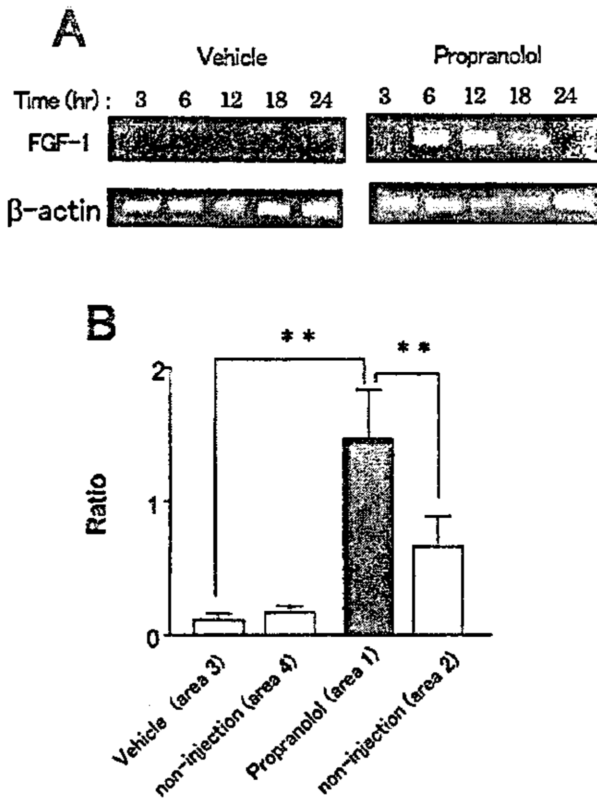

Fig. 8. Time-dependent (A) or site-specific (B) effects of propranolol injection on the expression of FGF-1 mRNA in the adult rat cerebral cortex. Vehicle (PBS) or propranolol, a blocker of adrenergic β-receptors, in PBS (5 μl, 100 μg/μl) was injected into the rat cerebral cortex as illustrated in Figure 7. A: Three animals were killed at the experimental times, and area 1 (propranolol) or 3 (vehicle) was dissected out and its total RNA was prepared. Each RNA was subjected to RT-PCR using primers specific for FGF-1 or β-actin, and PCR products were electrophoresed in a 2% agarose gel. Gels were stained with ethidium bromide, and photographs showing a representative result in each time were shown. B: Area 1, 2, 3, or 4 was dissected out 12 hr after the injection, and total RNA was prepared from these tissue samples. Each RNA was subjected to RT-PCR for FGF-1 or β-actin, and PCR products were analyzed in a 2% agarose gel. The bands corresponding to FGF-1 or β-actin mRNA-derived cDNA were densitometrically quantified, and the ratio of the FGF-1:β-actin band intensity was calculated. The values are expressed as the means ± SE of the five animals. Significant differences of the value of propranolol-injected tissue (area 1) from that of the vehicle-injected one (area 3), or that of the noninjected one of the ipsilateral side (area 2), were determined by Tukey's test. Significance, $*P < 0.05$ and $**P < 0.01$.

one of the ways of neurotransmitters to regulate brain function. Further investigations are necessary to clarify interactions between both signal pathways in FGF-1 gene expression.

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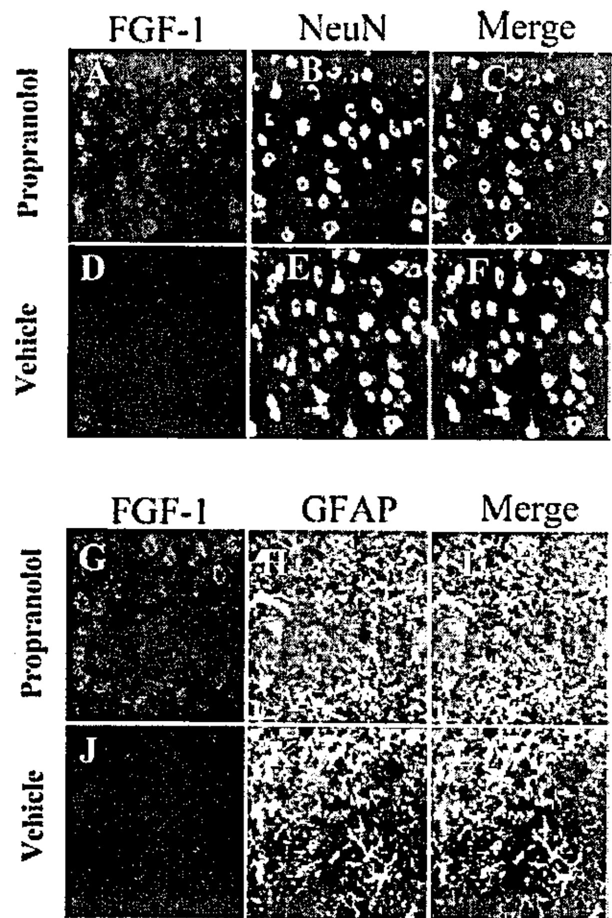

Fig. 9. Effects of propranolol injection on the expression of FGF-1 immunoreactivity in the adult rat cerebral cortex. Propranolol (a blocker of adrenergic β-receptors) or vehicle was injected into the rat cerebral cortex as indicated in Figure 7. Rats were anesthetized 24 hr after the injection, and cardio-perfused with 4% paraformaldehyde solution. The frozen brain tissues were cut into sections. The coronal sections including areas of 1 and 3 were reacted with antibody against FGF-1 (A, D, G, J), NeuN (B, E) or GFAP (H, K), and visualized as described in the text. The photographs of area 3 into which propranolol was administered (A–C, G–I) or area 1 into which vehicle was administered (D–F, J–L) are shown. The staining of FGF-1 (red color) and NeuN (green) are merged in C, F, I, and L. These experiments using three animals in each group were repeated three times. Scale bars = 20 μm.

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Hydrophobic Dipeptide Leu-Ile Protects Against Neuronal Death by Inducing Brain-Derived Neurotrophic Factor and Glial Cell Line-Derived Neurotrophic Factor Synthesis

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We investigated whether certain hydrophobic dipeptides, Leu-Ile, Leu-Pro, and Pro-Ile, which partially resemble the site on FK506 that binds to immunophilin, could stimulate glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) synthesis in cultured neurons and found only Leu-Ile to be an active dipeptide. Leu-Ile protected against the death of mesencephalic neurons from wild-type mice but not from mice lacking the BDNF or GDNF gene. Next, we examined the effects of i.p. or i.c.v. administration of Leu-Ile on BDNF and GDNF contents. Both types of administration increased the contents of BDNF and GDNF in the striatum of mice. Also, peripheral administration of Leu-Ile inhibited dopaminergic (DA) denervation caused by unilateral injection of 6-hydroxydopamine (6-OHDA) into the striatum of mice. The number of rotations following a methamphetamine challenge was lower in the Leu-Ile-treated group than in the nontreated group. Next, we compared the calcineurin activity and immunosuppressant activity of Leu-Ile with those of FK506. Leu-Ile was not inhibitory toward calcineurin cellular activity in cultured neuronal cells. Furthermore, Leu-Ile did not suppress concanavalin A (ConA)-induced synthesis/secretion of interleukin-2 by cultured spleen cells, suggesting that the immunosuppressant activity of Leu-Ile may be negligible when used as a therapeutic tool for neurodegenerative diseases. © 2004 Wiley-Liss, Inc.

