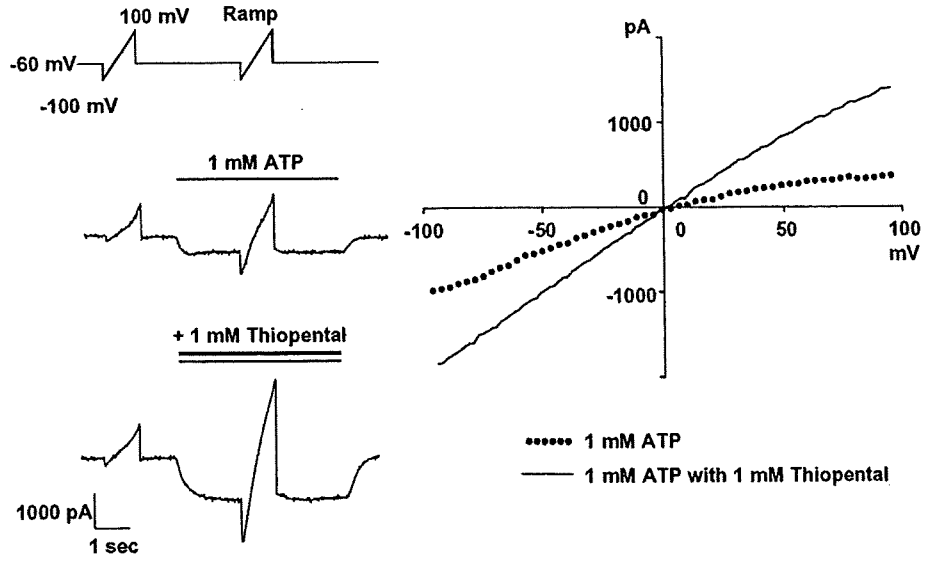


Figure 6. The current–voltage (I–V) relationship for $P2X_7Rs$-mediated currents in the absence and presence of thiopental. Voltage ramps from −100 to +100 mV were applied before (first ramp) and during the ATP application (second ramp) (left). I–V relationship of ATP-induced currents was obtained from the subtracted currents (right). Coapplication of 1-mM thiopental with 1-mM ATP increased both inward and outward currents without changes in the reversal potential.

at 300 μM ATP was also almost the same as that at 1 mM ATP (122% ± 10% with 30 μM propofol, 205% ± 34% with 100 μM propofol, $n = 3$ each). Thus, the effects of these IV anesthetics were not significantly different between the two doses of ATP. It should be noted that thiopental at low concentrations (3 and 10 μM) decreased, rather than increased, the $P2X_7Rs$-mediated currents. The reduction was slight but significant (13% ± 5% reduction with 3 μM, $P < 0.05$, $n = 12$; 13% ± 6% reduction with 10 μM, $P < 0.05$, $n = 12$).

To obtain the current–voltage (I–V) relationship, voltage ramps ranging from −100 to +100 mV were applied before and during application of ATP (Fig. 6, left). The data presented in Figure 6 are "pure " ATP (with or without thiopental) effects, derived by subtracting the control currents (first ramp) from the currents in the presence of ATP (second ramp). The I–V curve in the presence of 1 mM ATP (dotted line) had the reversal potential of approximately 0 mV, since $P2X_7Rs$ are nonselective cationic channels. Thiopental at 1 mM (continuous line), as well as 1 mM ketamine and 300 μM propofol (data not shown), increased both inward and outward conductances without changes in the reversal potential.

Effects of General Anesthetics on P2YRs

ATP increases the concentration of intracellular Ca^{2+} in microglia because of Ca^{2+} influx via P2XRs and Ca^{2+} release from the intracellular Ca^{2+} stores via P2Y receptors (10,35). To examine the effects of general anesthetics on P2YRs, the Ca^{2+} responses were measured in a low Ca^{2+} (100 nM) medium which avoided the P2XR-mediated Ca^{2+} influx. In each experiment, the standard external solution was replaced by the low Ca^{2+} solution a few minutes before application of anesthetics and/or ATP. The intracellular Ca^{2+} level was increased by application of 100 μM ATP and then it slowly decreased to the baseline level in the continuous presence of ATP (Fig. 7A). A second application of ATP 10 min later induced a considerably smaller response than the initial response (data not shown) (35), and therefore only the data obtained from the first application of test solution were analyzed. At the end of each experiment, the medium was switched back to the Ca^{2+} (1 mM) containing standard solution. The data from different preparations were normalized to the maximum increase determined by addition of 20 μM ionomycin, a Ca^{2+}-ionophore.

General anesthetics (330–990 μM sevoflurane, 10–100 μM ketamine, 30–300 μM propofol, and 30–100 μM thiopental) were pre-applied for 2 min before ATP (Fig. 7B). Anesthetics alone did not induce Ca^{2+} mobilization except for an extremely high concentration (300 μM) of propofol which increased the Ca^{2+} concentration (data not shown). None of the inhaled and IV anesthetics tested affected the peak response (Fig. 7C) and the time course (Figs. 7A and B) of the ATP-induced Ca^{2+} transients.

DISCUSSION

To the best of our knowledge, this is the first report investigating the effects of general anesthetics on ATP-induced responses in microglia. We assessed the actions of both inhaled and IV anesthetics on $P2X_7Rs$ and P2YRs using whole-cell clamp recording and single cell fluorometry of intracellular Ca^{2+}. P2YRs-mediated Ca^{2+} responses were not affected by all general anesthetics tested. IV anesthetics (ketamine, propofol, and thiopental), however, enhanced $P2X_7Rs$-mediated currents reversibly in a dose-dependent manner, whereas inhaled anesthetics had no effect on the $P2X_7Rs$ response.

