Pierce and Kalivas, 1997; Robinson and Becker, 1986). The activation of dopamine transmission in the nucleus accumbens (NAc) is implicated in mediating acute reinforcing effects of drugs of abuse (Everitt and Wolf, 2002; Robinson and Becker, 1986). Molecular mechanisms underlying long-term behavioural sensitization and tolerance remain poorly understood, although neural activity-dependent gene expression in the brain is implicated in such processes (Nestler, 2001; Norrholm et al., 2003; Toda et al., 2002).

Proteoglycans are components of the extracellular matrix that include glycosaminoglycans covalently attached to a core protein as side-chains (Iozzo, 1998). Multiple proteoglycan species with different structural features are expressed in a regulated manner during the development of central nervous tissues (Margolis et al., 1995; Oohira et al., 1994). There is much evidence that proteoglycans are involved in axonal outgrowth, synaptogenesis, and neuronal cell differentiation (Friedlander et al., 1994; Garwood et al., 1999; Small et al., 1996).

Neuroglycan C (NGC), a central nervous tissue-specific transmembrane chondroitin sulphate proteoglycan (CSPG), is expressed in the developing rat brain (Watanabe et al., 1995). This membrane-bound CSPG is present also in the cerebrum of various vertebrates, including humans, and is evolutionally conserved, indicating that NGC may be essential to nervous tissue development and maintenance (Yasuda et al., 1998). Although the exact function of NGC is unknown, an immunohistochemical study showed that NGC is expressed in Purkinje cells in the developing mouse cerebellum and present on thick dendrites on which the climbing fibres form synapses and not on the thin branches on which the parallel fibres form synapses, indicating that NGC may be involved in the formation of the neural network (Aono et al., 2000; Inatani et al., 2000). Interestingly, repeated exposure to cocaine or amphetamine increases the number of dendritic branch points and spines both of medium spiny neurons in the NAc and of pyramidal neurons in the medial frontal cortex both of which receive dopaminergic inputs (Robinson and Kolb, 1997, 1999). In contrast, chronic exposure to opiates decreases the size and calibre of dendrites and some dopamine neurons in the ventral tegmental area (VTA) (Sklair-Tavron et al., 1996).

Given its possible role in neural network formation, NGC could be involved in drug-induced neuroadaptation. In the present study, we investigated the effects of acute and chronic administration of MAP or morphine on NGC expression in various regions of the brain in rats using a real-time RT–PCR and immunoblotting methods.

Methods

Animals

Male Wistar rats (7 wk old; Charles River Japan, Yokohama, Japan) weighing 230 ± 10 g at the beginning of experiments were used in the study. They were housed three per cage with free access to food and water under controlled laboratory conditions (a 12 h light/dark cycle, lights on at 09:00 hours, 23 ± 0.5 °C, 50 ± 0.5% humidity). All animal care and use were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Nagoya University.

Drug treatment

MAP (Dainippon Pharmaceutical Co., Osaka, Japan), morphine (Shionogi Pharmaceutical Co., Osaka, Japan), naloxone, SCH-23390 and raclopride (Sigma, St. Louis, MO, USA) were used in this study. The drugs were dissolved in 0.9% saline. For acute experiments, rats were given a single subcutaneous injection of MAP (2 mg/kg) or morphine (10 mg/kg). Naloxone (5 mg/kg) was administered intraperitoneally 15 min before morphine. For sensitization experiments, rats were injected subcutaneously with 2 mg/kg of MAP once a day for 5 d. SCH-23390 (0.1 mg/kg) or raclopride (2 mg/kg) was administered intraperitoneally with MAP. For tolerance experiments, rats were subjected to a 5-d regimen in which increasing doses of morphine (10, 20, 30, 40 and 50 mg/kg) were injected subcutaneously twice a day (09:00 and 21:00 hours) and after that, rats were challenged with 10 mg/kg of morphine on day 6 (09:00 hours). Control rats were given the same volume of vehicle following the same schedule as used for the acute and chronic administration of drugs.

Measurement of locomotor activity

Locomotor activity was measured to assess motor function. Locomotor activity was measured for 60 min after drug administration on days 1, 3, and 5 using digital counters with infrared sensors (Scanet SV-10; Tokyo Sangyo, Toyama, Japan).

Antinociceptive test

The antinociceptive effect of morphine was determined by the hot-plate test (Mamiya et al., 1998). The

hot-plate latency represents the amount of time until the rats licked their hind paws and/or jumped after being placed on the hot-plate (55 ± 1 °C). The test was conducted 1 h after the injection of morphine.

Real-time RT–PCR

Rats were killed by decapitation 2 h after the last injection, and brains were quickly removed and placed on an ice-cold glass plate. Various regions (frontal cortex, NAc, striatum, hippocampus, VTA, amygdala) were rapidly dissected out, frozen, and stored in a deep freezer at −80 °C until assayed. Total RNA was extracted from brain tissues by a previously described method (Chamberlain and Burgoyne, 1996). The levels of NGC mRNA in brain tissues were determined by real-time RT-PCR using the TaqMan probe (Eder et al., 1999). The 18S ribosomal RNA kit (Applied Biosystems) was used as the internal control. Total RNA (1 μg) was converted into cDNA using the SuperScript™ First-Strand Synthesis System for RT–PCR kit (Life Technologies, Tokyo, Japan) in a total volume of 1/21 μl (RT reaction mixture). PCR was performed using 1/21 of the RT reaction mixture, 0.8 μM of each (forward and reverse) primer, 0.25 μM of TaqMan probe and 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) in a total reaction volume of 25 μl. The primers used were 5′-AGGGCTCCCATCCAAATGAT-3′ (forward) and 5′-CGAGTTTGGGTGACATGGAGTT-3′ (reverse), and the TaqMan probe was 5′-AAATCCAGGAAGCTCTCAAGTCCCGC-3′. The experimental amplification protocol consisted of an initial step (50 °C for 2 min and 95 °C for 10 min) and then 40 cycles of denaturation for 15 s at 95 °C, and annealing/extension for 1 min at 60 °C on the ABI PRISM 7700 sequencer detector. Signal was detected according to the manufacturer's instructions.

Immunoblot analysis

Rat brain tissues were homogenized in 50 μl of boiling lysis buffer (1% SDS, 1.0 mM sodium ortho-vanadate, and 10 mM Tris–HCl; pH 7.4) and microwaved for 15 s according to the protocol used for the immunoblotting with monoclonal antibodies (BD Transduction Laboratories, USA) (Linda et al., 2001). The homogenate was centrifuged (16 000 *g*, 15 °C) for 5 min to pellet the insoluble material. The protein concentration was determined using a Protein Assay Rapid kit (Wako, Osaka, Japan). The sample (protein concentration: 100 μg) was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 3% stacking gel and a 7.5% separating gel, and then transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was incubated in the blocking solution for 2 h at room temperature, and then with the mouse anti-NGC antibody (BD Transduction Laboratories). After washing, blots were incubated with goat anti-mouse IgG horseradish peroxidase-linked secondary antibody (Kirkegaard & Perry Laboratories, Inc., KPL, USA). Immunoreactive materials on the membrane were detected using the ECL Western blotting detection reagents (Amersham Pharmacia Biotech). The membranes were stripped and reprobed with goat anti-actin antibody (Santa Cruz Biotechnology Inc., Santa Crutz, CA, USA) and reacted with donkey anti-goat IgG antibody horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology Inc). The immunoreactive materials on the membrane were detected as described above. Bands were scanned using a computer-associated image analysis system (Cool Saver, Atto, Japan). Immunoblot values were corrected using their corresponding actin levels, and expressed as fold increases from the vehicle-treated control.