Key words: immunophilin; glial cell line-derived neurotrophic factor; brain-derived neurotrophic factor; FK506; Leu-Ile; dopamine; mice

The term *immunophilin* is used to designate receptors for immunosuppressant drugs, and FK506 is one of these drugs (Thomson, 1989; Srarzl et al., 1989). Immunosuppression is used therapeutically for a variety of purposes, one of the most important being the treatment of patients undergoing organ transplantation (Srarzl et al., 1989). Further additional action in the brain has been reported recently. FK506 can reduce ischemic brain damage in rats; for example, the drug can protect rats against quinolinate-induced excitotoxicity (Butcher et al., 1997). These findings suggest that the neuroprotective effects of FK506 may involve mechanisms distinct from N-methyl-D-aspatate (NMDA)-mediated signaling pathways (Butcher et al., 1997). FK506 administration diminished neural tissue damage following middle cerebral artery occlusion in rats (Phillips et al., 2002). Also, FK506 derivatives provided pronounced protection against neurotoxicity elicited by the β-amyloid peptide and serum derivation in cortical cultures (Lee et al., 1999). The ability of FK506 to

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block neurotoxicity in numerous models of important neurological diseases may have clinical relevance. FK 506 penetrates the blood–brain barrier reasonably well (Kochi et al., 1999). However, it would appear to be a poor therapeutic tool for neurodegenerative diseases because of its immunosuppressant activity. In this study, therefore, we looked for new neuroprotective immunophilin ligands without immunosuppressant activity and discovered the dipeptide Leu-Ile to be effective in neuroprotection. The neuroprotective mechanisms of this new ligand were shown to involve the induction of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). BDNF is one of the members of the neurotrophin family of proteins, which includes nerve growth factor (NGF), neurotrophin (NT)-3, NT-4/5, and NT-6 (Leibrock et al., 1989; Hohn et al., 1990; Hallbook et al., 1991). BDNF affects the survival or differentiation of cultured motor neurons, mesencephalic dopaminergic neurons (Knusel et al., 1991), and septal cholinergic neurons. In adult rats, BDNF mRNA is more widely distributed in the whole brain than the mRNA of NGF or NT-3 and is regulated by glutamate or γ-aminobutyric acid neurotransmission (Phillips et al., 1990). Enhanced expression occurs following the establishment of long-term potentiation (Zafra et al., 1990; Rutherford et al., 1997). BDNF thus seems to participate in various activity-dependent events, including synapse plasticity. On the other hand, GDNF produced by a glial cell line (rat B49) is a factor that can affect dopaminergic neurons (Schubert et al., 1974). GDNF can promote the survival and function of dopamine neurons in vivo, in both the intact rat brain and after nigrostriatal lesioning (Hoffer et al., 1994; Bowenkampt et al., 1995; Jonhansson et al., 1995; Linder et al., 1995; Tomac et al., 1995; Collins et al., 1996; Granholm et al., 1997a,b). It was also shown that GDNF is secreted in the target (striatum) and transported retrogradely to the DA cell bodies in the mesencephalon (Tomac et al., 1996). These results suggest that GDNF may be effective against dopaminergic degeneration. Therefore, GDNF is expected to be useful as a therapeutic tool for dopaminergic neurological disorders.

BDNF and GDNF would seem to be very useful proteins for the treatment of various neurological disorders. However, there is an important obstacle to their therapeutic application; BDNF and GDNF are macromolecules that cannot pass through the blood–brain barrier, so it is difficult to deliver them from the periphery to the brain. Previously we reported on some stimulators for the synthesis of neurotrophic factors (Nitta et al., 1993, 1994, 1999a, 2002). Because the dipeptide Leu-Ile, designed from a part of FK506, provided neuroprotection attributable to induction of BDNF and GDNF, we also examined its immunosuppressant action and found it to have none. Therefore, this peptide appears to be a promising therapeutic tool for the treatment of various neurological disorders.

MATERIALS AND METHODS

Materials

FK506, GDNF, and BDNF were donated by Fujisawa Pharmaceutical Co., Ltd. (Tokyo, Japan), Amgen (Thousand Oaks, CA), and Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan), respectively. Dipeptides, including Leu-Ile, Leu-Pro, and Pro-Ile, which partially resemble the binding site of FK506, were purchased from Kokusan Chemical Co. Ltd. (Tokyo, Japan). All other materials used were of reagent grade. Rats and mice were purchased from Nippon SLC (Shizuoka, Japan). Mice lacking the BDNF gene were purchased from the Jackson Laboratory (Bar Harbor, ME) and mated. The generation of GDNF knockout mice was described elsewhere (Picher et al., 1996). All animals were treated according to the Guideline of Experimental Animal Care issued from the Office of the Prime Minister of Japan.

Cell Culture

Dopaminergic neurons were cultured from 13-day-old rat embryos as described previously (Nitta et al., 1999a,c). We selected the mesencephalic neuron population, which is rich in doapminergic neurons, because BDNF and GDNF have neuroprotective effects on and are synthesized by the dopaminergic neurons (Knusel et al., 1991; Hoffer et al., 1994; Bowenkampt et al., 1995; Jonhansson et al., 1995; Linder et al., 1995; Tomac et al., 1995; Collins et al., 1996; Granholm et al., 1997a,b). Antibody specific for tyrosine hydroxylase (TH; Chemicon, Temecula, CA) was used to identify dopaminergic neurons.

Enzyme Immunoassay

BDNF content was measured by an enzyme immunoassay (EIA) method (Nitta et al., 1999b,c). The EIA system for GDNF was based on the method originally developed for the EIA of NGF, BDNF, and NT-3 (Furukawa et al., 1983; Kaechi et al., 1993; Nitta et al., 1999b).

Antibodies against GDNF were produced by immunizing rabbits with purified human recombinant GDNF. GDNF protein (0.5 mg each) in phosphate-buffered saline (PBS; 5 ml) was emulsified with an equal volume of Freund's adjuvant and injected intradermally into rabbits four times at 2-week intervals. All blood was collected 1 week after the final injection. Antiserum (1 ml) was loaded onto a GDNF-linked column (1-ml bed volume; Affi-Gel 10; Bio-Rad, Hercules, CA). After extensive sequential washing with three types of loading buffer, i.e., 1) 0.1 M Tris-HCl (pH 7.4) containing 0.9% NaCl, 2) 0.05 M borate buffer (pH 8.0), and 3) 0.05 mM sodium acetate buffer (pH 5.0), the bound antibodies were eluted with 0.1 M glycine-HCl buffer (pH 2.0). A part of the purified anti-GDNF antibody preparation was eluted, biotinylated, and used as the secondary antibody.

