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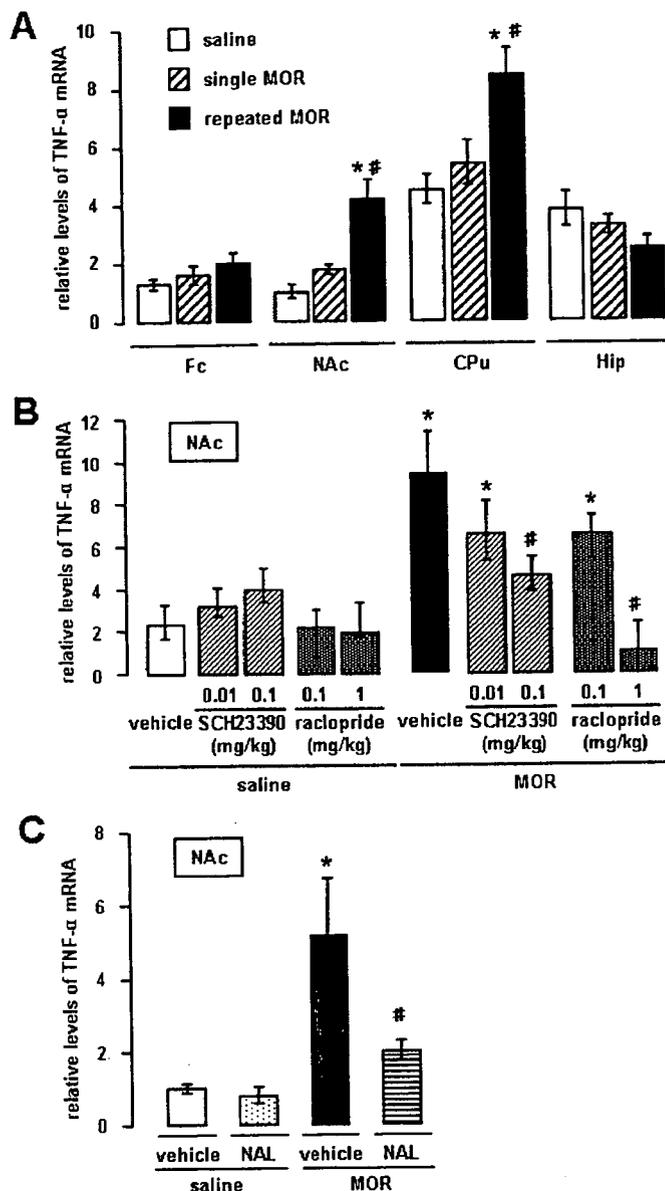
Figure 2. Change of expression of TNF-α mRNA after repeated MOR treatment. **(A)** Change of expression of TNF-α mRNA in the various regions of the mouse brain after single and repeated MOR treatment. Mice were decapitated 2 hours after single or repeated MOR (10 mg/kg, SC) treatment for 1 day or 9 days,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

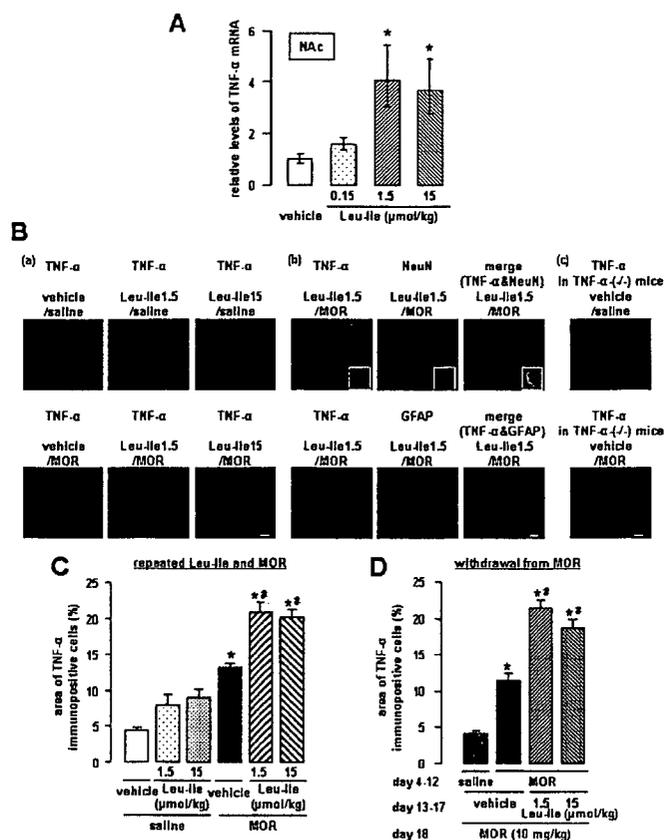We investigated the dose-response effect of Leu-Ile on the expression of TNF-α mRNA in the NAc. Leu-Ile (1.5 and 15 μmol/kg, IP) increased the levels of TNF-α mRNA compared with the vehicle-treated mice [$F(3,38) = 10.615$, $p < .05$, one-way ANOVA] (Figure 3A).

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, $F(9,70) = 68.815$

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 × 10⁴ μm² 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.

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

A

B
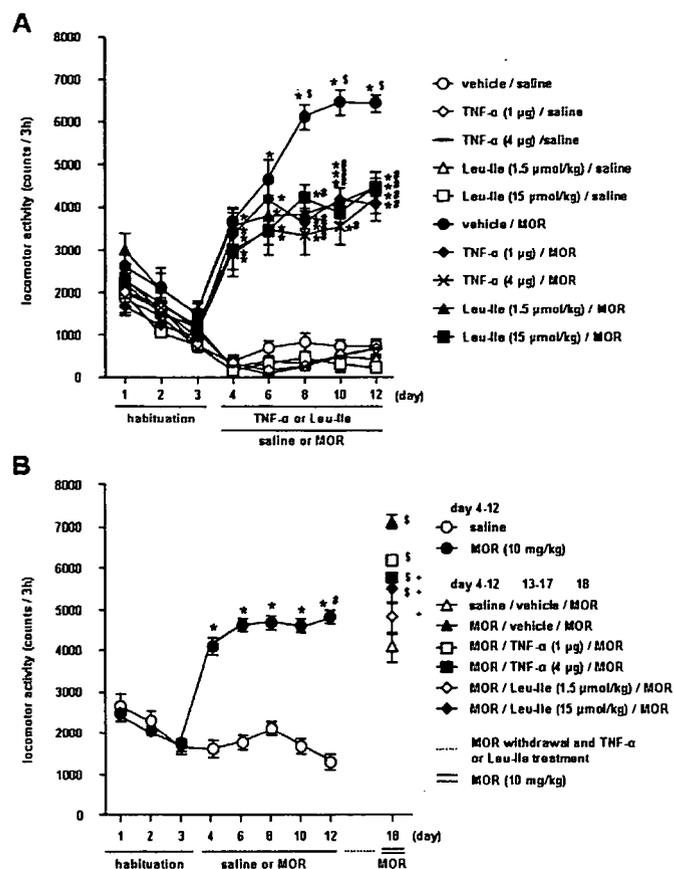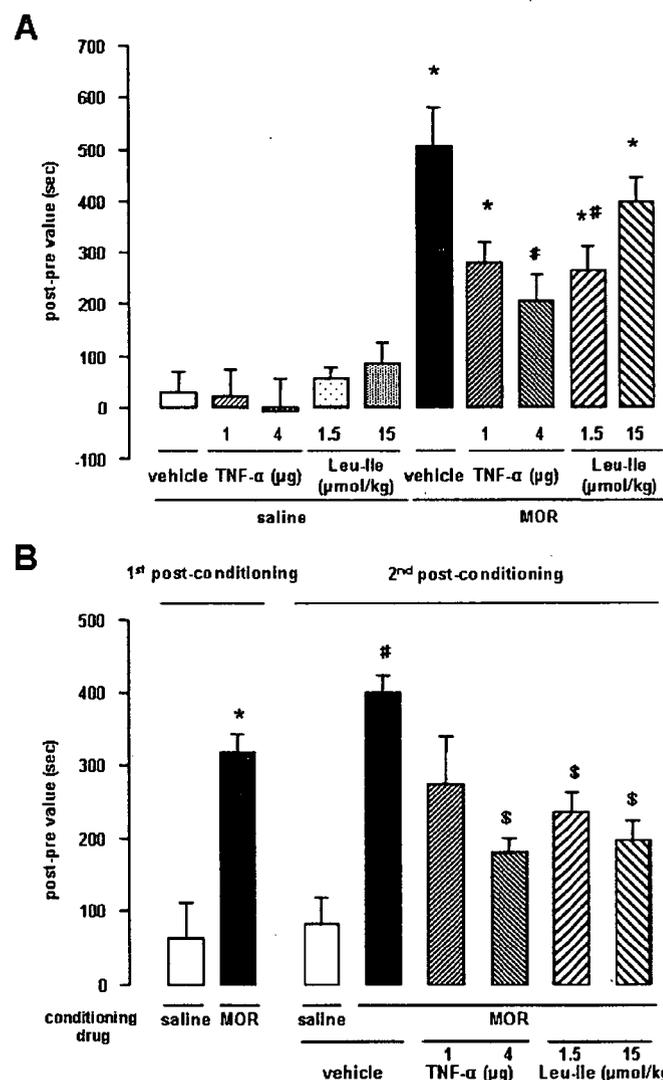

Figure 4. Effects of TNF-α or Leu-Ile on MOR-induced hyperlocomotion/sensitizaton before and after the development of sensitization. **(A)** Effect of TNF-α or Leu-Ile on MOR-induced hyperlocomotion and sensitization in wild-type mice. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and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