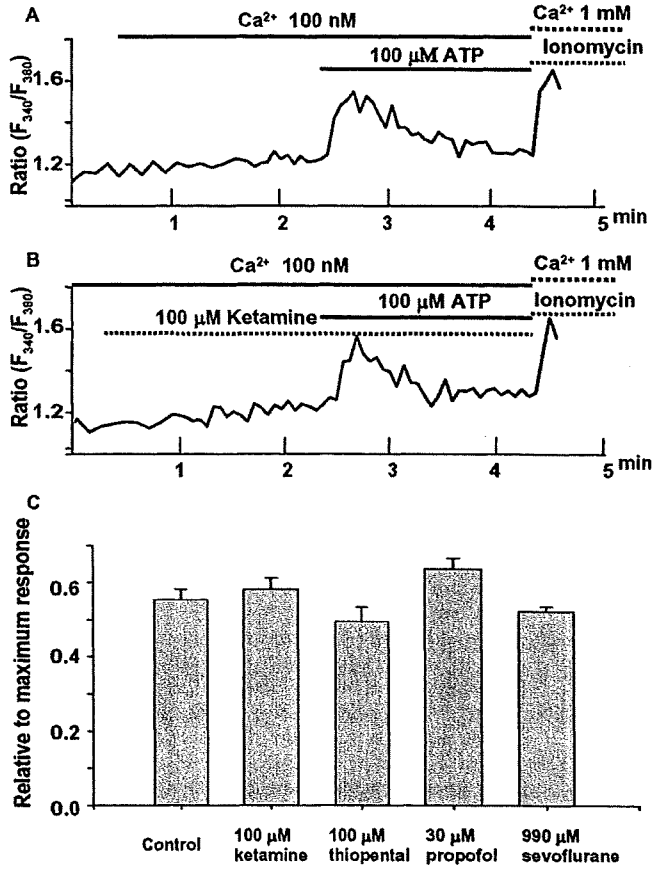


Figure 7. ATP-induced increase in intracellular Ca^{2+} concentration. Intracellular Ca^{2+} level was measured in a low Ca^{2+} solution (100 nM). ATP was applied for approximately 2 min. (A) A rapid transient increase in intracellular Ca^{2+} produced by application of ATP (100 μM). At the end of each experiment, the medium was switched back to the Ca^{2+} (1 mM) containing standard solution with 20 μM ionomycin for calibration of ATP response. (B) ATP-induced Ca^{2+} mobilization in the presence of 100 μM ketamine. Anesthetics were pre-applied for approximately 2 min before application of ATP. (C) Effects of general anesthetics on ATP-induced increase in intracellular Ca^{2+} level. There was no significant difference between ATP-induced Ca^{2+} increases in control and those with general anesthetics. Data are given as mean ± SEM.

In the present study, the ATP-induced nondesensitizing currents were characterized by activation with Bz-ATP (an agonist for $P2X_7Rs$), blockade with oATP (an antagonist for $P2X_7Rs$), the reversal potential closed to approximately 0 mV, and flow in both inward and outward directions. These properties were consistent with those of $P2X_7Rs$ in rat microglia in primary culture (10).

The potencies of IV anesthetics for enhancing $P2X_7Rs$ were not correlated with the octanol/buffer partition coefficients (approximately 60 for ketamine, approximately 390 for thiopental, and approximately 4000 for propofol). This finding may suggest that the actions of these anesthetics on $P2X_7Rs$ are not explained by nonspecific hydrophobic interactions. Further study is required to elucidate the mechanisms of the enhancement of microglia $P2X_7Rs$ by IV anesthetics. Interestingly, thiopental at low concentrations (3 and 10 μM) slightly decreased the $P2X_7Rs$-mediated currents (by approximately 13%), suggesting dual actions of thiopental on $P2X_7Rs$.

Clinically relevant concentrations of ketamine, propofol, and thiopental range from 2 to 7 μM, from 0.4 to 3 μM, and from 40 to 80 μM, respectively (33). The peak plasma concentrations increased up to 60 μM with ketamine, 56 μM with propofol, and 120 μM with thiopental after bolus administration (36,37). Therefore, IV anesthetics, in particular propofol and thiopental, have a potential to modulate the microglial $P2X_7R$-mediated response at concentrations which can be reached after bolus injection.

ATP is important in mediating interactions among various cell types in the brain, including synaptic transmission and inflammatory glial activation (5,38). Microglial $P2X_7Rs$ are involved in modulating neuronal damage caused by oxygen/glucose deprivation and focal cerebral ischemia (23,24). The activation of microglia $P2X_7Rs$ has been suggested to contribute to neuronal damage in certain neurodegenerative diseases, such as Alzheimer's disease (17,39). Thus, enhancement of microglial $P2X_7Rs$ by anesthetics could aggravate the pathological condition. Our results suggest that care should be taken when large doses of propofol and thiopental are administered in patients with brain infarction, traumatic brain injury, and certain neurodegenerative diseases. Further study is needed to clarify the role and mechanisms of $P2X_7Rs$ enhancement by IV anesthetics in the pathological condition of the brain.

An *in vivo* study (22) using transcranial two-photon microscopy elegantly demonstrated that extracellular ATP released from damaged tissues and astrocytes induced microglial migration through stimulation of P2YRs. In our study, inhaled and IV anesthetics had no effect on the P2YRs-mediated mobilization of intracellular Ca^{2+}, suggesting that these anesthetics do not affect P2YRs-related microglial activation and motility.

P2XRs have been found in cells in the whole body; $P2X_1Rs$ in smooth muscle, $P2X_{2-5}Rs$ in neurons, $P2X_5Rs$ in heart, thymus, and skeletal muscle (40). $P2X_7Rs$ are found in most cells of the immune system and share limited homology with other members. Microglia are macrophage-lineage phagocytic cells. Functional properties of macrophage $P2X_7Rs$ are similar to those of microglia and mediate biological processes, such as the release of cytokine, and the killing of pathogenic bacteria (41). Although pharmacological properties of macrophage $P2X_7Rs$ may not be necessarily the same as those of microglia, our results suggest the possibility that propofol and thiopental may affect macrophage function via $P2X_7Rs$ modulation.

The action of several inhaled and IV anesthetics on P2X receptors has been reported in a variety of cells. Sevoflurane (0.5 mM) apparently inhibited P2XRs-mediated currents in locus coeruleus neurons in rats

(42). Propofol at relatively higher concentrations (50, 100 μM) slightly inhibited P2XRs in vagus nerve neurons (43) whereas it enhanced $P2X_4Rs$ expressed in HEK cells (44). At clinically relevant concentrations, thiopental and propofol had no effect on P2XRs in PC12 cells (45,46). Our experiments showed that IV anesthetics augmented $P2X_7Rs$-mediated currents. These various effects of anesthetics may be attributed at least partly to involvement of different types of P2XRs among cells.

In summary, it is suggested that IV anesthetics, propofol and thiopental in particular, may modulate microglial immunological responses through $P2X_7Rs$ in pathological conditions.

ACKNOWLEDGMENTS

The authors thank Junko Kawawaki for technical assistance.

