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

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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

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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

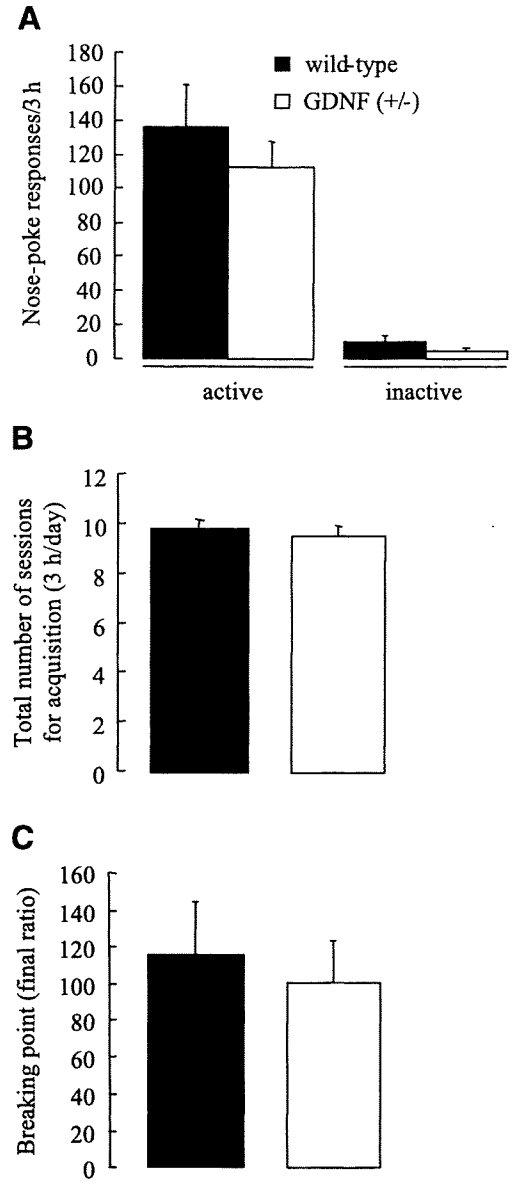

Figure 1. Food-reinforced operant behavior and motivation in GDNF (+/−) and wild-type mice. *A)* Active and inactive nose-poke responses for food reinforcement in a 3 h session under FR2 schedule during stable phase. *B)* Total number of training sessions needed to acquire stable active nose-poke responses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

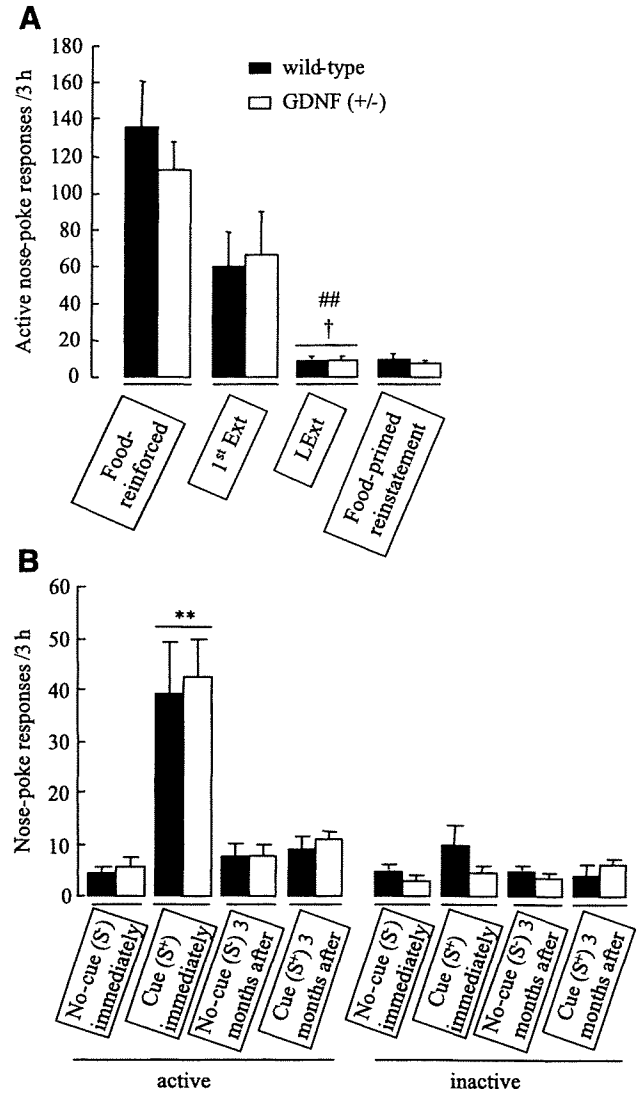

Figure 2. Extinction and reinstatement of food-seeking behavior in GDNF(+/−) and wild-type mice. *A)* Active nose-poke responses during stable phase of food-reinforced operant responding, extinction training, and food-primed reinstatement. *B)* Nose-poke responses in food-associated cue-induced reinstatement 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

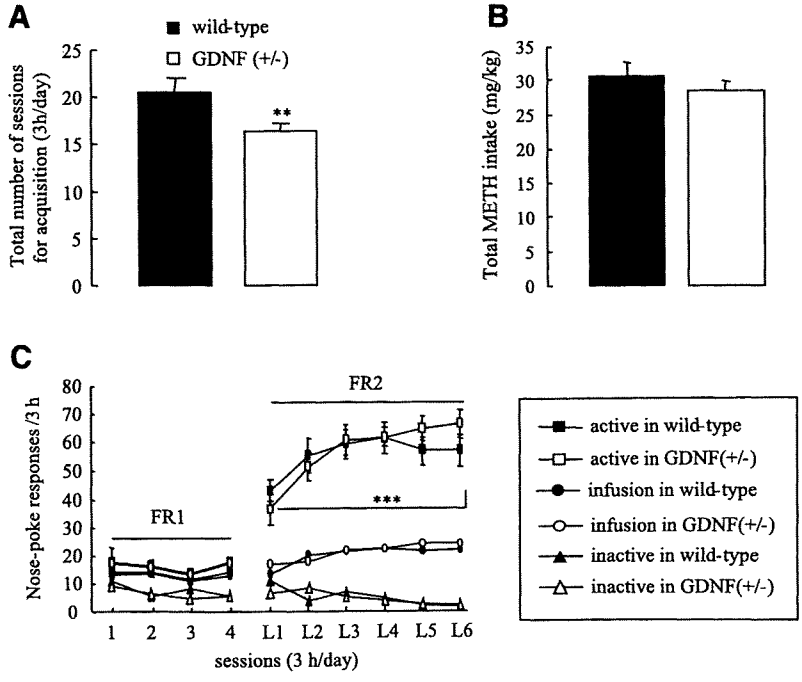

Figure 3. Acquisition of stable METH (0.1 mg/kg/infusion) self-administration behavior in GDNF (+/−) and wild-type mice. *A)* Total number of training sessions needed to acquire stable METH self-administration behavior for GDNF (+/−) and wild-type mice. $^{**}P < 0.01$ *vs.* wild-type littermates. *B)*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

