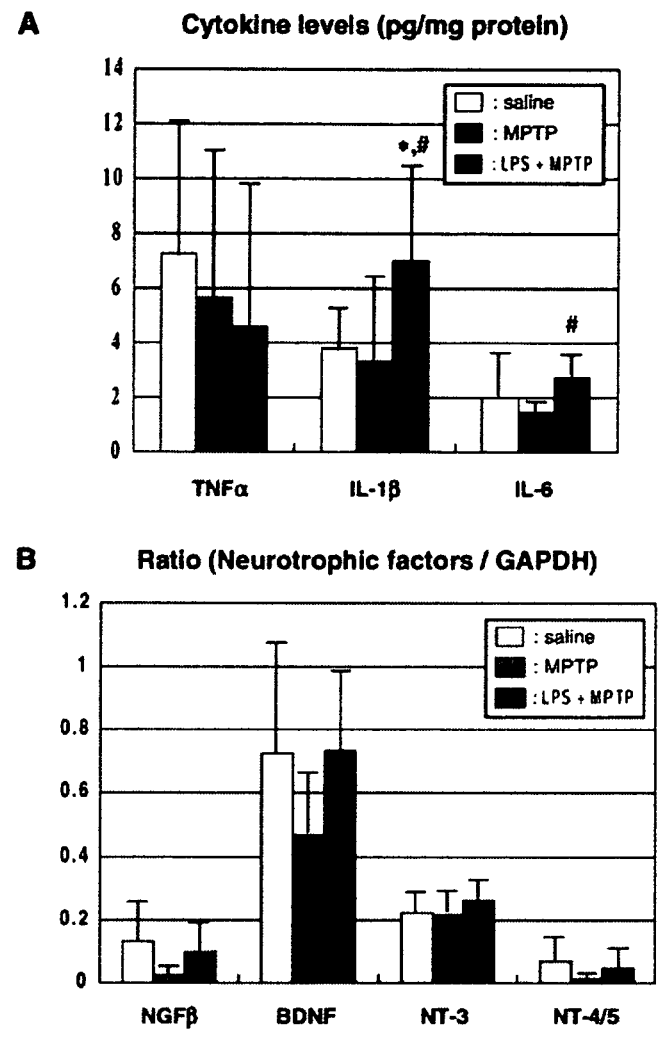


Fig. 4. Analysis of the levels of pro-inflammatory cytokines and neurotrophic factors in the midbrain of MPTP-treated neonatal mice. **A:** As to the pro-inflammatory cytokines, the TNFα level was not different among the three groups, but the IL-1β and IL-6 levels in the LPS-MPTP group were significantly 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. * 0.05 vs. saline group; # 0.05 vs. MPTP group (unpaired Student's -test) (= 8–9, pro-inflammatory cytokines; = 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-