A

B

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,

A

B
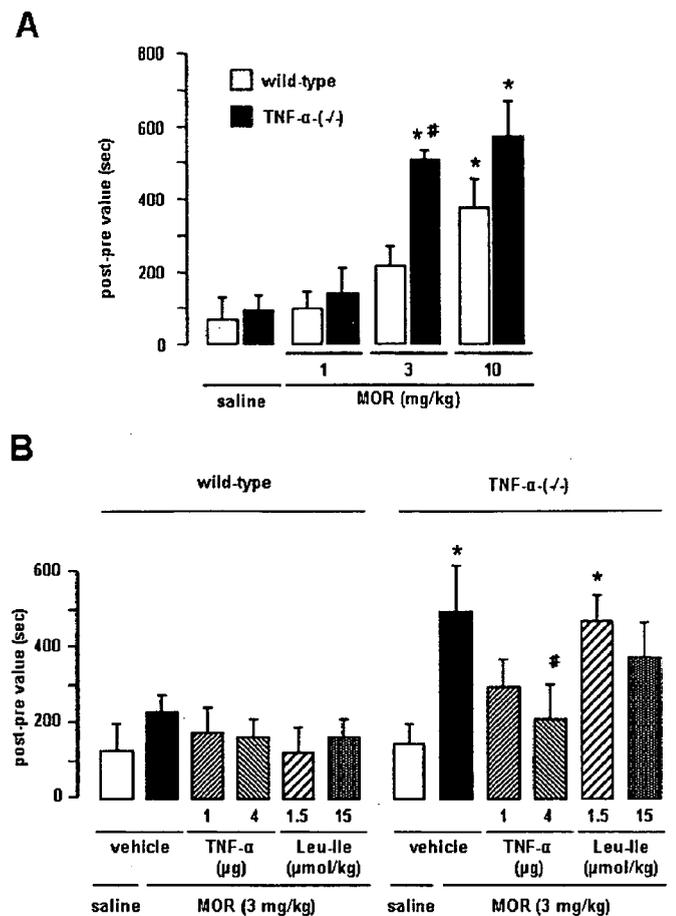
Figure 6. Effects of TNF-α or Leu-Ile on the rewarding effects of MOR in TNF-α-(-/-) mice. **(A)** Morphine-induced place preference in wild-type and TNF-α-(-/-) mice. Mice were treated with MOR (1, 3, and 10 mg/kg, SC) or saline during the conditioning. Values are means ± SEM (n = 8). *$p < .05$ versus 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
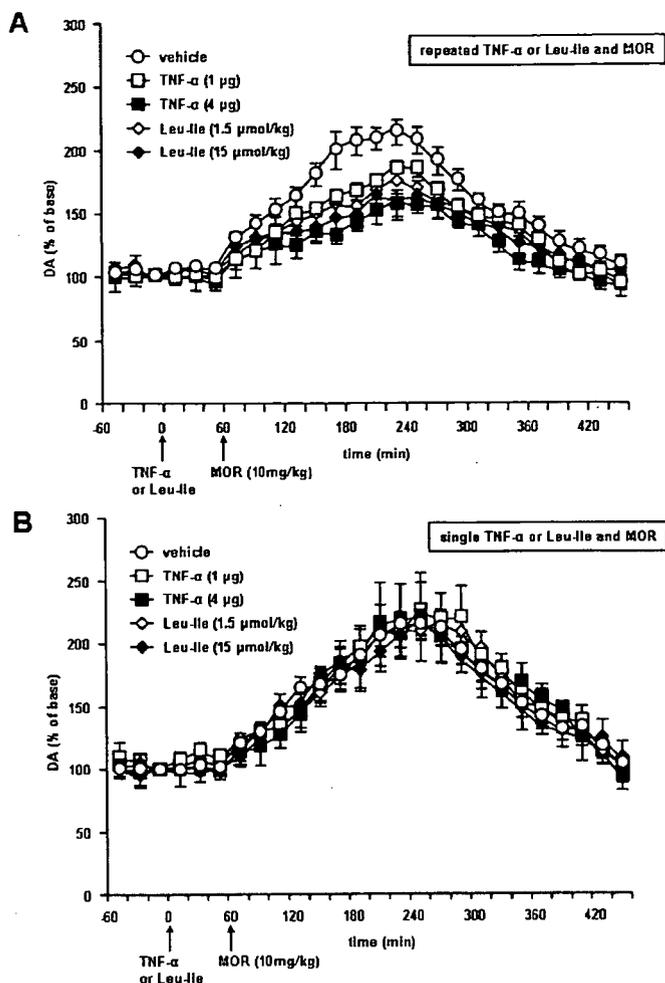
Figure 7. Effects of TNF-α or Leu-Ile on MOR-induced increase in extracellular DA levels. **(A)** Effect of exogenous TNF-α or Leu-Ile on the repeated MOR treatment-induced increase in extracellular DA levels. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before 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
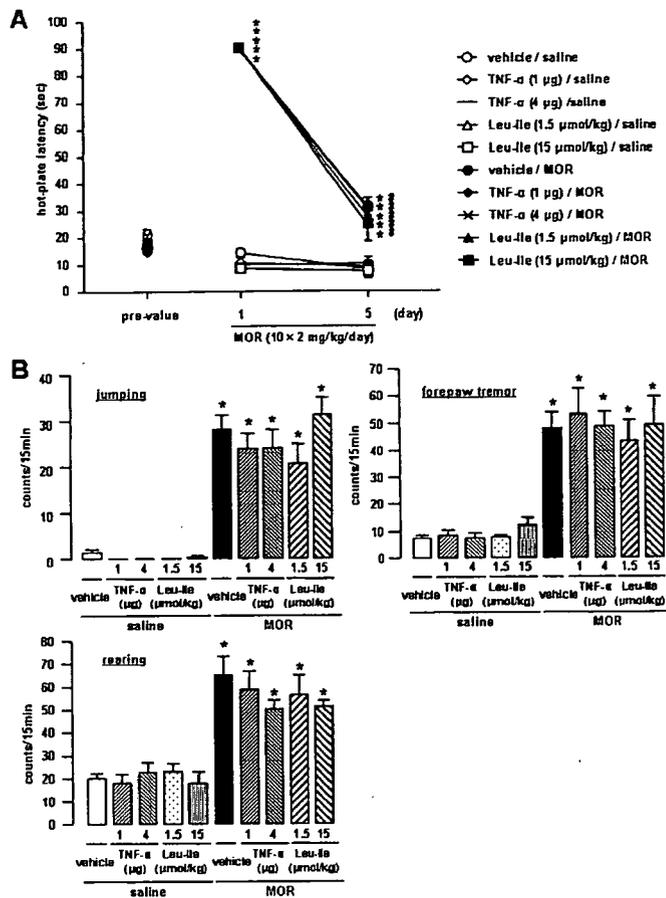
Figure 8. Effects of TNF-α or Leu-Ile on the MOR-induced antinociceptive effects and symptoms of withdrawal. **(A)** Effects of TNF-α or Leu-Ile on the antinociceptive effect and tolerance induced by repeated MOR treatment. Mice were treated with TNF-α (1 and 4 μg, IP) or Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before MOR (10 mg/kg, SC) twice a day for 5 days. The analgestic 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.

Albensi BC, Mattson MP (2000): Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. *Synapse* 35:151–159.

Aloe L, Fiore M (1997): TNF-α expressed in the brain of transgenic mice lowers central tyrosine hydroxylase immunoreactivity and alters grooming behavior. *Neurosci Lett* 238:65–68.

Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von Zastrow M, et al. (2002): Control of synaptic strength by glial TNF alpha. *Science* 295:2282–2285.

Bluthe RM, Pawlowski M, Suarez S, Parnet P, Pittman Q, Kelley KW, et al. (1994): Synergy between tumor necrosis factor alpha and interleukin-1 in the induction of sickness behavior in mice. *Psychoneuroendocrinology* 19:197–207.

Bonci A, Williams JT (1997): Increased probability of GABA release during withdrawal from morphine. *J Neurosci* 17:796–803.

Boudreau AC, Wolf ME (2005): Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. *J Neurosci* 25:9144–9151.

Brase DA, Loh HH, Way EL (1977): Comparison of the effects of morphine on locomotor activity, analgesia and primary and protracted physical dependence in six mouse strains. *J Pharmacol Exp Ther* 201:368–374.

Cen X, Nitta A, Ohya S, Zhao Y, Ozawa N, Mouri A, et al. (2006): An analogue of dipeptide-like structure of FK506 increases GDNF expression through CREB activated by Hsp90/Akt signaling pathway. *J Neurosci* 26:3335–3344.

Chavkin C, Goldstein A (1984): Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. *Proc Natl Acad Sci U S A* 81:7253–7257.

Di Chiara G, North RA (1992): Neurobiology of opioid abuse. *Trends Pharmacol Sci* 13:185–193.

Everitt BJ, Wolf ME (2002): Psychomotor stimulant addiction: A neural systems perspective. *J Neurosci* 22:3312–3320.

Franklin KBJ, Paxinos G (1997): *The Mouse Brain in Stereotaxic Coordinates.* San Diego: Academic press.

Funada M, Suzuki T, Narita M, Misawa M, Nagase H (1993): Blockade of morphine reward through the activation of κ-opioid receptors in mice. *Neuropharmacology* 32:1315–1323.

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

Guha M, Bai W, Nadler JL, Natarajan R (2000): Molecular mechanisms of tumor necrosis factor alpha gene expression in monocytic cells via hyperglycemia-induced oxidant stress-dependent and -independent pathways. *J Biol Chem* 275:17728–17739.

Hamdy MM, Noda Y, Miyazaki M, Mamiya T, Nozaki A, Nitta A, et al. (2004): Molecular mechanisms in dizocilpine-induced attenuation of development of morphine dependence: An association with cortical Ca^{2+}/calmodulin-dependent signal cascade. *Behav Brain Res* 152:263–270.

Heikkila RE, Orlansky H, Cohen G (1975): Studies on the distinction between uptake inhibition and release of ^{3}H-dopamine in rat brain tissue slices. *Biochem Pharmacol* 24:847–852.

Itoh A, Shiotani T, Nakayama S, Mamiya T, Hasegawa T, Noda Y, et al. (2000): Attenuation of the development of morphine dependence/tolerance by nefiracetam: Involvement of adenosine 3′:5′-cyclic monophosphate system. *Behav Brain Res* 115:65–74.

Johnson SM, Fleming WW (1989): Mechanisms of cellular adaptive sensitivity changes: Applications to opioid tolerance and dependence. *Pharmacol Rev* 41:435–488.

Johnson SW, North RA (1992): Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J Neurosci* 12:483–488.

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

Koob GF (1992): Drugs of abuse: Anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci* 13:177–184.

Koob GF, Nestler EJ (1997): The neurobiology of drug addiction. *J Neuropsychiatry Clin Neurosci* 9:482–497.

Koob GF, Sanna PP, Bloom FE (1998): Neuroscience of addiction. *Neuron* 21:467–476.

Kuwahara M, Sugimoto M, Tsuji S, Miyata S, Yoshida A (1999): Cytosolic calcium changes in a process of platelet adhesion and cohesion on a von Willebrand factor-coated surface under flow conditions. *Blood* 94:1149–1155.

Laakso A, Mohn AR, Gainetdinov RR, Caron MG (2002): Experimental genetic approaches to addiction. *Neuron* 36:213–228.

Law PY, Hom DS, Loh HH (1982): Loss of opiate receptor activity in neuroblastoma X glioma NG108-15 hybrid cells after chronic opiate treatment. A multiple-step process. *Mol Pharmacol* 22:1–4.

Maier SF, Watkins LR (1998): Cytokines for psychologists: Implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychol Rev* 105:83–107.

Mamiya T, Noda Y, Ren X, Hamdy M, Furukawa S, Kameyama T, et al. (2001): Involvement of cyclic AMP systems in morphine physical dependence in mice: Prevention of development of morphine dependence by rolipram, a phosphodiesterase 4 inhibitor. *Br J Pharmacol* 132:1111–1117.

Matthews RT, German DC (1984): Electrophysiological evidence for excitation of rat ventral tegmental area dopamine neurons by morphine. *Neuroscience* 11:617–625.

Messer CJ, Eisch AJ, Carlezon WA Jr, Whisler K, Shen L, Wolf DH, et al. (2000): Role for GDNF in biochemical and behavioral adaptations to drugs of abuse. *Neuron* 26:247–257.

Miyamoto Y, Yamada K, Nagai T, Mori H, Mishina M, Furukawa H, et al. (2004): Behavioral adaptations to addictive drugs in mice lacking the NMDA receptor ε1 subunit. *Eur J Neurosci* 19:151–158.

Mizoguchi H, Yamada K, Mizuno M, Mizuno T, Nitta A, Noda Y, et al. (2004): Regulations of methamphetamine reward by extracellular signal-regulated kinase 1/2/ets-like gene-1 signaling pathway via the activation of dopamine receptors. *Mol Pharmacol* 65:1293–1301.

Nagai T, Kamei H, Ito M, Hashimoto K, Takuma K, Nabeshima T, et al. (2005a): Modification by the tissue plasminogen activator-plasmin system of morphine-induced dopamine release and hyperlocomotion, but not anti-nociceptive effect in mice. *J Neurochem* 93:1272–1279.

Nagai T, Noda Y, Ishikawa K, Miyamoto Y, Yoshimura M, Ito M, et al. (2005b): The role of tissue plasminogen activator in methamphetamine-related reward and sensitization. *J Neurochem* 92:660–667.

Nagai T, Yamada K, Yoshimura M, Ishikawa K, Miyamoto Y, Hashimoto K, et al. (2004): The tissue plasminogen activator-plasmin system participates in the rewarding effect of morphine by regulating dopamine release. *Proc Natl Acad Sci U S A* 101:3650–3655.

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

Narita M, Funada M, Suzuki T (2001): Regulations of opioid dependence by opioid receptor types. *Pharmacol Ther* 89:1–15.

Nestler EJ (2001): Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neurosci* 2:119–128.

Nestler EJ (2002): From neurobiology to treatment: Progress against addiction. *Nat Neurosci* 5:1076–1079.

Nitta A, Nishioka H, Fukumitsu H, Furukawa Y, Sugiura H, Shen L, et al. (2004): Hydrophobic dipeptide Leu-Ile protects against neuronal death by inducing brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor synthesis. *J Neurosci Res* 78:250–258.