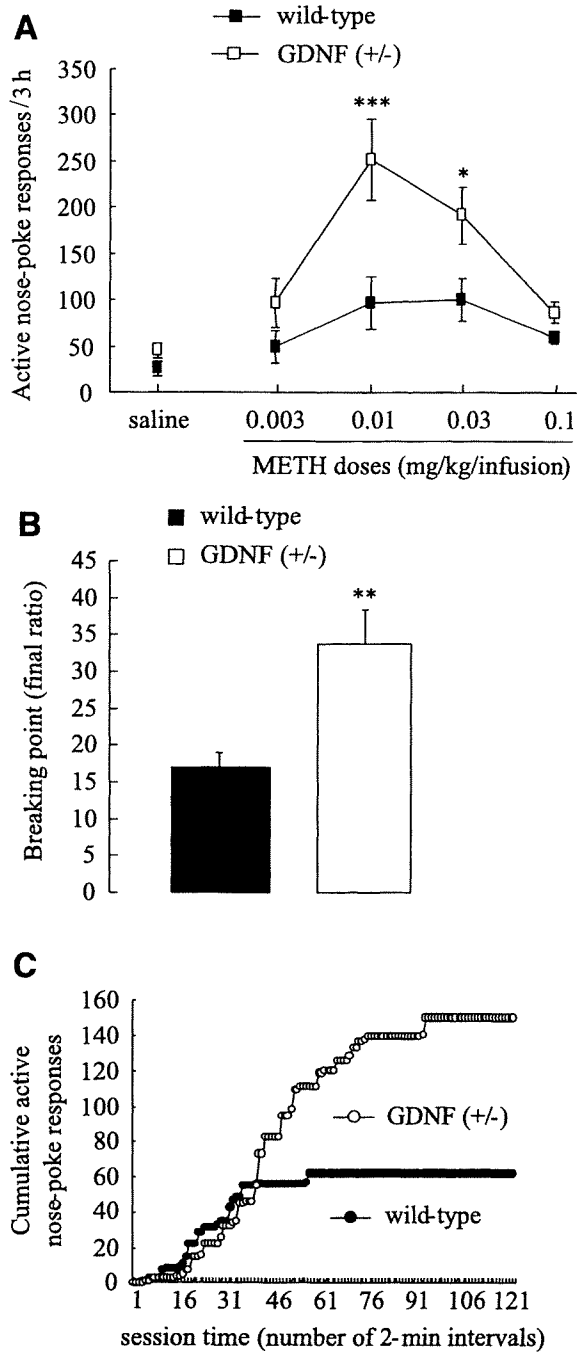

Figure 4. Dose responses and motivation for METH self-administration in GDNF (+/−) and wild-type mice. *A)* Dose-response function for METH self-administration under an FR2 schedule of reinforcement. *B)* Breaking points (final ratio) under a PR schedule of reinforcement. *C)* Representative curves for 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

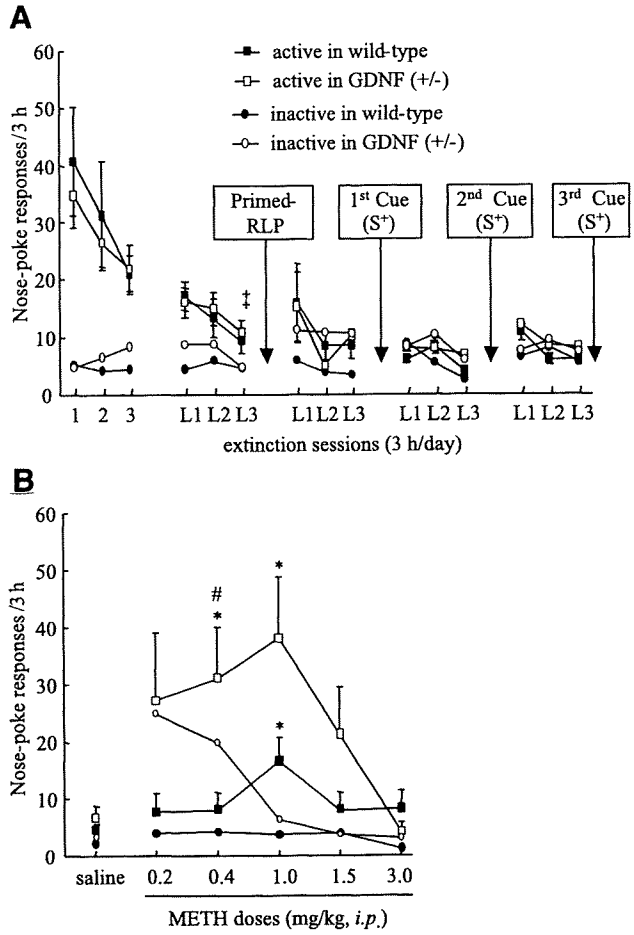

Figure 5. Nose-poke responses during extinction training (*A*) and METH-primed reinstatement of drug-seeking behavior (*B*; Primed-RLP) in GDNF (+/−) and wild-type mice. In *A*, data are from first 3 daily 3 h sessions (indicated by 1–3) and last 3 daily 3 h sessions (indicated by L1-L3) during 6–10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

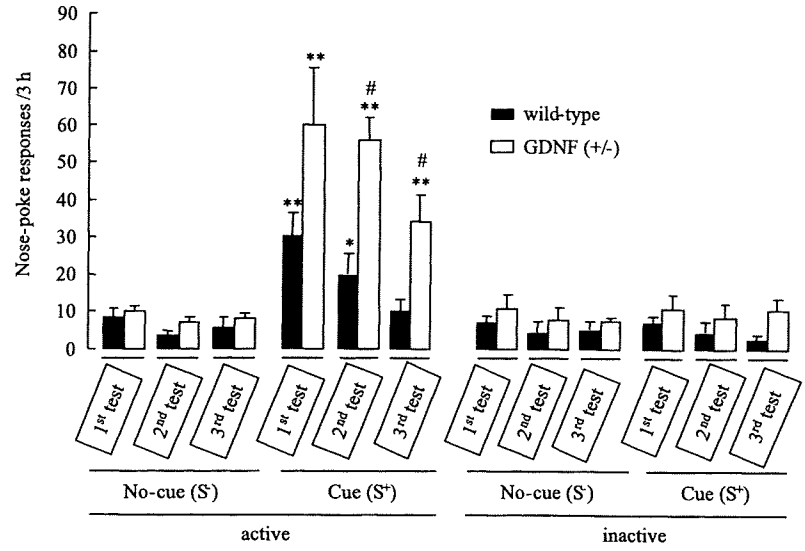

Figure 6. Active and inactive nose-poke responses during cue-induced reinstatement of METH-seeking behavior in GDNF (+/−) and wild-type mice. Tests for cue-induced reinstatement were conducted immediately (without withdrawal, 1st test), 3 months (2nd test), and 6 months (3rd test) after withdrawal from 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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A Novel Molecule "Shati" Is Involved in Methamphetamine-Induced Hyperlocomotion, Sensitization, and Conditioned Place Preference

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Drug addiction places an enormous burden on society through its repercussions on crime rate and healthcare. Repeated exposure to drugs of abuse causes cellular adaptations in specific neuronal populations that ultimately can lead to a state of addiction. In the present study, we have identified a novel molecule "shati" from the nucleus accumbens (NAc) of mice treated with methamphetamine (METH) using the PCR-select complementary DNA subtraction method. Moreover, we investigated whether shati is involved in METH-induced hyperlocomotion, sensitization, and conditioned place preference (CPP). METH induced expression of shati mRNA dose dependently via dopamine (DA) receptors. We prepared antibodies against shati and, using them, found shati to be expressed in neuronal cells of the mouse brain. Treatment with the shati antisense oligonucleotide (shati-AS), which significantly inhibited the expression of shati mRNA, enhanced the acute METH response, METH-induced behavioral sensitization, and CPP. Blockage of shati mRNA by shati-AS potentiated the METH-induced increase of DA overflow in the NAc and the METH-induced decrease in synaptosomal and vesicular DA uptake in the midbrain. These results suggest that a novel molecule shati is involved in the development of METH-induced hyperlocomotion, sensitization, and CPP. The functional roles of shati in METH-regulated behavioral alternations are likely to be mediated by its inhibitory effects on the METH-induced increase of DA overflow in the NAc and the METH-induced decrease in DA uptake in the midbrain.

