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.

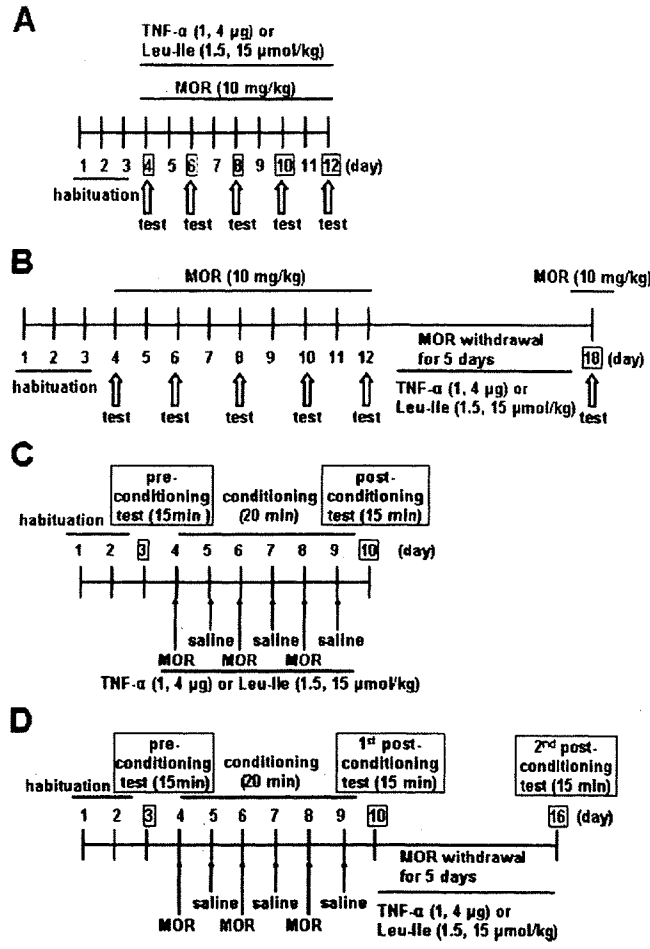

Figure 1. Experimental schedules. **(A)** Experimental schedule for the measurement of locomotor activity to investigate effects of TNF-α or Leu-Ile on MOR-induced sensitization. Mice were habituated to apparatus 240 min a day for 3 days and then co-treated with TNF-α or Leu-Ile and MOR for 9 days. Mice 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) (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, 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.

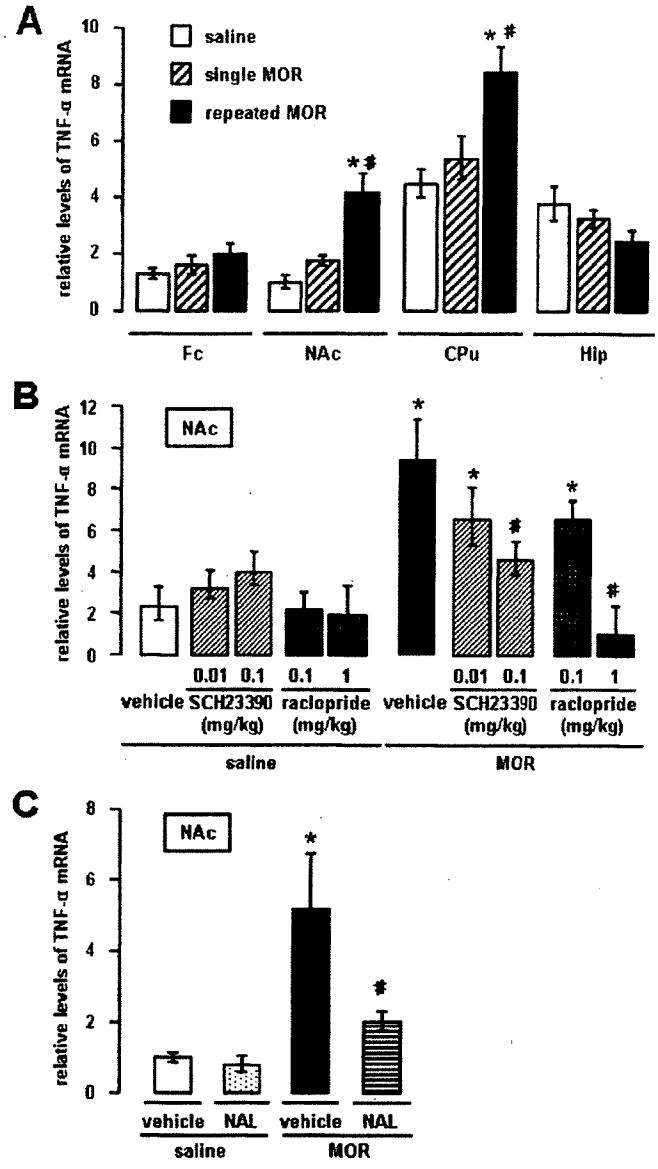

Figure 2. Change of expression of TNF-α mRNA after repeated MOR treatment. **(A)** Change of expression of TNF-α mRNA in the various regions of the mouse brain after single and repeated MOR treatment. Mice were decapitated 2 hours after single or repeated MOR (10 mg/kg, SC) treatment for 1 day or 9 days, 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 (Boudreau and Wolf 2005; Robinson and Berridge 2000). 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.

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

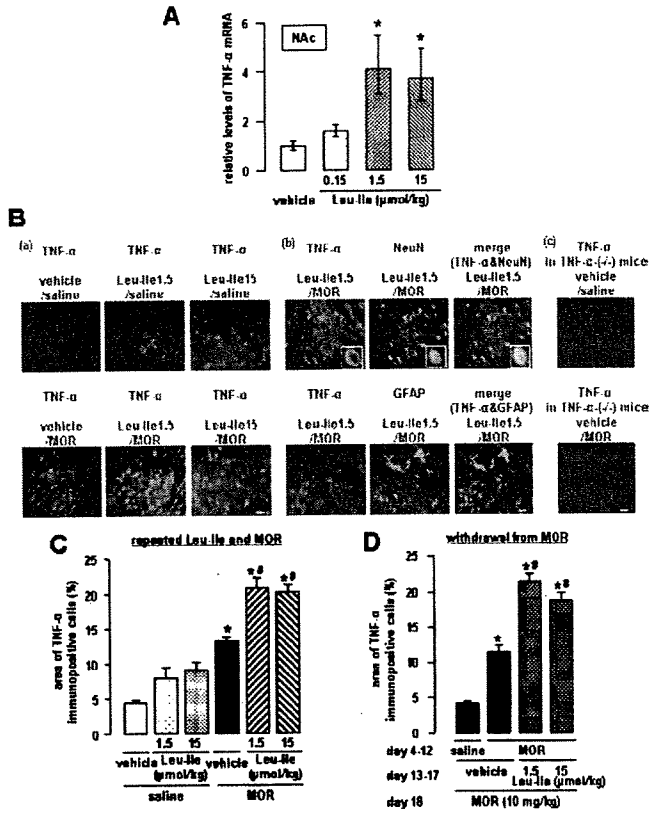

Figure 3. Change of TNF-α expression after the treatment with Leu-Ile and/or MOR. **(A)** The dose-response effect of Leu-Ile on the expression of TNF-α mRNA in the NAc. Mice were treated with Leu-Ile (.15, 1.5, and 15 μmol/kg, IP) for 9 days and decapitated 2 hours after the last injection. Values are 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.

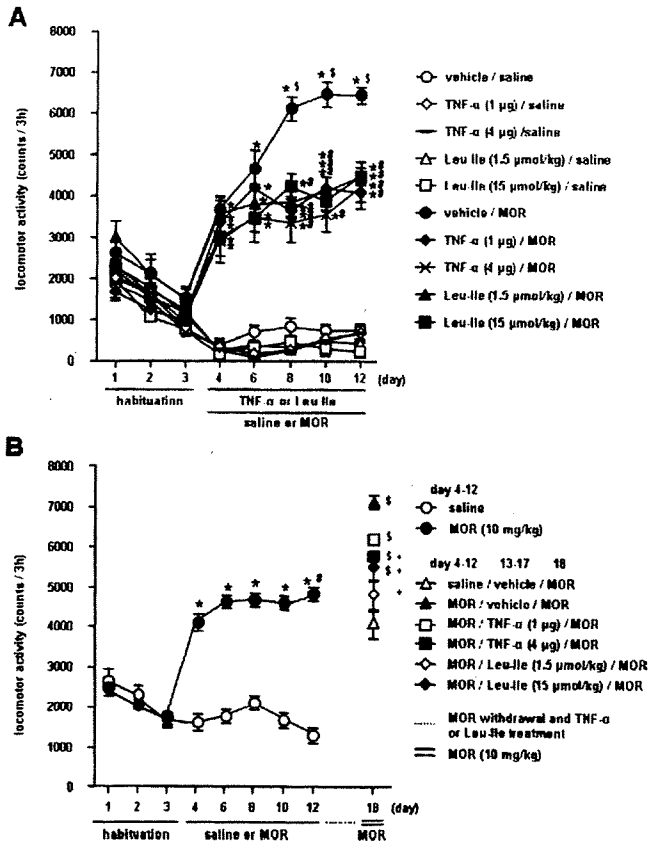

Figure 4. Effects of TNF-α or Leu-Ile on MOR-induced hyperlocomotion/sensitizaton before and after the development of sensitization. **(A)** Effect of TNF-α or Leu-Ile on MOR-induced hyperlocomotion and sensitization in wild-type mice. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 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

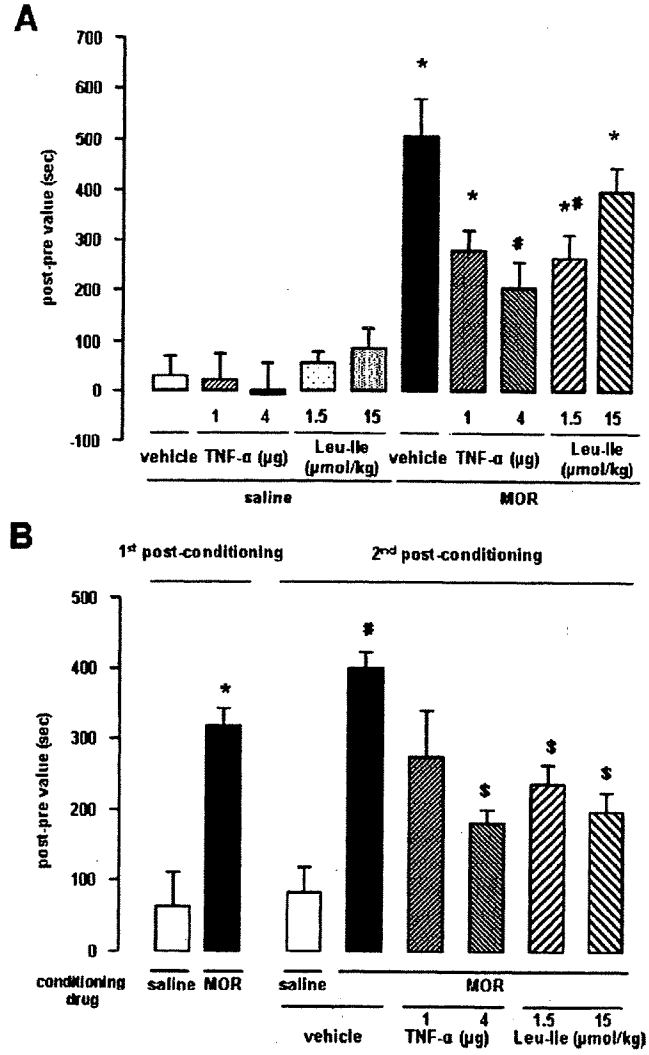

Figure 5. Effects of TNF-α or Leu-Ile on MOR-induced place preference before and after its development. **(A)** Effects of TNF-α or Leu-Ile on development of MOR-induced place preference in wild-type mice. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before 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.

before MOR, the development of MOR-induced place preference was significantly attenuated [$F(9,120) = 13.067$, $p < .05$, one-way ANOVA] (Figure 5A). Tumor necrosis factor-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) itself failed to affect place preference in mice (second, third, fourth, and fifth columns in Figure 5A). We confirmed that Leu-Ile at the lower dose, 1.5 μmol/kg, which could inhibit the rewarding effects of MOR, increased TNF-α expression in combination with MOR in the conditioned place preference (CPP) paradigm. On the contrary, Leu-Ile at the higher dose, 15 μmol/kg, which could not inhibit the rewarding effects of MOR, failed to increase TNF-α expression in combination with MOR in the CPP paradigm [data not shown; $F(5,30) = 35.937$ for co-treatment with Leu-Ile and MOR in CPP paradigm].