Niwa M, Nitta A, Yamada Y, Nakajima A, Saito K, Seishima M, et al.(in press): An inducer for glial cell line-derived neurotrophic factor and tumor necrosis factor-α protects methamphetamine-induced reward and sensitization. *Biol Psychiatry*.

Noda Y, Miyamoto Y, Mamiya T, Kamei H, Furukawa H, Nabeshima T (1998): Involvement of dopaminergic system in phencyclidine-induced place preference in mice pretreated with phencyclidine repeatedly. *J Pharmacol Exp Ther* 286:44–51.

Oliverio A, Castellano C, Eleftheriou BE (1975): Morphine sensitivity and tolerance: A genetic investigation in the mouse. *Psychopharmacologia* 42:219–224.

Puttfarcken PS, Werling LL, Cox BM (1988): Effects of chronic morphine exposure on opioid inhibition of adenylyl cyclase in 7315c cell membranes: A useful model for the study of tolerance at μ opioid receptors. *Mol Pharmacol* 33:520–527.

Rahman I, MacNee W (2000): Regulation of redox glutathione levels and gene transcription in lung inflammation: Therapeutic approaches. *Free Radic Biol Med* 28:1405–1420.

Ren X, Noda Y, Mamiya T, Nagai T, Nabeshima T (2004): A neuroactive steroid, dehydroepiandrosterone sulfate, prevents the development of morphine dependence and tolerance via c-fos expression linked to the extracellular signal-regulated protein kinase. *Behav Brain Res* 152:243–250.

Robinson TE, Berridge KC (2000): The psychology and neurobiology of addiction: An incentive-sensitization view. *Addiction* 95(suppl 2):S91–S117.

Seiden LS, Sabol KE, Ricaurte GA (1993): Amphetamine: Effects on catecholamine systems and behavior. *Annu Rev Pharmacol Toxicol* 33:639–677.

Taniguchi T, Tanaka M, Ikeda A, Momotani E, Sekikawa K (1997): Failure of germinal center formation and impairment of response to endotoxin in tumor necrosis factor α deficient mice. *Lab Invest* 77:647–658.

Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Crain SM, Nestler EJ (1991): A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res* 548:100–110.

Tsuji S, Sugimoto M, Miyata S, Kuwahara M, Kinoshita S, Yoshioka A (1999): Real-time analysis of mural thrombus formation in various platelet aggregation disorders: Distinct shear-dependent roles of platelet receptors and adhesive proteins under flow. *Blood* 94:968–975.

Vassali P (1992): The pathophysiology of tumor necrosis factors. *Annu Rev Immunol* 10:411–452.

Vezina P, Stewart J (1984): Conditioning and place-specific sensitization of increases in activity induced by morphine in VTA. *Phamacol Biochem Behav* 20:925–934.

Wada R, Tifft CJ, Proia RL (2000): Microglial activation precedes acute neurodegeneration in Sandhoff disease and is suppressed by bone marrow transplantation. *Proc Natl Acad Sci U S A* 97:10954–10959.

Wise RA (1996): Neurobiology of addiction. *Curr Opin Neurobiol* 6:243–251.

Yamada K, Iida R, Miyamoto Y, Saito K, Sekikawa K, Sedishima M, et al. (2000): Neurobehavioral alternations in mice with a targeted deletion of the tumor necrosis factor-alpha gene: Implication for emotional behavior. *J Neuroimmunol* 111:131–138.

Yamada K, Nagai T, Nabeshima T (2005): Drug dependence, synaptic plasticity, and tissue plasminogen activator. *J Pharmacol Sci* 97:157–161.
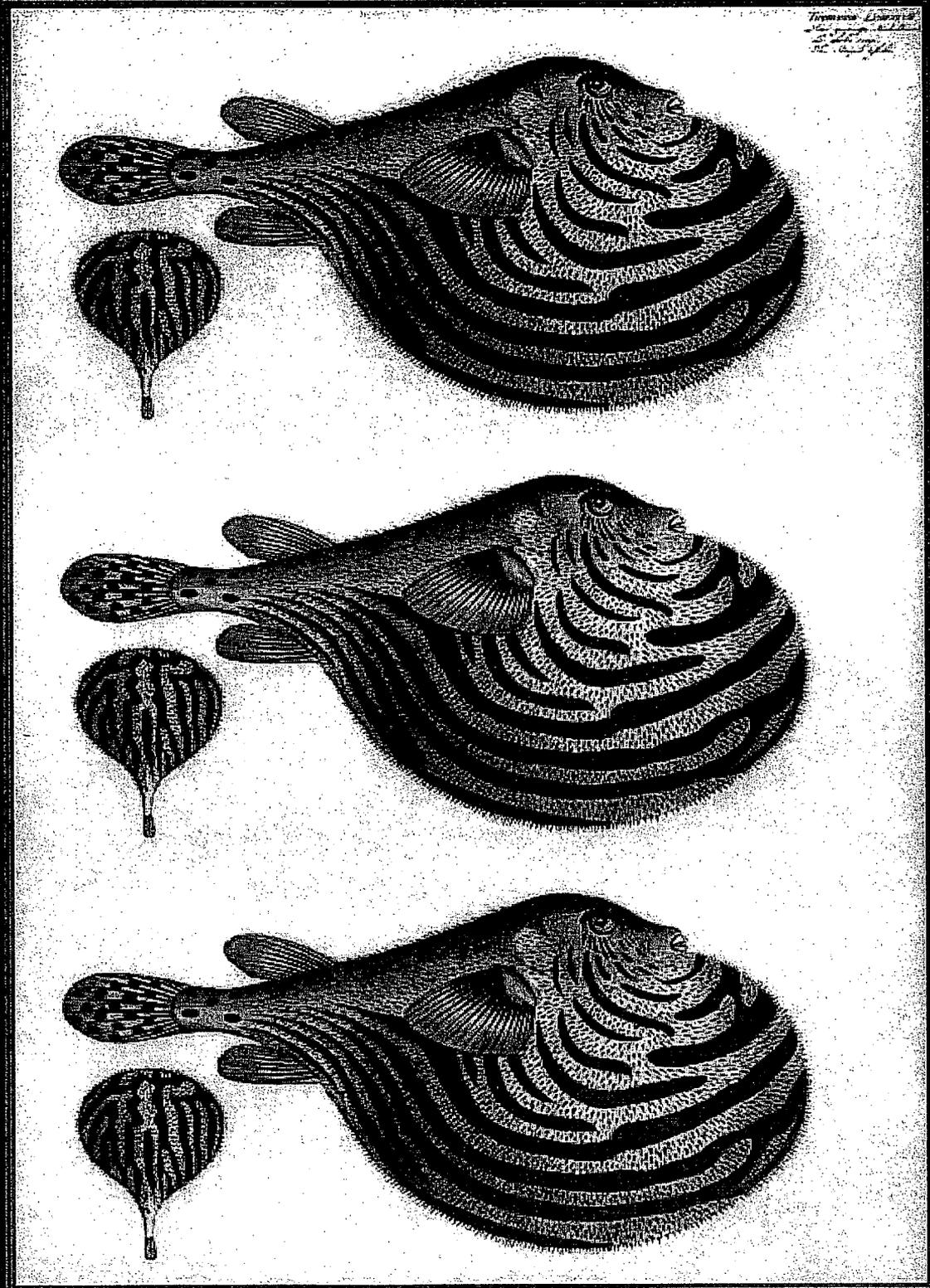
Gene Model
for Obesity
in Fish

p. 2042

Enduring vulnerability to reinstatement of methamphetamine-seeking behavior in glial cell line-derived neurotrophic factor mutant mice

Yijin Yan,* Kiyofumi Yamada,*,† Minae Niwa,* Taku Nagai,* Atsumi Nitta,* and Toshitaka Nabeshima*,1

*Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan; and †Laboratory of Neuropsychopharmacology, Kanazawa University Graduate School of Natural Science and Technology, Kanazawa, Japan

ABSTRACT   Genetic factors are considered to play an important role in drug dependence/addiction including the development of drug dependence and relapse. With the use of a model of drug self-administration in mutant mice, several specific genes and proteins have been identified as potentially important in the development of drug dependence. In contrast, little is known about the role of specific genes in enduring vulnerability to relapse, a clinical hallmark of drug addiction. Using a mouse model of reinstatement, which models relapse of drug-seeking behavior in addicts, we provide evidence that a partial reduction in the expression of the glial cell line-derived neurotrophic factor (GDNF) potentiates methamphetamine (METH) self-administration, enhances motivation to take METH, increases vulnerability to drug-primed reinstatement, and prolongs cue-induced reinstatement of extinguished METH-seeking behavior. In contrast, there was no significant difference in novelty responses, METH-stimulated hyperlocomotion and locomotor sensitization, food-reinforced operant behavior and motivation, or reinstatement of food-seeking behavior between GDNF heterozygous knockout mice and wild-type littermates. These findings suggest that GDNF may be associated with enduring vulnerability to reinstatement of METH-seeking behavior and a potential target in the development of therapies to control relapse.—Yan, Y., Yamada, K., Niwa, M., Nagai, T., Nitta, A., Nabeshima, T. Enduring vulnerability to reinstatement of methamphetamine-seeking behavior in glial cell line-derived neurotrophic factor mutant mice. *FASEB J.* 21, 1994–2004 (2007)

Key Words: *GDNF mutant mice · METH self-administration · relapse*

GENETIC FACTORS ARE CONSIDERED TO PLAY an important role in drug dependence/addiction and alcoholism (1–7). In animal models, vulnerability to self-administration and reinstatement in the taking of different addictive substances has been suggested to share common genetic determinants (8). By using a model of drug self-administration in mutant mice, several specific genes or proteins have been identified as potentially involved in the development of drug dependence (9–14). However, a good model of relapse in mutant mice has yet to be established. Thus, few lines of direct evidence have been obtained for an association between specific genes and vulnerability to relapse of drug-seeking behavior, which is a major challenge in the clinical treatment of addiction (15, 16).

Glial cell line-derived neurotrophic factor (GDNF) was originally purified from a rat glioma cell-line supernatant as a trophic factor for embryonic midbrain dopamine neurons (17). As a potential therapeutic agent for the treatment of Parkinson's disease, GDNF has been widely tested (18, 19). It is well established that dopaminergic transmission in the cortico-limbic system is crucial for the development of drug dependence/addiction (20–25). Given that GDNF is considered an important modulator for dopaminergic neuronal function (17, 26), it is reasonable to postulate that GDNF may be involved in drug addiction. Although direct evidence of a clinical association between GDNF and drug dependence/addiction has yet to be obtained, GDNF has been identified in the development of drug dependence in animal models (27–30). Manipulations that modulate GDNF content in the brain affected cocaine-induced conditioned place preference and cocaine or ethanol self-administration in rats (27–30). GDNF (+/−) heterozygous knockout mice [GDNF (+/−) mice] showed greater morphine, cocaine, and methamphetamine (METH) conditioned place preference (27, 31). However, the role of GDNF in vulnerability to relapse of drug-seeking behavior remains unclear. Using animal models of drug self-administration and relapsing behavior recently established in our laboratory (32, 33), which represent drug-taking and relapse of drug-seeking behavior in

addicts (34), we provided evidence that a partial loss of GDNF expression not only facilitated the acquisition of METH self-administration, resulted in an upward shift in the dose-response curve, and increased motivation to take METH, but also led to increased vulnerability to METH-primed reinstatement and enduring cue-induced reinstatement of extinguished drug-seeking behavior.