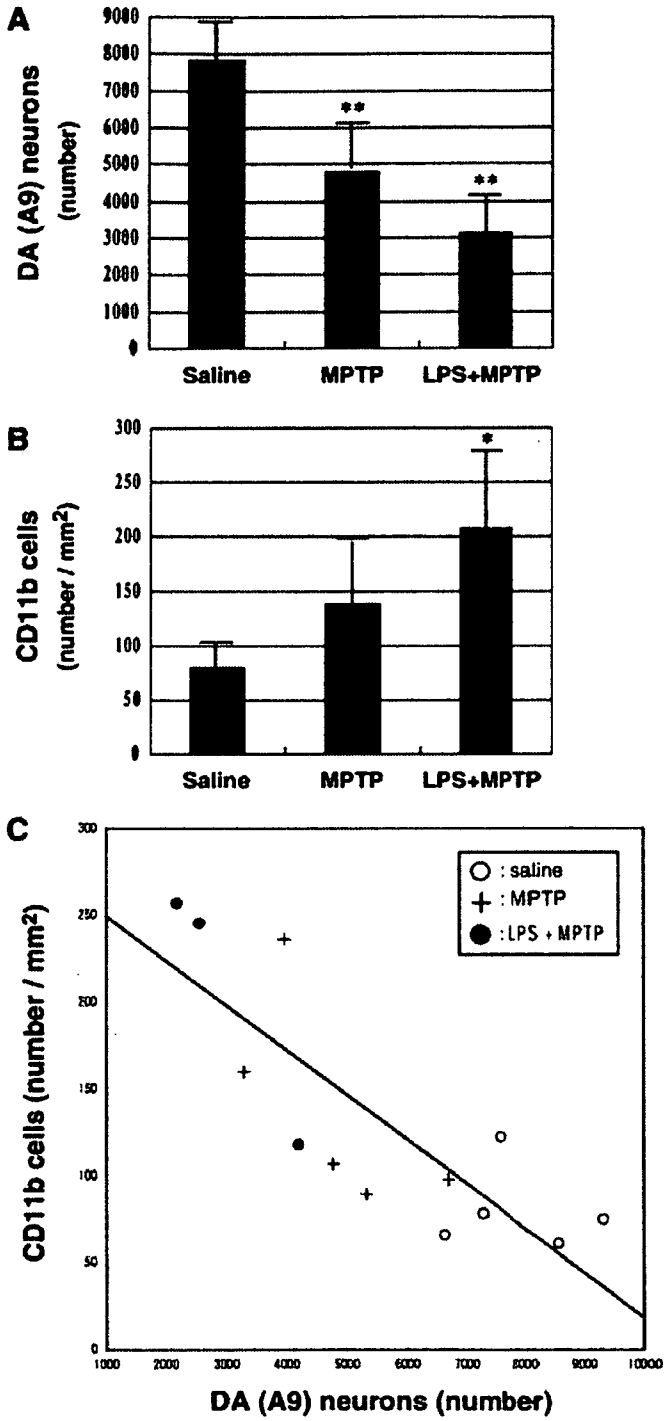


Fig. 5. Effects of microglial activation by LPS treatment on numbers of DA neurons and microglia in SN of 60-week-old (aged) mice treated with MPTP. **A:** Number of DA (A9) neurons in saline, MPTP, and LPS-MPTP groups. The number in the MPTP and LPS-MPTP groups was decreased significantly. **B:** Number 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. * 0.05; ** 0.01 vs. saline group (unpaired Student's -test) (= 3–5). **C:** Relationship between activated microglia and DA (A9) neurons in the SNc of MPTP-treated aged mice. An inverse correlation (= 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 (= 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.

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Antidepressants inhibit interferon-γ-induced microglial production of IL-6 and nitric oxide

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Abstract

Circumstantial evidence has suggested that activated microglia may be associated with the pathogenesis of depression. Pro-inflammatory cytokines may also be involved. Therefore, we examined the effects of various types of antidepressants, as well as the mood-stabilizer lithium chloride, on interferon-γ (IFN-γ)-induced microglial production of the pro-inflammatory mediators interleukin-6 (IL-6) and nitric oxide (NO). Treatment of the murine microglial 6-3 cells with 100 U/ml of IFN-γ resulted in an eightfold increase in IL-6 and a tenfold increase in NO into the culture medium. Pretreatment with the selective serotonin reuptake inhibitor fluvoxamine, the relatively selective noradrenaline reuptake inhibitor reboxetine, or the non-selective monoaminergic reuptake inhibitor imipramine, significantly inhibited IL-6 and NO production in a dose-dependent manner. These inhibitions were reversed significantly by SQ 22536, a cyclic adenosine monophosphate (cAMP) inhibitor, and, except for reboxetine, by the protein kinase A (PKA) inhibitor Rp-adenosine3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-3′,5′-cAMPS). Lithium chloride, which is believed to act by inhibiting the calcium-dependent release of noradrenaline, had a different spectrum of action on microglial 6-3 cells. It enhanced IFN-γ-stimulated IL-6 production and inhibited NO production. The inhibitory effect of lithium chloride was not reversed by either SQ 22536 or Rp-3′,5′-cAMPS. These results suggest that antidepressants have inhibitory effects on IFN-γ-activated microglia and these effects are, at least partially, mediated by the cAMP-dependent PKA pathway. On the other hand, the mood stabilizer and anti-manic agent lithium chloride has mixed effects on IFN-γ-induced microglial activation.

