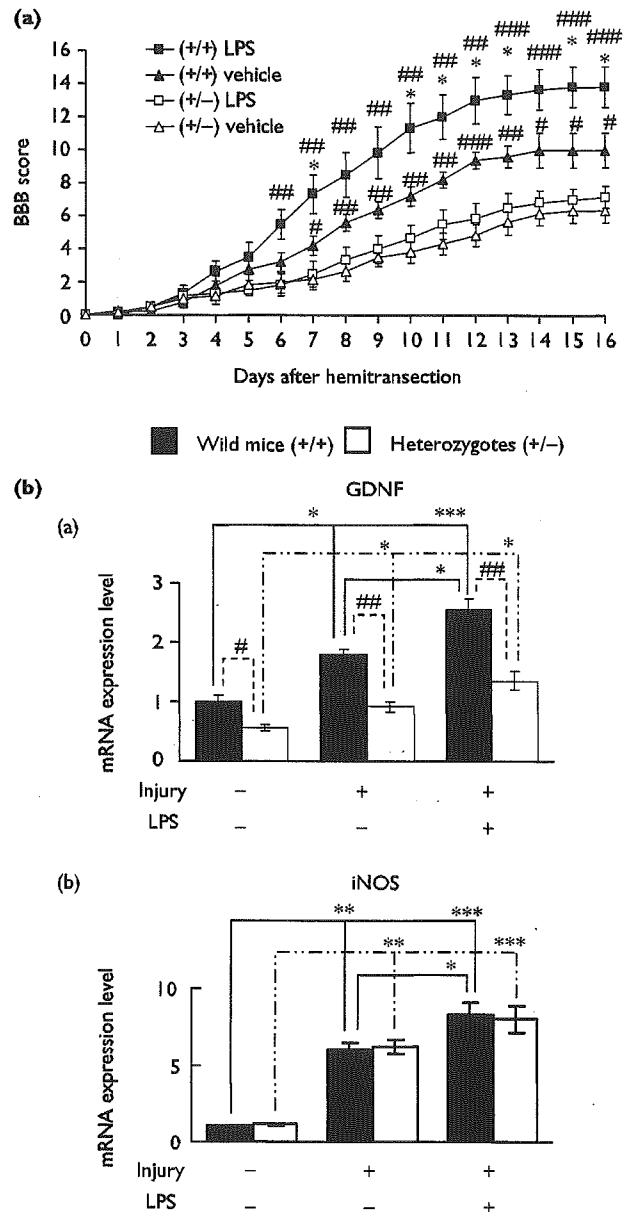


**Fig. 2.** Expression of GDNF and iNOS mRNAs (a) and colocalization of GDNF-ir in the CD11b-positive cells (b) in the injured rat spinal cord to which LPS or vehicle was administered. (a) LPS (10 µg, squares) or vehicle (triangles) was injected into the hemitransection site (at the level of the 9th thoracic vertebra) of the spinal cord immediately after the operation, and the spinal cord was removed. Total RNA was prepared from combined tissues of both rostral and caudal areas (5 mm length) just adjacent to the injury site, and subjected to RT-PCR for mRNA of GDNF (a) and iNOS (b). The ratio of the band intensity of GDNF or iNOS cDNA products to that of GAPDH cDNA products was calculated. The values are expressed as the means ± s.e. (n=3) of the fold-increase over the value of the control group in which total RNA samples were prepared immediately after the administration of LPS or vehicle. Significant differences from the value of the corresponding vehicle group were determined by means of Student's *t*-test. \**p* < 0.05. (b) LPS (10 µg) was injected into the hemitransection site of spinal cord immediately after the operation. At 12 h post injection, the spinal cord was removed from the fixed animal and processed. Sagittal sections were double stained with mouse antibody against CD11b and rabbit antibody against GDNF and visualized with rhodamine-conjugated anti-mouse IgG for CD11b (a) and FITC-conjugated anti-rabbit IgG for GDNF (b). a and b are merged in (c). Bar=50 µm.

difference in iNOS mRNA level between the mice of both genetic types. The combined action of these two types of regulators, i.e., GDNF and iNOS, with the former predominating, restored the locomotor function damaged by the spinal cord injury, suggesting that GDNF is involved in restoration processes via properly activated microglia/macrophages, i.e., via optimal inflammation reactions.