Statistical analysis

All data were expressed as the means ± S.E.M. In the analysis of locomotion, statistical differences between groups were determined by one-way analysis of variance (ANOVA) with repeated measures. The hot-plate latency and biochemical data were analysed by ANOVA, followed by Bonferroni's test when F ratios were significant ($p < 0.05$).

Results

Sensitization of MAP-induced hyperactivity and tolerance to morphine-induced analgesic effect in rats

As shown in Figure 1a, acute MAP-treatment significantly increased locomotor activity on day 1. After repeated administration of MAP for 5 d, MAP-induced hyperlocomotion was significantly potentiated compared with that on day 1 ($p < 0.05$), and chronic MAP treatment resulted in behavioural sensitization. Repeated vehicle-treated rats did not show any changes in spontaneous locomotor activity. As shown in Figure 1b, acute morphine administration significantly increased hot-plate latency compared to treatment with the vehicle alone ($p < 0.05$). Chronic morphine administration significantly decreased hot-plate latency compared to acute morphine treatment ($p < 0.05$), indicating the development of tolerance. Furthermore, it

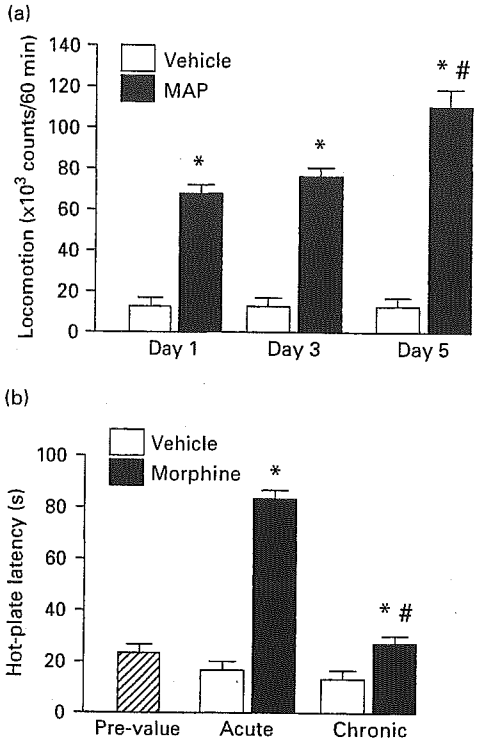

Figure 1. Sensitization of MAP-induced hyperactivity (a) and tolerance to morphine-induced analgesic effect (b) in rats. Rats were administered MAP (2 mg/kg) repeatedly once a day for 5 d. Locomotor activity was measured for 1 h after MAP treatment. Analgesic effect was evaluated in a hot-plate test 1 h after morphine (10 mg/kg) administration. In the chronic morphine-treated group, rats were subjected to a 5-d regimen in which increasingly higher doses of morphine (10, 20, 30, 40, and 50 mg/kg) were injected twice a day, and then challenged with morphine (10 mg/kg) 12 h after the last injection. Values are means ± S.E.M. ($n=6$). * $p<0.05$ vs. corresponding vehicle-treated groups; # $p<0.05$ vs. MAP-treated group on day 1 (a) or acute morphine-treated group (b).

also simultaneously induced locomotor sensitization (data not shown).

MAP- or morphine-induced NGC mRNA expression in the rat brain

We examined NGC gene expression in various areas of the rat brain after acute and chronic drug treatment using real-time RT–PCR. As shown in Figure 2a, acute MAP administration significantly decreased the levels of NGC mRNA in the frontal cortex, VTA, and amygdala, but had no effect in other areas, including the NAc, striatum, and hippocampus. Chronic MAP administration, which induced locomotor sensitization, significantly increased levels of NGC mRNA in the NAc, striatum, and hippocampus compared with levels in the vehicle-treated group. In addition to these areas, NGC mRNA levels in the frontal cortex, VTA, and amygdala of chronic MAP-treated rats increased significantly compared with those in the acute MAP-treated group. There was no difference in the levels of NGC mRNA between acute and chronic vehicle-treated groups (data not shown), and therefore, the results were combined to represent a single vehicle-treated group. Chronic MAP administration (1.0 and 2.0 mg/kg) induced NGC mRNA expression in the NAc with a bell-shaped dose-dependent curve, but not at 4.0 mg/kg (Figure 2b). The NGC gene expression induced by chronic MAP administration was antagonized by pretreatment with SCH-23390 (D_1 receptor antagonist) (Anderson and Haaren, 2000) or raclopride (D_2 receptor antagonist) (Anderson and Haarren, 2000) which itself did not have any effect on NGC mRNA in the NAc (Figure 2c) and significantly decreased MAP-induced hyperlocomotion compared with vehicle-treated rats (data not shown). As shown in Figure 2d, acute morphine administration significantly increased levels of NGC mRNA in the NAc, striatum, hippocampus, VTA, and amygdala. Chronic morphine administration, which induced tolerance, significantly decreased levels of NGC mRNA in the NAc, striatum, VTA, and amygdala compared with the levels in the acute morphine-treated group. There was no difference in the level of NGC mRNA between acute and chronic vehicle-treated groups (data not shown), and therefore, the results were combined to represent a single vehicle-treated group. The NGC gene expression induced by acute morphine administration was antagonized by treatment with naloxone (Mamiya et al., 2001; Nagai et al., 2004) which itself did not have any effect on NGC mRNA (Figure 2e).

NGC is produced in the NAc after MAP or morphine administration

We examined the changes in NGC protein levels in the NAc of rats after acute and chronic MAP or morphine treatment. NGC-immunopositive bands were detectable by immunoblot analysis. As shown in Figure 3, there was no significant alteration of NGC protein levels, as measured by the intensity of the 120–150 kDa immunopositive band, between the acute MAP-treated and vehicle-treated groups. In contrast, chronic MAP administration increased by 46% the intensity of the band over the control levels. Acute morphine administration increased by 32% the intensity of the band over the vehicle levels. In the chronic

