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Anatomical substrates for the discriminative stimulus effects of methamphetamine in rats

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Abstract

Methamphetamine is a psychostimulant drug acting on central monoaminergic neurons to produce both acute psychomotor stimulation and long-lasting behavioral effects including addiction and psychosis. Drug discrimination procedures have been particularly useful in characterizing subjective effects of addictive drugs. In the present study, to identify potential anatomical substrates for the discriminative stimulus effects of methamphetamine, we investigated the drug discrimination-associated Fos expression in Sprague–Dawley rats trained to discriminate methamphetamine from saline under a two-lever fixed ratio 20 (FR-20) schedule of food reinforcement. The rats that fulfilled the criteria for learning the discrimination were anesthetized and perfused 2 h after the drug discrimination test, and Fos immunoreactivity was examined in 15 brain regions. Fos expression in the brains of rats that discriminate methamphetamine from saline was significantly increased in the nucleus accumbens (NAc) and the ventral tegmental area (VTA), but not in other areas including the cerebral cortex, caudate putamen, substantia nigra, hippocampus, amygdala and habenulla, as compared with the expression in control rats that were maintained under the FR-20 schedule. The present findings suggest a role for the VTA and NAc as possible neuronal substrates in the discriminative stimulus effects of methamphetamine.

Keywords: addiction, c-Fos, drug discrimination, nucleus accumbens, rat, ventral tegmental area.

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Methamphetamine is an addictive drug with a wide range of behavioral actions that appear to be mainly mediated by the dopaminergic neuronal system (Ujike *et al.* 1989; Seiden *et al.* 1993; Giros *et al.* 1996; Munzar and Goldberg 2000). Acute methamphetamine treatment in rodents causes an increase in locomotor activity at low doses and stereotyped behavior at high doses. These behavioral effects of methamphetamine are associated with an increase in extracellular dopamine (DA) levels in the brain, by facilitating the release from presynaptic nerve terminals in addition to inhibiting the reuptake of DA (Kalivas and Stewart 1991; Seiden *et al.* 1993; Melega *et al.* 1995; Giros *et al.* 1996).

The discriminative stimulus effects of psychostimulants are related to aspects of drug actions that result in their subjective effects in humans (Schuster and Johanson 1988). In addition, drug discrimination studies reveal similar drug classifications between animals and humans (Kamien *et al.* 1993). Therefore, the drug discrimination procedure in animals has been used to elucidate the mechanism of action underlying the subjective effects of the different drugs of abuse (Callahan *et al.* 1997; Munzar and Goldberg 2000; Mori *et al.* 2001). So far, only pharmacological studies have been conducted to identify potential anatomical substrates of discriminative stimulus effects of addictive drugs: the microinjection of test compounds such as a specific receptor antagonist through indwelling catheters into specific brain regions has been conducted to map the brain circuitry that mediates the discriminative stimulus effects (Callahan *et al.* 1994; De La Garza *et al.* 1998; Filip *et al.* 2003). Alternatively, reassessment of the dose–response relationship for the

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Abbreviations used: DA, dopamine; FR, fixed ratio; NAc, nucleus accumbens; VTA, ventral tegmental area.

training drug following localized injury to specific neurotransmitter systems provides insight into the relevant neural circuitry (Nielsen and Scheel-Kruger 1986; Wood and Emmett-Oglesby 1989; Callahan *et al.* 1997).

Quantification of the changes in expression of the immediate early gene *c-fos* has proven to be a very useful method by which the distribution of neurons that are activated by physiological and pharmacological stimuli may be mapped (Sagar *et al.* 1988; Morgan and Curran 1991; Andre *et al.* 1998; Georges *et al.* 2000). Immunohistochemistry has indicated that acute methamphetamine dose-dependently produces Fos-like immunoreactivity in a wide area of the brains including the nucleus accumbens and striatum (Umino *et al.* 1995), and that chronic methamphetamine or amphetamine abolishes the inducibility of c-*fos* in the striatum (Cole *et al.* 1995; Namima *et al.* 1998). In the present study, to identify potential anatomical substrates of the discriminative stimulus effects of methamphetamine in rats, we investigated the drug discrimination-associated Fos expression in rats trained to discriminate methamphetamine from saline under a two-lever fixed ratio 20 (FR-20) schedule of food reinforcement.

Materials and methods

Animals

Male Sprague–Dawley rats (7 weeks old, Charles River Japan, Yokohama, Japan) weighing 230 ± 10 g at the beginning of experiments were used in the study. Their body weights were gradually reduced to approximately 80% of the free-feeding weight by limiting daily access to food. Water was available *ad libitum*. The animals were housed three per cage under controlled laboratory conditions (a 12-h light/dark cycle with lights on at 09:00 h, 23 ± 0.5°C, 50 ± 0.5% humidity).

All animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine.

Apparatus

Experiments were conducted in a standard operant-conditioning chamber (Neuroscience Co., Tokyo, Japan) set in a ventilated and sound-attenuated box. The chamber was equipped with two response levers, spaced 16 cm apart, with a food pellet trough mounted midway between levers. A houselight was located over the trough. Reinforcement consisted of a 45 mg food pellet (Bio Serv. Inc., Frenchtown, NJ, USA). Scheduling of reinforcement contingencies, reinforcement delivery and data recording were controlled by a computer system.

Methamphetamine discrimination procedure

Rats were initially trained to press each of the two levers under a fixed ratio (FR) 1 schedule of food reinforcement. The FR response requirement for food delivery was gradually increased from 1 to 20. After the response under the FR-20 schedule of food reinforcement had stabilized, drug discrimination training was begun (Mori *et al.* 2001; Nakajima *et al.* 2004). Discrimination training sessions were conducted 5 days per week under a double alternation schedule (i.e. MMSSMMSS, etc., M = methamphetamine, S = saline).

Rats were injected 10 min before the session with either saline or methamphetamine [0.5 mg/kg, subcutaneously (s.c.)]. After administration of methamphetamine, 20 consecutive responses (FR-20) on one lever produced a food pellet, whereas after saline administration, 20 consecutive responses on the other lever produced a food pellet. Responding on the incorrect lever reset the FR requirement for the correct lever. For half the rats, the right lever was the drug lever and, for the other half, the left lever was the drug lever. Each session ended after 20 food pellets were delivered or 20 min had elapsed, whichever occurred first. The criteria for learning the discrimination were three consecutive sessions with: (i) more than 85% correct-lever responding before the first reinforcement; (ii) more than 90% correct-lever responding throughout the session. The rats that fulfilled the criteria in a training session for three consecutive training sessions were used to test the dose–response effect of methamphetamine. Test sessions were identical to training sessions except that 20 consecutive responses on either lever resulted in delivery of a food pellet. Lever selection was examined after the administration of various doses of methamphetamine (0.1–0.5 mg/kg). After testing the dose–response effects of methamphetamine, rats were returned to daily training sessions.

Fos immunohistochemistry

A total of 11 groups of animals were prepared. Four groups of rats were prepared to investigate the neural circuitry underlying the discriminative stimulus effects of methamphetamine: naïve rats ($n = 3$) that were subjected to food restriction without lever pressing and drug discrimination training, control rats ($n = 4$) that were maintained on the FR-20 schedule of food reinforcement without drug discrimination training, and saline- ($n = 4$) and methamphetamine-injected trained rats ($n = 4$) that had met the criteria for learning the methamphetamine discrimination. Control rats were subjected to the FR-20 schedule of food reinforcement, while saline- and methamphetamine-injected rats were subjected to the test session of methamphetamine discrimination. Accordingly, the three groups of animals except naïve rats obtained the same number (20 pellets) of food reinforcement by almost equal numbers of lever pressing. The saline- and methamphetamine-injected rats had the same drug history during the drug discrimination training sessions, but received different drug treatments (methamphetamine vs. saline) on the test day for Fos immunohistochemistry. Because Fos expression was shown to occur from 1 to 4 h after a single short stimulation (Herdegen and Leah 1998), rats were killed 2 h after the drug discrimination test.

Four groups of rats were prepared to examine the effects of acute and chronic intermittent methamphetamine treatment on Fos expression. The conditions of age and food-restriction used in these groups were the same with the animals used to examine the discriminative effects of methamphetamine as described above. Two groups of rats ($n = 5$ and 4, respectively) received the same methamphetamine injection regimen with methamphetamine discrimination trained rats (intermittent methamphetamine treatment at a dose of 0.5 mg/kg under a double alternation schedule, i.e. MMSSMMSS, etc., M = methamphetamine, S =saline), but they did not receive any discrimination training.

The animals received 30 injections of methamphetamine because the average number of methamphetamine injections in rats that received discrimination training was 30. On the final day of the intermittent methamphetamine treatment, five rats were injected with methamphetamine 0.5 mg/kg ($n = 5$), while four rats were treated with saline ($n = 4$), and killed 2 h after the treatment. Another two groups of rats ($n = 5$ and 4, respectively) were injected daily saline to examine the acute effects of methamphetamine. On the final day, five rats were injected with methamphetamine 0.5 mg/kg ($n = 5$), while four rats were treated with saline ($n = 4$), and killed 2 h after the treatment.

A separate set of three groups of rats were prepared to examine the dose-dependent effects of acute methamphetamine treatment on Fos expression. Male Sprague–Dawley rats (7 weeks old, Charles River Japan) were used in the study. Food and water were provided *ad libitum*. After 1-week habituation, rats were treated with single saline or methamphetamine (0.5 mg/kg or 2 mg/kg, s.c.), and killed 2 h after the treatment.

Rats were deeply anesthetized with pentobarbital (50 mg/kg) and transcardially perfused with ice-cold saline, followed by 4% paraformaldehyde in phosphate buffer. The brains were removed, postfixed in the same fixative for 2 h and then cryoprotected in 30% sucrose in phosphate buffer. The brains were cut into 50-μm coronal sections on a cryostat, and free-floating sections were used for Fos immunohistochemistry (He *et al.* 2002). The sections were incubated with 5% goat serum and 0.3% Triton X-100 in 0.1 M phosphate buffer, and then incubated with rabbit anti-Fos antibody (1: 2000; sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 48 h at

