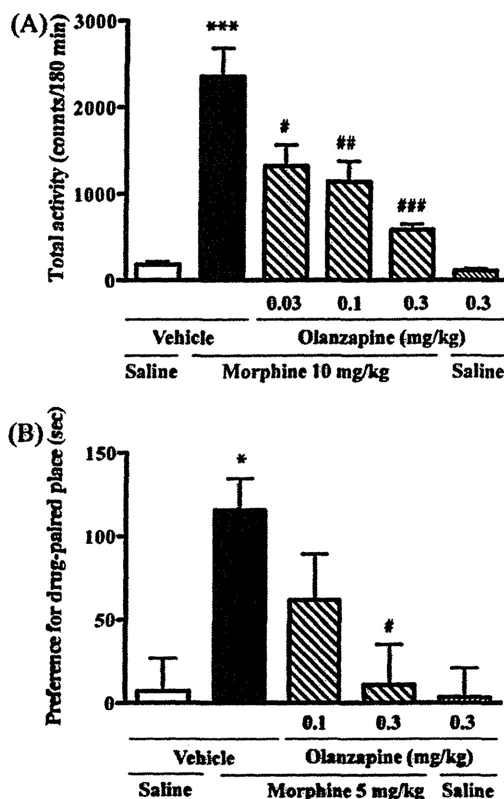

Fig. 2. Effects of olanzapine on the morphine-induced hyperlocomotion (A) and place preference (B) in mice. A: Each column represents the mean total activity for 180 min ± S.E.M. of 8–10 mice. B: Ordinate: mean differences between time spent in the postconditioning and preconditioning tests. Each column represents the mean ± S.E.M. of 6–8 mice. $*P < 0.05$, $***P < 0.001$ vs. vehicle-saline. $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. vehicle-morphine.

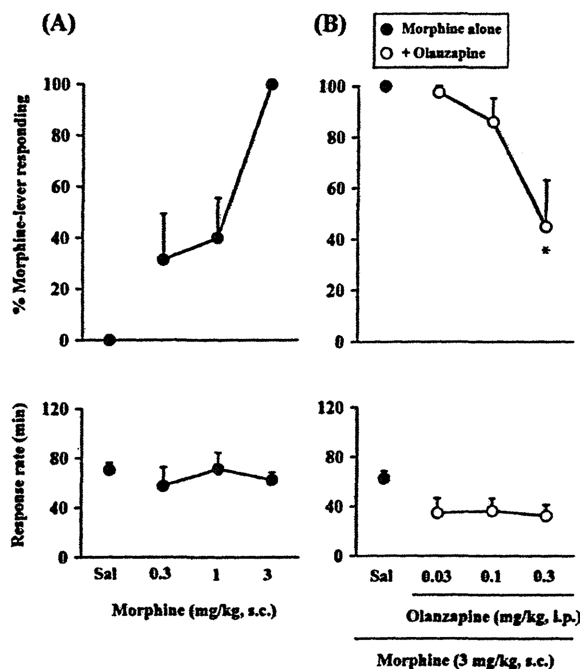

Fig. 3. A: Dose-response curve for % morphine-lever responses (upper panel) and response rates (bottom panel) in rats that had been trained to discriminate between 3 mg/kg morphine and saline. B: Effects of olanzapine on the discriminative stimulus effects (upper panel) and response rates (bottom panel) for morphine in rats that had been trained to discriminate between 3 mg/kg morphine and saline. Each point represents the mean percentage of morphine-appropriate responding with S.E.M. of 5–7 animals. $*P < 0.05$ compared with the saline pretreatment.

produced a significant place preference in mice. A significant place preference or place aversion was not observed by olanzapine itself at the dose of 0.3 mg/kg with saline. Under these conditions, pretreatment with olanzapine at 0.3 mg/kg (s.c.) significantly suppressed the rewarding effects of morphine (Fig. 2B).