**Fig. 3.** Effects of LPS on locomotor function after spinal cord injury (a) and effects of LPS and/or spinal cord injury on the expression of GDNF and iNOS mRNAs (b) of wild-type mice (+/+) and GDNF-gene heterozygous mutant mice (+/-). The left side of the spinal cord was transected or not at the level of the 9th thoracic vertebra, and LPS (2 µg) or vehicle was injected into the injury site immediately after the operation. (a) Locomotor function of the hind limb on the injured side was evaluated for +/+ (solid) and +/- (open) mice daily after injection of LPS (squares) or vehicle (triangles). The values are expressed as the means ± s.e. (n=6). Significance, \**p* < 0.05 vs the value of the vehicle group of the corresponding genotype, #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs the value of similarly treated +/+ mice (Student's *t*-test). (b) mRNA expression of GDNF (a) and iNOS (b) in the injury site of the spinal cord of +/+ (closed columns) and +/- (open columns) mice was examined by RT-PCR 12 h after the injection of LPS (2 µg) or vehicle into the hemitransection site. The ratio of the intensity of GDNF or iNOS cDNA products to that of GAPDH cDNA products was calculated. The values are expressed as the means ± s.e. (n=3) of the fold-increase over the value of vehicle-administered +/+ mice. Statistically significant differences between groups are indicated by brackets: \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001 (ANOVA with Newman-Keuls *post hoc* test); #*p* < 0.05, ##*p* < 0.01 (Student's *t*-test).

## DISCUSSION

Inflammatory reactions have opposing roles via production of neurotoxic or neurotrophic molecules. Previous reports described the activation or increase in the number of macrophages by LPS and inhibition of secondary injury after spinal cord injury by daily LPS injection [18,19]. Our present results demonstrate that high-dose LPS led to functional recovery of locomotion (Fig. 1), which we interpreted to be due to the increase in GDNF expression by macrophages activated and increased in number in the injury site (Fig. 2). Moreover, although the optimal dose of LPS enhanced iNOS mRNA expression, the GDNF mRNA level was sustained higher than normal for a much longer time (Fig. 2a), suggesting that properly-controlled inflammatory reactions favor neurotrophic influences by GDNF rather than neurotoxic ones due to iNOS. As exogenous GDNF exerts neuroprotection after spinal cord injury [20], LPS-induced GDNF would seem to contribute positively to nerve regeneration.

As GDNF enhances the phagocytic activity of cultured macrophages [17], GDNF synthesized by macrophages would activate macrophages in an autocrine manner. Therefore, not only neuroprotection but also activation of macrophages would result from macrophage-secreted GDNF in the injured spinal cord. Such GDNF might further upregulate GDNF synthesis in the macrophages, which would promote more and more restorative macrophage function. GDNF may be involved in an activation loop to form an adequate neurotrophic environment for nerve regeneration. Inflammatory reactions are sometimes harmful because of the production of NO, reactive oxygen species, and cytokines [6–8]; and therefore anti-inflammatory therapies are effective for spinal cord injury [21,22]. However, such therapies may simultaneously reduce the level of neurotrophic factors. Thus a proper balance is critical for establishing circumstances favorable for nerve regeneration.

Functional recovery of the GDNF-gene deficient heterozygous mice (+/–) was inferior to that of the wild-type mice (+/+) irrespective of LPS administration (Fig. 3). GDNF mRNA expression of the +/- mutant mice was about a half of that of the +/+ ones regardless of the treatment, whereas there was no difference in iNOS mRNA expression between both genetic mice. That is, GDNF produced by microglia/macrophages after spinal cord injury was important for recovery of locomotor function, except in the following case: Namely, LPS administration to the +/- mice enhanced their GDNF mRNA expression, but did not facilitate their functional recovery. This may have been due to insufficient expression of GDNF to elicit neurotrophic effects.