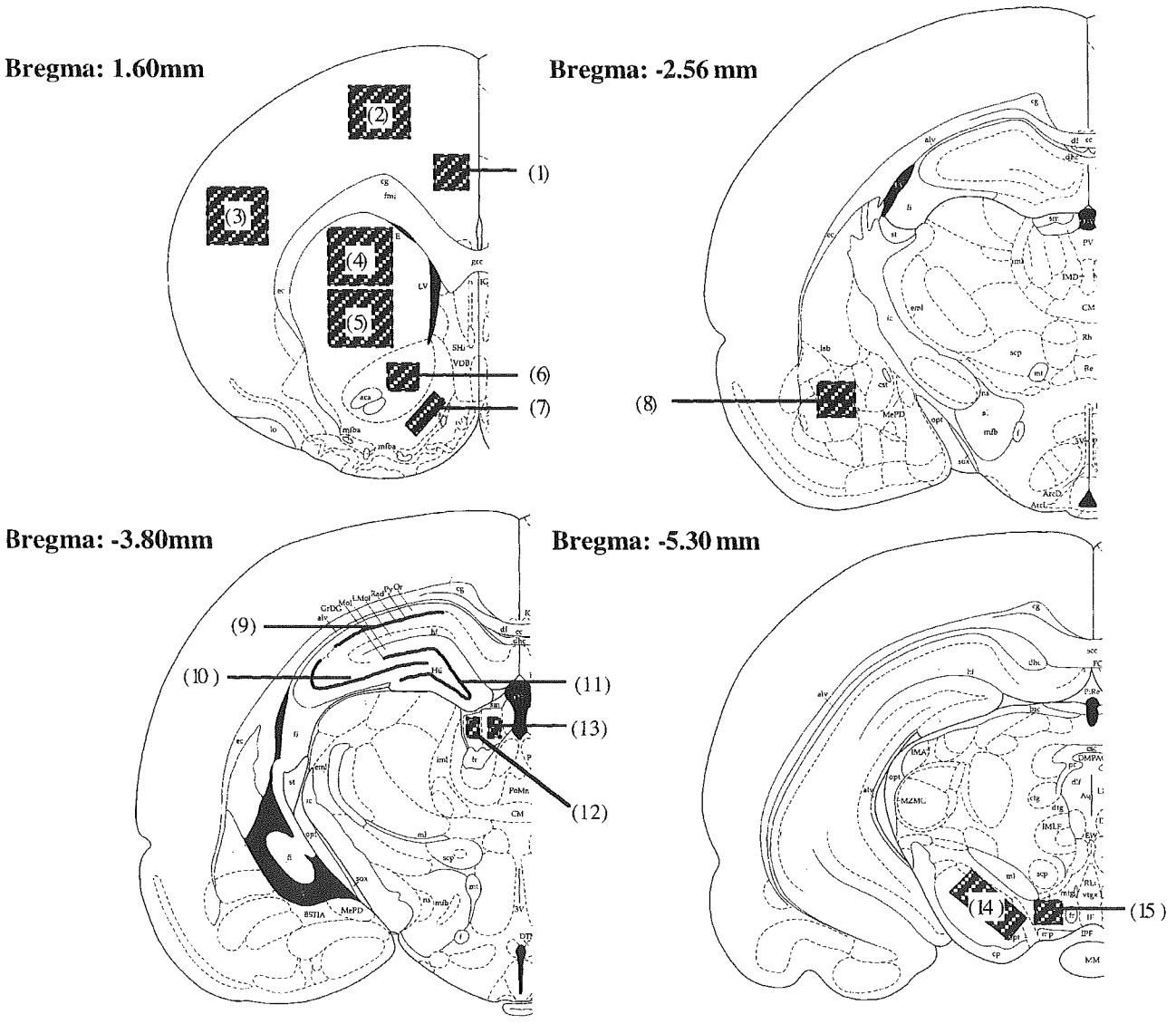

Fig. 1 Diagrammatic representation of the brain areas examined for Fos immunohistochemistry. The areas examined for enumerating Fos-positive cells include the cingulate (1), motor (2) and somatosensory cortex (3), the dorsal (4) and ventral caudate putamen (5), the core (6) and shell (7) of the nucleus accumbens, the amygdala (8) regions CA1 (9), CA3 (10) and the dentate gyrus (11) of the dorsal hippocampus, the medial (12) and lateral (13) habenula, the substantia nigra (14) and the ventral tegmental area (15).

4°C with constant rotation. They were then washed with phosphate buffer containing 0.3% Triton X-100 and incubated with biotinylated goat anti-rabbit antibody at 23°C for 2 h. Sections were washed and processed with avidin-biotinylated horseradish peroxidase complex (Vector ABC kit, Vector Laboratories, Burlingame, CA, USA), and the reaction was visualized using diaminobenzidine.

Quantitative analysis of c-Fos immunohistochemistry

To quantify the number of Fos-stained cells in the brain, we examined the sections, blind to the animal's treatment, with a computer-assisted image analysis system (C. Imaging Systems; Compix Inc., Mars, PA, USA) attached to a light microscope (Olympus BX60-FLB-3, Olympus, Tokyo, Japan), as described previously (Yamada *et al.* 1996; He *et al.* 2002). Both right and left hemispheres of four sequential sections for each selected area, located according to the atlas of Paxinos and Watson (1982), were examined for the counting of Fos-positive cells. This procedure resulted in a total of eight determinations of the number of Fos-positive cells within a specified area for each rat brain (Fig. 1). The average of the eight determinations was used for statistical analysis. Selected brain areas (mm^2) were as follows: cingulate (0.45 mm^2), motor (1.32 mm^2) and somatosensory cortex (1.32 mm^2), dorsal (1.32 mm^2) and ventral (1.32 mm^2) caudate putamen, and the core (0.31 mm^2) and shell (0.31 mm^2) of the nucleus accumbens (NAc) in sections at a level of +1.60 mm from the bregma; amygdala (0.45 mm^2) in sections at a level of −2.56 mm from the bregma; regions CA1, CA3 and the dentate gyrus of the dorsal hippocampus, and the medial (0.10 mm^2) and lateral (0.10 mm^2) habenula in sections at a level of −3.80 mm from the bregma, ventral tegmental area (0.31 mm^2; VTA) and the substantia nigra (0.89 mm^2) in sections at a level of −5.30 mm from the bregma. In the subfields of the hippocampus, the total number of Fos-positive cells was counted.

Statistical analysis

Results were expressed as the mean ± SE. The significance of differences was determined by a one-way analysis of variance (ANOVA), and individual post-hoc comparisons were made using Fisher's least squares difference (FLSD) test. p-values of less than 0.05 were regarded as statistically significant.

Results

Establishment of discriminative stimulus effects of methamphetamine

Rats reliably discriminated methamphetamine from saline after an average of 60 training sessions (range 50–70 sessions). The average number of methamphetamine injections was 30, and the amount of methamphetamine was 15 mg/kg. Once the training criterion was reached, methamphetamine discrimination stabilized and was maintained with a high degree of accuracy (> 95%) in all the subjects for the remainder of the investigation. In a dose–response test, methamphetamine produced a dose-related increase in methamphetamine-appropriate responding, while the response rate was stable at doses examined (Fig. 2).

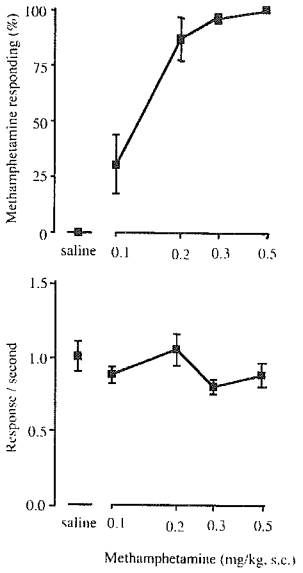

Fig. 2 Dose-dependent discriminative stimulus effects of methamphetamine in rats. Each point represents the mean ± SE (n = 8).

Fos expression associated with the discriminative stimulus effects of methamphetamine

To determine the neural circuitry underlying the discriminative stimulus effects of methamphetamine, rats were

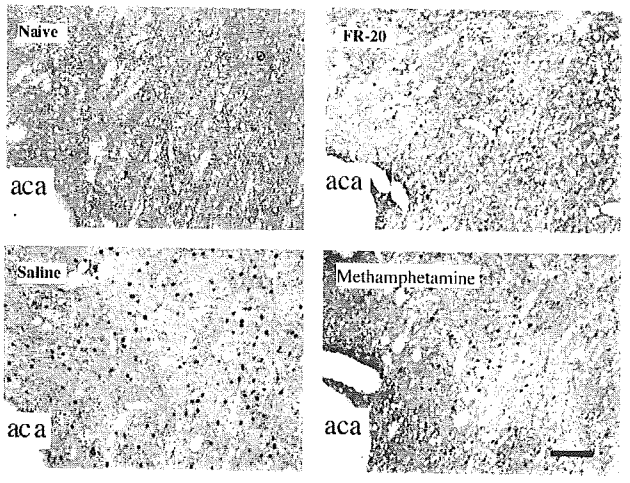

Fig. 3 Representative photomicrographs of Fos immunostaining of the NAc core in rats subjected to the drug discrimination test for methamphetamine. Rats were trained to discriminate methamphetamine (0.5 mg/kg) from saline under the two-lever FR-20 schedule of food reinforcement. The trained rats were subjected to the drug discrimination test after either saline or methamphetamine (0.5 mg/kg) treatment. Control rats were maintained under the FR-20 schedule of food reinforcement without drug discrimination training. Naïve rats were subjected to food restriction without lever pressing and drug discrimination training. aca: anterior commissure anterior part. Scale bar, 100 μm.

killed and examined for Fos immunohistochemistry, 2 h after the test session of methamphetamine (0.5 mg/kg) discrimination. It is plausible that the regional differences of Fos expression in the brain between control and trained rats reflect the neural circuitry for methamphetamine discrimination and the difference between methamphetamine- and saline-injected trained rats may indicate the mechanisms behind the discriminative stimulus effect of methamphetamine.

Representative photomicrographs of Fos staining in the NAc core and VTA are shown in Figs 3 and 4, respectively, and summaries of Fos expression observed in the core and shell of the NAc and VTA are ahown in Fig. 5. Summaries of Fos expression observed in other areas of the brain are shown in Table 1. Among the various regions examined, an ANOVA indicated significant differences in Fos expression among the four groups of rats in seven areas, the NAc core ($F_{3,11} = 27.376$, $p < 0.0001$), NAc shell ($F_{3,11} = 21.437$, $p < 0.0001$), VTA ($F_{3,11} = 38.162$, $p < 0.0001$), cingulate cortex ($F_{3,11} = 5.1364$, $p = 0.0184$), somatosensory cortex ($F_{3,11} = 5.0976$, $p = 0.0188$), amygdala ($F_{3,11} = 4.3290$, $p = 0.0303$) and substantia nigra ($F_{3,11} = 6.7381$, $p = 0.00076$; Fig. 5 and Table 1). Post-hoc analysis with the FLSD test revealed a marked difference in Fos expression between control and trained groups in the core and shell of the NAc, and the VTA (Fig. 5). Moreover, the number of Fos-positive cells was significantly smaller in the NAc core of methamphetamine-injected trained rats than saline-injected trained rats, whereas it was increased in the VTA of methamphetamine-injected trained rats compared with saline-injected trained rats (Fig. 5). No alteration in Fos expression was observed in other areas of the brain such as the motor cortex, dorsal and ventral caudate putamen, regions CA1, CA3 and dentate gyrus of dorsal hippocampus, and lateral habenula (Table 1).