REFERENCES

1. Nakajima K, Kohsaka S. Microglia: neuroprotective and neurotrophic cells in the central nervous system. Curr Drug Targets Cardiovasc Haematol Disord 2004;4:65–84.
2. Liu B, Hong JS. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. J Pharmacol Exp Ther 2003; 304:1–7.
3. Giulian D, Li J, Leara B, Keenen C. Phagocytic microglia release cytokines and cytotoxins that regulate the survival of astrocytes and neurons in culture. Neurochem Int 1994; 25:227–33.
4. Takeuchi A, Isobe KI, Miyaishi O, et al. Microglial NO induces delayed neuronal death following acute injury in the striatum. Eur J Neurosci 1998;10:1613–20.
5. Inoue K. Microglial activation by purines and pyrimidines. Glia 2002;40:156–63.
6. Nieber K, Eschke D, Brand A. Brain hypoxia: effects of ATP and adenosine. Prog Brain Res 1999;120:287–97.
7. Wang X, Arcuino G, Takano T, et al. P2X7 receptor inhibition improves recovery after spinal cord injury. Nat Med 2004;10:821–7.
8. Melani A, Turchi D, Vannucchi MG, et al. ATP extracellular concentrations are increased in the rat striatum during in vivo ischemia. Neurochem Int 2005;47:442–8.
9. Boucsein C, Zacharias R, Farber K, et al. Purinergic receptors on microglial cells: functional expression in acute brain slices and modulation of microglial activation in vitro. Eur J Neurosci 2003;17:2267–76.
10. Visentin S, Renzi M, Frank C, et al. Two different ionotropic receptors are activated by ATP in rat microglia. J Physiol 1999;519:723–36.
11. Moller T. Calcium signaling in microglial cells. Glia 2002;40: 184–94.
12. Tsuda M, Shigemoto-Mogami Y, Koizumi S, et al. $P2X_4$ receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 2003;424:778–83.
13. Di Virgilio F, Sanz JM, Chiozzi P, Falzoni S. The $P2Z/P2X_7$ receptor of microglial cells: a novel immunomodulatory receptor. Prog Brain Res 1999;120:355–68.
14. Inoue K, Nakajima K, Morimoto T, et al. ATP stimulation of Ca^{2+}-dependent plasminogen release from cultured microglia. Br J Pharmacol 1998;123:1304–10.
15. Hide I, Tanaka M, Inoue A, et al. Extracellular ATP triggers tumor necrosis factor-α release from rat microglia. J Neurochem 2000;75:965–72.
16. Gendron FP, Chalimoniuk M, Strosznajder J, et al. P2X7 nucleotide receptor activation type II nitric oxide synthase activity in BV-2 microglial cells. J Neurochem 2003;87:344–52.
17. Parvathenani LK, Tertyshnikova S, Greco CR, et al. $P2X_7$ mediates superoxide production in primary microglia and is upregulated in a transgenic mouse model of Alzheimer's disease. J Biol Chem 2003;278:13309–17.
18. Ferrari D, Los M, Bauer M, et al. P2Z purinoceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. FEBS Lett 1999;447: 71–5.
19. Honda S, Sasaki Y, Ohsawa K, et al. Extracellular ATP or ADP induce chemotaxis of cultured microglia through $G_{i/o}$-coupled P2Y receptors. J Neurosci 2001;21:1975–82.
20. Sasaki Y, Hoshi M, Akazawa C, et al. Selective expression of Gi/o-coupled ATP receptor $P2Y_{12}$ in microglia in rat brain. Glia 2003;44:242–50.
21. Franke H, Gunther A, Grosche J, et al. $P2X_7$ receptor expression after ischemia in the cerebral cortex of rats. J Neuropathol Exp Neurol 2004;63:686–99.
22. Davalos D, Grutzendler J, Yang G, et al. ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 2005;8:752–8.
23. Cavaliere F, Dinkel K, Reymann K. Microglia response and P2 receptor participation in oxygen/glucose deprivation-induced cortical damage. Neuroscience 2005;136:615–23.
24. Melani A, Amadio S, Gianfriddo M, et al. $P2X_7$ receptor modulation on microglia cells and reduction of brain infarct caused by middle cerebral artery occlusion in rat. J Cereb Blood Flow Metab 2006;26:974–82.
25. Kawaguchi M, Furuya H, Patel PM. Neuroprotective effects of anesthetic agents. J Anesth 2005;19:150–6.
26. Sawada M, Imai F, Suzuki H, et al. Brain-specific gene expression by immortalized microglial cell-mediated gene transfer in the mammalian brain. FEBS Lett 1998;433:37–40.
27. Inoue H, Sawada M, Ryo A, et al. Serial analysis of gene expression in a microglial cell line. Glia 1999;28:265–71.
28. Salimi K, Moser K, Zassler B, et al. Glial cell line-derived neurotrophic factor enhances survival of GM-CSF dependent rat GMIR1-microglial cells. Neurosci Res 2002;43:221–9.
29. Morihata H, Kawawaki J, Sakai H, et al. Temporal fluctuation of voltage-gated proton currents in rat spinal microglia via pH-dependent and-independent mechanisms. Neurosci Res 2000;38: 265–71.
30. Mori T, Zhao X, Zuo Y, et al. Modulation of neuronal nicotinic acetylcholine receptors by halothane in rat cortical neurons. Mol Pharmacol 2001;59:732–43.
31. Yamashita M, Mori T, Nagata K, et al. Isoflurane modulation of neuronal nicotinic acetylcholine receptors expressed in human embryonic kidney cells. Anesthesiology 2005;102:76–84.
32. Morihata H, Nakamura F, Tsutada T, Kuno M. Potentiation of a voltage-gated proton current in acidosis-induced swelling of rat microglia. J Neurosci 2000;20:7220–7.
33. Franks NP, Lieb WR. Molecular and cellular mechanisms of general anesthesia. Nature 1994;367:607–14.
34. Wachtel RE, Wegrzynowicz ES. Kinetics of nicotinic acetylcholine ion channels in the presence of intravenous anaesthetics and induction agents. Br J Anaesth 1992;106:623–7.
35. McLarnon JG, Zhang L, Goghari V, et al. Effects of ATP and elevated K^+ on K^+ currents and intracellular Ca^{2+} in human microglia. Neuroscience 1999;91:343–52.
36. Fragen RJ, Avram MJ. Barbiturates. In: Miller RD, ed. Anesthesia. Philadelphia: Churchill Livingstone, 2000:209–27.
37. Reves JG, Glass PSA, Lubarsky DA. Nonbarbiturate intravenous anesthesia. In: Miller RD, ed. Anesthesia. Philadelphia: Churchill Livingstone, 2000;228–72.
38. Illes P, Klotz KN, Lohse MJ. Signaling by extracellular nucleotides and nucleosides. Naunyn Schmiedebergs Arch Pharmacol 2000;362:295–8.
39. Rampe D, Wang L, Ringheim GE. $P2X_7$ receptor modulation of β-amyloid- and LPS-induced cytokine secretion from human macrophages and microglia. J Neuroimmunol 2004;147:56–61.
40. Girdler G, Khakh BS. ATP-gated P2X channels. Curr Biol 2004;14:R6.
41. Le Feuvre RA, Prough D, Iwakura Y, et al. Priming of macrophages with lipopolysaccharide potentiates $P2X_7$-mediated cell death via a caspase-1-dependent mechanism, independently of cytokine production. J Biol Chem 2002;277:3210–18.
42. Masaki E, Kawamura M, Kato F. Reduction by sevoflurane of adenosine 5'-triphosphate-activated inward current of locus coeruleus neurons in pontine slices of rats. Brain Res 2001; 921:226–32.

43. Patten D, Foxon GR, Martin KF, Halliwell RF. An electrophysiological study of the effects of propofol on native neuronal ligand-gated ion channels. Clin Exp Pharmacol Physiol 2001; 28:451–8.
44. Tomioka A, Ueno S, Kohama K, et al. Propofol potentiates ATP-activated currents of recombinant P2X(4) receptor channels expressed in human embryonic kidney 293 cells. Neurosci Lett 2000;284:167–70.
45. Furuya R, Oka K, Watanabe I, et al. The effects of ketamine and propofol on neuronal nicotinic acetylcholine receptors and P2x purinoceptors in PC12 cells. Anesth Analg 1999;88: 174–80.
46. Andoh T, Furuya R, Oka K, et al. Differential effects of thiopental on neuronal nicotinic acetylcholine receptors and P2X purinergic receptors in PC12 cells. Anesthesiology 1997; 87:1199–209.

