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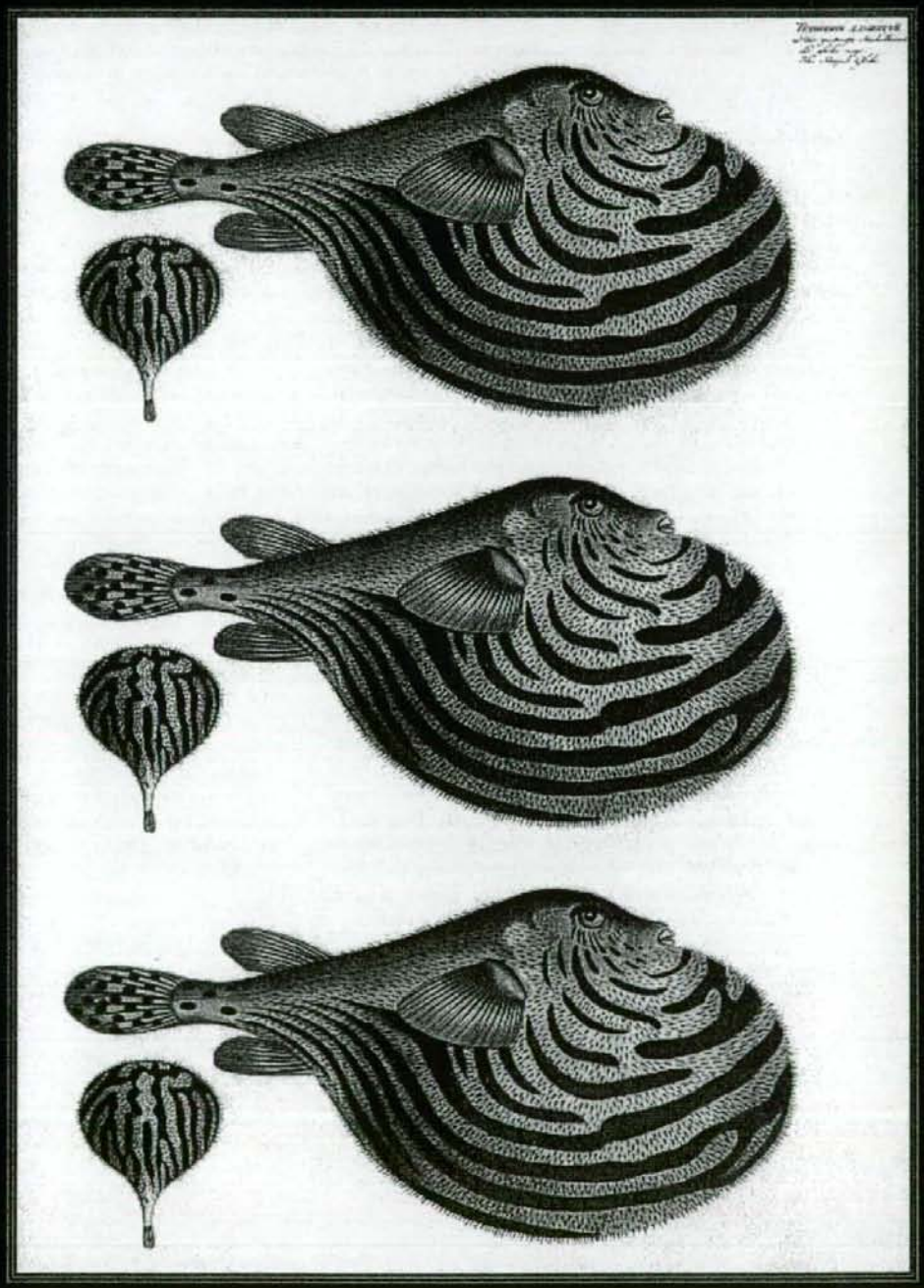

Gene Model for Obesity in Fish

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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

[1] Correspondence: Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466-8560, Japan. E-mail: tnabeshi@med.nagoya-u.ac.jp

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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. 1*A***). Also, there was no significant difference in the number of training sessions to acquire stable operant behavior between GDNF (+/−) and wild-type mice (Fig. 1*B*). Furthermore, GDNF (+/−) and wild-type mice showed similar breaking points under the PR schedule (Fig. 1*C*). These findings indicate that a partial loss of GDNF expression does not

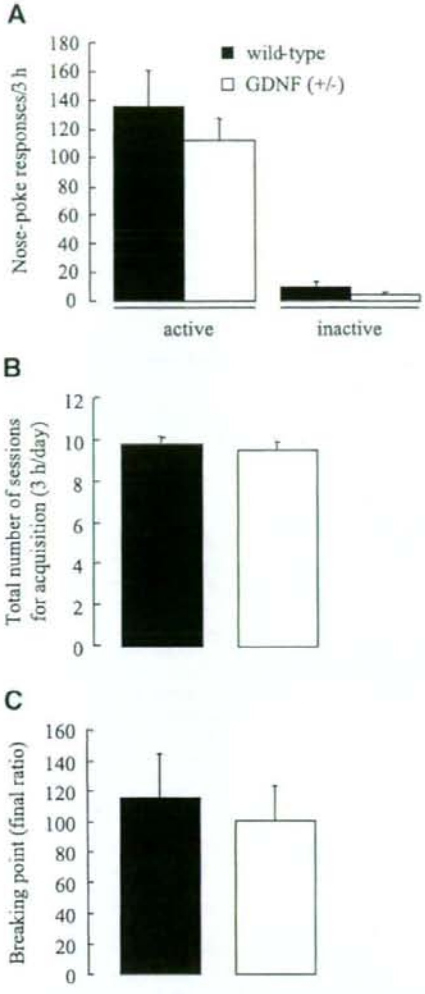

Figure 1. Food-reinforced operant behavior and motivation in GDNF (+/−) and wild-type mice. *A)* Active and inactive nose-poke responses for food reinforcement in a 3 h session under FR2 schedule during stable phase. *B)* Total number of training sessions needed to acquire stable active nose-poke responses 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. 2***A*; $F_{(5,48)}=27.83$; $P<0.001$). However, the priming of food pellets failed to reinstate food-seeking behavior in either genotype (Fig. 2*A*). 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-