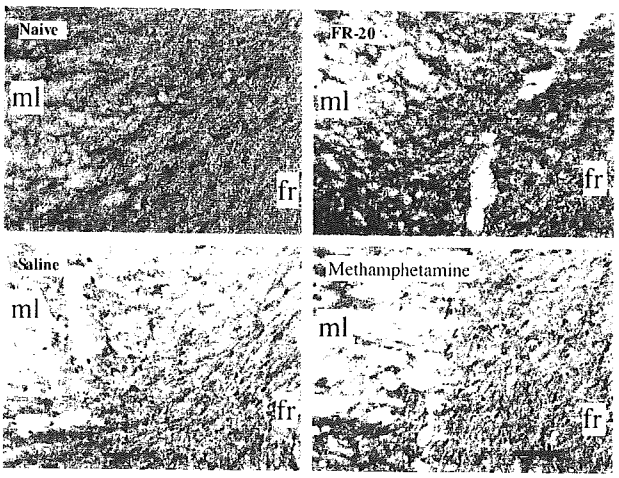

Fig. 4 Representative photomicrographs of Fos immunostaining of the VTA in rats subjected to the drug discrimination test for methamphetamine. ml, medial lemniscus; fr, fasciculus retroflexus (habenulointerpeduncular tract). Scale bar, 100 μm.

Effects of acute and chronic intermittent methamphetamine treatment on Fos expression

To confirm that the changes in Fos expression in the NAc and VTA are specifically attributed to the discriminative stimulus effects of methamphetamine, we examined the effects of acute and chronic intermittent methamphetamine treatment without the discrimination training on Fos expression. We

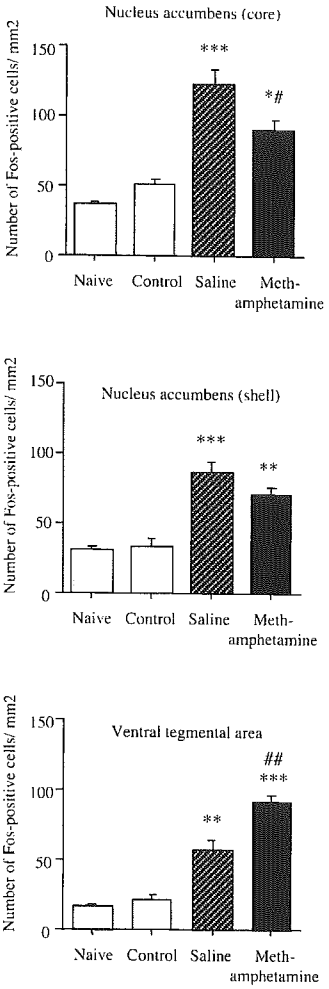

Fig. 5 Changes in Fos expression induced by discriminative stimulus effects of methamphetamine in the core and shell of the NAc and VTA. Rats were trained to discriminate methamphetamine (o.5mg/kg) from saline under the two-lever FR 20 schedule of food reinforcement. The trained rats were subjected to the drug discrimination test after either saline (n=4) or methamphetamine (0.5mg/kg, n=4) treatment. Control rats (n=4) were maintained under the FR 20 schedule of food reinforcement without drug discrimination training. Naïve rats (n=3) were subjected to neither food restriction nor the methamphetamine discrimination training. Each value represents the mean ±SE. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control. $\#p < 0.05$, $\#\#p < 0.01$ versus saline.

Table 1 Changes in Fos expression in various brain areas induced by discriminative stimulus effects of methamphetamine

Brain area	Naïve ($n = 3$)	Control ($n = 4$)	Saline ($n = 4$)	Methamphetamine ($n = 4$)
Cerebral cortex				
cingulate	72.3 ± 2.8*	102.4 ± 5.9	110.0 ± 3.4	115.7 ± 6.9
motor	54.1 ± 3.2	66.7 ± 4.5	68.3 ± 3.4	68.0 ± 1.9
somatosensory	38.8 ± 3.5*	48.7 ± 1.8	51.4 ± 1.2	49.2 ± 2.7
Caudate putamen				
dorsal	12.2 ± 2.2	13.4 ± 1.5	16.4 ± 1.5	15.5 ± 1.4
ventral	17.7 ± 2.8	18.8 ± 2.4	22.8 ± 1.9	22.0 ± 1.6
Amygdala	44.9 ± 2.8*	54.8 ± 5.4	64.7 ± 7.2	78.5 ± 8.4
Lateral habenula nucleus				
medial	49.1 ± 17.4	49.4 ± 10.1	34.4 ± 8.9	38.5 ± 4.6
lateral	11.3 ± 1.6	15.2 ± 2.5	16.6 ± 4.1	15.6 ± 2.8
Hippocampus				
CA1	2.5 ± 0.6	4.1 ± 0.7	3.6 ± 0.4	3.7 ± 0.6
CA2-3	20.4 ± 2.1	22.6 ± 2.3	29.9 ± 2.4	27.3 ± 2.8
dentate gyrus	20.8 ± 2.6	24.2 ± 0.7	25.5 ± 2.0	26.5 ± 1.0
Substantia nigra	6.4 ± 1.2*	16.9 ± 3.8	18.5 ± 2.5	24.3 ± 2.0

Fos expression in each area is indicated as the number of Fos-positive cells per mm^2, except in the hippocampus where the total number of cells is indicated. Each value represents the mean ± SE. *$p < 0.05$ versus control.

Table 2 Changes in Fos expression in the NAc and the VTA induced by acute and chronic intermittent methamphetamine treatment without discrimination training

Intermittent treatment	saline		methamphetamine	
Final treatment	saline	methamphetamine	saline	methamphetamine
Brain area	($n = 4$)	($n = 5$)	($n = 4$)	($n = 5$)
Nucleus accumbens				
core	13.7 ± 2.2	25.6 ± 3.3*	17.0 ± 2.3	25.9 ± 3.3*
shell	9.6 ± 0.7	11.1 ± 1.2	14.0 ± 2.7	15.0 ± 0.7
Ventral tegmental area	4.3 ± 1.2	12.5 ± 3.1	6.2 ± 3.5	17.8 ± 4.5

Methamphetamine 0.5 mg/kg was injected intermittently under a double alternation schedule, and the total number of methamphethamine injection was 30. Fos expression in each area is indicated as the number of Fos-positive cells per mm^2, Each value represents the mean ± SE. *$p < 0.05$ versus saline-saline control group.

chose the NAc and VTA for Fos immunohistochemistry, because Fos expression in the rats that were trained to discriminate methamphetamine from saline was significantly increased in the NAc and VTA. The summaries of Fos expression are shown in Table 2. The number of Fos-positive cells in the NAc core of acute methamphetamine-treated rats was significantly higher than that of saline-treated rats ($F_{3,14} = 4.2073, p = 0.0256$). Chronic intermittent methamphetamine 0.5 mg/kg does not lead to sensitization or desensitization of Fos expression in response to a methamphetamine injection. Furthermore, no alteration of Fos expression was observed in the NAc shell and VTA in all groups ($F_{3,14} = 2.9445$, $p = 0.0695$, $F_{3,14} = 2.7370$, $p = 0.0861$, respectively; Table 2).

We also examined the dose-dependent effects of acute methamphetamine treatment on Fos expression in the NAc and VTA. Methamphetamine (0.5–2 mg/kg) produced a dose-dependent increase in the number of Fos-positive cells in the NAc core, shell and VTA ($F_{2,8} = 899.84, p < 0.0001$, $F_{2,8} = 471.51$, $p < 0.0001$, $F_{2,8} = 7.0336$, $p = 0.0173$, respectively; Table 3). The number of Fos-positive cells in control group was less than that of naïve or control group in Table 1 or control rats that received chronic saline treatment in Table 2. This might reflect the difference of conditions of age, food restriction and treatment.

Discussion

In the present study, we demonstrated immunohistochemically that the act of discriminating methamphetamine from saline in rats is associated with a selective increase in Fos expression in the VTA and NAc. It is unlikely that this

Table 3 Changes in Fos expression in the NAc and the VTA induced by single methamphetamine treatment

	methamphetamine (mg/kg)		
Brain area	0 ($n = 4$)	0.5 ($n = 4$)	2 ($n = 3$)
Nucleus accumbens			
core	2.3 ± 0.5	11.2 ± 2.0**	87.5 ± 1.5***
shell	1.5 ± 0.2	5.1 ± 0.5	52.3 ± 2.6***
Ventral tegmental area	2.0 ± 0.0	18.6 ± 4.6*	32.1 ± 10.4*

Fos expression in each area is indicated as the number of Fos-positive cells per mm^2, Each value represents the mean ± SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control.

activation is due to lever press behavior or food reinforcement as Fos expression in these brain areas did not increase in the control group maintained on the FR-20 schedule of food reinforcement (Fig. 5). It is also unlikely that the activation is due to chronic intermittent methamphetamine treatment, as chronic intermittent methamphetamine treatment without discrimination training did not increase Fos expression in the VTA and NAc (Table 2).

It is well known that DA plays a major role in the discriminative stimulus effects of methamphetamine (Sasaki *et al.* 1995; Tidey and Bergman 1998; Munzar *et al.* 1999; Munzar and Goldberg 2000). In discrimination tests in rats, DA uptake inhibitors such as cocaine and nomifensine fully substituted for methamphetamine, and DA D1 and D2 receptor agonists also substituted for methamphetamine, whereas DA D1 and D2 receptor antagonists completely blocked the discriminative stimulus effects (Munzar and Goldberg 2000). Furthermore, microinjections of cocaine and amphetamine into the NAc have been shown to substitute for the effects of systemically administered psychostimulants (Callahan *et al.* 1997).

The VTA gives rise to dopaminergic pathways that innervate numerous limbic (e.g. NAc and amygdala) and cortical structures (e.g. prefrontal cortex; Fallon and Moore 1978; Beckstead *et al.* 1979). The NAc is a heterogenous structure with at least two anatomically and functionally distinct subregions: a medial and ventral shell region and a more lateral core region (Zahm and Heimer 1990; Heimer *et al.* 1991; Jones *et al.* 1996; David *et al.* 1998). Dopaminergic projections from the VTA to the NAc are involved in investigatory behavior evoked by novel stimuli (Ljungberg *et al.* 1992), and the reinforcement of adaptive investigatory approaches evoked by naturally occurring rewards (Hollerman and Schultz 1998; Schultz 1998) and by addictive drugs (Wise 1996). Collectively, our findings suggest that the development of methamphetamine discrimination is associated with a selective activation of the VTA-NAc, probably the dopaminergic neuronal system, and that both the core and shell region of the NAc are important neuroanatomical substrates, because the increase in Fos expression was observed in both the core and shell region of the NAc after methamphetamine discrimination test.

The NAc, which is positioned to integrate signals arising from limbic and cortical areas, participates in high-order brain functions, including reward, motivation, learning and memory (Apicella *et al.* 1991). A prominent excitatory glutamatergic input to the NAc arises from the ventral subiculum of the hippocampus (Groenewegen *et al.* 1987), and such inputs are in close apposition to the dopaminergic input from the VTA (Totterdell and Smith 1989; Sesack and Pickel 1990). The DA transmission in the VTA exerts a strong modulatory influence over the inputs from the hippocampus to the NAc (Yang and Mogenson 1987; Gonon and Sundstrom 1996). Conversely, the NAc modulates dopaminergic neuronal activity in the VTA by both a direct projection to the VTA and an indirect projection via the ventral pallidum (Zahm and Heimer 1990). Recent studies showed that glutamatergic afferents from the hippocampus to the NAc exert a potent excitatory effect on VTA DA neurons (Legault *et al.* 2000; Floresco *et al.* 2001). Therefore, it is also possible that the NAc-VTA pathway sets the methamphetamine discrimination in motion.