ERRATUM

In the March 2007 issue of *Anesthesia & Analgesia*, in the article by Feng et al., "Hydroxyethyl Starch, but Not Modified Fluid Gelatin, Affects Inflammatory Response in a Rat Model of Polymicrobial Sepsis with Capillary Leakage" (Anesth Analg 2007;104:624–30), on page 1138, two authors were omitted. The author apologizes for the error. The corrected author list should be:

Xiaomei Feng, PhD, MD*, Wei Yan, PhD†, Zhaomin Wang, PhD‡, Jian Liu, PhD*, Min Yu, PhD*, Sihai Zhu, MD*, and Jianguo Xu, MD*

From the *Departments of Anesthesiology and †Neurosurgery, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, and the ‡Department of Anaesthesia, Shanghai First People's Hospital, Shanghai Jiaotong University, Shanghai, China.

In addition, the corresponding author should be changed to Jianguo Xu.

DOI: 10.1213/01.ane.0000265603.36078.e3

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

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

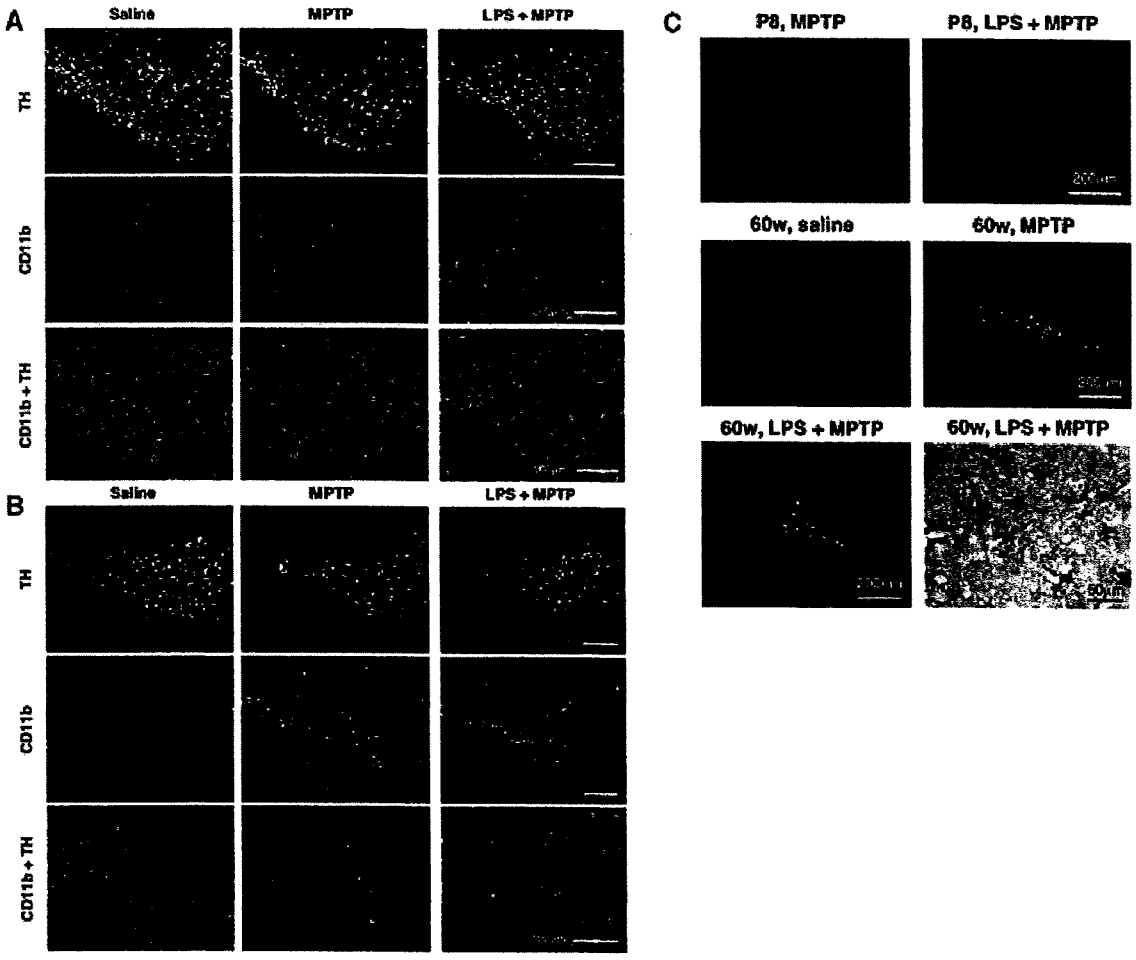


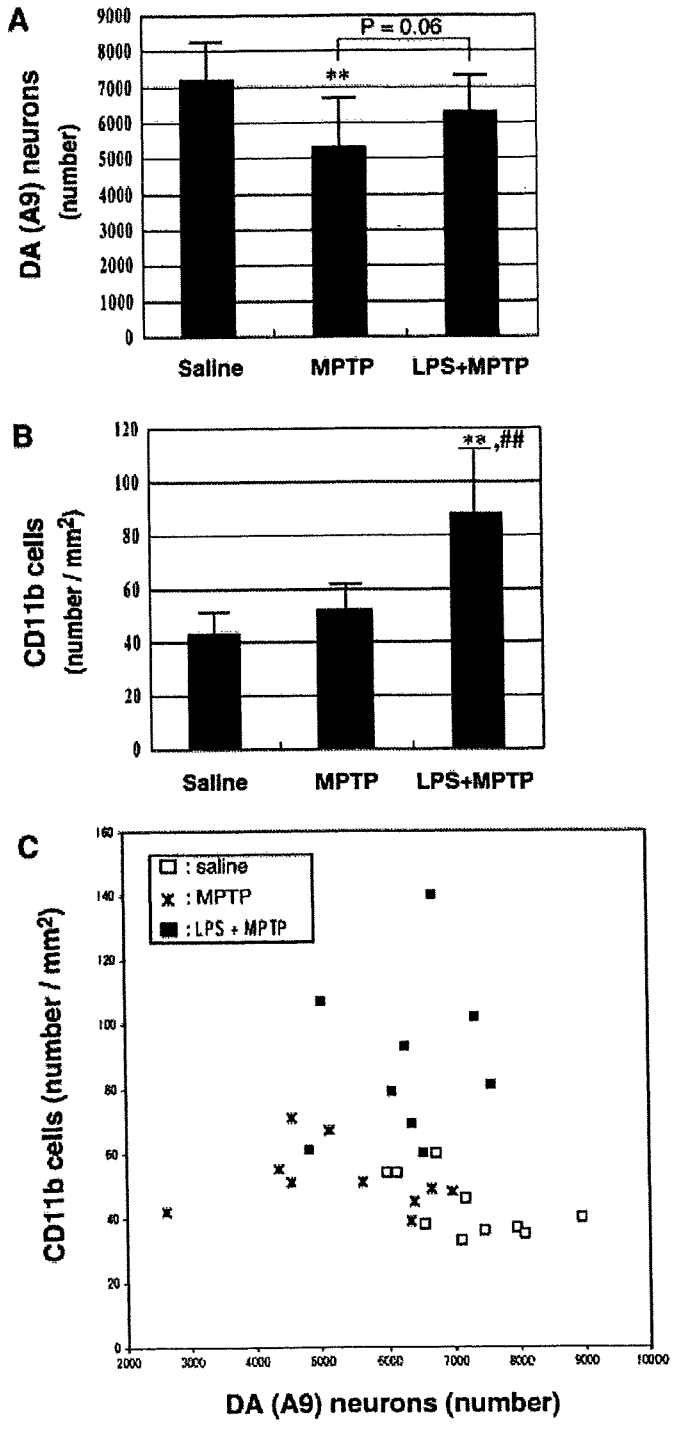
Fig. 1. Morphological changes due to MPTP administration in the SN. Immunostaining for tyrosine hydroxylase (TH)-positive dopamine (DA) (A9) neurons (merged, green) and CD 11b-positive activated microglia (merged, red) in the SN from mice treated with saline, MPTP, and LPS-MPTP are shown. **A:** In neonatal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