## CONCLUSIONS

Properly activated microglia/macrophages in the injured spinal cord synthesize and secrete the neurotrophic factor GDNF, which is involved in restoring locomotor function damaged by a spinal cord injury.

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# Effects of single and repeated administration of methamphetamine or morphine on neuroglycan C gene expression in the rat brain

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## Abstract

The rearrangement of neural networks associated with the behavioural sensitization and tolerance induced by psychostimulants is poorly understood. We have investigated the effects of repeated administration of methamphetamine (chronic MAP), which induces behavioural sensitization, or morphine (chronic morphine), which induces tolerance to its antinociceptive effect, on the mRNA levels of neural network-related genes in the rat brain. A gene of special interest was that for neuroglycan C (NGC), a neural tissue-specific transmembrane chondroitin sulphate proteoglycan. Single MAP (acute MAP) administration significantly decreased NGC mRNA levels in the frontal cortex, ventral tegmental area (VTA), and amygdala compared to vehicle-treated groups. Repeated MAP (chronic MAP) administration significantly increased NGC mRNA levels in the frontal cortex, nucleus accumbens (NAc), striatum, hippocampus, VTA, and amygdala compared to acute MAP treatment. Single morphine (acute morphine) administration significantly increased NGC mRNA levels in the NAc, striatum, hippocampus, VTA, and amygdala compared to vehicle-treated groups. Chronic morphine administration significantly decreased NGC mRNA levels in the NAc, striatum, VTA, and amygdala compared to acute treatment. In addition, the NGC protein level in the NAc was increased after chronic MAP and acute morphine treatment. Dopamine and opioid receptor antagonists attenuated the effect of MAP and morphine respectively on NGC mRNA levels. These results suggest that the sensitization to MAP is associated with up-regulation of NGC gene expression, while the tolerance to the morphine-induced analgesic effect is associated with the down-regulation of NGC gene expression.

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**Key words:** Methamphetamine, morphine, neuroglycan C, sensitization, tolerance.

## Introduction

The repeated, intermittent, passive administration of drugs of abuse, including cocaine, methamphetamine (MAP) and morphine, can induce a progressive increase in the sensitization of certain behavioural

effects of these drugs in laboratory rodents (Kalivas et al., 1998; Pierce and Kalivas, 1997), and the behavioural alterations persist for an extremely long time. Furthermore, repeated administration of morphine results in the development of both tolerance to its antinociceptive effects and locomotor sensitization (Eitan et al., 2003). Drug addiction, which constitutes a serious threat to public health, is a multifaceted disorder involving tolerance, dependence, craving, and relapse (Nestler, 2002). Some current theories of drug addiction argue that the cellular neuroadaptations mediating behavioural sensitization are relevant to certain aspects of drug addiction (Kalivas et al., 1998; A Q1 A Q1

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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  $230 \pm 10$  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,  $23 \pm 0.5$  °C,  $50 \pm 0.5\%$  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 ( $55 \pm 1$  °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 ( $1 \mu\text{g}$ ) was converted into cDNA using the SuperScript™ First-Strand Synthesis System for RT-PCR kit (Life Technologies, Tokyo, Japan) in a total volume of  $1/21 \mu\text{l}$  (RT reaction mixture). PCR was performed using  $1/21$  of the RT reaction mixture,  $0.8 \mu\text{M}$  of each (forward and reverse) primer,  $0.25 \mu\text{M}$  of TaqMan probe and  $12.5 \mu\text{l}$  of TaqMan Universal PCR Master Mix (Applied Biosystems) in a total reaction volume of  $25 \mu\text{l}$ . The primers used were 5'-AGGGC-TCCCATCCAAATGAT-3' (forward) and 5'-CGAG-TTTGGGTGACATGGAGTT-3' (reverse), and the TaqMan probe was 5'-AAATCCAGGAAGCTCTCA-AGTCCCGC-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  $50 \mu\text{l}$  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 ( $16\,000 \text{ g}$ ,  $15$  °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:  $100 \mu\text{g}$ ) 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 reprobbed with goat anti-actin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, 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.