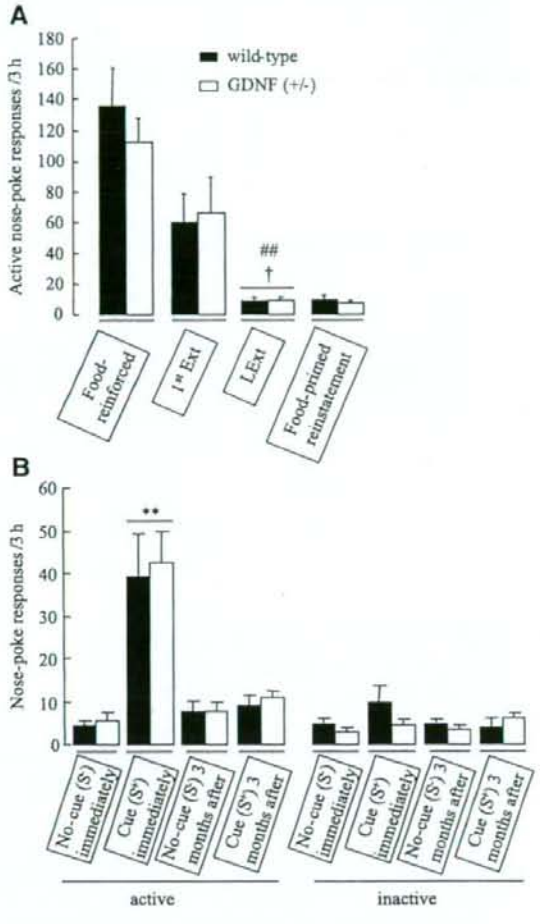

Figure 2. Extinction and reinstatement of food-seeking behavior in GDNF(+/−) and wild-type mice. *A)* Active nose-poke responses during stable phase of food-reinforced operant responding, extinction training, and food-primed reinstatement. *B)* Nose-poke responses in food-associated cue-induced reinstatement 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; $^{\dagger}P < 0.05$ *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. 2*B*; $F_{(1,24)}=33.44$; $P<0.001$). However, no significant difference in cue-induced reinstatement behavior was observed between GDNF (+/−) and wild-type mice (Fig. 2*B*). 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. 2*B*). 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. 3*A***, $P<0.01$). 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. 3*B*). In the early phase of METH self-administration under the FR1 schedule (Fig. 3*C*, 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. 3*C*, 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. 4*A***; $F_{(1,55)}=12.43$, $P<0.001$). 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

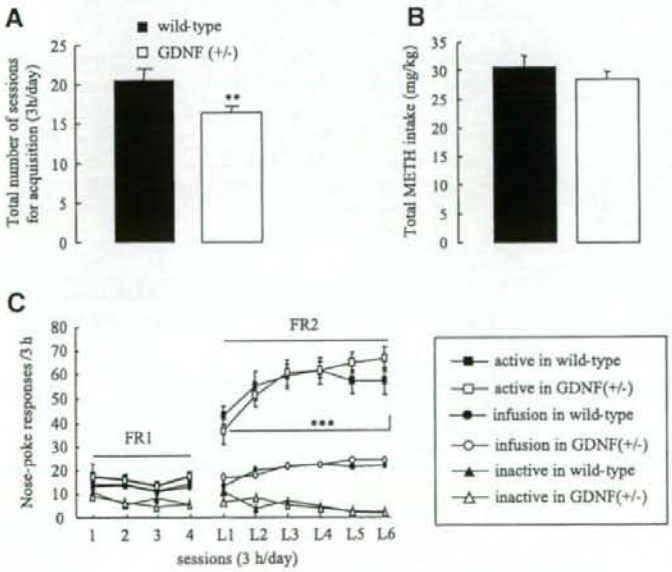

Figure 3. Acquisition of stable METH (0.1 mg/kg/infusion) self-administration behavior in GDNF (+/−) and wild-type mice. *A)* Total number of training sessions needed to acquire stable METH self-administration behavior for GDNF (+/−) and wild-type mice. $^{**}P < 0.01$ *vs.* wild-type littermates. *B)* 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.

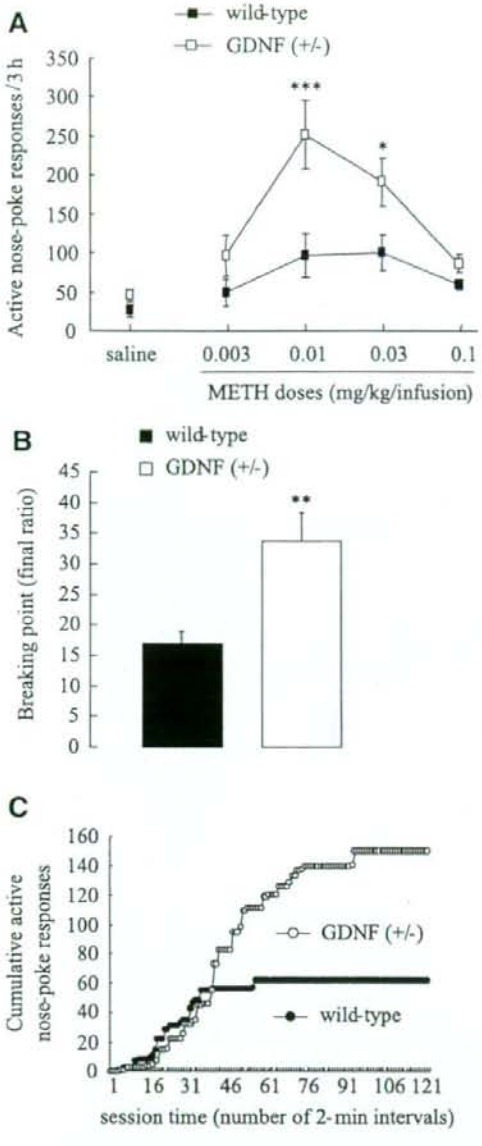

Figure 4. Dose responses and motivation for METH self-administration in GDNF (+/−) and wild-type mice. *A)* Dose-response function for METH self-administration under an FR2 schedule of reinforcement. *B)* Breaking points (final ratio) under a PR schedule of reinforcement. *C)* Representative curves for 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. 5*A***, 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. 5*A*), 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. 5*B*; $F_{(1,\ 77)}=12.72$; $P<0.001$), 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

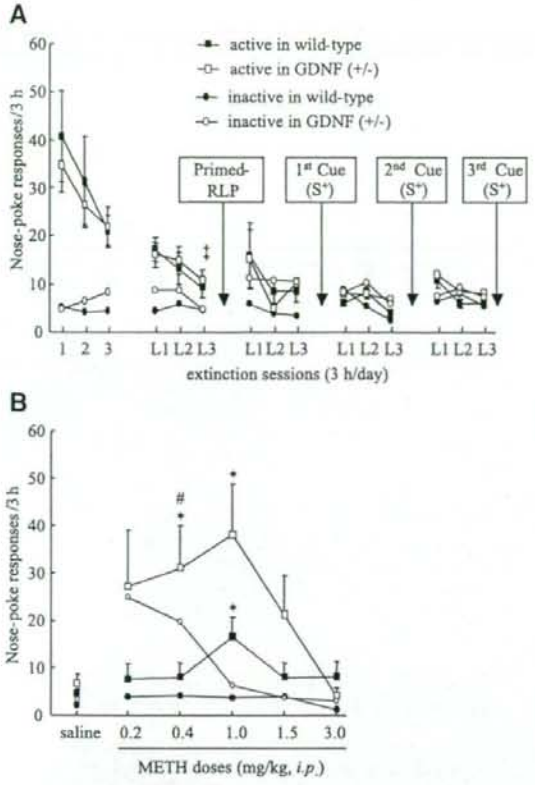

Figure 5. Nose-poke responses during extinction training (*A*) and METH-primed reinstatement of drug-seeking behavior (*B*; Primed-RLP) in GDNF (+/−) and wild-type mice. In *A*, data are from first 3 daily 3 h sessions (indicated by 1–3) and last 3 daily 3 h sessions (indicated by L1-L3) during 6–10 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. 5*A*). 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 withdrawal, 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