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. 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.

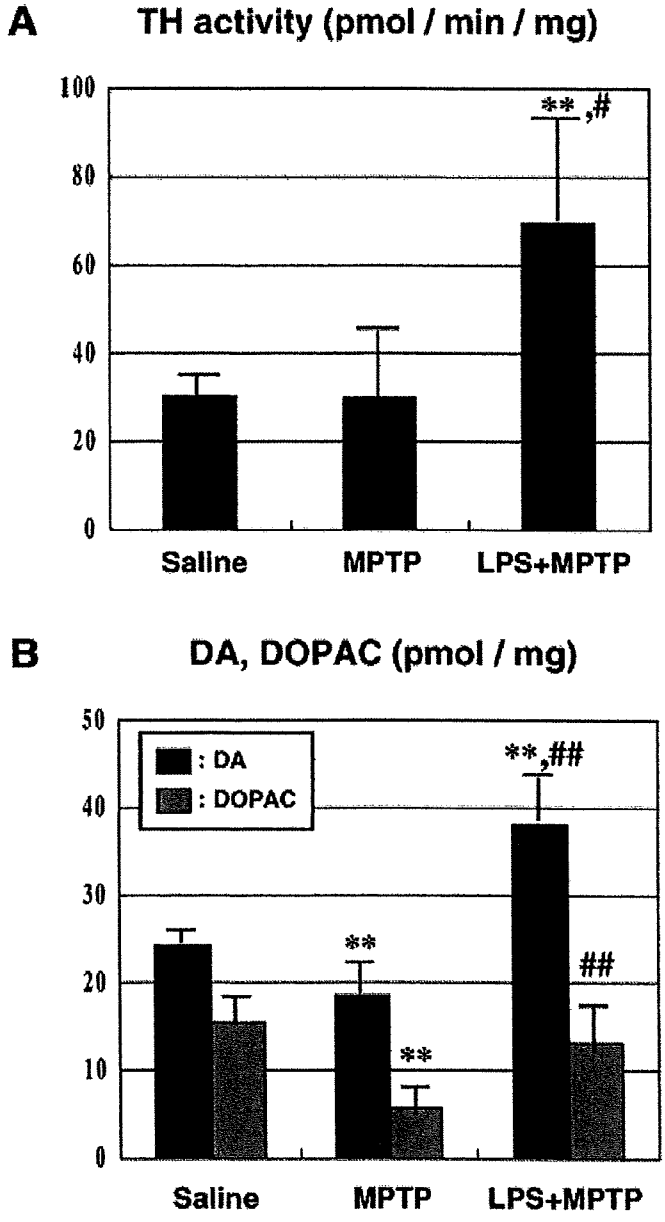


Fig. 3. Biochemical analyses of TH activity and DA and DOPAC contents in MPTP-treated neonatal mice. **A:** TH activity was measured in each of the three groups, and the LPS-MPTP group showed increased activity in the midbrain compared with the other two groups. **B:** Contents of DA and DOPAC in the 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).

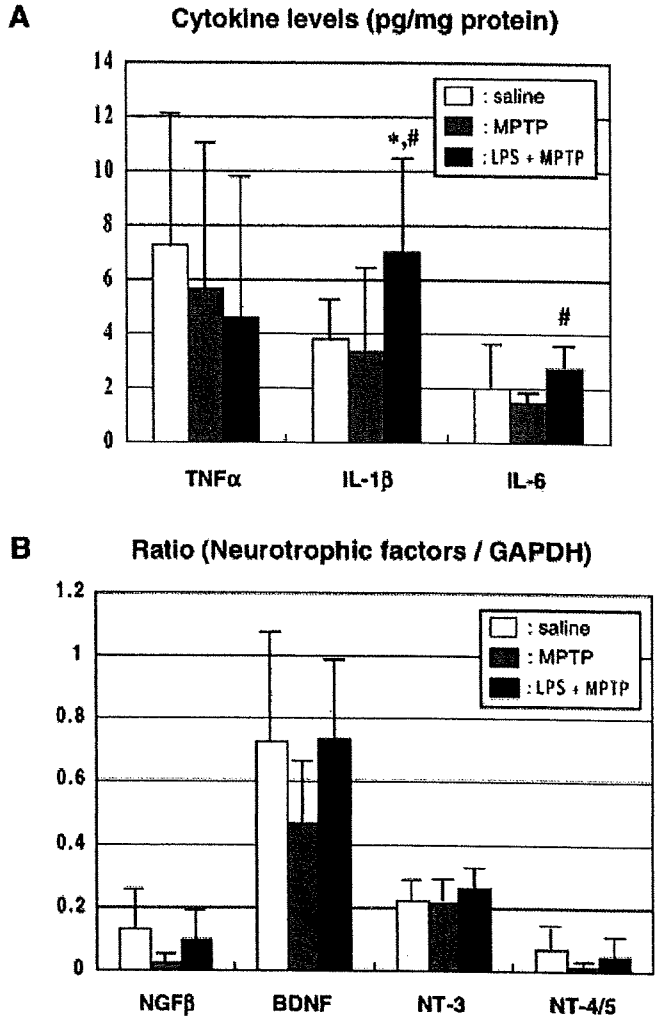


Fig. 4. Analysis of the levels of pro-inflammatory cytokines and neurotrophic factors in the midbrain of MPTP-treated neonatal mice. **A:** As to the pro-inflammatory cytokines, the TNFα level was not different among the three groups, but the IL-1β and IL-6 levels in the LPS-MPTP group were significantly 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-