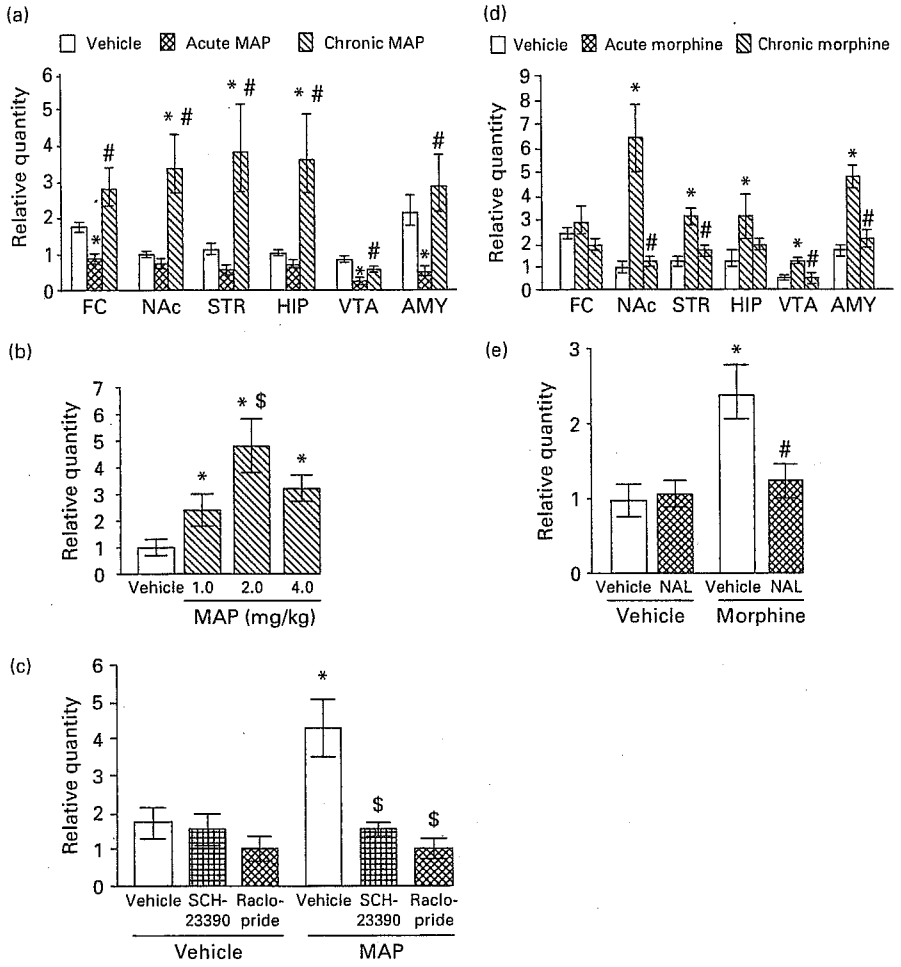

Figure 2. NGC mRNA levels in various brain regions after acute and chronic MAP (a) or morphine (d) treatment, at different doses of MAP (b), and in combination with dopamine (c) and opioid (e) antagonists in the NAc. The acute MAP-treated group was killed 2 h after a single MAP (2 mg/kg) treatment while the chronic MAP-treated group was killed 2 h after the 5th injection of MAP. The acute morphine-treated group was killed 2 h after a single morphine (10 mg/kg) treatment while the chronic morphine-treated group was killed 2 h after the 5-d injection of morphine. Rats were administered SCH-23390 (0.1 mg/kg) or raclopride (2 mg/kg) intraperitoneally with MAP. Naloxone (NAL) (5 mg/kg) was administered intraperitoneally 15 min before morphine. * $p<0.05$ vs. vehicle-treated group; # $p<0.05$ vs. acute MAP- or morphine-treated group; \$ $p<0.05$ vs. chronic MAP (1.0 mg/kg) (b) or chronic MAP (2.0 mg/kg) (c) treatment. Each value represents the mean ± S.E.M. ($n=7$). FC, frontal cortex; NAc, nucleus accumbens; STR, striatum; HIP, hippocampus; VTA, ventral tegmental area; AMY, amygdala.

morphine-treated groups, no changes were observed in the relative abundance of NGC.

Discussion

Repeated exposure to a psychostimulant drug such as MAP results in many neurobehavioural adaptations that outlast the acute effects of the drug. We have utilized cDNA microarray analysis to determine key factors of drug dependence, since this approach has been used successfully to identify the possible role of gene expression in the molecular events induced by drug addiction (Bibb et al., 2001). The gene for NGC was isolated as one of the unique genes associated with neural adaptation to chronic MAP or morphine administration. We demonstrated in the present study that chronic MAP administration induced the expression of locomotor sensitization and significantly increased NGC mRNA levels in the frontal cortex, NAc, striatum, hippocampus, VTA and amygdala

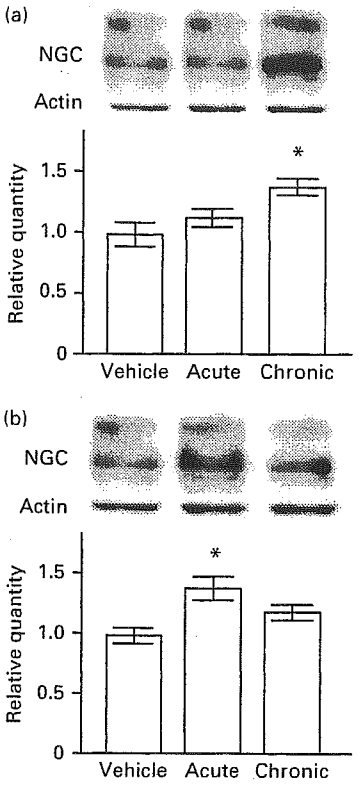

Figure 3. Immunoblot analysis of NGC protein levels in the nucleus accumbens after MAP (a) or morphine (b) administration. Rats were killed 2 h after acute or chronic administration of MAP and morphine respectively. NGC-immunopositive bands (100–200 kDa) were detectable by immunoblot analysis. The relative abundance of the immunoblots normalized to actin was determined by a computer-associated image analysis system. * $p<0.05$ vs. vehicle-treated group. Each value represents the mean ± S.E.M. ($n=7$).

compared with those in acute MAP-treated rats. Furthermore, chronic morphine administration induced tolerance to the antinociceptive effect of morphine and locomotor sensitization, and significantly decreased NGC mRNA levels in the NAc, striatum, VTA, and amygdala, compared with levels in the acute morphine-treated group. Pharmacological studies with receptor antagonists have revealed that the effects of MAP and morphine on NGC mRNA levels are mediated by dopamine and opioid receptors respectively. Furthermore, NGC protein levels in the NAc were significantly increased in chronic MAP-treated rats, indicating that chronic MAP administration modulates NGC expression at the transcriptional level. In contrast, acute morphine treatment increased NGC protein levels, but the effect was markedly attenuated after chronic treatment. Taken together, it is likely that the sensitization to MAP is associated with an up-regulation of NGC gene expression, while the tolerance to the morphine-induced analgesic effect and sensitization is associated with a down-regulation of NGC gene expression. It is suggested that the effect of MAP on NGC expression may contribute to locomotor sensitization via influencing dopamine release in the NAc. Opioid receptors are ubiquitously present in excitatory synapses. Both endogenous and exogenous opioids can modulate the morphology and function of dendritic spines. Endogenous opioids may maintain the normal density of dendritic spines by preventing abnormal overgrowth of dendritic spines, whereas exogenous opiates cause such spines to collapse (Liao et al., 2005). The latter might have a role in the pathological basis for drug addiction. In addition to acting on μ-opioid receptors in GABAergic synapses, opioids may act directly on μ-opioid receptors in glutamatergic synapses as shown in Liao et al. (2005). The interaction between these two distinct mechanisms is not yet known. One possibility is that the acute effect of morphine is mediated mainly through changes in GABAergic synapses, whereas the chronic effect is mediated through changes in glutamatergic synapses (Liao et al., 2005). An increased p42/44 mitogen-activated protein kinase activation in the posterior basolateral amygdala (BLA) represents a selective, sensitized signalling response to morphine that parallels the locomotor sensitization, but not analgesic tolerance (Eitan et al., 2003). This could reflect an increase in excitatory transmission in the NAc that contributes to this behaviour, because excitatory projections from the BLA synapse on dopaminergic neurons of the NAc (Johnson et al., 1994). Recent work shows that tetanic stimulation of the BLA results in potentiation of evoked firing in the NAc (Floresco et al., 2001). Moreover, high-frequency stimulation of the BLA, but not the central amygdala, produces a long-lasting glutamate receptor-dependent increase in dopamine efflux into the NAc (Howland et al., 2002). Together, this suggests that the effect of morphine on the BLA may contribute to locomotor sensitization via influencing dopamine release in the NAc. Thus, brain circuitry and biochemical changes may contribute specifically to each behaviour induced by chronic MAP or morphine administration.