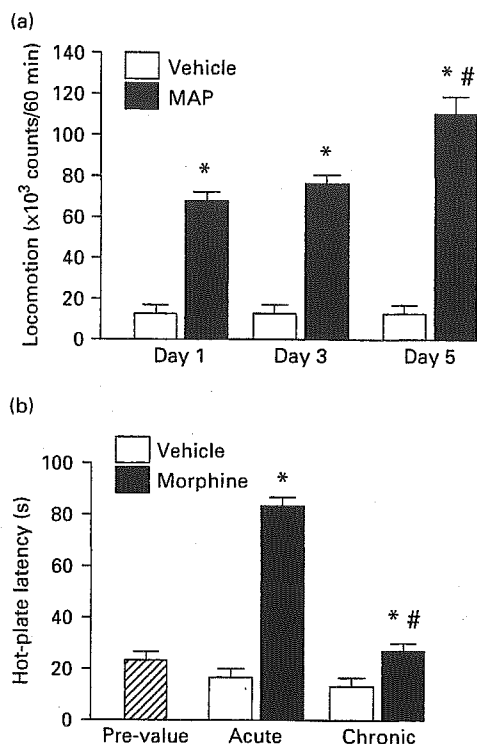
#### Statistical analysis

All data were expressed as the means  $\pm$  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  $\pm$  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).

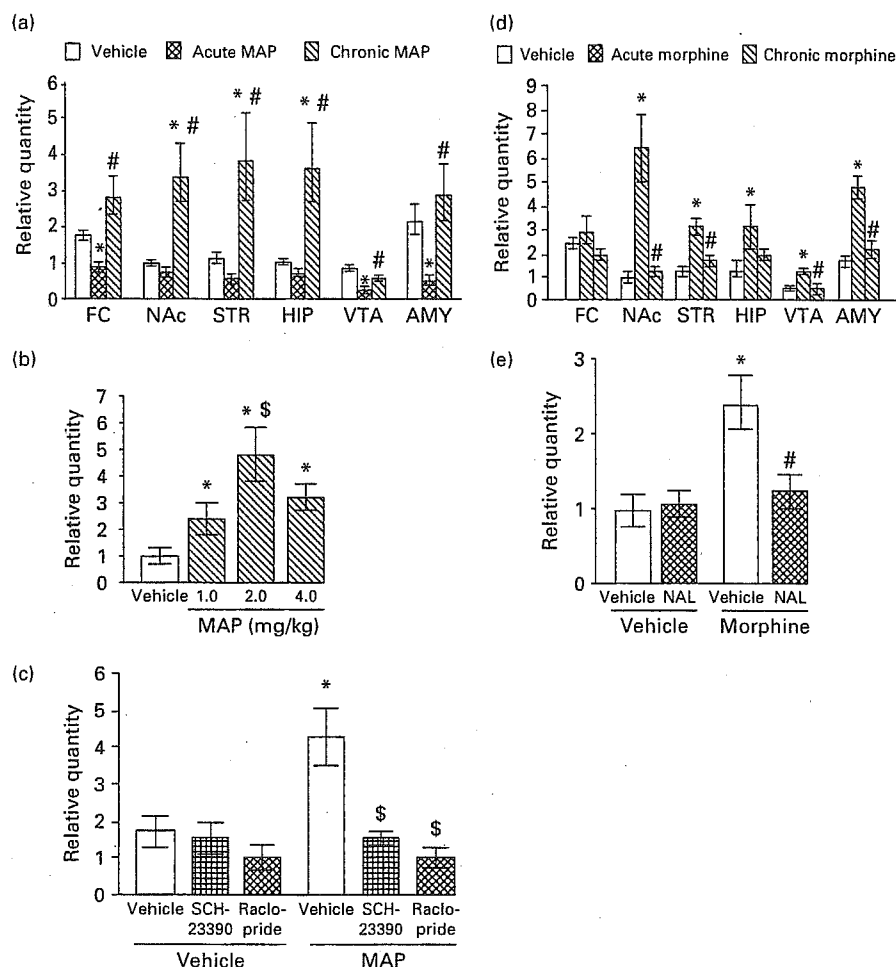
#### MAP- or morphine-induced NGC mRNA expression in the rat brain