For the determination of BDNF and GDNF contents in the conditioned medium and brain tissue, multiwell plates (Falcon 3910) were incubated with 5 μl of anti-BDNF or GDNF antibody in 0.1 M Tris-HCl buffer (pH 9.0, 10 μg/ml) per well overnight and washed with washing buffer [0.1 M Tris-HCl, pH 7.4, containing 1% (w/v) skim milk]. Sample or standard in washing buffer was then added to each antibody-coated well, and the plate was incubated for 12–18 hr at 4°. The well was

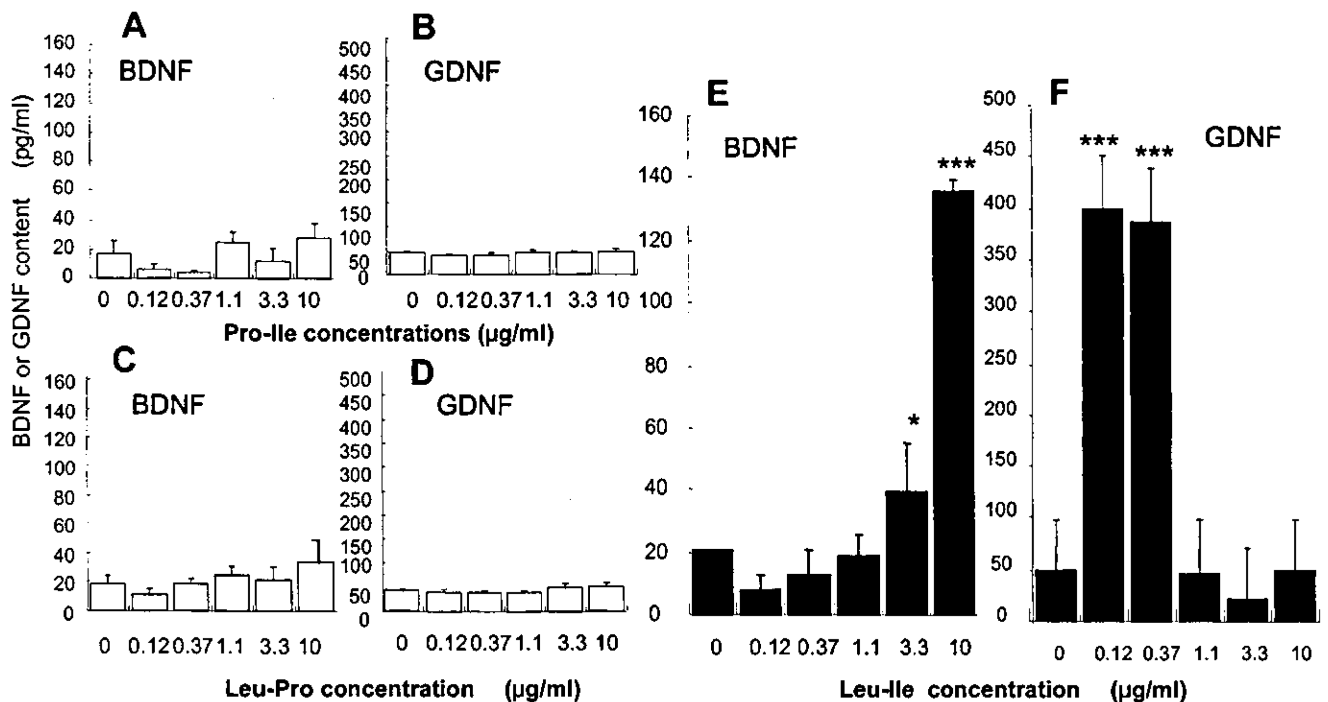

Fig. 1. Effects of dipeptides Pro-Ile, Leu-Pro, and Leu-Ile on BDNF and GDNF contents in medium from cultures of mesencephalic neurons. Neurons of the mesencephalon of 13-day-old rat embryos were cultured in the serum-free defined medium containing various concentrations of dipeptides. Conditioned media were taken 24 hr after the addition of each dipeptide, and their BDNF and GDNF contents were measured by EIAs. Values (n = 6) are expressed as mean ± SE. Three peptides, Pro-Ile (**A,B**), Leu-Pro (**C,D**), and Leu-Ile (**E,F**), were estimated for their inducing effects on the synthesis of BDNF (A,C,E) and GDNF (B,D,F). *$P < 0.05$, ***$P < 0.005$ vs. nontreated neurons (Kruskal-Wallis test).

then washed with the washing buffer and incubated with the biotinylated secondary antibodies for 5 hr at 4°. After further washing with the washing buffer, avidin-conjugated β-galactosidase (Roche) was added to each well, and incubation was carried out for 1 hr. Then, after thorough washing with the washing buffer, the enzyme activity retained in each well was measured by incubation with a fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside (100 μM), dissolved in the washing buffer. The intensity of fluorescence was monitored with 360-nm excitation and 488-nm emission. The detection limit of the EIAs was as low as 1 pg/ml.

Preparation of Samples for EIA

The conditioned medium from cultured neurons was directly applied to the EIA system. Brain tissue from mice was added to sonication buffer (0.1 M Tris-HCl, pH 7.4, containing 1 M NaCl, 2% bovine serum albumin, 2 mM EDTA, 0.2% Na_3N) at the ratio of 1 g wet weight per 19 ml buffer, pulse-sonicated for 30 sec, and centrifuged at 100,000*g* for 30 min. The supernatant was then mixed vigorously with 100 μl chloroform and centrifuged at 20,000*g* for 15 min, after which the aqueous phase and cells were taken and kept in a deep freeze and used for the EIA measurements.

Unilateral 6-Hydroxydopamine-Induced Lesions

Twenty mice received 6-hydroxydopamine (6-OHDA; 20 μg/2 μl/mouse, calculated as free base; Sigma, St. Louis, MO) dissolved in ascorbate-saline (0.05%), and injected into the right substantia nigra. The injection rate was 0.4 μl/min, and the tip of the microsyringe (Hamilton 3020) was left in place for an additional 3 min before it was slowly retracted.

Behavioral Analysis

All rotational testing was in a glass cylinder (diameter was 20 cm). Control and lesioned mice were allowed to rest for 15 min to adapt to the testing environment and then were injected i.p. with 10 mg/kg methamphetamine sulfate (Dainippon Co. Pharmaceutical Ltd., Osaka, Japan) dissolved in PBS. Measurement of rotational activity began 10 min after the injection and lasted for 10 min. The number of rotations was recorded during the test period. Clockwise turns (ipsilateral to the lesion) were counted as turns. After the behavioral test, the brains of these mice were used for the immunostaining for TH.