To investigate the therapeutic effect of TNF-α or Leu-Ile, mice were administered TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days without MOR treatment after the development of MOR-induced place preference. The experimental schedule is described in Figure 1E. In this experiment, the second postconditioning was carried out 5 days after the first postconditioning. Although MOR-induced (10 mg/kg) place preference was maintained for 5 days after the first postconditioning in wild-type mice, it was attenuated by the treatment with TNF-α (4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days between the first and second postconditionings [$F(5,84) = 8.561$, $p < .05$, one-way ANOVA] (Figure 5C). We confirmed that Leu-Ile at doses of 1.5 and 15 μmol/kg, which could inhibit the rewarding effects of MOR, increased TNF-α expression after withdrawal from repeated MOR treatment in the CPP paradigm [data not shown; $F(3,20) = 60.346$ for Leu-Ile treatment during withdrawal from MOR in CPP paradigm].

Effects of TNF-α or Leu-Ile on the Rewarding Effects of MOR in TNF-α-(-/-) Mice

The role of endogenous TNF-α in the rewarding effects of MOR was determined using TNF-α-(-/-) mice. To confirm the involvement of TNF-α in the rewarding effects of MOR, the effect of Leu-Ile on the MOR-induced place preference was also examined in TNF-α-(-/-) mice. The experimental schedule is described in Figure 1D. Although at a dose of 10 mg/kg MOR induced place preference in both wild-type and TNF-α-(-/-) mice, at 3 mg/kg it failed to induce place preference in wild-type mice (Figures 6A and 6B). On the other hand, TNF-α-(-/-) mice developed place preference even at the dose of 3 mg/kg [$F(7,56) = 9.711$, $p < .05$, one-way ANOVA] (Figure 6A). When TNF-α (4 μg, IP) was administered 1 hour before MOR, it inhibited MOR-induced place preference in TNF-α-(-/-) mice [$F(11,84) = 5.730$, $p < .05$, one-way ANOVA] (Figure 6B). Leu-Ile (1.5 and 15 μmol/kg, IP) treatment 1 hour before MOR failed to inhibit the MOR-induced place preference in TNF-α-(-/-) mice (Figure 6B).

Effects of TNF-α or Leu-Ile on MOR-Induced Increase in Extracellular DA Levels

The dopaminergic system is important not only for the rewarding effects but also for the locomotor-stimulating effects of MOR and METH (Nagai *et al.* 2004, 2005a, 2005b; Nakajima *et al.* 2004; Niwa *et al*, in press). To clarify the mechanisms of the inhibitory effects of TNF-α or Leu-Ile on MOR-induced rewarding effect and sensitization, the influence of TNF-α or Leu-Ile on the repeated or single MOR-induced increase in extracellular DA levels was examined in the NAc of mice, by using an in vivo microdialysis technique. Repeated and single MOR (10 mg/kg,

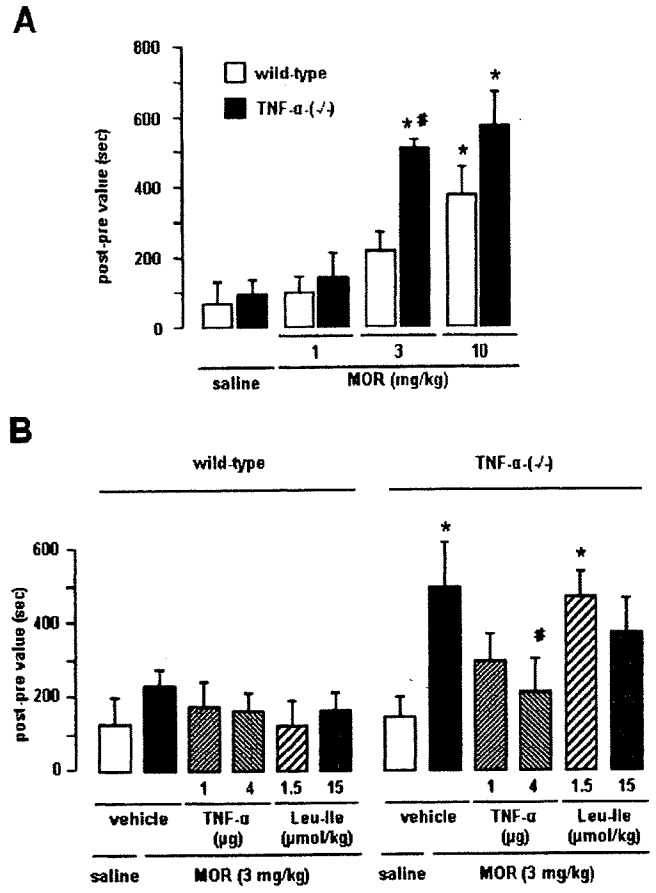

Figure 6. Effects of TNF-α or Leu-Ile on the rewarding effects of MOR in TNF-α-(-/-) mice. **(A)** Morphine-induced place preference in wild-type and TNF-α-(-/-) mice. Mice were treated with MOR (1, 3, and 10 mg/kg, SC) or saline during the conditioning. Values are means ± SEM ($n = 8$). $^{*}p < .05$ versus saline-treated wild-type mice. $^{\#}p < .05$ versus MOR-treated wild-type mice. **(B)** Effect of TNF-α or Leu-Ile treatment on MOR-induced place preference in wild-type and TNF-α-(-/-) mice. Mice were treated with MOR (3 mg/kg, SC) or saline during the conditioning. Values are means ± SEM ($n = 8$). $^{*}p < .05$ versus vehicle/saline-treated TNF-α-(-/-) mice. $^{\#}p < .05$ versus vehicle/MOR-treated TNF-α-(-/-) mice. TNF-α, tumor necrosis factor-α; MOR, morphine; SC, subcutaneous.

SC) treatment caused a marked increase in extracellular DA levels in the NAc on day 9 (repeated treatment) and day 1 (single treatment), respectively (Figures 7A and 7B). Peaks of extracellular DA levels in repeated and single treatments were 2.2-fold and 2.1-fold of the baseline, respectively. Treatment with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) for 9 days significantly inhibited the repeated MOR-induced increase in extracellular DA levels [$F(4,23) = 5.118$, $p < .05$, repeated ANOVA] (Figure 7A). On the other hand, pretreatment with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before the single MOR treatment failed to inhibit the increase in extracellular DA levels in mice (Figure 7B).

Effects of TNF-α or Leu-Ile on MOR-Induced Antinociceptive Effects and Withdrawal Symptoms

Tolerance refers to a situation where increasingly higher doses of a drug become necessary to elicit an equivalent physiological response. This behavioral manifestation is usually

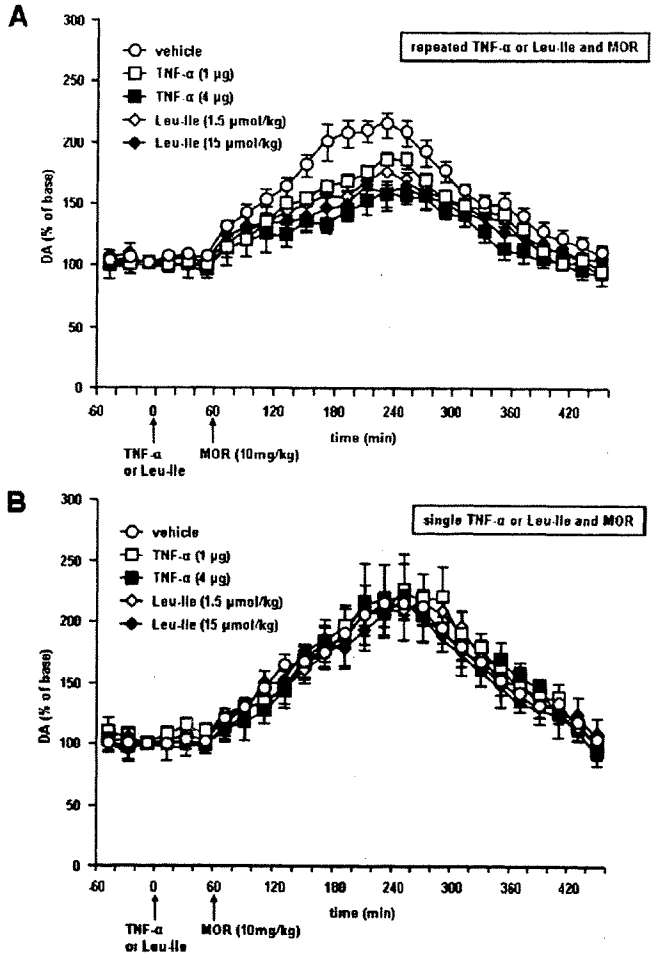

Figure 7. Effects of TNF-α or Leu-Ile on MOR-induced increase in extracellular DA levels. **(A)** Effect of exogenous TNF-α or Leu-Ile on the repeated MOR treatment-induced increase in extracellular DA levels. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before MOR (10 mg/kg, SC) once a day for 9 days. Extracellular levels of DA were measured in the NAc by in vivo microdialysis. Basal extracellular DA levels were 3.1 ± .4, 3.0 ± .4, 2.8 ± .3, 2.8 ± .4, and 3.0 ± .5 pg/20 μL for the vehicle/MOR-, TNF-α 1 μg/MOR-, TNF-α 4 μg/MOR-, Leu-Ile 1.5 μmol/MOR-, and Leu-Ile 15 μmol/MOR-treated mice, respectively. Values are means ± SEM (n = 5–7). Analysis of variance with repeated measures revealed significant differences in extracellular DA levels. **(B)** Effect of exogenous TNF-α or Leu-Ile on the single MOR treatment-induced increase in extracellular DA levels. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) once 1 hour before MOR (10 mg/kg, SC). Extracellular levels of DA were measured in the NAc by in vivo microdialysis. Basal extracellular DA levels were 2.1 ± .4, 1.9 ± .2, 2.0 ± .2, 1.9 ± .2, and 1.8 ± .2 pg/20 μL for the vehicle/MOR-, TNF-α 1 μg/MOR-, TNF-α 4 μg/MOR-, Leu-Ile 1.5 μmol/MOR-, and Leu-Ile 15 μmol/MOR-treated mice, respectively. Values are means ± SEM (n = 5–6). Analysis of variance with repeated measures revealed no significant differences in extracellular DA levels. TNF-α, tumor necrosis factor-α; MOR, morphine; DA, dopamine; IP, intraperitoneal; SC, subcutaneous; NAc, nucleus accumbens.

best exemplified when studying the antinociceptive or rewarding effects of MOR (Di Chiara and North 1992; Laakso *et al.* 2002).