Suppression of the drug discriminative effect of morphine by olanzapine at doses that failed to induce motor dysfunction

Rats required 23 sessions to acquire morphine–saline discrimination. Once rats attained the criterion, morphine–saline discrimination stabilized and was maintained with a high degree of accuracy. During the dose-response tests, morphine at 0.3–3.0 mg/kg (s.c.) produced a dose-dependent increase in morphine-appropriate responses in all of the rats (Fig. 3A). In the combination test, olanzapine dose-dependently and significantly attenuated the discriminative stimulus effects of the training dose (3.0 mg/kg) of morphine without affecting the response rates ($P = 0.0294$ vs. saline pretreatment) (Fig. 3B).

DISCUSSION

In humans, morphine induces several kinds of subjective and psychotic effects, while in animals morphine clearly has reinforcing/rewarding effects. The place-conditioning procedure is used to evaluate rewarding and aversive effects (Suzuki et al., 1991). In addition, the evaluation of hyperlocomotion induced by morphine has also been used to assess the pharmacological mechanisms that underlie the abuse of morphine (Narita et al., 1993). A large and growing body of evidence has demonstrated that activation of the dopaminergic, especially mesolimbic, system plays a crucial role in the expression of both hyperlocomotion and the reinforcing/rewarding effects of opioids (Bonci and Williams, 1997; Johnson and North, 1992; Narita et al., 2001). In the present study, we clearly confirmed that morphine at a range of 5–10 mg/kg induced significant hyperlocomotion and conditioned place preference in mice, which reflected the abuse potential of morphine in rodents.

Although μ-opioids have potential for abuse and/or addiction, clinical studies have shown that abuse and addiction do not usually occur when μ-opioids are used appropriately to control pain (Eisenberg et al., 2005). We have proposed that the abuse potential of morphine is potently suppressed under chronic pain in rodents (Suzuki et al., 1996; Narita et al., 2005; Niikura et al., 2010). However, over the past decade, there has been some concern regarding the abuse of and addiction to μ-opioids caused by the inappropriate use, misuse, or overdose of prescription opioids (Woodcock, 2009). Dopamine receptor antagonists have been commonly used to reduce the adverse reactions to μ-opioids, including delusions and hallucinations (McNicol et al., 2003). Olanzapine is an atypical antipsychotic that is clinically indicated for schizophrenia and mania. It blocks multiple neurotransmitters (Glazer, 1997; Tohen and Grundy, 1999). In the present study, we found that olanzapine at 0.3 mg/kg, which did not induce motor coordination significantly suppressed the hyperlocomotion and rewarding effects induced by morphine. In our preliminary study, treatment with olanzapine at 0.3 mg/kg failed to reduce the antinociceptive effect of morphine. Furthermore, the delay in colonic expulsion induced by morphine was not affected by 0.3 mg/kg of olanzapine, which indicated that olanzapine did not exacerbate morphine-induced constipation. Taken together, these findings support the idea that olanzapine may have a wide margin of safety when used as an adjuvant for μ-opioids.

In a previous binding study in brain tissue, we found that olanzapine exhibited the highest affinity for muscarinic M_1 receptors and also showed affinity toward serotonin 5-$HT_{2A/2C}$, 5-HT_3, histamine H_1, dopamine D_2, dopamine D_4, and 5-HT_4 receptors. In light of this multiple binding property, we previously documented in an *in vivo* study that olanzapine dose-dependently decreased morphine-induced nausea and vomiting that are caused through various mechanisms (Torigoe et al., in press).

Muscarinic M_1 receptors have been suggested to be responsible for the enhancement of opioid-stimulated dopaminergic transmission related to the aggravation of drug addiction (Tanda et al., 2007). Since olanzapine showed the highest affinity toward muscarinic M_1 receptors, it is reasonable to wonder if olanzapine could aggravate the abuse potential of μ-opioids. This contention can be supported by the fact that the selective muscarinic M_1 receptor antagonist trihexyphenidyl significantly enhanced the morphine-induced increase in the release of dopamine in the nucleus accumbens (our preliminary study; data not shown), which indicates that M_1 receptors play an important role in opioid addiction. However, in the present study, olanzapine did not enhance either the hyperlocomotion or place preference produced by morphine. Although the exact mechanism by which olanzapine suppresses morphine's abuse profile remains unclear, this phenomenon may result from the fact that olanzapine acts not only on muscarinic M_1 receptors but also partly on serotonin 5-$HT_{2A/2C}$, 5-HT_3, histamine H_1, dopamine D_2, dopamine D_4, and 5-HT_4 receptors as an antagonist.