Keywords: Antidepressants; Microglia; Lithium chloride; cAMP; PKA; SQ 22536

Introduction

Increasing evidence indicates that microglial activation and inflammatory processes play important roles in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Itagaki et al., 1989; McGeer and McGeer, 2004). So far, there is no direct evidence that activated microglia are involved in the pathogenesis of mood disorders. However, there is circumstantial evidence of such a role from recent studies. Elevated microglial density has been observed in patients with depression or schizophrenia who committed suicide (Steiner et al., in press). In addition, Ekdahl et al. (2003) and Monje et al. (2003) have demonstrated that the neuroinflammation associated with microglial activation inhibits hippocampal neurogenesis. It has been suggested that this impaired hippocampal neurogenesis contributes to the pathogenesis of depression (Duman, 2004). Furthermore, these investigators have shown that indometha-

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cin, a conventional non-steroidal anti-inflammatory drug, and minocycline, a specific inhibitor of microglial activation, can restore the impaired neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). Conversely, it has been demonstrated that antidepressant treatment increases neurogenesis in adult hippocampus (Duman, 2004; Malberg et al., 2000; Santarelli et al., 2003) and the behavioral effects of antidepressants may, in part, be due to stimulation of such hippocampal neurogenesis (Santarelli et al., 2003). This interpretation is supported by a preliminary report that the serotonin reuptake inhibitor fluoxetine improved cognition in patients with mild cognitive impairment (Mowla et al., 2007). Therefore, it is tempting to speculate that antidepressants have inhibitory effects on activated microglia and that these play a pivotal role in the inflammation-induced impairment of hippocampal neurogenesis.

Also, pro-inflammatory cytokines may be involved in the pathogenesis of depression and in the mechanism of action of antidepressants (Castanon et al., 2002; Miller and O'Callaghan, 2005; Schiepers et al., 2005; Smith, 1991). Clinically, it is well known that the pro-inflammatory cytokine interferon-alpha (IFN-α) is used to treat patients with hepatitis C. Such treatment frequently induces depressive symptoms as a side effect. In animal experiments, chronic administration of pro-inflammatory cytokines has been shown to induce symptoms similar to depression. These are referred to as sickness behavior, which includes appetite loss, insomnia and lack of interest (De La Garza, 2005). In addition, pro-inflammatory mediators released by activated microglia have been documented to block hippocampal neurogenesis (Monje et al., 2003). Especially, interleukin-6 (IL-6) has been suggested to be a key regulator (Monje et al., 2003; Vallieres et al., 2002). In the periphery, several *ex vivo/in vitro* studies have shown that antidepressants reduce the production of pro-inflammatory cytokines in human whole blood (Kubera et al., 2001; Maes et al., 1999, 2005); in human peripheral blood mononuclear cells (PBMCs) (Szuster-Ciesielska et al., 2003); and in human monocytes and T cells (Xia et al., 1996).

To our knowledge, only a few previous studies have examined the effect of antidepressants on microglial production of pro-inflammatory cytokines. Obuchowicz et al. (2006) examined the effects of amitriptyline and its metabolite nortriptyline on the release of IL-1β and tumor necrosis factor-alpha (TNF-α) from mixed glial and microglial cultures stimulated with lipopolysaccharide (LPS). They found the antidepressants inhibited the release of both cytokines. In the present study, we used three types of antidepressants and a mood stabilizer to further clarify the effects on activated microglia. Specifically, we treated microglial cells with fluvoxamine, a selective serotonin reuptake inhibitor (SSRI), reboxetine, a relatively selective noradrenaline reuptake inhibitor, and imipramine, a non-selective monoaminergic reuptake inhibitor. We also utilized the anti-manic mood-stabilizer lithium chloride. We then measured changes in IFN-γ-induced microglial production of the inflammatory mediators IL-6 and nitric oxide (NO). Because an *ex vivo/in vitro* study has shown that antidepressant treatment increases the levels of intracellular cyclic adenosine monophosphate (cAMP) in human monocytes and T cells (Xia et al., 1996), we furthermore examined whether or not the cAMP-dependent protein kinase A (PKA) pathway is involved in the actions of antidepressants on microglia by investigating the effects of SQ 22536, a cAMP inhibitor, and Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-3′,5′-cAMPS), a PKA inhibitor.