Key words: shati; methamphetamine; behavioral sensitization; conditioned place preference; dopamine; addiction

Introduction

In terms of lost lives and productivity, drug dependence remains one of the most serious threats to the public health of a nation (Nestler, 2002). Drugs of abuse, including methamphetamine (METH), modulate the activity of mesolimbic dopaminergic neurons, projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Koob, 1992; Wise, 1996b; Koob et al., 1998). The psychostimulatory effects of METH are associated with an increase in extracellular dopamine (DA) levels in the brain, by facilitating the release of DA from presynaptic nerve terminals and inhibiting reuptake (Heikkila et al., 1975; Seiden et al., 1993; Giros et al., 1996). In rodent, augmentation of behavioral responses to psychostimulants is observed during and after their repeated administration. Therefore, it has been proposed that activity-dependent synaptic plasticity and remodeling of the mesolimbic dopaminergic system may play a crucial role in drug dependence (Nestler, 2001; Yamada and Nabeshima, 2004).

Using cDNA microarrays, changes in the mRNA expression profile in relevant brain regions (e.g., NAc) have been assessed after chronic administration of abused drugs (Douglass and Daoud, 1996; Cha et al., 1997; Wang et al., 1997). Evidence from this line of research has implicated nuclear factor-κB (Ang et al., 2001) and ΔFosB (Zachariou et al., 2006) in signal transduction pathways that modulate behavioral effects induced by drugs and contribute to long-term neuronal changes associated with dependence (Laakso et al., 2002). To elucidate the mechanism, caused by chronic drug abuse, of stable changes in the brain that play a role in the long-lasting behavioral abnormalities of dependent subjects, the candidates for drug-dependence-related genes

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whose expression was altered by repeated administration of METH or morphine (MOR) were screened by using cDNA microarray. Recently, there are many studies that showed that cytokines/neurotrophic factors and extracellular matrix/proteases play critical roles in activity-dependent synaptic plasticity and remodeling of the mesocorticolimbic dopaminergic system (Horger et al., 1999; Messer et al., 2000; Mizoguchi et al., 2007). We found that tumor necrosis factor-α (TNF-α) plays a neuroprotective role in METH-induced dependence and neurotoxicity (Nakajima et al., 2004) and reduces MOR-induced rewarding effects and behavioral sensitization (Niwa et al., 2007a,d). Furthermore, the rewarding effects and sensitization induced by METH and MOR are attenuated by Leu-Ile, an inducer of TNF-α, and glial cell line-derived neurotrophic factor (GDNF) (Niwa et al., 2007a–d). The tissue plasminogen activator (tPA)–plasmin system potentiates the rewarding and locomotor-stimulating effects of METH, MOR, and nicotine by regulating release of DA (Nagai et al., 2004, 2005a,b, 2006). However, the exact neuronal circuits and molecular cascade essential for drug dependence remain unclear. Therefore, we attempt to explore the novel molecules that play more critical roles in drug dependence, because the functions of molecules targeted by DNA microarray screening have been already well known.

In the present study, we identified a novel molecule "shati" from the NAc of mice treated with METH using the PCR-select cDNA subtraction method, which is a differential and epochal cloning technique. Moreover, we demonstrated that shati is involved in the METH-induced hyperlocomotion, sensitization, and conditioned place preference (CPP).

Materials and Methods

Animals. The male C57BL/6J inbred mice were obtained from SLC Japan (Hamamatsu, Japan). Animals were housed in plastic cages and kept in a temperature-, humidity-, and light-controlled room (23 ± 1°C; 50 ± 5% humidity; 12 h light/dark cycle starting at 8:00 A.M.) and had *ad libitum* access to food and water, except during behavioral experiments. All animal care and use was in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the *Guidelines of Experimental Animal Care* issued from the Office of the Prime Minister of Japan.

PCR-select cDNA subtraction. Mice were administered METH (2 mg/kg, s.c.) or saline for 6 d and took NAc 2 h after the last injection of METH. PCR-select cDNA subtraction (Clontech, Palo Alto, CA) was performed using a previously established procedure (Diatchenko et al., 1996; Gurskaya et al., 1996) to detect the genes in the NAc affected by METH treatment. Briefly, they involve hybridization of cDNA from one population (tester; METH-treated NAc) to excess of mRNA (cDNA) from other population (driver; saline-treated NAc) and then separation of the unhybridized fraction (target) from hybridized common sequences. Total RNAs were extracted by RNeasy Max (Qiagen, Hilden, Germany). For each subtraction, we performed two PCR amplifications. Products from the secondary PCRs were inserted into pCRII using a T/A cloning kit (Invitrogen, Carlsbad, CA). Plasmid or cosmid DNAs were prepared using QIAwell 8 Plus kit (Qiagen) according to the protocol of the manufacturer. Nucleic acid homology searches were performed using the BLAST (basic local alignment search tool) program through e-mail servers at the National Center for Biotechnology Information (NCBI) (National Institutes of Health, Bethesda, MD).

Structure models. Homology modeling for C-terminal domain of shati was established using Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, Quebec, Canada). Molecular mechanics calculations were performed by using an MMFF94x force field. Docking simulations of acetyl-CoA or ATP with shati protein were also examined using MOE software (Chemical Computing Group) to calculate the interactive potential energy of molecules.

Table 1. Primers sequences and their targets for RT-PCR

Primer	Sequence	Target (bp)
1		
Forward	5′-CTTGCCTCCCCAGCCCATCA-3′	1987–2006
Reverse	5′-CTGGGGGCCAGGGTTCTGCT-3′	2147–2166
2		
Forward	5′-GGGTGGCCGGGTAGGTGGAA-3′	2909–2928
Reverse	5′-GGCAGTGCCCAGCCCTTCCT-3′	3073–3092
3		
Forward	5′-TGTACATTCCTCCCTGGTGGTG-3′	3521–3542
Reverse	5′-AAATCTGAGAGCTGCAAGAAAATAGGG-3′	3594–3620

The amplification consisted of an initial step (95°C for 5 min) and then 35 cycles of denaturation for 30 s at 94 °C and annealing for 1 min at 70, 71, and 65°C.

Reverse transcription-PCR and real-time reverse transcription-PCR. Mice were administered METH (0.3, 1, and 2 mg/kg, s.c., once a day for 3 or 6 d) and decapitated 2 h after the last injection of METH. In the real-time reverse transcription (RT)-PCR experiment on the antagonism of METH-induced shati mRNA expression, mice were treated with the DA D_1-like receptor antagonist *R*(+)-SCH23390 [*R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine] (0.1 mg/kg, i.p.) or DA D_2-like receptor antagonist raclopride (2 mg/kg, i.p.) 30 min before METH (2 mg/kg, s.c.) once a day for 6 d. Functionally, *R*(+)-SCH23390 (0.1–0.5 mg/kg) is a potent blocker of stereotyped behaviors and increased locomotion induced by amphetamine or apomorphine (Christensen et al., 1984; Napier et al., 1986). The increase in TNF-α or tPA mRNA expression in the NAc induced by METH is inhibited by pretreatment with either *R*(+)-SCH23390 (0.1 or 0.5 mg/kg, i.p.) or raclopride (2 mg/kg, i.p.) (Nakajima et al., 2004; Nagai et al., 2005a). *R*(+)-SCH23390 at the dose of 0.1 mg/kg, not 0.03 mg/kg, significantly inhibits the hyperphosphorylation of extracellular signal-regulated kinase 1/2 in the NAc and striatum evoked by METH-induced CPP as well as the expression of CPP in METH-treated animals (Mizoguchi et al., 2004). Depending on these evidences, we selected the doses of *R*(+)-SCH23390 at 0.1 mg/kg and raclopride at 2 mg/kg.

