MOR-induced place preference in C57BL/6J mice [14]. The involvement of GDNF in the rewarding effects of MOR and the inhibitory effects of Leu-Ile on MOR-induced place preference were examined in GDNF-(+/−) mice. The experimental schedule is described in Fig. 2A. As shown in Fig. 2B, at the low dose of MOR (3 mg/kg, s.c.), GDNF-(+/−) mice developed place preference, although littermate control GDNF-(+/+) mice did not ($F_{(7,86)} = 7.6, p < 0.05$, one-way ANOVA). When Leu-Ile (1.5 and 15 μmol/kg, i.p.) was administered 1 h before MOR, it failed to exhibit a significant effect on the action of MOR in GDNF-(+/−) mice (Fig. 2B). We measured the GDNF levels in the NAc after CPP test using EIA methods. As shown in Fig. 2C, in the NAc of GDNF-(+/+) mice, administration of MOR (3 mg/kg, s.c.) during conditioning phase in CPP test increased GDNF levels compared with vehicle/saline-treated mice ($F_{(7,32)} = 12.1$, $p < 0.05$, one-way ANOVA). Moreover, Leu-Ile increased GDNF levels in combination with MOR (3 mg/kg, s.c.) in CPP paradigm compared with vehicle/saline-treated GDNF-(+/+) mice (Fig. 2C). Conversely, in the NAc of GDNF-(+/−) mice, we confirmed that administration of MOR (3 mg/kg, s.c.) during conditioning phase in CPP test, which could develop place preference, failed to increase GDNF levels. Moreover, Leu-Ile, which could not inhibit rewarding effects of MOR, also failed to increase GDNF levels in combination with MOR in CPP paradigm (Fig. 2C). These results suggest that GDNF acts to negate the rewarding effects of MOR and is involved in the effects of Leu-Ile on the rewarding effects.

4. Discussion

GDNF enhances the survival and maintains the differentiated properties of dopaminergic neurons in cell cultures. The use of GDNF appears to be a promising strategy to promote the survival and function of the nigrostriatal dopaminergic pathway damaged in Parkinson's disease [6]. Transplantation of simian virus-40 glial cells, which produces and secretes GDNF, or delivery of GDNF-conjugated nanoparticles into dorsal and ventral striatum impairs the acquisition of cocaine self-administration in rats [3,4]. The upregulation of the GDNF pathway in the midbrain, is the molecular mechanism by which the putative anti-addiction drug ibogaine mediates its desirable action of reducing ethanol consumption [5]. Infusion of GDNF into the VTA blocks certain biochemical adaptations (induction of TH, NR1 subunit of *N*-methyl D-aspartate receptors, ΔFosB and protein kinase A catalytic subunit) to chronic cocaine or MOR treatment as well as cocaine-induced place preference [10]. Conversely, responses to cocaine are enhanced in rats by intra-VTA infusion of anti-GDNF antibody and in GDNF-(+/−) mice [10]. GDNF has pronounced effects on the dopaminergic system *in vivo*, including neuroprotective effects against METH-induced neurotoxicity [20]. However, as said at the beginning of this article, GDNF cannot be used directly as a therapeutic tool for drug dependence.

Recently, we have demonstrated that Leu-Ile, which induces the expression of TNF-α and GDNF, inhibits METH and MOR-induced sensitization and rewarding effects [14,15]. In the present study, to extend our findings, we examined the involvement of GDNF in the inhibitory effects of Leu-Ile on MOR-induced sensitization and rewarding effects.

GDNF levels in the striatum are increased by the intracerebroventricular administration of Leu-Ile in rats [13]. Expression levels of GDNF mRNA are significantly elevated 24 h after Leu-Ile treatment in cultured neurons compared with the control group and GDNF levels after the co-administration of Leu-Ile and METH are significantly increased compared with those in the vehicle/METH-treated mice [15]. Leu-Ile inhibits the MOR-induced locomotor sensitization, at least in part, through the action in the NAc, since it has inhibitory effects on the repeated MOR treatment-induced increase in extracellular DA levels [14]. In the present study, GDNF levels in the NAc were determined after the co-administration of Leu-Ile and MOR using the EIA method. Leu-Ile potentiated MOR-induced increase in GDNF levels (Fig. 1) in addition to TNF-α [14] in the NAc. GDNF inhibits the drug-induced upregulation of tyrosine hydroxylase activity [10]. TNF-α activates plasmalemmal and vesicular DA transporter [11]. Thereby, we suggest that GDNF and TNF-α induced by Leu-Ile attenuate the MOR-induced increase in extracellular DA levels in the NAc and then inhibit MOR-induced sensitization. In addition, Leu-Ile treatment in combination with METH or MOR and after withdrawal from repeated treatment with METH or MOR inhibits place preference and sensitization to METH or MOR [14,15]. GDNF acts to negate the rewarding effects of MOR, since GDNF-(+/−) mice showed greater MOR-induced place preference compared with littermate control mice (Fig. 2A and B). GDNF could be involved in the inhibitory effects of Leu-Ile on the rewarding effects of MOR, since no effects of Leu-Ile were observed in the GDNF-(+/−) mice (Fig. 2A and B) and Leu-Ile failed to increase GDNF levels in combination with MOR in CPP paradigm in the NAc of GDNF-(+/−) mice (Fig. 2C). GDNF blocks the biochemical and behavioral responses to chronic cocaine or MOR exposure [10]. GDNF decreases TH levels in normal animals, suggesting an active down-regulation of the synthesis of this enzyme [9]. These results suggest that Leu-Ile plays an inhibitory role in the rewarding effects and sensitization induced by MOR in addition to METH *via* the induction of GDNF expression.

Our previous findings indicated that Leu-Ile inhibits MOR-induced sensitization and rewarding effects by attenuating the MOR-induced increase in extracellular DA levels *via* the induction of TNF-α expression [14]. In the present study, we demonstrated that GDNF is also involved in the inhibitory effects of Leu-Ile on MOR-induced sensitization and rewarding effects. Taken together, Leu-Ile inhibits MOR-induced sensitization and rewarding effects *via* the induction of not only TNF-α, but also GDNF, expression. Leu-Ile could be a novel therapeutic agent for MOR-induced dependence.

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An Inducer for Glial Cell Line-Derived Neurotrophic Factor and Tumor Necrosis Factor-α Protects Against Methamphetamine-Induced Rewarding Effects and Sensitization

Minae Niwa, Atsumi Nitta, Yuichiro Yamada, Akira Nakajima, Kuniaki Saito, Mitsuru Seishima, Liya Shen, Yukihiro Noda, Shoei Furukawa, and Toshitaka Nabeshima

Background: *There are few efficacious medications for drug dependence. We investigated the potential of Leu-Ile, which induces the expression of glial cell line-derived neurotrophic factor (GDNF) and tumor necrosis factor-α (TNF-α), as a novel therapeutic agent for methamphetamine (METH)-induced dependence.*

Methods: *The levels of GDNF and TNF-α messenger RNA (mRNA) were determined by real-time reverse transcription polymerase chain reaction. Enzyme immunoassays and immunohistochemistry were employed to determine levels of these proteins. Effects of Leu-Ile on METH-induced rewarding effects and sensitization were investigated with conditioned place preference and locomotor activity tests. Extracellular dopamine (DA) levels and DA uptake into synaptosomes were examined with an in vivo microdialysis and trititated thymidine ([^{3}H]) DA uptake assay.*

Results: *Leu-Ile induced the expression of not only GDNF but also TNF-α. Pretreatment with Leu-Ile blocked the acquisition of METH-induced place preference and sensitization. Interestingly, post-treatment with Leu-Ile attenuated them even after their development. An inhibitory effect of Leu-Ile on METH-induced place preference was observed in neither GDNF heterozygous nor TNF-α knockout mice. Leu-Ile inhibited DA release in the nucleus accumbens and the decrease in synaptosomal DA uptake in the midbrain induced by repeated METH treatment.*

Conclusions: *These results suggest that Leu-Ile inhibits METH-induced rewarding effects and sensitization by regulating extracellular DA levels via the induction of GDNF and TNF-α expression.*

Key Words: Methamphetamine (METH), glial cell line-derived neurotrophic factor (GDNF), tumor necrosis factor-α (TNF-α), dopamine (DA), rewarding effects, sensitization

The abuse of substances such as psychostimulants, opiates, nicotine, and alcohol has become a significant social and public health concern worldwide. Activation of the mesocorticolimbic dopamine (DA) system has been implicated in the positive reinforcing (rewarding) effects of drugs of abuse (Robbins and Everitt 1999; Yamada and Nabeshima 2004). The psychostimulant effects of methamphetamine (METH), a typical drug of abuse, are associated with an increase in extracellular DA levels in the brain, by facilitating the release of DA from presynaptic nerve terminals and inhibiting reuptake (Giros et al 1996; Heikkila et al 1975; Seiden et al 1993).

Neurotropic factors and cytokines, which are known to influence synaptic transmission and neuronal morphology (Boulanger and Poo 1999; Connor and Dragunow 1998; Neumann et al 2002), might be involved in alterations of the morphology of dendrites and dendritic spines in the nucleus accumbens (NAc) and prefrontal cortex after repeated injection of psychostimulants (Robinson and Kolb 1997, 1999; Yamada et al 2000). Glial cell line-derived neurotrophic factor (GDNF) inhibits the cocaine-induced upregulation of tyrosine hydroxylase activity in the ventral tegmental area (VTA) and blocks behavioral responses to cocaine (Messer et al 2000). Furthermore, we have previously demonstrated that tumor necrosis factor-α (TNF-α) inhibits METH-induced dependence (Nakajima et al 2004; Yamada and Nabeshima 2004). Taken together, GDNF and TNF-α would be candidates for therapeutic agents against drug dependence. However, there are serious obstacles to their therapeutic application: it is difficult to deliver GDNF from the periphery to the brain, because it is a macromolecule that cannot penetrate the blood-brain barrier (BBB) (Lin et al 1993) and is easily broken down by proteases in the blood stream. In addition, TNF-α, an inflammatory cytokine, damages the peripheral tissues, because it triggers the expression of other cytokines (Bluthe et al 1994). Therefore, GDNF and TNF-α cannot be used directly as therapeutic tools for drug dependence. We hypothesized that a low-molecular-weight compound that induces production of GDNF and TNF-α in the brain could be a novel therapeutic agent for drug dependence. Previous study has demonstrated that inflammatory stimuli such as TNF-α and lipopolysaccharide induces the synthesis of GDNF in cultured astrocytes from mouse brain (Appel et al 1997). Furthermore, Leu-Ile, a hydrophobic dipeptide, induces GDNF synthesis both in vivo and in vitro (Nitta et al 2004). Taken together, Leu-Ile is expected to induce the production of not only GDNF but also TNF-α and to inhibit drug dependence.

In the present study, we examined: 1) whether Leu-Ile induces production of TNF-α, and 2) the effects of Leu-Ile on the

From the Department of Neuropsychopharmacology and Hospital Pharmacy (MN, AN, YY, AN, YN, TN), Nagoya University Graduate School of Medicine; Division of Clinical Science in Clinical Pharmacy Practice (YN), Management and Research, Faculty of Pharmacy, Meijo University, Nagoya, Japan; Department of Informative Clinical Medicine (KS, MS), Gifu University Graduate School of Medicine; Laboratory of Molecular Biology (SF), Gifu Pharmaceutical University, Gifu, Japan; and Laboratory of Cellular Carcinogenesis and Tumor Promotion (LS), Center for Cancer Research, National Cancer Institute, Bethesda, Maryland.

Address reprint requests to Toshitaka Nabeshima, Ph.D., Nagoya University Graduate School of Medicine, Department of Neuropsychopharmacology and Hospital Pharmacy, Tsurumai-cho 65, Showa-ku Nagoya 466-8560, Japan; E-mail: tnabeshi@med.nagoya-u.ac.jp.

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rewarding actions and the sensitization to the locomotor-stimulating effects of METH and on the increase in extracellular DA levels and the decrease in DA uptake induced by METH.

Methods and Materials

Reagents

Glial cell line-derived neurotrophic factor and TNF-α were donated by Amgen (Thousand Oaks, California) and Dainippon Pharmaceutical (Osaka, Japan), respectively. Leu-Ile was purchased from Kokusan Chemical (Tokyo, Japan). All other materials used were of reagent grade.

Animals

Animals were housed in plastic cages and kept in a temperature-, humidity-, and light-controlled room (23° ± 1°C; 50% ± 5% humidity; 12-hour light/dark cycle starting at 8:00 AM) and had ad libitum access to food and water, except during behavioral experiments. Animal care and use was in accordance with the Principles of Laboratory Animal Care (National Institutes of Health Publication 85-123, 1983) and was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the Guidelines of Experimental Animal Care issued from the Office of the Prime Minister of Japan. The behavioral experiment's schedule is shown in Figure 1.

The wild-type C57BL/6 mice were obtained from Slc Japan (Hamamatsu, Japan).

Male C57BL/6-GDNF heterozygous [GDNF-(±)] mice and C57BL/6-TNF-α knockout (TNF-α-[−/−]) mice, 8–12 weeks of age, were used in the experiments. The GDNF-(±) and TNF-α-(−/−) mice were generated as described previously (Nakajima et al 2004; Pichel et al 1996; Taniguchi et al 1997); GDNF (−/−) homozygous knockout mice die shortly after birth (postnatal 7 days), but GDNF (±) mice are viable. Glial cell line-derived neurotrophic factor levels in the frontal cortex (Fc), NAc, striatum, and hippocampus (Hip) of GDNF-(±) mice are 54.8%, 65.4%, 59.0%, and 66.8 %, respectively, of those in littermate GDNF-(+/+) mice (Table 1). Littermate GDNF-(+/+) mice were used as control subjects in the behavioral experiments.

Drug Treatment

Mice were administered Leu-Ile (1.5 and 15 μmol/kg, IP) once/day 1 hour before METH (1 mg/kg, SC) treatment for 9 days. In the withdrawal experiment, mice were administered Leu-Ile or vehicle for 5 days after the withdrawal from METH after 9 successive days of METH administration. To determine trititated thymidine ([^{3}H]) DA uptake, messenger RNA (mRNA) expression, and protein levels, mice were decapitated 1, 2, and 24 hours after the last METH injection, respectively.

Enzyme Immunoassay of GDNF

Glial cell line-derived neurotrophic factor levels were measured with an enzyme immunoassay (EIA) with a minor modification (Nitta et al 1999a, 1999b, 2004). Homogenate buffer (.1 mol/L Tris-HCl [pH 7.4] containing 1 mol/L sodium chloride (NaCl), 2% bovine serum albumin, 2 mmol/L ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), and .2% sodium nitride [Na3N]) was added to brain tissue at a ratio of 1 g wet weight / 19 mL of buffer, pulse-sonicated for 100 sec, and centrifuged at 100,000 *g* for 30 min. The supernatant was collected and used for the EIA.