Materials and methods

Chemicals and reagents

Imipramine, fluvoxamine, reboxetine, SQ 22536 and Rp-3′,5′-cAMPS were purchased from Sigma Chemicals (St. Louis, MO, USA). Lithium chloride was purchased from Tomiyama Pure Chemical Industries (Tokyo, Japan). Recombinant mouse granulocyte–macrophage colony-stimulating factor (GM-CSF) and recombinant IFN-γ were purchased from R&D systems (Minneapolis, MN, USA). Imipramine, fluvoxamine, reboxetine and lithium chloride were dissolved in phosphate buffered saline (150 mM NaCl, 5 mM phosphate, pH 7.4). SQ 22536 and Rp-3′,5′-cAMPS were dissolved in sterile distilled–deionized water.

Microglial cell cultures

The murine microglial cell line, 6-3, was originally established by one of us from neonatal C57BL/6J(H-2b) mice using a non-enzymatic and non-virus-transformed procedure (Kanzawa et al., 2000). The 6-3 cells closely resemble primary cultured microglia. We cultured the 6-3 cells according to the methods described previously (Hashioka et al., 2007). Briefly, the 6-3 cells were maintained in Eagle's minimal essential medium, 0.3% $NaHCO_3$, 2 mM glutamine, 0.2% glucose, 10 μg/ml insulin and 10% fetal calf serum, and maintained at 37 °C with 10% CO_2, 90% air atmosphere. One ng/ml mouse recombinant GM-CSF was added as a supplement in the culture medium because these cells stop proliferating without GM-CSF. The culture media were renewed twice per week.

IL-6 quantification

6-3 Microglial cells were plated on 96-well tissue culture plates at 5×10^4 cells per well and then cultured at 37 °C overnight. Afterwards, the media were replaced with serum-free fresh media in the presence or absence of the indicated concentrations of antidepressants or lithium chloride. In studying the effects of cAMP/PKA inhibitors, 6-3 cells were treated with 10 μM of SQ 22536 or Rp-3′,5′-cAMPS for 20 min before the addition of 50 μM of antidepressants or 1 mM of lithium chloride. After 24 h of pretreatment with antidepressants or lithium chloride at 37 °C, 6-3 cells were stimulated by 100 U/ml IFN-γ. After 24 h of incubation at 37 °C, the media were collected and centrifuged. The cell-free supernatants were then assayed for IL-6 accumulation using a mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kit

(Biosource International, Camarillo, CA, USA). The assay was carried out according to the manufacturer's protocol.

NO quantification

Accumulation of NO_2^- was assayed by the Griess reaction which is extensively used as an indicator of NO production by cultured cells. 6-3 Microglial cells were plated on 96-well tissue culture plates at 5×10^4 cells per well and then cultured at 37 °C overnight. Afterwards, the media were replaced with phenol red-free fresh media in the presence or absence of the indicated concentrations of antidepressants or lithium chloride. In the case of cAMP/PKA inhibitors, 6-3 cells were treated with 10 μM of SQ 22536 or Rp-3′,5′-cAMPS for 20 min before the addition of 50 μM of antidepressants or 1 mM of lithium chloride. After 24 h of pretreatment with antidepressants or lithium chloride at 37 °C, 6-3 microglial cells were stimulated by 100 U/ml IFN-γ. After 48 h of incubation at 37 °C, the media collected were centrifuged and the cell-free supernatants were mixed with one tenth the amount of fluorescent Griess reagent (Dojindo, Kumamoto, Japan). Samples were incubated at room temperature for 15 min and subsequently the fluorescence was read at an excitation of 380 nm and an emission of 450 nm using a fluorescent plate reader.