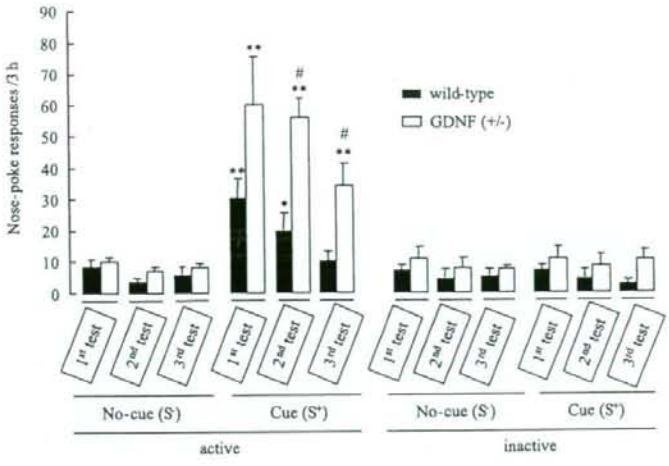

Figure 6. Active and inactive nose-poke responses during cue-induced reinstatement of METH-seeking behavior in GDNF (+/−) and wild-type mice. Tests for cue-induced reinstatement were conducted immediately (without withdrawal, 1st test), 3 months (2nd test), and 6 months (3rd test) after withdrawal from 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. 5*A*), 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. 5*A*), 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. 3*C*). 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. 2*B*). 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

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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-β 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

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Reduction of methamphetamine-induced sensitization and reward in matrix metalloproteinase-2 and -9-deficient mice

Hiroyuki Mizoguchi,*,† Kiyofumi Yamada,*,† Minae Niwa,* Akihiro Mouri,* Tomoko Mizuno,* Yukihiro Noda,*,‡ Atsumi Nitta,* Shigeyoshi Itohara,§ Yoshiko Banno¶ and Toshitaka Nabeshima*

**Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan*

†Laboratory of Neuropsychopharmacology, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Japan

‡Division of Clinical Science in Clinical Pharmacy Practice, Management and Research, Faculty of Pharmacy, Meijo University, Nagoya, Japan

§Laboratory for Behavioral Genetics, RIKEN Brain Science Institute, Wako, Japan

¶Department of Cell Signaling, Gifu University Graduate School of Medicine, Gifu, Japan

Abstract

Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) function to remodel the pericellular environment. Their activation and regulation are associated with synaptic physiology and pathology. Here, we investigated whether MMP-2 and MMP-9 are involved in the rewarding effects of and sensitization to methamphetamine (METH) in animals, in which the remodelling of neural circuits may play a crucial role. Repeated METH treatment induced behavioural sensitization, which was accompanied by an increase in MMP-2 and MMP-9 activity in the brain. In MMP-2- and MMP-9-deficient mice [MMP-2-(–/–) and MMP-9-(–/–)], METH-induced behavioural sensitization and conditioned place preference, a measure of the rewarding effect, as well as METH-increased dopamine release in the nucleus accumbens (NAc) were attenuated compared with those in wild-type mice. In contrast, infusion of purified human MMP-2 into the NAc significantly potentiated the METH-increased dopamine release. The [^{3}H]dopamine uptake into striatal synaptosomes was reduced in wild-type mice after repeated METH treatment, but METH-induced changes in [^{3}H]dopamine uptake were significantly attenuated in MMP-2-(–/–) and MMP-9-(–/–) mice. These results suggest that both MMP-2 and MMP-9 play a crucial role in METH-induced behavioural sensitization and reward by regulating METH-induced dopamine release and uptake in the NAc.

Keywords: dopamine, drug dependence, matrix metalloproteinases, methamphetamine.

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Drug dependence is a complex phenomenon with important psychological and social causes and consequences, which result from adaptations in specific brain circuits caused by repeated exposure to drugs of abuse (Berke and Hyman 2000; Yamada *et al.* 2005). It has been proposed that cellular and molecular mechanisms for drug dependence involve processes similar to those operating in other forms of synaptic plasticity such as learning and memory (Shippenberg and Heidbreder 1995; Mizoguchi *et al.* 2004). Methamphetamine (METH), a typical drug of abuse, has both acute and long-lasting effects on psychomotor behaviours. The effects of METH are associated with an increase in extracellular dopamine levels in the brain through redistri-

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Address correspondence and reprint requests to Toshitaka Nabeshima PhD, Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466–8560, Japan. E-mail: tnabeshi@med.nagoya-u.ac.jp or Kiyofumi Yamada PhD, Laboratory of Neuropsychopharmacology, Division of Life Science, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Japan.
E-mail: kyamada@p.kanazawa-u.ac.jp

Abbreviations used: DAT, dopamine transporter; ECM, extracellular matrix; Fc, frontal cortex; GFAP, glial fibrillary acidic protein; METH, methamphetamine; MMP, matrix metalloproteinase; NAc, nucleus accumbens; NeuN, neuron-specific nuclear antigen; SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; TIMP, tissue inhibitors of MMP; tPA, tissue plasminogen activator.

bution of dopamine from synaptic vesicles to the cytosol and promotion of reverse transport (Sulzer *et al.* 1995; Nakajima *et al.* 2004). Although the involvement of endogenous substances such as tumour necrosis factor (TNF)-α (Nakajima *et al.* 2004), tissue plasminogen activator (tPA: Nagai *et al.* 2004, 2005) and brain-derived neurotrophic factor (Nestler 2001), which are known to play a role in synaptic plasticity, has been implicated in the development of METH dependence (Robinson and Kolb 1997; Nestler 2001), the mechanism underlying the enduring brain dysfunction associated with the dependence are poorly understood.

Matrix metalloproteinases (MMPs) function to remodel the pericellular environment, primarily through the cleavage of extracellular matrix (ECM) proteins (Yong *et al.* 2001) and cell-surface components. MMPs constitute a family of enzymes with more than 20 members identified to date, which require Zn^{2+} for their enzymatic activity. Gelatinases (MMP-2 and MMP-9) are capable of cleaving collagens IV and V, laminin, and chondroitin sulfate proteoglycan, which were associated with cell adhesion, and have been implicated specifically in cerebral ischaemia (Lo *et al.* 2002), kainate-induced neuronal injury (Szklarczyk *et al.* 2002) and hippocampal long-term potentiation and memory (Nagy *et al.* 2006). MMP activity is regulated by interaction with tissue inhibitors of MMP (TIMPs), and thus the MMP/TIMP system may be involved in brain development (Vaillant *et al.* 1999; Wright *et al.* 2002). The consequences of proteolytic cleavage of target molecules by MMPs are varied and complex and are thought broadly to include both changes in physical constraints of the pericellular environment as well as signalling through liberation of normally sequestered molecules such as growth factors, or exposure of latent bioactive peptide fragments (Nagase and Woessner 1999).

We have recently demonstrated that tPA, an extracelluar serine protease, is involved in morphine and METH dependence (Nagai *et al.* 2004, 2005; Yamada *et al.* 2005). The tPA/plasmin system is one of the regulators of MMP (Wright *et al.* 2002; Wang *et al.* 2003) and could play a pivotal role in ECM degradation (Baricos *et al.* 1995). In this study, we investigated whether the MMP-2 and MMP-9 are involved in METH-induced behavioural sensitization and reward in an animal model. Here, we show that the expression of MMP-2 and MMP-9 is induced by repeated METH treatment in the brain, and that the MMP expression is involved in the development of METH-induced sensitization and place preference by regulating METH-induced dopamine release and uptake in the nucleus accumbens (NAc).