Morphine has subjective effects (e.g., "strength of drug effect," "sedated," "heavy or sluggish feeling," and "high") in healthy volunteers (Zacny et al., 1994). Furthermore, morphine can induce drowsiness, hallucination, and delirium, which have been considered to be important cues for the subjective effects of morphine (Adunsky et al., 2002; Maddocks et al., 1996). To assess the subjective effects in humans, animal models for studying the components of drug action that bear on the subjective effects have been developed. A methodology that has considerable potential in this regard is the drug discrimination procedure (Schuster and Johanson, 1988). However, little is known about the mechanism of the discriminative stimulus effects of morphine in animals. In the present study, olanzapine at 0.3 mg/kg significantly attenuated the discriminative stimulus effects of the training dose of morphine in rats. It should be emphasized that neither the D_1 receptor antagonist SCH23390, the D_2 receptor antagonist haloperidol nor their combination affected the discriminative stimulus effects of morphine in rats that had been trained to discriminate between 3.0 mg/kg morphine and saline (Suzuki et al., 1995). Therefore, we hypothesize that the blockade of neurotransmitters other than dopamine receptors by olanzapine at this dose may contribute to attenuate the discriminative stimulus effects of morphine.

In conclusion, we found that olanzapine at a dose that failed to induce motor dysfunction suppresses the hyperlocomotion, place preference, and discriminative stimulus effect induced by morphine. These results further provide evidence that cotreatment with olanzapine may be very useful as an adjuvant for pain control by μ-opioids.

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Addiction Biology

PRECLINICAL STUDY: BRIEF REPORT doi:10.1111/j.1369-1600.2010.00219.x

Epigenetic modulation at the CCR2 gene correlates with the maintenance of behavioral sensitization to methamphetamine

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ABSTRACT

The intermittent administration of methamphetamine produces behavioral sensitization to methamphetamine. In the limbic forebrain, mainly including the nucleus accumbens, of mice that had been intermittently treated with methamphetamine, we found a significant increase in mRNA of a chemokine, CCR2. This increase was accompanied by a significant increase in histone H3 lysine 4 (H3K4) trimethylation at its promoter. Interestingly, the maintenance of sensitization to methamphetamine-induced hyperlocomotion was significantly decreased in CCR2 knockout mice. These findings suggest that increased CCR2 associated with epigenetic modification after the intermittent administration of methamphetamine may be associated with the maintenance of sensitization to methamphetamine-induced hyperlocomotion.

Keywords CCR2, drug abuse, epigenetics, histone modification, methamphetamine, sensitization.

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Methamphetamine is a strongly addictive psychostimulant that dramatically affects the central nervous system (CNS) and is highly abused worldwide. In rodents, it has been shown consistently that repeated exposure to psychostimulants results in a progressive and enduring enhancement of the motor stimulant effect elicited by a subsequent drug challenge, which is called behavioral sensitization. Many studies have suggested that the mesolimbic dopaminergic system, which projects from the ventral tegmental area to the nucleus accumbens, is critical for the initiation of methamphetamine-induced hyperlocomotion (Vanderschuren & Kalivas 2000).

A growing body of evidence suggests that the behavioral sensitization induced by psychostimulants may be accompanied by long-lasting neural plasticity (Robinson & Kolb 1999). The neuronal plasticity has been believed to require diverse alterations in gene expression. Although some of the candidate genes that are involved in behavioral sensitization to psychostimulants have been identified (Ujike *et al.* 2002; Sokolov, Polesskaya & Uhl 2003), an important step toward unraveling the complex machinery of psychostimulant-induced behavioral sensitization is a multiplex analysis for both gene expression profiling and epigenetic modifications, which exert lasting control over gene expression without altering the genetic code.