Cell viability assessment

We confirmed that antidepressants at the highest concentrations used in this study (i.e. 100 μM) did not affect cell viability using both the Trypan blue exclusion test and electron spin resonance assay. In the former, the number of intact 6-3 cells treated with 100-μM antidepressants for 48 h was comparable to that of control not treated with antidepressants. In the latter, the intensity of superoxide adducts generated by phorbol 12-myristate-13-acetate (PMA)-stimulated 6-3 cells with 24-h pretreatment of 100-μM antidepressants was comparable to that of controls not pretreated with antidepressants (data not shown).

Statistics

All values are expressed as the means±standard error of mean (SEM). Comparisons were made with the Student's *t*-test or a one-way analysis of variance (ANOVA) followed by a post hoc Fisher's protected least significant difference (PLSD) test. The significance was established at a level of $p<0.05$.

Results

IL-6 and NO production by IFN-γ-activated microglia

First, in order to confirm the potency of IFN-γ to induce microglial activation, we measured the IL-6 and NO production by IFN-γ-stimulated 6-3 microglial cells using ELISA and the Griess reaction, respectively. The treatment of the 6-3 cells with 100 U/ml of IFN-γ resulted in significant increases in both IL-6 and NO production. As shown in Fig. 1A, the IFN-γ-induced increase of IL-6 was approximately eightfold more than the non-stimulated control. The IFN-γ-induced increase of NO was approximately tenfold more than the non-stimulated control (Fig. 1B).

Effects of antidepressants on IL-6 production by IFN-γ-activated microglia

We next investigated the effects of various antidepressants on IL-6 production by IFN-γ-activated microglia. We confirmed that neither imipramine nor fluvoxamine induced microglial production of IL-6 (data not shown). We observed that 24-h pretreatment with 10–100 μM of imipramine (Fig. 2A) or fluvoxamine (Fig. 2B) suppressed microglial IL-6 production in a dose-dependent manner (Fig. 2A). Reboxetine