Preparation of Splenic Lymphocytes

The preparation of splenic lymphocytes and measurement of interleukin-2 were carried out as described earlier (Sugiura et al. 2000). All procedures were conducted under aseptic conditions. Each group included four mice. Mice were sacrificed by cervical dislocation under ether anesthesia, and each single-cell suspension was prepared by pressing the spleen between two side glasses. The cell suspensions were passed through a 200-gauge stainless-steel sieve and then allowed to stand for 10 min

to remove tissue fragments. The cell suspensions were then centrifuged (600g for 10 min) and resuspended gently in fetal calf serum (FCS)-RPMI 1640 (Sigma) to a concentration of 4.0×10^6 viable cells/ml. The viability of the spleen cells, as determined by the trypan blue dye exclusion test, was greater than 95%.

Production of IL-2

One hundred microliters of spleen cell suspension at a concentration of 4.0×10^6 cells/ml were incubated with concanavalin A (ConA; 5 μg/ml) for 24 hr in an incubator at 37° with 5% CO_2 in humidified air. The IL-2 content in cultured supernatants was measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit for mouse IL-2 (Genzyme, Cambridge, MA).

Calcineurin Cellular Activity Assay

Calcineurin activity was measured by use of a calcineurin cellular activity assay kit (Calbiochem-Novabiochem Corporation, La Jolla, CA) as specified by the manufacturer. The cultured hippocampal neurons 5 days after the start were used for the measurement of calcineurin activity. Two hours after the addition of FK506 or Leu-Ile to the cultures, the cells were harvested and used as samples to be measured for calcineurin activity.

Statistical Analysis

Data are expressed as the mean ± SEM. ANOVA with Kruskal-Wallis test was used to establish statistical significance, set at $P < 0.05$.

RESULTS

Inducing Effects of Dipeptides on the Production of BDNF and GDNF in Cultured Hippocampal Neurons

In neuronal cultures (cell density 10^7 cells/mm^2), BDNF and GDNF contents were 16.8 ± 2.3 pg/ml and 45.2 ± 3.5 pg/ml, respectively, in medium conditioned for 1 day without any dipeptides. As shown in Figure 1A–D, neither Pro-Ile nor Leu-Pro affected BDNF and GDNF contents in the conditioned media from the cultured neurons 24 hr after the treatment, whereas Leu-Ile significantly increased both of them (Fig. 1E,F). The BDNF content was increased to 133.1 ± 23 pg/ml by Leu-Ile at the concentration of 10 μg/ml (Fig. 1E). As for GDNF, 0.12 and 0.37 μg/ml Leu-Ile increased its content to an amount about eight times that of the nontreated cells, whereas higher concentrations were ineffective (Fig. 1F).

Neuroprotective Effects of Leu-Ile Against Natural Neuronal Cell Death Among the Cultured Mesencepalic Cells

Neuronal cells seeded at a low density (3×10^4 cells/cm^2) were cultured for 3 days; during this period, they gradually died, the number of surviving cells decreasing to 50% of that at the beginning of the culture (Fig. 2). In the mesencephalic cells cultures, Leu-Ile protected against neuronal cell death in terms of both total cells and TH-positive cells (Fig. 2A). This result suggests that Leu-Ile can protect both dopaminergic and nondopaminergic neurons. Leu-Ile provided no neuroprotection

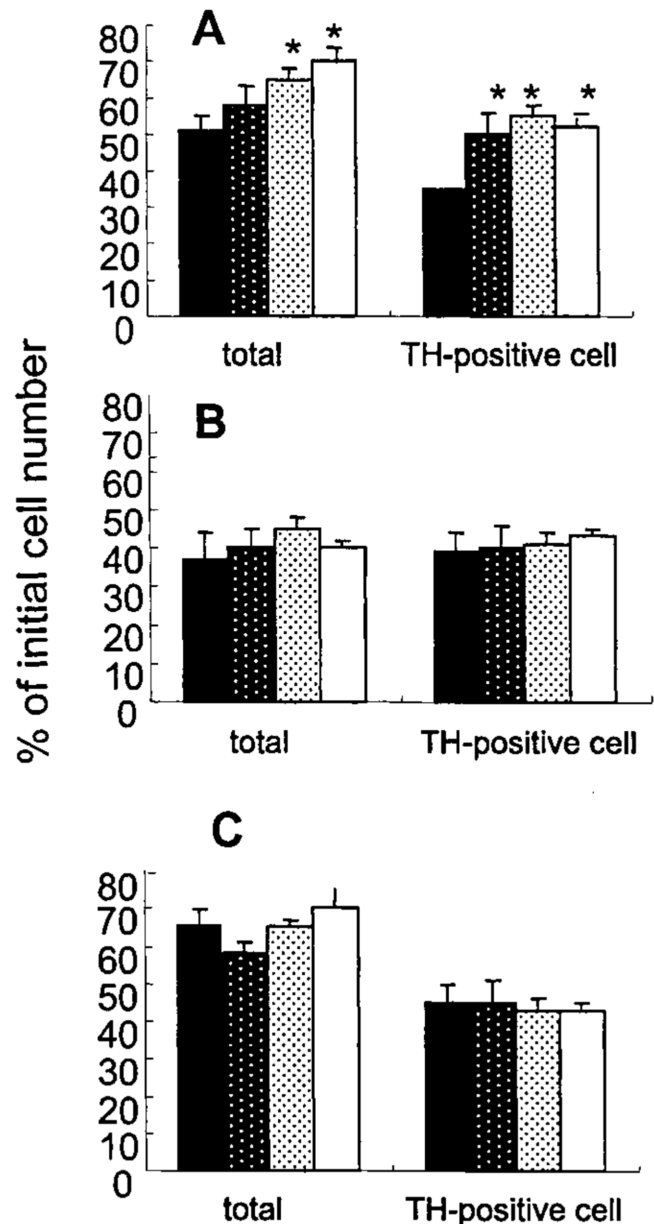

Fig. 2. Effects of Leu-Ile on the survival of mesencephalic neurons. Three-day cultures of mesencephalon from normal rats (**A**) or rats lacking the BDNF (**B**) or GDNF (**C**) gene were incubated with Leu-Ile for 24 hr. The number of surviving cells was then counted in eight arbitrarily selected fields (0.3 mm^2 each). The number in each field was averaged for each culture dish and was expressed as the mean ± SE (n = 5) percentage of the value of the initial cell numbers. Solid bars, Leu-Ile, 0 μM; hatched bars, Leu-Ile, 1 μM; stippled bars, Leu-Ile, 10 μM; open bars, Leu-Ile, 100 μM. *$P < 0.05$ vs. Leu-Ile 0 μM treated neurons (Kruskal-Wallis test).