Total RNA was isolated using an RNeasy kit (Qiagen) and converted into cDNA using a SuperScript First-Strand System for RT-PCR kit (Invitrogen). The primers used for RT-PCR were as follows: 5′-CTTGCCTCCCCAGCCCATCA-3′ (forward-1; base pairs 1987–2006) and 5′-CTGGGGGCCAGGGTTCTGCT-3′ (reverse-1; base pairs 2147–2166) for set of sequences 1; 5′-GGGTGGCCGGGTAGGTGGAA-3′ (forward-2; base pairs 2909–2928) and 5′-GGCAGTGCCCAGCCCTTCCT-3′ (reverse-2; base pairs 3073–3092) for set of sequences 2; and 5′-TGTACATTCCTCCCTGGTGGTG-3′ (forward-3; base pairs 3521–3542) and 5′-AAATCTGAGAGCTGCAAGAAAATAGGG-3′ (reverse-3; base pairs 3594–3620) for set of sequences 3 (Table 1). The amplification consisted of an initial step (95°C for 5 min) and then 35 cycles of denaturation for 30 s at 94°C and annealing for 1 min at 70, 71, and 65°C in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The levels of shati and TNF-α mRNA were determined by real-time RT-PCR using a TaqMan probe. The 18S ribosomal RNA was used as the internal control (PE Applied Biosystems, Foster City, CA). The mouse shati primers used for real-time RT-PCR were as follows: 5′-TGTAAACACCCCTAAAGTGCCCT-3′ (forward; base pairs 2967–2989) and 5′-TCAATCCTGCATACAAGGAATCAA-3′ (reverse; bare pairs 3022–3045); and TaqMan probe, 5′-CACAGTCTGTGAGGCTCAGGTTGCCC-3′ (probe; base pairs 2995–3020). The amplification consisted of an initial step (95°C for 5 min) and then 40 cycles of denaturation for 30 s at 95°C and annealing for 1 min at 59°C in an iCycle iQ Detection System (Bio-Rad, Hercules, CA). The expression levels were calculated as described previously (Wada et al., 2000).

Immunohistochemistry. Two antibodies against the peptide of the hypothetical protein, CNTAFRGLRQHPRTQLL (S-3) and CMSVDSRFRGKGIAKALG (S-4), unique to shati were generated. These peptides were conjugated to the keyhole limpet hemocyanin and injected into rabbits six times at 1 week intervals. Serum was taken from the rabbits 1

week after the final injection of these peptides. The serum was diluted 200 times used for the immunostaining.

For immunohistochemical analysis, mice were killed 24 h after repeated treatment with METH (2 mg/kg, s.c., once a day for 6 d). The brains were sliced at 20 μm in the cryostat. Polyclonal rabbit anti-S-3 or S-4 antibody (1:200), monoclonal mouse anti-neuron-specific nuclear antigen (NeuN) antibody (1:200; Chemicon, Temecula, CA), and monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:200; Chemicon) served as primary antibodies. Goat anti-mouse Alexa Fluor 546 (1:1000; Invitrogen) and goat anti-rabbit Alexa Fluor 488 (1:1000; Invitrogen) were used as secondary antibodies. Each stained slice was observed under a fluorescence microscope (Axioskoop 2 plus; Zeiss, Jena, Germany) and checked with Axiovision 3.0 systems (Zeiss).

Shati-antisense oligonucleotide treatment. Mice were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic apparatus. The infusion cannula was connected to a miniosmotic pump (total capacity was 90 μl, Alzet 1002; Alza, Palo Alto, CA) filled with shati-antisense oligonucleotide (shati-AS) and -scramble oligonucleotide (shati-SC) and was implanted into the right ventricle [anteroposterior (AP) −0.5 mm, mediolateral (ML) +1.0 mm from the bregma, and dorsoventral (DV) −2.0 mm from the skull, according to the atlas of Franklin and Paxinos (1997)]. No histological or mechanical disruption was produced by implantation of the infusion cannula (data not shown). Phosphorothionate oligonucleotides were custom synthesized at Nisshinbo Biotechnology (Tokyo, Japan) and dissolved in artificial CSF (in mM: 147 NaCl, 3 KCl, 1.2 $CaCl_2$, and1.0 $MgCl_2$, pH 7.2). We used shati-SC as a control of shati-AS, because we should deny the secondary effects on other genes or toxic effects, and we selected the design of shati-AS, which does not affect the other genes and already have been identified. The oligonucleotides were phosphorothionated at the three bases of both 5′ and 3′ ends, which results in increased stability and less toxicity. The sequences of shati-AS and shati-SC were 5′-TCTTCGTCTCGCAGACCATGTCG-3′ and 5′-GGTCTGCTACACTGCTGCTAGTC-3′, respectively. Shati-AS and shati-SC were continuously infused into the cerebral ventricle at a dose of 1.8 nmol/6 μl per day (flow rate, 0.25 μl/h). Additionally, shati-SC was used as a control. Three days after the start of oligonucleotide infusion, mice were subjected to METH treatment for sensitization.

Locomotor activity. Locomotor activity was measured using an infrared detector (Neuroscience Company, Tokyo, Japan) in a plastic box (32 × 22 × 15 cm high) and determined as described previously (Nakajima et al., 2004; Niwa et al., 2007b,d). One day after the start of oligonucleotide infusion, mice were habituated for 3 h in the box for 2 d and then administered METH (1 mg/kg, s.c.) or saline once a day for 5 d. Locomotor activity was measured for 2 h immediately after the METH or saline administration.

In vivo *microdialysis.* Mice were anesthetized with sodium pentobarbital, and a guide cannula (AG-8; EICOM, Kyoto, Japan) was implanted into the NAc (AP +1.7 mm, ML +0.8 mm mediolateral from the bregma, and DV −4.0 mm from the skull) according to the atlas of Franklin and Paxinos (1997) and secured to the skull using stainless steel screws and dental acrylic cement. Mice were administered METH (1 mg/kg, s.c.) 3 d after implantation of the guide cannula and the start of oligonucleotide infusion. One day after METH treatment for 2 d, a dialysis probe (AI-8-1, 1 mm membrane length; EICOM) was inserted through the guide cannula and perfused continuously with CSF (in mM: 147 NaCl, 4 KCl, and 2.3 $CaCl_2$) at a flow rate of 1.0 μl/min. Dialysate was collected in 20 min fractions and injected into the HPLC system (EICOM) for the measurement of DA levels. Three samples were used to establish baseline levels of DA before the administration of METH (1 mg/kg, s.c.).

Synaptosomal [^{3}H]DA uptake. Three days after the start of oligonucleotide infusion, mice were subjected to METH treatment once a day for 3 d. Mice were decapitated 1 h after the final METH treatment. Midbrain synaptosomal [^{3}H]DA uptake was determined as described previously (Fleckenstein et al., 1997; Nakajima et al., 2004; Niwa et al., 2007b). The final concentration of [^{3}H]DA (PerkinElmer, Wellesley, MA) was 5 nM. Samples were incubated at 37°C for 4 min, and then ice-cold Krebs–Ringer's solution containing 10 μM GBR12909 [1-(2[bis(4-fluorophenyl-)methoxy]ethyl)-4-(3-phenylpropyl)piperazine] bimesylate hydrate] (Sigma, St. Louis, MO), a specific DA uptake inhibitor, was added. Nonspecific values were determined in the presence of 100 μM GBR12909 during the incubation. The radioactivity trapped on filters was measured with a liquid scintillation counter (Beckman Coulter, Fullerton, CA).

Vesicular [^{3}H]DA uptake. Vesicular [^{3}H]DA uptake was determined as described by Erickson et al. (1990). Synaptosomes were prepared as described by Nakajima et al. (2004). Vesicular [^{3}H]DA uptake was performed by incubating synaptic vesicle samples (15 μg protein/100 μl) at 30°C for 4 min in assay buffer (in mM: 25 HEPES, 100 potassium tartrate, 1.7 ascorbic acid, 0.05 EGTA, 0.1 EDTA, and 2 ATP-Mg^{2+}, pH 7.0) in the presence of 30 nM [^{3}H]DA (PerkinElmer). The reaction was terminated by the addition of 1 ml of cold wash buffer (assay buffer containing 2 mM $MgSO_4$ substituted for the ATP-Mg^{2+}, pH 7.0) and rapid filtration. Nonspecific values were determined by measuring vesicular [^{3}H]DA uptake at 4°C. The radioactivity was measured with a liquid scintillation counter (Beckman Coulter).