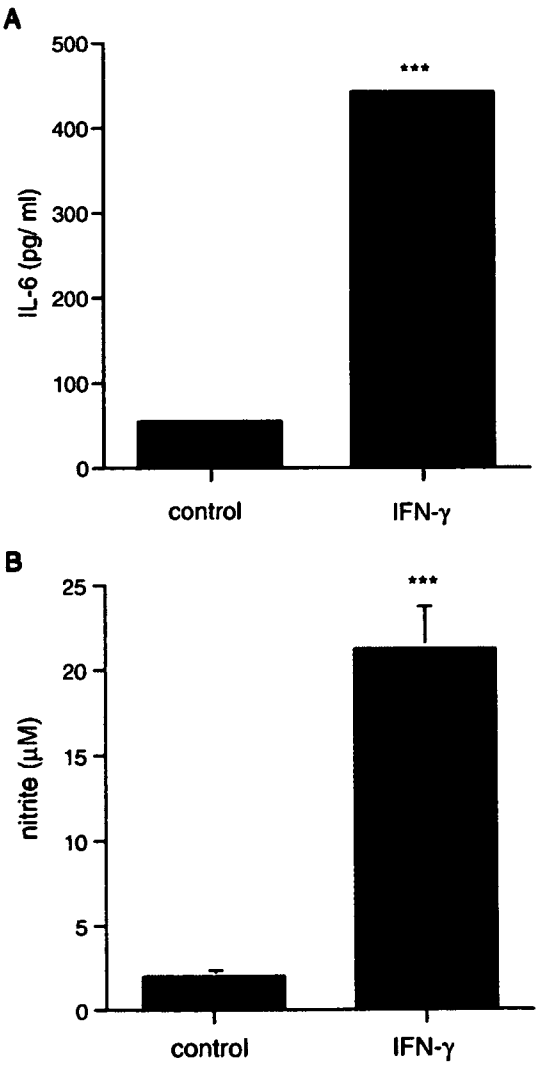

Fig. 1. IL-6 and NO production by IFN-γ-activated microglia. 6-3 microglial cells were treated with or without 100 U/ml of IFN-γ. After 24 h, the cell-free media were collected and IL-6 accumulation was measured by ELISA (A) or after 48 h, the cell-free media were collected and NO accumulation was measured by Griess reaction (B). Values are the means±SEM of 9 samples. ***$p<0.0001$, compared with control (medium). Comparisons were made with the Student's *t*-test.