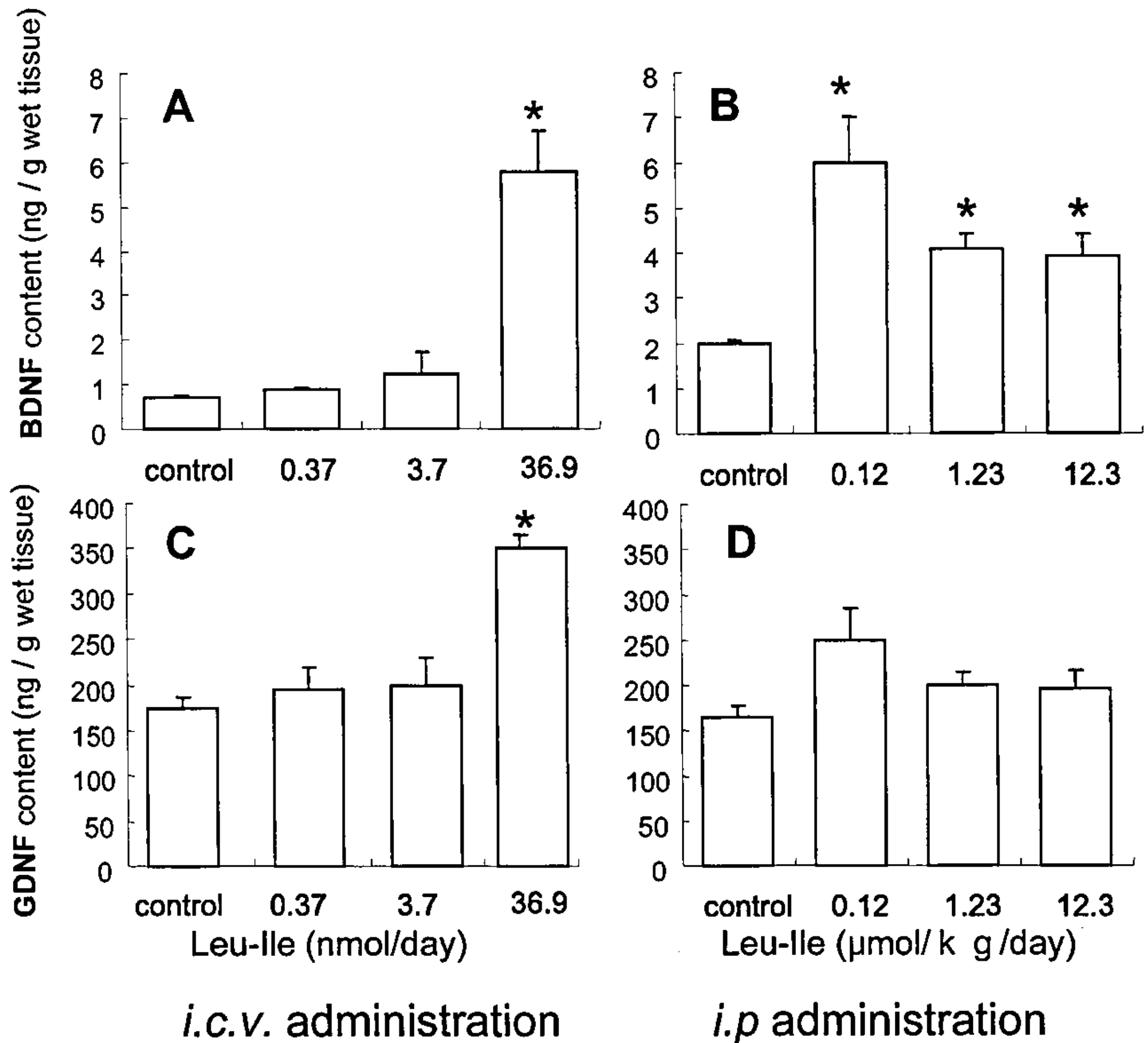

Fig. 3. Effects of Leu-Ile on the BDNF (**A,B**) and GDNF (**C,D**) contents in the rat striatum after i.c.v. (A,C) or i.p. (B,D) injections given once per day for 5 days. Each rat was decapitated 24 hr after the last injection, and the striatum was removed to prepare samples for measurements of BDNF and GDNF. $*P < 0.05$ vs. control (Kruskal-Wallis test).

of the mesencephalic neurons from mice lacking the BDNF or GDNF gene (Fig. 2B or C, respectively).

Effects of i.c.v. and i.p. Administration of Leu-Ile on the BDNF and GDNF Contents in the Mouse Striatum

Next, we investigated the inducing effect of Leu-Ile on BDNF and GDNF contents in the mouse striatum after i.p. or i.c.v. administration of the dipeptide once per day for 5 days (Fig. 3). Both routes of administration of Leu-Ile increased the BDNF and GDNF contents in the striatum. The i.p. administration required a much higher dose of Leu-Ile than the i.c.v. administration for the same effects. With i.c.v. administration, BDNF and GDNF contents in the striatum were increased dose dependently by Leu-Ile. However, by the i.p. route, 0.12 μmol/kg/day Leu-Ile increased the content of both, whereas higher concentrations were less stimulatory.

Effects of Leu-Ile on the Rotation Behavior Caused by 6-OHDA-Induced Dopaminergic Neuronal Degeneration

Mice with 6-OHDA-induced damaged to the dopaminergic neuronal system in their striatum were allowed to rest for 15 min to adapt to the testing environment and then were injected i.p. with 10 mg/kg methamphetamine