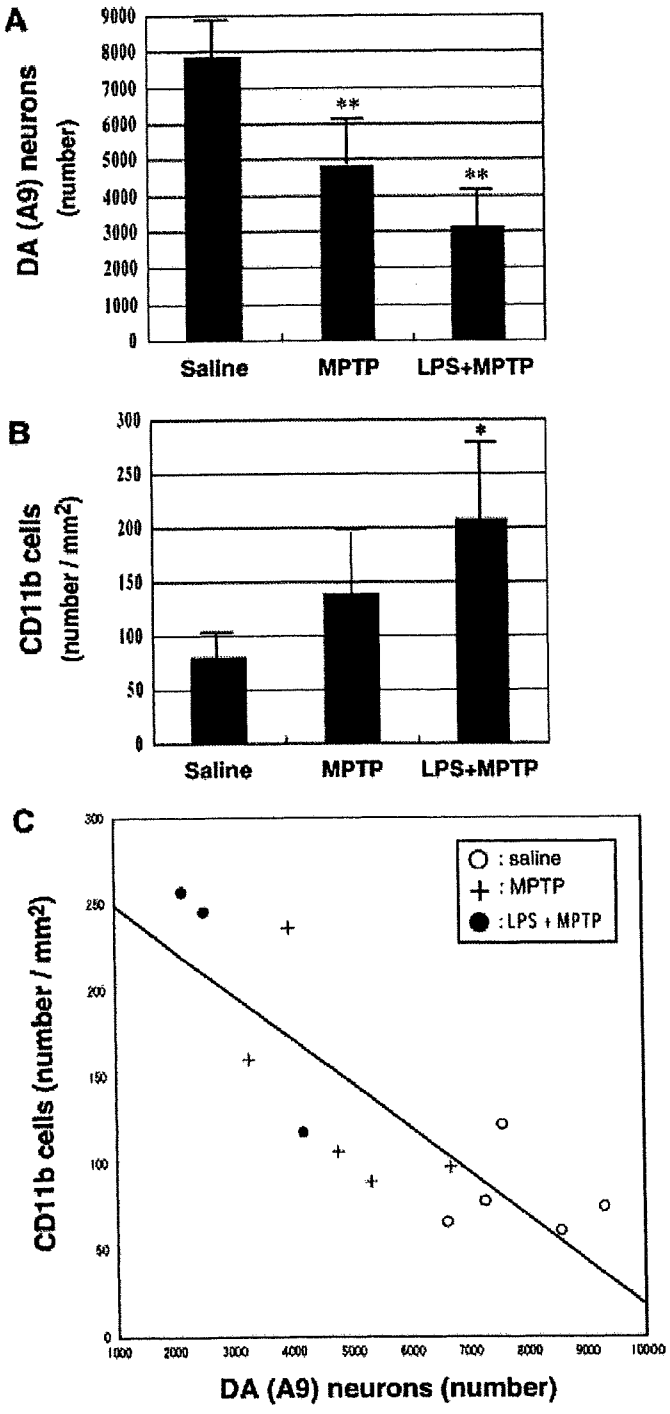


Fig. 5. Effects of microglial activation by LPS treatment on numbers of DA neurons and microglia in SN of 60-week-old (aged) mice treated with MPTP. **A:** Number of DA (A9) neurons in saline, MPTP, and LPS-MPTP groups. The number in the MPTP and LPS-MPTP groups was decreased significantly. **B:** Number 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.

Available online at www.sciencedirect.com
ScienceDirect
Experimental Neurology 206 (2007) 33–42
Experimental Neurology
www.elsevier.com/locate/yexnr

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

Sadayuki Hashioka [a,b,*], Andis Klegeris [b], Akira Monji [a,*], Takahiro Kato [a], Makoto Sawada [c], Patrick L. McGeer [b], Shigenobu Kanba [a]

[a] *Department of Neuropsychiatry, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka, 812-8582 Japan*
[b] *Kinsmen Laboratory of Neurological Research, Department of Psychiatry, Faculty of Medicine, The University of British Columbia, 2255 Wesbrook Mall, Vancouver, BC, Canada V6T 1W5*
[c] *Department of Brain Function, Research Institute of Environmental Medicine, Nagoya University, Japan*

Received 9 February 2007; revised 17 March 2007; accepted 23 March 2007
Available online 30 March 2007

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-

* Corresponding authors. S. Hashioka is to be contacted at Kinsmen Laboratory of Neurological Research, Department of Psychiatry, Faculty of Medicine, The University of British Columbia, 2255 Wesbrook Mall, Vancouver, BC, Canada V6T 1W5. Fax: +1 604 822 7086. A. Monji, fax: +81 92 642 5644.
E-mail addresses: hashioka@interchange.ubc.ca (S. Hashioka), amonji@hf.rim.or.jp (A. Monji).

0014-4886/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2007.03.022

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

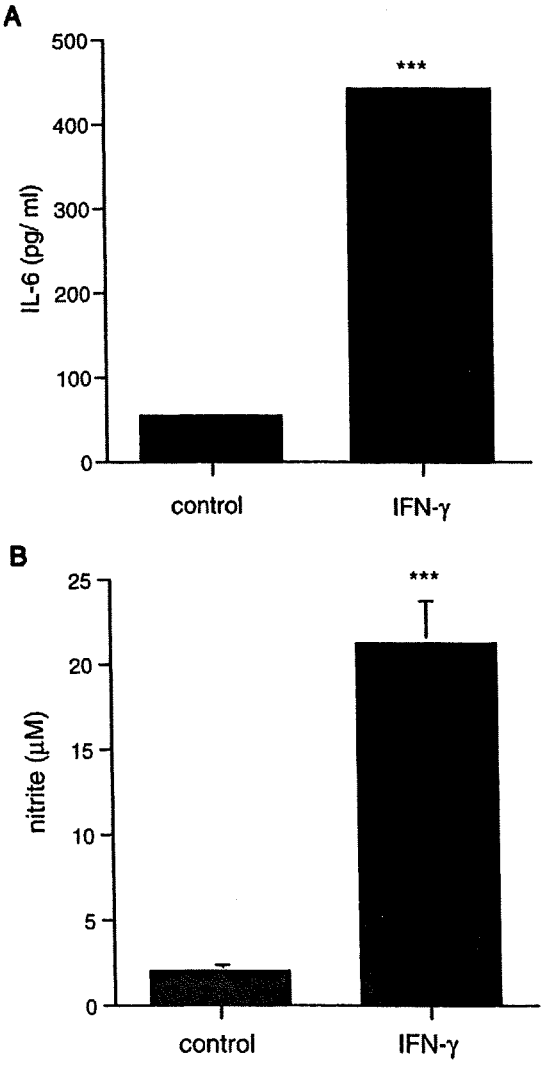


Fig. 1. IL-6 and NO production by IFN-γ-activated microglia. 6-3 microglial cells were treated with or without 100 U/ml of IFN-γ. After 24 h, the cell-free media were collected and IL-6 accumulation was measured by ELISA (A) or after 48 h, the cell-free media were collected and NO accumulation was measured 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.