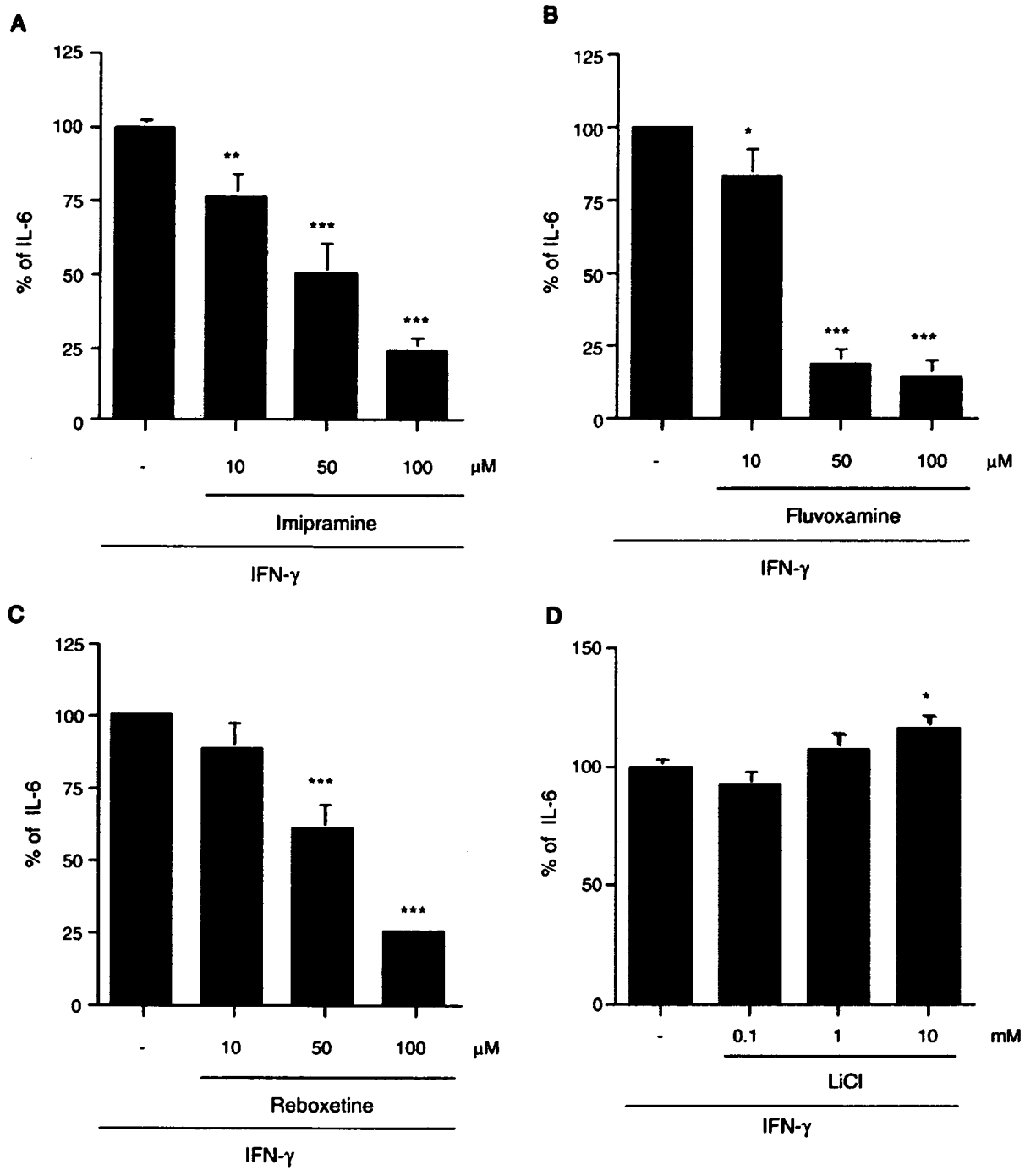

Fig. 2. Effects of antidepressants or lithium on the IFN-γ-activated microglial production of IL-6. 6-3 microglial cells were pre-incubated for 24 h with or without indicated concentrations of imipramine (A), fluvoxamine (B), reboxetine (C) or lithium chloride (D). Afterwards, the cells were stimulated by 100 U/ml of IFN-γ. After 24 h, the media collected were assayed for IL-6 accumulation using ELISA. Values are the means ± SEM of 6–12 samples and expressed as percentage control, where 100% is the value obtained from IFN-γ alone. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.0001$, compared with control (IFN-γ). Comparisons were made with ANOVA followed by the Fisher's PLSD.

was slightly less effective. At a dose of 50 μM, it significantly reduced microglial IL-6 production, but at 10 μM the reduction did not reach significance (Fig. 2C). We also tested lithium chloride, since it is used clinically to stabilize manic behavior, an opposite purpose to antidepressant medication. In contrast to the antidepressants, lithium chloride was a weak promoter of IL-6 production, reaching significance at a dose of 10 mM (Fig. 2D).

Effects of antidepressants on the NO production by IFN-γ-activated microglia

Subsequently, we studied the effects of antidepressants on NO production by IFN-γ-activated microglia. We found that all four agents significantly reduced the production (Fig. 3). In the case of imipramine, significance was reached at 50 μM (Fig. 3A). In the cases of fluvoxamine (Fig. 3B) and reboxetine (Fig.