Molecular mechanisms of NGC gene expression and regulation of NGC protein levels in the brain, however, remain to be determined. NGC may play a role in selective synaptogenesis as a receptor for an unidentified ligand-like molecule (Aono et al.,

2000). A recent study demonstrated a novel function for NGC as a growth factor that binds directly to the ErbB3 tyrosine kinase classified as the sixth member (neuregulin 6) of the neuregulin family (Kinugasa et al., 2004). It has been speculated that the change of NGC gene expression in the corticolimbic circuitry contributes to chronic cocaine-induced behavioural changes (Toda et al., 2002). Chronic treatment with psychostimulants such as cocaine and amphetamine produces different effects on neuronal morphology and synaptic connectivity in the mesolimbic neuronal system as does morphine. Repeated exposure to amphetamine produces an increase in the dendritic surface and in the number of dendritic spines on medium spiny neurons located in the shell and core subregions of the NAc (Robinson and Kolb, 1997, 1999) while chronic morphine treatment decreases the size of dendrites and some VTA dopamine neurons (Sklair-Tavron et al., 1996). It is hypothesized that the alterations of synaptic connectivity in these structures represent the neural basis for long-term behavioural consequences of the repeated use of addictive drugs, including amphetamine psychosis and addiction, and the dysphoria of drug withdrawal (Nestler, 2001). Accordingly, various molecules that regulate synaptic structure and connectivity might play a crucial role in drug addiction. Examples are neurotrophic factors, including brain-derived neurotrophic factor (BDNF) (Horger et al., 1999) neurotrophin-3 (Adamson et al., 2002), and glial cell line-derived neurotrophic factor (Fine et al., 2002). BDNF for instance, enhances locomotor activity and conditioned reward in response to cocaine. We also found that tumour necrosis factor-α plays a neuroprotective role in MAP-induced drug dependence and neurotoxicity (Nakajima et al., 2004) and the tissue plasminogen activator–plasmin protease system participated in the rewarding effects of morphine and MAP in the NAc (Nagai et al., 2004, 2005).

We suggest that NGC is one of the molecules that contributes to the long-lasting neural and behavioural plasticity associated with drug addiction. Molecular information on drug addiction might aid in elucidating the precise nature of such a stable neural and behavioural plasticity. Our findings provide an association between drug treatment/gene expression/behaviour-induced psychostimulants. Further studies on the NGC gene product are in progress.

Acknowledgements

This study was supported in part by Special Coordination Funds for Promoting Science and Technology, Target-Oriented Brain Science Research Programme from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant-in-Aid for Health Sciences Research from the Ministry of Health, Labour and Welfare of Japan. Also acknowledged are grants from the Smoking Research Foundation.

Statement of Interest

None.

References

Adamson CL, Reid MA, Davis RL (2002). Opposite actions of brain-derived neurotrophic factor and neurotrophin-3 on firing features and ion channel composition of murine spiral ganglion neurons. *Journal of Neuroscience 22*, 1385–1396.

Anderson KG, Haaren F (2000). Effects of SCH-23390 and raclopride on cocaine discrimination in male and female Wistar rats. *Pharmacology, Biochemistry and Behavior 65*, 671–675.

Aono S, Keino H, Ono T, Yasuda Y, Tokita Y, Matsui F, Taniguchi M, Sonta S, Oohira A (2000). Genomic organization and expression pattern of mouse neuroglycan C in the cerebellar develpoment. *Journal of Biological Chemistry 275*, 337–342.

Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Snyder GL, Yan Z, Segawa ZK, Ouimet CC, Naim AC, Nestler EJ, Greengard P (2001). Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature 410*, 376–380.

Chamberlain LH, Burgoyne RD (1996). Identification of a novel cysteine string protein variant and expression of cysteine string proteins in non-neuronal cells. *Journal of Biological Chemistry 271*, 7320–7323.

Eder M, Battmer K, Kafert S, Stucki A, Ganser A, Hertenstein B (1999). Monitoring of BCR-ABL expression using real-time RT-PCR in CML after bone marrow or peripheral blood stem cell transplantation. *Leukemia 13*, 1383–1389.

Eitan S, Bryant CD, Saliminejad N, Yang YC, Vojdani E, Keith Jr. D, Polakiewicz R, Evans CJ (2003). Brain region-specific mechanisms for acute morphine-induced mitogen-activated protein kinase modulation and distinct patterns of activation during analgesic tolerance and locomotor sensitization. *Journal of Neuroscience 23*, 8360–8369.

Everitt BJ, Wolf ME (2002). Psychomotor stimulant addiction: a neural systems perspective. *Journal of Neuroscience 22*, 3312–3320.

Fine EG, Decosterd I, Papaloizos M, Zurn AD, Aebischer P (2002). GDNF and NGF released by synthetic guidance channels support sciatic nerve regeneration across a long gap. *European Journal of Neuroscience 15*, 589–601.

Floresco SB, Blaha CD, Yang CR, Phillips AG (2001). Dopamine D1 and NMDA receptors mediate potentiation

of basolateral amygdala-evoked firing of nucleus accumbens neurons. *Journal of Neuroscience 21*, 6370–6376.

Friedlander DR, Milev P, Karthikeyan L, Margolis RK, Margolis RU, Grument M (1994). The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth. *Journal of Cell Biology 125*, 669–680.

Garwood J, Schnadelbach O, Clement A, Schutte K, Bach A, Faissner A (1999). DSD-1-proteoglycan is the mouse homolog of phosphacan and displays opposing effects on neurite outgrowth dependent on neuronal lineage. *Journal of Neuroscience 19*, 3888–3899.

Horger BA, Lyasere CA, Berhow MT, Messer CJ, Nestler EJ, Taylor JR (1999). Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. *Journal of Neuroscience 19*, 4110–4122.

Howland JG, Taepavarapruk P, Phillips AG (2002). Glutamate receptor-dependent modulation of dopamine efflux in the nucleus accumbens by basolateral, but not central, nucleus of the amygdala in rats. *Journal of Neuroscience 22*, 1137–1145.

Inatani M, Tanibara H, Oohira A, Otori Y, Nishida A, Honjo M, Kida N, Honda Y (2000). Neuroglycan C, a neural tissue-specific transmembrane chondroitin sulfate proteoglycan, in retinal neural network formation. *Investigative Ophthalomology & Visual Science 41*, 4338–4346.

Iozzo RV (1998). Matrix proteoglycans: from molecular design to cellular function. *Annual Review of Biochemistry 67*, 609–652.

Johnson LR, Aylward RL, Hussain Z, Totterdell S (1994). Input from the amygdala to the rat nucleus accumbens: its relationship with tyrosine hydroxylase immunoreactivity and identified neurons. *Neuroscience 61*, 851–865.

Kalivas PW, Duffy P, DuMars LA, Skinner C (1998a). Behavioral and neurochemical effects of acute and daily cocaine administration in rats. *Journal of Pharmacology and Experimental Therapeutics 245*, 485–492.

Kalivas PW, Pierce RC, Cornish J, Sorg BA (1998b). A role for sensitization in craving and relapse in cocaine addiction. *Journal of Psychopharmacology 12*, 49–53.

Kinugasa Y, Ishiguro H, Tokita Y, Oohira A, Ohmoto H, Higashiyama S (2004). Neuroglycan C, a novel member of the neuregulin family. *Biochemical and Biophysical Research Communications 321*, 1045–1049.