It is of interest that both saline and methamphetamine treatment in rats that fulfilled the criteria for the discrimination led to an increase in Fos expression in the VTA and the core and shell of the NAc. These results suggest that once the animals acquired the ability to discriminate methamphetamine from saline, these brain areas were selectively activated even after saline treatment. Our data, however, do not exclude the possibility that the changes in Fos expression are not specific for methamphetamine, but instead reflect processes involved in the learning of a discrimination. Thus, further investigations for other drugs such as morphine or nicotine and non-drug discriminative stimulus are needed to clarify the changes in Fos expression in this study are unique to methamphetamine.

Moreover, our study demonstrated that Fos expression in the VTA was significantly increased in methamphetamine-treated rats compared with saline-treated rats, and an inverse relation was found in the core of the NAc. There is much evidence that DA inhibits cell firing in the NAc via DA D1 and D2 receptors (Hu and White 1994; Chang *et al.* 1994; Kiyatkin and Rebec 1999; Nicola *et al.* 2000), and that DA controls the firing pattern of DA neurons via a network feedback mechanism (Paladini *et al.* 2003). DA neurons can affect many target nuclei that have direct or indirect reciprocal connections with DA neurons. For example, DA neurons project to γ-aminobutyric acid (GABA)ergic neurons in the striatum (Deniau *et al.* 1978; Guyenet and Aghajanian 1978), which in turn project back to the DA neurons in the midbrain (Somogyi *et al.* 1981; Paladini *et al.* 1999). Accordingly, it is important to determine whether the modulation of cell firing

by DA plays a role in a pattern of Fos expression in the VTA and the NAc after methamphetamine treatment.

Fos expression in the cingulate and somatosensory cortex, amygdala and substantia nigra of both control and methamphetamine discrimination-trained rats was increased compared with naïve group (Table 1). It has been suggested that the cingulate cortex and amygdala are involved in the incentive motivational effects (Neisewander *et al.* 2000), and that the substantia nigra is related to motor functions in motivated behaviors (Ono *et al.* 2000). Therefore, the increase in Fos expression in these areas might reflect the motivational effect of food reinforcement and motivational aspects of motor function. The somatosensory cortex is involved in exploratory behavior and texture discrimination, which are important for spatial orientation and learning (Van der Zee *et al.* 1994; Le Foll *et al.* 2002). Because the operant chamber differs from home-cages by floor texture, recognized with vibrissae, the increase in Fos expression in the somatosensory cortex is most likely due to the sensory stimulation of the vibrissae that occurs to recognize the operant chamber. There were no changes in Fos expression in the other areas of the brain examined, including the motor cortex, dorsal and ventral caudate putamen, and CA1, CA3 and dentate gyrus of dorsal hippocampus. These areas are well known to participate in the effects of methamphetamine. For example, intraperitoneal injection of methamphetamine (1.6–4.8 mg/kg) induced a widespread Fos-like immunoreactivity in the brain including the neocortex, amygdala, NAc, striatum and VTA (Umino *et al.* 1995). Destruction of dentate granule cells in the hippocampus was reported to potentiate methamphetamine-induced hyperlocomotion and Fos expression in the NAc (Tani *et al.* 2001). Accordingly, although Fos expression in these areas was not affected by methamphetamine discrimination, we cannot rule out their involvement. The expression of other genes should also be determined.

In conclusion, Fos expression in the VTA and NAc is selectively increased after either methamphetamine or saline treatment in rats that discriminate methamphetamine from saline. Methamphetamine treatment in the trained rats resulted in a significant increase in Fos expression in the VTA, and a decrease in the core of NAc, as compared to saline treatment. Our findings suggest a role for the VTA and NAc as possible neuronal substrates in the discriminative stimulus effects of methamphetamine. To support the hypothesis, an experiment which manipulates or blocks Fos expression in the VTA and NAc would be necessary to draw a causal relationship.

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Involvement of Glial Cell Line-Derived Neurotrophic Factor in Activation Processes of Rodent Macrophages

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The physiological roles of glial cell line-derived neurotrophic factor (GDNF) expressed in the microglia/macrophages of the injured spinal cord have not yet been clarified. mRNA expression of chemokines, including monocyte chemoattractant protein (MCP)-1, was evoked within 1 hr after transection of the spinal cord, and GDNF mRNA expression was similarly up-regulated. Immunohistochemical analysis showed that GDNF was coexpressed with MCP-1 in the CD11b-positive cells. Therefore, we examined further the effects of GDNF on cultured rat peritoneal macrophages. GDNF enhanced the phagocytic activity of the macrophages via GFRα-1, glycosylphosphatidylinositol-anchored specific binding site of GDNF, in a c-Ret-independent manner. The influence of autocrine and/or paracrine GDNF synthesis was evaluated by performing activation experiments using macrophages cultured from heterozygous (+/-) GDNF gene-deficient mice or wild-type (+/+) mice. There were no morphological differences dependent on genetic types or stimulators. However, the GDNF mRNA level, but not the MCP-1 or GFRα-1 mRNA level, was substantially lower in the mutant macrophages than in the +/+ cells irrespective of stimulation with MCP-1 or lipopolysaccharide (LPS). The phagocytic activity enhanced by MCP-1 or LPS was significantly lower in the mutant cells (+/-) than in the +/+ ones, demonstrating the involvement of endogenous GDNF in the activation processes of macrophages in vitro and suggesting that not only neuroprotective function but also activation of macrophages is effected by the GDNF produced after a spinal cord injury.

Key words: macrophages; phagocytic activity; spinal cord; glial cell line-derived neurotrophic factor; GDNF; monocyte chemoattractant protein-1; MCP-1

Several experimental strategies have been employed to minimize tissue damage and to enhance axonal growth and regeneration after injury to the central nervous system (CNS). The failure of axonal regeneration after CNS injury is thought to result in part from the expression of molecules inhibitory for axonal growth (Fawcett and Asher, 1999), the lack of neurotrophic factors (Widenfalk et al., 2001), and/or inflammatory reactions (Franzen et al., 1998).

Inflammation is one of the responses occurring immediately after spinal cord injury and is likely to cause secondary injury that magnifies the primary injury and facilitates neuronal dysfunction. This inflammatory response is characterized by the invasion of leukocytes into the injury site and the activation of resident glial cells, which is regulated by chemokines in the case of various injuries and diseases of the CNS (Glabinski et al., 1995; Adams and Lloyd, 1997; Rollins, 1997). Several studies have shown that accumulation of macrophages around the lesion site is correlated with the secondary injury that appears after a spinal cord injury (Blight, 1985, 1992; Popovich et al., 1997; Zhang et al., 1997). On the other hand, inflammation is also thought to contribute to the induction of neurotrophic factors beneficial to damaged neurons. It has been shown that glial cell line-derived neurotrophic factor (GDNF), one of the most potent neurotrophic factors for motoneurons (Lin et al., 1993; Henderson et al., 1994), is expressed in leukocytes and especially in activated microglia/macrophages of the injured brain (Batchelor et al., 1999; Wei et al., 2000). There is also a report showing that the activated microglia/macrophages in the injured spinal cord express GDNF (Satake et al., 2000; Widenfalk et al., 2001). How-

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ever, the interaction between neurotrophic factors and chemokines in the injured spinal cord and the physiological significance of GDNF expression in microglia/macrophages have not been studied.

In this study, we found that 1) microglia/macrophages recruited into the transected spinal cord coexpressed both GDNF and one of the chemokines, monocyte chemoattractant protein (MCP)-1; 2) cultured peritoneal macrophages responded to exogenous GDNF by enhancing their phagocytic activity and expression of MCP-1 mRNA; and 3) macrophages cultured from the heterozygous (+/−) GDNF gene-deficient mice were activated by MCP-1 or lipopolysaccharide (LPS) to a much lesser extent than those cultured from the +/+ mice. These results suggest that GDNF induced in activated macrophages behaves as a cytokine for macrophages and participates in the development of inflammation in the injured CNS in addition to functioning in its role as a neurotrophic factor.

MATERIALS AND METHODS

Animals and Surgery

Animals were cared for according to the guidelines for experimental animal care issued from the Office of the Prime Minister of Japan. Adult male Wistar rats (7–8 weeks old, 150–200 g; Nippon SLC, Shizuoka, Japan) were anesthetized by an intraperitoneal injection with pentobarbital (30 mg/kg). After laminectomy at the level of the ninth thoracic vertebra, the exposed spinal cords were completely transected with a razor blade. Afterward, the incision sites were sutured. The generation of the GDNF gene-deficient mice has been described elsewhere (Picher et al., 1996).

Reverse Transcription-Polymerase Chain Reaction

The spinal cords were dissected out from the anesthetized animals 0, 1, 3, 6, 12, 24, or 48 hr after the spinal cord transection, and segments (5 mm in length) of each spinal cord just rostral or caudal to the transected site were collected and pooled. RNA was prepared from the collected tissues by using Isogen (Nippon Gene, Tokyo, Japan), which is basically composed of guanidine isothiocyanate. The reverse transcription-polymerase chain reaction (RT-PCR) was performed with a SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) according to the instructions. In brief, 500 ng of RNA was denatured for 2 min at 70°C and reverse transcribed with MMLV reverse transcriptase for 1 hr at 42°C in the presence of 1 μM CDS primer. Afterward, the transcriptase was inactivated by heating for 7 min at 72°C. The synthesized cDNA was amplified with each pair of primers by PCR. The amplification was carried out with the thermal cycler at 94°C for 3 min, followed by 20–36 cycles consisting of 94°C for 30 sec, 52–60°C for 30 sec, and 72°C for 30 sec. The products were electrophoresed on 2% agarose gels. The predicted sizes of amplified cDNA fragments, the numbers of PCR cycles, and the annealing temperatures are indicated in Table I. β-Actin mRNA was used as an internal control. The intensity of the bands was analyzed by use of image-analysis software (Mac BAS; Fuji Film, Tokyo, Japan). RNA from rat peritoneal macrophages cultured in the presence of GDNF, MCP-1 (Prepro-Tech, Rocky Hill, NJ), or LPS (Sigma, St. Louis, MO) was prepared and analyzed by RT-PCR as described above.

Immunohistochemical Study

Two days after the spinal cord transection, animals were anesthetized with pentobarbital (30 mg/kg) and then cardioperfused with cold 4% (w/v) paraformaldehyde solution prepared in 0.1 M phosphate buffer, pH 7.3 (the fixative). The spinal cord was postfixed with the same fixative for 2 hr, soaked in 20% (w/v) sucrose for 1 day, and frozen in embedding compound (Tissue-Tec; Miles, Elkhart, IN). Coronal sections of 30 μm thickness were cut with a cryostat (model CM 1800; Leica, Deerfield, IL) and thawed on coverslips bearing covalently linked amino groups (Sumitomo Bakelite, Tokyo, Japan). The tissue sections were next dried and subsequently soaked in the fixative for 5 min to cross-link covalently the tissue sections to the coverslips. Then, they were rinsed in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.3% (v/v) Triton X-100 (TT buffer) at 37°C for 30 min to make the cell membrane permeable to antibodies. Next, the sections were incubated with TT buffer containing anti-GDNF rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution or anti-MCP-1 rabbit antibody (Biogenesis, Poole, United Kingdom) at a 1:1,000 dilution or antiglial fibrillary acidic protein mouse antibody (GFAP; Boehringer Mannheim Biochemica, Indianapolis, IN), anti-CD11b mouse antibody (Chemicon, Temecula, CA), and anti-NeuN mouse antibody (Chemicon) at a 1:1,000 dilution at 4°C overnight, and subsequently incubated at room temperature for 2 hr with TT buffer containing both fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG and rhodamine-labeled anti-mouse IgG antibodies (Chemicon). After having been washed with TT buffer, the sections were observed for fluorescence with a confocal laser microscope (Zeiss model L SM510).