Conditioned place preference. The apparatus used for the place conditioning task consisted of two compartments: a transparent Plexiglas box and a black Plexiglas box (both 15 × 15 × 15 cm high). To enable mice to distinguish easily the two compartments, the floors of the transparent and black boxes were covered with white plastic mesh and black frosting Plexiglas, respectively. Each box could be divided by a sliding door (10 × 15 cm high). The place conditioning paradigm was performed by using a previously established procedure with a minor modification (Noda et al., 1998; Schechter and Calcagnetti, 1998; Niwa et al., 2007a,b,d). In the preconditioning test, the sliding door was opened, and the mouse was allowed to move freely between both boxes for 15 min once a day for 3 d. On the third day of the preconditioning test, we measured the time that the mouse spent in the black and transparent boxes by using a Scanet SV-20 LD (Melquest, Toyama, Japan). The box in which the mouse spent the most time was referred to as the "preferred side" and the other box as the "nonpreferred side." Conditioning was performed during 6 successive days. Mice were given METH or saline in the apparatus with the sliding door closed. That is, a mouse was subcutaneously given METH and put in its nonpreferred side for 20 min. On the next day, the mouse was given saline and placed opposite the drug conditioning site for 20 min. These treatments were repeated for three cycles (6 d). In the postconditioning test, the sliding door was opened, and we measured the time that the mouse spent in the black and transparent boxes for 15 min, using the Scanet SV-20 LD. Place conditioning behavior was expressed by Post-Pre, which was calculated as: [(postvalue) − (prevalue)], where postvalue and prevalue were the difference in time spent at the drug conditioning and the saline conditioning sites in the postconditioning and preconditioning tests, respectively.

Statistical analysis. All data were expressed as means ± SE. Statistical differences between two groups were determined with Student's *t* test. Statistical differences among more than three groups were determined using a one-way ANOVA, two-way ANOVA, or an ANOVA with repeated measures (two or three-factor), followed by the Bonferroni's multiple comparison test (Bonferroni's correction; 3, 6, 15, and 36 comparisons in 3, 4, 6, and 9 groups, respectively). $p < 0.05$ was regarded as statistically significant.

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

Results

Identification of shati

The reasons why we pursued shati for intensive investigation arose from our preliminary findings with the PCR-select cDNA subtraction method to detect the genes in the NAc affected by METH treatment: mice were administrated METH (2 mg/kg, s.c.) or saline for 6 d, and shati mRNA production in the NAc was found to increase by 640% in METH-treated mice with robust behavioral sensitization compared with saline-treated mice (data not shown). The sequence of cDNA was completely matched to accession number NM_001001985 of NCBI gene bank (the gene

record was replaced by accession number NM_001001985.2 on April, 10, 2005). The sequence has been identified by the Mammalian Gene Collection Program Team (Strausberg et al., 2002). Blackshaw et al. (2004) has demonstrated the extended cDNA sequence by serial analysis of gene expression methods, which provides an unbiased and nearly comprehensive readout of gene expression and that the gene was for one of the proteins related to the retina development. We named this novel molecule shati after the symbol at Nagoya castle in Japan. The sequence is translated to a protein LOC269642 (accession number is NP_001001985.1 and 2; 001001985.1 was a part of 001001985.2) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Characterization of shati

Homology modeling for C-terminal domain of shati was established using MOE software (Chemical Computing Group) (Fig. 1*A*,*B*). Red character in Figure 1*A* showed homology modeling of shati. From motif analysis of shati, shati contained the sequence of GCN5-related *N*-acetyltransferase (GCAT) (Fig. 1*C*). Underlined character in Figure 1*A* showed GNAT motif. Docking simulations of acetyl-CoA or ATP with shati protein were also examined using MOE software (Chemical Computing Group) to calculate the interactive potential energy of molecules. Shati also contained acetyl-CoA binding or ATP binding site, because the analysis showed the lowest interactive potential energy of shati with acetyl-CoA or ATP, −301 and −322 kcal, respectively (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Docking simulations of shati with DA, DNA binding site, and nuclear localization signals showed too high interactive potential energy of molecules or no domain.

Expression of shati mRNA

As shown in Figure 2*A*, RT-PCR analysis revealed that shati is expressed at high levels in the cerebrum, cerebellum, liver, kidney, and spleen. We amplified and analyzed its three different target sequences by RT-PCR (Table 1). Similar results of RT-PCR were obtained with three different sets of primers (Fig. 2*A*).

We performed a series of experiments to validate the results of cDNA subtraction. Repeated METH treatment (2 mg/kg, s.c.) for 6 d significantly elevated the mRNA levels of the target sequences of shati in the NAc (Fig. 2*B*).

METH-induced expression of shati mRNA in the brain

As an initial step in assessing the relationship between shati and METH-induced sensitization and dependence, we examined whether single and repeated METH treatment altered the expression of shati mRNA in the mouse brain using the real-time RT-PCR method. The effects of repeated METH treatment (0.3, 1 and 2 mg/kg, s.c. for 3 d) on shati mRNA expression in the NAc were dose dependent ($F_{(3,28)} = 5.503$; $p < 0.01$, one-way ANOVA) (Fig. 3*A*). The levels of shati mRNA were significantly increased 2, 6, and 24 h after the last METH treatment and then returned to control value 1 week after the treatment ($F_{(6,41)} = 4.444$; $p < 0.01$, one-way ANOVA) (Fig. 3*B*). Single METH treatment (2 mg/kg, s.c.) remarkably induced shati mRNA expression in the NAc and hippocampus (Hip). METH (2 mg/kg, s.c.) or saline challenge on day 6 after repeated administration of METH (2 mg/kg, s.c.) for 5 d remarkably induced shati mRNA expression in the frontal cortex (Fc), NAc, and caudate–putamen (CPu) (repeated drug administration, $F_{(1,32)} = 20.368$, $p < 0.01$ for Fc; single administration, $F_{(1,32)} = 0.005$, $p = 0.942$ for Fc; repeated drug administration × single administration, $F_{(1,32)} = 1.643$, $p = 0.209$ for Fc; repeated drug administration, $F_{(1,31)} = 14.436$, $p <$