Recent evidence has suggested that epigenetic mechanisms contribute to drug-induced transcriptional and behavioral changes (Renthal & Nestler 2008). Such epigenetic modulation is mainly controlled by histone modification. Histones are modified at many sites. Previously published reports have indicated that the increased acetylation of histone H3 or methylation of H3 at K4 (lysine 4) highly predicts gene activation, while increased methylation of H3 at K9 or K27 (lysine 9 or 27) is predictives of gene repression. The triggering of signaling cascades in target neurons leads to more long-lasting effects, including changes in gene expression via the control of transcription and thereby, chromatin remodeling.

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In the present study, we report for the first time that the intermittent administration of methamphetamine increases the mRNA level of a C-C chemokine, CCR2, with histone modification in the limbic forebrain including the nucleus accumbens. To address the functional relevance of this increased CCR2 expression, we also investigated whether the increased locomotion observed after the intermittent administration of methamphetamine could be affected in CCR2 knockout mice.

The locomotor activity of mice was measured by an ambulometer (ANB-M20, O'Hara, Tokyo, Japan) as described previously. Briefly, male C57BL/6J mice or CCR2 knockout mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and individually placed in a tilting-type round activity cage. To induce behavioral sensitization to methamphetamine-induced hyperlocomotion, the mice were given five intermittent treatments with methamphetamine (2 mg/kg, s.c.), once every 96 hours. To clarify the maintenance of behavioral sensitization, the mice were again administered methamphetamine (2 mg/kg, s.c.) after seven weeks of withdrawal.

The limbic forebrain area, mainly including the nucleus accumbens, was removed 24 hours after the last injection of methamphetamine. RNA preparation and semiquantitative analysis by reverse transcription polymerase chain reaction (RT-PCR) were performed as described previously. The methods are described in detail in the Supporting Information. The primers used are listed in Table S1.

Chromatin immunoprecipitation (ChIP) was performed as described previously with minor modifications. Soluble chromatin extracted from the mouse limbic forebrain was incubated with specific antibodies against acetylated histone H3 (Millipore; Billerica, MA, USA), H3K4 trimethylation (Wako Pure Chemicals, Osaka, Japan), H3K9 trimethylation (Millipore) and H3K27 trimethylation (Millipore) overnight at 4°C. The immunocomplex was collected by Dynabeads Protein A (Invitrogen Dynal AS, Oslo, Norway), and DNA was recovered by isopropanol precipitation. The methods are described in detail in the Supporting Information. The statistical analysis is also described in detail in the Supporting Information.