MATERIALS AND METHODS

Subjects and drugs

The generation of GDNF knockout mice was described elsewhere (35). GDNF (−/−) homozygous knockout mice die shortly after birth, but GDNF (+/−) mice are viable. After genomic DNA was purified from a 0.5–1.0 cm segment of tail, the mice were genotyped by PCR utilizing three sets of primers selective of the neomycin cassette: primer 1 (5′-GAC TGG CTT GGT TCT TTG CAT GCA TCC −3′); primer 2 (5′-ACC AAA GAA CGG AGC CGG TTG GCG C-3′), and primer 3 (5′-GAG AGG AAT CGG CAG GCT GCA GCT G-3′). To characterize the influence of the GDNF expression on the operant behavior, a colony of GDNF (+/−) mice was employed in the present study. In this colony, the levels of GDNF expression in corticolimbic areas of the brain are reduced to 54–66% of those in wild-type littermates, at the age of 8 wk (Supplemental Fig. 1). Wild-type littermates were used as a control of the GDNF (+/−) mice. GDNF (+/−) and wild-type mice were bred locally in the Laboratory Animal Center, Nagoya University Graduate School of Medicine in Japan. Male GDNF (+/−) and wild-type mice were 8-wk-old and weighed 25–30 g at the beginning of the experiments. All mice were kept in a regulated environment (23 ± 0.5°C; 50 ± 0.5% humidity) with a reversed 12-h light/dark cycle (lights on at 9:00 AM). Both water and food were available ad libitum throughout the experiments unless otherwise noted. All procedures followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Nagoya University School of Medicine Animal Care and Use Committee.

METH hydrochloride (Dainippon Pharmaceutical Ltd, Osaka, Japan) was dissolved in sterile saline and self-administered at a dose of 0.1 mg/kg/infusion over 5 s (infusion volume=2.1 μl). The unit dose for METH self-administration is based on our previous report (32).

Food-reinforced operant behavior and reinstatement of food-seeking behavior

Food-reinforced operant behavior and motivation

Food-reinforced operant behavior and motivation were tested in standard mouse operant conditioning chambers as described previously (32). Briefly, the chamber was equipped with two nose-poke sensors (ENV-313M, Med Associates) in two holes, two cue-lamps in and above each hole, and a food pellet dispenser (ENV-203-20, Med Associates, Georgia, VT, USA) connected to a rectangular opening (2.25 cm × 2.25 cm) between the two holes. The bottom of the opening was 5 mm above the chamber floor and was equidistant from the holes. A house light was located at the top of the chamber opposite the holes. During the tests for food-reinforced operant behavior and motivation, one hole was defined as active, and the other, as inactive. Nose-poke responses in the active hole resulted in the delivery of a single food pellet

(dustless precision pellets 20 mg, A Holton Industries Co., Frenchtown, NJ, USA) to the opening by the dispenser (ENV302M, Med Associates) and inactivation of the cue-lamp and hole-lamp for 5 s followed by a 5 s timeout period. Nose-poke responses in the active hole during the timeout period and in the inactive hole had no programmed consequences but were recorded by the software MED-PC for Windows (Med Associates).

Naive GDNF (+/−) and wild-type mice (n=7 for each genotype) were deprived of food for 20 h (water remained available ad libitum throughout the experiments). From the next day, both genotypes were daily subjected to nose-poke responding for food pellets in the standard operant chambers as mentioned above. During this phase, the mice were returned to their home cages and given unlimited amounts of food for 2 h immediately after each session of nose-poke responding for food pellets. The daily 3 h sessions of food-reinforced nose-poke responding in GDNF (+/−) and wild-type mice were initially performed under a fixed ratio (FR) 1 schedule. Once the mice showed stable nose-poke responding for food pellets (deviations of <15% of the mean of active responses in 3 consecutive training sessions), the reinforcement schedule was changed to FR2 until the same criterion as above was achieved. The same groups of mice were then subjected to nose-poke responding for food pellets under a progressive ratio (PR) schedule, in which the number of active nose-poke responses required to obtain a single food pellet escalates according to the following series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145,178, 219, 268, 328, 402, 492, 603, 737, etc. (36). This "breaking point," expressed as the final ratio (the number of active nose-poke responses needed to earn the last single food pellet), reflects the intensity of motivation for earning food pellets. Each session under the PR schedule lasted for 5 h or until mice failed to respond within 1 h. After 2–4 daily sessions, GDNF (+/−) and wild-type mice demonstrated stable active nose-poke responses for food pellets (deviations of <15% of the mean of total active responses in 2 consecutive sessions).

Extinction and reinstatement of food-seeking behavior

During this phase, both food and water were available ad libitum in the home cages. After the test for motivation to take food pellets under the PR schedule, the same groups of GDNF (+/−) and wild-type mice were then subjected to 6–10 daily 3 h sessions of extinction. Throughout the extinction session, the house light was on. The food-associated cue- and hole-lamps, and the system that delivers food pellets were turned off. Therefore, the nose-poke responses into the previously active hole resulted in neither the delivery of food pellets nor food-associated cues (cue- and hole-lamps). Once the mice met the criterion of extinction (<15 active responses or 25% of active responses in the stable phase of self-administration in 2 consecutive sessions), they were subjected to a 3 h session of the food-priming reinstatement test under the same conditions as in the extinction sessions (without either food-associated cues or the delivery of food pellets). As priming of food pellets, 12 food pellets were placed into the rectangular opening between the 2 holes before the food-priming reinstatement test. Nose-poke responses in the previously active or inactive hole were counted as active and inactive, respectively.

After the food-primed reinstatement test, the same groups of mice were subjected to 3–6 daily 3 h sessions of extinction immediately, and 3 months, after withdrawal from responding for food pellets. Once the mice met the extinction criterion as mentioned above, they were subjected to daily 3 h sessions of food-associated cue-induced reinstatement tests immediately, and 3 months, after the withdrawal. The food-

associated cue-induced reinstatement tests were performed under the same conditions as the food-reinforced operant behavioral test under the FR2 schedule, except that there was no delivery of food pellets after the nose-poke responses in a previously active hole. Nose-poke responses in the previously active or inactive hole were counted as active and inactive, respectively.

Surgery and apparatus for METH self-administration

Catheter implantation

New groups of naive GDNF (+/−) and wild-type mice were deprived of food for 20 h (water remained available *ad libitum*) and then trained to make nose-poke responses under the FR 1 schedule for food pellets in the operant chambers as mentioned above, except that both nose-poke holes were defined as active. Once a mouse had earned 30 food pellets, the session for nose-poke training ended (for 2–8 h, no difference between GDNF (+/−) and wild-type mice). After the training session, the mice were returned to their home cages, where both food and water were available *ad libitum* throughout the subsequent experiments. Two days later, the mice were anesthetized with pentobarbital sodium (50 mg/kg ip). Indwelling catheters were constructed of microsilicone tubing (inner diameter, 0.50 mm; outer diameter, 0.7 mm; IMG, Imamura Co., Ltd., Tokyo, Japan) and polyethylene tubing (inner diameter, 0.50 mm; outer diameter, 0.8 mm). Incisions were made on the skin of the head and ventral neck, and the right jugular vein was externalized. The end of the catheter was inserted into the jugular vein *via* a small incision and was secured to the vein and surrounding tissue with silk sutures. The exit port of the catheter passed subcutaneously to the top of the skull where it was attached to a modified 24-gauge cannula, which was secured to the mouse's skull with quick self-curing acrylic resin (Shofu Inc., Tokyo, Japan). To extend catheter patency, the catheters were flushed immediately after surgery, and in the morning and evening of the following days, with 0.03 ml of an antibiotic solution of cefmetazole sodium (20.0 mg/ml; Sankyo Co., Ltd., Tokyo, Japan) dissolved in heparinized saline (70 U/ml; Leo Pharmaceutical Products, Ltd., Tokyo, Japan). The patency of the catheter was usually confirmed once a week before operant behavior tests by infusion of a pentobarbital sodium solution

(6.0 mg/ml, 0.15 ml/mouse) into the jugular vein. If the mice could not be knocked down within 5 s, the corresponding data were excluded from the statistical analysis.

Apparatus for METH self-administration

METH self-administration was conducted in standard mouse operant conditioning chambers (ENV-307A, Med Associates) located within ventilated sound attenuation cubicles as described previously (32). Briefly, the chambers were equipped with nose-poke sensors (ENV-313M, Med Associates) in two holes located on one side of the chamber 1.0 cm above the floor, cue- and hole-lamps located, respectively, above and in each hole, and a red house light located on the top of the chamber opposite the holes. During the self-administration, one hole was defined as active, and the other, as inactive. Nose-poke responses in the active hole resulted in activation of the infusion pump (PHM-100, Med Associates) and inactivation of the cue-lamp and hole-lamp. Nose-poke responses in the inactive hole, and in the active hole during the timeout period, had no programmed consequences but were recorded. The components of the infusion line were connected to each other from the injector to the exit port of the mouse's catheter by joint FEP tubing (inner diameter=0.25 mm; outer diameter=0.55 mm; Eicom Co., Ltd., Japan), which was encased in steel spring leashes (Instech, Plymouth Meeting, PA). Swivels were suspended above the chamber. One pump/syringe set was used for each chamber located inside of the cubicle. The infusion pump/syringe set was outside of the chambers but inside of the cubicles.

METH self-administration and reinstatement of METH-seeking behavior

Outline

After recovering from the surgery to implant the catheter, GDNF (+/−) (*n*=28) and wild-type mice (*n*=26) were subjected to METH self-administration, extinction, and reinstatement of extinguished METH-seeking behavior according to the workflow shown in **Table 1**. During METH self-administration, nose-poke responses in the active hole resulted in an infusion of METH at a dose of 0.1 mg/kg/

TABLE 1. *Workflow for METH self-administration and reinstatement*

Step	Experiment	Number (n)	
		Wild-type	GDNF (+/−)
1	Training for self-administration	26 (−14)*	28 (−10)*
2.1	Dose-response	6	9 (−2)*
2.2	Motivation	6	9 (−2)*
3	Re-training for self-administration	12	14
4	Extinction	7	8
5	METH-primed reinstatement	7	8
6	Extinction	7	8
7	1st cue-induced reinstatement	7	8
8	Withdrawal until 3 months after the re-training	7	8
9	Extinction	7	8
10	2nd cue-induced reinstatement	7	8
11	Withdrawal until 6 months after the re-training	7	8
12	Extinction	7	8
13	3rd cue-induced reinstatement	7	8

*The number in the brackets indicates the number of mice excluded from the statistical analysis because of a failure of catheter patency during the METH self-administration, or death from infection before completion of the 3rd cue-induced reinstatement.

infusion over 5 s (infusion volume=2.1 μl) followed by a 5 s timeout period. Nose-poke responses in the inactive hole, and in the active hole during the timeout period, had no programmed consequences but were recorded.

Acquisition of METH self-administration under an FR schedule

METH self-administration was initially under the FR1 schedule. Once the mice could make a minimum of 60% nose-poke responses in the active hole and received no >10 infusions of METH <2 consecutive sessions (at least for 4 sessions), the METH reinforcement schedule was changed to FR2. Under the FR2 schedule, the mice gradually acquired stable METH self-administration behavior (deviations of <15% of the mean of active responses in 3 consecutive training sessions). After acquiring stable self-administration behavior, GDNF (+/−) and wild-type mice were each counterbalance-separated into two subgroups. One subgroup of GDNF (+/−) and wild-type mice were subjected to the test for dose responses. The others were subjected to METH self-administration under the PR schedule.

Dose responses for METH self-administration under an FR2 schedule

After acquiring stable self-administration behavior, one subgroup of GDNF (+/−) and wild-type mice were subjected to METH self-administration under the FR2 schedule of reinforcement in the dose range 0.003–0.1 mg/kg/infusion from the higher to lower dose. Each mouse was subjected to two to four daily 3 h sessions of METH self-administration at one dose until it demonstrated stable active nose-poke responses (deviations of <15% of the mean of total active responses in 2 consecutive sessions).

Motivation for METH self-administration under a PR schedule

After stable self-administration behavior was acquired, the other subgroup of GDNF (+/−) and wild-type mice were subjected to METH self-administration under the PR schedule. The "breaking point" is defined as the final ratio (the number of active nose-poke responses needed to earn the last infusion of METH) and reflects the intensity of motivation for taking the drug tested. Each session lasted for 5 h or until mice failed to respond within 1 h. Each mouse was subjected to two to five sessions of METH self-administration. Both genotypes of mice demonstrated stable active nose-poke responses for METH infusion (as described in the section of dose response) during the two to five sessions.