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<sub>1</sub> receptor antagonist) (Anderson and Haaren, 2000) or raclopride (D<sub>2</sub> receptor antagonist) (Anderson and Haaren, 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



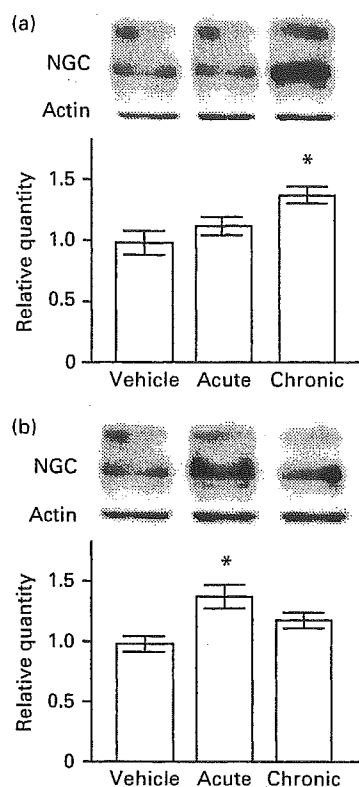
**Figure 2.** NGC mRNA levels in various brain regions after acute and chronic MAP (a) or morphine (d) treatment, at different doses of MAP (b), and in combination with dopamine (c) and opioid (e) antagonists in the NAc. The acute MAP-treated group was killed 2 h after a single MAP (2 mg/kg) treatment 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  $\pm$  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  $\pm$  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  $\mu$ -opioid receptors in GABAergic synapses, opioids may act directly on  $\mu$ -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- $\alpha$  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.

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#### Statement of Interest

None.

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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  $230 \pm 10$  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,  $23 \pm 0.5^\circ\text{C}$ ,  $50 \pm 0.5\%$  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.