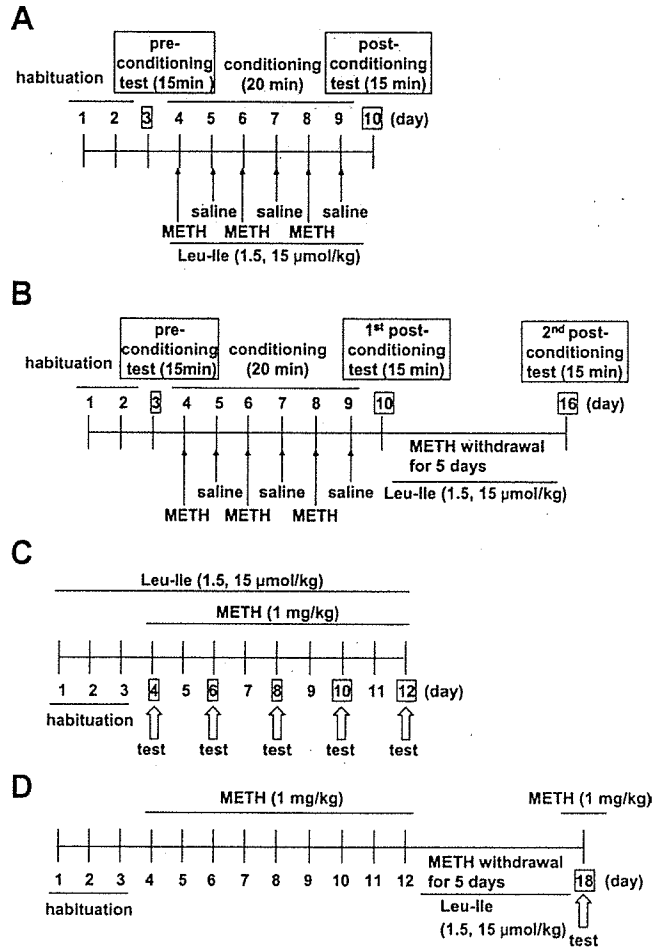

Figure 1. Experimental schedules. **(A)** Experimental schedule for the conditioned place preference task. Mice were co-treated with Leu-Ile and methamphetamine (METH) in the conditioning period. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before receiving METH (1 mg/kg, SC) or saline. Closed arrows indicate the days of METH or saline injection. **(B)** Experimental schedule for the conditioned place preference task to investigate the effects of Leu-Ile after the withdrawal from METH. Mice were not treated with Leu-Ile in the conditioning period. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after withdrawal from METH. **(C)** Experimental schedule for measurement of locomotor activity. Mice were treated with Leu-Ile during the habituation period for 3 days and then co-treated with Leu-Ile and METH for 9 days. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before the METH (1 mg/kg, SC) injection. Locomotor activity was measured for 2 hours after the METH treatment. Open arrows indicate the days when locomotor activity was measured. **(D)** Experimental schedule for measurement of locomotor activity to investigate the effects of Leu-Ile after the withdrawal from METH. Mice were treated with Leu-Ile after the establishment of METH (1 mg/kg, SC)-induced sensitization: mice were treated with METH for 9 days and then with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days without METH. On day 18, mice were administered only METH (1 mg/kg, SC), and locomotor activity was measured for 2 hours after the METH treatment.

Semi-Quantative mRNA Analysis by Real-Time Reverse Transcription Polymerase Chain Reaction

Corti-hippocampal neurons from 18-day-old rat embryos were cultured as previously described (Nitta et al 1999a, 1999b). More than 95% of the cells were positive for microtuble-associated protein-2 (MAP2) immunoreactivity. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free

Table 1. The Difference of GDNF Levels in the Brain (Fc, NAc, Str, Hip) Between GDNF-(+/+) and GDNF-(+/−) Mice

Brain Region	GDNF Levels (pg/g Wet Tissue)		
	GDNF-(+/+) Mice	GDNF-(+/−) Mice	% of GDNF-(+/+) Mice
Fc	1285.0 ± 254.3	704.0 ± 26.4*	54.8
NAc	1253.4 ± 58.5	820.2 ± 58.4*	65.4
Str	2083.1 ± 231.0	1229.4 ± 178.2*	59.0
Hip	782.2 ± 99.9	522.5 ± 20.9*	66.8

Mice were decapitated without any treatment, and the brains were quickly removed. Glial cell line-derived neurotrophic factor (GDNF) levels were measured using an enzyme immunoassay. Values are means ± SE ($n = 10$).

Fc, frontal cortex; Hip, hippocampus; NAc, nucleus accumbens; Str, striatum.

*$p < .05$ versus GDNF-(+/+) mice.

defined medium containing Leu-Ile (.037, .37, 3.7, and 37 μg/mL) or TNF-α (2, 20, 100, and 200 ng/mL). Total RNA was isolated with an RNeasy Mini Kit (Qiagen, Hilden, Germany) and converted into complementary DNA with a SuperScriptTM First-Strand System for RT-PCR Kit (Invitrogen Life Technologies, Carlsbad, California). The rat GDNF primers used were as follows: 5'-AGCTGCCAGCCCAGAGAATT-3' (forward) and 5'-GCACCCCCGATTTTTGC-3' (reverse), and TaqMan probe: 5'-CAGAGGGAAAGGTCGCAGAGGCC-3'. The rat TNF-α primers used were as follows: 5'-ATTTGGCCCCATCCTTCC-3' (forward) and 5'-GCCTCCATGGCAGAGCC-3' (reverse), and TaqMan probe: 5'-TCCCAGGACATCAGGACTCTGTTCCC-3'. The 18S ribosomal RNA Kit was used as the internal control (PE Applied Biosystems, Foster, California). The amplification consisted of an initial step (50°C for 2 min and 95°C for 2 min) and then 40 cycles of denaturation for 15 sec at 95°C and annealing for 1 min at 60°C in an iCycle iQ Detection System (Bio-Rad Laboratories, Tokyo, Japan). The expression levels were calculated as described previously (Wada et al 2000).

Immunohistochemical Analysis

Polyclonal rabbit anti-GDNF (1:50; sc-328; Santa Cruz Biotechnology, Santa Cruz, California), polyclonal goat anti-TNF-α (1:100; R&D Systems, Minneapolis, Minnesota), monoclonal mouse anti-MAP2 (1:200; Sigma-Aldrich, Saint Louis, Missouri), and monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:200, Chemicon International, Temecula, California) served as primary antibodies. Goat anti-rabbit Alexa Fluor 546 (1:1000, Molecular Probes, Eugene, Oregon) and goat anti-mouse Alexa Fluor 488 (1:1000, Molecular Probes) served as secondary antibodies for GDNF immunostaining. Donkey anti-goat Alexa Fluor 546 (1:1000, Molecular Probes) and rabbit anti-mouse Alexa Fluor 488 (1:1000, Molecular Probes) served as secondary antibodies for TNF-α immunostaining. Each stained tissue was observed under a fluorescence microscope (Axioskoop 2 plus: Carl Zeiss, Jena, Germany) and analyzed with Axiovision 3.0 systems (Carl Zeiss). The area with TNF-α-positive cells in the defined NAc region of mice was determined with the software WinROOF (Mitani, Fukui, Japan) (Kuwahara et al 1999; Tsuji et al 1999). We employed an immunostaining method with which one can analyze the distribution and levels of TNF-α protein in the present investigation, because it is too difficult to use Western blotting or an enzyme immunoassay to quantify the amount of TNF-α protein in brain tissue.

Measurement of the TNF-α Concentration in Peripheral Blood

Blood was collected into tubes containing 5% EDTA 1, 2, and 4 hours after the injection of TNF-α (4 μg/body, IP). Mice were treated with Leu-Ile (1.5 μmol/kg, IP), and their blood was collected into tubes containing 5% EDTA 0, 1, 2, 4, and 8 hours after the injection. The blood samples were centrifuged at 2000 × g for 20 min at 4°C. The supernatants were taken as the samples. The TNF-α concentration was assessed by using a specific human (QuantiGlo QTA00, R&D Systems) or mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Quantikine MTA00, R&D Systems), according to the instructions provided.

Behavioral Tests

Conditioned Place Preference. The place conditioning paradigm was performed by using previously established procedure with a minor modification (Nagai et al 2004; Nakajima et al 2004; Noda et al 1998). The experimental schedule for the conditioned place preference (CPP) task is shown in Figures 1A and 1B. The mouse was allowed to move freely between transparent and black boxes for 15 min once/day for 3 days (days 1–3) in the preconditioning. On day 3, the time the mouse spent in each box was measured. On days 4, 6, and 8, the mouse was treated with METH and confined in either the transparent or black box for 20 min. On days 5, 7, and 9, the mouse was given saline and placed opposite the METH-conditioning box for 20 min. On day 10, the postconditioning test was performed without drug treatment, and the time the mouse spent in each box was measured for 15 min.

Locomotor Activity. Locomotor activity was measured with an infrared detector (Neuroscience, Tokyo, Japan) in a plastic box (32 × 22 × 15 cm high). Mice were administered METH (1 mg/kg, SC) or saline 1 hour after the Leu-Ile treatment, and the locomotor activity was measured for 2 hours immediately after the METH or saline administration (Figure 1C). In the withdrawal experiment, mice were administered Leu-Ile or vehicle for 5 days after the withdrawal from METH (days 13–17) after 9 successive days of METH administration. On day 18, the mice were administered only METH (1 mg/kg, SC), and locomotor activity was measured for 2 hours immediately after the administration (Figure 1D).

In Vivo Microdialysis

Mice were anesthetized with sodium pentobarbital, and a guide cannula (AG-8, EICOM, Kyoto, Japan) was implanted into the NAc (+1.1 mm anteroposterior, +1.0 mm mediolateral from bregma, and −4.0 mm dorsoventral to dura) according to the atlas of Franklin and Paxinos (1997) and secured to the skull with stainless steel screws and dental acrylic cement. One day after the operation, a dialysis probe (AI-8-1; 1-mm membrane length, EICOM) was inserted through the guide cannula and perfused continuously with artificial cerebrospinal fluid (aCSF; 147 mmol/L NaCl, 4 mmol/L potassium chloride, and 2.3 mmol/L calcium dichloride) at a rate of 1.0 μL/min. Dialysate was collected in 20-min fractions and injected into the HPLC system (EICOM) for the measurement of DA levels. Three samples were used to establish baseline levels of extracellular DA before the administration of Leu-Ile and METH.

Synaptosomal [^{3}H] DA Uptake

Midbrain synaptosomal [^{3}H] DA uptake was determined as previously described (Fleckenstein et al 1997; Nakajima et al 2004). Samples were incubated at 37°C for 4 min, and then ice-cold Krebs-Ringer's solution containing 10 μmol/L GBR12909, a specific DA uptake inhibitor, was added. Nonspecific values were determined in the presence of 100 μmol/L GBR12909. The radioactivity trapped on Whatman GF/B filters (Brandel, Gaith-

ersburg, Maryland) was measured with a liquid scintillation counter.

Statistical Analysis

All data were expressed as means ± SEM. Statistical differences between two groups were determined with Student *t* test. Statistical differences among more than three groups were determined with a one-way analysis of variance (ANOVA) or a repeated ANOVA followed by the Bonferroni multiple comparison test; $p < .05$ was regarded as statistically significant.

Results

Effect of Leu-Ile on METH-Induced Increase in GDNF Levels

Levels of GDNF expression induced by Leu-Ile (.037, .37, 3.7, and 37 μg/mL) were determined in the cultured neurons with the EIA method. The GDNF levels were significantly increased 24 hours after the addition of Leu-Ile (.37 μg/mL) resulting in a bell-shaped dose response curve compared with the control group [$F(4,25) = 8.895$, $p < .05$, one-way ANOVA] (Figure 2A). Therefore, a dose of .37 μg/mL was used in this experiment. The time course of GDNF mRNA expression was determined 6, 12, and 24 hours after Leu-Ile (.37 μg/mL) treatment in the cultured neurons. The GDNF mRNA levels were significantly elevated 24 hours after Leu-Ile treatment in the cultured neurons compared with the control group [$F(5,42) = 7.627$, $p < .05$, one-way ANOVA] (Figure 2B).

Glial cell line-derived neurotrophic factor–positive cells were found among the neurons, which were immunopositive for MAP2, in the NAc of mice co-treated with Leu-Ile (1.5 μmol/kg, IP) and METH (1 mg/kg, SC); GDNF-positive cells were also found among astro glial cells, which were immunopositive for GFAP, in the NAc of mice co-treated with Leu-Ile (1.5 μmol/kg, IP) and METH (1 mg/kg, SC) (Figure 2C).

The GDNF levels in the NAc were determined after the co-administration of Leu-Ile and METH with the EIA method. Methamphetamine (1 mg/kg) increased GDNF levels in the NAc compared with those in the vehicle/saline-treated mice. The GDNF levels after the co-administration of Leu-Ile (1.5 and 15 μmol/kg, IP) and METH (1 mg/kg) were much more increased compared with those in the vehicle/METH-treated mice [$F(5,38) = 16.971$, $p < .05$, one-way ANOVA] (Figures 2C and 2D). Moreover, we determined GDNF levels in the NAc after Leu-Ile treatment during the withdrawal from METH after 9 successive days of METH administration. The schedule is described in Figure 1D. An acute challenge of METH in mice treated with vehicle for 5 days after the development of METH-induced sensitization increased GDNF levels in the NAc compared with levels in the saline/vehicle/METH-treated mice. An acute challenge of METH in mice treated with Leu-Ile (1.5 and 15 μmol/kg) for 5 days after the development of METH-induced sensitization resulted in a much greater increase compared with levels in the METH/vehicle/METH-treated mice [$F(3,20) = 23.777$, $p < .05$, one-way ANOVA] (Figure 2E).

Effect of Leu-Ile on METH-Induced Increase in TNF-α Levels

Expression levels of TNF-α mRNA induced by Leu-Ile (.037, .37, 3.7, and 37 μg/mL) were determined in the cultured neurons with the real-time reverse transcription polymerase chain reaction (RT-PCR) method, because TNF-α induces the synthesis of GDNF as described in the introductory section of this report. Levels of TNF-α mRNA were significantly increased 24 hours after the addition of Leu-Ile (.37 μg/mL), resulting in a bell-shaped dose response curve compared with the control group [$F(4,30) = 2.572$, $p < .05$, one-way ANOVA] (Figure 3A). The time course of TNF-α mRNA expression was determined 6, 12, and 24 hours after Leu-Ile (.37 μg/mL) treatment in the cultured neurons. The TNF-α mRNA levels were significantly elevated 24 hours after the Leu-Ile (.37 μg/mL) treatment in the cultured neurons compared with the control group [$F(5,42) = 6.067$, $p < .05$, one-way ANOVA] (Figure 3B).

Tumor necrosis factor-α–positive cells were found among the neurons that were immunopositive for MAP2 but not for GFAP in the NAc of mice co-treated with Leu-Ile (1.5 μmol/kg, IP) and METH (1 mg/kg, SC) (Figure 3C).