Cell Cultures

Macrophages were obtained from the peritoneal cavity of adult male Wistar rats or adult GDNF gene-deficient mice [wild-type mice (+/+) or heterozygous mice (+/−)] 2 days after an intraperitoneal injection of 200 μg of concanavalin A (Sigma) in 5 ml phosphate-buffered saline (PBS). The cells were washed with PBS and cultured in RPMI 1640 medium (Sigma) containing 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (Cansera Int., Rexdale, Canada); 1 hr later, the medium was aspirated to remove nonadherent cells and replaced with fresh medium.

Phagocytosis Assay

The FITC-labeled latex beads (1.0 μm in diameter; Polysciences, Warrington, PA) were opsonized by incubation with human IgG (Sigma) at 4°C overnight, washed, and suspended in PBS. Cells pretreated or not with phosphatidylinositol-specific phospholipase C (PI-PLC, 0.5 U/ml; Sigma) for 1 hr were cultured in the presence of GDNF, MCP-1, or LPS and then incubated in the medium containing 0.01% FITC-labeled latex beads for 30 min at 37°C. Excess beads were washed away with PBS, and incorporated beads were visualized with a fluorescence

TABLE I. Sequences of Oligonucleotide Primers Used in the RT-PCR

Gene	Upstream primer	Downstream primer	Product size (bases)	Annealing temperature (°C)	Cycle number
GDNF	GAGAGGAATCGGCAG GCTGCAGCTG	CAGATACATCCACAT CGTTTAGCGG	337	60	36
NGF	GGGGATCCTCCACCC ACCCAGTCTTCCAC	GGCAAGTCAGCCTCT TCTTGTAGCCTTCC	376	60	30
BDNF	GGAATTCGAGTGATG ACCATCCTTTTCCTTAC	CGGATCCCTATCTTC CCCTTTTAATGGTCAGTG	771	60	31
NT-3	TATGCAGAACATAAG AGTCAC	CTACGAGTTTGTTGT TTTCTG	294	55	36
MCP-1	TAGCATCCACGTGCT GTCTC	CATTCAAAGGTGCTG AAGTCC	299	55	25
MIP-1α	TGCCCTTGCTGTTCT TCTCT	AGATCTGCCGGTTTC TCTTG	200	55	30
MIP-1β	CTCTCTCCTCCTGCT TGTGG	CACAGATTTGCCTGC CTTTT	200	55	30
MIP-2	AGGGTACAGGGGTTG TTGTG	TTTGGACGATCCTCT GAACC	204	55	30
RANTES	ATATGGCTCGGACAC CACTC	CCCACTTCTTCTCTG GGTTG	167	55	30
CCR2	CTGCCCCTACTTGTC ATGGT	AACGCAGCAGTGTGT CATTC	264	55	30
GFRα-1	TCCAGCCACATAACC ACAAA	CTTCAACAGAAGCCC CTGAG	297	55	30
c-Ret	CGGCACACCTCTGCT CTATG	CTGGAGGAAGACGGT GAGCA	237	55	35
β-Actin	GTGGGCCGCCCTAGG CACCAG	CTCTTTAATGTCACG CACGAT	542	55	32

microscope. Phagocytic activity was quantified as the number of FITC-positive cells (phagocytosing macrophages) per total cells.

RESULTS

Injury-Induced mRNA Expression of Neurotrophic Factors

Time-dependent mRNA expression of GDNF or neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), was examined in the segment adjacent to the transection site of the rat spinal cord (Fig. 1). NGF, BDNF, and GDNF mRNA levels were significantly elevated after the injury, but the NT-3 mRNA level remained constant throughout the experimental period. The increase was greatest for NGF mRNA, followed by that for GDNF mRNA. The NGF mRNA level started to increase 1 hr after the transection, peaked at 6 or 12 hr, and gradually declined until 48 hr. The GDNF mRNA started to rise slightly later, at 3 hr after the injury, attained its maximal level 12 hr postoperation, and gradually declined until 48 hr. However, the change in the mRNA level of BDNF was relatively small, though significant; i.e., an increase was detected first at 6 hr and was sustained until 24 hr after the injury. These results for NGF and GDNF gene expression are in agreement with previous findings (Widenfalk et al., 2001; Murakami et al., 2002). We were particularly interested in GDNF with respect to spinal cord regeneration, because GDNF has potent neurotrophic activity on motoneurons and CNS neurons that send axons into the spinal cord.

Injury-Induced mRNA Expression of Chemokines

Various chemokines are up-regulated in the injured CNS, including the spinal cord (McTigue et al., 1998; Huang et al., 2000; Lee et al., 2000). They are thought to act as chemoattractants to recruit immune cells, such as leukocytes and microglia/macrophages, to the injury site, thus triggering inflammatory reactions. To compare the gene expression profile of GDNF with that of various chemokines, we monitored by RT-PCR the time-dependent mRNA expression of MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, and regulated upon activation normal T-cell expressed and secreted (RANTES) in the segment adjacent to the transection site of the spinal cord. The expression profiles of these mRNAs were similar to each other. Especially, the pattern of MIP-1β quite resembled that of MIP-1α, so the results for MIP-1β are not shown. The expression of all chemokines tested was evoked 1 hr after the injury, peaked at 6 or 12 hr, and was sustained at a significantly high level until 48 hr (Fig. 2). These patterns resembled the expression profile of GDNF mRNA, suggesting shared mechanisms for expression between chemokines and GDNF in the injured spinal cord.

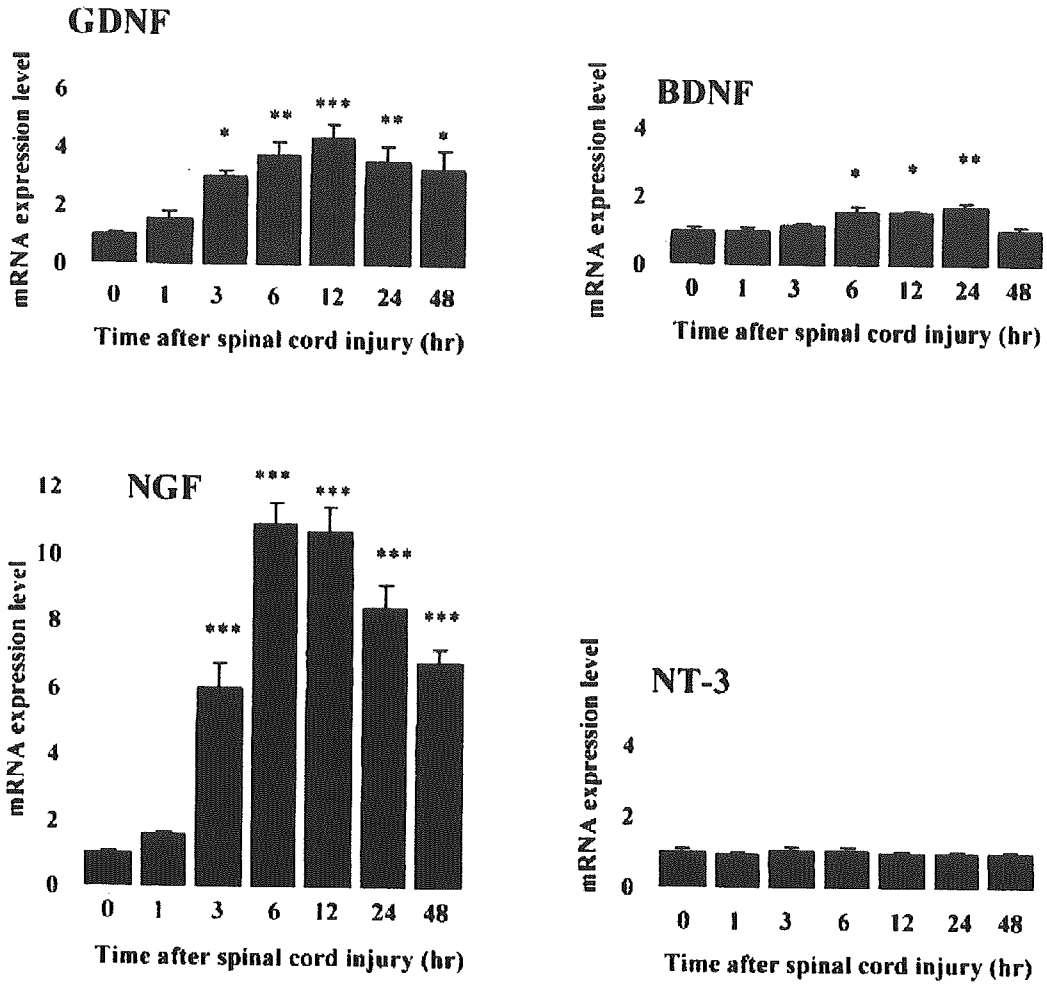

Fig. 1. Expression of neurotrophic factor mRNAs in the sites adjacent rostrally or caudally to the transection site of the rat spinal cord. The time-dependent mRNA expression of neurotrophic factors, including GDNF, NGF, BDNF, and NT-3, after spinal cord transection was monitored by RT-PCR. Total RNA of the tissue was subjected to RT-PCR using primers specific for the respective target genes (see Table I), and PCR products were applied to 2% agarose gels. The bands corresponding to neurotrophic factor mRNA- or β-actin mRNA-derived cDNA were densitometrically quantified, and the ratio of the intensity of each neurotrophic factor band to that of the β-actin band (ratio of neurotrophic factor/β-actin) was calculated. The values are expressed as the means ± SE (n = 3) of the -fold increase in the ratio of GDNF/β-actin, regarding the value of the mice of control group (0 hr) as 1.0. Significant differences from the value of the corresponding control group were determined by one-way ANOVA and Tukey test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Expression of GDNF and MCP-1 in the Injured Spinal Cord

Mac1/CD11b-positive cells, implying activated microglia/macrophages (Lu and Pitha, 2001; Babcock et al., 2003), appeared in a limited fashion in both rostral and caudal areas just adjacent to the injury site (data not shown). GDNF or MCP-1 immunoreactivity (-ir) was observed predominantly around the injury site and was colocalized with most of the Mac1/CD11b-positive cells (Fig. 3A–F). These results demonstrate that activated microglia/macrophages synthesized both GDNF and MCP-1 in the transected spinal cord. MCP-1-ir (Fig. 3G–I), but not GDNF-ir (data not shown), was colocalized with GFAP-ir in fibrous structures in the area over 5 mm distant from the injury site, implying the expression of MCP-1 in the processes of astrocytes, as reported previously (Che et al., 2001; Babcock et al., 2003). This finding may suggest that the physiological roles of GDNF are at least partially different from those of MCP-1, because glial production of MCP-1 was shown to be important for leukocyte responses to CNS axonal injury (Babcock et al., 2003). The MCP-1-ir-positive and GFAP-ir-negative cells were sparse in the same area distant from the injury site (Fig. 3I). They might resemble neurons morphologically, but they were likely to be activated microglia/