Materials and methods

Animals

Male Wistar rats (8 weeks old; Charles River Japan, Yokohama, Japan) weighing 270 ± 20 g at the beginning of experiments were used in the study. We also used MMP-2 heterozygous knockout [MMP-2-(+/−)], homozygous knockout [MMP-2-(−/−)] and their wild-type (C57BL/6 J) mice (10–12 weeks old). The mutant mice were backcrossed into the C57BL/6J strain more than 13 times, and the generation, genotyping, and characterization of MMP-2-(−/−) mice have been described (Itoh *et al.* 1997). MMP-9 homozygous knockout [MMP-9-(−/−)] mice (10–12 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Wild-type FVB/N [MMP-9-(+/−)] mice were obtained from the CLEA Japan (Fuji, Japan).

All experiments were performed in accordance with the Guidelines for Animal Experiments of the Nagoya University School of Medicine, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

METH treatment for behavioural sensitization

Rats were given saline or METH at a dose of 2 mg/kg (s.c.) for 5 days (Nakajima *et al.* 2004; Nagai *et al.* 2005). The animals were placed in their home cage for 1 h following injection and then locomotor activity was measured in an acrylic chamber (25 × 42 × 20 cm) for 1 h using behavioural analysis systems (SCANET SV-10; Neuroscience, Tokyo, Japan) on days 1, 3 and 5. In the experiments on mice, they were given saline or METH at a dose of 1 mg/kg (s.c.) for 7 days (Nakajima *et al.* 2004; Nagai *et al.* 2005). Locomotor activity was immediately measured for 1 h after METH treatment on days 1, 3, 5 and 7. After 7-day withdrawal of METH treatment (on day 14), the mice were given METH at a dose of 1 mg/kg (s.c.).

Conditioned place preference (CPP)

The apparatus used for the place conditioning task consisted of two compartments: a black Plexiglas box and a transparent Plexiglas box (both 15 × 15 × 15 cm high for mice) with a metal grid floor. The place conditioning paradigm was performed with a minor modification (Nagai *et al.* 2004; Nakajima *et al.* 2004). In the preconditioning test, the sliding door was opened and the animal was allowed to move freely between both boxes for 15 min once a day for 3 days. On the third day of the preconditioning test, we measured the pre-value using Scanet SV-10 LD (Tokyo Sangyo Co. Ltd, Toyama, Japan). On days 4, 6 and 8, a rat or mouse was given METH at 1 mg/kg and put on its non-preferred side for 20 min. On days 5, 7, and 9, the animal was given saline and placed on the opposite drug-conditioning side. In the post-conditioning test, the sliding door was opened, and we measured the post-value. Place conditioning behaviours were expressed as [(post-value)–(pre-value)].

Gel zymography

After the final administration of METH, rats were intracardially perfused with cold saline and then killed by rapid decapitation as described (Asahi *et al.* 2001; Lee *et al.* 2004). Various regions of the brain including the NAc and frontal cortex (Fc) were dissected out and immediately frozen and stored at −80°C until assayed. All dissection was made using brain matrix (NeuroScience Idea, Osaka, Japan) and based on the atlas of Paxinos and Watson (1982).

Sample preparation was made as described previously (Zhang and Gottschall 1997). Briefly, brain tissues were homogenized in

lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij35, and 0.02% NaN_3, pH 7.6) with 1% TritonX-100 and centrifuged at 12 000 *g* for 10 min to pellet insoluble material. The protein concentration in the supernatant was determined using a Protein Assay Rapid Kit. The supernatant was incubated with gelatin-sepharose 4B (GE Healthcare Bio-Science, Piscataway, NJ, USA) that had previously been washed three times with the lysis buffer, with constant shaking, for 24 h at 4°C. After centrifugation at 500 *g* for 2 min, the pellet was re-suspended in 500 μL of the lysis buffer and washed three times. The pellet was re-suspended in 150 μL of lysis buffer containing 10% dimethyl sulfoxide and shaken for 2 h, and used for assaying gelatinase activity and for western blotting of MMP-2 and MMP-9.

The samples, together with human pro-MMP-2 (1 ng/lane; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and cultured medium of the C6 cell line, were subjected to electrophoresis in 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) containing 0.1% gelatin under non-reducing conditions. The human pro-MMP-2 and C6 cell medium were used as positive controls. To detect the active lower molecular weight forms of these proteases, samples were incubated with 400 mM *p*-aminophenylmercuric acetate (APMA; Amersham Pharmacia Biotech), which is known to induce the autocatalytic cleavage of gelatinases. After incubation for 5 h with APMA, samples were subjected to gel zymography. Gels were washed twice for 30 min in 2.5% Triton X-100 to remove SDS, washed for 30 min in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 2 μM $ZnCl_2$, 200 mM NaCl, and 0.02% Briji35, pH 7.4) at 25 ± 2°C, and further incubated for 24 h in the same buffer at 37°C. Gels were then stained for 3 h in Coomassie blue (1% Coomassie Brilliant Blue G-250, 30% methanol, 10% acetic acid) and destained in 40% methanol/7% acetic acid until clear bands of gelatinolysis appeared on a dark background. Total activity including pro-MMP was analysed with the ATTO Densitograph Software Library Lane Analyzer (Atto Instruments, Tokyo, Japan).

Western blotting

The sample preparation of brain tissues was made as described above. Brain tissues were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij35, and 0.02% NaN_3, pH 7.6) with 1% TritonX-100. The supernatant was boiled in 2 × sample buffer (0.25% bromophenol blue/12% 2-mercaptothanol/20% glycerol/4% SDS/0.1 M Tris-HCl, pH 6.8) and electrophoresed by SDS–PAGE on a 4.75% stacking gel and 10% separating gel, and then transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, MA, USA; Mizoguchi *et al.* 2004). The same amount of protein per lane was loaded for all western blotting. The membrane was incubated in the blocking solution (5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20) for 2 h at 25 ± 2°C and then incubated with primary antibodies. After washing, blots were incubated with the secondary antibodies. Immunoreactive materials on the membrane were detected using enhanced chemiluminescene (ECL) western blotting detection reagents (GE Healthcare Bio-Science) and exposed to X-ray film. The band intensities of the film were analysed by densitometry. The primary antibodies used in the present study were polyclonal rabbit anti-MMP-2 (1 : 3000; AB809; Chemicon, Temecula, CA, USA) and anti-MMP-9 (1 : 1000; AB19016; Chemicon) antibodies. The secondary antibodies, used at a 1 : 2000 or 1 : 5000 dilution, were horseradish peroxidase-linked anti-rabbit (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA).

In situ zymography

Rats were intracardially perfused with cold saline before being frozen at −80°C using O.C.T. Compound (Sakura Finetechnical, Tokyo, Japan). The brains were sectioned at 20 μm in a cryostat. We adapted an *in situ* zymography method to localize net gelatinolytic activity in brain sections as described previously (Szklarczyk *et al.* 2002). Non-fixed sections were incubated for 24 h at 37°C in a humid dark chamber with a reaction buffer containing 0.5 M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, 2 mM sodium azide, pH 7.6 and 100 μg/mL FITC-labelled DQ-gelatin (Molecular Probes, Eugene, OR, USA) intramolecularly quenched. After incubation, sections were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde and mounted on slides. Some sections were incubated with the broad spectrum MMP inhibitor 1,10-O-phenanthroline (1 mM; Molecular Probes) or with 200 ng/mL human recombinant TIMP-2 (Daiichi FineChem., Takaoka, Japan). Samples were observed with a FITC filter, and the images were analysed using AXIOVISION 3.0 systems (Carl Zeiss, Jene, Germany). Gelatin–FITC cleavage by tissue gelatinases releases quenched fluorescence representative of net proteolytic activity. Sections incubated without DQ-gelatin were not fluorescent.