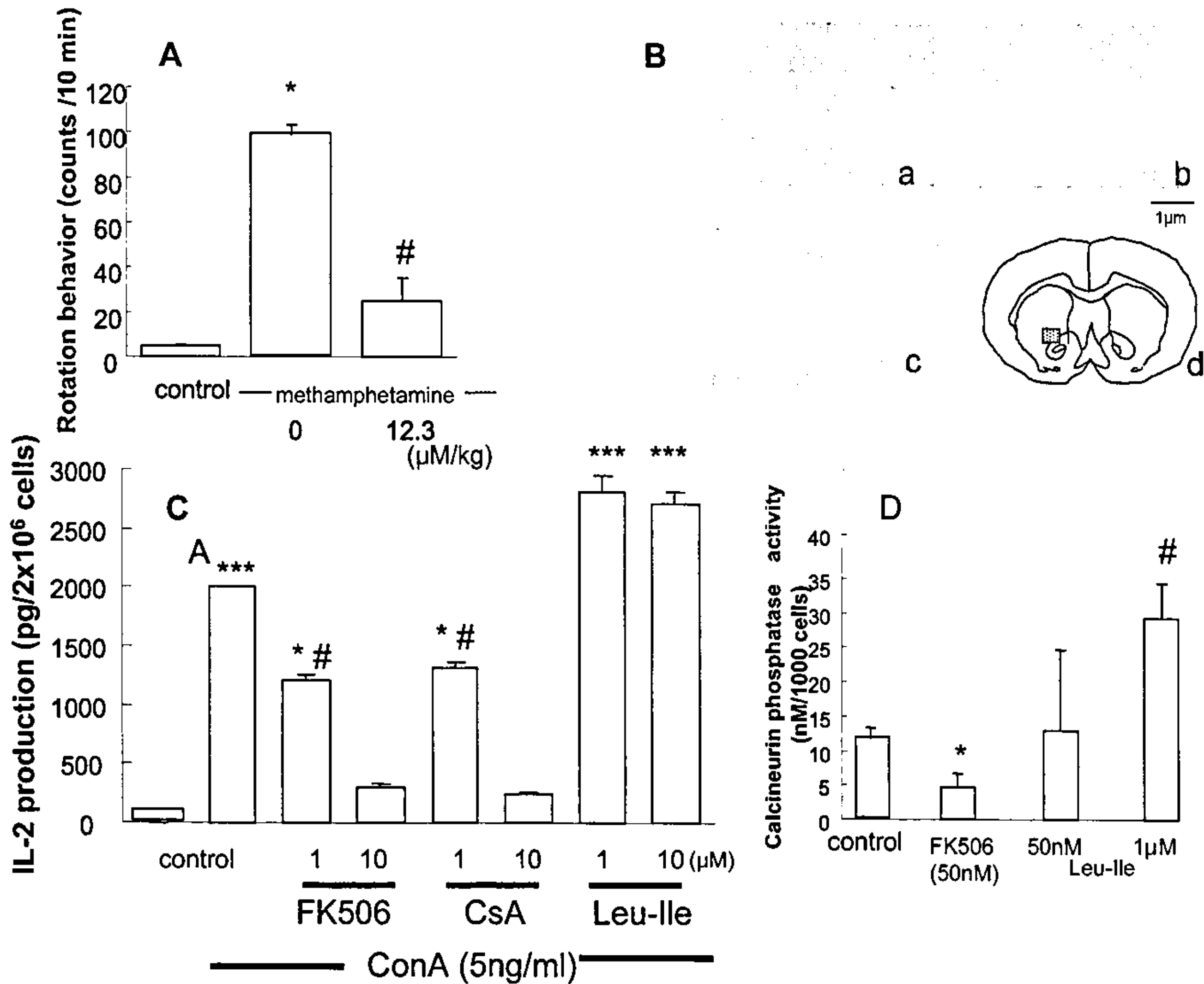

Fig. 4. **A:** Protective effects of Leu-Ile on mice lesioned with 6-OHDA. Each mouse received Leu-Ile i.p. for 10 days once per day after lesioning by 6-OHDA. Twenty-four hours after the last administration of Leu-Ile, the mice were challenged with methamphetamine (1.0 mg/kg). $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. 6-OHDA lesioned group (Kruskal-Wallis test). **B:** Effects of Leu-Ile on the dopaminergic denervation induced by 6-OHDA. The procedure was as described for A. a, Control; b, 6-OHDA injection and treatment of vehicle; c, 6-OHDA injection and treatment of Leu-Ile; d, indication of region for a–c. **C:** Effects of various immunophilin ligands on IL-2 produced by ConA-stimulated splenic lymphocytes. The procedure for preparation of the lymphocytes is described in Materials and Methods. The media were taken 24 hr after the stimulation with ConA. IL-2 concentration in the medium was measured by ELISA. $^{*}P < 0.05$ and $^{***}P < 0.005$ vs. control; $^{\#}P < 0.05$ vs. only ConA stimulation (Kruskal-Wallis test). **D:** Effects of Leu-Ile and FK506 on calcineurin activity in the cultured neurons. The cells were taken 30 min after the addition of FK506 or Leu-Ile. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. FK506-treated group (Kruskal-Wallis test).

dissolved in PBS. Measurement of rotation activity began 10 min after the injection. Leu-Ile reduced the number of methamphetamine-induced turns experienced by the animals 10 days after the striatum and substantia nigra lesions (25 ± 3), compared with the number for control animals that received the vehicle only (97 ± 4, Fig. 4A). Immnostaining for TH in the striatum (Fig. 4Bd) was carried out using the brains after the behavioral test. TH-positive cells were found in the striatum in the control mice (Fig. 4Ba). 6-OHDA decreased these positive cells 10 days after the injection (Fig. 4Bb). Repeated i.p. administration of Leu-Ile protected the reduction of dopaminergic neurons (Fig. 4Bc).

IL-2 Induction

Cultured splenic lymphocytes produced IL-2 at a concentration of less than 1 pg/ml under normal culture conditions. The content of IL-2 in the medium was dramatically increased to 2,000 pg/ml by 24 hr after ConA stimulation. Both FK-506 and cyclosporine A

(CsA) tested at 1 and 10 μM inhibited the induction of IL-2 dose dependently, whereas Leu-Ile (1 and 10 μM) was not inhibitory.

Calcineurin Activity

FK506 at a concentration of 50 nM inhibited calcineurin activity in the cultured neurons. This concentration of FK506 was earlier reported to be the lowest effective concentration (Gaymes et al., 1997). Leu-Ile did not inhibit this activity at a concentration of either 50 nM or 1 μM, the latter of which was the effective concentration for the stimulation of GDNF production by the cultured neurons.

DISCUSSION

Immunophilins are used to define receptors for immnunosuppressant drugs such as CsA and FK506. In clinical fields, immunosuppression is used therapeutically for a variety of purposes. We have already reported that FK506 induces the synthesis of NGF, BDNF, and GDNF (Nitta et al., 2002). However, FK506 itself cannot be used for neuronal disorders, such as Parkinson's disease or Alzheimer's disease, because of its immunosuppressant actions. For this study, we selected three hydrophobic peptides, i.e., Leu-Ile, Leu-Pro, and Pro-Ile, because they are resemble the binding site of FK506 for immunophilin or FK-binding protein (Schreiber et al., 1991). As shown in Figure 1, only Leu-Ile increased the contents of BDNF and GDNF in the medium of the cultured neurons. Cameron et al. (1997) reported that FKBP12 binds inositol-1,4,5-trisphosphate receptors (IP3R) at Leu-Pro of FK506 and anchors calcineurin to this FK506-like domain. Immunosuppression appears to stem from the binding of the FK506-FKBP complex to the calcium-activated phosphatase (calcineurin), which inhibits its catalytic activity and results in the accumulation of phosphorylated calcinurin substrate. However, the present results show that Leu-Pro had no effects on the induction of BDNF or GDNF. The induction of BDNF and GDNF appears to be independent of the calcinurin activity, in that our results show that Leu-Ile did not inhibit the calcineurin activity and was not immunosuppressive. We have already found that Leu-Ile binds some transcription factors and regulates the apoptosis pathway (Nitta et al., unpublished data). Furthermore, Leu-Ile cannot bind to FKBP12 (Nitta et al., unpublished data). Immunophilin ligands regulate the immunosuppressive action via inhibition of calcineurin activity. Leu-Ile has inducible effects of calcineurin activity, and then immunological activity was increased. At the beginning of this study, Leu-Ile was designed to resemble the binding site of FK506 for FKBP; however, our results show that it is not an immunophilin ligand.