Extinction

After the self-administration under the FR or PR schedule, the two subgroups of GDNF (+/−) and wild-type mice were subjected to METH (0.1 mg/kg/infusion) self-administration under the FR2 schedule until both genotypes showed stable (as described above) active nose-poke responses once again, and took approximately the same amount of METH. The mice were then subjected to 6–10 daily 3 h sessions of extinction before the METH-primed reinstatement test or 3–6 daily 3 h sessions of extinction before the cue-induced reinstatement test until they met the extinction criterion (<15 active responses or 25% of active responses in the stable phase of self-administration in 2 consecutive sessions). Throughout the extinction session, the house light was on. The METH-associated cue- and hole-lamps, and the pump for METH infusion, were turned off. Therefore, nose-poke responses into the previously active hole resulted in neither an

infusion of METH nor METH-associated cues (cue- and hole-lamps, and pump noise for METH infusion).

METH-primed reinstatement

Once the extinction criterion was met, the GDNF (+/−) and wild-type mice were firstly subjected to a 3 h session of the operant test 30 min after the injection (ip) of saline as a control for the METH-primed reinstatement. From the next day, the mice were consecutively subjected to METH-primed reinstatement tests 30 min after the intraperitoneal injection with increasing doses of METH (0.2, 0.4, 1.0, 1.5, or 3.0 mg/kg, each dose for 1 daily 3 h session). The METH-primed reinstatement tests were conducted under the same conditions as in the extinction sessions in which neither METH infusions nor METH-associated cues were available after nose-poke responses into a previously active hole. Nose-poke responses in the previously active or inactive hole were counted as active and inactive, respectively.

Cue-induced reinstatement

Once the extinction criterion was met, the same groups of mice were subjected to the cue-induced reinstatement tests immediately, 3 months, and 6 months after withdrawal from METH self-administration. The cue-induced reinstatement tests were conducted under the same conditions as the METH self-administration under the FR2 schedule, except that METH was unavailable throughout the testing session. Nose-poke responses in the previously active or inactive hole were counted as active and inactive, respectively.

Data analysis

All data are ± SE. A one- or two-way ANOVA with (or without) repeated measures was performed for the difference in locomotor activity and nose-poke responses between the two genotypes of mice during the self-administration training, dose-response function, and METH-primed and cue-induced reinstatement of drug-seeking behavior, followed post hoc by the Bonferroni/Dunn test. The Mann-Whitney test was used to analyze the breaking points under the PR schedule, whereas Student's t test was used to analyze the other two sets of data. In all cases, a significant difference was set at $P < 0.05$.

RESULTS

Food-reinforced operant behavior and motivation of GDNF (+/−) and wild-type mice

Naive GDNF (+/−) and wild-type mice were trained to make nose-poke responses for food reinforcement under the FR and PR schedules in daily 3 h sessions. GDNF (+/−) mice did not show any significant difference from wild-type littermates in either active or inactive responses under the FR schedule of food reinforcement (Fig. 1A). Also, there was no significant difference in the number of training sessions to acquire stable operant behavior between GDNF (+/−) and wild-type mice (Fig. 1B). Furthermore, GDNF (+/−) and wild-type mice showed similar breaking points under the PR schedule (Fig. 1C). These findings indicate that a partial loss of GDNF expression does not

A

B

C
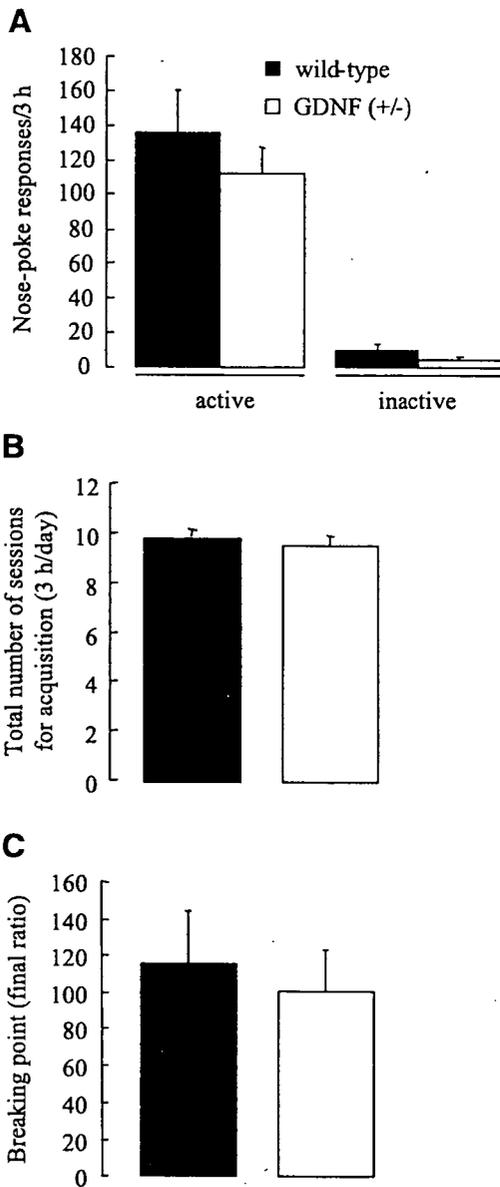
Figure 1. Food-reinforced operant behavior and motivation in GDNF (+/−) and wild-type mice. A) Active and inactive nose-poke responses for food reinforcement in a 3 h session under FR2 schedule during stable phase. B) Total number of training sessions needed to acquire stable active nose-poke responses for food reinforcement under FR schedule. C) Breaking points (final ratio) for food reinforcement under PR schedule. Data are mean ± SE. n = 7 for each genotype.

affect food-reinforced operant behavior and motivation in mice.

Reinstatement of food-seeking behavior in GDNF (+/−) and wild-type mice

To evaluate the reinstatement of food-seeking behavior in the mutant animals, the same groups of GDNF (+/−) and wild-type mice were subjected to extinction training after the tests for the food-reinforced operant behavior. After similar daily 3 h sessions of extinction

training, GDNF (+/−) and wild-type mice achieved the extinction criterion (**Fig. 2A**; $F_{(3,48)}=27.83$; $P<0.001$). However, the priming of food pellets failed to reinstate food-seeking behavior in either genotype (Fig. 2A). The same groups of mice were then subjected to extinction training once again. Once the extinction criterion was met, the food-associated cue-induced reinstatement tests were conducted immediately and 3 months after the end of tests for the food-reinforced operant behavior. Food-associated cues reliably trig-

A

B
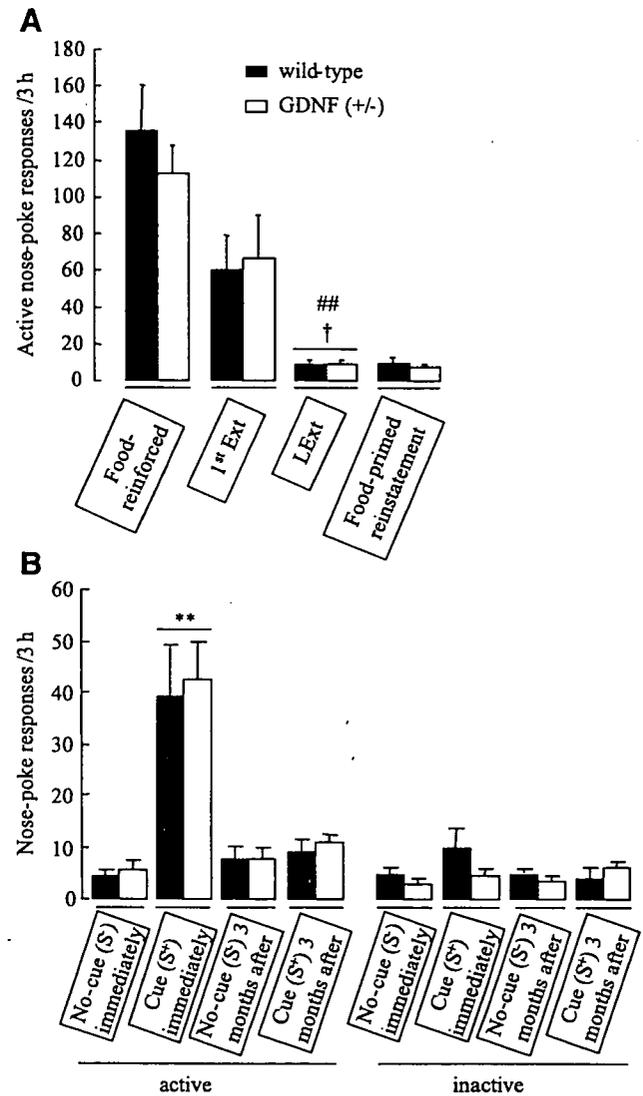
Figure 2. Extinction and reinstatement of food-seeking behavior in GDNF(+/−) and wild-type mice. A) Active nose-poke responses during stable phase of food-reinforced operant responding, extinction training, and food-primed reinstatement. B) Nose-poke responses in food-associated cue-induced reinstatement tests immediately, and 3 months, after withdrawal from food-reinforced operant behavior; Data are mean ± SE. n = 7 for each genotype. ##$P < 0.01$ vs. Food-reinforced in same genotype; †$P < 0.05$ vs. 1st Ext in same genotype; **$P < 0.01$ vs. No-cue (S−) in same genotype. Food-reinforced, stable food-reinforced operant behavior; 1st Ext, first session of extinction; LExt, last session of extinction; No-cue (S−), control for cue-induced reinstatement (without food-associated cues and food pellets); Cue (S+), food-associated cue-induced reinstatement.

gered reinstatement of food-seeking behavior in both GDNF (+/−) and wild-type mice immediately after withdrawal (Fig. 2B; $F_{(1,24)}=33.44$; $P<0.001$). However, no significant difference in cue-induced reinstatement behavior was observed between GDNF (+/−) and wild-type mice (Fig. 2B). Importantly, the food cue-induced reinstatement of food-seeking behavior disappeared within the period of a 3 month withdrawal in both genotypes of animals (Fig. 2B). These findings suggest that reinstatement of food-seeking behavior in both genotypes of animals is weak or transient and that the partial loss of GDNF expression does not affect extinction behavior, reinstatement of food-seeking behavior, or duration of food-associated cue-induced reinstatement behavior in mice.

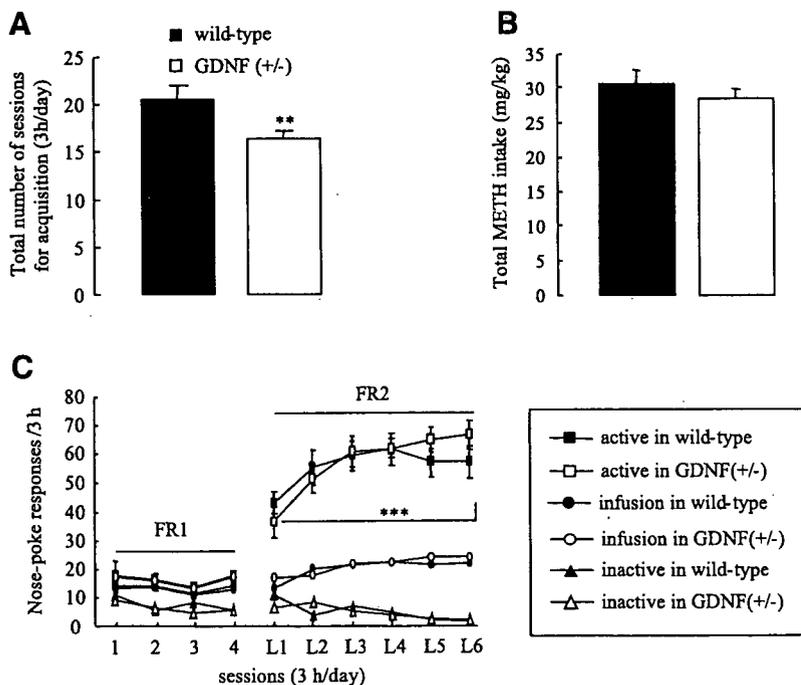## Facilitated acquisition of METH self-administration behavior in GDNF (+/−) mice

To investigate whether a partial loss of GDNF expression affects drug self-administration behavior in animals, separate groups of GDNF (+/−) and wild-type mice were subjected to METH self-administration training. GDNF (+/−) mice took less time than wild-type littermates to acquire stable METH self-administration behavior (Fig. 3A, $P<0.01$). However, there was no significant difference in total METH intake during the period of METH self-administration training between wild-type (30.7±2.0 mg/kg) and GDNF (+/−) (28.5±1.5 mg/kg) mice (Fig. 3B). In the early phase of METH self-administration under the FR1 schedule (Fig. 3C, day 1–4), neither genotype could discriminate active (METH-associated) from inactive (without METH infusion) nose-poke responses. Accordingly, there was no significant difference in active nose-poke responses for METH self-administration between

GDNF (+/−) and wild-type mice. The mice gradually demonstrated stable METH self-administration behavior under the FR2 schedule. Accordingly, they could discriminate active from inactive nose-poke responses to METH reinforcement (Fig. 3C, the last day 1–6 (L1-L6), $P<0.001$). However, there was no significant difference in active nose-poke responses for METH-taking between wild-type and GDNF (+/−) mice. These findings indicate that GDNF (+/−) mice are capable of METH self-administration.