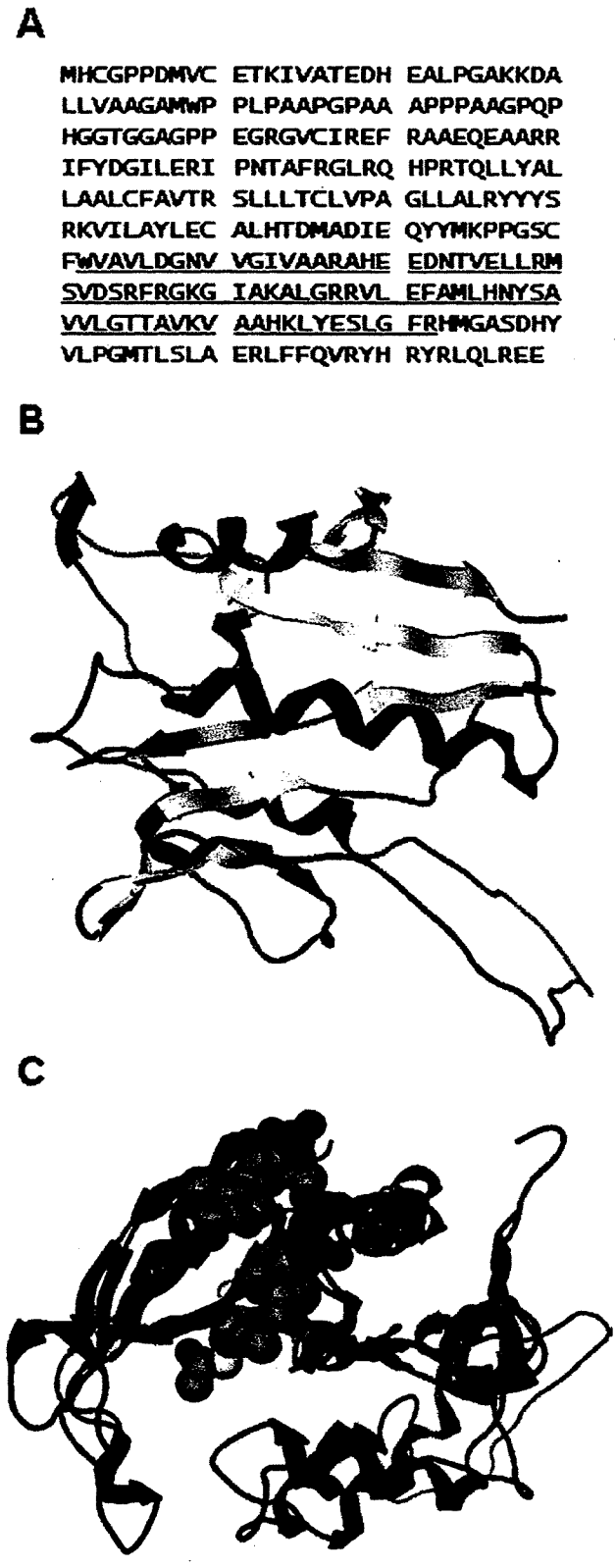


Figure 1. Characterization of shati. ***A***, The sequence of shati. The red character showed homology modeling of shati. The underlined character showed GCN5-related *N*-acetyltransferase motif. ***B***, Homology modeling for C-terminal domain of shati. ***C***, Homology modeling and motif analysis of shati. Shati has the sequence of GCAT. Red ribbon, Homology model of shati; sphere, acetyl-CoA analyzed by x-ray crystallography; green ribbon, *N*-acetyltransferase.

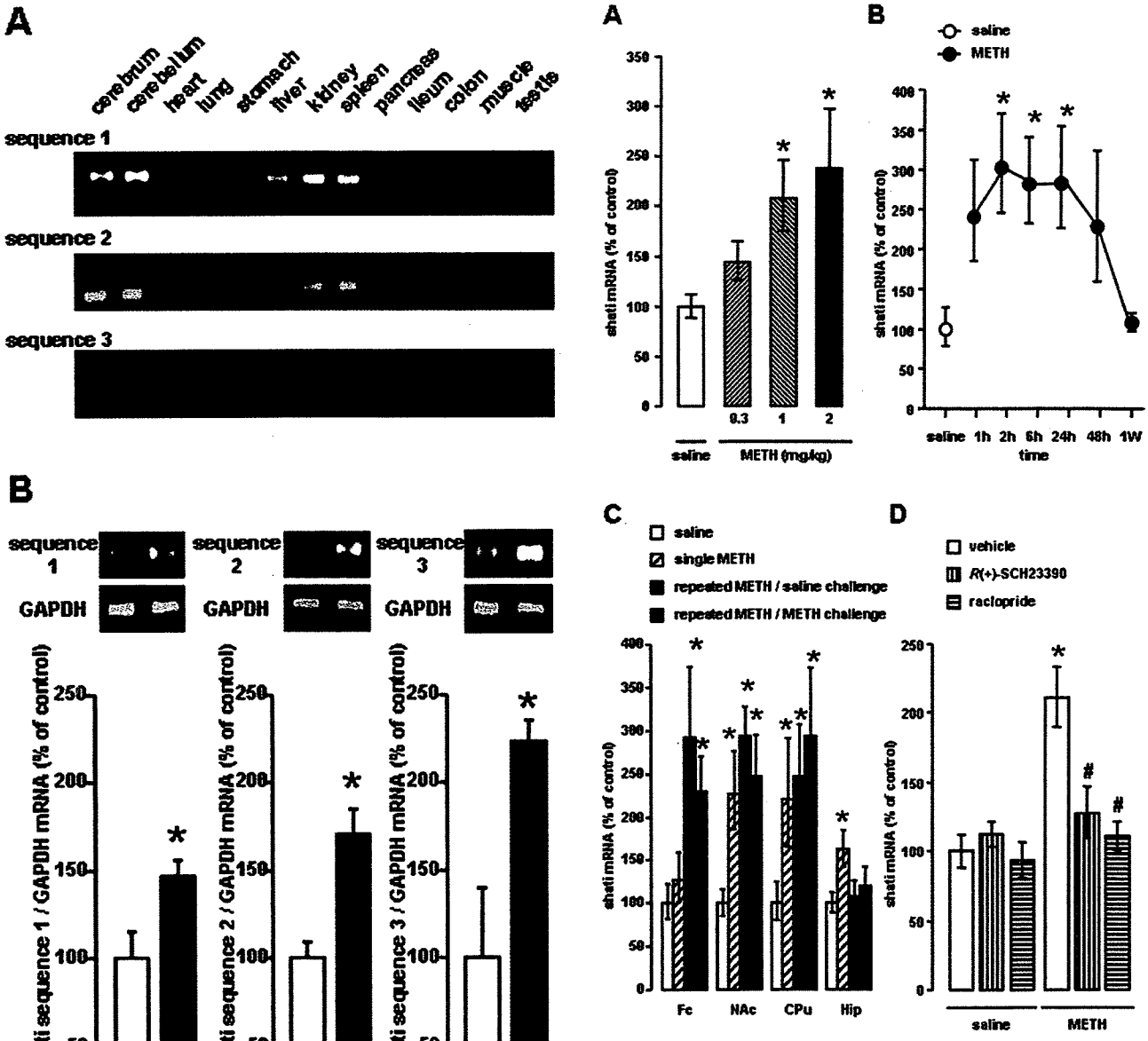
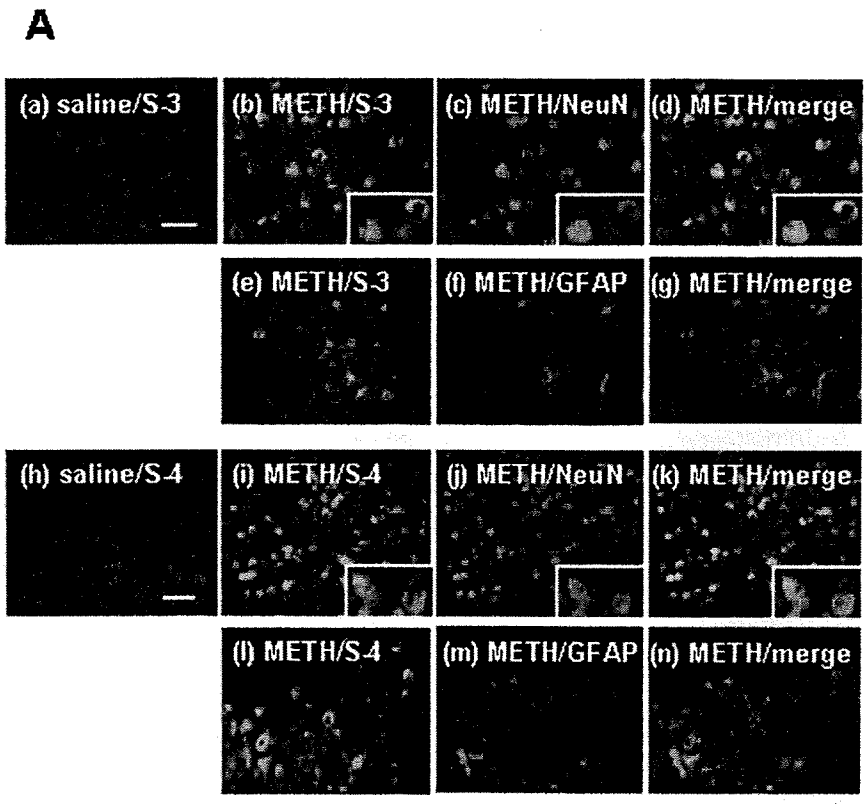


Figure 2. Expression of shati mRNA in the various organs of mice. ***A***, RT-PCR analysis of shati in the various organs in mice. Mice were decapitated without any treatment, and the brains were quickly removed. The sets of primers used for PCR are listed in Table 1. ***B***, Increase in the production of the three sets of target sequences of shati induced by repeated METH treatment in the NAc of mice. Mice were administered METH (2 mg/kg, s.c.) for 6 d and decapitated 2 h after the last METH treatment. Values are means ± SE ($n = 5$). *$p < 0.05$ versus saline-treated mice. The sets of primers used for PCR are listed in Table 1. To standardize the PCR products, we used primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control.