Next, we investigated the effects of Leu-Ile on the mouse brain after i.p. or i.c.v. injection. We had assumed that dipeptides such as Leu-Ile would not be stable in the blood stream and not be able to reach the brain tissue when they were peripherally administered. However, BDNF and GDNF contents in the striatum of mice were increased after repeated i.p. or i.c.v. injections. These results suggest that Leu-Ile can pass through the blood–brain barrier and initiate the synthesis of BDNF and GDNF in the brain. We attempted to obtain pharmacokinetic data by using Leu-Ile-labeled with a fluorescent marker at its N- or C-terminals; however, we were unable to detect it in the brain after i.p. injecton. The brain level of fluorescent Leu-Ile might have simply been too low to be detectable after the injection under physiological conditions.

The neuroprotective effects of Leu-Ile on the cultured neurons are at least partially the result of the induction of BDNF and GDNF, because the effect was not found when neurons from mice lacking the BDNF or GDNF gene were used. Some stimulators of neurotrophic factors have survival effects on cultured neurons (Nitta et al., 1997). For example interleukins-2, -4, and -5, all of which can induce NGF synthesis, also protect against neuronal death (Awatsuji et al., 1993ab). Furthermore, 4-methylcatechol (4MC), another stimulator of NGF and BDNF synthesis, also protects cultured neurons from dying, and 4MC enhances neuronal differentiation during brain development in rats (Fukumitsu et al., 1999; Nitta et al., 1999a; Sometani et al., 2002). The present data on mice lacking neurotrophic factor genes provide the first direct evidence that a stimulator of neurotrophic factors provides neuroprotection via such induction of neurotrophic factor synthesis.

The neurotrophic effects of the immunophilin ligands have been demonstrated in animal models of neurological disability. For example, FK506 stimulated regrowth of damaged sciatic nerves and functional recovery (Gold et al., 1994; Steiner et al., 1997a,b). We also demonstrated that FK506 had neuroprotective effects in mice with brain lesions induced by 6-OHDA (Nitta et al., 2002). Nonimmunosuppressive derivatives of FK506, e.g., GPI-1046, V-10,367 and L685,818, were also effective (Gold et al., 1997). Lesions made with 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) or 6-OHDA elicit massive destruction of nigral doapminergic neurons (Schreiber, 1991; Gerlach et al., 1991; Gold et al., 1997; Rose et al., 2001). These are the same neurons that degenerate in patients with Parkinson's disease, making MPTP and 6-OHDA treatments experimental paradigms for the study of this disorder. Treatment with GPI-1046 before, during, or after the MPTP lesions substantially restored the damaged dopaminergic neurons, as demonstrated by increased striatal TH staining and reversal of haloperidol-induced catalepsy and akinesia (Gerlach et al., 1991). In rats lesioned by the unilateral intranigral administration of 6-OHDA, GPI-1046 elicited both morphological and functional recovery, with increased striatal catecholamine levels and a reduction in amphetamine-induced rotations (Gerlach et al., 1991). These pharmacological actions of GPI-1046 are attributed to the blocking of the calcineurin activity without the induction of neurotrophic factors. In present study, Leu-Ile also protected the dopaminergic denervations induced by 6-OHDA. Both GPI-1046 and Leu-Ile are compounds

related to FK506. However, their neuroprotective actions result from the different pathways, insofar as Leu-Ile has no effect on calcineurin activity (Fig. 4D). Thus, Leu-Ile should not be considered an immunophilin ligand but should be defined as a novel neurotrophic ligand.

The major 28-amino-acid neuropeptide known as vasoactive intestinal peptide (VIP) provides neuroprotection against the neuronal cell death induced by β-amyloid proteins (Gozes et al., 1999). The results of structure–function analysis of VIP indicated that, for most activities, the entire sequence of the peptide is required for full biological function. An active site comprising four amino acids has been discovered, and it mimics the neuroprotective activity of the 28-amino-acid peptides. The sequence of this four-amino-acid site is Ser-Ile-Leu-Asn, suggesting that the pair of Leu and Ile might be necessary (Gozes et al., 1999). Taken together, these suggestions and our present results suggest that Leu and/or Ile may be essential, key amino acids for the neuroprotective effects of neuropeptides. Leu-Pro and Pro-Ile did not provide the neuroprotection against natural death of cultured neurons. However, these peptides might have a neuroprotective effects on damaged neurons.

In conclusion, Leu-Ile, resembling part of the binding site on FK506 for FKBP12, had neuroprotective effects both in vivo and in vitro attributable to the induction of BDNF and GDNF synthesis. This dipeptide may thus have therapeutic potential in a wide range of neurological diseases. Conceivable targets include diabetic neuropathy, spinal cord injury, amyotrophic lateral sclerosis, and stroke. BDNF and GDNF have been used for therapy of these neurological diseases. However, delivering their potency to the central nervous system is a quite different matter. Leu-Ile should prove to be very useful clinically.

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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.

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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