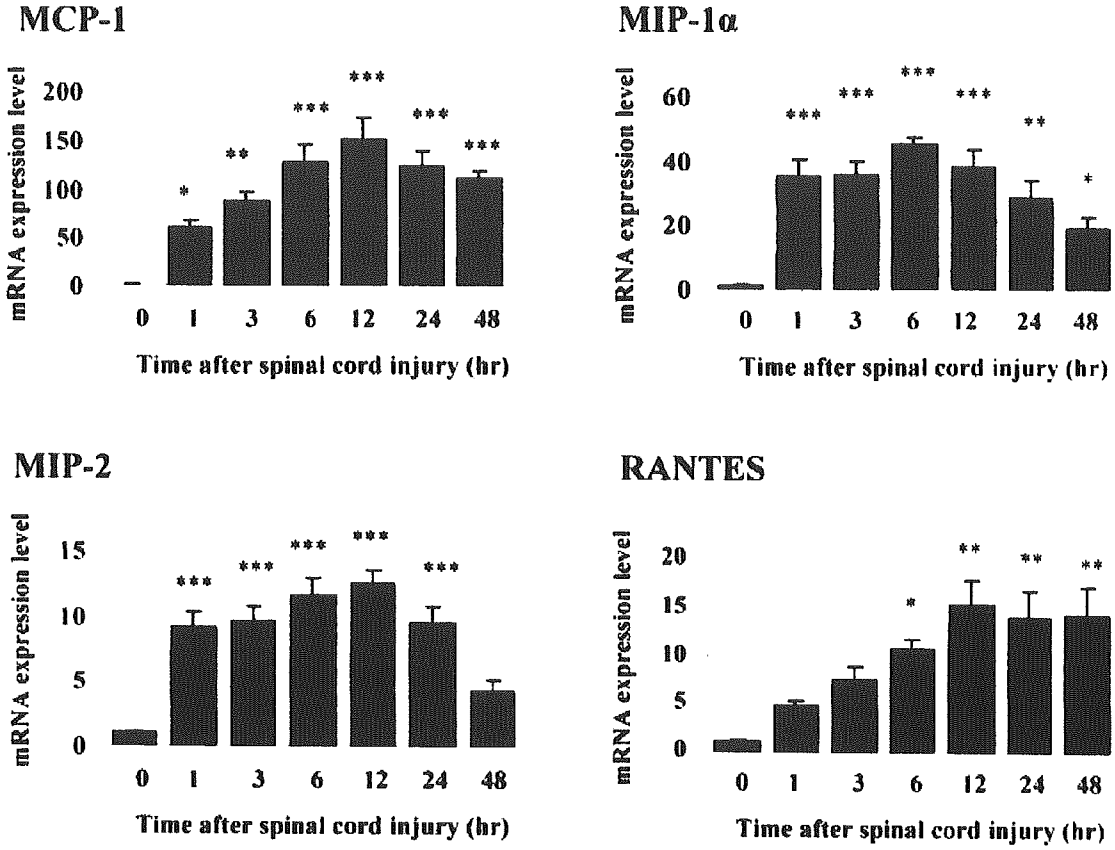

Fig. 2. Expression of chemokine mRNAs in the sites adjacent rostrally or caudally to the transected site of the rat spinal cord. The time-dependent mRNA expression of chemokines, including MCP-1, MIP-1α, MIP-2, and RANTES, after spinal cord transection was monitored by RT-PCR. The ratio of the intensity of chemokine cDNA products to that of β-actin cDNA products was calculated. The values are expressed as the means ± SE (n = 3) of -fold increase over the control group (0 hr). Significant differences from the value of the corresponding control group were determined by one-way ANOVA and Tukey test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

macrophages because of intensive MCP-1 expression, which is poor in neurons. Because MCP-1 expression has been shown to occur on injured neurons (Flugel et al., 2001), combined expression of MCP-1 and NeuN, an antigen specific for differentiated neurons, was examined in the areas just adjacent to the injury site. As demonstrated in Figure 3J–L, NeuN and MCP-1 were separately expressed, demonstrating that neurons in the injured spinal cord did not always express MCP-1.

Expression of Receptors for GDNF or MCP-1

To examine whether the activated microglia/macrophages can respond to GDNF, we analyzed by RT-PCR the mRNA expression of receptors for GDNF in macrophages cultured from the rat peritoneal cavity. In addition to the mRNA of MCP-1 receptor CCR2, that of GFRα-1, a glycosyl-phosphatidylinositol (GPI)-anchored binding site of GDNF, was detected, but not that of the tyrosine kinase c-Ret, which transduces the GDNF signal intracellularly (Fig. 4). Recent experiments showed that GDNF actions could be mediated by GFRα-1 in the absence of c-Ret (Paratcha et al., 2003). Therefore, the cultured macrophages used in this work hereinafter seem to respond to GDNF in an autocrine and/or local paracrine manner through GFRα-1 alone.

Effects of GDNF and MCP-1 on Phagocytosis Activity of Cultured Macrophages

Next, dose- and time-dependent effects of GDNF on the phagocytic activity of cultured rat peritoneal macrophages were examined (Fig. 5). Although relatively higher concentrations (100–1,000 ng/ml) and longer exposure times (over 12 hr) were necessary, GDNF significantly facilitated the phagocytosis of latex beads. On the other hand, MCP-1 was effective at a concentrarion as low as 10 ng/ml and showed potent activity as early as 1 hr after exposure. LPS caused a high level of phagocytic activity at 100 ng/ml. The GDNF activity was completely lost when the macrophages were pretreated with PI-PLC, which liberates GFRα-1, the GPI-anchored specific binding site of GDNF, demonstrating a specific and direct action of GDNF on macrophage phagocytosis via this

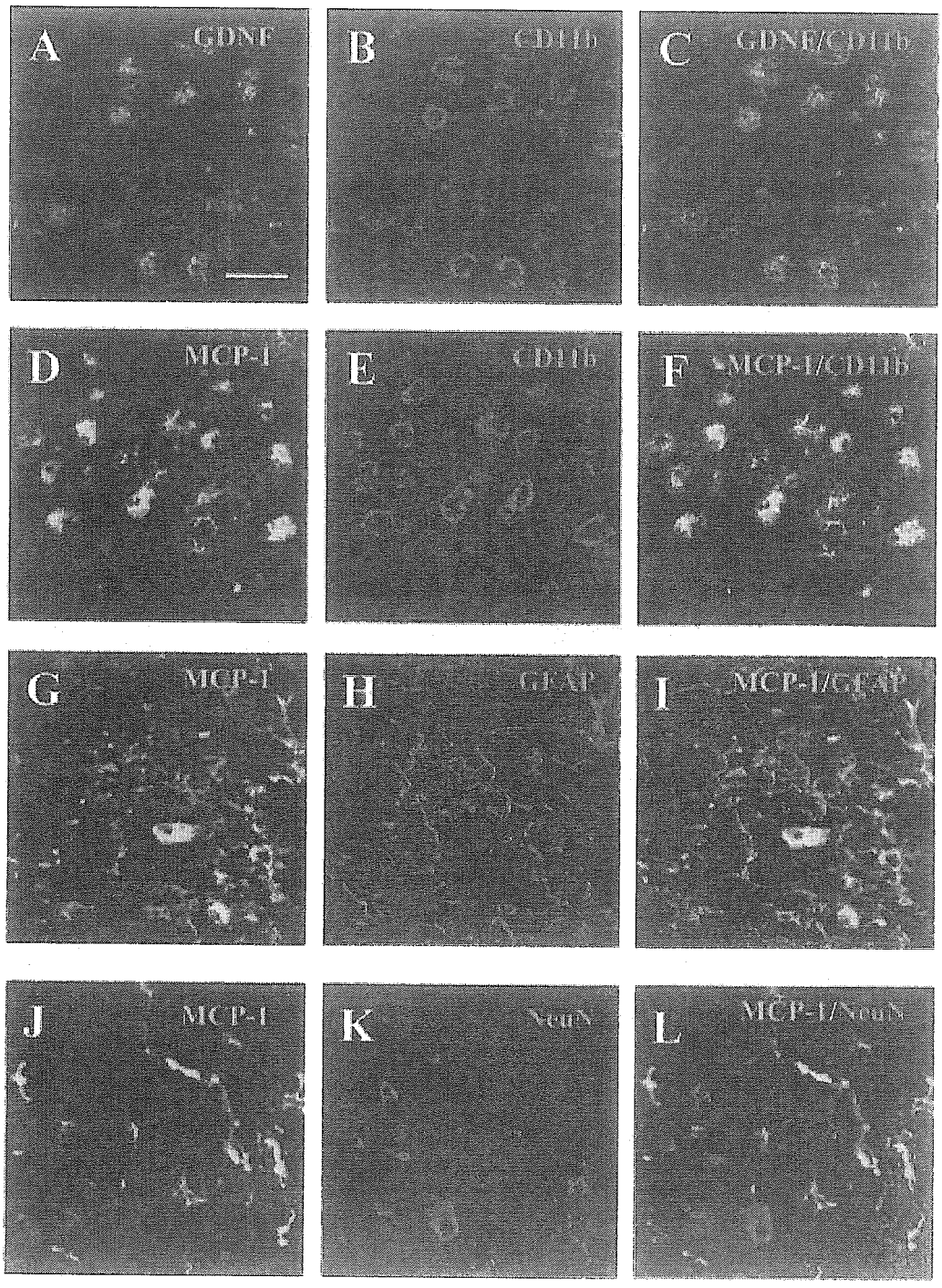

Fig. 3. Localization of GDNF- and MCP-1-ir in the injured rat spinal cord. Rats were anesthetized and cardioperfused 2 days after spinal cord transection. The spinal cord was dissected out, postfixed, and frozen in embedding compound. Coronal sections of 30 μm thickness were cut from the area 2 mm (**A–F**) or 5 mm (**G–L**) distant from the transection site of the spinal cord and used for immunostaining analysis. The sections were reacted with specific antibody against GDNF (A), MCP-1 (D,G,J), GFAP (H), NeuN(K) or CD11b (B,E) and visualized with FITC-conjugated anti-rabbit IgG (A,D,G,J) or rhodamine-conjugated anti-mouse IgG (B,E,H,K). A and B are merged in C, D and E in F,G and H in I, and J and K in L. Scale bar = 20 μm.