Upward shifted dose responses and increased motivation to take METH in GDNF (+/−) mice

GDNF (+/−) and wild-type mice showed significantly different dose responses to self-administer METH (Fig. 4A; $F_{(1,55)}=12.43$, $P<0.001$). In the dose range of 0.01–0.03 mg/kg/infusion, the number of active nose-poke responses for METH-taking was significantly higher in GDNF (+/−) mice than in wild-type littermates ($P<0.05$ and 0.001). There was no significant difference in active nose-poke responses to self-administer METH at 0.1 and 0.003 mg/kg/infusion between GDNF (+/−) and wild-type mice. When saline was substituted for METH, no significant difference was observed in self-administration behavior between GDNF (+/−) and wild-type mice. The upward shift of the dose-response function for METH self-administration in GDNF (+/−) mice suggests that the partial loss of GDNF expression may increase sensitivity to METH self-administration in mutant animals during the maintenance phase.

To further support this idea, the other subgroups of GDNF (+/−) and wild-type mice were subjected to METH (0.1 mg/kg/infusion) self-administration under the PR schedule. GDNF (+/−) mice demonstrated a

A

wild-type
GDNF (+/-)

Total number of sessions for acquisition (3h/day)

B

Total METH intake (mg/kg)

C

Nose-poke responses/3 h

FR2

FR1

1 2 3 4 L1 L2 L3 L4 L5 L6

sessions (3 h/day)

active in wild-type
active in GDNF(+/-)
infusion in wild-type
infusion in GDNF(+/-)
inactive in wild-type
inactive in GDNF(+/-)

Figure 3. Acquisition of stable METH (0.1 mg/kg/infusion) self-administration behavior in GDNF (+/−) and wild-type mice. A) Total number of training sessions needed to acquire stable METH self-administration behavior for GDNF (+/−) and wild-type mice. $**P < 0.01$ vs. wild-type littermates. B) Total METH intake during period of METH self-administration training for GDNF (+/−) and wild-type mice. C) Nose-poke responses and number of METH infusions during first 4 sessions (session 1–4) and last 6 sessions (session L1-L6) of METH self-administration under FR1 and FR2 schedules of reinforcement for GDNF (+/−) and wild-type mice. $***P < 0.001$ vs. inactive nose-poke responses in same genotype; Data are mean ± SE. $n = 12$–18 for each genotype.
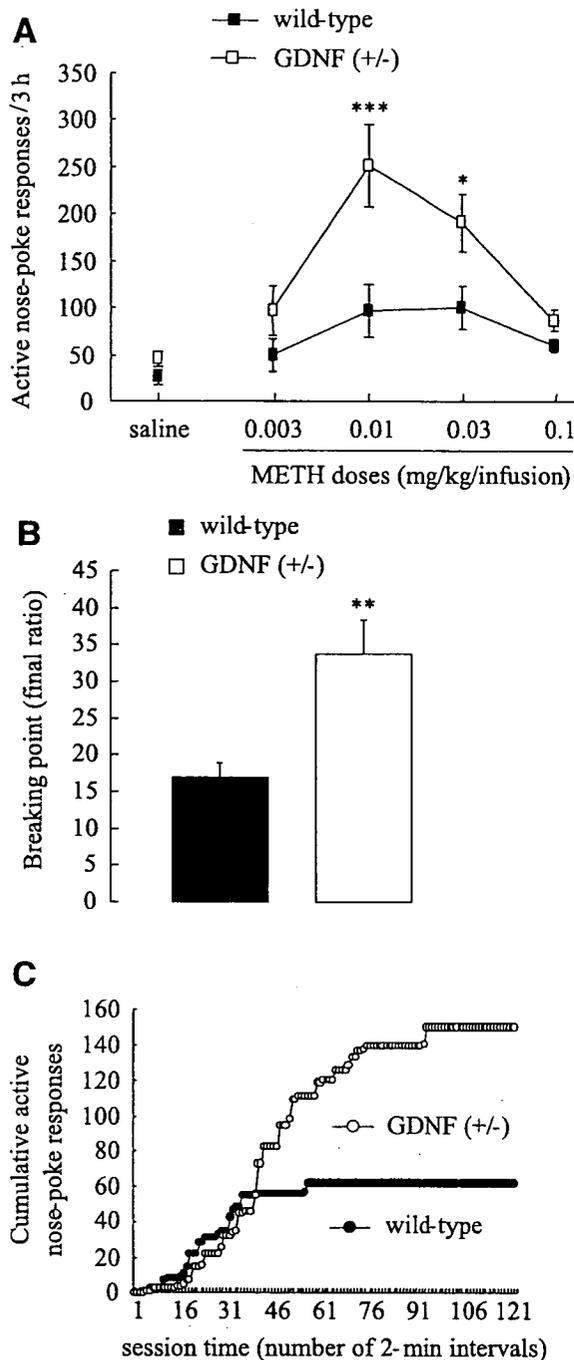
Figure 4. Dose responses and motivation for METH self-administration in GDNF (+/−) and wild-type mice. *A)* Dose-response function for METH self-administration under an FR2 schedule of reinforcement. *B)* Breaking points (final ratio) under a PR schedule of reinforcement. *C)* Representative curves for cumulative active nose-poke responses for METH-taking under PR schedule in mutant animals. Data are mean ± SE. $n = 6$–9 for each genotype. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. wild-type littermates.

significantly increased breaking point compared with wild-type littermates (Fig. 4*B*; $P<0.01$), suggesting that the partial loss of GDNF expression leads to greater motivation to take METH. Representative curves for the two genotypes of animals are illustrated in Fig. 4*C*.

Increased vulnerability to METH-primed reinstatement of drug-seeking behavior in GDNF (+/−) mice

We further investigated the performance of GDNF (+/−) mice in the reinstatement of extinguished METH-seeking behavior. After once again acquiring stable METH self-administration behavior, during which wild-type and GDNF (+/−) mice had taken similar amounts of METH (43.9±3.2 and 50.1±4.1 mg/kg, respectively), the two genotypes were exposed to extinction training for 6–10 daily 3 h sessions. There was no significant difference between GDNF (+/−) and wild-type mice in the number of nose-poke responses into previously active holes [METH-associated; **Fig. 5A**, session 1–3 and the last session 1–3 (L1-L3)] or the number of extinction training sessions to achieve the extinction criterion (data not shown). During the last two extinction sessions, neither group of animals could discriminate active (previously associated with METH self-administration) from inactive (previously without METH self-administration) nose-poke responses, similar to the early stage of METH self-administration. These findings suggested that in GDNF (+/−) and wild-type mice, purposely active nose-poke responses acquired during METH self-administration had been extinguished.

Once the mice met the extinction criterion (Fig. 5A), a drug-primed reinstatement test was carried out 30 min after treatment with either saline or a different dose of METH. Wild-type and GDNF (+/−) mice showed different active nose-poke responses (Fig. 5B; $F_{(1, 77)}=12.72$; $P<0.001$), although there was no significant difference in inactive nose-poke responses between GDNF (+/−) and wild-type mice. In wild-type littermates, both lower (0.2 and 0.4 mg/kg) and higher (1.5 and 3.0 mg/kg) doses of METH-priming failed to reinstate drug-seeking behavior. However, a moderate dose of METH (1.0 mg/kg) reliably triggered the reinstatement behavior. In contrast, both lower and moderate doses of METH (0.4 mg/kg and 1.0 mg/kg) reliably triggered the reinstatement of extinguished drug-seeking behavior in GDNF (+/−) mice ($P<0.05$), although higher doses of METH did not evoke the reinstatement behavior. The leftward shift of the dose-response curve for METH-primed reinstatement behavior suggests that the partial loss of GDNF expression may affect vulnerability to the reinstatement of extinguished METH-seeking behavior in mice.

Prolonged persistence of cue-induced reinstatement of drug-seeking behavior in GDNF (+/−) mice

To investigate the enduring vulnerability to cue-induced reinstatement of METH-seeking behavior in GDNF (+/−) mice, the same groups of mice were subjected to three to six extinction sessions, followed by three cue-induced reinstatement tests, which were conducted immediately, 3 months, and 6 months after METH withdrawal. During the extinction sessions, neither genotype showed any significant difference in

A

B
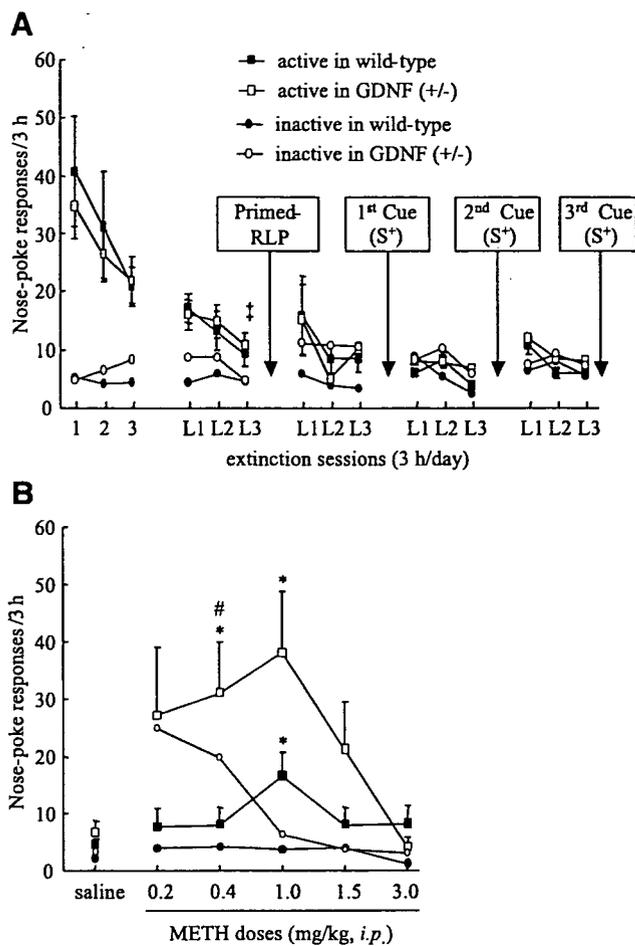
Figure 5. Nose-poke responses during extinction training (A) and METH-primed reinstatement of drug-seeking behavior (B; Primed-RLP) in GDNF (+/−) and wild-type mice. In A, data are from first 3 daily 3 h sessions (indicated by 1–3) and last 3 daily 3 h sessions (indicated by L1-L3) during 6–10 extinction training sessions before METH-primed reinstatement test (Primed-RLP), and last 3 daily 3 h sessions (indicated by L1-L3) during 3–6 sessions of extinction training before cue-induced reinstatement tests (1st –3rd Cue (S^+). Data are mean ± SE. n = 7–8 for each genotype. $^{\ddagger}P < 0.01$ vs. first session of extinction in same genotype. $^*P < 0.05$ vs. saline treatment in same genotype. $^{\#}P < 0.05$ vs. wild-type littermates during same reinstatement test. Primed-RLP, METH-primed reinstatement; 1st Cue (S^+), first test for METH-associated cue-induced reinstatement immediately after withdrawal; 2nd Cue (S^+), second test for METH-associated cue-induced reinstatement 3 months after withdrawal; 3rd Cue (S^+), third test for METH-associated cue-induced reinstatement 6 months after withdrawal.