Double immunostaining

Polyclonal rabbit anti-MMP-2 antibody (1 : 250; AB809, Chemicon), anti-MMP-9 antibody (1 : 250; AB19016, Chemicon), monoclonal mouse anti-neuron-specific nuclear antigen (NeuN) antibody (1 : 200; Chemicon) and anti-glial fibrillary acidic protein (GFAP) antibody (1 : 200; Chemicon) served as primary antibodies. Affinity-purified FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG served as secondary antibodies. Samples were observed with AXIOVISION 3.0 systems (Carl Zeiss).

In vivo microdialysis

Rats and mice were anaesthetized with sodium pentobarbital before stereotaxic implantation of a guide cannula into the NAc [AP +1.6, ML −1.0 from the bregma, DV −7 from the skull for rats (Paxinos and Watson 1982), AP −1.7, ML +0.8 from the bregma, DV −4 from the skull for mice (Franklin and Paxinos 1997)]. A dialysis probe (AI-8–1 or AI-6–1; 1-membrane length, Eicom, Kyoto, Japan) was inserted though the guide cannula and perfused with artificial cerebrospinal fluid (aCSF; 147 mM NaCl/4 mM KCl/2.3 mM $CaCl_2$) at a flow rate of 1.2 μL/min in rats and 2 μL/min in mice. Outflow fractions were collected every 20 min. After the collection of three baseline fractions, the animals were treated with METH (1 or 2 mg/kg, s.c.). Dopamine levels in the dialysates were analysed as described (Nakajima *et al.* 2004). To examine the effect of microinjection of MMP-2 into the NAc on METH-induced dopamine release in rats, a dialysis probe equipped with a microinjection tube (MI-AI-8–1; 1-mm membrane length, Eicom) was used (Nagai *et al.* 2004). After the collection of baseline fractions, a 10-ng dose of purified human MMP-2 (Chemicon) or its vehicle was injected into the NAc, at a volume of 1 μL during a 10-min period, through the

microinjection tube. METH at 2 mg/kg was injected s.c. 60 min after treatment with MMP-2. The probe placement was checked in each animal at the end of the *in vivo* microdialysis experiment, and the data of the mice in which tip of the probe was located outside the Nac were excluded for the data analysis.

Crude synaptosomal [^{3}H]dopamine uptake

Crude synaptosomal [^{3}H]dopamine uptake was determined as described (Nakajima *et al.* 2004). Tissue including the striatum and NAc was homogenized in ice-cold 0.32 M sucrose and centrifuged at 1000 *g* for 10 min at 4°C. The supernatant fractions were removed and centrifuged at 22 000 *g* for 15 min. The resulting pellet was re-suspended in an ice-cold modified Krebs–Ringer solution composed of (in mM) 125 NaCl, 4.8 KCl, 25 $NaHCO_3$, 1.2 $KH2PO_4$, 1.3 $MgCl_2$, 1.2 $CaCl_2$, 10 glucose, and 0.57 ascorbic acid gassed with 95% O_2 and 5% CO_2. Crude synaptosomal homogenate (100 μg synaptosomes protein) was contained 5 μM pargyline (Sigma, St Louis, MO, USA) in 200 μL of Krebs–Ringer solution. After pre-incubation for 10 min at 37°C, assays were initiated by the addition of [^{3}H]dopamine (60 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA, USA) in 1 mL of Krebs-Ringer solution. Samples were incubated at 37°C for 4 min, and then ice-cold Krebs-Ringer solution containing 10 μM GBR12909, a specific dopamine uptake inhibitor, was added. Samples were filtered through Whatman GF/B filters. Non-specific values were determined in the presence of 100 μM GBR12909. The radioactivity trapped on filters was measured using a liquid scintillation counter.

Statistical analyses

Results were expressed as the mean ± SE. The significance of differences was determined by one-way ANOVA, followed by the Student–Newman–Keuls test or by repeated ANOVA, followed by Sheffe's test for multigroup comparisons. Student's *t*-test was used for two-group comparison.

Results

METH increases MMP-2 and MMP-9 expression in the brain

Gelatinases, MMP-2 and MMP-9, have been implicated specifically in cerebral ischaemia and physiological tissue remodelling. For example, MMP-2 plays a principal role in establishing the growth-promoting properties of denervated peripheral nerve (Krekoski *et al.* 2002). MMP-9, but not MMP-2, is particularly involved in dendritic remodelling in the hippocampus of adult rat (Szklarczyk *et al.* 2002). Therefore, we examined whether repeated METH treatment altered the protein levels and enzymatic activities of MMP-2 and MMP-9 in the rat brain.

MMP-2 protein levels were increased in the Fc and NAc by repeated METH treatment for 5 days (Fig. 1a), which induced behavioural sensitization. In the Fc, the increase was evident as early as 2 h after the last administration of METH, whereas the change in the NAc was manifested 24 h after the last injection. Gel zymography revealed that MMP-2 activity

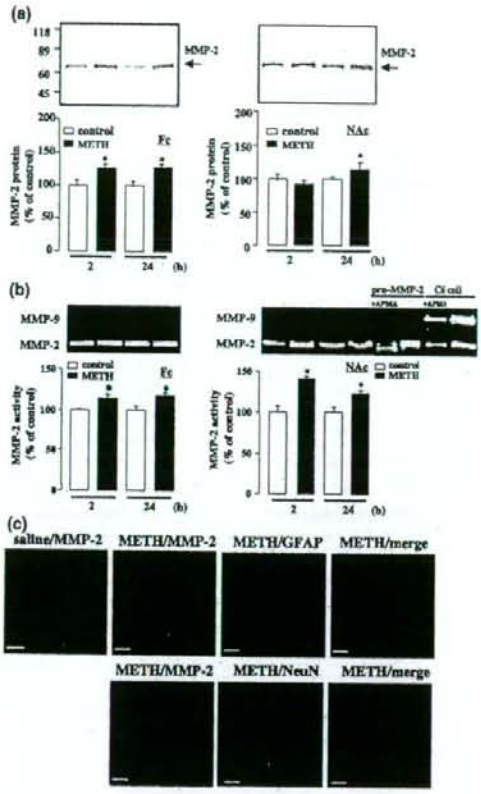

Fig. 1 METH-induced MMP-2 expression. Changes in protein levels (a) and MMP-2 activity (b) in Fc and NAc after repeated METH treatment. Rats were given METH at a dose of 2 mg/kg for 5 days and killed 2 or 24 h after the final administration. Control rats were given saline. The human pro-MMP-2 and C6 cell line was used as a positive control. Incubation for 5 h with APMA induced the autocleavage of pro-MMP-2 to active-MMP-2. *$p < 0.05$ versus control group. Values are the means ± SE (a, $n = 4$–6; b, $n = 3$–5). Double immunostaining for MMP-2 and the neuronal marker NeuN or GFAP in the Fc (c). Rats were killed 2 h after the final administration of METH (2 mg/kg, for 5 days). Scale bar, 20 μm.

was enhanced in the Fc and NAc by repeated METH treatment 2 and 24 h after the last injection (Fig. 1b). The apparent discrepancy of the METH effects on MMP-2 protein and activity in the NAc observed 2 h after the last administration may be as a result of the different sensitivities of the methods used in the experiments. Treatment of pro-MMP-2 with APMA induced the autocatalysis of the gelatinases, resulting in lower molecular weight bands likely to represent active forms of gelatinases (Fig. 1b). Compared with the band of APMA-treated pro-MMP-2, no active-MMP-2 was detected in control or METH-treated rats.