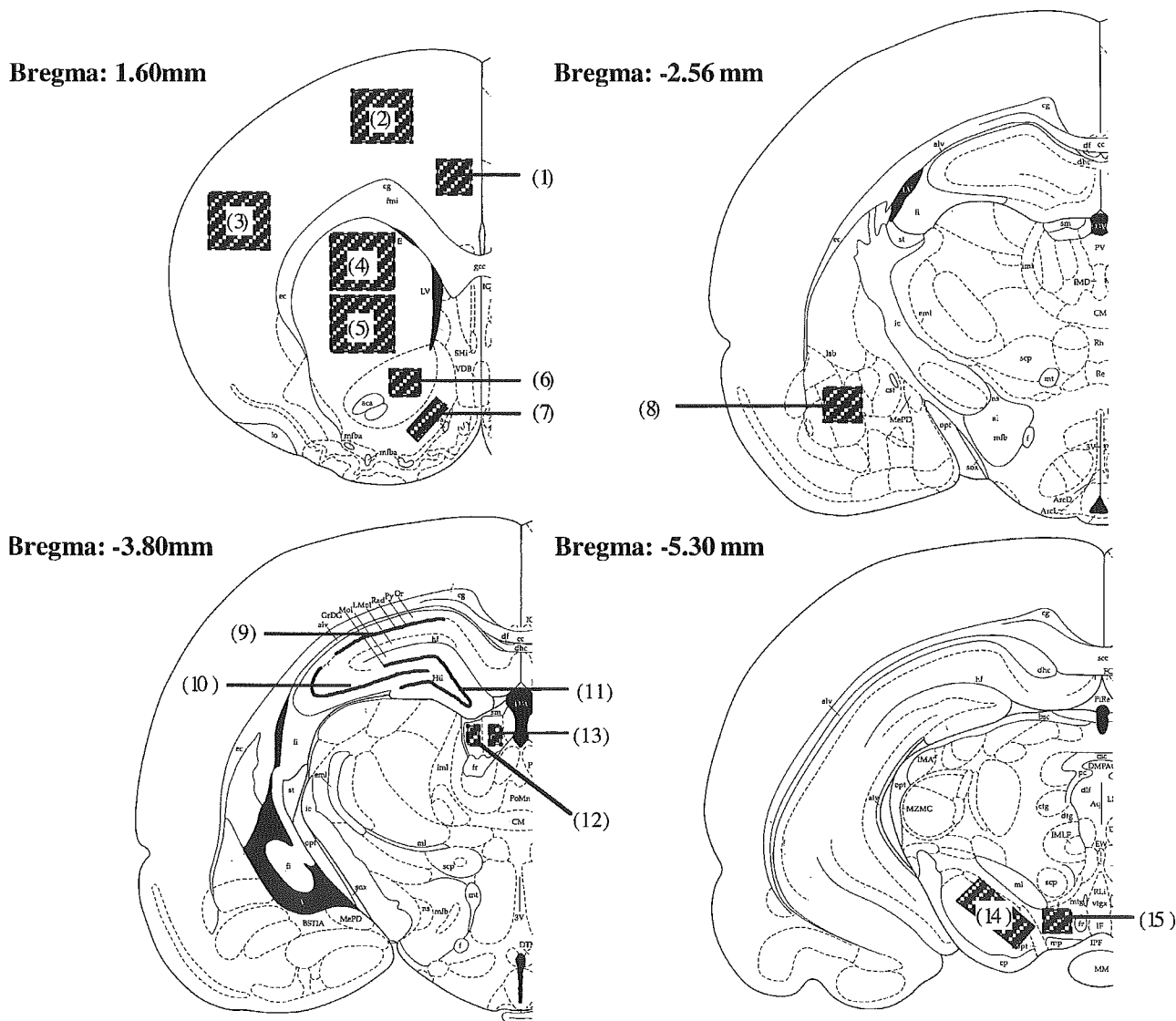
Four groups of rats were prepared to examine the effects of acute and chronic intermittent methamphetamine treatment on Fos expression. The conditions of age and food-restriction used in these groups were the same with the animals used to examine the discriminative effects of methamphetamine as described 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- $\mu$ m 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<sup>2</sup>) were as follows: cingulate (0.45 mm<sup>2</sup>), motor (1.32 mm<sup>2</sup>) and somatosensory cortex (1.32 mm<sup>2</sup>), dorsal (1.32 mm<sup>2</sup>) and ventral (1.32 mm<sup>2</sup>) caudate putamen, and the core (0.31 mm<sup>2</sup>) and shell (0.31 mm<sup>2</sup>) of the nucleus accumbens (NAc) in sections at a level of +1.60 mm from the bregma; amygdala (0.45 mm<sup>2</sup>) 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<sup>2</sup>) and lateral (0.10 mm<sup>2</sup>) habenula in sections at a level of -3.80 mm from the bregma, ventral tegmental area (0.31 mm<sup>2</sup>; VTA) and the substantia nigra (0.89 mm<sup>2</sup>) 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  $\pm$  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).

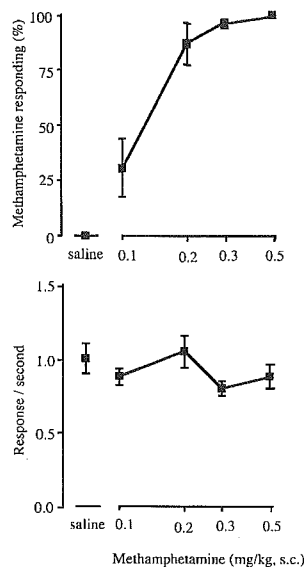


Fig. 2 Dose-dependent discriminative stimulus effects of methamphetamine in rats. Each point represents the mean  $\pm$  SE ( $n = 8$ ).