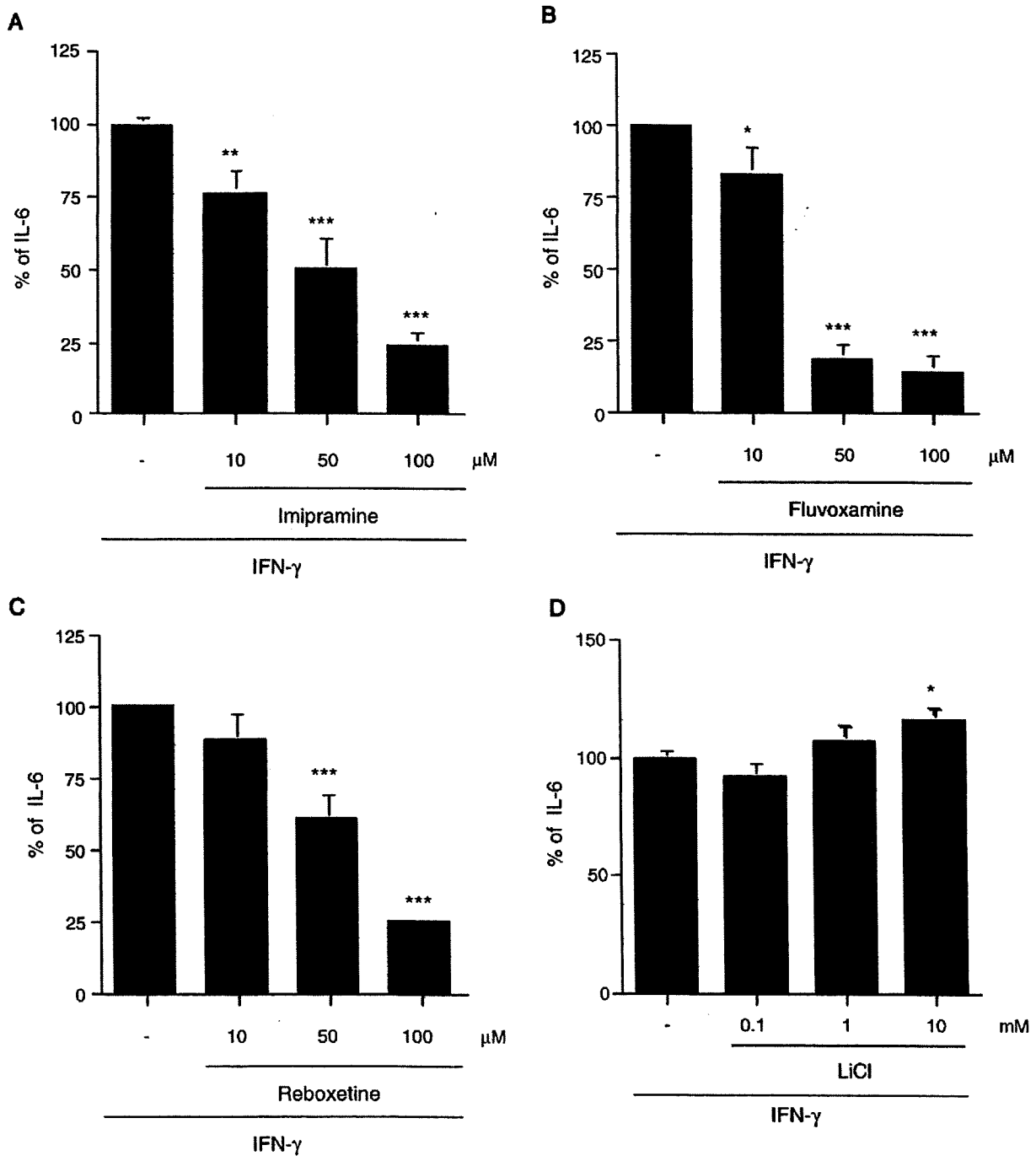


Fig. 2. Effects of antidepressants or lithium on the IFN-γ-activated microglial production of IL-6. 6-3 microglial cells were pre-incubated for 24 h with or without indicated concentrations of imipramine (A), fluvoxamine (B), reboxetine (C) or lithium chloride (D). Afterwards, the cells were stimulated 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.