Repeated METH treatment also increased MMP-9 protein levels and activity in the Fc and NAc (Figs 2a and b, and Fig. 1b showing the MMP-9 bands). It should be noted that repeated METH treatment produced a lower molecular weight band which may be an active MMP-9 form (Fig. 1b). Acute METH treatment had no effect on MMP-2 or MMP-9 activity in the Fc (MMP-2 100 ± 9.9 of control, $n = 5$–6, $p > 0.05$; MMP-9 100 ± 17.6 of control, $n = 5$–6, $p > 0.05$) and NAc (MMP-2 100 ± 7.2 of control, $n = 5$–6, $p > 0.05$; MMP-9 100 ± 7.3 of control, $n = 5$–6, $p > 0.05$) 2 h after the treatment.

To determine the cell types in which the expressions of MMP-2 and MMP-9 are induced by repeated METH treatment, double immunostaining for MMP-2 and MMP-9 with NeuN, a neuronal marker, or GFAP, a glial marker, was performed. The findings indicated the co-localization of MMP-2 immunoreactivity with NeuN and GFAP immunoreactivity, suggesting the neuronal and glial localization of MMP-2 (Fig. 1c). Similar results were found in double immunostaining for MMP-9 and NeuN or GFAP (Fig. 2c). These results indicate that MMP-2 and MMP-9 expression is induced by METH in both neuronal and glial cells in Fc.

Spatial changes in MMP proteolytic activity in the brain after repeated METH treatment

We analysed the spatial changes in gelatinase activity in the brain following repeated METH treatment by *in situ* zymography. Brain sections were incubated with gelatin conjugated to a quenched fluorescence dye. Cleavage of gelatin results in an increase in fluorescence. The signal was inhibited by the zinc chelator phenantroline (Phe), broad spectrum MMP inhibitor, indicating that the fluorescence is associated with MMP activity (Fig. 3b compared with 3a). In control sections, gelatinase activity was observed in layer II–V of the cingulate and prelimbic cortex, the CA1–CA4 layers and dentate gyrus of the hippocampus, striatum and the shell of the NAc. Two

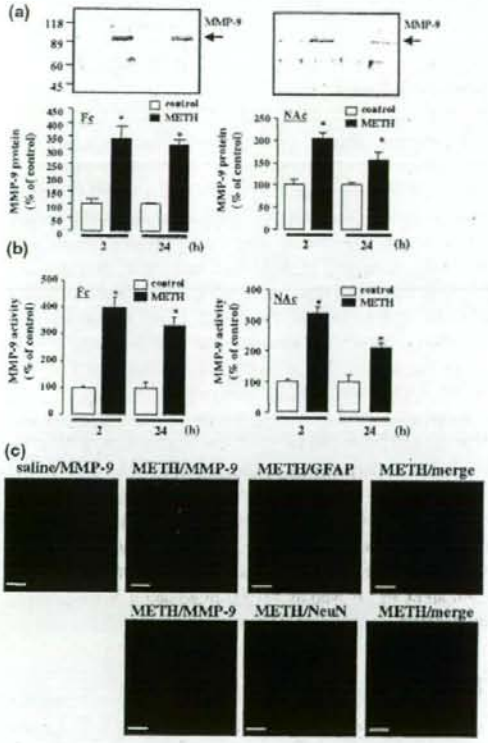

Fig. 2 METH-induced MMP-9 expression. Changes in protein levels (a) and MMP-9 activity (b) in Fc and NAc after repeated METH treatment. Rats were given METH at a dose of 2 mg/kg for 5 days and killed 2 or 24 h after the final administration. Control rats were given saline. The C6 cell line was used as a positive control. Incubation for 5 h with APMA induced the autocleavage of pro-MMP-9 to active-MMP-9 (Fig. 1b). $^*p < 0.05$ versus control group. Values are the means ± S.E. (a, $n = 4$–6; b, $n = 3$–4). Double immunostaining for MMP-9 and NeuN or GFAP in Fc (c). Rats were killed 2 h after the final administration of METH (2 mg/kg, for 5 days). Scale bar, 20 μm.

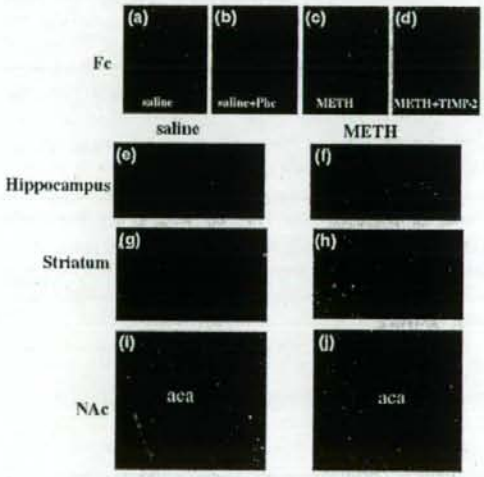

Fig. 3 Spatial changes in METH-induced MMP activity in the brain. *In situ* zymography detected gelatinase activity in Fc (a–d), hippocampus (e, f), striatum (g, h) and NAc (i, j). Rats were given saline (a, b, e, g, i) or METH (c, d, f, h, j) at a dose of 2 mg/kg for 5 days, and killed 2 h after the final administration. Brain sections were incubated with fluorescent gelatin. Cleavage of the gelatin by proteinases resulted in the unblocking of quenched fluorescence and an increase in fluorescence. Gelatinase activity was attenuated by either the zinc chelator 1,10-O-phenantroline (Phe, b) or TIMP-2 (200 ng/mL, d). Photomicrographs are representative of observations made in at least three animals per group. Photomicrographs were representative of observations made from at least three animals per group.

hours after the final administration of METH, a strong signal was visualized in the Fc compared with the saline group (Fig. 3c compared with 3a). METH-increased gelatinase activity in the Fc was inhibited by TIMP-2 at 200 ng/mL (Fig. 3d). In addition, the MMP activity was increased markedly by repeated METH treatment in the striatum and NAc, but moderately in the hippocampus (Figs 3e–j).