The areas occupied by TNF-α-immunopositive cells were measured to estimate the effects of Leu-Ile on the production of TNF-α protein. The area with TNF-α immunoreactive cells was determined in the NAc by using the software WinROOF (Mitani Shoji, Fukui, Japan). Methamphetamine (1 mg/kg, SC) potentiated the immunoreactivity to TNF-α in the NAc. After the co-administration of Leu-Ile (1.5 and 15 μmol/kg, IP) and METH (1 mg/kg, SC), immunoreactivity was much more increased in the NAc [$F(5,32) = 26.836$, $p < .05$, one-way ANOVA] (Figures 3C and 3D). Moreover, we determined levels of TNF-α protein in the NAc after Leu-Ile treatment during the withdrawal from METH after 9 successive days of METH administration. The schedule is described in Figure 1D. An acute challenge of METH in mice treated with vehicle for 5 days after the development of METH-induced sensitization increased TNF-α levels in the NAc compared with those in the saline/vehicle/METH-treated mice. An acute challenge of METH in mice treated with Leu-Ile (1.5 and 15 μmol/kg) for 5 days after the development of METH-induced sensitization markedly increased TNF-α levels compared with those in the METH/vehicle/METH-treated mice [$F(3,22) = 26.800$, $p < .05$, one-way ANOVA] (Figure 3E).

We checked whether the concentration of TNF-α in venous blood was changed after the intraperitoneal injection of Leu-Ile in mice. In venous blood, the TNF-α concentration was not changed 1, 2, 4, and 8 hours after the Leu-Ile treatment (1.5 μmol/kg, IP). In contrast, the concentration was dramatically increased 1, 2, and 4 hours after the TNF-α treatment (4 μg/body, IP) (Figure 3F).

Regulatory Effect of TNF-α on GDNF Expression in Cultured Neurons

With the real-time RT-PCR method, levels of GDNF mRNA stimulated by TNF-α (20 and 100 ng/mL) were determined in the cultured neurons. The mRNA levels were significantly elevated by TNF-α (100 ng/mL) compared with the control group [$F(2,11) = 7.826$, $p < .05$, one-way ANOVA] (Figure 4A). Next, the time course of GDNF mRNA expression was determined 6, 12, and 24 hours after TNF-α (100 ng/mL) treatment. The mRNA levels were significantly elevated 24 hours after the treatment with TNF-α (100 ng/mL) compared with the control group [$F(5,46) = 6.114$, $p < .05$, one-way ANOVA] (Figure 4B). The GDNF levels showed a significant increase 24 hours after the addition of TNF-α (100 and 200 ng/mL) compared with the control group [$F(4,25) = 12.372$, $p < .05$, one-way ANOVA] (Figure 4C).

Effects of Leu-Ile on METH-Induced Place Preference and Hyperlocomotion/Sensitization in Wild-Type Mice

We investigated the effects of Leu-Ile on the behavioral responses to METH. First, the effects of Leu-Ile on the rewarding effects of METH were examined in CPP paradigm, in which animals learn the association of an environment paired with drug

exposure; CPP is, therefore, considered a measure of the rewarding properties of drugs of abuse. The experimental schedule is described in Figure 1A. As shown in Figure 5A, METH (1 mg/kg, SC) produced place preference in mice. When mice were treated with Leu-Ile (1.5 μmol/kg, IP) 1 hour before receiving METH, the METH-induced place preference was significantly attenuated [$F(5,53) = 5.338$, $p < .05$, one-way ANOVA] (Figure 5A). Leu-Ile (1.5 and 15 μmol/kg, IP) itself failed to induce place preference in mice (Figure 5A second and third columns in saline-treated group).

We next examined the effects of Leu-Ile on METH-induced hyperlocomotion and sensitization. The sensitization to the locomotor-stimulating effects is argued to reflect one neuroadaptive process associated with dependence. The experimental schedule is described in Figure 1C. As shown in Figure 5B, acute METH treatment at a dose of 1 mg/kg caused a marked increase in locomotor activity, and repeated administration for 9 days resulted in an enhancement of the locomotor-stimulating effect of METH (sensitization) [$F(4,45)=2.919$, $p < .05$, one-way ANOVA]. Leu-Ile (1.5 and 15 μmol/kg, IP) did not inhibit acute METH-induced hyperlocomotion on day 4, although it inhibited METH-induced hyperlocomotion and sensitization on days 8–12 [$F(5,54) = 24.374$ at day 8, $F(5,54) = 20.738$ at day 10, $F(5,54) = 30.956$ at day 12, $p < .05$, one-way ANOVA] (Figure 5B). Leu-Ile (1.5 and 15 μmol/kg, IP) did not affect spontaneous locomotor activity in saline-treated mice (Figure 5B).

Effects of Leu-Ile Treatment After the Development of Place Preference and Sensitization Induced by METH in Wild-Type Mice

The therapeutic effects of Leu-Ile on METH-induced place preference were examined. The experimental schedule is described in Figure 1B. Mice were administered Leu-Ile (1.5 and 15 μmol/kg, IP) after the development of METH-induced place preference for 5 days without METH treatment. In this experiment, the second post-conditioning was carried out 5 days after the first post-conditioning. Although METH (1 mg/kg)-induced place preference was maintained for 5 days after the first post-conditioning in wild-type mice, it was attenuated by Leu-Ile treatment (1.5 μmol/kg, IP) for 5 days between the first and second post-conditionings [$F(5,58) = 14.407$, $p < .05$, one-way ANOVA] (Figure 5C).

Next, the therapeutic effects of Leu-Ile on METH-induced sensitization were examined. The experimental schedule is described in Figure 1D. The repeated administration of METH (1 mg/kg, SC) for 9 days resulted in sensitization of METH. Sensitization was observed on day 18 of the administration of METH (1 mg/kg, SC) 5 days after withdrawal from METH [$F(5,154) = 23.107$, $p < .05$, one-way ANOVA]. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days during the withdrawal period. Leu-Ile (1.5 and 15 μmol/kg, IP) inhibited METH (1 mg/kg, SC)-induced sensitization on day 18 [$F(3,36) = 21.840$, $p < .05$, one-way ANOVA] (Figure 5D).

We confirmed that Leu-Ile at the low dose, 1.5 μmol/kg, which could inhibit METH-induced rewarding effects, increased GDNF and TNF-α both in combination with METH and after withdrawal from repeated treatment with METH in CPP paradigm. In contrast, Leu-Ile at the higher dose, 15 μmol/kg, which could not inhibit METH-induced rewarding effects, failed to increase them [data not shown; $F(5, 30) = 12.387$ and 19.764 for GDNF and TNF-α levels, respectively, after co-treatment of Leu-Ile and METH in CPP paradigm; $F(3,20) = 12.260$ and 16.670 for GDNF and TNF-α levels, respectively, after Leu-Ile treatment during withdrawal from METH in CPP paradigm]. The results of GDNF and TNF-α contents in the NAc after locomotor test are described in Figures 2D, 2E, 3D, and 3E.

Effects of Leu-Ile on the Rewarding Effects of METH in GDNF-(±) and TNF-α-(−/−) Mice

To confirm the involvement of GDNF and TNF-α in the rewarding effects of METH, the effect of Leu-Ile on METH-induced place preference was examined in GDNF-(±) and TNF-α-(−/−) mice. The experimental schedule is described in Figure 1A. As shown in Figure 6, although at a low dose of METH (.3 mg/kg), GDNF-(+/+) and wild-type mice did not develop place preference, GDNF-(±) and TNF-α-(−/−) mice did [GDNF-(±): $F(7,64) = 6.493$; TNF-α-(−/−): $F(7,56) = 9.514$, $p < .05$, one-way ANOVA]. When Leu-Ile (1.5 and 15 μmol/kg, IP) was administered 1 hour before METH, it failed to exhibit a significant effect on the action of METH in GDNF-(±) and TNF-α-(−/−) mice (Figure 6). These results suggest that GDNF and TNF-α act to negate the rewarding effects of METH and are involved in the effects of Leu-Ile on these rewarding effects.

Effects of Leu-Ile on METH-Induced DA Responses

To explore the mechanisms of the inhibitory effects of Leu-Ile on METH-induced rewarding effects and sensitization, the effects of Leu-Ile on the increase in extracellular DA levels induced by repeated or single METH treatment were examined in the NAc of mice, by using an in vivo microdialysis technique. Repeated and single METH (1 mg/kg, SC) treatment caused a marked increase in extracellular DA levels in the NAc on day 9 and day 1, respectively (Figures 7A and 7B). Peaks of extracellular DA levels were 2.5- and 2.0-fold the baseline, respectively. Treatment with Leu-Ile (1.5 μmol/kg, IP) for 9 days significantly inhibited the repeated METH-induced increase in extracellular DA levels [$F(1,7) = 5.227$, $p < .05$, repeated ANOVA] (Figure 7A). In contrast, pretreatment with Leu-Ile (1.5 μmol/kg, IP) 1 hour before the single METH treatment failed to inhibit the increase in extracellular DA levels (Figure 7B).

We examined the therapeutic effect of Leu-Ile on the METH-induced increase in extracellular DA levels in the NAc of mice after the repeated treatment with METH. As shown in Figure 7C, an acute challenge with METH (1 mg/kg, SC) 5 days after withdrawal caused a marked increase in extracellular DA levels in the NAc of mice treated with vehicle for 5 days during the withdrawal from METH (1 mg/kg, SC). The peak of extracellular DA levels was 2.0-fold the baseline. Leu-Ile (1.5 μmol/kg, IP) treatment for 5 days during the withdrawal from METH significantly attenuated the METH-induced increase in extracellular DA levels [$F(1,14) = 1.689$, $p < .05$, repeated ANOVA] (Figure 7C).

Next, we examined the effect of Leu-Ile on the METH-induced decrease in synaptosomal DA uptake in the midbrain. Repeated METH treatment (1 mg/kg, SC) caused a decrease in [^{3}H] DA uptake by 38% compared with the vehicle/saline-treated mice. Leu-Ile (1.5 μmol/kg, IP) inhibited the METH-induced decrease in synaptosomal [^{3}H] DA uptake [$F(3,28) = 12.477$, $p < .05$, one-way ANOVA] (Figure 7D).

Finally, we examined the therapeutic effect of Leu-Ile on the METH-induced decrease in synaptosomal DA uptake in the midbrain. As shown in Figure 7E, an acute challenge with METH (1 mg/kg, SC) after treatment with vehicle for 5 days during withdrawal from METH caused a decrease in [^{3}H] DA uptake by 51% in the midbrain compared with the saline/vehicle/METH-treated mice. Leu-Ile (1.5 μmol/kg, IP) treatment for 5 days

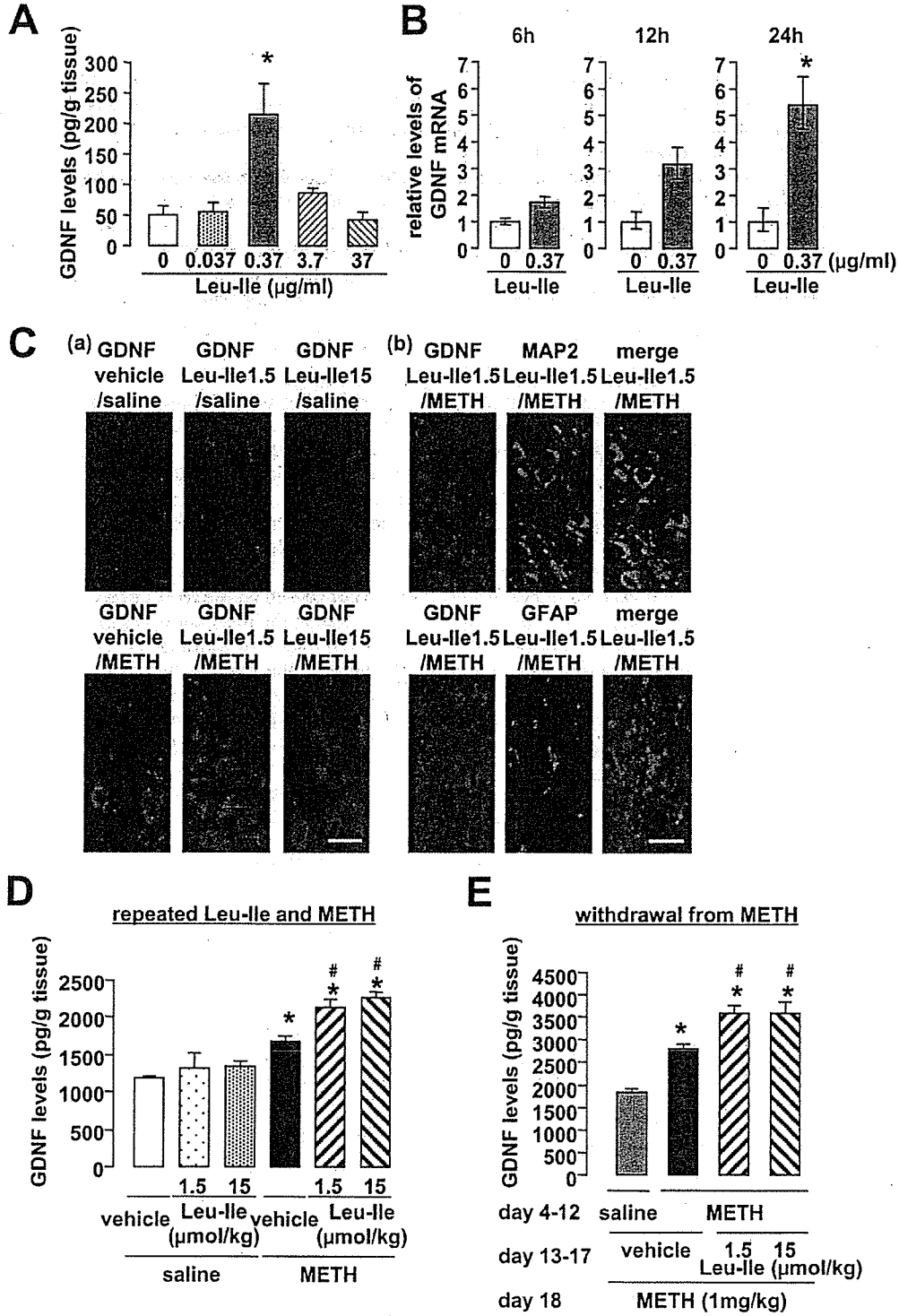

Figure 2. Effect of Leu-Ile on methamphetamine (METH)-induced increase in glial cell line-derived neurotrophic factor (GDNF) levels. **(A)** The dose-response stimulatory effects of Leu-Ile on GDNF levels in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing Leu-Ile (.037, .37, 3.7, and 37 μg/mL). The GDNF levels were determined 24 hours after the addition of Leu-Ile by enzyme immunoassay. Values are means ± SEM ($n = 5–10$). *$p < .05$ versus control subjects. **(B)** The time-course stimulatory effects of Leu-Ile on the expression of GDNF messenger RNA in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing Leu-Ile (.37 μg/mL). Total RNA was prepared 6, 12, and 24 hours after the addition of Leu-Ile. Values are means ± SEM ($n = 8$). *$p < .05$ versus control subjects. **(C)** Immunostaining of GDNF in the nucleus accumbens (NAc) after the administration of Leu-Ile and/or METH **(a)**. Double-labeling fluorescence photomicrographs for GDNF and microtuble-associated protein-2 (MAP2) or glial fibrillary acidic protein (GFAP) **(b)**. The GDNF-immunoreactive cells (red) were colocalized with MAP2-positive and GFAP-positive cells (green) in the NAc. Double immunostaining for GDNF and MAP2 or GFAP in the NAc reveals GDNF expression in neuronal and astro glial cells. Scale bars, 20 μm. **(D)** Change of GDNF levels in the NAc after the administration of Leu-Ile and/or METH. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before METH (1 mg/kg, SC) once/day for 9 days and decapitated 24 hours after the last METH injection. Values are means ± SEM ($n = 6–8$). *$p < .05$ versus vehicle/saline-treated mice. #$p < .05$ versus vehicle/METH-treated mice. **(E)** Change of GDNF levels in the NAc after Leu-Ile treatment during the withdrawal from METH. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after the development of METH (1 mg/kg, SC)-induced sensitization. The next day, the mice were administered only METH (1 mg/kg, SC) and decapitated 24 hours after the administration. Values are means ± SEM ($n = 6$). *$p < .05$ versus saline/vehicle/METH-treated mice. #$p < .05$ versus METH/vehicle/METH-treated mice.