We examined the influence of TNF-α or Leu-Ile on the antinociceptive effect of MOR in a hot plate test. As shown in Figure 8A, there was no difference in hot plate latency by co-administration of vehicle, TNF-α (1 and 4 μg, IP), or Leu-Ile (1.5 and 15 μmol/kg, IP) with single and repeated MOR (10

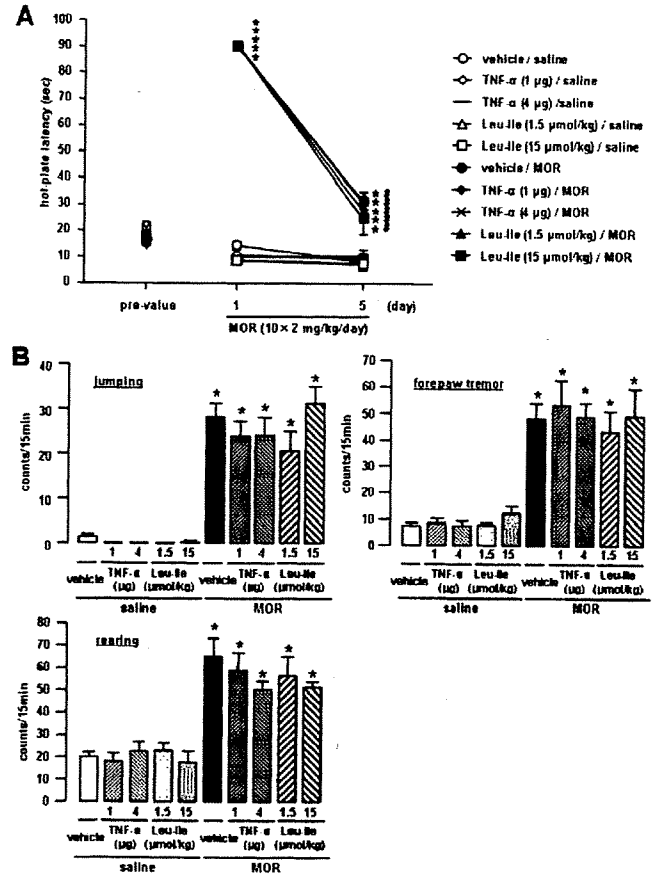

Figure 8. Effects of TNF-α or Leu-Ile on the MOR-induced antinociceptive effects and symptoms of withdrawal. **(A)** Effects of TNF-α or Leu-Ile on the antinociceptive effect and tolerance induced by repeated MOR treatment. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before MOR (10 mg/kg, SC) twice a day for 5 days. The analgesic effect of MOR was determined 60 min after the first MOR treatment on day 1 and the second MOR treatment on day 5. Values are means ± SEM (n = 6–10). *$p < .05$ versus vehicle/saline-treated mice on the first day. #$p < .05$ versus vehicle/MOR-treated mice on the first day. **(B)** Effects of repeated co-administration of TNF-α or Leu-Ile with MOR on naloxone-precipitated (NAL; 5 mg/kg, IP) withdrawal symptoms. 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) twice a day for 5 days. On the sixth day, mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) and NAL (5 mg/kg, IP) 1 hour before and 2 hours after MOR (10 mg/kg, SC) treatment, respectively. Immediately after the NAL treatment, NAL-precipitated MOR withdrawal symptoms (jumping, forepaw tremor, rearing) were enumerated manually for 15 min. Saline-precipitated MOR withdrawal symptoms: [jumping] .00 ± .00 (vehicle/saline), .00 ± .00 (TNF-α 1 μg/saline), .00 ± .00 (TNF-α 4 μg/saline), .00 ± .00 (Leu-Ile 1.5 μmol/saline), .00 ± .00 (Leu-Ile 15 μmol/saline), .10 ± .00 (vehicle/MOR), .00 ± .00 (TNF-α 1 μg/MOR), .00 ± .00 (TNF-α 4 μg/MOR), .00 ± .00 (Leu-Ile 1.5 μmol/MOR), .00 ± .00 (Leu-Ile 15 μmol/MOR); [forepaw tremor] 9.00 ± 1.28 (vehicle/saline), 10.00 ± 1.06 (TNF-α μg/saline), 12.83 ± 1.64 (TNF-α 4 μg/saline), 9.70 ± 1.40 (Leu-Ile 1.5 μmol/saline), 13.81 ± 1.80 (Leu-Ile 15 μmol/saline), 14.60 ± 2.24 (vehicle/MOR), 12.67 ± 1.74 (TNF-α 1 μg/MOR), 13.50 ± 2.32 (TNF-α 4 μg/MOR), 13.60 ± 1.97 (Leu-Ile 1.5 μmol/MOR), 12.00 ± 2.63 (Leu-Ile 15 μmol/MOR); [rearing] 34.30 ± 2.38 (vehicle/saline), 29.33 ± 4.47 (TNF-α 1 μg/saline), 30.00 ± 3.57 (TNF-α 4 μg/saline), 28.60 ± 3.25 (Leu-Ile 1.5 μmol/saline), 32.17 ± 1.89 (Leu-Ile 15 μmol/saline), 42.60 ± 5.48 (vehicle/MOR), 38.50 ± 2.72 (TNF-α 1 μg/MOR), 39.17 ± 3.27 (TNF-α 4 μg/MOR), 38.40 ± 3.01 (Leu-Ile 1.5 μmol/MOR), 32.50 ± 2.79 (Leu-Ile 15 μmol/MOR). Values are means ± SEM (n = 6–10). *$p < .05$ versus vehicle/saline-treated mice. TNF-α, tumor necrosis factor-α; MOR, morphine; IP, intraperitoneal; SC, subcutaneous; NAL, naloxone.

mg/kg, SC). In addition, a tolerance in the analgesic effects of MOR to thermal stimuli was developed in all MOR-treated mice [$F(2,27) = 383.976$, $F(2,15) = 277.874$, $F(2,15) = 723.622$, $F(2,27) = 517.466$, $F(2,15) = 112.064$, $p < .05$, one-way ANOVA] (Figure 8A).

Finally, we investigated the effects of repeated co-administration of TNF-α or Leu-Ile with MOR on NAL-precipitated withdrawal. Withdrawal symptoms (jumping, forepaw tremor, rearing) after treatment with NAL (5mg/kg, IP) were shown in all repeated MOR-treated (10 × 2 mg/kg/day for 5 days) mice [$F(9,66) = 22.846$, $F(9,66) = 13.938$, $F(9,66) = 10.676$, $p < .05$, one-way ANOVA]. There was no difference in the NAL-precipitated withdrawal syndrome by co-administration of vehicle, TNF-α (1 and 4 μg/day for 5 days, IP), or Leu-Ile (1.5 and 15 μmol/kg/day for 5 days, IP) with repeated MOR (10 mg/kg, SC) (Figure 8B).

Discussion

Drugs of abuse are able to elicit compulsive drug-seeking behaviors on repeated administration, which ultimately leads to the phenomenon of addiction (Laakso *et al.* 2002). In terms of lost lives and productivity, drug addiction remains one of the most serious threats to the nation's public health (Nestler 2002).

Recently, we have demonstrated that TNF-α or Leu-Ile, which induces GDNF production via TNF-α synthesis, inhibits METH-induced dependence (Nakajima *et al.* 2004; Niwa *et al*, in press). Morphine is a drug of abuse like METH, although they have opposite effects, acting as a psychosedative and psychostimulant, respectively. In the present study, to extend our findings, we examined the effects of TNF-α or Leu-Ile on the rewarding effect, the sensitization to the locomotor-stimulating effects, and the increase in extracellular DA levels induced by MOR.

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 and CPu (Figure 2A). Moreover, we confirmed TNF-α protein was not increased after single treatment of MOR using immunostaining method (data not shown). We suggest that the induction of TNF-α by MOR requires repeated treatment. The MOR-induced increase in the expression of TNF-α mRNA in the NAc was completely inhibited by pretreatment with the DA D1 receptor antagonist SCH23390, the D2 receptor antagonist raclopride (Figure 2B), and the specific opioid receptor antagonist NAL (Figure 2C), suggesting that the activation of DA D1, D2, and opioid receptors is attributable to MOR-induced gene expression of TNF-α. It is likely that activation of DA transmission in neurons, where TNF-α specifically acts (Figure 3B), is necessary for MOR-induced TNF-α expression. The expression of TNF-α is induced through the activation of transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) by the activation of JNK/p38 MAPK (Guha *et al.* 2000; Rahman and MacNee 2000). Further, TNF-α acts on mitochondria to generate reactive oxygen species (ROS), which are involved in the activation of AP-1 and NF-κB (Rahman and MacNee 2000). Changes in transcription factors may result in long-term changes in gene expression, thereby contributing to neuronal adaptations that underlie behavioral sensitization (Nestler 2001).

Tumor necrosis factor-α induces GDNF expression (Niwa *et al*, in press) and blocks METH-induced dependence (Nakajima *et al.* 2004). Tumor necrosis factor-α or Leu-Ile treatment, both in combination with MOR and after withdrawal from repeated treatment with MOR, inhibited place preference and sensitization to MOR (Figures 4 and 5). Therefore, we investigated whether Leu-Ile, which is a GDNF inducer, induces the expression of TNF-α. Leu-Ile increased TNF-α mRNA levels in the NAc (Figure 3A). Leu-Ile treatment, both in combination with MOR and after withdrawal from repeated treatment with MOR, also increased TNF-α levels (Figures 3B, 3C, and 3D) in the brain but not in the peripheral blood stream (Niwa *et al*, in press). Leu-Ile can penetrate the blood-brain barrier (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. Therefore, we suggest that Leu-Ile plays an inhibitory role in rewarding effects and sensitization induced by MOR via the induction of TNF-α expression.

Tumor necrosis factor-α-(-/-) mice showed marked conditioned place preference at the low dose of MOR, which failed to establish place preference in wild-type mice (Figure 6A). Morphine-induced place preference in TNF-α-(-/-) mice was significantly attenuated by the administration of TNF-α (Figure 6B). These results suggest that TNF-α acts to negate the rewarding effects of MOR.

From the point of view of pharmacotherapy, Leu-Ile would be better than TNF-α itself, since TNF-α damages the peripheral tissues by triggering the expression of other cytokines (Bluthe *et al.* 1994). Tumor necrosis factor-α could be involved in the inhibitory effects of Leu-Ile on MOR-induced rewarding effects, since no effect of Leu-Ile was observed in the TNF-α-(-/-) mice (Figure 6B). Our results showed that Leu-Ile, which induces GDNF production via TNF-α synthesis, inhibited MOR-induced place preference and sensitization not only during, but also after, their development (Figures 4 and 5), as in the case of METH (Niwa *et al*, in press). Glial cell line-derived neurotrophic factor levels in the NAc after the co-administration of Leu-Ile and MOR were much more increased compared with those in the vehicle/MOR-treated mice (Niwa *et al.* 2006, unpublished observations). Glial cell line-derived neurotrophic factor could be involved in the inhibitory effects of Leu-Ile on MOR-induced rewarding effects, since no effect of Leu-Ile was observed in the GDNF heterozygous mice (Niwa *et al.* 2006, unpublished observations). These results suggest that GDNF acts to negate the rewarding effects of MOR and are involved in the effects of Leu-Ile on rewarding effects. Glial cell line-derived neurotrophic factor blocks biochemical adaptations to the chronic use of cocaine or MOR, as well as the rewarding effects of cocaine (Messer *et al.* 2000). Therefore, Leu-Ile may induce production of GDNF as a result of TNF-α expression to inhibit drug-induced rewarding effects and sensitization, although another pathway should be considered—that Leu-Ile upregulates GDNF expression by activating heat shock protein 90 (Hsp90)/Akt/cyclic adenosine 3', 5'-monophosphase (cAMP) response element binding protein (CREB) signaling (Cen *et al.* 2006).

Leu-Ile inhibited MOR-induced place preference (Figures 5A) in bell-shaped response curves. We confirmed that Leu-Ile at the lower dose, 1.5 μmol/kg, which could inhibit the rewarding effects of MOR, increased TNF-α expression both in combination with MOR and after withdrawal from repeated MOR treatment in the CPP paradigm. On the contrary, Leu-Ile at the higher dose, 15 μmol/kg, which could not inhibit the rewarding effects of MOR, failed to increase TNF-α expression in combination with MOR in the CPP paradigm (data not shown). These results suggest involvement of induction of TNF-α expression in inhibitory effect of Leu-Ile on the rewarding effects and sensitization of MOR.