Liao D, Lin H, Law PY, Loh HH (2005). Mu-opioid receptors modulate the stability of dendritic spines. *Proceedings of the National Academy of Sciences USA 102*, 1725–1730.

Linda CH, Leigh W, Jennifer N, Thomas EE (2001). 15-Lipoxygenase-1 metabolites down-regulate peroxisome proliferator-activated receptor γ via the MAPK signaling pathway. *Journal of Biological Chemistry 276*, 34545–34552.

Mamiya T, Noda Y, Nishi M, Takeshima H, Nabeshima T (1998). Enhancement of spatial attention in nociceptin/orphanin FQ receptor-knockout mice. *Brain Research 783*, 236–240.

Mamiya T, Noda Y, Ren X, Hamdy M, Furukawa S, Kameyama T, Yamada K, Nabeshima T (2001). Involvement of cyclic AMP systems in morphine physical dependence in mice: prevention of development of morphine dependence by rolipram, a phoshodiesterase 4 inhibitor. *British Journal of Pharmacology 132*, 1111–1117.

Margolis RK, Rauch U, Maurel P, Margolis RU (1995). Neurocan and phosphacan: two major nervous tissue-specific chondroitin sulfate proteoglycans. *Perspectives on Developmental Neurobiology 3*, 273–290.

Nagai T, Noda Y, Ishikawa K, Miyamoto Y, Yoshimura M, Yamada K, Nabeshima T (2005). The role of tissue plasminogen activator in methamphetamine-related reward and sensitization. *Journal of Neurochemistry 92*, 660–667.

Nagai T, Yamada K, Yoshimura M, Ishikawa K, Miyamoto Y, Hashimoto K, Noda Y, Nitta A, Nabeshima T (2004). The tissue plasminogen activator-plasmin system participates in the rewarding effect of morphine by regulating dopamine release. *Proceedings of the National Academy of Sciences USA 101*, 3650–3655.

Nakajima A, Yamada K, Nagai T, Uchiyama T, Miyamoto Y, Mamiya T, He J, Nitta A, Mizuno M, Tran MH, et al. (2004). Role of tumor necrosis factor-α in methamphetamine-induced drug dependence and neurotoxicity. *Journal of Neuroscience 24*, 2212–2225.

Nestler EJ (2001). Molecular basis of long-term plasticity underlying addiction. *Nature Reviews Neuroscience 2*, 119–127.

Nestler EJ (2002). From neurobiology to treatment: progress against addiction. *Nature Neuroscience 5*, 1076–1079.

Norrholm SD, Bibb JA, Nestler EJ, Ouimet CC, Taylor JR, Greengard P (2003). Cocaine-induced proliferation of dendritic spines in nucleus accumbens is dependent on the activity of cyclin-dependent kinase 5. *Neuroscience 116*, 19–22.

Oohira A, Katoh-Semba R, Watanabe E, Matsui F (1994). Brain development and multiple molecular species of proteoglycan. *Neuroscience Research 20*, 195–207.

Pierce RC, Kalivas PW (1997). A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. *Brain Research Reviews 25*, 192–216.

Robinson TE, Becker JB (1986). Enduring changes in brain and behavior produced by chronic amphetamine administration: a review and evaluation of animal models of amphetamine psychosis. *Brain Research 396*, 157–198.

Robinson TE, Kolb B (1997). Persistent structural modifications in nucleus accumbens and prefrontal cortex neurons produced by previous experience with amphetamine. *Journal of Neuroscience 17*, 8491–8497.

Robinson TE, Kolb B (1999). Alteration in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. *European Journal of Neuroscience 11*, 1598–1604.

Sklair-Tavron L, Shi WX, Lane SB, Harris HW, Bunney BS, Nestler EJ (1996). Chronic morphine induces visible changes in the morphology of mesolimbic dopamine

neurons. *Proceedings of the National Academy of Sciences USA 93*, 11202–11207.

Small DH, Mok SS, Williamson TG, Nurcombe V (1996). Role of proteoglycans in neural development, regeneration, and the aging brain. *Journal of Neurochemistry 67*, 889–899.

Toda S, McGinty JF, Kalivas PW (2002). Repeated cocaine administration alters the expression of genes in corticolimbic circuitry after a 3-week withdrawal: a DNA macroarray study. *Journal of Neurochemistry 82*, 1290–1299.

Watanabe E, Maeda N, Matsui F, Kushima Y, Noda M, Oohira A (1995). Neuroglycan C, a novel membrane-spanning chondroitin sulfate proteoglycan that is restricted to the brain. *Journal of Biological Chemistry 270*, 26876–26882.

Yasuda Y, Tokita Y, Aono S, Matsui F, Ono T, Sonta S, Watanabe E, Nakanishi Y, Oohira A (1998). Cloning and chromosomal mapping of the human gene of neuroglycan C (NGC), a neural transmembrane chondroitin sulfate proteoglycan with an EGF module. *Neuroscience Research 32*, 313–322.

Journal of Neurochemistry, 2004, **91**, 308–317 doi:10.1111/j.1471-4159.2004.02705.x

Anatomical substrates for the discriminative stimulus effects of methamphetamine in rats

Akira Nakajima,* Kiyofumi Yamada,*,† Jue He,* Nan Zeng,* Atsumi Nitta* and Toshitaka Nabeshima*

**Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan*

†Laboratory of Neuropsychopharmacology, Division of Life Sciences, Kanazawa University Graduate School of Natural Science & Technology, Kakuma-machi, Kanazawa, Japan

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.

J. Neurochem. (2004) **91**, 308–317.

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

Received April 6, 2004; revised manuscript received June 10, 2004; accepted June 14, 2004.

Address correspondence and reprint requests to Toshitaka Nabeshima, Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466–8560, Japan. E-mail: tnabeshi@med.nagoya-u.ac.jp