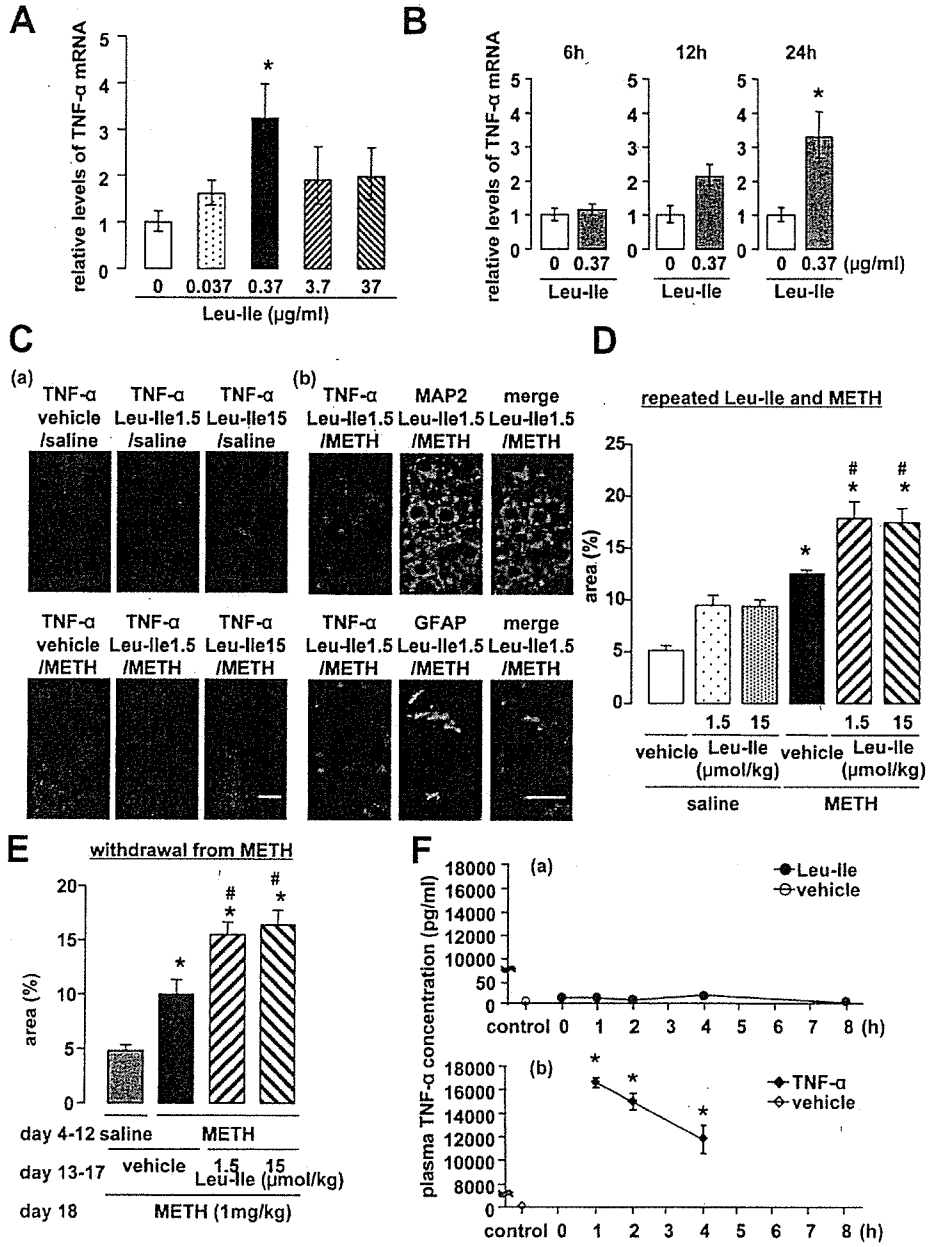

Figure 3. Effect of Leu-Ile on METH-induced increase in tumor necrosis factor-α (TNF-α) levels. **(A)** The dose-response stimulatory effects of Leu-Ile on the expression of TNF-α messenger RNA (mRNA) in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing Leu-Ile (.037, .37, 3.7, and 37 μg/mL). Total RNA was prepared 24 hours after the addition of Leu-Ile. Values are means ± SEM ($n = 7$). *$p <$ .05 versus control subjects. **(B)** The time-course stimulatory effects of Leu-Ile on the expression of TNF-α mRNA in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing Leu-Ile (.37 μg/mL). Total RNA was prepared 6, 12, and 24 hours after the addition of Leu-Ile. Values are means ± SEM ($n = 8$). *$p <$ .05 versus control subjects. **(C)** Immunostaining of TNF-α in the NAc after the administration of Leu-Ile and/or METH **(a)**. Double-labeling fluorescence photomicrographs for TNF-α and MAP2 or GFAP **(b)**. The TNF-α-immunoreactive cells (red) were colocalized with MAP2-positive cells (green) in the NAc. Double immunostaining for TNF-α and MAP2 in the NAc reveals TNF-α expression in neuronal cells. Scale bars, 100 **(a)** and 20 **(b)** μm. **(D)** Change of TNF-α expression in the NAc after the administration of Leu-Ile and/or METH. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before METH (1 mg/kg, SC) for 9 days and decapitated 24 hours after the last METH injection. The area of TNF-α positive cells in 3.8×10^4 μm^2 was estimated with the software WinRoof. Values are means ± SEM ($n = 6–8$). *$p <$ .05 versus vehicle/saline-treated mice. #$p <$ .05 versus vehicle/METH-treated mice. **(E)** Change of TNF-α expression in the NAc after Leu-Ile treatment during the withdrawal from METH. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after the development of METH (1 mg/kg, SC)-induced sensitization. The next day, the mice were administered only METH (1 mg/kg, SC) and decapitated 24 hours after the administration. Values are means ± SEM ($n = 6–8$). *$p <$ .05 versus saline/vehicle/METH-treated mice. #$p <$ .05 versus METH/vehicle/METH-treated mice. **(F)** Tumor necrosis factor-α concentration in peripheral blood after the treatment with Leu-Ile. Mice were treated with Leu-Ile (1.5 μmol/kg, IP), and venous blood was collected 0, 1, 2, 4, and 8 hours after the injection **(a)**. Values are means ± SEM ($n = 4$) (upper panel). Mice were treated with TNF-α (4 μg/body, IP), and venous blood was collected 1, 2, and 4 hours after the injection **(b)**. Values are means ± SEM (n = 3–4) (lower panel). The concentration of TNF-α was measured with a mouse TNF-α (upper panel) or human TNF-α enzyme-linked immunosorbent assay (ELISA) kit (lower panel). *$p <$ .05 versus vehicle-treated mice. Other abbreviations as in Figure 2.

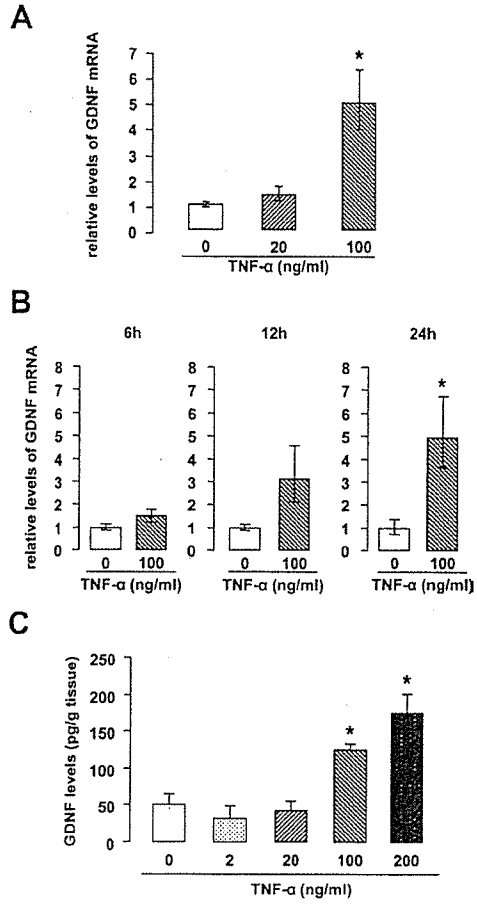

Figure 4. Regulatory effect of TNF-α on GDNF expression in cultured neurons. **(A)** The dose-response stimulatory effects of TNF-α on the expression of GDNF messenger RNA (mRNA) in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing TNF-α (20 and 100 ng/mL). Total RNA was prepared 24 hours after the addition of TNF-α. Values are means ± SEM (n = 4–5). *$p < .05$ versus control subjects. **(B)** The time-course stimulatory effects of TNF-α on the expression of GDNF mRNA in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing TNF-α (100 ng/mL). Total RNA was prepared 6, 12, and 24 hours after the addition of TNF-α. Values are means ± SEM (n = 8–12). *$p < .05$ versus control subjects. **(C)** The dose-response stimulatory effects of TNF-α on GDNF levels in the cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing TNF-α (2, 20, 100, and 200 ng/mL). The GDNF levels were determined 24 hours after the addition of TNF-α by enzyme immunoassay. Values are means ± SEM (n = 5–10). *$p < .05$ versus control subjects. Other abbreviations as in Figure 2.

during the withdrawal from METH inhibited the METH-induced decrease in synaptosomal [^{3}H] DA uptake [$F(2,21) = 7.544$, $p < .05$, one-way ANOVA] (Figure 7E).

Discussion

There are currently few efficacious medications for drug dependence. Recently, it has been reported that an opioid κ receptor agonist, TRK-820, inhibits not only the rewarding effects of morphine and cocaine but also a mecamylamine-precipitated nicotine-withdrawal aversive effect (Mori et al 2002; Tsuji et al 2001). A DA D3 receptor partial agonist, BP897affects cocaine-associated stimulus-induced drug-seeking behavior in rats (Cervo et al 2003). These medications should be effective even when they are administered after the development of drugs of abuse. In this study, the METH-induced place preference and sensitization that formed before Leu-Ile treatment were attenuated by Leu-Ile (Figures 5C and 5D), by regulating extracellular DA levels (Figures 7C and 7E). These results suggest that Leu-Ile could be a novel therapeutic agent for dependence on METH.

Leu-Ile increased GDNF levels in the cultured neurons (Figures 2A and 2B). In vivo, Leu-Ile treatment both in combination with METH and after withdrawal from repeated treatment with METH also increased GDNF levels (Figures 2D and 2E). We have previously demonstrated that Leu-Ile targets the Hsc70/Hsp90 cochaperone and, thus, triggers Akt/CREB signaling, resulting in an upregulation of GDNF expression (Cen et al 2006). In addition, Leu-Ile treatment, both in combination with METH and after withdrawal from repeated treatment with METH, inhibited place preference and sensitization to METH (Figure 5). Glial cell line-derived neurotrophic factor blocks the ability of cocaine and morphine to increase levels of tyrosine hydroxylase in the VTA and blunts the biochemical and behavioral responses to chronic cocaine or morphine exposure (Messer et al 2000). Glial cell line-derived neurotrophic factor decreases tyrosine hydroxylase levels in normal animals, suggesting an active down-regulation of the synthesis of this enzyme (Lu and Hagg 1997). These results suggest that Leu-Ile plays an inhibitory role in the rewarding effects and sensitization induced by METH via the induction of GDNF expression.

As described at the beginning of this article, TNF-α induces GDNF expression and blocks METH-induced dependence. Therefore, we investigated whether Leu-Ile induces the expression of TNF-α. Leu-Ile increased TNF-α mRNA levels in the cultured neurons (Figures 3A and 3B). In vivo, Leu-Ile treatment, both in combination with METH and after withdrawal from repeated treatment with METH, also increased TNF-α levels (Figures 3D and 3E) in the brain but not in the peripheral blood stream (Figure 3F). Leu-Ile can penetrate the BBB and initiate the synthesis of GDNF in the brain (Nitta et al 2004). Therefore, we suggest that Leu-Ile penetrates the BBB and induces TNF-α expression only in the brain. The expression of TNF-α is induced through the activation of transcription factors such as activator protein-1 and nuclear factor-κB (NF-κB) (Guha et al 2000; Rahman and MacNee 2000). Changes in transcription factors might result in long-term changes in gene expression, thereby contributing to neuronal adaptations that underlie behavioral sensitization to chronic psychostimulant treatment (Nestler 2001). Furthermore, TNF-α influences synaptic strength and transmission (Albensi and Mattson 2000; Beattie et al 2002). Collectively, these observations lead to the hypothesis that Leu-Ile might have inhibitory effects on long-lasting behavioral changes induced by repeated METH treatment via the induction of TNF-α expression.