nose-poke responses or in the number of training sessions needed to achieve the extinction criterion (Fig. 5A). Once the extinction criterion was achieved, the mice were subjected to cue-induced reinstatement tests. GDNF (+/−) and wild-type mice initially demonstrated a cue-triggered reinstatement of METH-seeking behavior (Fig. 6; 1st test; $P<0.001$). Importantly, there was a clear tendency for GDNF (+/−) mice to show more active nose-poke responses than wild-type littermates when exposed to the METH-associated cues (Fig. 6; $F_{(1, 26)}=3.99$; $P=0.056$). With a prolonged with-

drawal, GDNF (+/−) and wild-type mice showed significantly different responses (Fig. 6, $F_{(1, 150)}=26.1$; $P<0.001$). In wild-type littermates, the cue-induced reinstatement behavior was still observed 3 months after the withdrawal (Fig. 6; 2nd test; $P<0.05$) but disappeared after a 6 month withdrawal (3rd test). In contrast, GDNF (+/−) mice maintained the cue-induced reinstatement behavior even after a 6-month withdrawal (Fig. 6; 3rd test; $P<0.01$). In addition, there was no significant difference in inactive nose-poke responses during any of the tests for cue-induced reinstatement behavior between GDNF (+/−) and wild-type mice.

DISCUSSION

With the use of mouse models of METH self-administration and reinstatement of METH-seeking behavior, the present study demonstrated that a partial loss of GDNF expression resulted in a facilitated acquisition of METH self-administration behavior, upward shifted dose responses and enhanced motivation to take METH, increased vulnerability to drug-primed reinstatement, and prolonged cue-induced reinstatement of extinguished METH-seeking behavior. In contrast, there was no significant difference in food-reinforced operant behavior and motivation, locomotor activity, or novelty responses between the two genotypes of animals. These findings may provide evidence that GDNF is associated with vulnerability to relapse of METH-seeking behavior.

Acquisition and maintenance of METH self-administration behavior

It has been documented that GDNF (+/−) mice demonstrate increased morphine, cocaine, and METH-conditioned place preference (27, 31). In the present study, GDNF (+/−) mice took a shorter period of time to acquire stable METH self-administration behavior, with an upward shift of dose responses to METH-taking, compared with wild-type littermates. Furthermore, GDNF (+/−) mice showed greater motivation (breaking point) for METH self-administration. In contrast, there was no significant difference in food-reinforced operant behavior (including acquisition and maintenance) and motivation between GDNF (+/−) and wild-type mice. Thus, GDNF (+/−) mice may represent a phenotype susceptible to the rewarding and reinforcing effects of addictive drugs although the precise molecular mechanisms underlying this phenomenon remain unclear. It has been documented that the extracellular dopamine concentration, FosB levels, and deltaFosB expression are elevated in the nucleus accumbens and striatum of GDNF (+/−) mice as compared with wild-type littermates (37). In addition, it is well established that GDNF is an important modulator for dopaminergic neuronal function (e.g., refs. 17, 26). Thus, it seems reasonable to postulate that

Figure 6. Active and inactive nose-poke responses during cue-induced reinstatement of METH-seeking behavior in GDNF (+/−) and wild-type mice. Tests for cue-induced reinstatement were conducted immediately (without withdrawal, 1st test), 3 months (2nd test), and 6 months (3rd test) after withdrawal from METH self-administration. Data are mean ± SE. $n = 7$–8 for each genotype. *$P < 0.05$, **$P < 0.01$ vs. No-cue (S⁻) groups in same reinstatement test of same genotype. #$P < 0.05$ vs. wild-type littermates in same reinstatement test. No-cue (S⁻): control for reinstatement test (without either METH-associated cues or METH infusion). Cue (S⁺): METH-associated cue-induced reinstatement (with METH-associated cues but no METH infusion).
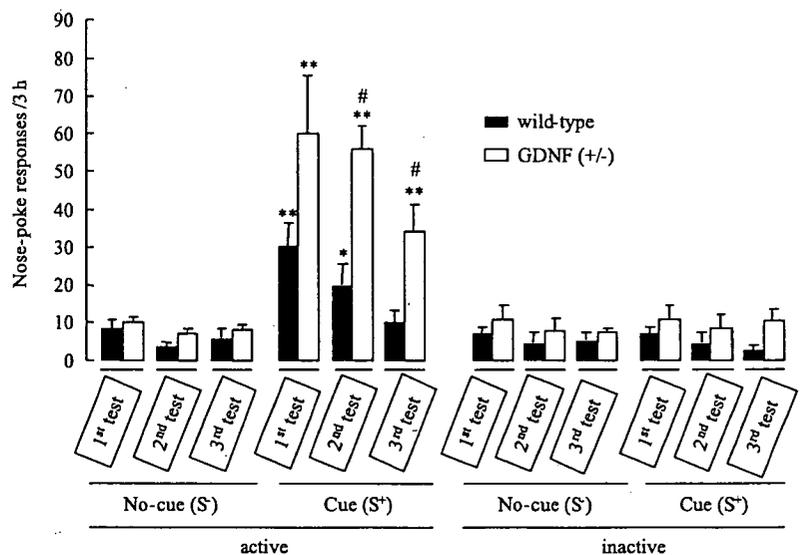
the reduced GDNF content causes an increase in the extracellular dopamine concentration, FosB levels, and deltaFosB expression, leading to greater morphine-, cocaine-, or METH-conditioned place preference (27, 31, 37), facilitated acquisition of METH self-administration behavior, upward shifted dose responses, and enhanced motivation to take METH (in the present study).

It seems unlikely that the differences in acquisition of self-administration behavior, dose responses, and motivation to take METH between GDNF (+/−) and wild-type mice are due to nonspecific physiological adaptations or compensatory effects during the development of mutant animals. Firstly, in our colony of GDNF (+/−) mice, the levels of GDNF expression in cortico-limbic areas of the brain were reduced 34–46% (see Supplemental Fig. 1). This is consistent with previous reports that striatal GDNF contents are reduced in GDNF (+/−) mice (37, 38). Secondly, both cocaine-conditioned place preference and cocaine- or ethanol-reinforced self-administration are reduced by an increased level of GDNF in the animal brain (27, 28, 30). Thirdly, cocaine-conditioned place preference and ethanol self-administration are potentiated by a decrease in the amount of GDNF in the brain through local delivery of anti-GDNF neutralizing antibodies (27, 30). A previous report has demonstrated an impairment of water-maze learning in GDNF (+/−) mice (39). In the present study, there was no significant difference in the acquisition of, retention of, and motivation for food-reinforced operant behavior between GDNF (+/−) and wild-type mice. Thus, it seems difficult to explain the alterations in METH-reinforced self-administration behavior and motivation, based on the learning and memory deficits in GDNF (+/−) mice. It has been suggested that responses to novelty in animals are associated with the propensity for drug self-administration (40). Given that there was no significant difference in exploratory behavior in the open field test, locomotor activities during habituation to the testing box environment, and locomotor responses to METH between GDNF (+/−) and wild-type mice (see

Supplemental Figs. 2, 3), it is unlikely that differences in novelty responses or locomotor activity during METH self-administration contribute to alterations in METH-reinforced self-administration and motivation between GDNF (+/−) and wild-type mice.

Vulnerability to METH-primed reinstatement of drug-seeking behavior in GDNF (+/−) mice

No significant difference was observed in active or inactive nose-poke responses (Fig. 5A), and the number of extinction training sessions needed to achieve the extinction criterion (data not shown) between GDNF (+/−) and wild-type mice during the period of extinction training. This phenomenon indicates that a primary reinforcer (METH) or secondary reinforcer (METH-associated cues) may be necessary for the effects of GDNF on the development of METH-reinforced self-administration behavior. Indeed, GDNF (+/−) mutant mice showed a leftward and upward shifted dose-response curve for reinstatement of extinguished drug-seeking behavior after a priming injection of the primary reinforcer METH, whereas neither genotype showed reinstatement of food-seeking behavior after priming with food pellets. It has been shown that drug-primed reinstatement and drug self-administration share similar anatomical neural substrates (cortico-limbic system) and neural transmission (dopamine) in the brain (22, 41). Thus, possible mechanisms underlying enhanced METH-reinforced self-administration and motivation may contribute to the vulnerability to METH-primed reinstatement behavior in GDNF (+/−) mice. It seems unlikely that the vulnerability to METH-primed reinstatement behavior is due to the different experiences of METH self-administration. First, wild-type littermates took longer to acquire stable METH self-administration behavior than GDNF (+/−) mice (Fig. 5A), whereas there was no significant difference in active nose-poke responses for METH during the early and stable phases of METH self-

administration between GDNF (+/−) and wild-type mice (Fig. 3C). Second, there was no significant difference in total METH intake before the test for METH-primed reinstatement between wild-type and GDNF (+/−) mice (43.9±3.2 and 50.1±4.1 mg/kg, respectively). In addition, there was no significant difference in novelty seeking behavior and in METH-stimulated hyperlocomotion and locomotor sensitization between GDNF (+/−) and wild-type mice (see Supplemental Figs. 2, 3). This phenomenon is consistent with a previous report that the effects of acute and repeated treatment of cocaine on locomotor activity are similar between GDNF (+/−) and wild-type mice (37). For similar reasons to those mentioned above, it is unlikely that vulnerability to METH-primed reinstatement behavior in GDNF (+/−) mice reflects nonspecific increases in motor activity or novelty responses.

Prolonged cue-induced reinstatement of METH-seeking behavior in GDNF (+/−) mice

In the present study, GDNF (+/−) mice demonstrated a stronger and more persistent cue-induced reinstatement of extinguished METH-seeking behavior than did wild-type littermates. Moreover, cue-induced reinstatement behavior in GDNF (+/−) mice could be observed even after a 6 month withdrawal when the cue-induced reinstatement in wild-type littermates had disappeared. In contrast, there was no significant difference in the transient cue-induced reinstatement of food-seeking behavior between GDNF (+/−) and wild-type mice (Fig. 2B). The more severe and persistent cue-induced reinstatement of extinguished METH-seeking behavior in GDNF (+/−) mice suggests that the partial loss of GDNF expression may lead to vulnerability to and persistence of cue-induced reinstatement of drug-seeking behavior, without affecting food-seeking behavior. It has been reported that striatal synaptic plasticity is crucial for the formation of an addictive habit or cue-controlled drug-seeking behavior (42–44) and that deltaFosB, once expressed, persists in the brain for a relatively long period of time in the absence of further drug exposure and acts as a sustained molecular switch for addiction (45–47). Thus, the enduring vulnerability to cue-induced reinstatement may be attributable to higher levels of deltaFosB in striatal brain areas of GDNF (+/−) mice (37). In addition, the occurrence of drug-seeking behavior after a delay of several weeks in rats (48) seems inconsistent with our finding that cue-induced reinstatement of drug-seeking behavior was reduced with the time of withdrawal from METH self-administration in GDNF (+/−) and wild-type mice. This discrepancy may be because the cue-induced reinstatement behavior in the present study was examined after repeated cycles of extinction training (a with-subjects design), since repeated extinction training decreases the propensity for a relapse of extinguished drug-seeking behavior (49).