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

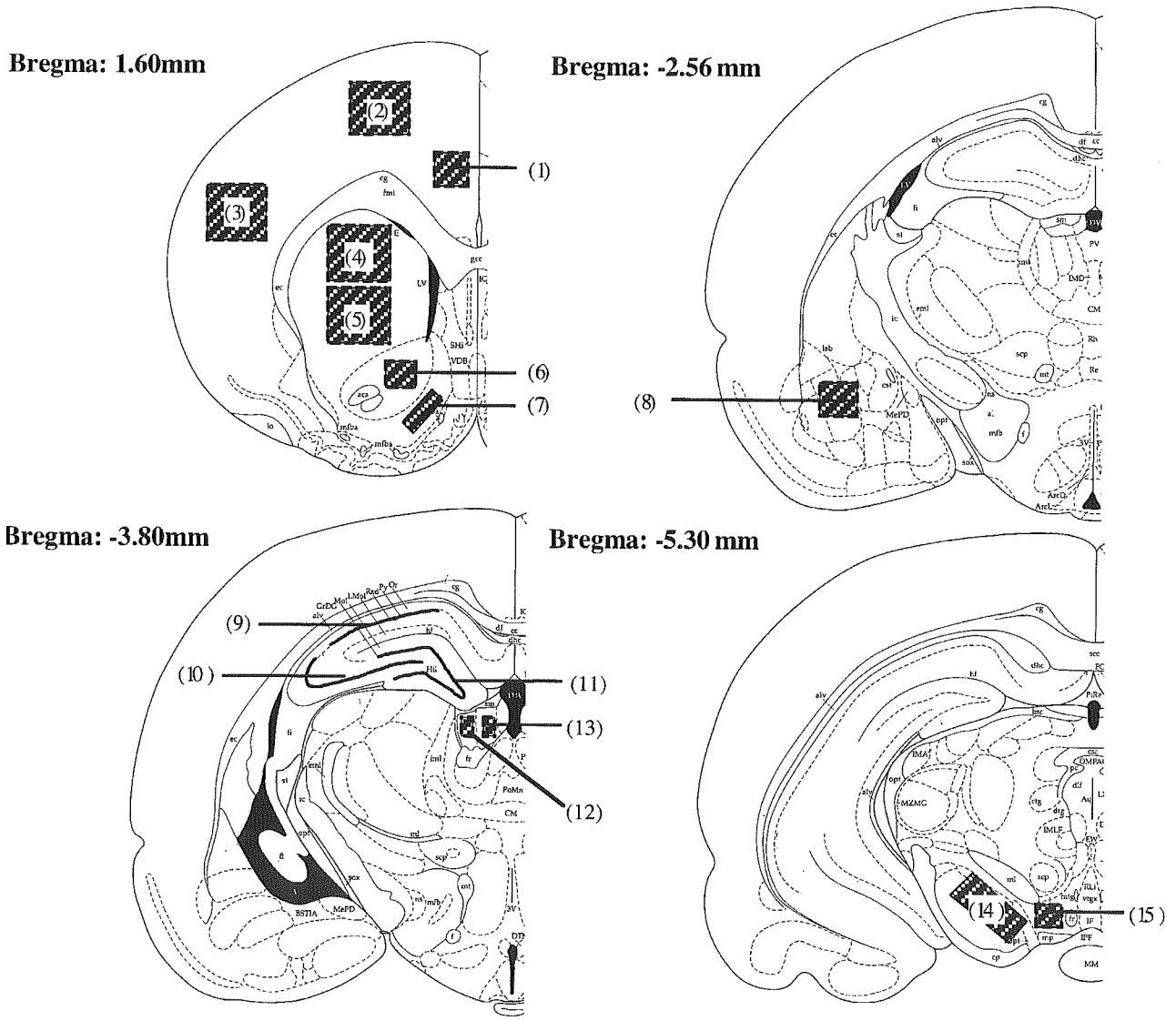

Fig. 1 Diagrammatic representation of the brain areas examined for Fos immunohistochemistry. The areas examined for enumerating Fos-positive cells include the cingulate (1), motor (2) and somatosensory cortex (3), the dorsal (4) and ventral caudate putamen (5), the core (6) and shell (7) of the nucleus 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).

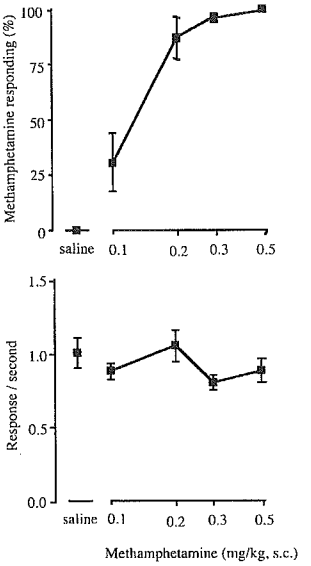

Fig. 2 Dose-dependent discriminative stimulus effects of methamphetamine in rats. Each point represents the mean ± SE ($n = 8$).

Fos expression associated with the discriminative stimulus effects of methamphetamine

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

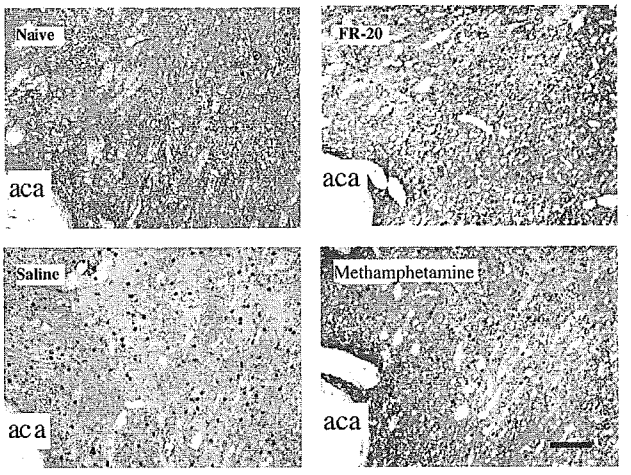

Fig. 3 Representative photomicrographs of Fos immunostaining of the NAc core in rats subjected to the drug discrimination test for methamphetamine. Rats were trained to discriminate methamphetamine (0.5 mg/kg) from saline under the two-lever FR-20 schedule of food reinforcement. The trained rats were 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).

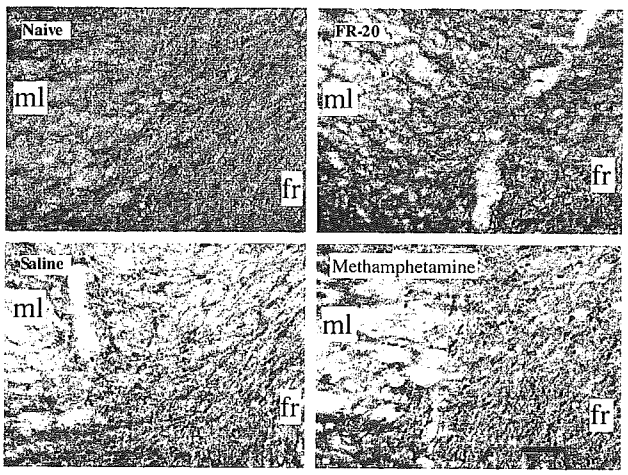

Fig. 4 Representative photomicrographs of Fos immunostaining of the VTA in rats subjected to the drug discrimination test for methamphetamine. ml, medial lemniscus; fr, fasciculus retroflexus (habenulointerpeduncular tract). Scale bar, 100 μm.

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

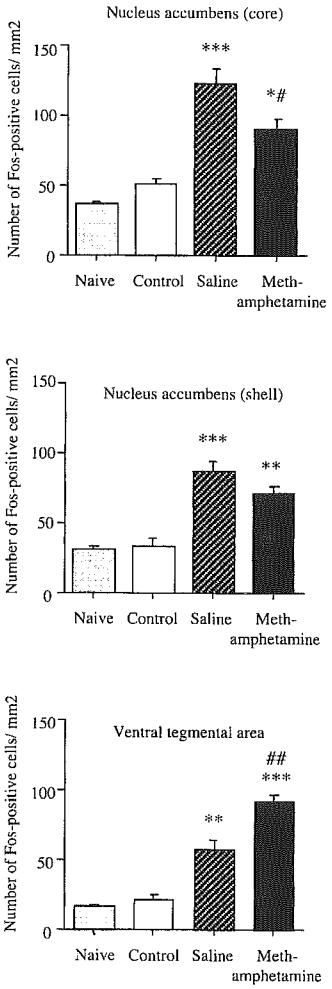

Fig. 5 Changes in Fos expression induced by discriminative stimulus effects of methamphetamine in the core and shell of the NAc and VTA. Rats were trained to discriminate methamphetamine (o.5mg/kg) from saline under the two-lever FR 20 schedule of food reinforcement. The trained rats were subjected to 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.