Tumor necrosis factor-α was expressed in the neurons of the NAc after the co-administration of Leu-Ile with METH (Figure 3C), whereas GDNF was expressed in the neuronal and astro glial cells (Figure 2C). Therefore, TNF-α expression induced by Leu-Ile might regulate GDNF expression in neuronal cells, although little is known about the regulation of GDNF synthesis in the brain. It has been reported that GDNF expression in astrocytes can be induced by inflammatory stimuli such as TNF-α and lipopolysaccharide (Appel et al 1997). These previous reports have suggested that the induction of GDNF expression might be regulated through production of TNF-α. In the present study, TNF-α increased GDNF expression in the cultured neu-

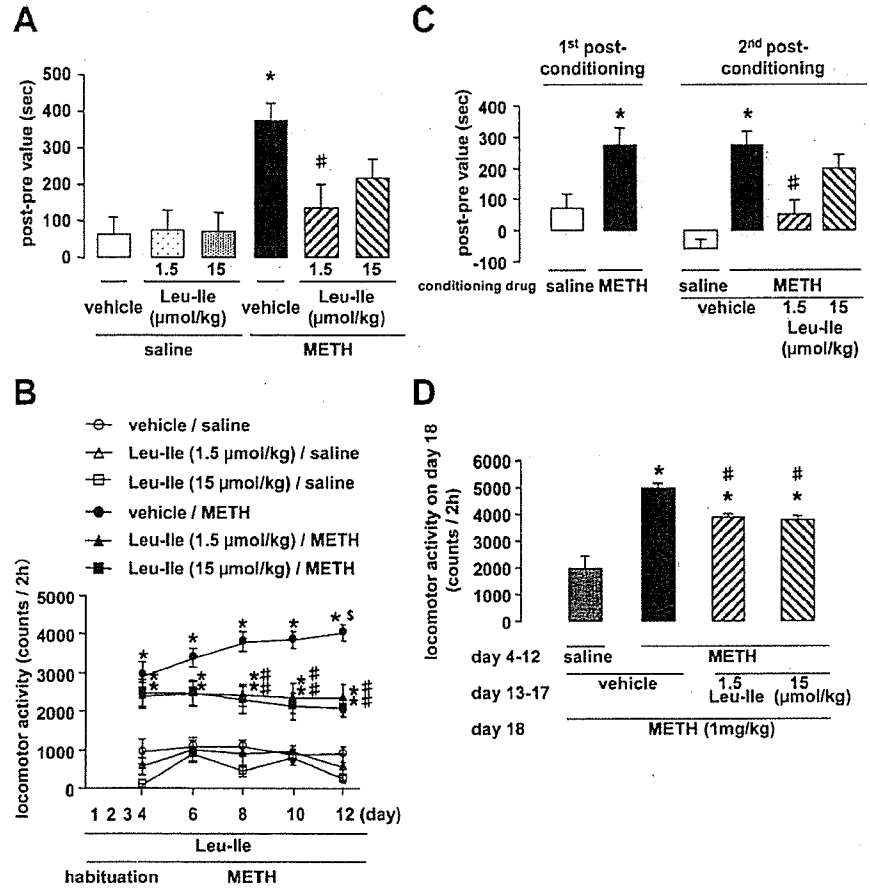

Figure 5. Effects of Leu-Ile on methamphetamine (METH)-induced place preference and hyperlocomotion/sensitization in wild-type mice. **(A)** Effect of Leu-Ile on METH-induced place preference in wild-type mice. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before receiving METH (1 mg/kg, SC) or saline during the conditioning. Values are means ± SEM (n = 9–10). $^{*}p < .05$ versus vehicle/saline-treated mice. $^{\#}p < .05$ versus vehicle/METH-treated mice. **(B)** Effect of Leu-Ile on METH-induced hyperlocomotion and sensitization in wild-type mice. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before the METH (1 mg/kg, SC) injection. Values are means ± SEM (n = 10). Locomotor activity was measured for 2 hours after the METH treatment. Analysis of variance with repeated measures revealed significant differences in the locomotor activity [$F(5,54) = 59.1278$, $p < .05$]. $^{*}p < .05$ versus vehicle/saline-treated mice. $^{\#}p < .05$ versus vehicle/METH-treated mice. $^{\$}p < .05$ versus vehicle/METH-treated mice on day 4. **(C)** Effect of Leu-Ile treatment after the development of place preference induced by METH in wild-type mice. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after withdrawal from METH. Values are means ± SEM (n = 7–10). $^{*}p < .05$ versus saline/vehicle-treated mice. $^{\#}p < .05$ versus METH/vehicle-treated mice. **(D)** Effect of Leu-Ile treatment after the development of METH-induced sensitization in wild-type mice. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after the development of METH (1 mg/kg, SC)-induced sensitization. Values are means ± SEM (n = 10). Locomotor activity was measured for 2 hours after the METH treatment on day 18. $^{*}p < .05$ versus saline/vehicle/METH-treated mice. $^{\#}p < .05$ versus METH/vehicle/METH-treated mice.

rons (Figure 4). Furthermore, Leu-Ile induced GDNF and TNF-α expression (Figures 2 and 3). Therefore, Leu-Ile might induce GDNF as a result of TNF-α expression to inhibit METH-induced rewarding effects and sensitization, although another signal pathway should be considered—that Leu-Ile upregulates GDNF expression by activating Hsp90/Akt/CREB signaling (Cen et al 2006).

Leu-Ile increased GDNF and TNF-α expression in the cultured neurons (Figures 2A and 3A) and inhibited METH-induced place preference (Figures 5A and 5C) in bell-shaped response curves. It has been reported that TNF-α, reactive oxygen species (H_2O_2), and β-amyloid activate NF-κB in bell-shaped dose-response curves (Kaltschmidt et al 1999). Rasagiline, an anti-Parkinson drug, activates NF-κB and increases GDNF in bell-shaped dose-response curves (Maruyama et al 2004). We confirmed that Leu-Ile at the lower dose, 1.5 μmol/kg, which could inhibit the rewarding effects of METH, increased GDNF and TNF-α expression both in combination with METH and after withdrawal from repeated METH treatment in the CPP paradigm. Conversely, Leu-Ile at the higher dose, 15 μmol/kg, which could not inhibit the rewarding effects of METH, failed to increase GDNF and TNF-α levels (data not shown). These results suggest involvement of induction of GDNF and TNF-α expression in inhibitory effect of Leu-Ile on the rewarding effects and sensitization of METH.

Leu-Ile attenuated the METH-induced place preference (Figure 5A). Glial cell line-derived neurotrophic factor and TNF-α could be involved in the inhibitory effects of Leu-Ile on the rewarding effects of METH, because no effects of Leu-Ile were observed in the GDNF-(±) and TNF-α-(−/−) mice (Figure 6). These findings support that GDNF and TNF-α play important roles in METH-induced behavioral changes and suggest that Leu-Ile attenuates rewarding effects via the induction of GDNF and TNF-α expression.

The mesolimbic dopaminergic pathway projecting from the VTA to NAc is considered to play a major role in mediating the rewarding effects of electrical stimulation of the brain and drugs of abuse (Everitt and Wolf 2002; Koob 1992; Koob et al 1998). This pathway is important not only for the rewarding effects but also for the locomotor-stimulating effects of METH (Mizoguchi et al 2004; Nagai et al 2005b; Nakajima et al 2004). Leu-Ile inhibited METH-induced hyperlocomotion and sensitization (Figures 5B and 5D), at least in part, through the NAc, because it showed inhibitory effects on the increase in extracellular DA levels induced by repeated METH treatment (Figures 7A and 7C) and the decrease in synaptosomal DA uptake (Figures 7D and 7E) in the NAc. Although Leu-Ile failed to inhibit the hyperlocomotion induced by single METH treatment, it inhibited repeated METH-induced sensitization (Figure 5B). These results suggest that Leu-Ile has inhibitory effects on neuronal plasticity induced by repeated METH treatment but not on single METH-induced hyperlocomotion or the increase in extracellular DA levels (Figure 7B). Because acute treatment of Leu-Ile failed to inhibit single METH-induced hyperlocomotion and the increase in extracellular DA levels, the induction of GDNF and TNF-α expression requires repeated treatment of Leu-Ile.

We have previously reported that TNF-α attenuates the METH-induced increase in extracellular DA levels and potentiates DA uptake into synaptic vesicles and negates METH-induced inhibition of DA uptake in the striatum (Nakajima et al 2004). We

have also demonstrated that the tissue plasminogen activator-plasmin system accelerates the release of DA, which is involved in the rewarding effects of METH and morphine (Nagai et al 2004, 2005a, 2005b). In the present study; we demonstrated that Leu-Ile attenuated the increase in extracellular DA levels in the NAc induced by repeated METH treatment (Figures 7A and 7C) and negated METH-induced inhibition of DA uptake in the midbrain (Figures 7D and 7E). The inhibition of the METH-induced increase in extracellular DA levels and decrease of DA uptake by repeated Leu-Ile treatment might be one plausible mechanism by which Leu-Ile inhibits METH-induced chronic behavioral changes via the induction of TNF-α expression. One might consider that TNF-α could attenuate the rewarding effects and sensitization of other drugs of abuse if it activates DA uptake and thereby attenuates the METH-induced increase in extracellular DA levels. Our results have shown that TNF-α or Leu-Ile inhibits morphine-induced place preference and sensitization by regulating extracellular DA levels (Niwa M, Nitta A, Yamada Y, Nakajima A, Saito K, Seishima M, Noda Y, and Nabeshima T, unpublished observations). These observations support our hypothesis about effect of Leu-Ile on drug abuse.

It has been reported that Leu-Ile induces the expression of

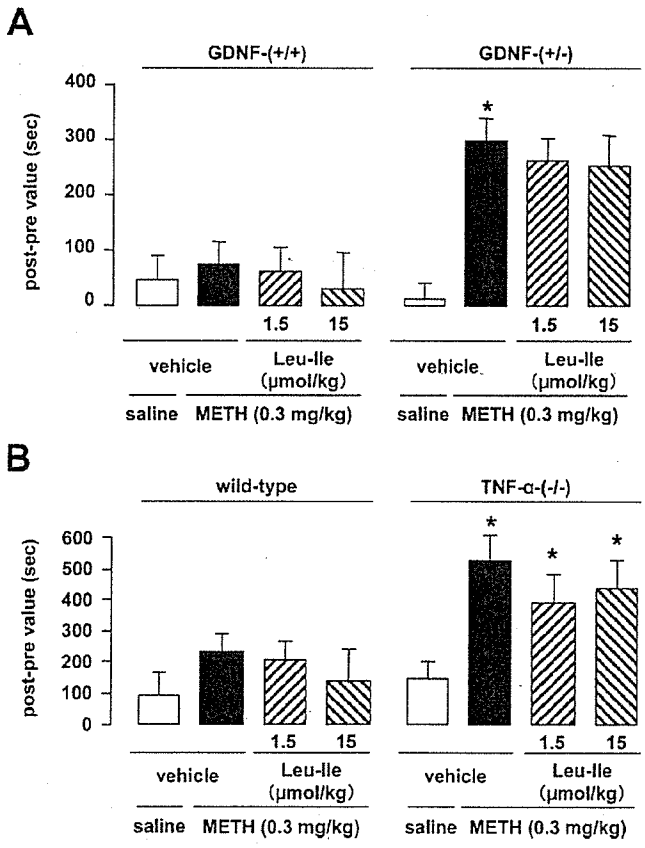

Figure 6. Effect of Leu-Ile on METH-induced place preference in GDNF-(±) and TNF-α-(−/−) mice. **(A)** Effect of Leu-Ile treatment on METH-induced place preference in GDNF-(±) mice. Mice were treated with METH (.3 mg/kg, SC) or saline during the conditioning. Values are means ± SEM (n = 7–12). *$p < .05$ versus vehicle/METH-treated GDNF-(+/+) mice. **(B)** Effect of Leu-Ile treatment on METH-induced place preference in TNF-α-(−/−) mice. Mice were treated with METH (.3 mg/kg, SC) or saline during the conditioning. Values are means ± SEM (n = 8). *$p < .05$ versus vehicle/METH-treated wild-type mice. Abbreviations as in Figure 2.

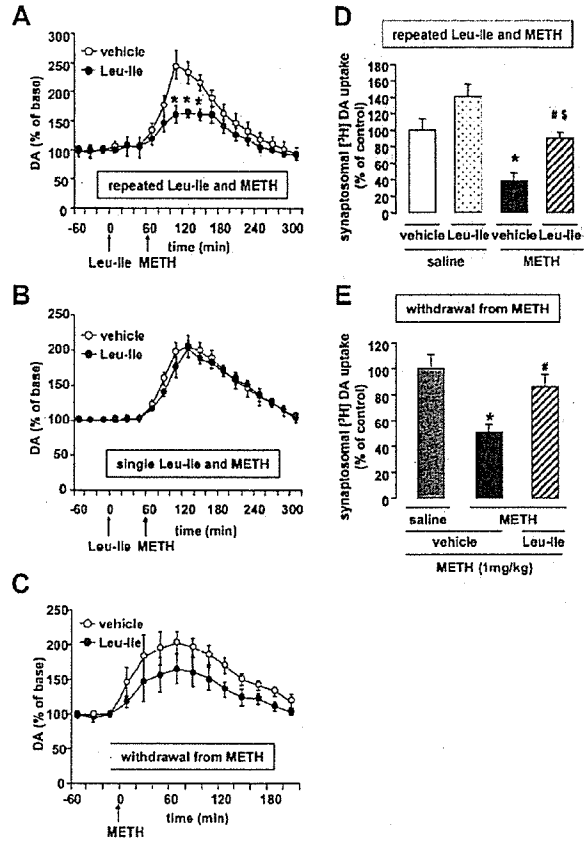

Figure 7. Effect of Leu-Ile on methamphetamine (METH)-induced dopamine (DA) responses. **(A)** In vivo effect of Leu-Ile on the increase in extracellular DA levels induced by repeated METH treatment. Mice were treated with Leu-Ile (1.5 μmol/kg, IP) 1 hour before METH (1 mg/kg, SC) once/day for 9 days. Basal extracellular DA levels were .50 ± .10 and .81 ± .35 nmol/L for the vehicle/METH- and Leu-Ile/METH-treated mice, respectively. Values are means ± SEM (n = 4–5). *$p < .05$ versus vehicle/METH-treated mice. **(B)** In vivo effect of Leu-Ile on the increase in extracellular DA levels induced by single METH treatment. Mice were treated with Leu-Ile (1.5 μmol/kg, IP) once, 1 hour before receiving METH (1 mg/kg, SC). Basal extracellular DA levels were .81 ± .09 and .80 ± .12 nmol/L for the vehicle/METH- and Leu-Ile/METH-treated mice, respectively. Values are means ± SEM (n = 5). **(C)** In vivo effect of Leu-Ile treatment during the withdrawal from METH on the METH-induced increase in extracellular DA levels. Mice were treated with Leu-Ile (1.5 μmol/kg, IP) for 5 days during the withdrawal from METH (1 mg/kg, SC). Basal extracellular DA levels were .27 ± .02 and .24 ± .01 nmol/L for the METH/vehicle- and METH/Leu-Ile-treated mice, respectively. Values are means ± SEM (n = 8). **(D)** Effect of Leu-Ile on the decrease in synaptosomal DA uptake induced by repeated METH treatment. Mice were treated with Leu-Ile (1.5 μmol/kg, IP) 1 hour before METH (1 mg/kg, SC) once/day for 9 days and decapitated 1 hour after the last METH treatment. The synaptosomal [^{3}H] DA uptake was .47 ± .10, .61 ± .10, .13 ± .03, and .39 ± .05 pmol/4-min/mg protein for vehicle/saline-treated, Leu-Ile/saline-treated, vehicle/METH-treated, and Leu-Ile/METH-treated mice, respectively. The final concentration of [^{3}H] DA was 5 nmol/L. Values are means ± SEM (n = 8). *$p < .05$ versus vehicle/saline-treated mice. $^{\#}p < .05$ versus Leu-Ile/saline-treated mice. $^{\$}p < .05$ versus vehicle/METH-treated mice. **(E)** The therapeutic effect of Leu-Ile on the decrease in synaptosomal DA uptake induced by repeated METH treatment. Mice were treated with Leu-Ile (1.5 μmol/kg, IP) for 5 days after the development of METH (1 mg/kg, SC)-induced sensitization. The next day, the mice were administered only METH (1 mg/kg, SC) and decapitated 1 hour after the administration. The synaptosomal [^{3}H] DA uptake was .50 ± .06, .25 ± .03, and .43 ± .05 pmol/4-min/mg protein for saline/vehicle/METH-treated, METH/vehicle/METH-treated, and METH/Leu-Ile/METH-treated mice, respectively. The final concentration of [^{3}H] DA was 5 nmol/L. Values are means ± SEM (n = 8). *$p < .05$ versus saline/vehicle/METH-treated mice. $^{\#}p < .05$ versus METH/vehicle/METH-treated mice.

brain-derived neurotropic factor (BDNF) (Nitta et al 2004) in addition to that of GDNF and TNF-α. Infusion of BDNF into the NAc enhances the stimulation of locomotor activating by cocaine in rats, whereas the development of sensitization and CPP is delayed in heterozygous BDNF knockout mice compared with wild-type littermates (Hall et al 2003; Horger et al 1999). These results suggest a possible role for BDNF in long-term adaptations of the brain to cocaine (Yamada and Nabeshima 2004). In the present study, we targeted anti-addictive factors like GDNF and TNF-α but not pro-addictive factors like BDNF to find a new therapeutic agent for drug dependence. As shown in bell-shaped dose-response curves described previously, the narrow effective dose range of Leu-Ile might be due to the balance of level between anti- and pro-addictive factors induced by Leu-Ile.