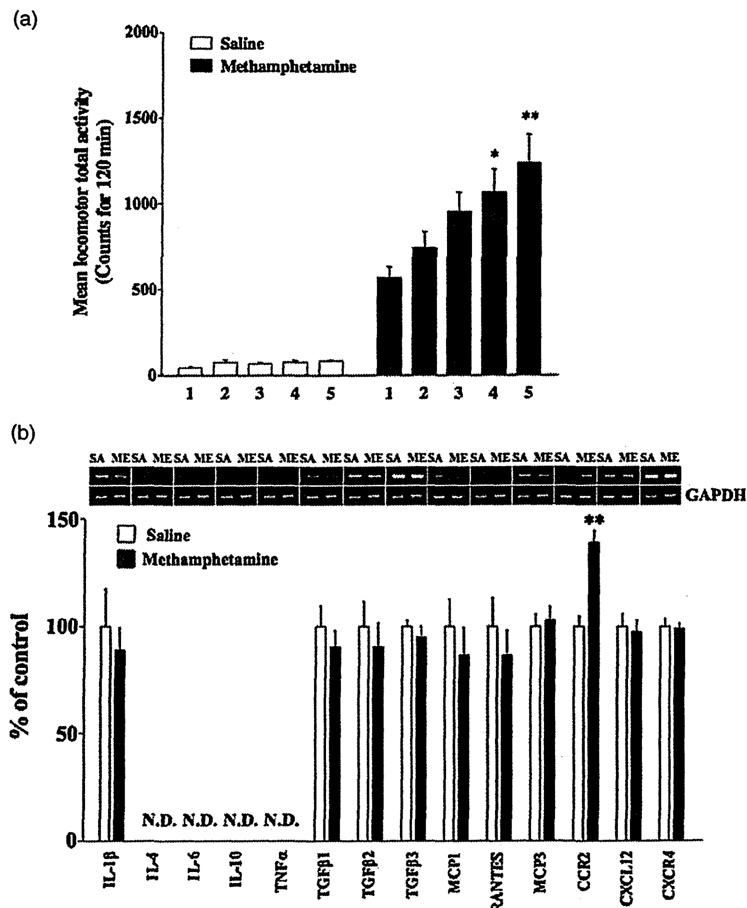

Figure 1 (a) Development of sensitization to methamphetamine in mice. Methamphetamine (2 mg/kg, s.c.) or saline was repeatedly given five times to mice every 96 hours. Total activity was counted for 120 minutes after each injection. Each column represents the mean total counts for 120 minutes with S.E.M. of 20 mice. (*$P<0.01$, **$P<0.001$ versus METH 1st). (b) Upper: Representative RT-PCR with 35 cycles for IL1β, IL-4, IL-6, IL-10, TNFα, TGFβ1, TGFβ2, TGFβ3, MCP1, RANTES, MCP3, CCR2, CXCL12 and CXCR4 mRNAs in the limbic forebrain of mice that have shown behavioral sensitization to methamphetamine (ME). The limbic forebrain sample was prepared 24 hours after the last injection of saline (SA) or ME. Lower: The intensity of the aforementioned bands was semi-quantified using Image J software. The value for mRNA was normalized by that for the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The value for mice treated with methamphetamine is expressed as a percentage of the increase in mice treated with saline. Each column represents the mean ± SEM ($n=3$ animals per group; three independent experiments). N.D., not detectable. **$P<0.01$ versus saline-treated mice

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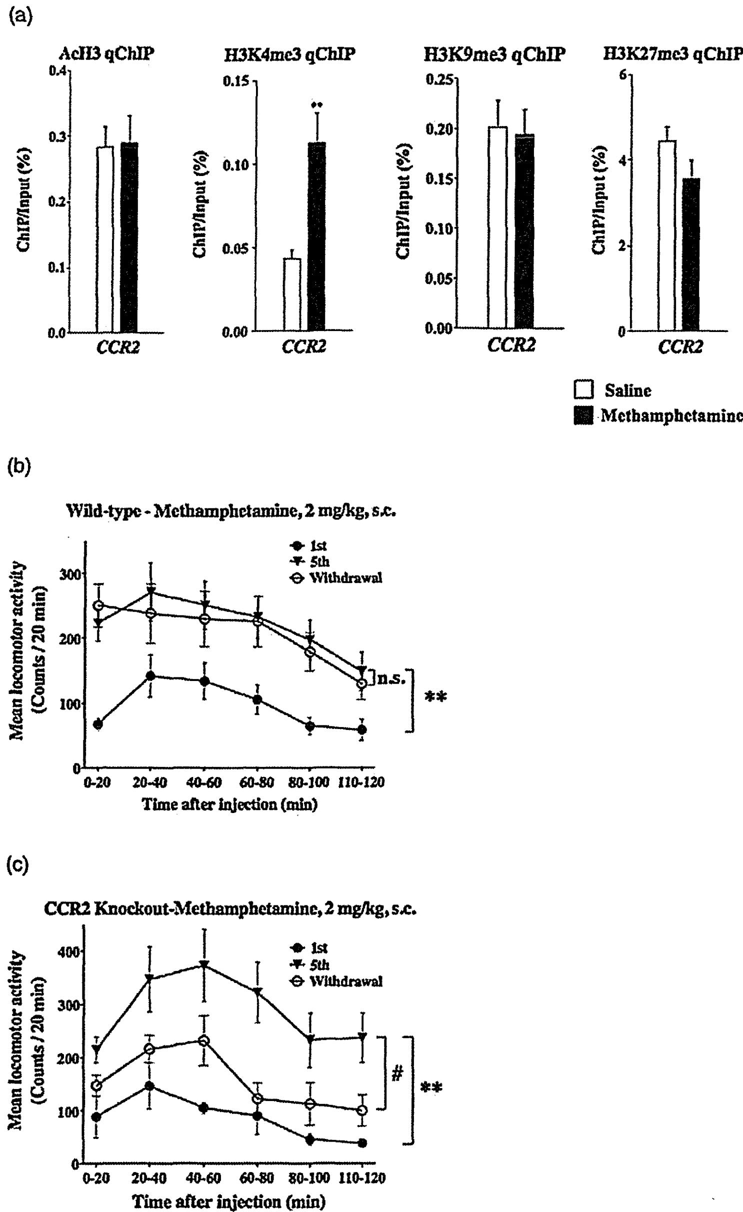