There has been considerable progress in identifying the mechanisms that contribute to the long-lasting neural and behav-

ioral plasticity related to addiction, including drug-induced changes in gene transcription, in RNA and protein processing, and in synaptic structure (Nestler 2001). Although a single administration of TNF-α or Leu-Ile failed to inhibit the single treatment-induced hyperlocomotion, it inhibited the sensitization to hyperlocomotion induced by repeated treatment with MOR (Figures 4A and 4B). These results suggest that TNF-α or Leu-Ile has inhibitory effects on neuronal plasticity induced by repeated MOR treatment but not on hyperlocomotion or the increase in extracellular DA levels induced by single MOR treatment (Figure 7B). Several reports have suggested that TNF-α influences synaptic strength and transmission (Albensi and Mattson 2000; Beattie *et al.* 2002). Further, the expression of TNF-α is induced through the activation of transcription factors such as AP-1 and NF-κB (Guha *et al.* 2000; Rahman and MacNee 2000). Our results have shown that Leu-Ile binds heat shock cognate protein (Hsc70) and triggers the phosphorylation of NF-κB and CREB via a pathway involving Hsp90/Akt and induces GDNF expression (Cen *et al.* 2006). We suggest that the induction of TNF-α and GDNF by Leu-Ile requires repeated treatment, and these molecules inhibit MOR-induced rewarding effects and sensitization.

The mesolimbic dopamine system 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 (Koob *et al.* 1998). The VTA and NAc have been shown to be the key brain regions that underlie the actions of opioids (e.g., MOR) and psychostimulants (e.g., METH and cocaine) (Koob 1992). It is well recognized that the rewarding effects of opioids and psychomotor stimulants depend on the mesocorticolimbic dopamine system innervating the NAc (Everitt and Wolf 2002; Koob *et al.* 1998; Mizoguchi *et al.* 2004). It has been suggested that the enhancement of DA release in the NAc is an essential process related to the rewarding effects of MOR (Matthews and German 1984). Further, the NAc is involved in the locomotor-stimulating effect of MOR (Brase *et al.* 1977; Oliverio *et al.* 1975), which is regarded as a result of the increase in extracellular DA levels (Koob and Nestler 1997; Matthews and German 1984). We have recently demonstrated that the tissue plasminogen activator (tPA)-plasmin system participates in the rewarding and locomotor-stimulating effects induced not only by MOR but also by METH by triggering the release of dopamine in the NAc (Nagai *et al.* 2004, 2005a, 2005b; Yamada *et al.* 2005). Leu-Ile inhibited the sensitization of hyperlocomotion induced not only by MOR (Figures 4B and 4C) but also by METH (Niwa *et al*, in press), at least in part, through the action in the NAc, since it had inhibitory effects on the repeated MOR treatment-induced increase in extracellular DA levels (Figure 7A). Leu-Ile induces the expression of not only TNF-α (Figure 3A) but also GDNF (Niwa *et al*, in press). Tumor necrosis factor-α induced by Leu-Ile activates plasmalemmal and vesicular DA transporter (Nakajima *et al.* 2004). Glial cell line-derived neurotrophic factor induced by Leu-Ile inhibits the drug-induced upregulation of tyrosine hydroxylase activity (Messer *et al.* 2000). Thereby, TNF-α and GDNF induced by Leu-Ile attenuate the MOR-induced increase in extracellular DA levels (Figure 7A) and then inhibit MOR-induced rewarding effect and sensitization (Figures 4 and 5).

Chronic use of an opioid results in tolerance to and dependence on the drug (Chavkin and Goldstein 1984; Law *et al.* 1982; Puttfarcken *et al.* 1988). Dependence is defined by a number of abnormal responses after the abrupt withdrawal of a drug (Johnson and Flemming 1989). Tumor necrosis factor-α or Leu-Ile has no effect on MOR-induced tolerance and physical dependence (Figure 8). Tumor necrosis factor-α or Leu-Ile regulates dopaminergic neurons, at least in part, through action in the NAc, whereas the cortex is the terminal/intermedial area for noradrenergic neurons associated with drug addiction and plays a key role in NAL-precipitated MOR withdrawal (Terwilliger *et al.* 1991). Therefore, the mechanism by which TNF-α or Leu-Ile inhibits MOR-induced rewarding effect and sensitization is different from that of the NAL-precipitated MOR withdrawal syndrome.

Our findings suggest that TNF-α inhibits MOR-induced rewarding effect and sensitization by attenuating the MOR-induced increase in extracellular DA levels, and Leu-Ile inhibits them via the induction of TNF-α expression. Leu-Ile could be a novel therapeutic agent for MOR-induced dependence.

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A novel molecule 'shati' increases dopamine uptake via the induction of tumor necrosis factor-α in pheochromocytoma-12 cells

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Abstract

The psychostimulant properties of methamphetamine (METH) are associated with an increase in extracellular dopamine (DA) levels in the brain, via facilitation of DA's release from pre-synaptic nerve terminals and inhibition of its reuptake through DA transporter. Recently, we have demonstrated that tumor necrosis factor-α (TNF-α) increases DA uptake and inhibits METH dependence. Moreover, we have clarified 'shati' identified in the nucleus accumbens of mice treated with METH is involved in METH dependence. In the present study, we investigated the effects of TNF-α on DA uptake in PC12 cells and established a PC12 cell line transfected with a vector containing shati cDNA to examine the precise mechanism behind the role of shati in DA uptake. Moreover, we examined the relationship between shati and TNF-α. TNF-α increased DA uptake via the mitogen-activated protein kinase kinase pathway and inhibited the METH-induced decrease in DA uptake in PC12 cells. Transfection of the vector containing shati cDNA into PC12 cells, induced the expression of shati and TNF-α mRNA, accelerated DA uptake, and inhibited the METH-induced decrease in DA uptake. These results suggest that the functional roles of shati in METH-regulated behavioral changes are mediated through inhibition of the METH-induced decrease in DA uptake via TNF-α.

Keywords: addiction, dopamine (DA) uptake, methamphetamine, shati, tumor necrosis factor-α (TNF-α).

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The abuse of methamphetamine (METH) has significant psychiatric and medical consequences, including dependence, psychosis, overdose, and even death (Rawson *et al.* 2002). Drugs of abuse, including METH, modulate the activity of mesolimbic dopaminergic neurons, projecting from the ventral tegmental area to the nucleus accumbens

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Abbreviations used: AMPH, amphetamine; ANOVA, analysis of variance; AP, anteroposterior; cDNA, complementary DNA; CNS, central nervous system; CSF, cerebrospinal fluid; D1-R, dopamine D1 receptor; D2-R, dopamine D2 receptor; DA, dopamine; DAT, dopamine transporter; DV, dorsoventral; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; GBR 12909, [1-(2[bis(4-fluorophenyl-) methoxy]ethyl)-4-(3-phenylpropyl)piperazine] bimesylate hydrate; GFP, green fluorescent protein; GNAT, GCN5-related N-acetyltransferase; JNK, c-Jun N-termial kinase; MAO, monoamine oxidase; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; METH, methamphetamine; ML, mediolateral; NAc, nucleus accumbens; NF-κB, nuclear factor-κB; p38, p38 mitogen-activated protein kinase; PC12, pheochromocytoma-12; PCR, polymerase chain reaction; PD98059, 2-(2-amino-3-methyoxyphenyl)-4H-1-benzopyran-4-one; RIP, serine and threonine protein kinase receptor-interacting protein; RT-PCR, reverse transcription-polymerase chain reaction; shati-AS, shati antisense oligonucleotide; shati-SC, shati-scrambled oligonucleotide; SLC6, solute carrier 6; TH, tyrosine hydroxylase; TNFR I, tumor necrosis factor type I receptor; TNF-α, tumor necrosis factor-α; TRAF2, TNF receptor-associated factor 2; VMAT-2, vesicular monoamine transporter; VTA, ventral tegmental area.

(NAc) (Koob 1992; Wise 1996; Koob *et al.* 1998). The principal target for the action of METH is believed to be the dopamine transporter (DAT), which is a member of the solute carrier 6 (SLC6) gene family of Na^+/Cl^- coupled transporters that also includes the neurotransmitter transporters of norepinephrine, serotonin, glycine, and γ-aminobutyric acid (GABA) (Amara and Kuhar 1993; Torres *et al.* 2003; Chen *et al.* 2004). The DAT controls dopaminergic signaling by the rapid reuptake of dopamine (DA) from synaptic clefts. As a substrate, METH not only competitively inhibits DA uptake and thereby increases synaptic DA but also promotes the reverse transport of nonvesicular DA, resulting in an efflux of DA via the DAT (Sulzer *et al.* 2005; Fog *et al.* 2006). This efflux results in a dramatic increase in extracellular DA and is believed to be of major importance for the psychostimulant properties of METH (Sulzer *et al.* 2005; Fog *et al.* 2006). However, the exact neuronal circuits and molecular cascade essential for drug dependence are still poorly understood. Moreover, the molecules related to the METH-induced increase in DA efflux are unclear.

Tumor necrosis factor-α (TNF-α) plays an important role in a variety of infectious, inflammatory, and autoimmune conditions (Vassalli 1992). TNF-α also affects the 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. Recently, we have demonstrated that TNF-α activates synaptosomal and vesicular DA uptake (Nakajima *et al.* 2004). Moreover, we have reported that TNF-α and its inducer diminish METH and morphine-induced behavioral sensitization and rewarding effects by promoting plasmalemmal and vesicular DA uptake as well as attenuating the METH and morphine-induced increase in overflow of DA in the NAc (Nakajima *et al.* 2004; Niwa *et al.* 2007b,d; Niwa *et al.* 2008). TNF-α modulates cellular responses through the extracellular signal-regulated kinase 1/2 (ERK1/2) and nuclear factor-κB (NF-κB) signaling pathways (van Vliet *et al.* 2005). ERK1/2 regulates the surface expression and capacity of DAT (Morón *et al.* 2003). However, the mechanisms by which TNF-α regulates the uptake of DA are poorly understood.

Recently, we have identified a novel molecule 'shati' in the NAc of mice treated with METH repeatedly using the polymerase chain reaction (PCR)-select complementary DNA (cDNA) substraction method, which is a differential and epochal cloning technique. Further, we have demonstrated that shati, which contains the sequence of GCN5-related *N*-acetyltransferase (GNAT), acetyl-CoA-binding sites, and ATP-binding sites, is involved in METH-induced hyperlocomotion, sensitization, and conditioned place preference (Niwa *et al.* 2007a). Blockage of shati expression by shati antisense oligonucleotide (shati-AS) potentiates not only the increase in extracellular DA levels, but also the decrease in synaptosomal and vesicular DA uptake in the NAc induced by repeated METH treatment, resulting in potentiation of the METH-induced dependence (Niwa *et al.* 2007a).

Pheochromocytoma-12 (PC12) cells are useful as a model of the neuronal system and have DATs. In the present study, we investigated the effects of TNF-α on DA uptake in PC12 cells and the involvement of the mitogen-activated protein kinase kinase (MEK) pathway in the effects of TNF-α on DA uptake. Moreover, we succeeded in the transfection of a vector containing shati cDNA into PC12 cells, investigated the involvement of shati in DA uptake and the METH-induced decrease in DA uptake, and examined the relationship between shati and TNF-α by using these PC12 cells.

Materials and methods

Cell culture and transfection

PC12 cells purchased from the Riken cell bank (No. RCB0009) were cultured on poly-ornithine-coated coverslips in Dulbecco's modified Eagle's medium (Sigma-Aldrich St Louis, MO, USA) supplemented with 10% heat inactivated horse serum and 5% fetal bovine serum (Loder and Melikian 2003). We made the vector containing shati cDNA with the suggested sequence of NM_001001985 using the plasmid pcDNA-DEST53 (Invitrogen, Carlsbad, CA, USA) as an expression vector with green fluorescent protein (GFP), although N-terminal of seven amino acids of shati was missing (CDS 882-1760) in this vector. For transient expression, the cells were transfected with the plasmid expressing shati using Lipofectamine 2000 (Invitrogen).