MMP-2-deficient [MMP-2-(–/–)] and MMP-9-deficient [MMP-9-(–/–)] mice show reduced responses to METH in behavioural sensitization, CPP and dopamine release in the NAc

We investigated a role of MMP-2 and MMP-9 in METH-induced behavioural sensitization and reward using MMP-2 and MMP-9 gene knockout mice. As shown in Fig. 4(a), there was no difference in spontaneous locomotor activity or single METH-induced hyperlocomotion among wild-type, MMP-2 heterozygous knock-out [MMP-2-(+/–)] and homozygous knock-out [MMP-2-(–/–)] mice. However, they showed significantly different responses to repeated METH treatment. Repeated ANOVA revealed that both MMP-2-(+/–) and MMP-2-(–/–) mice showed significantly reduced locomotor activity during repeated METH treatment compared with wild-type mice (Fig. 4a, $F_{5,68} = 17.8$; $p < 0.05$). On day 14 when METH (1 mg/kg) was challenged, the attenuation of the METH-induced sensitization in MMP-2 mutant mice was reconfirmed (Fig. 4a, $F_{5,68} = 43.5$; $p < 0.05$; one-way ANOVA). It was also indicated by repeated ANOVA that both METH-induced hyperlocomotion and repeated METH-induced sensitization were markedly decreased in MMP-9 homozygous knockout [MMP-9-(–/–)] mice compared with those in wild-type mice (Fig. 4b, $F_{3,49} = 50.8$; $p < 0.05$). Furthermore, the expression of METH-induced sensitization on day 14 was significantly attenuated in MMP-9-(–/–) mice compared with wild-type mice ($F_{3,49} = 57.6$; $p < 0.05$; one-way ANOVA).

Regarding METH reward, METH-induced CPP was significantly attenuated in MMP-2-(–/–) and MMP-9-(–/–) mice compared with wild-type mice (Fig. 4c, $F_{3,44} = 11.3$; $p < 0.05$; one-way ANOVA; Fig. 4d, $F_{3,46} = 5.45$; $p < 0.05$; one-way ANOVA). There was no difference in place preference between saline-treated wild-type mice and mutant mice.

Consistent with behavioural changes, METH-induced increase in extracellular dopamine levels in the NAc was markedly reduced in MMP-2-(–/–), MMP-2-(–/–) and MMP-9-(–/–) mice compared with wild-type mice (Fig. 4e, $F_{2,27} = 4.99$; $p < 0.05$; repeated ANOVA, and $p < 0.05$ by post-hoc test; Fig. 4f, $F_{1,11} = 8.45$; $p < 0.05$; repeated ANOVA, and $p < 0.05$ by post-hoc test. The gene dose-related changes in METH-induced increase in extracellular dopamine levels were evident among wild-type, MMP-2-(+/–) and MMP-2-(–/–) mice (Fig. 4e, $p < 0.05$).

Effect of MMP-2 infusion into the NAc on METH-induced dopamine release

We also investigated the effect of microinjection of MMP-2 into the NAc on the METH-induced increase in the extracellular dopamine levels in rats. Acute METH treatment increased the peak extracellular dopamine levels to approximately 500–600% of the basal levels in the NAc (Fig. 5). Infusion of purified human MMP-2 protein at a dose of 0.01 μg into the NAc had no effect on basal dopamine levels, but it significantly potentiated METH-induced increase in the extracellular dopamine levels compared with the response in vehicle-treated control group 40–60 min after acute METH treatment (Fig. 5, $p < 0.05$ by t-test). These results suggest that an increase in MMP-2 activity in the NAc leads to the potentiation of METH-induced dopamine release.

Dopamine transporter function in MMP-2-(–/–) and MMP-9-(–/–) mice

Finally, we examined the role of endogenous MMP on [^{3}H]dopamine uptake into a crude striatal synaptosome preparation. There was no difference in [^{3}H]dopamine uptake between wild-type mice and MMP-2-(–/–) or MMP-9-(–/–) mice, suggesting no changes in [^{3}H]dopamine uptake activity under normal conditions. The [^{3}H]dopamine uptake was markedly decreased in wild-type mice 1 h after the last administration of repeated METH treatment on day 7 (Figs 6a and b). In MMP-2-(–/–) mice, no changes in [^{3}H]dopamine uptake was observed after repeated METH treatment, and thereby a significant difference in [^{3}H]dopamine uptake was evident between two groups of mice (Fig. 6a, $F_{3,25} = 5.33$; $p < 0.05$; one-way ANOVA). A similar changes in [^{3}H]dopamine uptake was observed in MMP-9-(–/–) mice after METH treatment (Fig. 6b, $F_{3,19} = 21.8$; $p < 0.05$; one-way ANOVA). These results suggest that both mutant mice exhibit the resistance to the inhibitory effect of METH on dopamine transport activity, which may be associated with the impairment in METH-induced sensitization and CPP, as well as METH-increased dopamine release in MMP-2-(–/–) and MMP-9-(–/–) mice.

Discussion

Repeated treatment with psychostimulants such as cocaine and amphetamine produces changes in neural morphology and synaptic connectivity in the mesolimbic neuronal system (Nestler 2001). It has been hypothesized that alterations in synaptic connectivity in these structures might be involved in the long-lasting behavioural consequences of repeated treatment with drugs of abuse such as amphetamine psychosis and dependence, and dysphoria during drug withdrawal. Accordingly, various molecules that regulate synaptic structure and connectivity may play a crucial role in METH dependence. In this study, we demonstrated for the first time