Figure 2 (a) qChIP analysis of acetylated histone H3 (AcH3), histone H3 trimethylated at lysine 4 (H3K4me3), lysine 9 (H3K9me3), and lysine 27 (H3K27me3) at CCR2 loci in the limbic forebrain of mice that had been intermittently treated with methamphetamine. Each column represents the mean ± SEM ($n = 4$ animals per group; three independent experiments). $^{**}P < 0.01$ versus saline-treated mice. (b) Change in locomotor activity (per 20 minutes time intervals) following intermittent administration of methamphetamine (2 mg/kg, s.c.) in wild-type mice (B-i) or CCR2 knockout mice (B-ii). Mice were treated intermittently with methamphetamine every 96 hours for five sessions. '1st' represents the 1st injection group, whereas 5th shows the 5th injection group. Mice described as 'withdrawal' were again administered methamphetamine after seven weeks of withdrawal. $^{**}P < 0.01$, 1st versus 5th, $\#P < 0.05$, 5th versus withdrawal (two-way ANOVA). n.s., not significant. Each point represents the mean ± SEM ($n = 4$–17 mice)

As shown in Fig. 1a, intermittent injection of methamphetamine produced a progressive increase in methamphetamine-induced locomotion, indicating the development of sensitization to methamphetamine (Fig. 1a, $F_{(4, 95)} = 4.940$, $P < 0.01$, first session versus fifth session).

As shown in Fig. 1b, a significant increase in mRNA of CCR2, but not of IL-1β, IL-4, IL-6, IL-10, TNFα, TGFβ1, TGFβ2, TGFβ3, MCP-1, RANTES, MCP-3, CXCL12 or CXCR4, was observed in the limbic forebrain, mainly including the nucleus accumbens, of the mice that had shown behavioral sensitization to methamphetamine (Fig. 1b, $P < 0.01$ versus the saline-treated mice). CCR2 is a seven-transmembrane-spanning Gαi protein-coupled receptor for a member of the C-C chemokine family, MCP-1, and is considered to regulate various brain disorders (Yong & Rivest 2009).

To gain further insight into these phenomena, we next studied histone modifications at the promoter regions of the CCR2 gene (Iida *et al.* 2008). A ChIP assay, where tissue is lightly fixed to crosslink DNA with histones and other DNA-binding proteins and then immunoprecipitated for a protein of interest, can be used to assess the extent to which a given gene is associated with these markers of activation or repression. In this study, we analyzed two active histone modifications [acetylation of

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histone H3 and trimethylation of lysine 4 on histone H3 (H3K4)] and two repressive histone modifications [trimethylation of lysine 9 on histone H3 (H3K9) and trimethylation of lysine 27 on histone H3 (H3K27)] at the CCR2 gene promoter in the limbic forebrain of mice that had been intermittently treated with methamphetamine. As a result, intermittent treatment with methamphetamine caused a significant increase in the level of H3K4 trimethylation at the CCR2 promoter in the mouse limbic forebrain (Fig. 2a, $P < 0.01$ versus the saline-treated mice; Figs S1 and S2). Methamphetamine did not produce other histone modifications at the CCR2 gene promoter (Fig. 2a). To the best of our knowledge, the present data are the first to indicate that intermittent treatment with methamphetamine induces a dramatic increase in the expression of the CCR2 gene along with epigenetic modifications in the nucleus accumbens.