Figure 3. METH induced expression of shati mRNA in the brain. ***A***, Dose-dependent effect of repeated METH treatment on shati mRNA expression in the NAc. Mice were administered METH (0.3, 1, and 2 mg/kg, s.c.) for 3 d. Mice were decapitated 2 h after the last METH treatment. Values are means ± SE ($n = 8$). *$p < 0.05$ versus saline-treated mice. ***B***, Time course changes in the expression of shati mRNA after repeated METH treatment in the NAc. Mice were administered METH (2 mg/kg, s.c.) for 6 d and decapitated 1, 2, 6, 24, and 48 h and 1 week after the last METH treatment. Values are means ± SE ($n = 6–7$). *$p < 0.05$ versus saline-treated mice. ***C***, Changes in the expression of shati mRNA in the various brain regions (Fc, NAc, CPu, and Hip) of the mice after single and repeated METH treatment. Mice were administered METH (2 mg/kg, s.c.) for 5 d and challenged with METH (2 mg/kg, s.c.) or saline on day 6. Mice were decapitated 2 h after last treatment of METH (2 mg/kg, s.c.) or saline challenge. Values are means ± SE ($n = 8–10$). *$p < 0.05$ versus saline-treated mice. ***D***, The effects of the DA D_1-like receptor antagonist *R*(+)-SCH23390 or D_2-like receptor antagonist raclopride on METH-induced expression of shati mRNA in the NAc. Mice were treated with *R*(+)-SCH23390 (0.1 mg/kg, s.c.) or raclopride (2 mg/kg, s.c.) 30 min before daily METH (2 mg/kg, s.c. for 6 d) treatment. Mice were decapitated 2 h after the last METH treatment. Values are means ± SE ($n = 6–8$). *$p < 0.05$ versus vehicle/saline-treated mice. #$p < 0.05$ versus vehicle/METH-treated mice.

0.01 for NAc; single administration, $F_{(1,31)} = 4.917$, $p < 0.05$ for NAc; repeated drug administration × single administration, $F_{(1,31)} = 10.545$, $p < 0.01$ for NAc; repeated drug administration $F_{(1,32)} = 8.023$, $p < 0.01$ for CPu; single administration, $F_{(1,32)} = 4.833$, $p < 0.05$ for CPu; repeated drug administration × single administration, $F_{(1,32)} = 1.669$, $p = 0.206$ for CPu; repeated drug administration, $F_{(1,32)} = 0.628$, $p = 0.434$ for Hip; single administration, $F_{(1,32)} = 6.464$, $p < 0.05$ for Hip; repeated drug administration × single administration, $F_{(1,32)} = 2.496$, $p = 0.124$ for Hip; two-way ANOVA) (Fig. 3*C*). The increase caused by METH in the NAc was inhibited by pretreatment with either the DA



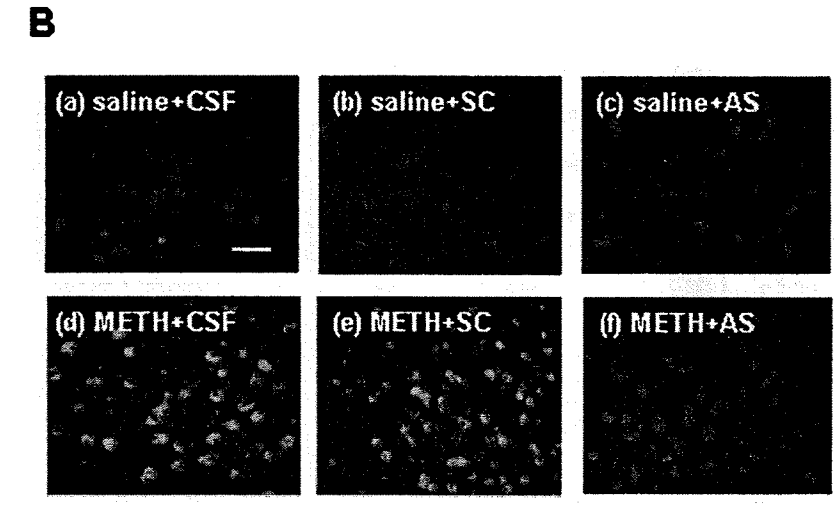


Figure 4. Immunostaining of shati in the NAc after repeated treatment with METH. Mice were administered METH (2 mg/kg, s.c.) for 6 d and decapitated 24 h after the last treatment. ***A***, Double-labeling fluorescence photomicrographs for shati and NeuN or GFAP. The shati-immunopositive cells (green) were colocalized with NeuN-immonopositive cells (red). Double immunostaining for S-3 or S-4 and NeuN in the NAc reveals shati expression in neuronal cells. Scale bars, 20 μm. ***B***, Effect of shati-AS on METH-induced increase in shati expression. METH-induced increase in shati expression in the NAc was inhibited by shati-AS. Scale bar, 20 μm.

D_1-like receptor antagonist *R*(+)-SCH23390 (0.1 mg/kg, i.p.) or the D_2-like receptor antagonist raclopride (2 mg/kg, i.p.) (agonist, $F_{(1,34)} = 18.649$, $p < 0.01$; antagonist, $F_{(2,34)} = 5.554$, $p < 0.01$; agonist × antagonist, $F_{(2,34)} = 5.382$, $p < 0.01$; two-way ANOVA) (Fig. 3*D*), although neither antagonists had an effect on shati mRNA expression in the saline-treated mice. These results indicate that METH induces the expression of shati mRNA in the brain through the activation of both DA D_1 and D_2 receptors.

Localization of shati in the brain of mice treated with METH

There were few shati-immunopositive cells in saline-treated mouse brain (Fig. 4*Aa*,*Ah*). METH (2 mg/kg, s.c. for 6 d) increased the number of shati-immunopositive cells in the NAc compared with that in saline-treated mice (Fig. 4*A*). The shati-immunopositive cells were diminished when the antibodies were absorbed by S-3 or S-4 antigen (data not shown). The shati-immunopositive cells were colocalized with the cells that were immunopositive for NeuN, a neuronal marker, but not for GFAP, an astroglial marker, in the NAc of mice (Fig. 4*Ab–Ag*,*Ai–An*). The repeated METH treatment-induced increase in the numbers of shati-immunopositive cells in the NAc was abolished by shati-AS treatment, although shati-SC had no effect (Fig. 4*Ba–Bf*).

Roles of shati in METH-induced hyperlocomotion and sensitization

To examine the role of shati in the behavioral and neurochemical phenotype in response to METH, we used an AS strategy, which widely used to manipulate gene expression in the brain via intracerebroventricular infusion (Taubenfeld et al., 2001; Bowers et al., 2004). The experimental schedules are shown in Figure 5, *A* and *C*. The AS downregulated the expression of shati mRNA in the NAc (Fig. 5*B*). The increase in the levels of shati mRNA expression evoked by repeated METH treatment in the NAc was significantly and completely abolished by shati-AS, although shati-SC had no effect. Moreover, shati mRNA expression in the NAc of saline-treated mice was also reduced by shati-AS, whereas shati-SC did not affect the expression in saline-treated mice (drug, $F_{(1,42)} = 72.765$, $p < 0.01$; intracerebroventricular treatment, $F_{(2,42)} = 14.104$, $p < 0.01$; drug × intracerebroventricular treatment, $F_{(2,42)} = 0.092$, $p = 0.912$; two-way ANOVA) (Fig. 5*B*), indicating that shati-AS has an ability to reduce effectively the expression of shati mRNA. We also examined the effect of shati-AS on tPA expression as one of drug-dependence-related other proteins, because tPA–plasmin system potentiates the rewarding and locomotor-stimulating effects of METH, MOR, and nicotine by regulating release of DA (Nagai et al., 2004, 2005a,b, 2006). The increase in the levels of tPA mRNA expression in the NAc was not abolished by shati-AS (drug, $F_{(1,47)} = 62.530$, $p < 0.01$; intracerebroventricular treatment, $F_{(2,47)} = 0.148$, $p = 0.862$; drug × intracerebroventricular treatment, $F_{(2,47)} = 0.803$, $p = 0.454$; two-way ANOVA). Moreover, tPA mRNA expression in the NAc of saline-treated mice was not also reduced by shati-AS, indicating that shati-AS has no ability to

reduce effectively the expression of tPA mRNA (data not shown). Therefore, shati-AS is considered to have no secondary effects.