[^{3}H] DA uptake in PC12 cells

The uptake of [^{3}H] DA in PC12 cells was performed as described before (Melikian and Buckley 1999). The cells were washed in Krebs-Ringers- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer twice before the assay. Uptake was initiated by adding 1 μM [^{3}H] DA (Perkin Elmer, Waltham, MA, USA) containing 10^{-5} M pargyline and 10^{-5} M ascorbic acid. Uptake proceeded for 10 min at 23°C and was terminated with three rapid washes in ice-cold Krebs-Ringers-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The amount of [^{3}H] DA accumulated was determined by liquid scintillation counting (Beckman Coulter, Inc., Fullerton, CA, USA). Non-specific uptake was defined in the presence of 10 μM [1-(2[bis(4-fluorophenyl-) methoxy]ethyl)-4-(3-phenylpropyl)piperazine] bimesylate hydrate (GBR 12909). The cells were pre-treated with TNF-α (0.1, 1, and 10 ng/mL) for 40 min, and assayed for [^{3}H] DA uptake. To neutralize TNF-α in PC12 cells, the cells were pre-treated with polyclonal goat anti-TNF-α antibody (R&D Systems Ltd., Minneapolis, MN, USA; Ab; 1, 10, 50, and 100 ng/mL) or soluble TNF receptor I (R&D Systems Ltd, sRI; 0.1, 0.5, 1, and 10 ng/mL) 10 min before the treatment with TNF-α (10 ng/mL, 40 min) (Barone *et al.* 1997), and assayed for [^{3}H] DA uptake. The function of TNF-α is mediated through two distinct cell surface receptors, TNF receptor I and TNF receptor II. The majority of TNF functions are mediated primarily through TNF receptor I, whereas TNF receptor II seems to play a role in only a limited number of

TNF responses (Hsu *et al.* 1995). Moreover, it has been reported that immunoreactivity for TNF receptor I is found in cell bodies and process of dopaminergic neurons (Boka *et al.* 1994). Therefore, we have used soluble TNF receptor I for neutralization for TNF-α. To examine the involvement of the MEK pathway in the TNF-α-induced increase in DA uptake in PC12 cells, the cells were pre-treated with a selective MEK inhibitor 2-(2-amino-3-methyoxy-phenyl)-4H-1-benzopyran-4-one (Calbiochem, San Diego, CA, USA; PD98059; 1, 10, 100, and 500 μM) 10 min before their treatment with TNF-α (10 ng/mL, 40 min), and assayed for [^{3}H] DA uptake. PD98059 was dissolved in dimethyl sulfoxide to give a concentration of 50 mM, stored in aliquots at −80°C, and diluted in Dulbecco's modified Eagle's medium to 1–500 μM immediately prior to use. To examine the effects of TNF-α on the METH-induced decrease in DA uptake in PC12 cells, the cells were pre-treated with TNF-α (10 ng/mL) 10 min before being treated with METH (1 μM, 30 min), and assayed for [^{3}H] DA uptake, following previous observations (Nakajima *et al.* 2004). Cen *et al.* (2008) have reported that METH (1 μM) decreases plasmmalemmal DAT expression in time-dependent manner (0, 5, 15, 30, 60 min), which is paralleled with the decrease in [^{3}H] DA uptake. Since treatment of METH (1 μM) for 30 min significantly decreases DA uptake compared with control group (Cen *et al.*, 2008), we have selected this time point for treatment of METH before the uptake assay. To examine the involvement of TNF-α in the shati-induced increase in [^{3}H] DA uptake in the shati-over-expressing PC12 cells, the cells were pre-treated with polyclonal goat anti-TNF-α antibody (R&D Systems Ltd, Ab; 50 ng/mL) or soluble TNF receptor I (R&D Systems Ltd, sR I; 1 ng/mL) 10 min before their treatment with METH (1 μM, 30 min), and assayed for [^{3}H] DA uptake.

Immunocytochemistry

Two antibodies against the peptide of the hypothetical protein, CNTAFRGLRQHPRTQLL (S-3) and CMSVDSRFRGKGIAKALG (S-4) unique to shati were generated. These peptides were conjugated to keyhole limpet hemocyanin and injected into rabbits six times at 1-week intervals. Serum was taken from the rabbits 1 week after the final injection. The serum was diluted 200 times for immunostaining (Niwa *et al.* 2007a).

Transfected PC12 cells attached to glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min, and then blocked in 3% normal sera and 0.1% Triton X-100 for 1 h. The coverslips were incubated with primary antibodies at 4°C overnight, washed with phosphate-buffered saline, and then incubated with appropriate secondary antibodies for 2 h. Polyclonal rabbit anti-S-3 or anti-S-4 antibody (1 : 200), monoclonal mouse anti-tyrosine hydroxylase (TH) antibody (1 : 200, Chemicon, Temecula, CA, USA), monoclonal mouse anti-GFP antibody (1 : 500, Chemicon), polyclonal goat anti-rat TNF-α antibody (1 : 100, R&D Systems Ltd), and polyclonal rabbit anti-GFP antibody (1 : 100, Chemicon) served as primary antibodies. Goat anti-mouse Alexa Fluor 546 (1 : 1000, Invitrogen), donkey anti-goat Alexa Fluor 546 (1 : 1000, Invitrogen), rabbit anti-mouse Alexa Fluor 488 (1 : 1000, Invitrogen), and donkey anti-rabbit Alexa Fluor 488 (1 : 1000, Invitrogen) were used as secondary antibodies. After being washed and mounted, stained cells were observed under a fluorescence microscope (Axioskoop 2 plus). Because similar results were obtained using the anti S-3 and anti-S-4 antibodies in the immunohistochemical experiments, only the data obtained with the anti-S-4 antibody is described.

Real time reverse transcription-polymerase chain reaction

Total RNA was isolated using an RNeasy Kit (Qiagen, Hilden, Germany) and converted into cDNA using a SuperScript™ First-Strand System for RT-PCR Kit (Invitrogen). The levels of shati and TNF-α mRNA were determined by real-time RT-PCR using a TaqMan probe. The 18S ribosomal RNA was used as the internal control (Applied Biosystems, CA, USA). The shati primers used for real-time RT-PCR were as follows: 5′-TGTAAACACCCCTAA AGTGCCCT-3′ (forward; bp 2967–2989) and 5′-TCAATCCTGC ATACAAGGAATCAA-3′ (reverse; bp 3022–3045), and the Taq-Man probe was 5′-CACAGTCTGTGAGGCTCAGGTTGCCC-3′ (probe; bp 2995–3020). The amplification consisted of an initial step (95°C for 5 min) and then 40 cycles of denaturation for 30 sec at 95°C, annealing for 40 s at 59°C, and the extension time for 1 min at 72°C in an iCycle iQ Detection System (Bio-Rad Laboratories, Inc., CA, USA) (Niwa *et al.* 2007a). The expression levels were calculated as described previously (Wada *et al.* 2000).

Animals

The male C57BL/6J- wild-type mice were obtained from Slc Japan (Hamamatsu, Japan). Animals were housed in plastic cages and kept in a temperature-, humidity-, and light-controlled room (23 ± 1°C; 50 ± 5% humidity; 12 : 12 h light/dark cycle starting at 8:00 AM) and had free access to food and water, except during behavioral experiments. All animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and 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 Japanese Pharmaceutical Society.

Shati-antisense oligonucleotide (shati-AS) treatment

Mice were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic apparatus. The infusion cannula was connected to a miniosmotic pump (total capacity was 90 μL, Alzet 1002; Alza, Palo Alto, CA, USA) filled with shati-antisense oligonucleotide (shati-AS) or -scrambled oligonucleotide (shati-SC). The pump was implanted into the right ventricle [anteroposterior (AP) −0.5 mm, mediolateral +1.0 mm from the bregma, and dorsoventral −2.0 mm from the skull, according to the atlas of Franklin and Paxinos (1997)]. Phosphorothionate oligonucleotides were custom-synthesized at Nisshinbo Biotechnology (Tokyo, Japan) and dissolved in artificial CSF (147 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.2). The oligonucleotides were phosphorothioated at the first three bases of both the 5′- and 3′-ends, which results in increased stability and less toxicity. The sequences of shati-AS and -SC were 5′-TCTTCGTCTCGCAGAC CATGTCG-3′ and 5′-GGTCTGCTACACTGCTGCTAGTC-3′, respectively. Shati-AS and -SC were continuously infused into the cerebral ventricle at a dose of 1.8 nmol/6 μL/day (flow rate, 0.25 μL/h). Additionally, shati-SC was used as a control. Three days after the start of oligonucleotide infusion, mice were

administered METH (1 mg/kg, s.c.) for 5 days and decapitated 2 h after the final treatment (Niwa *et al.* 2007a).

Statistical analysis

All data were expressed as means ± SE. Statistical differences between two groups were determined with Student's *t*-test. Statistical differences among three groups or more were determined using a one-way analysis of variance (ANOVA), two-way ANOVA, or three-way ANOVA, followed by the Bonferroni multiple comparison test. $p < 0.05$ was regarded as statistically significant.

Nucleotide sequences

The DNA Data Bank of Japan/GenBank/European Molecular Biology Laboratory accession number for the primary nucleotide sequence of shati is DQ174094.

Results

Effect of TNF-α on DA uptake in PC12 cells

First, we investigated the effects of TNF on DA uptake in PC12 cells, since we have recently demonstrated that TNF-α activates synaptosomal and vesicular DA uptake in mice (Nakajima *et al.* 2004).

TNF-α (10 ng/mL, 40 min) increased [^{3}H] DA uptake compared with the control group ($F_{3,28} = 4.933$, $p < 0.01$, one-way ANOVA) (Fig. 1a). Moreover, we investigated whether the TNF-α-induced increase was antagonized by the anti-TNF-α antibody and soluble TNF receptor in PC12 cells. Pre-treatment with the antibody (10, 50, and 100 ng/

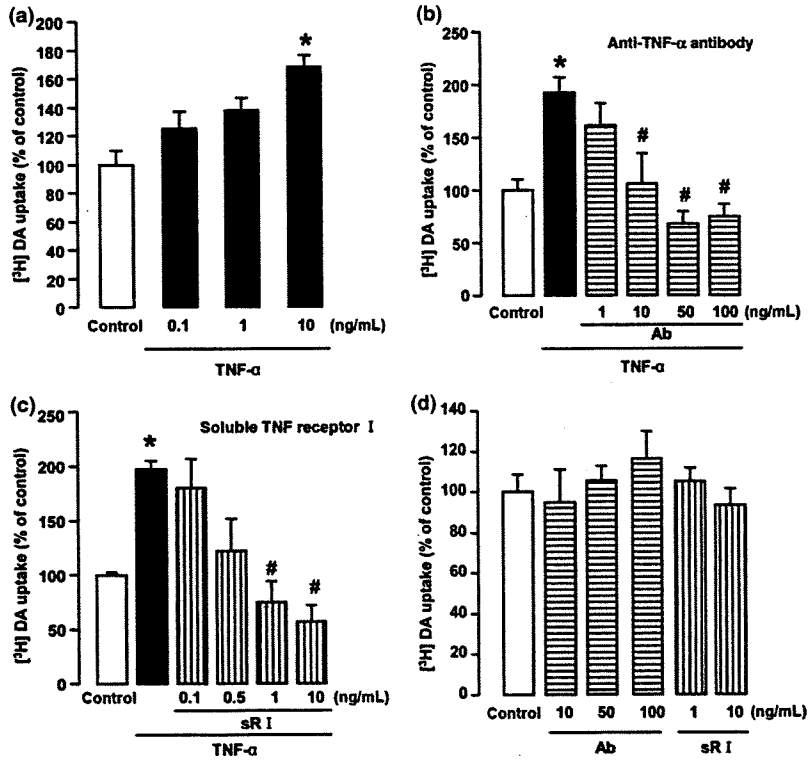

Fig. 1 Effects of anti-TNF-α antibody (Ab) or soluble TNF receptor I (sR I) on TNF-α-induced increase in [^{3}H] DA uptake in PC12 cells. (a) The cells were pre-treated with TNF-α (0.1, 1, and 10 ng/mL) for 40 min, and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.14 ± 0.01 pmol/10 min for control. The final concentration of [^{3}H] DA was 20 nM. Value are means ± SE ($n = 8$). $^*p < 0.05$ versus control. (b) Effects of anti-TNF-α antibody (Ab) on TNF-α-induced increase in [^{3}H] DA uptake in PC12 cells. The cells were pre-treated with anti-TNF-α antibody (1, 10, 50, and 100 ng/mL) 10 min before their treatment with TNF-α (10 ng/mL, 40 min), and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.10 ± 0.02 pmol/10 min for the control. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE ($n = 6$–7). $^*p < 0.05$ versus control. $^\#p < 0.05$ versus TNF-α-treated cells. (c) Effects of soluble TNF receptor I (sR I) on TNF-α-induced increase in [^{3}H] DA uptake in PC12 cells. The cells were pre-treated with soluble TNF receptor I (0.1, 0.5, 1, and 10 ng/mL) 10 min before being treated with TNF-α (10 ng/mL, 40 min), and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.06 ± 0.00 pmol/10 min for the control. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE ($n = 6$–7). $^*p < 0.05$ versus control. $^\#p < 0.05$ versus TNF-α-treated cells. (d) Effects of anti-TNF-α antibody (Ab) or soluble TNF receptor I (sR I) on [^{3}H] DA uptake in PC12 cells. The cells were pre-treated with anti-TNF-α antibody (10, 50, and 100 ng/mL) or soluble TNF receptor I (1 and 10 ng/mL) for 50 min, and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.08 ± 0.01 pmol/10 min for the control. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE ($n = 6$–8).