The present series of experiments demonstrated an association between specific genes or proteins, for example, the expression of the GDNF gene, and vulnerability to relapse of drug-seeking behavior, suggesting that GDNF may be critically involved in the acquisition and maintenance of METH self-administration, vulnerability to METH-primed reinstatement, and persistent cue-induced reinstatement of extinguished drug-seeking behavior. In line with previous reports (27–30) and our present findings, GDNF may be a potential target of therapeutic agents not only for the prevention of drug dependence but also for the control of relapse of drug-seeking behavior. □FJ

REFERENCES

1. Tsuang, M. T., Lyons, M. J., Doyle, T., Eisen, S. A., Goldberg, J., True, W., Lin, N., Toomey, R., and Eaves, L. (1998) Co-occurrence of abuse of different drugs in men: the role of drug-specific and shared vulnerabilities. Arch. Gen. Psychiatry 55, 967–972

2. Prescott, C. A., and Kendler, K. S. (1999) Genetic and environment contributions to alcohol abuse and dependence in a populated-based sample of male twins. Am. J. Psychiatry 156, 34–40

3. Nestler, E. J. (2000) Genes and addiction. Nat. Genet. 26, 277–281

4. Kendler, K. S., Jacobson, K. C., Prescott, C. A., and Neale, M. C. (2003) Specificity of genetic and environmental risk factors for use and abuse/dependence of cannabis, cocaine, hallucinogens, sedatives, stimulants, and opiates in male twins. Am. J. Psychiatry 160, 687–695

5. Uhl, G. R., and Grow, R. W. (2004) The burden of complex genetics in brain disorders. Arch. Gen. Psychiatry 61, 223–229

6. Volkow, N. D., and Li, T. K. (2004) Drug addiction: the neurobiology of behaviour gone awry. Nat. Rev. Neurosci. 5, 963–970

7. Kreek, M. J., Nielsen, D. A., Butelman, E. R., LaForge, K. S. (2005) Genetic influences on impulsivity, risk taking, stress responsivity and vulnerability to drug abuse and addiction. Nat. Neurosci. 8, 1450–1457

8. Le, D. A., Li, Z., Funk, D., Shram, M., Li, T. K., and Shaham, Y. (2006) Increased vulnerability to nicotine self-administration and relapse in alcohol-naive offspring of rats selectively bred for high alcohol intake. J. Neurosci. 26, 1872–1879

9. Picciotto, M. R., Zoli, M., Rimondini, R., Lena, C., Marubio, L. M., Pich, E. M., Fuxe, K., and Changeux, J. P. (1998) Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature 391, 173–177

10. Rocha, B. A., Scearce-Levie, K., Lucas, J. J., Hiroi, N., Castanon, N., Crabbe, J. C., Nestler, E. J., and Hen, R. (1998) Increased

vulnerability to cocaine in mice lacking the serotonin-1B receptor. *Nature* **393**, 175–178

11. Chiamulera, C., Epping-Jordan, M. P., Zocchi, A., Marcon, C., Cottiny, C., Tacconi, S., Corsi, M., Orzi, F., and Conquet, F. (2001) Reinforcing and locomotor stimulant effects of cocaine are absent in mGluR5 null mutant mice. *Nat. Neurosci.* **4**, 873–874

12. Caine, S. B., Negus, S. S., Mello, N. K., Patel, S., Bristow, L., Kulagowski, J., Vallone, D., Saiardi, A., and Borrelli, E. (2002) Role of dopamine D2-like receptors in cocaine self-administration: studies with D2 receptor mutant mice and novel D2 receptor antagonists. *J. Neurosci.* **22**, 2977–2988

13. Elmer, G. I., Pieper, J. O., Rubinstein, M., Low, M. J., Grandy, D. K., and Wise, R. A. (2002) Failure of intravenous morphine to serve as an effective instrumental reinforcer in dopamine D2 receptor knock-out mice. *J. Neurosci.* **22**, 1–6

14. Szumlinski, K. K., Dehoff, M. H., Kang, S. H., Frys, K. A., Lominac, K. D., Klugmann, M., Rohrer, J., Griffin, W., III, Toda, S., Champtiaux, N. P., *et al.* (2004) Homer proteins regulate sensitivity to cocaine. *Neuron* **43**, 401–413

15. Hyman, S. E. (2005) Addiction: a disease of learning and memory. *Am. J. Psychiatry* **162**, 1414–1422

16. Kalivas, P. W., and Volkow, N. D. (2005) The neural basis of addiction: a pathology of motivation and choice. *Am. J. Psychiatry* **162**, 1403–1413

17. Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S., and Collins, F. (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130–1132

18. Choi-Lundberg, D. L., Lin, Q., Chang, Y. N., Chiang, Y. L., Hay, C. M., Mohajeri, H., Davidson, B. L., and Bohn, M. C. (1997) Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science* **275**, 838–841

19. Mandel, R. J., Spratt, S. K., Snyder, R. O., and Leff, S. E. (1997) Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 14083–14088

20. Nestler, E. J., and Aghajanian, G. K. (1997) Molecular and cellular basis of addiction. *Science* **278**, 58–63

21. Koob, G. F., Sanna, P. P., and Bloom, F. E. (1998) Neuroscience of addiction. *Neuron* **21**, 467–476

22. Self, D. W. (2004) Regulation of drug-taking and -seeking behaviors by neuroadaptations in the mesolimbic dopamine system. *Neuropharmacology* **47**, 242–255

23. Mizoguchi, H., Yamada, K., Mizuno, M., Mizuno, T., Nitta, A., Noda, Y., and Nabeshima, T. (2004) Regulations of methamphetamine reward by extracellular signal-regulated kinase 1/2/ets-like gene-1 signaling pathway via the activation of dopamine receptors. *Mol. Pharmacol.* **65**, 1293–1301

24. Nagai, T., Yamada, K., Yoshimura, M., Ishikawa, K., Miyamoto, Y., Hashimoto, K., Noda, Y., Nitta, A., and Nabeshima, T. (2004) The tissue plasminogen activator-plasmin system participates in the rewarding effect of morphine by regulating dopamine release. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 3650–3655

25. Stuber, G. D., Wightman, R. M., and Carelli, R. M. (2005) Extinction of cocaine self-administration reveals functionally and temporally distinct dopaminergic signals in the nucleus accumbens. *Neuron* **46**, 661–669

26. Tomac, A., Lindqvist, E., Lin, L. F., Ogren, S. O., Young, D., Hoffer, B. J., and Olson, L. (1995) Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature* **373**, 335–339

27. Messer, C. J., Eisch, A. J., Carlezon, W. A. Jr., Whisler, K., Shen, L., Wolf, D. H., Westphal, H., Collins, F., Russell, D. S., and Nestler, E. J. (2000) Role for GDNF in biochemical and behavioral adaptations to drugs of abuse. *Neuron* **26**, 247–257

28. Green-Sadan, T., Kinor, N., Roth-Deri, I., Geffen-Aricha, R., Schindler, C. J., and Yadid, G. (2003) Transplantation of glial cell line-derived neurotrophic factor-expressing cells into the striatum and nucleus accumbens attenuates acquisition of cocaine self-administration in rats. *Eur. J. Neurosci.* **18**, 2093–2098

29. Green-Sadan, T., Kuttner, Y., Lublin-Tennenbaum, T., Kinor, N., Boguslavsky, Y., Margel, S., and Yadid, G. (2005) Glial cell line-derived neurotrophic factor-conjugated nanoparticles suppress acquisition of cocaine self-administration in rats. *Exp. Neurol.* **194**, 97–105

30. He, D. Y., McGough, M. N., Ravindranathan, A., Jeanblanc, J., Logrip, M. L., Phamluong, K., Janak, P. H., and Ron, D. (2005)

Glial cell line-derived neurotrophic factor mediates the desirable actions of the anti-addiction drug ibogaine against alcohol consumption. *J. Neurosci.* **25**, 619–628

31. Niwa, M., Nitta, A., Yamada, Y., Nakajima, A., Saito, K., Seishima, M., Shen, L., Noda, Y., Furukawa, S., and Nabeshima, T. (In press) An inducer for glial cell line-derived neurotrophic factor and tumor necrosis factor-f_z protects against methamphetamine-induced rewarding effects and sensitization. *Biol. Psychiatry*

32. Yan, Y., Nitta, A., Mizoguchi, H., Yamada, K., and Nabeshima, T. (2006) Relapse of methamphetamine seeking behavior demonstrated by a reinstatement procedure involving self-administration. *Behav. Brain. Res.* **168**, 137–143

33. Yan, Y., Yamada, K., Nitta, A., and Nabeshima, T. (2006) Transient drug-primed but persistent cue-induced reinstatement of extinguished methamphetamine-seeking behavior in mice. *Behav. Brain Res.* **177**, 261–268

34. Epstein, D. H., Preston, K. L., Stewart, J., and Shaham, Y. (2006) Toward a model of drug relapse: an assessment of the validity of the reinstatement procedure. *Psychopharmacology* **189**, 1–16

35. Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., *et al.* (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73–76

36. Roberts, D. C., and Bennett, S. A. (1993) Heroin self-administration in rats under a progressive ratio schedule of reinforcement. *Psychopharmacology* **111**, 215–218

37. Airavaara, M., Planken, A., Gaddnas, H., Piepponen, T. P., Saarma, M., and Ahtee, L. (2004) Increased extracellular dopamine concentrations and FosB/DeltaFosB expression in striatal brain areas of heterozygous GDNF knockout mice. *Eur. J. Neurosci.* **20**, 2336–2344

38. Griffin, W. C., III, Boger, H. A., Granholm, A. C., and Middaugh, L. D. (2006) Partial deletion of glial cell line-derived neurotrophic factor (GDNF) in mice: Effects on sucrose reward and striatal GDNF concentrations. *Brain Res.* **1068**, 257–260

39. Gerlai, R., McNamara, A., Choi-Lundberg, D. L., Armanini, M., Ross, J., Powell-Braxton, L., and Phillips, H. S. (2001) Impaired water maze learning performance without altered dopaminergic function in mice heterozygous for the GDNF mutation. *Eur. J. Neurosci.* **14**, 1153–1163

40. Piazza, P. V., Deminiere, J.-M., Le, Moal. M., and Simon, H. (1989) Factors that predict vulnerability to amphetamine self-administration. *Science* **245**, 1511–1513

41. Zavala, A. R., Weber, S. M., Rice, H. J., Alleweireldt, A. T., and Neisewander, J. L. (2003) Role of the prelimbic subregion of the medial prefrontal cortex in acquisition, extinction, and reinstatement of cocaine-conditioned place preference. *Brain. Res.* **990**, 157–164

42. Gerdeman, G. L., Partridge, J. G., Lupica, C. R., and Lovinger, D. M. (2003) It could be habit forming: drugs of abuse and striatal synaptic plasticity. *Trends Neurosci.* **26**, 184–192

43. Everitt, B. J., and Robbins, T. W. (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nat. Neurosci.* **8**, 1481–1489

44. Vanderschuren, L. J., Di Ciano, P., and Everitt, B. J. (2005) Involvement of the dorsal striatum in cue-controlled cocaine seeking. *J. Neurosci.* **25**, 8665–8670

45. Nestler, E. J., Barrot, M., and Self, D. W. (2001) DeltaFosB: a sustained molecular switch for addiction. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 11042–11046

46. Colby, C. R., Whisler, K., Steffen, C., Nestler, E. J., and Self, D. W. (2003) Striatal cell type-specific overexpression of DeltaFosB enhances incentive for cocaine. *J. Neurosci.* **23**, 2488–2493

47. Ulery, P. G., Rudenko, G., and Nestler, E. J. (2006) Regulation of DeltaFosB stability by phosphorylation. *J. Neurosci.* **26**, 5131–5142

48. Lu, L., Grimm, J. W., Hope, B. T., Shaham, Y. (2004) Incubation of cocaine craving after withdrawal: a review of preclinical data. *Neuropharmacology.* **47 (Suppl)**, 214–226

49. Sutton, M. A., Schmidt, E. F., Choi, K. H., Schad, C. A., Whisler, K., Simmons, D., Karanian, D. A., Monteggia, L. M., Neve, R. L., Self, D. W. (2003) Extinction-induced upregulation in AMPA receptors reduces cocaine-seeking behaviour. *Nature* **421**, 70–75