Repeated METH administration leads to a progressive augmentation of many behavioral effects of the drug (behavioral sensitization). Sensitization is of interest as a model for drug-induced neuroplasticity in neuronal circuits important for addiction. It is well established that the induction of sensitization involves complex neuronal circuitry (Wolf, 1998). In rodent, sensitization is observed as a progressive augmentation of locomotor activity that may relate to an increase in the incentive to obtain drugs (Robinson and Berridge, 1993; Lorrain et al., 2000). There is also evidence of sensitization in human drug users (Satel et al., 1991) and normal subjects (Strakowski and Sax, 1998). Repeated METH treatment (1 and 2 mg/kg, s.c.) for 5 d produced behavioral sensitization [$F_{(2,12)} = 7.404$ for METH (1 mg/kg) plus shati-AS-treated mice; $F_{(2,18)} = 5.593$ for METH (1 mg/kg) plus shati-SC-treated mice; $F_{(2,18)} = 30.917$ for METH (1 mg/kg) plus CSF-treated mice; $F_{(2,12)} = 7.453$ for METH (2 mg/kg) plus shati-AS-treated mice, $F_{(2,12)} = 4.243$ for METH (2 mg/kg) plus shati-SC-treated mice; $F_{(2,15)} = 8.569$ for METH (2 mg/kg) plus CSF-treated mice; $p < 0.05$, one-way ANOVA] (Fig. 5*D*). As shown in Figure 5*D*, the shati-AS treatment potentiated the METH (1 mg/kg, s.c.)-induced hyperlocomotion and sensitization compared with shati-SC- or CSF-treated mice (drug, $F_{(2,141)} = 291.696$, $p < 0.01$; intracerebroventricular treatment, $F_{(2,141)} = 28.223$, $p < 0.01$; time, $F_{(2,141)} = 17.154$, $p < 0.01$; drug × intracerebroventricular treatment, $F_{(4,141)} = 12.432$, $p < 0.01$; drug × time, $F_{(4,141)} = 12.913$, $p < 0.01$; intracerebroventricular treatment × time, $F_{(4,141)} = 0.156$, $p = 0.960$; drug × intracerebroventricular treatment × time, $F_{(8,141)} = 0.427$, $p = 0.903$; three-factor repeated ANOVA), whereas the shati-AS, shati-SC, or CSF treatment had no effect on spontaneous locomotor activity (Fig. 5*D*). The sensitization was observed on day 10 after challenge administration of METH (0.3 mg/kg, s.c.). Shati-AS-treated mice showed a marked potentiation of METH (0.3 mg/kg, s.c.)-induced sensitization on day 10 compared with shati-SC- or CSF-treated mice ($F_{(2,13)} = 6.974$, $p < 0.05$, one-way ANOVA), although shati-AS-treated mice did not show a potentiation of METH (2 mg/kg, s.c.)-induced hyperlocomotion and sensitization compared with shati-SC- or CSF-treated mice on days 1–5 (Fig. 5*D*).

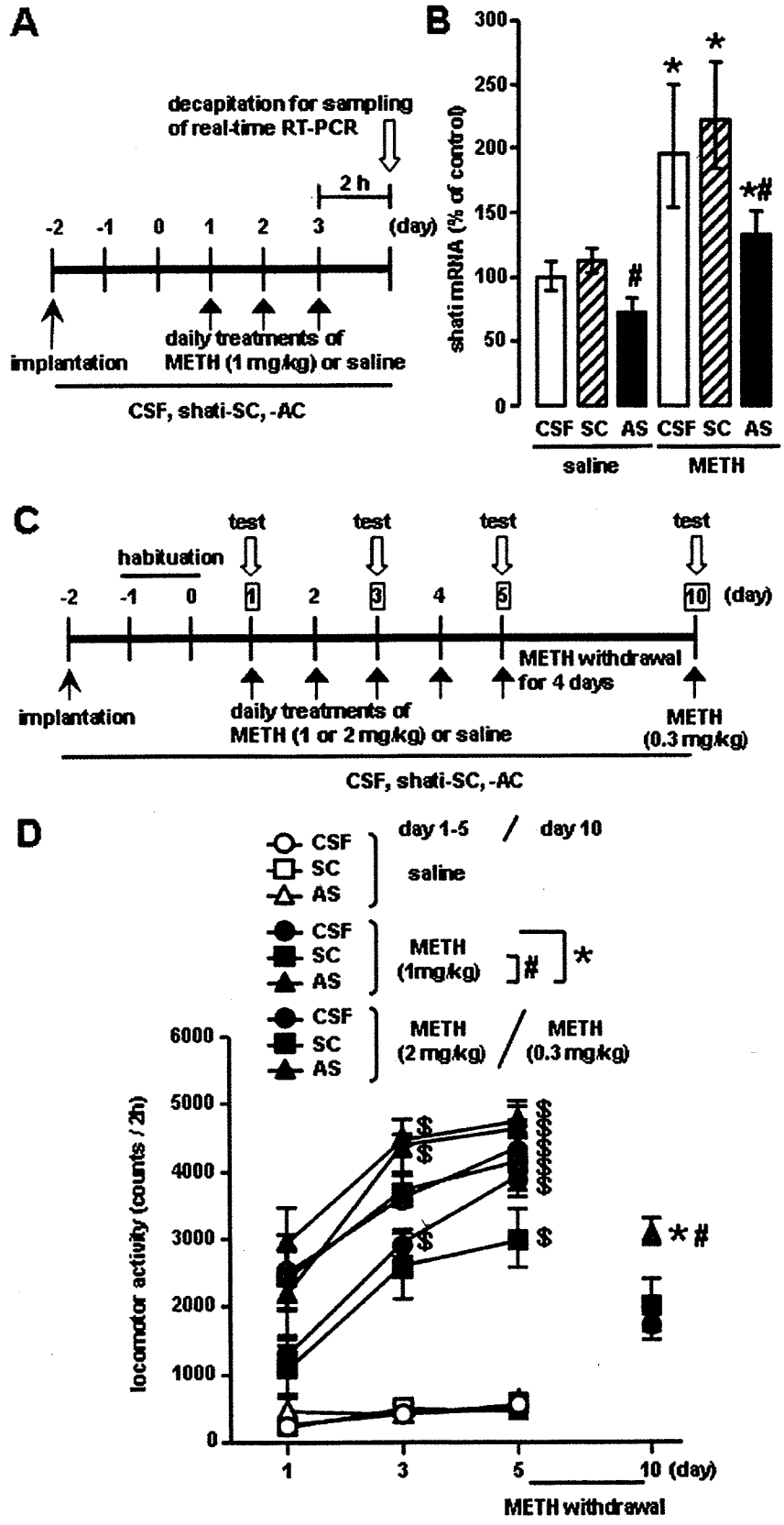


Figure 5. Roles of shati in METH-induced hyperlocomotion and sensitization. An osmotic minipump was used to deliver a continuous infusion of shati-AS (1.8 nmol/6 μl per day), shati-SC (1.8 nmol/6 μl per day), or CSF into the right ventricle (AP −0.5 mm, ML +1.0 mm from bregma, and DV −2.0 mm from the skull). *A*, Experimental schedule for the real-time RT-PCR using shati-AS. *B*, Effect of shati-AS on shati mRNA expression. Mice were administered METH (1 mg/kg, s.c.) for 3 d and decapitated 2 h