Our findings suggest that Leu-Ile has inhibitory effects on METH-induced rewarding effects and sensitization by negating the METH-induced inhibition of DA uptake as well as attenuating the METH-induced increase in extracellular DA levels in the NAc via the induction of GDNF and TNF-α expression. Leu-Ile could be a novel therapeutic agent for METH-induced dependence.

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Tumor Necrosis Factor-α and Its Inducer Inhibit Morphine-Induced Rewarding Effects and Sensitization

Minae Niwa, Atsumi Nitta, Yuichiro Yamada, Akira Nakajima, Kuniaki Saito, Mitsuru Seishima, Yukihiro Noda, and Toshitaka Nabeshima

Background: *Tumor necrosis factor-α (TNF-α) is emerging as an important modulator of the function of the central nervous system (CNS). We have demonstrated that TNF-α or Leu-Ile, a TNF-α inducer, inhibits methamphetamine-induced rewarding effects and sensitization. In this study, we investigated the effects of TNF-α or Leu-Ile on morphine (MOR)-induced rewarding effects and sensitization.*

Methods: *Levels of TNF-α messenger RNA (mRNA) and protein were determined by real-time reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry. Effects of TNF-α or Leu-Ile on MOR-induced rewarding effects and sensitization were investigated by conditioned place preference and locomotor activity tests. Extracellular dopamine levels were examined using in vivo microdialysis. Effects of TNF-α or Leu-Ile on MOR-induced antinociceptive effect and withdrawal symptoms were examined by hot plate test and naloxone-preciptated withdrawal.*

Results: *Morphine induced TNF-α mRNA expression via dopamine and opioid receptors. Posttreatment with TNF-α or Leu-Ile attenuated the MOR-induced place preference and sensitization even after their development, as well as pretreatment with TNF-α or Leu-Ile blocked them. An inhibitory effect of Leu-Ile on MOR-induced place preference was not observed in TNF-α knockout mice. Tumor necrosis factor-α or Leu-Ile inhibited the increase in extracellular dopamine levels in the nucleus accumbens induced by repeated MOR treatment.*

Conclusions: *These results suggest that TNF-α inhibits MOR-induced rewarding effect and sensitization by regulating extracellular dopamine levels, and Leu-Ile inhibits them via the induction of TNF-α.*

Key Words: dopamine (DA), Leu-Ile, morphine (MOR), rewarding effect, sensitization, tumor necrosis factor-α (TNF-α)

Tumor necrosis factor-α (TNF-α) plays an important role in a variety of infectious, inflammatory, and autoimmune conditions (Vassali 1992). Tumor necrosis factor-α also affects the central nervous system (CNS) directly or indirectly through the stimulation of vagal afferents (Maier and Watkins 1998). Thus, this proinflammatory cytokine is emerging as a modulator of CNS function. Regarding the behavioral effects of TNF-α, transgenic mice expressing high levels of TNF-α in the brain showed several changes in exploratory activity and emotional behavior in association with reduced tyrosine hydroxylase (TH) immunoreactivity in the caudate putamen (CPu) without neuronal cell death (Aloe and Fiore 1997). On the other hand, TNF-α knockout mice show anxiogenic-like behavior accompanied by an increase in serotonin metabolism (Yamada et al 2000).

Recently, we have demonstrated that TNF-α plays a neuroprotective role in methamphetamine (METH)-induced drug dependence and neurotoxicity by inhibiting the METH-induced increase in extracellular dopamine (DA) levels through activation of plasmalemmal dopamine transporter (DAT) as well as vesicular monoamine transporter-2 (Nakajima et al 2004). Furthermore, we have demonstrated that Leu-Ile, which induces glial cell line-derived neurotrophic factor (GDNF) production via TNF-α synthesis, inhibits METH-induced rewarding effect and sensitization by regulating extracellular DA levels in the nucleus accumbens (NAc) (Niwa et al, in press).

The psychostimulative effects of METH are associated with an increase in extracellular DA levels in the brain by facilitating the release of DA from presynaptic nerve terminals and inhibiting reuptake (Giros et al 1996; Heikkila et al 1975; Kalivas and Stewart 1991; Seiden et al 1993). It is well known that drugs of abuse, including METH and morphine (MOR), modulate the activity of mesolimbic dopaminergic neurons, projecting from the ventral tegmental area (VTA) of the midbrain to the NAc (Koob 1992, 1998; Wise 1996). Brain DA systems have also been focused on in histochemical, biochemical, and pharmacological research into psychological dependence on opioids, such as MOR (Funada et al 1993; Narita et al 2001). Morphine increases dopaminergic neurotransmission in the NAc via the activation of DA cells in the VTA, an area with a high density of μ-opioid receptors. This activation results mainly from the disinhibition of inhibitory γ-aminobutyric acid (GABA)ergic interneurons in the VTA (Bonci and Williams 1997; Johnson and North 1992). Various studies have provided substantial evidence to support roles for mesolimbic dopaminergic transmission in the rewarding effects of and behavioral sensitization to opioids (Vezina and Stewart 1984). Further, it has been proposed that activity-dependent synaptic plasticity and remodeling of the mesolimbic dopaminergic system play a crucial role in the development of drug dependence (Nestler 2001).

We hypothesized that those genes whose expression was altered by repeated administration of METH and MOR could be candidates for drug-dependence-related genes, because both METH and MOR increase dopaminergic neurotransmis-

From the Department of Neuropsychopharmacology and Hospital Pharmacy (MN, AN, YY, AN, YN, TN), Nagoya University Graduate School of Medicine, Nagoya, Japan; Department of Informative Clinical Medicine (KS, MS), Gifu University Graduate School of Medicine, Gifu, Japan; and Division of Clinical Science in Clinical Pharmacy Practice (YN), Management and Research, Faculty of Pharmacy, Meijo University, Nagoya, Japan.

Address reprint requests to Dr. Toshitaka Nabeshima, Ph.D., Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan; E-mail: tnabeshi@med.nagoya-u.ac.jp.

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sion in the NAc (Nagai et al 2004, 2005a, 2005b; Niwa et al, in press).

To build on our findings, in the present study we examined the roles of TNF-α and Leu-Ile in the rewarding effect and the sensitization to the locomotor-stimulating effects of MOR.

Methods and Materials

Reagents

Tumor necrosis factor-α was donated by Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Leu-Ile was purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). All other materials used were of reagent grade.

Animals

Animals were housed in plastic cages and kept in a temperature-, humidity-, and light-controlled room (23 ± 1°C; 50 ± 5% humidity; 12:12 hour light-dark cycle starting at 8:00 AM) and had free access to food and water, except during behavioral experiments. All animals' care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animials and were approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the Guidelines of Experimental Animal Care issued from the Office of the Prime Minister of Japan. The behavioral experimental schedule is shown in Figure 1.

The male C57BL/6- wild-type mice were obtained from Slc Japan (Hamamatsu, Japan). The generation of TNF-α knockout mice, TNF-α-(-/-), was conducted as reported (Taniguchi et al 1997; Nakajima et al 2004). Tumor necrosis factor-α-(-/-) mice were bred locally in the Laboratory Animal Center, University of Nagoya University Graduate School of Medicine in Japan, on the C57BL/6 background. Male C57BL/6- wild-type and C57BL/6-TNF-α-(-/-) mice, 8 to 12 weeks of age, were used in the experiments.

Drug Treatment

Mice were administered TNF-α (1 and 4 μg, intraperitoneal [IP]), Leu-Ile (1.5 and 15 μmol/kg, IP), or vehicle once a day 1 hour before MOR (10 mg/kg, subcutaneous [SC]) treatment for 9 days (Figure 1A). Tumor necrosis factor-α/MOR, Leu-Ile/MOR, or vehicle/MOR indicate coadministration of TNF-α (1 and 4 μg, IP), Leu-Ile (1.5 and 15 μmol/kg, IP), or vehicle and MOR (10 mg/kg, SC). In the withdrawal experiment, mice were administered TNF-α (1 and 4 μg, IP), Leu-Ile (1.5 and 15 μmol/kg, IP), or vehicle without MOR for 5 days after the withdrawal from MOR following 9 successive days of MOR administration (Figure 1B). Morphine/TNF-α/MOR, MOR/Leu-Ile/MOR, or MOR/vehicle/MOR indicate an acute challenge of MOR in mice treated with TNF-α (1 and 4 μg, IP), Leu-Ile (1.5 and 15 μmol/kg, IP), or vehicle for 5 days after the development of MOR-induced sensitization. To determine messenger RNA (mRNA) expression and protein levels, mice were decapitated 2 and 24 hours after the last MOR injection, respectively. In the real-time reverse transcription polymerase chain reaction (RT-PCR) experiment on the antagonism of MOR-induced TNF-α expression, mice were treated with DA D1 receptor antagonist *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2, 3, 4, 5-tetrahydro-1*H*-3-benzazepine (*R*[+]-SCH23390) (.01 and .1 mg/kg, IP), DA D2 receptor antagonist raclopride (.1 and 1 mg/kg, IP), and the specific opioid receptor antagonist naloxone (NAL) (5 mg/kg, IP) 30, 30, and 15 min before MOR (10 mg/kg, SC) once per day for 9 days, respectively.

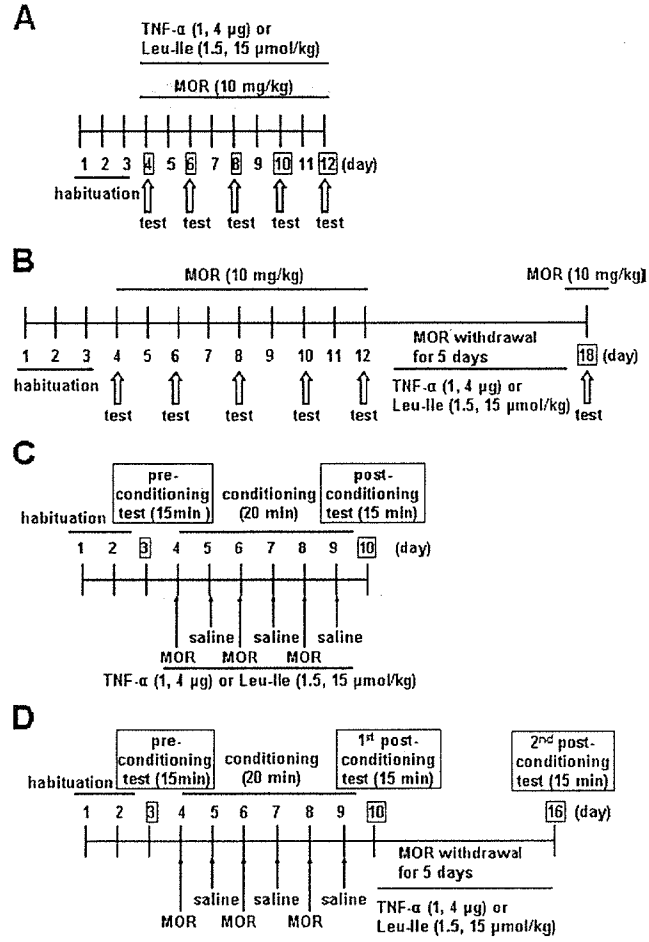

Figure 1. Experimental schedules. **(A)** Experimental schedule for the measurement of locomotor activity to investigate effects of TNF-α or Leu-Ile on MOR-induced sensitization. Mice were habituated to apparatus 240 min a day for 3 days and then co-treated with TNF-α or Leu-Ile and MOR for 9 days. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before MOR (10 mg/kg, SC). Locomotor activity was measured for 3 hours after the MOR treatment. Open arrows indicate the days when locomotor activity was measured. **(B)** Experimental schedule for the measurement of locomotor activity to investigate the effects of TNF-α or Leu-Ile after the withdrawal from MOR. Mice were treated with TNF-α or Leu-Ile after the development of MOR-induced (10 mg/kg, SC) sensitization: Mice were treated with MOR for 9 days and then with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days without MOR. On day 18, mice were administered only MOR (10 mg/kg, SC), and locomotor activity was measured for 3 hours after the MOR treatment. Open arrows indicate the day when locomotor activity was measured. **(C)** Experimental schedule for the conditioned place preference task to investigate effects of TNF-α or Leu-Ile on MOR-induced place preference. Mice were co-treated with TNF-α or Leu-Ile and MOR in the conditioning period. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before MOR (10 mg/kg, SC) or saline. The postconditioning test was carried out 1 day after the conditioning. Closed arrows indicate the days of MOR or saline injection. **(D)** Experimental schedule for the conditioned place preference task to investigate the effects of TNF-α or Leu-Ile after the withdrawal from MOR. Mice were not treated with TNF-α or Leu-Ile in the conditioning period. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after withdrawal from MOR. The first postconditioning test was carried out 1 day after conditioning. The second postconditioning test was carried out 5 days after the first postconditioning test. Closed arrows indicate the days of MOR or saline injection. TNF-α, tumor necrosis factor-α; MOR, morphine; IP, intraperitoneal; SC, subcutaneous.