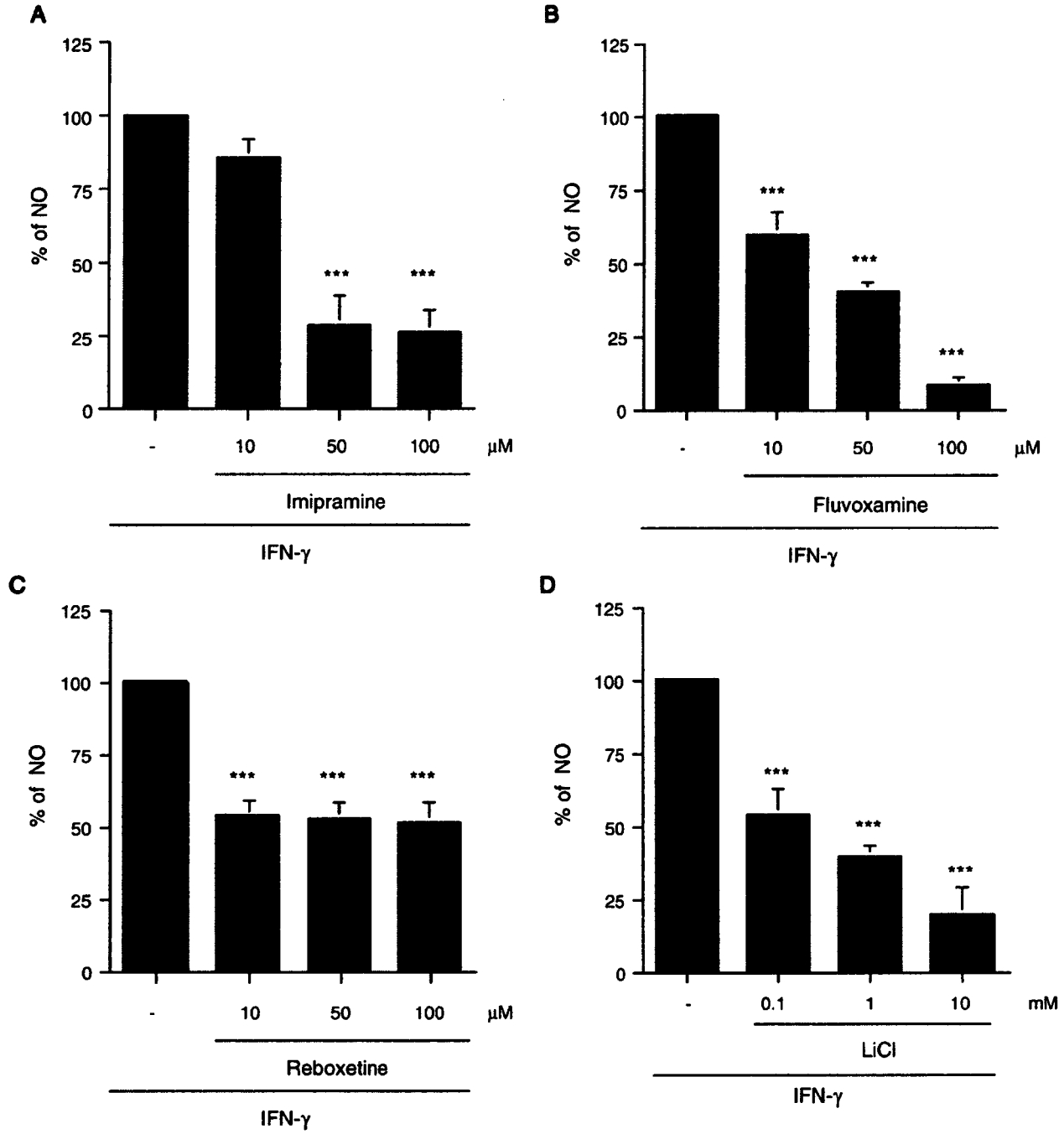

Fig. 3. Effects of antidepressants or lithium on the IFN-γ-activated microglial production of NO. 6-3 microglial cells were pre-incubated for 24 h with or without indicated concentrations of imipramine (A), fluvoxamine (B), reboxetine (C) or lithium chloride (D). Afterwards, the cells were stimulated by 100 U/ml of IFN-γ. After 48 h, the media collected were assayed for NO accumulation using Griess reaction. Values are the means ± SEM of 6–12 samples and expressed as percentage control, where 100% is the value obtained from IFN-γ alone. ***$p<0.0001$, compared with control (IFN-γ). Comparisons were made with ANOVA followed by the Fisher's PLSD.

3C), it was reached at 10 μM. In contrast to the promotive effect of lithium chloride on IL-6 production, lithium chloride inhibited the microglial NO production in a dose-dependent manner reaching significance at a dose of 0.1 mM (Fig. 3D).

Effects of cAMP/PKA inhibitors on the antidepressants-induced IL-6 suppression

In order to clarify whether or not the cAMP-dependent PKA pathway is involved in the inhibitory effects of antidepressants on IFN-γ-induced microglial IL-6 production, we tested SQ 22536 as a cAMP inhibitor and Rp-3′,5′-cAMPS as a PKA inhibitor, for their effects on antidepressant-induced IL-6 suppression. We observed that, when administered alone, neither 10 μM of SQ 22536 nor 10 μM of Rp-3′,5′-cAMPS significantly affected the IFN-γ-induced microglial IL-6 production (data not shown).

Fig. 4 shows that both SQ 22536 (10 μM) and Rp-3′,5′-cAMPS (10 μM), at a concentration of 50 μM of both imipramine (Fig. 4A) and fluvoxamine (Fig. 4B), reversed