mL, 50 min) or soluble TNF receptor I (1 and 10 ng/mL, 50 min) significantly inhibited the TNF-α-induced increase in [^{3}H] DA uptake ($F_{5,34}$ = 7.370 for anti-TNF-α antibody; $F_{5,34}$ = 7.526 for soluble TNF receptor I, $p < 0.01$, one-way ANOVA) (Fig. 1b and c), although the anti-TNF-α antibody (10, 50, and 100 ng/mL, 50 min) or soluble TNF receptor I (1 and 10 ng/mL, 50 min) itself had no effect on DA uptake (Fig. 1d). These results suggest that TNF-α activates DA uptake in PC12 cells.

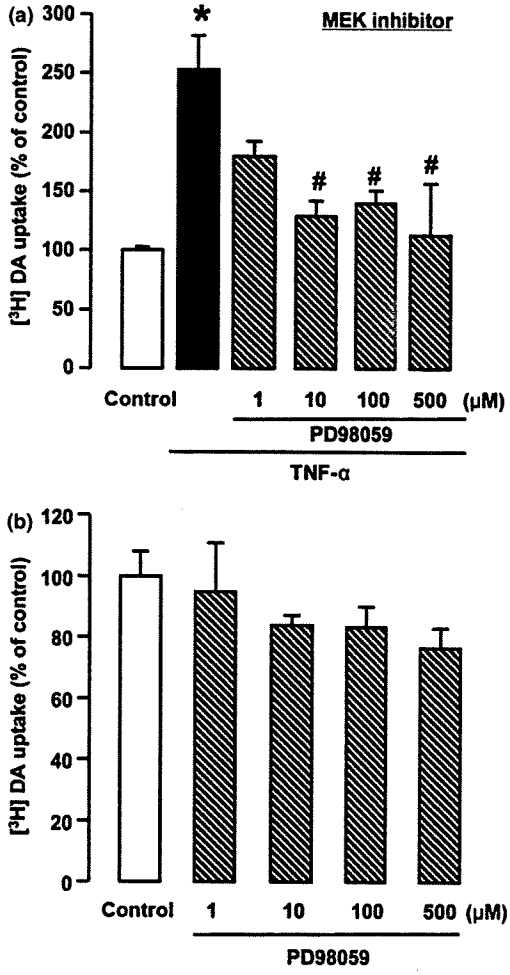

Fig. 2 Effects of MEK inhibitor on TNF-α-induced increase in [^{3}H] DA uptake in PC12 cells. (a) Effects of the MEK inhibitor PD98059 on TNF-α-induced increase in [^{3}H] DA uptake in PC12 cells. The cells were pre-treated with PD98059 (1, 10, 100, and 500 μM) 10 min before their treatment with TNF-α (10 ng/mL, 40 min), and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.10 ± 0.00 pmol/10 min for control. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE (n = 4). *$p < 0.05$ versus control. #$p < 0.05$ versus TNF-α-treated cells. (b) Effects of PD98059 on [^{3}H] DA uptake in PC12 cells. The cells were pre-treated with PD98059 (1, 10, 100, and 500 μM) for 50 min, and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.12 ± 0.00 pmol/10 min for the control. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE (n = 4).

Effects of mitogen-activated protein kinase kinase inhibitor on TNF-α-induced increase in DA uptake in PC12 cells

TNF-α modulates cellular responses through the ERK1/2 signaling pathway (van Vliet *et al.* 2005). Therefore, we investigated whether the TNF-α-induced increase in DA

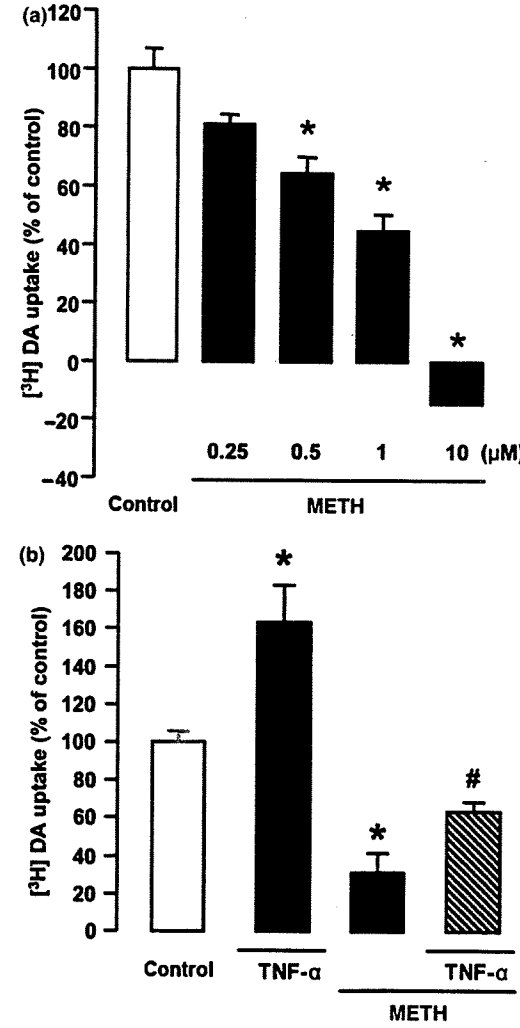

Fig. 3 Effects of TNF-α on METH-induced decrease in [^{3}H] DA uptake in PC12 cells. (a) Dose-response effects of METH on [^{3}H] DA uptake in PC12 cells. The cells were pre-treated with METH (0.25, 0.5, 1, and 10 μM) for 30 min, and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.28 ± 0.02 pmol/10 min for the control. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE (n = 4). *$p < 0.05$ versus control. (b) Effects of TNF-α on METH-induced decrease in [^{3}H] DA uptake in PC12 cells. The cells were pre-treated with TNF-α (10 ng/mL) 10 min before being treated with METH (1 μM, 30 min), and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.19 ± 0.01 pmol/10 min for the control. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE (n = 5). *$p < 0.05$ versus control. #$p < 0.05$ versus METH-treated cells.

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uptake was antagonized by the MEK inhibitor PD98059 in PC12 cells.

Pre-treatment with PD98059 (10, 100, and 500 μM, 50 min) significantly inhibited the TNF-α-induced increase in [^{3}H] DA uptake ($F_{5,18} = 5.961, p < 0.01$, one-way ANOVA) (Fig. 2a), although PD98059 (1, 10, 100, and 500 μM, 50 min) itself had no effect on the uptake (Fig. 2b). These results suggest that TNF-α activates DA uptake via the MEK signaling pathway in PC12 cells.

Effects of TNF-α on METH-induced decrease in DA uptake in PC12 cells

We have previously demonstrated that TNF-α and its inducer diminish the METH-induced decrease in DA uptake and inhibit the rewarding effects of and sensitization to METH (Nakajima *et al.* 2004; Niwa *et al.* 2007c, e). Therefore, we confirmed the effects of TNF-α on the METH-induced decrease in DA uptake in PC12 cells.

METH (0.5, 1, and 10 μM, 30 min) decreased [^{3}H] DA uptake compared with the control group in a dose-dependent manner ($F_{4,15} = 83.675$, $p < 0.01$, one-way ANOVA) (Fig. 3a). Moreover, TNF-α (10 ng/mL, 40 min) inhibited the METH-induced decrease in [^{3}H] DA uptake (TNF-α, $F_{1,16} = 14.759$, $p < 0.01$; METH, $F_{1,16} = 45.994$, $p < 0.01$; TNF-α·METH $F_{1,16} = 1.573$, $p = 0.228$; two-way ANOVA) (Fig. 3b). These results suggest that TNF-α inhibits the METH-induced decrease in DA uptake in PC12 cells (Fig. 3) as well as promoting plasmalemmal and vesicular DA uptake to diminish METH and morphine-induced behavioral sensitization and rewarding effects (Nakajima *et al.* 2004; Niwa *et al.* 2007b; Niwa *et al.* 2008).

Transfection of the vector containing shati cDNA into PC12 cells

We established a PC12 cell line transfected with the vector containing shati cDNA to examine the role of shati in DA uptake and the METH-induced decrease in DA uptake.

We used immunostaining for TH to check morphological changes of the PC12 cells after the transfection of the vector containing shati cDNA. Morphological changes to the cells were not observed after the transfection compared with mock-transfected or non-transfected PC12 cells (Fig. 4a). To confirm the transfection of the vector containing shati cDNA, we checked for immunostaining against S-4 and GFP, co-expressed with shati. No immunoreactivity for S-4 or GFP was found in the cells that were mock-transfected, which express neither shati nor GFP [Fig. 4b (i)]. The cells mock-transfected (=expression vector [pcDNA-DEST53]), which express GFP, but not shati, were immunopositive for GFP, but not S-4 [Fig. 4b (ii)]. The cells transfected with the vector containing shati cDNA, which express both shati and GFP, were immunopositive for S-4 and GFP [Fig. 4b (iii)]. The cells immunopositive for S-4 were merged with those positive for GFP. These results indicated that shati was certainly expressed in PC12 cells and transfection did not affect cell survival or morphology.

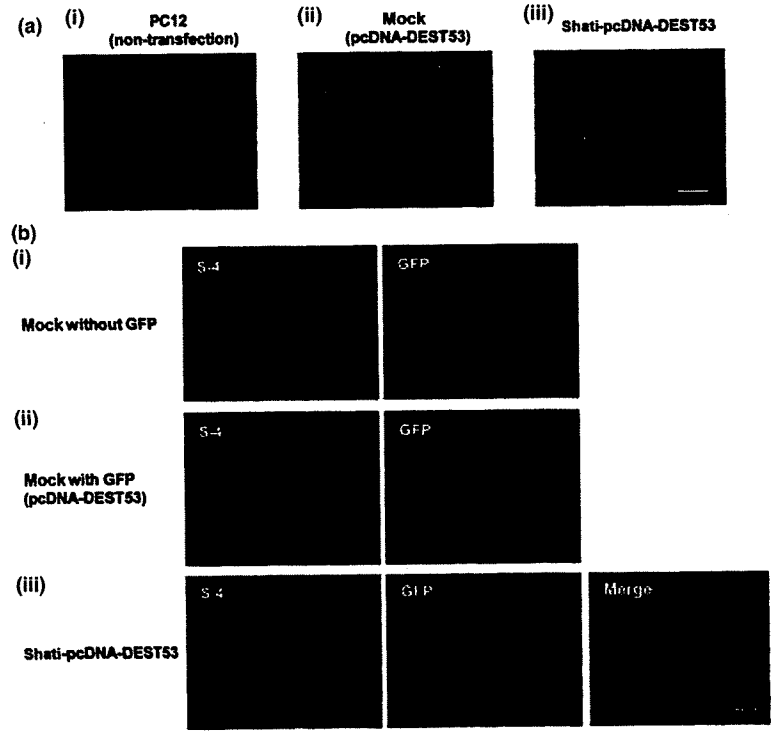


Fig. 4 Transfection of the vector containing shati cDNA into PC12 cells. (a) The morphological changes of the PC12 cells after transfection of the expression vector (pcDNA-DEST53) (ii) or vector containing shati cDNA (iii). The expression vector alone (mock-transfection) (ii), or the vector containing shati cDNA (iii) was introduced into PC12 cells. There were no changes in survival or morphology in the transfected PC12 cells. Scale bar: 20 μm. (b) Immunostaining of shati in PC12 cells transfected with the vector containing shati cDNA. pENTR/TEV/D-TOPO (without shati recombination and green fluorescent protein (GFP) site) (i), pcDNA-DEST53 with GFP (mock-transfection) (ii), or the vector containing shati cDNA and GFP (iii), was introduced into PC12 cells. The shati-immunopositive cells (green) were colocalized with GFP-immunopositive cells (red). Double immunostaining for S-4 and GFP in PC12 cells transfected with the vector containing shati cDNA reveals overexpression of shati in PC12 cells (iii). Scale bar: 20 μm.