Acknowledgements

This study was supported in part by Special Coordination Funds for Promoting Science and Technology, the Target-oriented Brain Science Research Program and a Grant-in-Aid for Scientific Research (No. 14658249), from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by a Grant-in-Aid for Health Sciences Research from the Ministry of Health, Labour and Welfare of Japan, by the JSPS Joint Research Project under the Japan-Korea Basic Scientific Cooperation Program, and by a SRF Grant for Biomedical Research, and by NOVARTIS Foundation (Japan) for the Promotion of Science.

References

Andre V., Pineau N., Motte J. E., Marescaux C. and Nehlig A. (1998) Mapping of neural networks underlying generalized seizures induced by increasing doses of pentylenetetrazol in the immature and adult rat: c-Fos immunohistochemical study. *Eur. J. Neurosci.* **10**, 2094–2106.

Apicella P., Ljungberg T., Scarnati E. and Schultz W. (1991) Responses to reward in monkey dorsal and ventral striatum. *Exp. Brain. Res.* **85**, 491–500.

Beckstead R. M., Domesick V. B. and Nauta W. J. (1979) Efferent connections of the substantia nigra and ventral tegmental area in the rat. *Brain. Res.* **175**, 191–217.

Callahan P. M., De La Garza R. II and Cunningham K. A. (1994) Discriminative stimulus properties of cocaine: modulation by dopamine D1 receptors in the nucleus accumbens. *Psychopharmacology* **115**, 110–114.

Callahan P. M., De La Garza R. II and Cunningham K. A. (1997) Mediation of the discriminative stimulus properties of cocaine by mesocorticolimbic dopamine systems. *Pharmacol. Biochem. Behav.* **57**, 601–607.

Chang J. Y., Sawyer S. F., Lee R. S. and Woodward D. J. (1994) Electrophysiological and pharmacological evidence for the role of the nucleus accumbens in cocaine self-administration in freely moving rats. *J. Neurosci.* **14**, 1224–1244.

Cole R. L., Konradi C., Douglass J. and Hyman S. E. (1995) Neuronal adaptation to amphetamine and dopamine molecular mechanisms of prodynorphin gene regulation in rat striatum. *Neuron* **14**, 813–823.

David D. J., Zahniser N. R., Hoffer B. J. and Gerhardt G. A. (1998) *In vivo* electrochemical studies of dopamine clearance in subregions of rat nucleus accumbens: differential properties of the core and shell. *Exp. Neurol.* **153**, 277–286.

De La Garza R. II, Callahan P. M. and Cunningham K. A. (1998) The discriminative stimulus properties of cocaine, a 5-HT1A agonist or antagonist, into the ventral tegmental area. *Psychopharmacology* **137**, 1–6.

Deniau J. M., Hammond C., Riszk A. and Feger J. (1978) Electrophysiological properties of identified output neurons of the rat substantia nigra (pars compacta and pars reticulata): evidences for the existence of branched neurons. *Exp. Brain. Res.* **32**, 409–422.

Fallon J. H. and Moore R. Y. (1978) Catecholamine innervation of basal forebrain IV. Topography of the dopamine projection to the basal forebrain and striatum. *J. Comp. Neurol.* **180**, 545–580.

Filip M., Papla I., Nowak E., Czepiel K. and Przegalinski E. (2003) Effects of 5-HT1B receptor ligands microinjected into the ventral tegmental area on cocaine discrimination in rats. *Eur. J. Pharmacol.* **459**, 239–245.

Floresco S. B., Todd C. L. and Grace A. A. (2001) Glutamatergic afferents from the hippocampus to the nucleus accumbens regulate activity of ventral tegmental area dopamine neurons. *J. Neurosci.* **21**, 4915–4922.

Georges F., Stinus L. and Le Moine C. (2000) Mapping of c-fos gene expression in the brain during morphine dependence and

precipitated withdrawal, and phenotypic identification of the striatal neurons involved. *Eur. J. Neurosci.* **12**, 4475–4486.

Giros B., Jaber M., Jones S. R., Wightman R. M. and Caron M. G. (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379**, 606–612.

Gonon F. and Sundstrom L. (1996) Excitatory effects of dopamine released by impulse flow in the rat nucleus accumbens *in vivo*. *Neuroscience* **75**, 13–18.

Groenewegen H. J., Vermeulen-Van der Zee E., Te Kortschot A. and Witter M. P. (1987) Organization of the projections from the subiculum to the ventral striatum in the rat. A study using anterograde transport of *Phaseolus vulgarus* leucoagglutinin. *Neuroscience* **23**, 103–120.

Guyenet P. G. and Aghajanian G. K. (1978) Antidromic identification of dopaminergic and other output neurons of the rat substantia nigra. *Brain Res.* **150**, 69–84.

He J., Yamada K. and Nabeshima T. (2002) A role of Fos expression in the CA3 region of the hippocampus in spatial memory formation in rats. *Neuropsychopharmacology* **26**, 259–268.

Heimer L., Zahm D. S., Churchill L., Kalivas P. W. and Wohltmann C. (1991) Specificity in the projection patterns of accumbal core and shell in the rat. *Neuroscience* **41**, 89–125.

Herdegen T. and Leah J. D. (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res. Rev.* **28**, 370–490.

Hollerman J. R. and Schultz W. (1998) Dopamine neurons report an error in the temporal prediction of reward during learning. *Nat. Neurosci.* **1**, 304–309.

Hu X. T. and White F. J. (1994) Loss of D1/D2 dopamine receptor synergisms following repeated administration of D1 or D2 receptor selective antagonists: electrophysiological and behavioral studies. *Synapse* **17**, 43–61.

Jones S. R., O'Dell S. J., Marshall J. F. and Wightman R. M. (1996) Functional and anatomical evidence for different dopamine dynamics in the core and shell of the nucleus accumbens in slices of rat brain. *Synapse* **23**, 224–231.

Kalivas P. W. and Stewart J. (1991) Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. *Brain Res. Rev.* **16**, 223–244.

Kamien J. B., Bickel W. K., Hughes J. R., Higgins S. T. and Smith B. J. (1993) Drug discrimination by humans compared to nonhumans: Current status and future directions. *Psychopharmacology* **111**, 259–270.

Kiyatkin E. A. and Rebec G. V. (1999) Striatal neuronal activity and responsiveness to dopamine and glutamate after selective blockade of D1 and D2 dopamine receptors in freely moving rats. *J. Neurosci.* **19**, 3594–3609.

Le Foll B., Frances H., Diaz J., Schwartz J. C. and Sokoloff P. (2002) Role of the dopamine D3 receptor in reactivity to cocaine-associated cues in mice. *Eur. J. Neurosci.* **15**, 2016–2026.

Legault M., Rompre P. P. and Wise R. A. (2000) Chemical stimulation of the ventral hippocampus elevates nucleus accumbens dopamine by activating dopaminergic neurons of the ventral tegmental area. *J. Neurosci.* **20**, 1635–1642.

Ljungberg T., Apicella P. and Schultz W. (1992) Responses of monkey dopamine neurons during learning of behavioral reactions. *J. Neurophysiol.* **67**, 145–163.

Melega W. P., Williams A. E., Schmitz D. A., DiStefano E. W. and Cho A. K. (1995) Pharmacokinetic and pharmacodynamic analysis of the actions of d-amphetamine and d-methamphetamine on dopamine terminal. *J. Pharmacol. Exp. Ther.* **274**, 90–96.

Morgan J. I. and Curran T. (1991) Stimulus-transcription coupling in the nervous system: involvement of inducible proto-oncogenes fos and jun. *Annu. Rev. Neurosci.* **14**, 421–451.