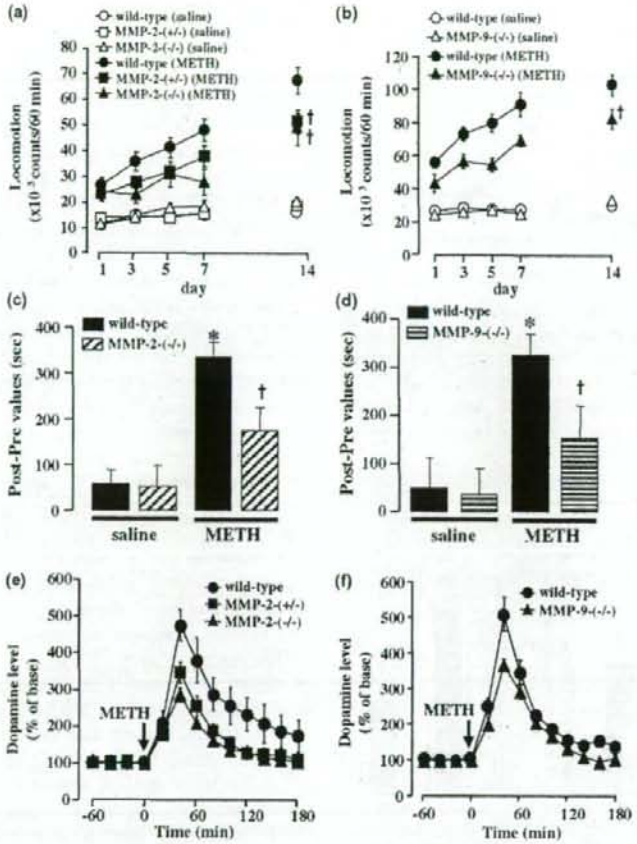

Fig. 4 Sensitization of METH-induced (a, b) hyperlocomotion, (c, d) reward and (e, f) dopamine release in the NAc in (a, c, e) MMP-2(–/–), (a, e) MMP-2(+/–) and (b, d, f) MMP-9(–/–). (a, b): Mice were given saline or METH at a dose of 1 mg/kg for 7 days and, after withdrawal for 7 days, they were given saline or METH (1 mg/kg) on day 14. Repeated-measures ANOVA revealed a significant difference in repeated METH-induced sensitization of MMP-2(+/–), MMP-2(–/–) and MMP-9(–/–) compared with wild-type mice. Values are the means ± SE (a, $n = 10$–14; b, $n = 13$–14). †$p < 0.05$ versus wild-type (METH). (c, d): Mice were trained for CPP to METH (1 mg/kg) to examine the METH reward in the MMP-2-(–/–) and MMP-9-(–/–) mice. Values are the means ± SE (c, $n = 12$; d, $n = 12$–13). *$p < 0.05$ versus wild-type (saline). †$p < 0.05$ versus wild-type (METH). (e, f): Mice were given METH at a dose of 1 mg/kg for 7 days. On day 7, extracellular dopamine release in the NAc was measured for 3 h after the administration of METH. Basal extracellular dopamine levels were 0.41 ± 0.06 nM for the wild-type, 0.50 ± 0.11 nM for MMP-2-(+/–) and 0.30 ± 0.06 nM for MMP-2(–/–). Basal extracellular dopamine levels were 0.42 ± 0.08 nM for the wild-type, 0.28 ± 0.03 nM for MMP-9(–/–). Values are the means ± SE (e, $n = 6$–8; f, $n = 6$–7). Repeated-measures ANOVA revealed a significant difference in METH-increased dopamine release in the NAc of MMP-2-(+/–), MMP-2-(–/–) and MMP-9-(–/–) mice compared with wild-type mice (Fig. 4e, $F_{2,27} = 4.99$; repeated ANOVA, and $p < 0.05$ by post-hoc test; Fig. 4f, $F_{1,11} = 8.45$; repeated ANOVA, and $p < 0.05$ by post-hoc test.

that repeated administration of METH led to behavioural sensitization which was accompanied by the induction of MMP-2 and MMP-9 expression in the Fc and NAc. No active-MMP-2 was detected, however, in METH-treated rats by gel zymography, indicating that repeated METH treatment increased the expression of pro-MMP-2. Although active-MMP-2 was not detected in the gel zymography, it is possible that the activity of MMP-2 may be affected at some point by repeated METH treatment.

Our data showed that MMP-2 and MMP-9 were expressed in neurons as well as glial cells in the Fc. Some previous studies suggested that the MMP/TIMP system is expressed by adult CNS neurons and glial cells, and the expression level and cellular localization may be regulated according to

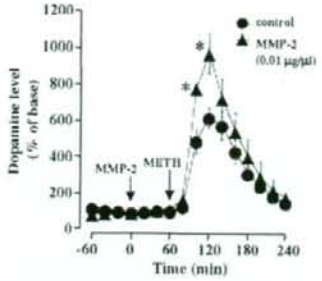

Fig. 5 Effect of MMP-2 on the METH-induced increase in extracelluar dopamine levels in the NAc of rats. Purified human MMP-2 (0.01 μg) was microinjected into the NAc in a volume of 1.0 μL 1 h before METH (2 mg/kg) treatment. Basal extracellular dopamine levels were 0.21 ± 0.07 nM for the control and 0.29 ± 0.11 nM for the MMP-2-infused group. Values are the means ± SE (n = 4–5). *p < 0.05 versus vehicle-treated control group (p < 0.05 by t-test).

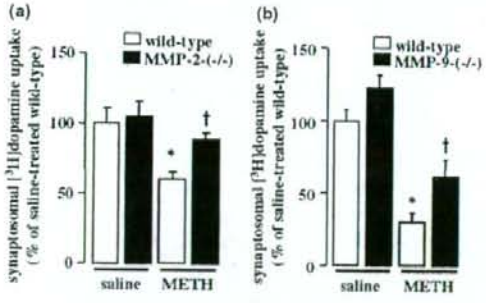

Fig. 6 Changes in METH-induced reduction of the activity of synaptosomal dopamine uptake in (a) MMP-2-(–/–) and (b) MMP-9-(–/–) mice. Mice were given saline or METH at a dose of 1 mg/kg for 7 days, and were killed 1 h after the final administration. (a) The [^{3}H]dopamine uptake activity in the saline-treated wild-type mice was 2.66 ± 0.2 pmol/μg protein/4 min. (b) The [^{3}H]dopamine uptake in the saline-treated wild-type mice was 1.34 ± 0.1 pmol/μg protein/4 min. Values are means ± SE (a, n = 7–8; b, n = 5–6). *p < 0.05 versus saline-treated wild-type mice. †p < 0.05 versus METH-treated wild-type mice.

the developmental and/or functional status of the brain (Vaillant *et al.* 1999; Szklarczyk *et al.* 2002). For instance, in the process of axonal extension, MMP is located at the growth cone tips, permitting attachment/detachment between the neurons and matrix substratum (Monard 1988), and oligodendrocytes use MMP to extend their processes (Uhm *et al.* 1998), suggesting that MMP regulation is likely to provide guidance during the proliferation of new synapses. Accordingly, it is plausible that the expression of MMP-2 and MMP-9 may take part in the structural and functional alterations in the brain following repeated exposure to METH.

We also demonstrated that METH-induced behavioural sensitization and CPP were markedly attenuated in MMP-2-(–/–) and MMP-9-(–/–) mice compared with those in wild-type, suggesting that METH-induced expression of the MMP in the brain plays a role in the development of METH-induced sensitization and CPP, which may be associated with neuronal plasticity and remodelling. Indeed, the inhibition of MMP alters functional and structural correlations of deafferentation-induced sprouting, such as remodelling in the dentate gyrus of the hippocampus (Reeves *et al.* 2003). In a behavioural study, MMP-9 knockout mice display impairments in long-term potentiation and hippocampal-dependent memory in a fear-conditioning memory task (Nagy *et al.* 2006). As learning/memory mechanisms are considered to overlap with and are involved in the development of drug dependence that occurs with chronic administration of drugs of abuse (Berke and Hyman 2000; Mizoguchi *et al.* 2005), MMP-2 and MMP-9 expression may play a crucial role in the acquisition of METH-induced CPP.

Behavioural changes induced by METH are linked to its capacity to elevate extracellular dopamine levels through the redistribution of dopamine from synaptic vesicles to the cytosol, promotion of reverse transport (Sulzer *et al.* 1995; Nakajima *et al.* 2004) and the internalization of dopamine transporter (DAT; Zahniser and Sorkin 2004; Cervinski *et al.* 2005). The microinjection of purified human MMP-2 directly into the NAc significantly potentiated the acute METH-induced increase in extracellular dopamine levels in NAc, without affecting basal dopamine levels. Moreover, in MMP mutant mice [MMP-2-(+/–), MMP-2-(–/–) and MMP-9-(–/–)], METH-induced dopamine release in the NAc was significantly decreased compared with the response in wild-type mice. These findings demonstrate a previously undescribed function of the MMP in the regulation of dopamine release in the NAc.

It is unlikely that changes in repeated METH-induced behavioural sensitization and dopamine release in the MMP-2-(–/–) and MMP-9-(–/–) mice are as a result of the alteration of basal DAT activity as there were no differences in basal [^{3}H]dopamine uptake *in vitro* between wild-type and MMP mutant mice. Furthermore, we observed no changes in dopamine D_1 agonist SKF-81297 or D_2 agonist quinpirole–stimulated [^{35}S]GTPγS binding between wild-type and MMP-2-(–/–) or MMP-9-(–/–) mice (data not shown), indicating no changes in dopamine D_1 and D_2 receptor activity in MMP-2-(–/–) and MMP-9-(–/–) mice. However, we cannot rule out a possibility that the observed behavioural and neurochemical changes induced by METH in the MMP-2-(–/–) and MMP-9-(–/–) mice may be as a result of the consequence of developmental compensation in the mutant mice.