Effect of over-expressed shati on DA uptake in PC12 cells

We have previously demonstrated that shati-AS, which inhibits the expression of shati mRNA, significantly potentiates the METH-induced decrease in synaptosomal and vesicular [^{3}H] DA uptake compared with that in the shati-SC or CSF-treated mice (Niwa *et al.* 2007a). Moreover, [^{3}H] DA uptake in saline-treated mice was also decreased by shati-AS compared with that in the CSF-treated mice, although shati-SC had no effect on [^{3}H] DA uptake (Niwa *et al.* 2007a). Given the results for synaptosomal and vesicular [^{3}H] DA uptake using shati-AS, we concluded that shati plays a critical role in modulating DA uptake. To address this issue, we investigated the role of shati in DA uptake in PC12 cells transfected with the vector containing shati cDNA.

Transfection of the vector containing shati cDNA increased shati mRNA expression compared with the mock-transfection, suggesting that shati was over-expressed in these cells (Fig. 5a left two columns). The increase in the levels of shati mRNA expression evoked by METH treatment (1 μM, 30 min) in mock-transfected cells was significantly potentiated by shati over-expression in PC12 cells (drug, $F_{1,28} = 20.917$, $p < 0.01$; transfection, $F_{1,28} = 247.684$, $p < 0.01$; drug × transfection, $F_{1,28} = 0.003$, $p = 0.955$; two-way ANOVA) (Fig. 5a right two columns).

We examined the *in vitro* effect of over-expressed shati on [^{3}H] DA uptake in PC12 cells. Shati-over-expressing cells themselves showed increased [^{3}H] DA uptake compared with the mock-transfected cells, suggesting that shati itself promotes DA uptake (Fig. 5b left two columns). We pre-treated PC12 cells with METH (1 μM) for 30 min, and then assayed the uptake of [^{3}H] DA. As shown in Fig. 5b, METH (1 μM, 30 min) decreased [^{3}H] DA uptake compared with the mock-transfected control cells. In the shati-over-expressing cells, the METH-induced decrease in [^{3}H] DA uptake was significantly inhibited compared with that in the mock-transfected cells (drug, $F_{1,40} = 45.807$, $p < 0.01$; transfection, $F_{1,28} = 21.551$, $p < 0.01$; drug × transfection, $F_{1,28} = 0.001$, $p = 0.971$; two-way ANOVA) (Fig. 5b right two columns). These results indicated that shati could attenuate METH-induced inhibition of DA uptake.

Regulation of TNF-α expression by shati

TNF-α activates synaptosomal and vesicular DA uptake (Nakajima *et al.* 2004). TNF-α and its inducer diminish the METH-induced decrease in DA uptake and inhibit the METH-induced dependence (Nakajima *et al.* 2004; Niwa *et al.* 2007c, e). Moreover, given the findings on [^{3}H] DA uptake obtained using shati-AS (Niwa *et al.* 2007a) and shati-over-expressing cells (Fig. 5b), we hypothesized that shati increased DA uptake by regulating TNF-α. To address this issue, we examined expression levels of TNF-α mRNA after transfection of the vector containing shati cDNA or treatment with shati-AS.

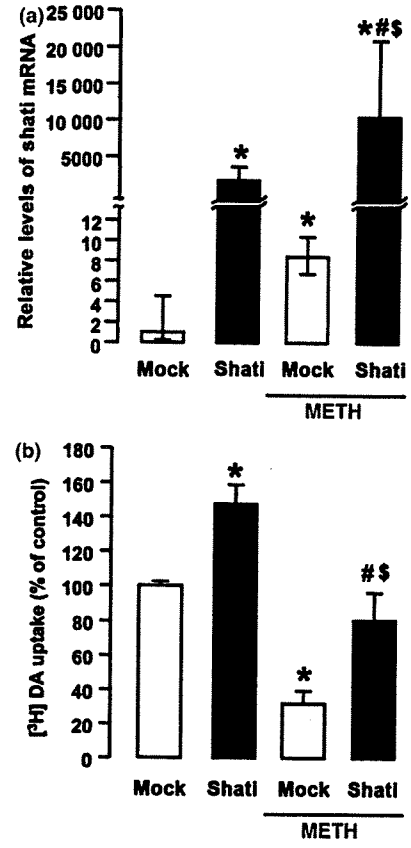
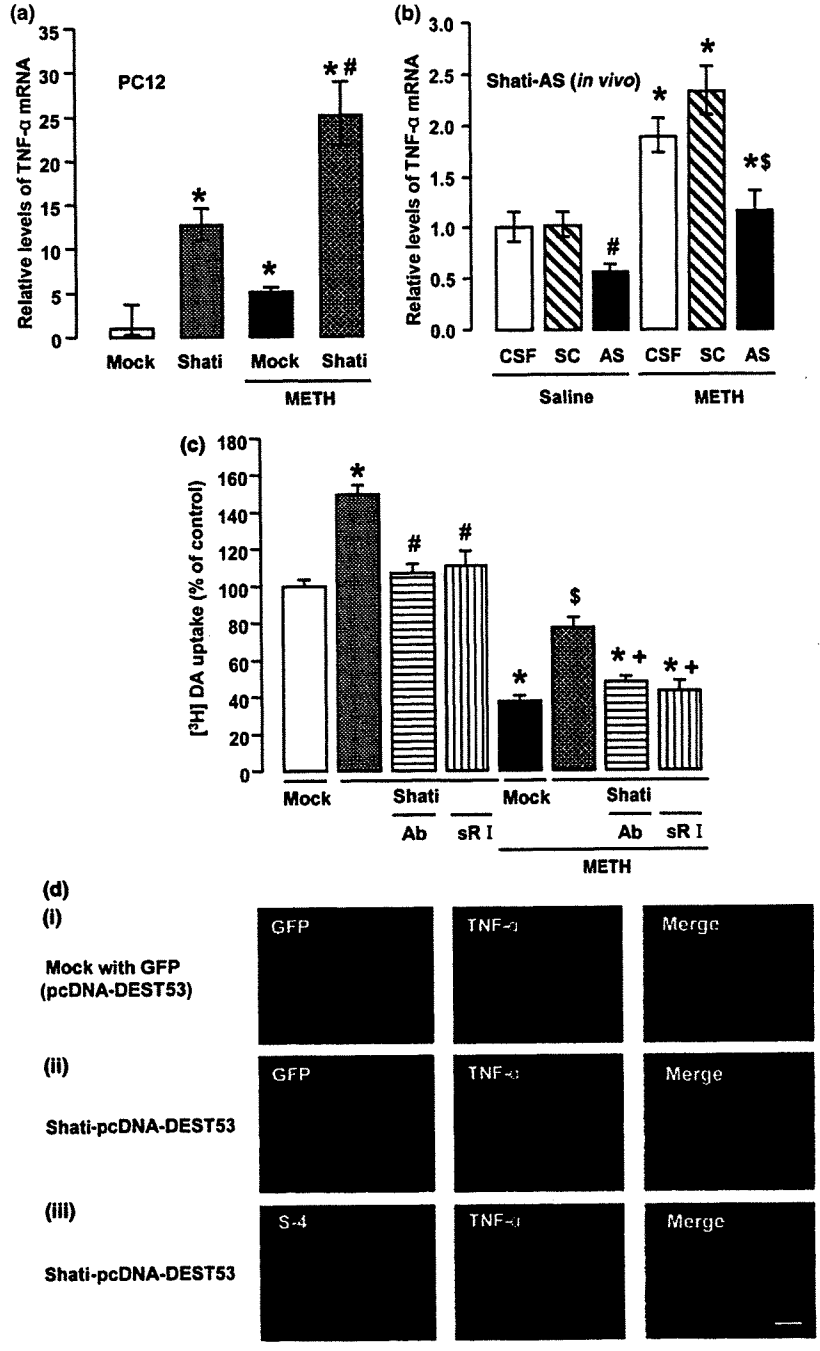


Fig. 5 Effect of overexpression of shati on DA uptake in PC12 cells. (a) Shati mRNA expression in PC12 cells transfected with the vector containing shati cDNA. The mock construct (pcDNA-DEST53), or the vector containing shati cDNA was introduced into PC12 cells. These cells were treated with 1 μM METH for 30 min. Values are means ± SE ($n = 8$). $^*p < 0.05$ versus mock-transfected cells. $^\#p < 0.05$ versus the vector containing shati cDNA-transfected cells. $^\$p < 0.05$ versus METH + mock-transfected cells. (b) Effect of over-expression of shati on [^{3}H] DA uptake in PC12 cells. The mock construct (pcDNA-DEST53), or the vector containing shati cDNA was introduced into PC12 cells. The cells were pre-treated with 1 μM METH for 30 min, and [^{3}H] DA uptake was measured. The [^{3}H] DA uptake was 0.12 ± 0.02 pmol/10 min for the mock-transfected cells. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE ($n = 10$–12). $^*p < 0.05$ versus mock-transfected cells. $^\#p < 0.05$ versus the vector containing shati cDNA-transfected cells. $^\$p < 0.05$ versus METH + mock-transfected cells.

Shati-over-expressing cells themselves had increased TNF-α mRNA expression compared with the mock-transfected cells (Fig. 6a left two columns), suggesting that shati regulates expression of TNF-α in PC12 cells. The increase in TNF-α mRNA expression evoked by METH treatment (1 μM, 30 min) in mock-transfected cells was significantly potentiated by overexpression of shati *in vitro* (drug, $F_{1,28} = 21.000$, $p < 0.01$; transfection, $F_{1,28} = 65.860$,

$p < 0.01$; drug × transfection, $F_{1,28} = 3.557$, $p = 0.070$; two-way ANOVA) (Fig. 6a right two columns). As shown in Fig. 6b right three columns, the increase in TNF-α mRNA expression evoked by repeated METH treatment in the NAc was significantly abolished by shati-AS, although shati-SC had no effect. Moreover, TNF-α mRNA expression in the NAc of saline-treated mice was also inhibited by shati-AS, although not by shati-SC (drug, $F_{1,47} = 48.473$, $p < 0.01$; intracerebroventricular treatment, $F_{2,47} = 15.670$, $p < 0.01$; drug × intracerebroventricular treatment, $F_{2,47} = 0.239$, $p = 0.788$; two-way ANOVA) (Fig. 6b left three columns), indicating that shati-AS decreases effectively the expression of TNF-α mRNA through the down-regulation of shati mRNA expression.