#### Fos expression associated with the discriminative stimulus effects of methamphetamine

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

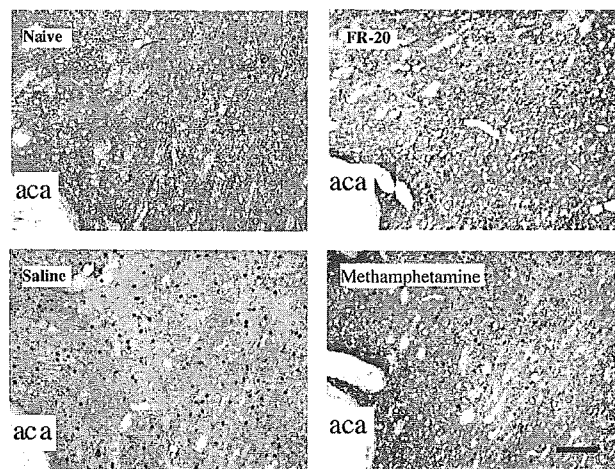
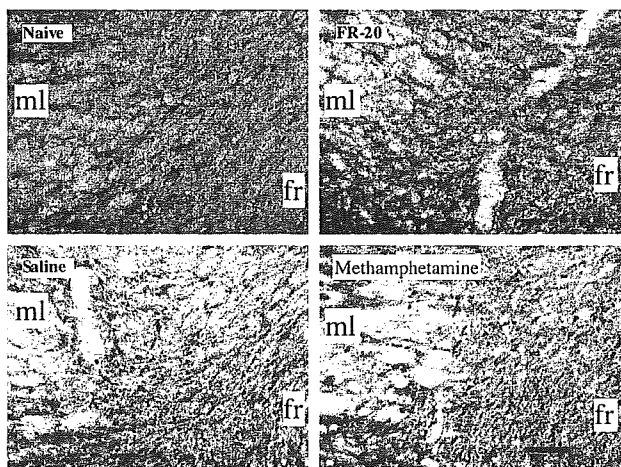


Fig. 3 Representative photomicrographs of Fos immunostaining of the NAc core in rats subjected to the drug discrimination test for methamphetamine. Rats were trained to discriminate methamphetamine (0.5 mg/kg) from saline under the two-lever FR-20 schedule of food reinforcement. The trained rats were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

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 shown 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,

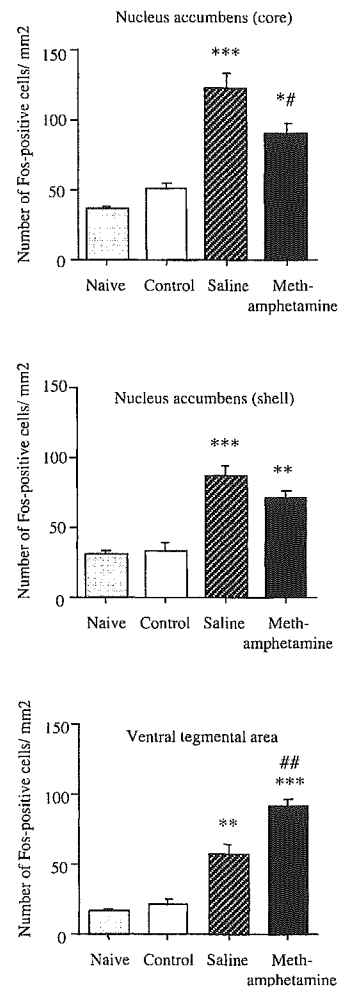


**Fig. 4** Representative photomicrographs of Fos immunostaining of the VTA in rats subjected to the drug discrimination test for methamphetamine. ml, medial lemniscus; fr, fasciculus retroflexus (habenulointerpeduncular tract). Scale bar, 100  $\mu$ m.

regions CA1, CA3 and dentate gyrus of dorsal hippocampus, and lateral habenula (Table 1).

#### 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 (0.5mg/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. Naive rats ( $n=3$ ) were subjected to neither food restriction nor the methamphetamine discrimination training. Each value represents the mean  $\pm$ 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<sup>2</sup>, 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 Final treatment Brain area	saline		methamphetamine	
	saline (n = 4)	methamphetamine (n = 5)	saline (n = 4)	methamphetamine (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 methamphetamine injection was 30. Fos expression in each area is indicated as the number of Fos-positive cells per mm<sup>2</sup>. 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

Brain area	methamphetamine (mg/kg)		
	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<sup>2</sup>. 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 heterogeneous 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  $\gamma$ -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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