Semiquantative mRNA Analysis by Real-Time RT-PCR

Mice were decapitated 2 hours after the last injection of MOR, and the brains were quickly removed. Various regions including the frontal cortex (Fc), NAc, CPu, and hippocampus (Hip) were rapidly dissected out, frozen, and stored in a deep freezer at −80°C until the assays. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The levels of TNF-α mRNA were determined by real-time RT-PCR (Bio-Rad Laboratories Inc., Hercules, California) using a TaqMan probe. The 18S ribosomal RNA was used as the internal control (PE Applied Biosystems, Foster, California). Total RNA was converted into complementary DNA (cDNA) using a SuperScript First-Strand System for RT-PCR Kit (Invitrogen Life Technologies, Carlsbad, California) in a total volume of 21 μL (reverse transcriptase [RT]-reaction mixture). Polymerase chain reaction (PCR) was performed using 1 μL of the RT-reaction mixture, 10 nm of each (forward and reverse) primer, 80 nm of TaqMan probe, and 12.5 μL of TaqMan Universal PCR Master Mix (PE Applied Biosystems) in a total reaction volume of 25 μL. The mouse TNF-α primers used were as follows: 5'-CCCTTGCCCAGCCAGAA-3' (forward) and 5'-CCCCCTAAAAGACACGAAGATG-3' (reverse) and TaqMan probe: 5'-AGCTTGATGTCATCTCTTCGTGGGCT-3'. Partial cDNA sequences of mouse TNF-α have been deposited in the National Center for Biotechnology Information (NCBI) GenBank database (accession number: NM_013693). The amplification consisted of an initial step (50°C for 2 min and 95°C for 2 min) and then 40 cycles of denaturation for 15 sec at 95°C and annealing for 1 min at 60°C in an iCycle iQ Detection System (Bio-Rad Laboratories, Inc., Tokyo, Japan). The signal was detected according to the manufacturer's instructions. The expression levels were calculated as described previously (Wada et al 2000).

Immunohistochemical Analysis

For samples for immunohistochemical analysis, mice were killed 24 hours after repeated treatment with Leu-Ile (1.5 μmol/kg, IP) 1 hour before the administration of MOR (10 mg/kg, SC, once a day for 9 days). The brains were sliced at 20 μm in the cryostat. Slices were rinsed briefly with phosphate-buffered saline (PBS) containing .01% Triton X-100 (washing buffer; Sigma-Aldrich, St. Louis, Missouri). They were incubated with primary antibodies in washing buffer overnight at 4°C. After being washed, slices were incubated with secondary antibodies for 2 hours. Polyclonal goat anti-TNF-α antibody (1:100; R&D Systems Ltd., Minneapolis, Minnesota), monoclonal mouse anti-neuron-specific nuclear antigen (NeuN) antibody (1:200; Chemicon, Temecula, California), and monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:200; Chemicon International, Inc., Temecula, California) served as primary antibodies. Donkey anti-goat Alexa Fluor 546 (1:1000; Molecular Probes, Inc., Engene, Oregon) and rabbit anti-mouse Alexa Fluor 488 (1:1000; Molecular Probes, Inc.) were used as secondary antibodies for TNF-α immunostaining. Each stained tissue was observed under a fluorescence microscope (Axioskoop 2 plus; Carl Zeiss, Jena, Germany) and analyzed with Axiovision 3.0 systems (Carl Zeiss).

The area with TNF-α-positive cells in the defined NAc region of mice was determined using the software WinROOF (Mitani Co., Ltd., Fukui, Japan) (Tsuji et al 1999; Kuwahara et al 1999). We employed an immunostaining method with which one can analyze the distribution and levels of TNF-α protein in the present investigation, since it is too difficult to use Western blotting or an enzyme immunoassay to quantify the amount of TNF-α protein in brain tissue.

Locomotor Activity

Locomotor activity was measured using an infrared detector (Neuroscience Co., Ltd., Tokyo, Japan) in a plastic box (32 × 22 × 15 cm high). Mice were administered Leu-Ile (1.5 and 15 μmol/kg, IP) or vehicle and habituated for 1 hour in the box. Mice were administered MOR (10 mg/kg, SC) or saline 1 hour after the TNF-α or Leu-Ile treatment, and the locomotor activity was measured for 2 hours immediately after the MOR or saline administration (Figures 1A and 1B). Leu-Ile and MOR were injected once a day for 9 days (days 4–12). In the experiment with withdrawal, mice were administered TNF-α, μ Leu-Ile, or vehicle for 5 days (days 13–17) after the withdrawal from MOR following 9 successive days of MOR administration. On day 18, the mice were administered only MOR (10 mg/kg, SC), and locomotor activity was measured for 2 hours immediately after the administration (Figure 1C).

Conditioned Place Preference

The apparatus used for the place-conditioning task consisted of two compartments: a transparent Plexiglas box and a black Plexiglas box (both 15 × 15 × 15 cm high). To enable mice to distinguish easily the two compartments, the floors of the transparent and black boxes were covered with white plastic mesh and black frosting Plexiglas, respectively. Each box could be divided by a sliding door (10 × 15 cm high). The place-conditioning paradigm was performed by using a previously established procedure with a minor modification (Noda et al 1998; Mizoguchi et al 2004; Niwa et al, in press). The experimental schedule for the condition place preference task is shown in Figures 1C and 1D. In the preconditioning test, the sliding door was opened, and the mouse was allowed to move freely between both boxes for 15 min once a day for 3 days. On the third day of the preconditioning test, we measured the time that the mouse spent in the black and transparent boxes by using a Scanet SV-20 LD (Melquest Co., Ltd., Toyama, Japan). The box in which the mouse spent the most time was referred to as the "preferred side," and the other box as the "nonpreferred side." Conditioning was performed during 6 successive days. Mice were given MOR or saline in the apparatus with the sliding door closed. That is, a mouse was subcutaneously given MOR and put in its nonpreferred side for 20 min. On the next day, the mouse was given saline and placed opposite the drug-conditioning site for 20 min. These treatments were repeated for three cycles (6 days). In the postconditioning test, the sliding door was opened, and we measured the time that the mouse spent in the black and transparent boxes for 15 min, using the Scanet SV-20 LD. Place-conditioning behavior was expressed by Post-Pre, which was calculated as: ([postvalue] − [prevalue]), where postvalues and prevalues were the difference in time spent at the drug-conditioning and the saline-conditioning sites in the postconditioning and preconditioning tests, respectively.

In Vivo Microdialysis

Mice were anesthetized with sodium pentobarbital, and a guide cannula (AG-8, EICOM, Kyoto, Japan) was implanted into the NAc (+1.1 mm anteroposterior, +1.0 mm mediolateral from the bregma, and -4.0 mm dorsoventral to the dura) according to the atlas of Franklin and Paxinos (1997) and secured to the skull using stainless steel screws and dental acrylic cement. One day after the operation, a dialysis probe (AI-8-1; 1-mm mem-

brane length, EICOM) was inserted through the guide cannula and perfused continuously with artificial cerebrospinal fluid (aCSF; 147 mmol/L sodium chloride [NaCl], 4 mmol/L potassium chloride [KCl], and 2.3 mmol/L calcium chloride [$CaCl_2$]) at a rate of 1.0 μL/min. Dialysate was collected in 20-min fractions and injected into the high-performance liquid chromatography (HPLC) system (EICOM) for the measurement of DA levels. Three samples were used to establish baseline levels of extracellular DA before the administration of TNF-α or Leu-Ile and MOR.

Hot Plate Test

To develop tolerance, mice received MOR (10 mg/kg) subcutaneously twice a day for 5 days.

Nociceptive threshold was assessed as the response time to the hot plate (55 ± 1°C). The response was defined by the animal either licking its forepaws or hindpaws or flicking its hindpaws. Licking of the hindpaws was taken as the end point. The cutoff time (90 sec) was set to minimize injury to the mouse. The antinociceptive effect of MOR was determined 60 min after the first treatment on day 1 and the second treatment on day 5 (Hamdy et al 2004; Miyamoto et al 2004).

Induction of Withdrawal Syndrome

Withdrawal was precipitated on day 6 by injecting NAL (5 mg/kg, IP) 2 hours after the final administration of MOR, according to previous reports (Itoh et al 2000; Mamiya et al 2001). Twenty minutes before the observation, mice were placed in a transparent acrylic cylinder (20 cm in diameter, 35 cm high) to habituate to the new environment. Immediately after the NAL challenge, each mouse was placed gently again in the cylinder, and the frequency of NAL-precipitated withdrawal signs (jumping, forepaw tremor, and rearing) was recorded for 15 min (Hamdy et al 2004; Ren et al 2004).

Statistical Analysis

All data were expressed as means ± SEM. Statistical differences between two groups were determined with Student *t* test. Statistical differences among more than three groups were determined using a one-way analysis of variance (ANOVA) or a repeated ANOVA, followed by the Bonferroni multiple comparison test; $p < .05$ was regarded as statistically significant.

Results

Change of Expression of TNF-α mRNA After MOR Treatment

As an initial step in assessing the relationship between TNF-α and MOR, the effects of single and repeated MOR administration on the expression of TNF-α mRNA in various regions of the mouse brain were measured 2 hours after the final MOR treatment using the real-time RT-PCR method. Although single MOR treatment did not induce expression of TNF-α mRNA in any regions examined, repeated MOR treatment remarkably induced it in the NAc [$F(2,21) = 17.108$, $p < .05$, one-way ANOVA] and CPu [$F(2,21) = 8.300$, $p < .05$, one-way ANOVA] (Figure 2A). The increase in TNF-α mRNA expression caused by MOR in the NAc was inhibited by pretreatment with the DA D1 receptor antagonist SCH23390 (.1 mg/kg, IP), the D2 receptor antagonist raclopride (1 mg/kg, IP) [$F(9,40) = 5.390$, $p < .05$, one-way ANOVA] (Figure 2B), and the specific opioid receptor antagonist, naloxone (5 mg/kg, IP) [$F(3,28) = 14.301$, $p < .05$, one-way ANOVA] (Figure 2C), although neither antagonist itself had an effect on TNF-α mRNA expression in the control group, suggesting the involvement of DA D1, D2, and opioid receptors in the MOR-induced increase in TNF-α mRNA expression.

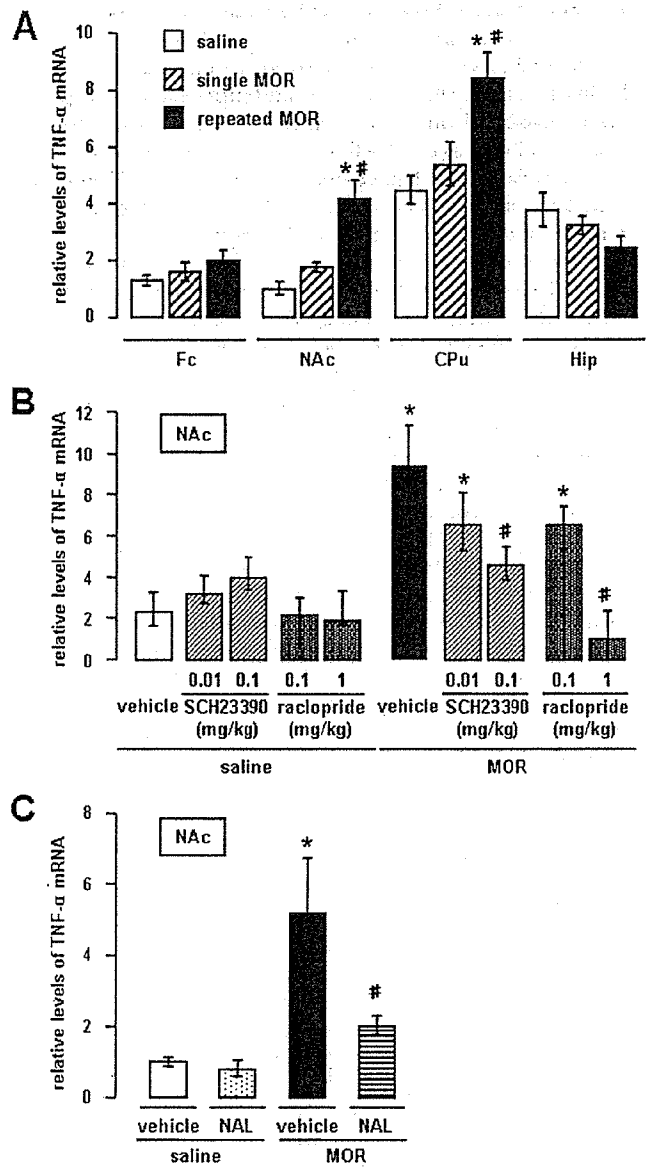

Figure 2. Change of expression of TNF-α mRNA after repeated MOR treatment. **(A)** Change of expression of TNF-α mRNA in the various regions of the mouse brain after single and repeated MOR treatment. Mice were decapitated 2 hours after single or repeated MOR (10 mg/kg, SC) treatment for 1 day or 9 days, respectively. Values are means ± SEM ($n = 8$). *$p < .05$ versus saline-treated mice. #$p < .05$ versus single MOR-treated mice. **(B)** Effects of the DA D1 receptor antagonist SCH23390 and D2 receptor antagonist raclopride on MOR-induced expression of TNF-α mRNA in the NAc. Mice were treated with *R*(+)-SCH23390 (.01 and .1 mg/kg, IP) and raclopride (.1 and 1 mg/kg, IP) 30 min before MOR (10 mg/kg, SC) once per day for 9 days and decapitated 2 hours after the last MOR treatment. Values are means ± SEM ($n = 5$). *$p < .05$ versus vehicle/saline-treated mice. #$p < .05$ versus vehicle/MOR-treated mice. **(C)** Effect of naloxone (NAL) on MOR-induced expression of TNF-α mRNA in the NAc. Mice were treated with NAL (5 mg/kg, IP) 15 min before MOR (10 mg/kg, SC) once per day for 9 days and decapitated 2 hours after the last MOR treatment. Values are means ± SEM ($n = 8$). *$p < .05$ versus vehicle/saline-treated mice. #$p < .05$ versus vehicle/MOR-treated mice. Fc, frontal cortex; NAc, nucleus accumbens; CPu, caudate putamen; Hip, hippocampus; TNF-α, tumor necrosis factor-α; mRNA, messenger RNA; MOR, morphine; SC, subcutaneous; DA, dopamine; *R*(+)-SCH23390, *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2, 3, 4, 5-tetrahydro-1*H*-3-benzazepine; IP, intraperitoneal; NAL, naloxone.

Change of TNF-α Expression After the Treatment with Leu-Ile and/or MOR

We have reported that TNF-α mRNA levels were elevated in the NAc, CPu, Hip, and amygdala after repeated daily administration of METH (2 mg/kg for 5 days) in the rat brain (Nakajima et al 2004). Moreover, we have found that TNF-α mRNA, GDNF mRNA, and GDNF levels were significantly elevated 24 hours after treatment with Leu-Ile (.37 μg/mL) in cultured neurons compared with the control group (Niwa et al, in press).

We investigated the dose-response effect of Leu-Ile on the expression of TNF-α mRNA in the NAc. Leu-Ile (1.5 and 15 μmol/kg, IP) increased the levels of TNF-α mRNA compared with the vehicle-treated mice [$F(3,38) = 10.615$, $p < .05$, one-way ANOVA] (Figure 3A).