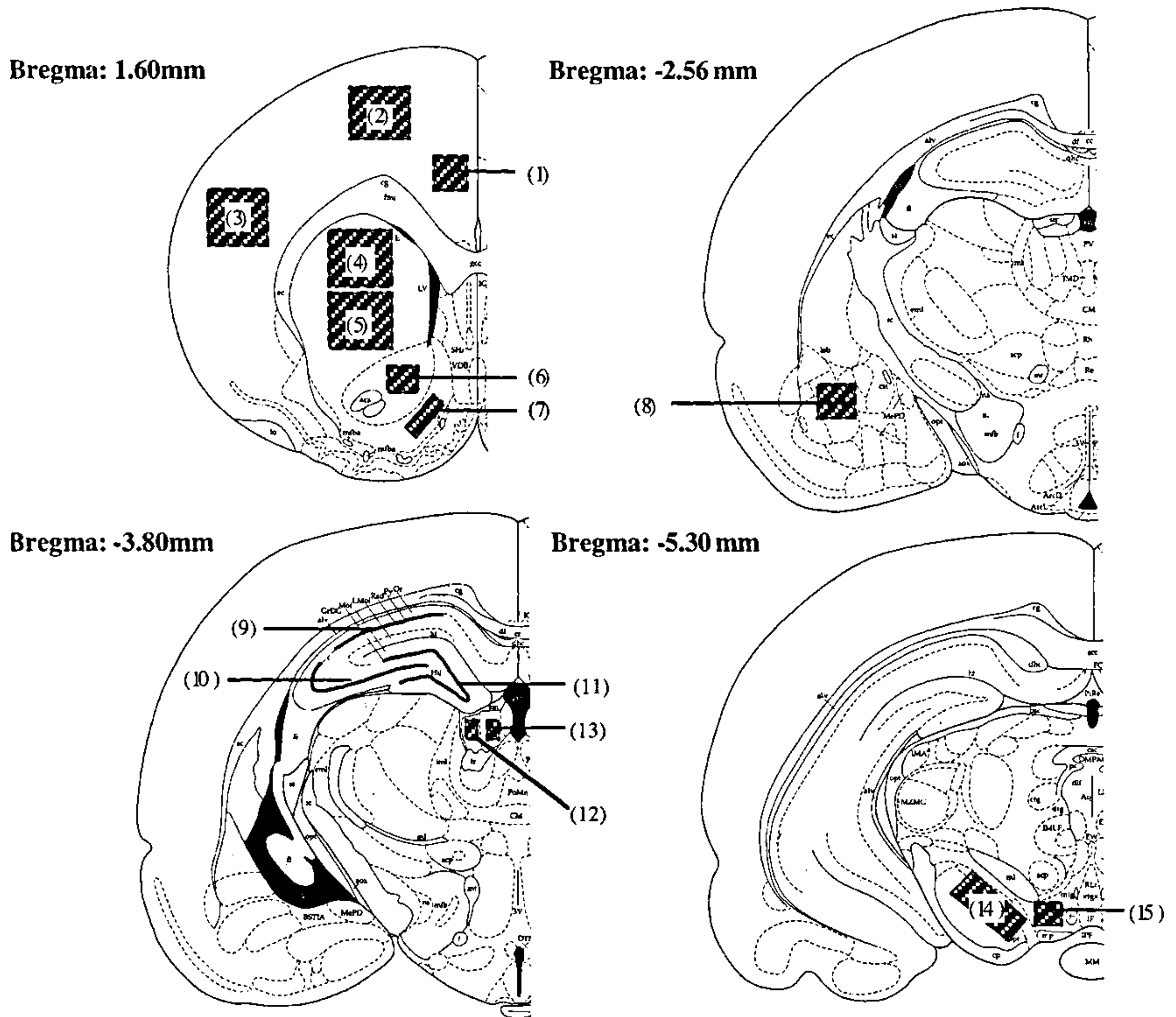

Fig. 1 Diagrammatic representation of the brain areas examined for Fos immunohistochemistry. The areas examined for enumerating Fos-positive cells include the cingulate (1), motor (2) and somatosensory cortex (3), the dorsal (4) and ventral caudate putamen (5), the core (6) and shell (7) of the nucleus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

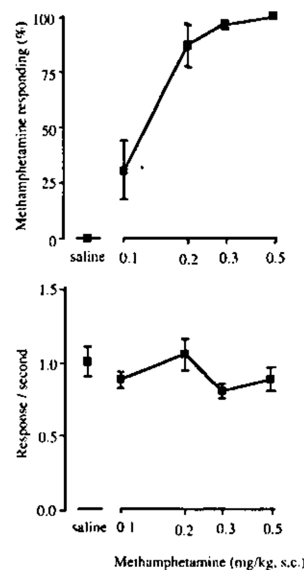

Fig. 2 Dose-dependent discriminative stimulus effects of methamphetamine in rats. Each point represents the mean ± SE ($n = 8$).

Fos expression associated with the discriminative stimulus effects of methamphetamine

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

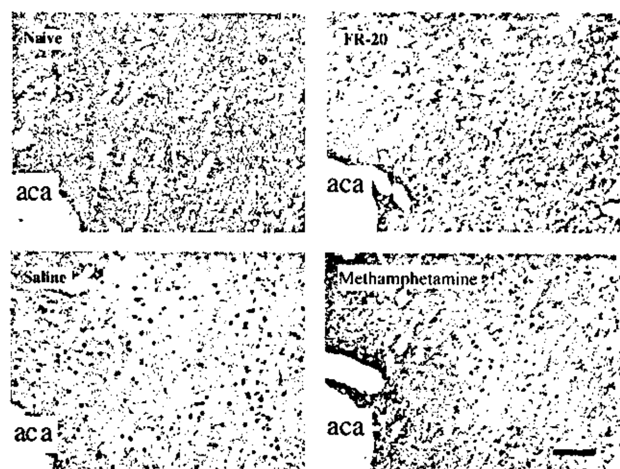

Fig. 3 Representative photomicrographs of Fos immunostaining of the NAc core in rats subjected to the drug discrimination test for methamphetamine. Rats were trained to discriminate methamphetamine (0.5 mg/kg) from saline under the two-lever FR-20 schedule of food reinforcement. The trained rats were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

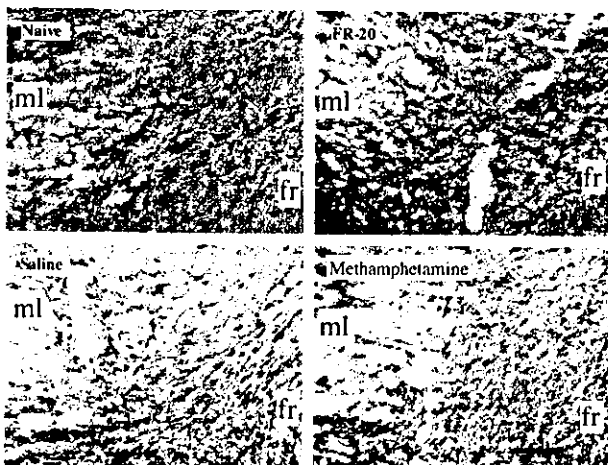

Fig. 4 Representative photomicrographs of Fos immunostaining of the VTA in rats subjected to the drug discrimination test for methamphetamine. ml, medial lemniscus; fr, fasciculus retroflexus (habenulointerpeduncular tract). Scale bar, 100 μm.

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

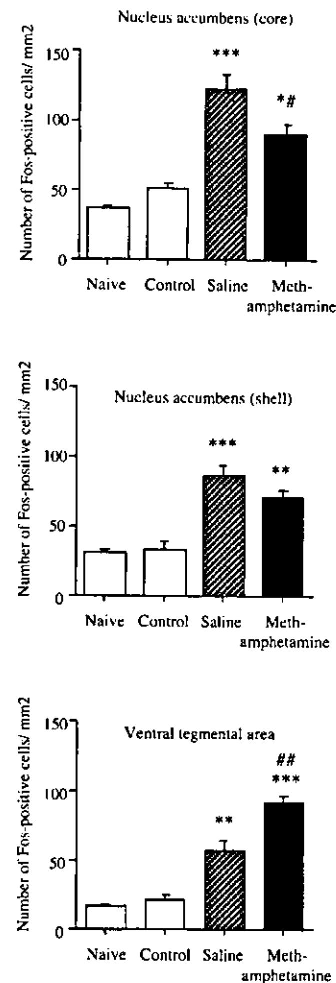

Fig. 5 Changes in Fos expression induced by discriminative stimulus effects of methamphetamine in the core and shell of the NAc and VTA. Rats were trained to discriminate methamphetamine (o.5mg/kg) from saline under the two-lever FR 20 schedule of food reinforcement. The trained rats were subjected to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

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