As shown in Fig. 6c, right four columns, the ameliorative effect of shati on the METH-induced decrease in DA uptake



was antagonized by treatment with the TNF-αantibody (50 ng/mL) or soluble TNF receptor I (1 ng/mL). The shati-induced potentiation of DA uptake was also inhibited by the treatments in shati-over-expressing cells (drug, $F_{1,72} = 296.090$, $p < 0.01$; transfection, $F_{1,72} = 13.864$, $p < 0.01$; neutralization, $F_{1,72} = 32.930$, $p < 0.01$; drug × transfection, $F_{1,72} = 0.189$, $p = 0.665$; drug × neutralization, $F_{1,72} = 1.496$, $p = 0.225$; transfection × neutralization, $F_{1,72} = 34.828$, $p < 0.01$; drug × transfection × neutralization, $F_{1,72} = 0.003$, $p = 0.958$; three-way ANOVA) (Fig. 6c left four columns). These results suggest that over-expression of shati increased DA uptake by regulating TNF-α in PC12 cells. To confirm the relationship between shati and TNF-α, we examined immunostaining for GFP, which is co-expressed with shati, or S-4 and TNF-α. The cells mock-transfected, which express GFP, but not shati, were immunopositive for GFP, but not TNF-α. The cells transfected with the vector containing shati cDNA, which express both GFP and shati, were immunopositive for GFP or S-4 and TNF-α. The cells immunopositive for S-4 were merged with those positive for TNF-α. These results indicated that shati was expressed in TNF-α-immunopositive cells (Fig. 6d).

Discussion

DA is the predominant catecholamine neurotransmitter in the CNS. Disruptions of DA signaling contribute to various psychiatric and neurological disorders, including drug addiction, schizophrenia, and Parkinson's disease (Self and Nestler 1995; Hyman 1996). Extracellular DA levels are primarily regulated by DAT, an integral membrane protein that is a member of the Na^+/Cl^--dependent co-transporter gene family (Amara and Kuhar 1993). By removing extracellular DA and recycling it back to the neuron, DAT plays an essential role in terminating DA signaling. Pharmacological blockage of DAT by psychostimulants inhibits the reuptake of DA from the extracellular space, resulting in increased extracellular DA levels and augmented receptor stimulation (Horn 1990). Although pharmacological and genetic ablation (Grace 1995; Jones *et al.* 1998) studies indicate a critical role for DAT in the maintenance of DA neuronal homeostasis, the endogenous mechanisms regulating DAT expression and activity are poorly understood.

The PC12 cell line is derived from the rat pheochromocytoma. It is often used as an *in vitro* model to understand the physiology of central DA neurons (Roda *et al.* 1980; Tischler 2002; Fornai *et al.* 2007). A number of factors contribute to the wide use of PC12 cells: they are inexpensive as well as easy to handle, and mimic many features of central DA neurons. In fact, PC12 cells produce catecholamines (Markey *et al.* 1980; Roda *et al.* 1980; Vaccaro *et al.* 1980). In particular, they contain DA (Greene and Rein 1978) as the main catecholamine and bear DA receptors on their external membrane (Sampath *et al.* 1994). In light of the presence of DA and DA receptors, as well as DA uptake mechanisms, PC12 cell lines are considered to be closer to DA terminals than their ancestors (i.e. chromaffin cells of the adrenal medulla). This concept is reinforced by the presence of monoamine oxidase type A, which also characterizes DA neurons (Finberg and Youdim 1983), in contrast with the established prevalence of monoamine oxidase type B within chromaffin cells of the adrenal medulla (Youdim 1991).

Recently, we have demonstrated that TNF-α and its inducer play a neuroprotective role in the behavioral sensitization to and rewarding effects of METH by activating plasmalemmal and vesicular DAT as well as by inhibiting the METH-induced increase in extracellular DA levels (Nakajima *et al.* 2004; Niwa *et al.* 2007c,e). TNF-α modulates cellular responses through the ERK1/2 and NF-κB signaling pathways (van Vliet *et al.* 2005). The adaptor protein TNF receptor-associated factor 2 (TRAF2) and the serine and threonine protein kinase receptor-interacting protein are required for optimal TNF-induced signaling through ERK1/2, c-Jun N-termial kinase (JNK) and p38 mitogen-activated

Fig. 6 Involvement of TNF-α in shati-induced increase in DA uptake in PC12 cells. (a) TNF-α mRNA expression in PC12 cells transfected with the vector containing shati cDNA. The expression vector alone (pcDNA-DEST53), or the vector containing shati cDNA was introduced into PC12 cells. The cells were treated with 1 μM METH for 30 min. Values are means ± SE ($n = 8$). $^*p < 0.05$ versus mock-transfected group. $^\#p < 0.05$ versus METH + mock-transfected group. (b) Effect of shati-AS on TNF-α mRNA expression. Mice were administered METH (1 mg/kg, s.c.) for 5 days and decapitated 2 h after the final treatment. Values are means ± SE ($n = 8$–10). $^*p < 0.05$ versus corresponding saline-treated mice. $^\#p < 0.05$ versus saline + CSF and saline + shati-SC-treated mice. $^\$p < 0.05$ versus METH + CSF and METH + shati-SC-treated mice. (c) Involvement of TNF-α in shati-induced increase in [^{3}H] DA uptake in PC12 cells. The expression vector alone (pcDNA-DEST53), or the vector containing shati cDNA was introduced into PC12 cells. The cells were pre-treated with anti-TNF-α antibody (Ab; 50 ng/mL) or soluble TNF receptor I (sR I; 1 ng/mL) 10 min before their treatment with METH (1 μM, 30 min), and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.15 ± 0.02 pmol/10 min for the mock-transfected group. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE ($n = 10$). $^*p < 0.05$ versus mock-transfected group. $^\#p < 0.05$ versus the vector containing shati cDNA-transfected group. $^\$p < 0.05$ versus METH + mock-transfected group. $^+p < 0.05$ versus METH + the vector containing shati cDNA-transfected group. (d) Immunostaining of shati and TNF-α in PC12 cells transfected with the vector containing shati cDNA. The expression vector alone (pcDNA-DEST53) (i), or the vector containing shati cDNA (ii) (iii) was introduced into PC12 cells. The GFP or shati-immunopositive cells (green) were co-localized with TNF-α-immunopositive cells (red) (ii) (iii). Double immunostaining for GFP or S-4 and TNF-α in PC12 cells transfected with the vector containing shati cDNA reveals expression of shati in TNF-α-immunopositive cells (ii) (iii). Scale bar: 20 μm.

protein kinase (p38) (Baud and Karin 2001; Devin *et al.* 2003). MEK inhibitor PD98059 significantly decreases phosphorylated ERK1/2 without affecting total ERK level, MEK-JNK, -p38, and -NF-κB, resulting in loss of DAT surface expression and DAT capacity. According these results, MEK-ERK pathway, but not MEK-JNK, -p38, or -NF-κB pathway, is important for intracellular trafficking and transport capacity of DAT (Morón *et al.* 2003). Therefore, we investigated the involvement of TNF-α in DA uptake and the METH-induced inhibition of DA uptake in PC12 cells. Moreover, we examined the involvement of MEK-ERK signaling in the effects of TNF on DA uptake. TNF-α increased DA uptake via the MEK-ERK signaling pathway in PC12 cells (Figs 1 and 2). The increase was antagonized by the anti-TNF-α antibody and soluble TNF receptor I (Fig. 1b and c), suggesting that TNF-α certainly increases DA uptake in PC12 cells. Moreover, TNF-α inhibited the METH-induced decrease in DA uptake in PC12 cells (Fig. 3b). We have previously reported that the kinetics of [^{3}H] DA uptake in the absence or presence of TNF-α (10 ng/mL). Lineweaver-burk plots show that TNF-α potentiates [^{3}H] DA uptake by increasing the affinity (Km) accompanied by reducing the maximum number of [^{3}H] DATs (Vmax) (Nakajima *et al.* 2004). We suggest that TNF-α modulates the function of DAT, although it also regulates the expression of DAT. The expression of TNF-α is induced through the activation of transcription factors such as activator protein-1 (AP-1) and NF-κB by the activation of JNK/p38 (Guha *et al.* 2000; Rahman and MacNee 2000). Further, TNF-α acts on mitochondria to generate reactive oxygen species, which are involved in the activation of AP-1 and NF-κB (Rahman and MacNee 2000). Changes in transcription factors may result in long-term changes in gene expression, thereby contributing to neuronal adaptations that underlie behavioral sensitization (Nestler 2001). Therefore, we hypothesized that TNF-α inhibits the METH-induced increase in extracellular DA levels in the NAc by promoting DA uptake and finally inhibits METH-induced sensitization and rewarding effects (Nakajima *et al.* 2004; Niwa *et al.* 2007c,e).

'Shati', named after the symbol for Nagoya castle, was identified among molecules whose expression was regulated in the NAc of mice treated with METH (Niwa *et al.* 2007a). Recently, we have demonstrated that blockage of shati expression by shati-AS potentiates the increase in extracellular DA levels in the NAc and the decrease in synaptosomal and vesicular DA uptake in the midbrain induced by repeated METH treatment (Niwa *et al.* 2007a). Both TNF-α and shati increase DA uptake and inhibit the METH-induced decrease in DA uptake (Nakajima *et al.* 2004; Niwa *et al.* 2007a). Therefore, we investigated the precise mechanism of the effects of shati on DA uptake, and the METH-induced inhibition of DA uptake in PC12 cells. Moreover, we examined the relationship between shati and TNF-α in PC12 cells. Over-expression of shati by transfection of the vector containing shati cDNA (Fig. 4) dramatically induced the expression of shati mRNA (Fig. 5a) and TNF-α mRNA (Fig. 6a) in PC12 cells. No histological or mechanical disruption was produced by transfection of the vector (Fig. 4a). Over-expression of shati (Fig. 5a), which occurs in TNF-α-immunopositive cells (Fig. 6d), potentiated DA uptake and inhibited the METH-induced decrease in DA uptake (Fig. 5b) in PC12 cells by regulating TNF-α expression (Fig. 6a), since these effects were antagonized by anti-TNF-α antibody and soluble TNF receptor I used for the neutralization of TNF-α (Fig. 6c; Barone *et al.* 1997). These findings strongly suggest that the over-expression of shati elicited by METH serves as a homeostatic mechanism that prevents behavioral sensitization and rewarding effects by attenuating the METH-induced increase in extracellular DA

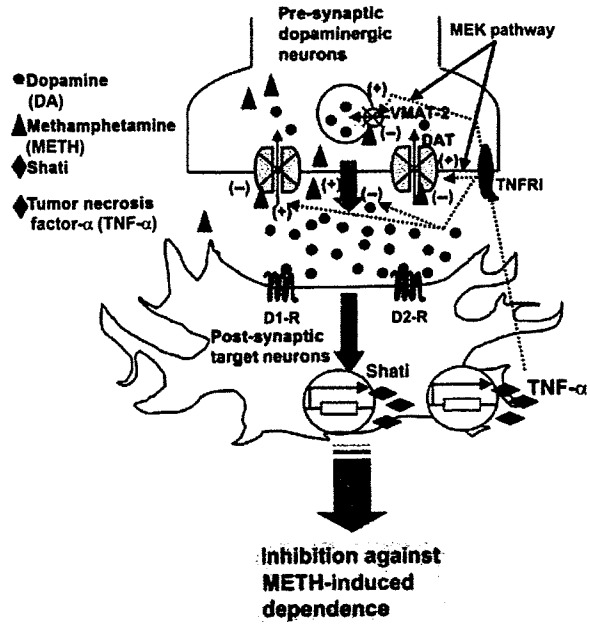


Fig. 7 Schema for regulation of TNF-α induced by shati on METH-induced DA responses. Under basal conditions, plasmalemmal DAT is involved in the reuptake of extracellular DA into the cytosol; subsequently the cytosolic DA is stored into synaptic vesicles via VMAT-2. Treatment of METH inhibits DA uptake through DA transporter and facilitates DA's release from pre-synaptic nerve terminals. METH is associated with an increase in extracellular DA levels in the brain, resulting in potentiation of the METH-induced dependence. METH induces shati and TNF-α expression in target neurons through the activation of DA receptors. TNF-α regulated by shati inhibits the METH-induced increase in extracellular DA levels in the nucleus accumbens by promoting DA uptake via MEK pathway and finally inhibits sensitization to and the rewarding effects of METH. DA: dopamine, METH: methamphetamine, TNF-α: tumor necrosis factor-α, D1-R: dopamine D1 receptor, D2-R: dopamine D2 receptor, DAT: dopamine transporter, VMAT-2: vesicular monoamine transporter-2, TNFR I: tumor necrosis factor type I receptor.