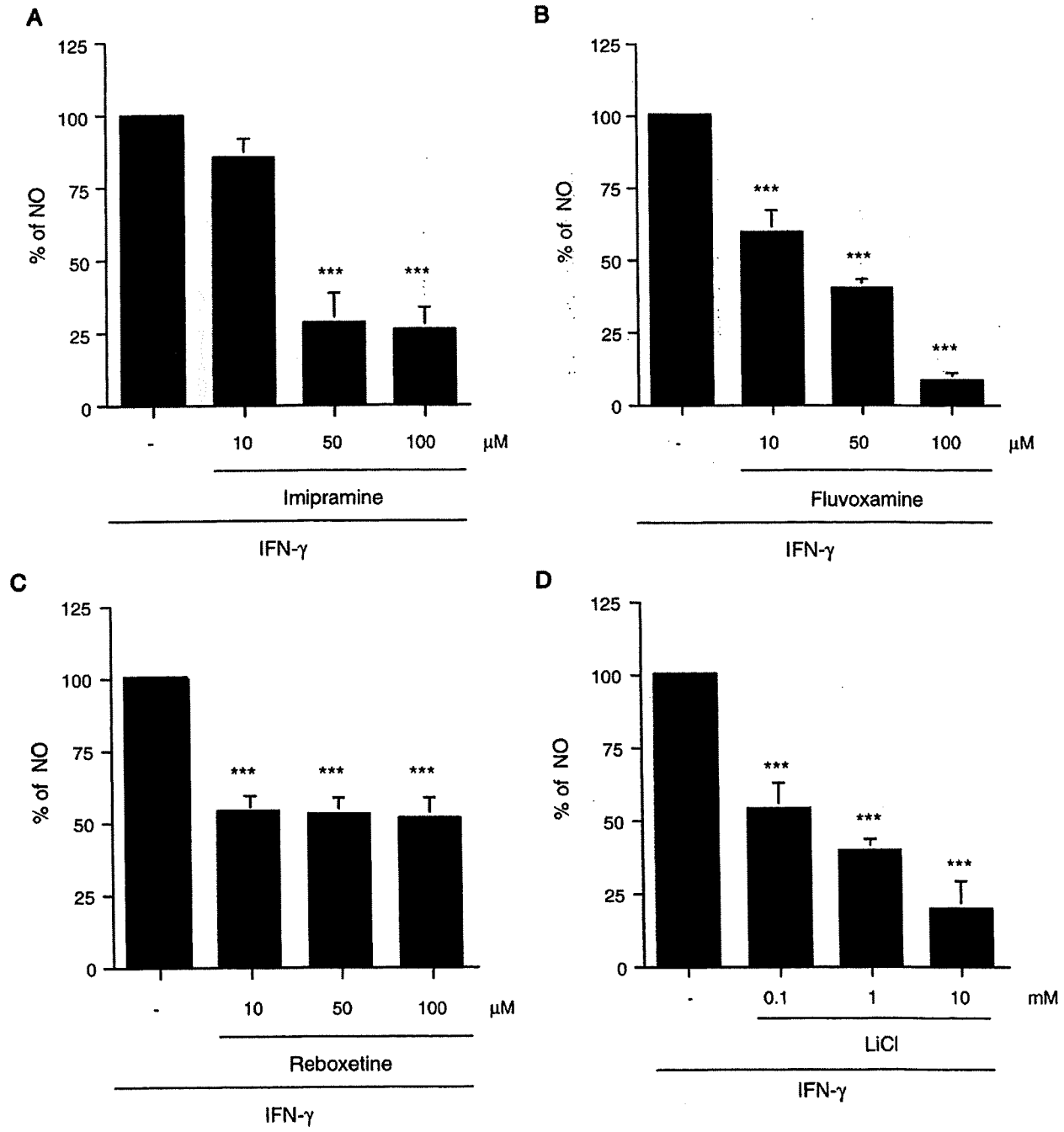


Fig. 3. Effects of antidepressants or lithium on the IFN-γ-activated microglial production of NO. 6-3 microglial cells were pre-incubated for 24 h with or without indicated concentrations of imipramine (A), fluvoxamine (B), reboxetine (C) or lithium chloride (D). Afterwards, the cells were stimulated 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), reverse

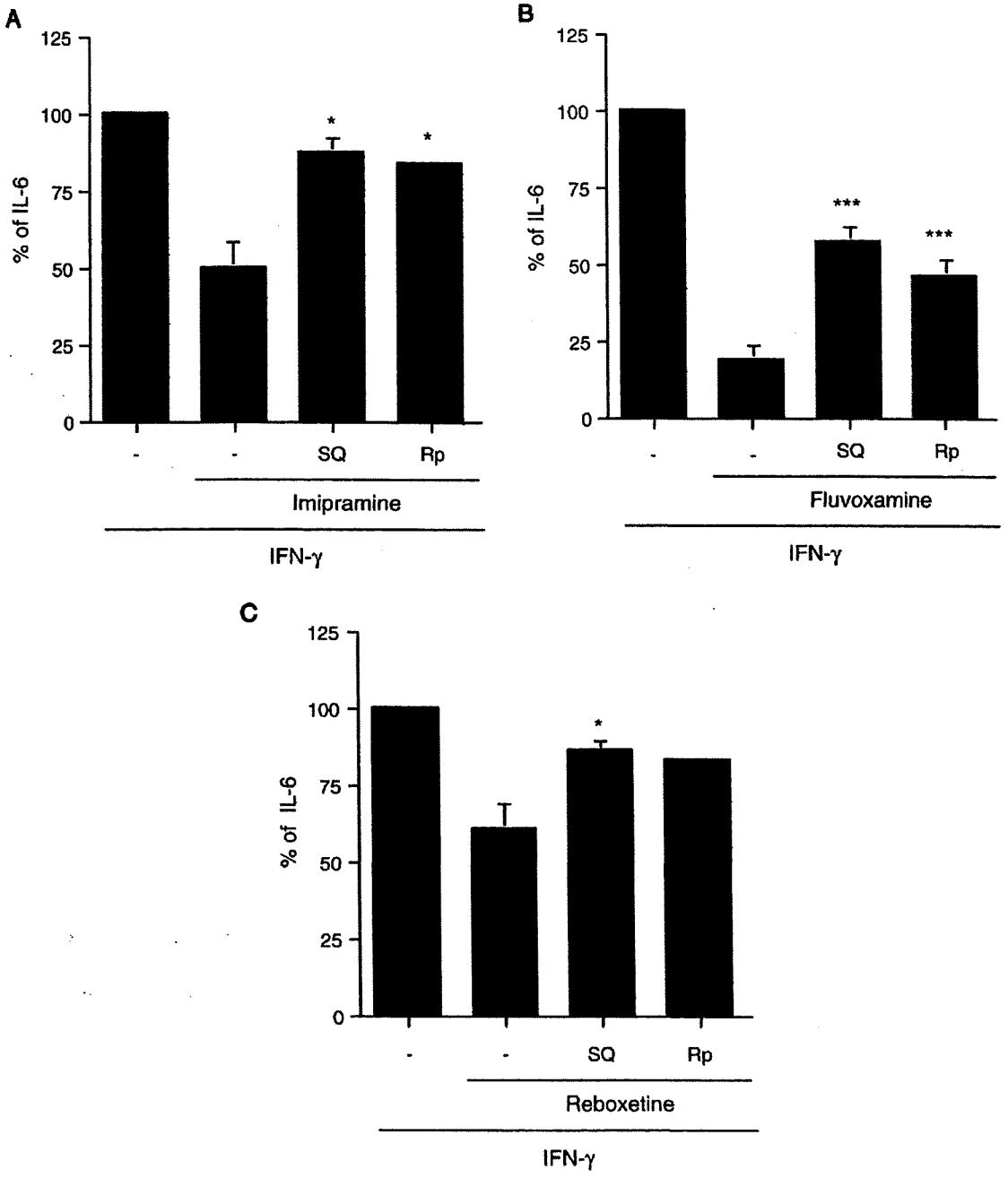


Fig. 4. Effects of cAMP/PKA inhibitors on the antidepressants-induced IL-6 suppression. 6-3 microglial cells were pre-incubated for 20 min with SQ 22536 (10 μM) or Rp-3′,5′-cAMPS (10 μM) before the addition of imipramine (50 μM) (A), fluvoxamine (50 μM) (B) or reboxetine (50 μM) (C). After 24 h of the pretreatment with antidepressants, 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 3–6 samples and expressed as percentage control, where 100% is the value obtained from IFN-γ alone. *$p<0.05$, ***$p<0.0001$, compared with imipramine (A), fluvoxamine (B) or reboxetine (C). Comparisons were made with ANOVA followed by the Fisher's PLSD. SQ, SQ 22536; Rp, Rp-3′,5′-cAMPS.

significantly those antidepressants-induced suppression of IL-6. In the case of reboxetine, the effect reached significance for SQ 22536 but only a non-significant trend was observed for Rp-3′,5′-cAMPS (Fig. 4C, $p=0.0558$).

Effects of cAMP/PKA inhibitors on the antidepressants-induced NO suppression

Finally, we investigated the effects of SQ 22536 and Rp-3′,5′-cAMPS on antidepressant or lithium chloride-induced NO suppression. We observed that 10 μM of SQ 22536 alone did not affect significantly the IFN-γ-induced microglial NO production, whereas 10 μM of Rp-3′,5′-cAMPS alone decreased significantly the IFN-γ-induced microglial NO production to 83.1±2.1% (data not shown).

Fig. 5 shows that both SQ 22536 and Rp-3′,5′-cAMPS at a dose of 10 μM significantly reversed the inhibitory effects of antidepressants on the IFN-γ-induced increase of microglial NO production. The effect is shown for imipramine (Fig. 5A), fluvoxamine (Fig. 5B) and reboxetine (Fig. 5C) tested at a dose