GPI-linked receptor component. On the other hand, MCP-1 was fully active, but LPS showed only half of its original activity toward the PI-PLC-treated macrophages. A part of the phagocytic activity elicited by LPS was shown to be mediated through GPI-anchored CD14 (Schiff et al., 1997). Therefore, the PI-PLC treatment liberated not only GFRα-1 but also CD-14 from the surface of the macrophages, resulting in the reduction of GDNF or LPS activity. Neither GDNF nor MCP-1, nor their combination, showed any mitotic activity on macrophages, as judged from the lack of increase in cell number (data not shown). These results demonstrate that GDNF has phagocytosis-stimulating activity, like MCP-1 and LPS, and suggest that GDNF behaves as a cytokine

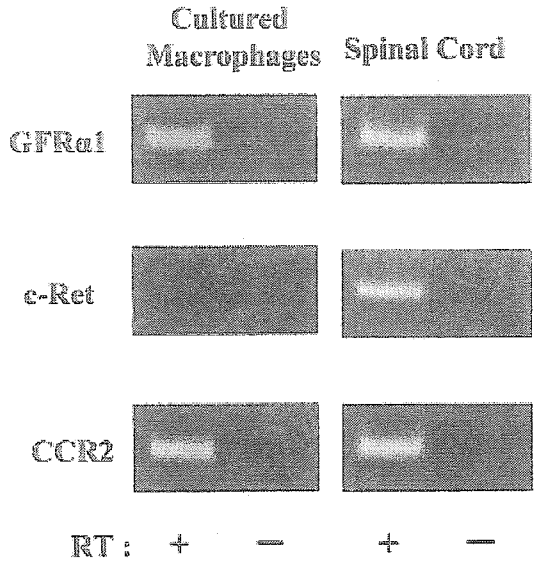

Fig. 4. Expression of GDNF and MCP-1 receptors in rat peritoneal macrophages. RT-PCR analysis detected mRNAs of GFRα-1 and CCR2 in total RNA prepared from cultured rat peritoneal macrophages but no c-Ret mRNA (RT^+). No signal was obtained when the RT reaction was conducted without reverse transcriptase (RT−).

that acts on microglia/macrophages in the injured spinal cord.

Effects of GDNF on mRNA Expression of MCP-1 in Cultured Macrophages

Next, the effects of GDNF on the mRNA expression of MCP-1 and its own mRNA were investigated. As shown in Figure 6, GDNF stimulated significantly the expression of MCP-1 mRNA at 100 and 1,000 ng/ml but did not induce any significant elevation of GDNF mRNA. MCP-1 markedly stimulated the expression of MCP-1 mRNA as low as 10 ng/ml, but that of GDNF mRNA only at higher concentrations 100 and 1,000 ng/ml. LPS stimulated both mRNAs at a concentration of 100 ng/ml. These results suggest that the phagocytosis-stimulating activity of GDNF is mediated by GDNF-induced MCP-1.

Activation of the Macrophages Cultured From GDNF Gene Mutants

To clarify the involvement of GDNF in the activation processes of macrophages, we tested the effects of agents that activate macrophages on the macrophages cultured from GDNF gene-deficient +/− or +/+ mice. Macrophages from homozygous mice (−/−) could not be used for this experiment, because the mice died immediately after birth. First, mRNA expression of GDNF, GFRα-1, and c-Ret gene in the cerebral cortex and cultured macrophages was analyzed by RT-PCR (Fig. 7A). GDNF, GFRα-1, and c-Ret mRNAs were expressed in the cerebral cortex of both mutant (+/−) and

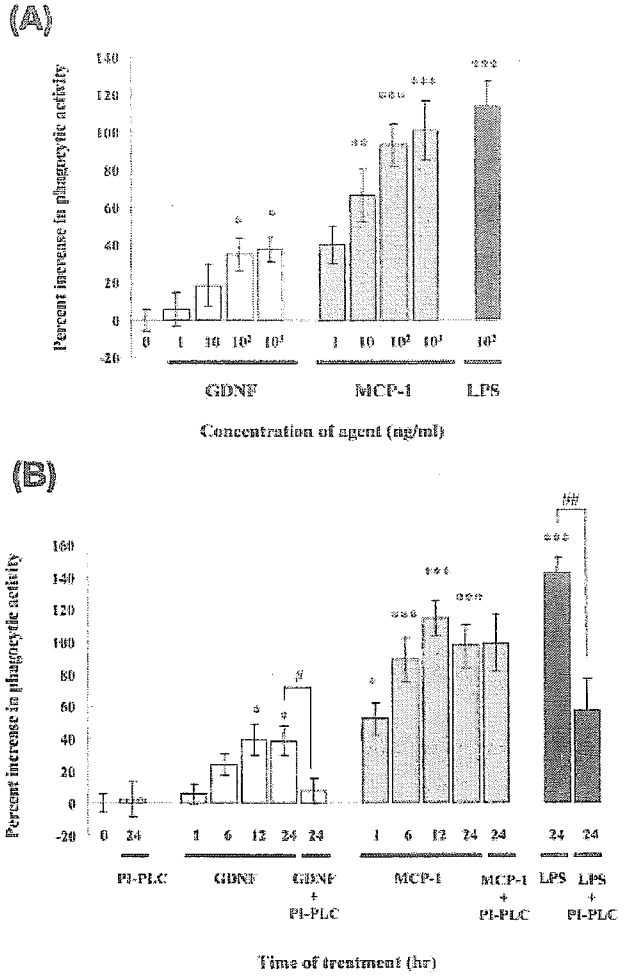

Fig. 5. Dose-dependent (**A**) and time-dependent (**B**) increase in phagocytic activity of cultured macrophages after exposure to GDNF, MCP-1, or LPS. Peritoneal macrophages were cultured in the presence of FITC-labeled latex beads after incubation with or without GDNF, MCP-1, or LPS at the indicated concentrations for 24 hr (**A**) or at 100 ng/ml for the indicated times (**B**). Some of the cells were pretreated with PI-PLC (0.5 U/ml) for 1 hr to liberate GPI-anchored binding sites, such as GFRα-1 for GDNF and CD14 for LPS. Phagocytic activity is expressed as percentage increase in the number of macrophages bearing FITC-labeled beads/total number of macrophages (mean ± SE, n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the value of the control group (0 hr, without any reagents; one-way ANOVA and Tukey test); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the value of the group treated with PI-PLC (Student's *t*-test).

+/+ littermates. However, c-Ret mRNA was not expressed in the macrophages of either genetic type, consistent with the data on cultured rat macrophages shown in Figure 4. The expression level of GDNF mRNA was always lower in the +/− than in the +/+ mice before and after the stimulation with MCP-1 or LPS (Fig. 7A,Ba). The level of GFRα-1 mRNA tended to be lower in the +/− than in the +/+ mice in both cerebral cortex and

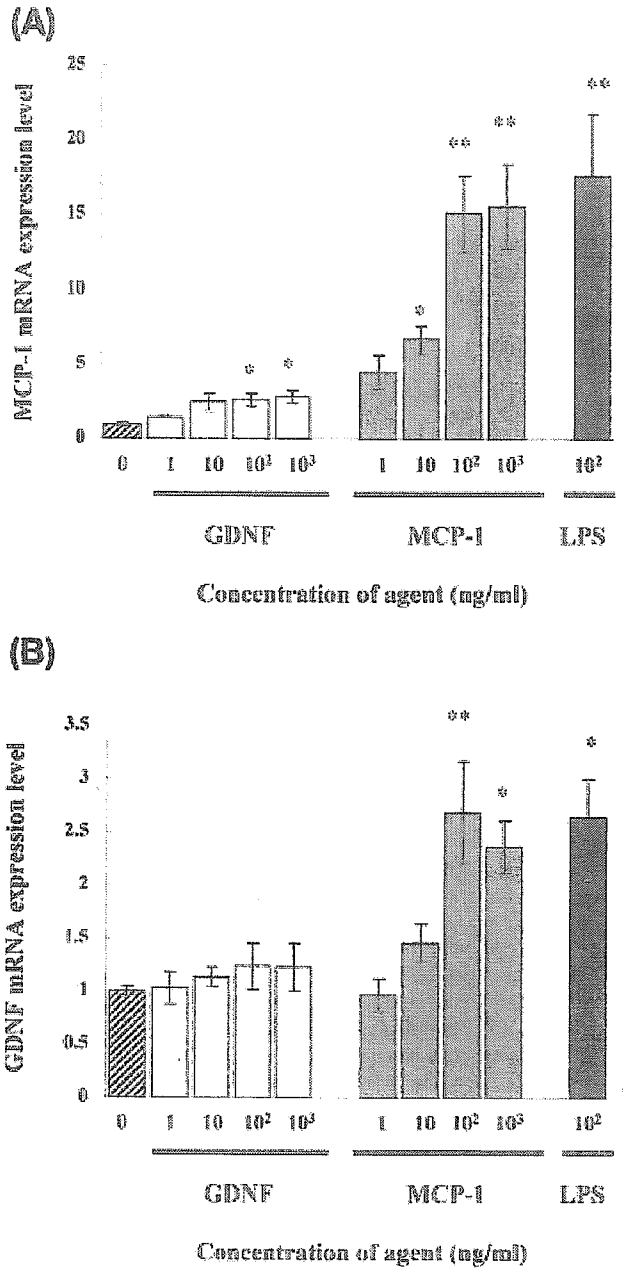

Fig. 6. Dose-dependent effects of GDNF, MCP-1, and LPS on the mRNA expression of MCP-1 (A) or GDNF (B) of cultured rat peritoneal macrophages. Macrophages were cultured with or without MCP-1, GDNF, or LPS at various concentrations for 24 hr, and then total RNA of the macrophages was prepared and subjected to RT-PCR with primers specific for MCP-1 (A) or GDNF (B). The ratio of the intensity of cDNA products to that of β-actin cDNA products was calculated. The values are expressed as mean ± SE (n = 3) of -fold increase over the control group (0 ng/ml). Significant differences from the value of the control group (0 ng/ml) were determined by one-way ANOVA and Tukey post hoc test. $*P < 0.05$, $**P < 0.01$.

macrophages (Fig. 7A), but the difference was not statistically significant (Fig. 7Bb). MCP-1 mRNA expression was markedly facilitated by the stimulation with MCP-1 or LPS but was the same for both genetic types (Fig. 7Bc).

There were no morphological differences in the macrophages with respect to the genetic type or stimulant (Fig. 8A). The phagocytosis activity of the +/+ macrophages was significantly enhanced by the treatment with GDNF, MCP-1, or LPS or with MCP-1 or LPS but not with GDNF, in the +/− cells (Fig. 8B). However, the value of the phagocytosis activity induced was significantly lower in the +/− than in the +/+ macrophages when the cells were treated with GDNF, MCP-1, or LPS. The data thus indicate that the GDNF signal is important and is involved in phagocytosis-stimulating processes, i.e., activation processes of macrophages.