Mori A., Noda Y., Mamiya T., Miyamoto Y., Nakajima A., Furukawa H. and Nabeshima T. (2001) Phencyclidine-induced discriminative stimulus is mediated via phencyclidine binding sites on the N-methyl-D-aspartate receptor-ion channel complex, not via sigmal receptors. *Behav. Brain Res.* **119**, 33–40.

Munzar P. and Goldberg S. R. (2000) Dopaminergic involvement in the discriminative-stimulus effects of methamphetamine in rats. *Psychopharmacology* **148**, 209–216.

Munzar P., Baumann M. H., Shoaib M. and Goldberg S. R. (1999) Effects of dopamine and serotonin-releasing agents on methamphetamine discrimination and self-administration in rats. *Psychopharmacology* **141**, 287–296.

Nakajima A., Yamada K., Nagai T. *et al.* (2004) Role of tumor necrosis factor-alpha in methamphetamine-induced drug dependence and neurotoxicity. *J. Neurosci.* **24**, 2212–2225.

Namima M., Sugihara K. and Okamoto K. (1998) Lithium inhibits the reverse tolerance and c-Fos expression induced by methamphetamine in mice. *Brain Res.* **782**, 83–90.

Neisewander J. L., Baker D. A., Fuchs R. A., Tran-Nguyen L. T., Palmer A. and Marshall J. F. (2000) Fos protein expression and cocaine-seeking behavior in rats after exposure to a cocaine self-administration environment. *J. Neurosci.* **20**, 798–805.

Nicola S. M., Surmeier J. and Malenka R. C. (2000) Dopaminergic modulation of neuronal excitability in the striatum and nucleus accumbens. *Annu. Rev. Neurosci.* **23**, 185–215.

Nielsen E. B. and Scheel-Kruger J. (1986) Cueing effects of amphetamine and LSD: Elicitation by direct microinjection of the drugs into the nucleus accumbens. *Eur. J. Pharmacol.* **125**, 85–92.

Ono T., Nishijo H. and Nishino H. (2000) Functional role of the limbic system and basal ganglia in motivated behaviors. *J. Neurol.* **247**, Suppl 5: V23–32.

Paladini C. A., Celada P. and Tepper J. M. (1999) Striatal, pallidal, and pars reticulata evoked inhibition of nigrostriatal dopaminergic neurons is mediated by GABA(A) receptors in vivo. *Neuroscience* **89**, 799–812.

Paladini C. A., Robinson S., Morikawa H., Williams J. T. and Palmiter R. D. (2003) Dopamine controls the firing pattern of dopamine neurons via a network feedback mechanism. *Proc. Natl Acad. Sci. USA* **100**, 2866–2871.

Paxinos G. and Watson C. (1982) *The Rat Brain in Stereotaxic Coordinates*. New York, Academic press.

Sagar S. M., Sharp F. R. and Curran T. (1988) Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science* **240**, 1328–1331.

Sasaki J. E., Tatham T. A. and Barrett J. E. (1995) The discriminative stimulus effects of methamphetamine in pigeons. *Psychopharmacology* **120**, 303–310.

Schultz W. (1998) Predictive reward signal of dopamine neurons. *J. Neurophysiol.* **80**, 1–27.

Schuster C. R. and Johanson C. E. (1988) Relationship between the discriminative stimulus properties and subjective effects of drugs. In: *Psychopharmacology, Series 4: Transduction Mechanisms of Drug Stimuli* (Colpaert F. C. and Balster R. L., eds), pp. 161–175. Springer, Berlin.

Seiden L. S., Sabol K. E. and Ricaurte G. A. (1993) Amphetamine: effects on catecholamine systems and behavior. *Annu. Rev. Pharmacol. Toxicol.* **33**, 639–677.

Sesack S. R. and Pickel V. M. (1990) In the rat medial nucleus accumbens, hippocampal and catecholaminergic terminals converge on spiny neurons and are in apposition to each other. *Brain Res.* **527**, 266–279.

Somogyi P., Bolam J. P., Totterdell S. and Smith A. D. (1981) Monosynaptic input from the nucleus accumbens-ventral striatum

region to retrogradely labelled nigrostriatal neurones. *Brain Res.* **217**, 245–263.

Tani K., Iyo M., Matsumoto H. *et al.* (2001) The effects of dentate granule cell destruction on behavioural activity and Fos protein expression induced by systemic methamphetamine in rats. *Br. J. Pharmacol.* **134**, 1411–1418.

Tidey J. W. and Bergman J. (1998) Drug discrimination in methamphetamine-trained monkeys: agonist and antagonist effects of dopaminergic drugs. *J. Pharmacol. Exp. Ther.* **285**, 1163–1174.

Totterdell S. and Smith A. D. (1989) Convergence of hippocampal and dopaminergic input onto identified neurons in the nucleus accumbens of the rat. *J. Chem. Neuroant.* **2**, 285–298.

Ujike H., Onoue T., Akiyama K., Hamamura T. and Otsuki S. (1989) Effects of selective D-1 and D-2 dopamine antagonists on development of methamphetamine-induced behavioral sensitization. *Psychopharmacology* **98**, 89–92.

Umino A., Nishikawa T. and Takahashi K. (1995) Methamphetamine-induced nuclear c-Fos in rat brain regions. *Neurochem. Int.* **26**, 85–90.

Van der Zee E. A., Douma B. R., Bohus B. and Luiten P. G. (1994) Passive avoidance training induces enhanced levels of immunoreactivity for muscarinic acetylcholine receptor and coexpressed PKC γ and MAP-2 in rat cortical neurons. *Cereb. Cortex* **4**, 376–390.

Wise R. A. (1996) Neurobiology of addiction. *Curr. Opin. Neurobiol.* **6**, 243–251.

Wood D. M. and Emmett-Oglesby M. W. (1989) Mediation in the nucleus accumbens of the discriminative stimulus produced by cocaine. *Pharmacol. Biochem. Behav.* **33**, 453–457.

Yamada K., Noda Y., Komori Y., Sugihara H., Hasegawa T. and Nabeshima T. (1996) Reduction in the number of NADPH-diaphorase-positive cells in the cerebral cortex and striatum in aged rats. *Neurosci. Res.* **24**, 393–402.

Yang C. R. and Mogenson G. J. (1987) Hippocampal signal transmission to pedunculopontine nucleus and its regulation by dopamine D2 receptors in the nucleus accumbens: an electrophysiological and behavioural study. *Neuroscience.* **23**, 1041–1055.

Zahm D. S. and Heimer L. (1990) Two transpallidal pathways originating in the nucleus accumbens. *J. Comp. Neurol.* **302**, 437–446.

Journal of Neuroscience Research 79:476–487 (2005)

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

Manabu Hashimoto,[1] Atsumi Nitta,[2] Hidefumi Fukumitsu,[1] Hiroshi Nomoto,[1] Liya Shen,[3] and Shoei Furukawa[1]*

[1]Laboratory of Molecular Biology, Gifu Pharmaceutical University, Gifu, Japan
[2]Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan
[3]Laboratory of Mammalian Genes and Development, National Institutes of Health, Bethesda, Maryland

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-

Contract grant sponsor: Ministry of Education, Science, and Culture of Japan; Contract grant number: 14658252.

*Correspondence to: Shoei Furukawa, Laboratory of Molecular Biology, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan. E-mail: furukawa@gifu-pu.ac.jp

Received 30 August 2004; Revised 8 October 2004; Accepted 13 October 2004

Published online 5 January 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20368

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