Tumor necrosis factor-α-positive cells were found among the neurons that were immunopositive for NeuN but not for GFAP, in Leu-Ile-treated (1.5 μmol/kg, IP) and MOR-treated (10 mg/kg, SC) mouse brain (Figure 3B). There was no observation of TNF-α immunopositive cells in TNF-α-(-/-) mice (Figure 3B[c]).

Areas with TNF-α immunoreactive cells were detected in the NAc using the software WinROOF (Mitani Co. Ltd., Fukui, Japan). The areas occupied by TNF-α-positive cells were measured to estimate the effects of Leu-Ile on the production of TNF-α protein. Morphine (10 mg/kg, SC) potentiated the immunoreactivity to TNF-α in the NAc. After the coadministration of Leu-Ile (1.5 and 15 μmol/kg, IP) and MOR (10 mg/kg, SC), immunoreactivity was much more increased in the NAc [$F(5,32) = 38.841$, $p < .05$, one-way ANOVA] (Figures 3B and 3C). Moreover, we determined levels of TNF-α protein in the NAc after Leu-Ile treatment during the withdrawal from MOR following 9 successive days of MOR administration. The schedule is described in Figure 1B. An acute challenge of MOR in mice treated with vehicle for 5 days after the development of MOR-induced sensitization increased TNF-α levels in the NAc compared with those in the saline/vehicle/MOR-treated mice. An acute challenge of MOR in mice treated with Leu-Ile (1.5 and 15 μmol/kg) for 5 days after the development of MOR-induced sensitization markedly increased TNF-α levels compared with those in the MOR/vehicle/MOR-treated mice [$F(3,22) = 75.245$, $p < .05$, one-way ANOVA] (Figure 3D).

Effects of TNF-α or Leu-Ile on MOR-Induced Hyperlocomotion/Sensitization Before and After the Development of Sensitization

We investigated the effects of TNF-α or Leu-Ile on the behavioral responses to MOR. First, we examined the effects of TNF-α or Leu-Ile on the development of MOR-induced hyperlocomotion and sensitization. Sensitization refers to the augmentation of behavioral responses to drugs of abuse that occurs during their repeated administration and persists long after drug exposure is discontinued (Robinson and Berridge 2000; Boudreau and Wolf 2005). The experimental schedule is described in Figure 1A. Tumor necrosis factor-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) did not affect spontaneous locomotor activity in saline-treated mice (Figure 4A). As shown in Figure 4A, single MOR treatment (day 4) at the dose of 10 mg/kg increased locomotor activity, and repeated administration for 9 days (days 4–12) resulted in an enhancement of the locomotor-stimulating effect of MOR (sensitization: days 8–12) [$F(4,35) = 15.402$, $p < .05$, one-way ANOVA]. Coadministration of TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) with MOR inhibited the development of MOR-induced hyperlocomotion and sensitization on days 8–12 [$F(9,70) = 65.020$ at day 8, $F(9,70) = 68.815$ at day 10, $F(9,70) = 85.493$ at day 12, $p < .05$, one-way ANOVA]. Tumor necrosis factor-α or Leu-Ile had no effect on days 4–6 (Figure 4A). These results demonstrate that TNF-α or Leu-Ile inhibits the development of MOR-induced hyperlocomotion and sensitization on repeated administration but not single administration.

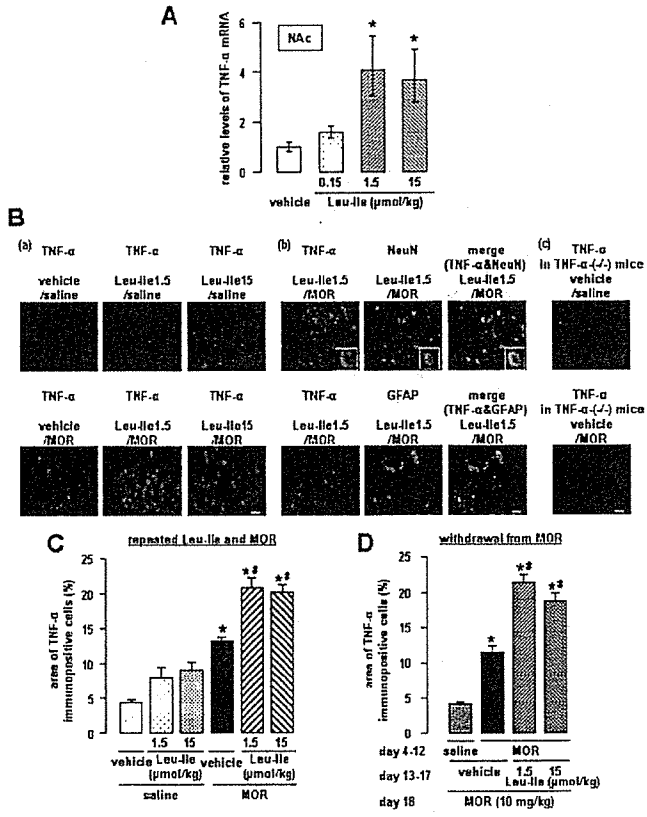

Figure 3. Change of TNF-α expression after the treatment with Leu-Ile and/or MOR. **(A)** The dose-response effect of Leu-Ile on the expression of TNF-α mRNA in the NAc. Mice were treated with Leu-Ile (.15, 1.5, and 15 μmol/kg, IP) for 9 days and decapitated 2 hours after the last injection. Values are means ± SEM ($n = 10–11$). *$p < .05$ versus vehicle-treated mice. **(B)** Immunostaining of TNF-α in the NAc after the repeated administration of Leu-Ile and/or MOR in wild-type mice (a). Double-labeling fluorescence photomicrographs for TNF-α and NeuN or GFAP in wild-type mice (b). Tumor necrosis factor-α-immunoreactive cells (red) were colocalized with NeuN-positive cells (green) in the NAc. Double immunostaining for TNF-α and NeuN in the NAc reveals TNF-α expression in neuronal cells. Immunostaining of TNF-α in the NAc after the repeated administration of vehicle and saline or MOR in TNF-α-(-/-) mice (c). Immunopositive cells of TNF-α were absent in TNF-α-(-/-) mice. Scale bar, 20 μm. **(C)** Change of TNF-α expression in the NAc after the administration of Leu-Ile and/or MOR. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before MOR (10 mg/kg, SC) for 9 days and decapitated 24 hours after the last MOR injection. The area of TNF-α positive cells in 3.8×10^4 μm^2 was estimated using the software WinROOF. Values are means ± SE ($n = 6–8$). *$p < .05$ versus vehicle/saline-treated mice. #$p < .05$ vs. vehicle/MOR-treated mice. **(D)** Change of TNF-α expression in the NAc after Leu-Ile treatment during the withdrawal from MOR. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) without MOR for 5 days after the development of MOR-induced (10 mg/kg, SC, for 9 days) sensitization. The next day, the mice were administered only MOR (10 mg/kg, SC) and decapitated 24 hours after the administration. Values are means ± SE ($n = 6–8$). *$p < .05$ versus saline/vehicle/MOR-treated mice. #$p < .05$ versus MOR/vehicle/MOR-treated mice. TNF-α, tumor necrosis factor-α; MOR, morphine; mRNA, messenger RNA; NAc, nucleus accumbens; IP, intraperitoneal; NeuN, neuron-specific nuclear antigen; GFAP, glial fibrillary acidic protein; SC, subcutaneous.

Next, the inhibitory effects of TNF-α (1 and 4 μg, IP) or

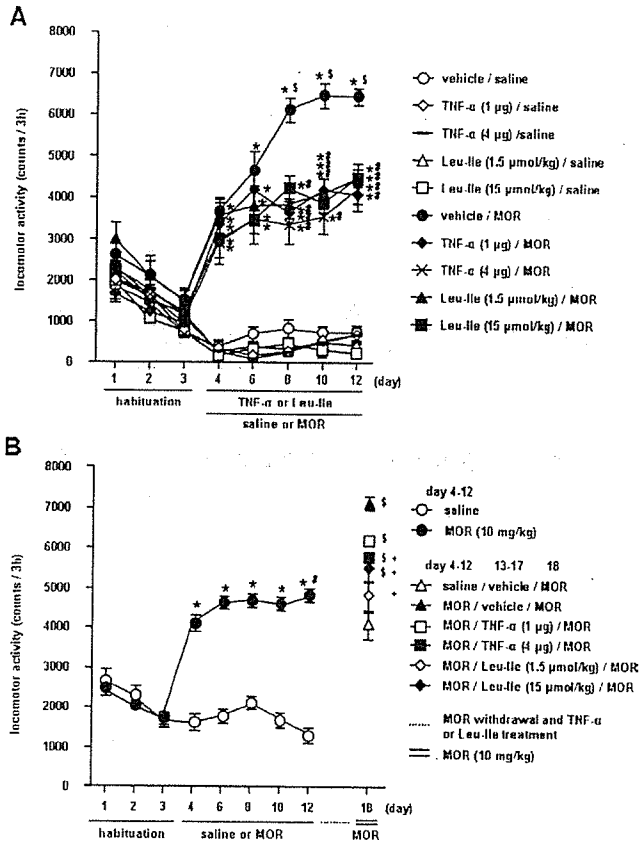

Figure 4. Effects of TNF-α or Leu-Ile on MOR-induced hyperlocomotion/sensitizaton before and after the development of sensitization. **(A)** Effect of TNF-α or Leu-Ile on MOR-induced hyperlocomotion and sensitization in wild-type mice. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before receiving the MOR (10 mg/kg, SC) injection. Values are means ± SEM ($n = 8$). Locomotor activity was measured for 3 hours after the MOR treatment. Analysis of variance with repeated measures revealed significant differences in locomotor activity. $^{*}p < .05$ versus vehicle/saline-treated mice. $^{\#}p < .05$ versus vehicle/MOR-treated mice. $^{\$}p < .05$ versus vehicle/MOR-treated mice on day 4. **(B)** Effect of TNF-α or Leu-Ile treatment after the development of MOR-induced sensitization in wild-type mice. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) without MOR for 5 days after the development of MOR-induced (10 mg/kg, SC, for 9 days) sensitization. Values are means ± SEM ($n = 10$). Locomotor activity was measured for 3 hours after the MOR treatment. $^{*}p < .05$ versus saline-treated mice. $^{\#}p < .05$ versus MOR-treated mice on day 4. $^{\$}p < .05$ versus saline/vehicle/MOR-treated mice. $^{+}p < .05$ versus MOR/vehicle/MOR-treated mice. TNF-α, tumor necrosis factor-α; MOR, morphine; IP, intraperitoneal; SC, subcutaneous.

Leu-Ile (1.5 and 15 μmol/kg, IP) were investigated after the development of MOR-induced (10 mg/kg) sensitization. The experimental schedule is described in Figure 1B. The repeated administration of MOR for 9 days again resulted in an enhancement of the locomotor-stimulating effect of MOR (sensitization) [$F(4,245) = 2.374$, $p < .05$, one-way ANOVA]. Sensitization was maintained on day 18, 5 days after withdrawal from MOR. To investigate the therapeutic effects of TNF-α and Leu-Ile, mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days during the withdrawal period. Tumor necrosis factor-α (4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) inhibited MOR-induced (10 mg/kg) sensitization on day 18 [$F(5,54) = 12.221$, $p < .05$, one-way ANOVA] (Figure 4C). The results of TNF-α levels in the NAc after locomotor test are described in Figures 3C and 3D.

Effects of TNF-α or Leu-Ile on MOR-Induced Place Preference Before and After Its Development

The effects of TNF-α or Leu-Ile on the rewarding effects of MOR were examined in a place-conditioning paradigm, in which animals learn the association of an environment paired with drug exposure. Place conditioning is, therefore, considered a measure of the rewarding properties of drugs of abuse. The experimental schedule is described in Figure 1D. As shown in Figure 5A, MOR (10 mg/kg, SC) produced place preference in mice. When TNF-α (4 μg, IP) or Leu-Ile (1.5 μmol/kg, IP) was administered 1 hour

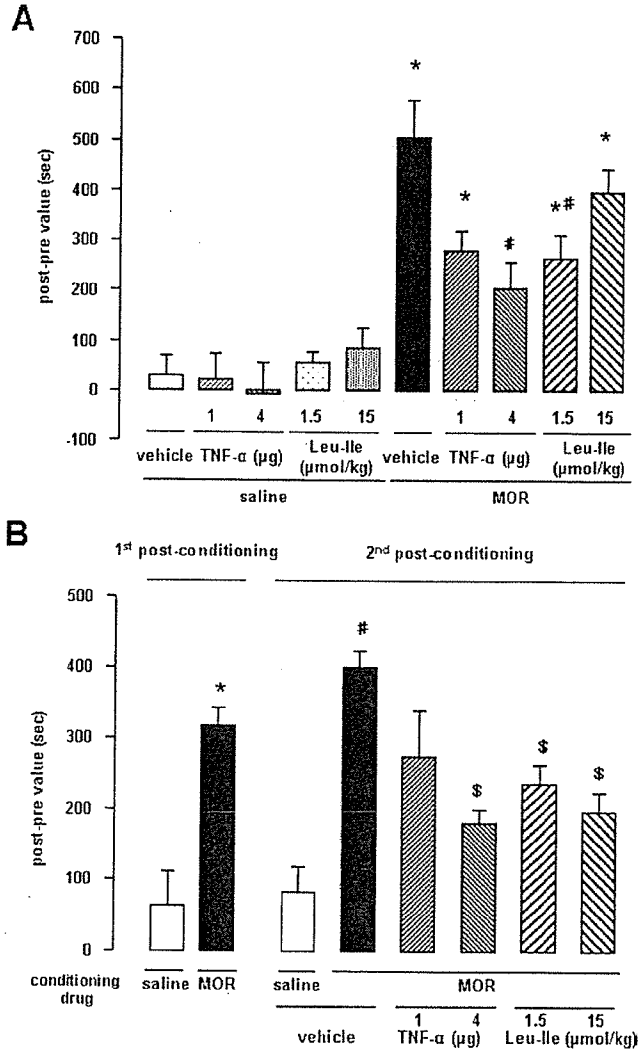

Figure 5. Effects of TNF-α or Leu-Ile on MOR-induced place preference before and after its development. **(A)** Effects of TNF-α or Leu-Ile on development of MOR-induced place preference in wild-type mice. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before MOR (10 mg/kg, SC) or saline during the conditioning. Values are means ± SEM ($n = 13$). $^{*}p < .05$ versus vehicle/saline-treated mice. $^{\#}p < .05$ versus vehicle/MOR-treated mice. **(B)** Effect of TNF-α or Leu-Ile treatment after the development of place preference induced by MOR in wild-type mice. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) without MOR for 5 days after withdrawal from MOR. Values are means ± SEM ($n = 15$). $^{*}p < .05$ versus saline-treated mice in first conditioning test. $^{\#}p < .05$ versus saline/vehicle-treated mice in second conditioning test. $^{\$}p < .05$ versus MOR/vehicle-treated mice in second conditioning test. TNF-α, tumor necrosis factor-α; MOR, morphine; IP, intraperitoneal; SC, subcutaneous.