DISCUSSION

Our present study was aimed at addressing the physiological significance of GDNF produced by macrophages in the injured CNS, and the results suggest that GDNF plays a role as a cytokine to activate microglia/macrophages in an autocrine/paracrine manner. Our observation that mRNAs of GDNF, NGF, BDNF, and all chemokines examined were up-regulated around the injury site of the rat spinal cord is essentially consistent with previous findings (McTigue et al., 1998; Lee et al., 2000; Satake et al., 2000; Widenfalk et al., 2001). However, detailed expression profiles of these factors had not been studied before. The mRNA expression profile of GDNF resembled very much that of NGF or various chemokines, including MCP-1 (Figs. 1, 2). Indeed, NGF is a well-known cytokine that stimulates phagocytosis and chemotaxis of macrophages (Susaki et al., 1996; Kobayashi and Mizisin, 2001). These findings prompted us to examine whether GDNF plays an inflammatory role following spinal cord injury. We focused on GDNF and compared its biological actions particularly on cultured macrophages with those of MCP-1, because GDNF and MCP-1 were coexpressed in microglia/macrophages around the transected site of the spinal cord (Fig. 3). Furthermore, Batchelor et al. (1999) reported that the synthesis of GDNF was increased in macrophages around the site of striatal injury, demonstrating a possibility that GDNF participates as a cytokine or chemokine to induce inflammatory reactions following spinal cord injury.

We initially examined the expression of the receptors of GDNF or MCP-1 in rat peritoneal macrophages, and we detected GFRα-1 mRNA, but not c-Ret mRNA (Figs. 4, 7). Generally, GDNF first binds to GFRα-1, and this complex then activates Ras/ERK and phosphatidylinositol 3-kinase/Akt pathways through the tyrosine kinase receptor c-Ret (Jing et al., 1996; Trupp et al., 1999). However, GDNF can also signal through activation of Src family kinase via GFRα-1 in the absence of c-Ret (Trupp et al., 1999; Pezeshki et al., 2001). In fact, exogenous GDNF enhanced phagocytic activity of the cultured macrophages via GFRα-1, a GPI-anchored GDNF binding protein (Fig. 5), demonstrating that GDNF could directly

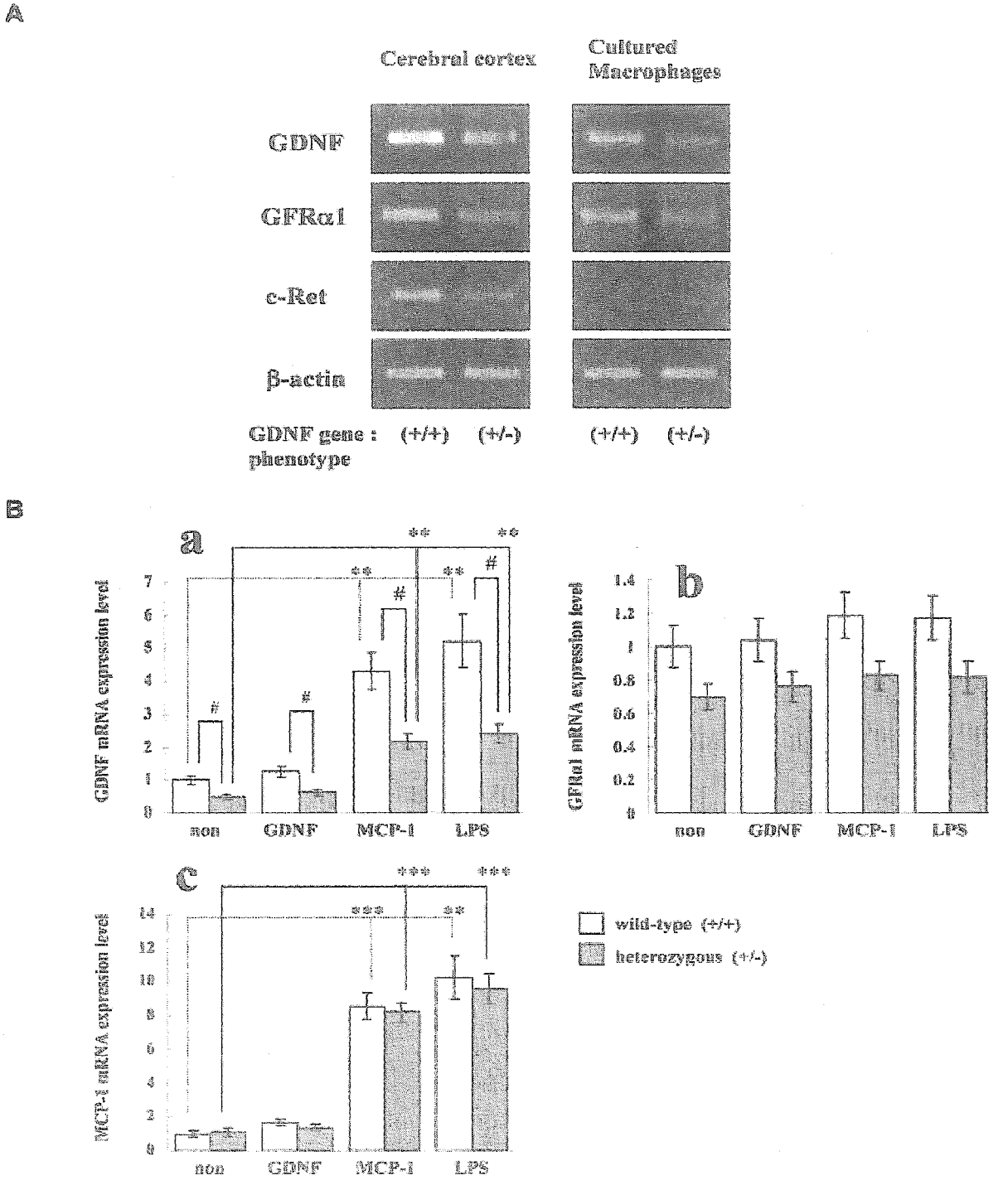

Fig. 7. Effects of GDNF, MCP-1, and LPS on mRNA expression of GDNF and its receptors, GFRα-1 and c-Ret, in the macrophages cultured from wild-type mice or GDNF gene mutants. **A:** mRNA expression of GDNF and of its receptors, GFRα-1 and c-Ret, in the cerebral cortex and cultured macrophages of GDNF gene-deficient heterozygous mice (+/−) and wild-type mice (+/+) was examined by RT-PCR. The macrophages were cultured with or without GDNF, MCP-1, or LPS (100 ng/ml) for 24 hr, after which total RNA of the cells was prepared and subjected to RT-PCR with primers specific for GDNF, GFRα-1, or MCP-1. **B:** The ratio of the intensity of cDNA products to that of the β-actin cDNA product was calculated. The values are expressed as mean ± SE (n = 3) of -fold increase over the nontreatment group of +/+ macrophages. Statistical significance was assessed using one-way ANOVA followed by Tukey post hoc test. Significance of differences as indicated by the brackets: $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$.

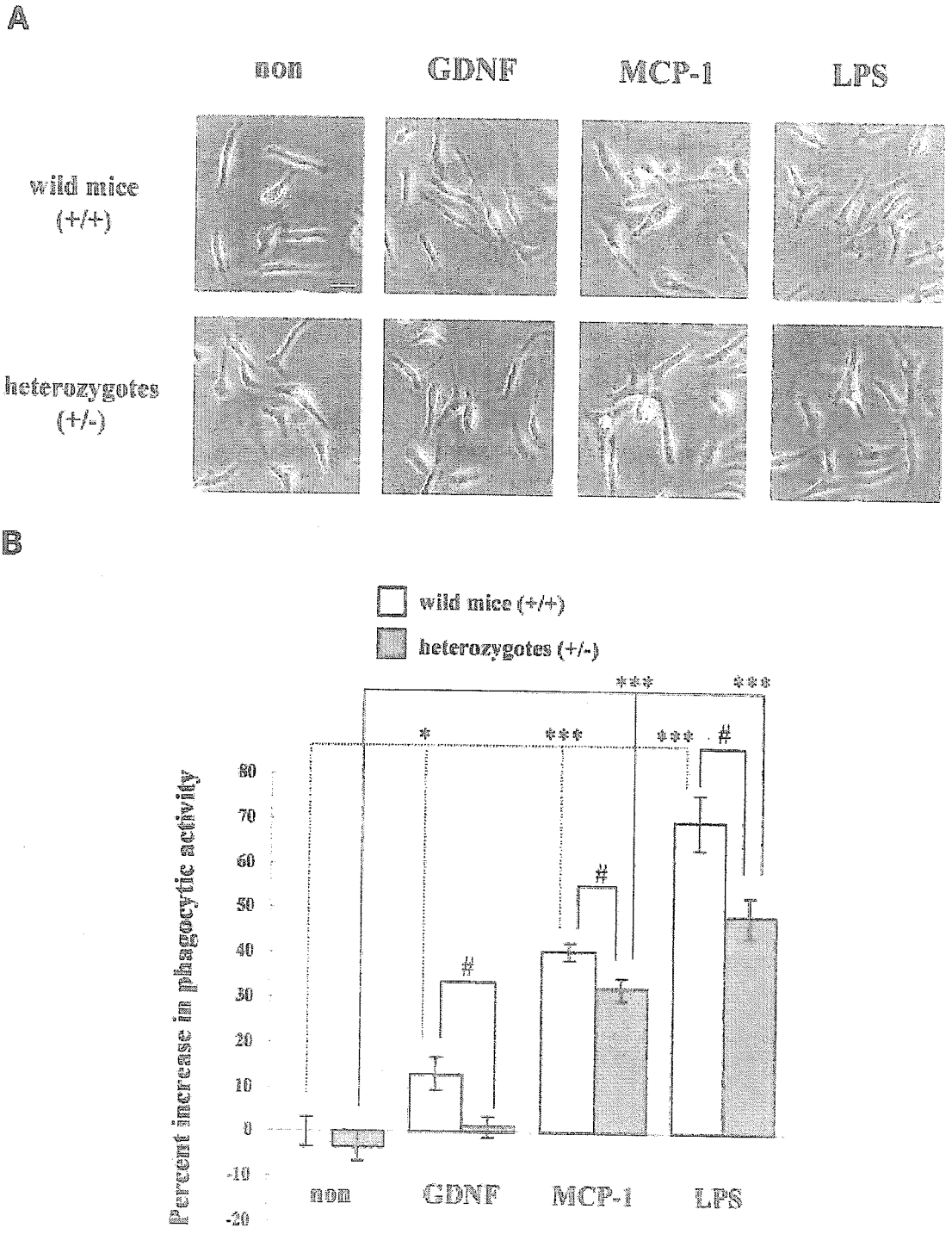

Fig. 8. Effects of GDNF, MCP-1, and LPS on the morphology (**A**) and phagocytic activity (**B**) of the macrophages cultured from wild-type mice or GDNF gene mutants. A: Macrophages from wild-type mice (+/+) or GDNF gene-deficient heterozygous mice (+/−) were cultured for 24 hr in medium supplemented or not with GDNF, MCP-1, or LPS (100 ng/ml), and then photographs of them were taken under a phase-contrast microscope. B: The +/+ or +/− macrophages were cultured for 30 min in medium containing FITC-labeled latex beads after incubation with or without 100 ng/ml of GDNF, MCP-1, or LPS for 24 hr. FITC-positive cells (phagocytosing macrophages) were counted under a fluorescence microscope, and the percentage of positive cells per total cells was calculated. The values are expressed as mean ± SE (n = 4) of the percentage increase over the control group (non). Statistical significance was assessed using one-way ANOVA followed by Tukey post hoc test. Significance of differences as indicated by the brackets: $^{*}P < 0.05$, $^{***}P < 0.001$, $^{\#}P < 0.05$. Scale bar = 20 μm.