To address the functional relevance of the increased CCR2 expression after the intermittent administration of methamphetamine, we next investigated whether the reduction of CCR2 expression could affect behavioral sensitization to methamphetamine using CCR2 knockout mice (Fig. S3). As shown in Fig. 2b, the fifth injection of methamphetamine produced a dramatic and significant increase in methamphetamine-induced hyperlocomotion compared with the first injection in both C57BL/6J (wild-type) and CCR2 gene knockout mice to the same degree (wild-type: first versus fifth, $F_{(1, 160)} = 12.39$, $P < 0.01$, CCR2 knockout: first versus fifth, $F_{(1, 30)} = 20.00$, $P < 0.01$), indicating that lack of the CCR2 gene had little or no effect on the development of sensitization to methamphetamine-induced hyperlocomotion. Intriguingly, the sensitization to methamphetamine was maintained even after seven weeks of withdrawal following intermittent administration of methamphetamine in the wild-type mice (fifth versus withdrawal, $F_{(1, 110)} = 0.05$, no significant). However, the methamphetamine-induced sensitization was almost reversed after seven weeks of withdrawal in CCR2 knockout mice (fifth versus withdrawal, $F_{(1, 30)} = 8.50$, $P < 0.05$, Fig. 2b). These results indicate that CCR2 is implicated in the maintenance of behavioral sensitization to methamphetamine.

In conclusion, the present study suggests that the intermittent administration of methamphetamine increases the mRNA level of CCR2 in association with epigenetic modification at its promoter in the limbic forebrain including the nucleus accumbens, and this may correlate with the maintenance of sensitization to methamphetamine-induced hyperlocomotion.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 qChIP analysis of acetylation of histone H3 (AcH3), H3K4me3, H3K9me3, and H3K27me3 in the limbic forebrain of mice that had been intermittently treated with methamphetamine (2 mg/kg, s.c., 5 times). Tubulin was used as a control for AcH3 and H3K4me3. High levels of AcH3 and H3K4me3 at the TATA box binding protein (Tbp) gene, which is transcriptionally activated within neurons, H3K9 and K27 trimethylation at silenced genes marker major satellite DNA, which probably comprises the functional centromere, and Gbx2 promoter (a homeobox-containing family of DNA-binding transcription factors) are seen in the limbic forebrain of methamphetamine-treated mice. Each column represents the mean ± S.E.M. ($n = 4$ animals per group; three independent experiments)

Figure S2 Representative PCR product with 40 cycles for CCR2 DNA in the limbic forebrain of mice that have shown behavioral sensitization to methamphetamine (ME). The limbic forebrain sample was prepared 24 hours after the last injection of saline (SA) or ME

Figure S3 Analysis of CCR2 mRNA expression by RT-PCR in the mouse whole brain from wild-type (WT) and CCR2 knockout (KO) mice

Table S1 Comprehensive list of all primer sequences used.

Appendix S1 Supplemental methods

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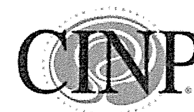

Intrastriatal gene delivery of GDNF persistently attenuates methamphetamine self-administration and relapse in mice

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Abstract

Relapse of drug abuse after abstinence is a major challenge to the treatment of addicts. In our well-established mouse models of methamphetamine (Meth) self-administration and reinstatement, bilateral microinjection of adeno-associated virus vectors expressing GDNF (AAV-Gdnf) into the striatum significantly reduced Meth self-administration, without affecting locomotor activity. Moreover, the intrastriatal AAV-Gdnf attenuated cue-induced reinstatement of Meth-seeking behaviour in a sustainable manner. In addition, this manipulation showed that Meth-primed reinstatement of Meth-seeking behaviour was reduced. These findings suggest that the AAV vector-mediated Gdnf gene transfer into the striatum is an effective and sustainable approach to attenuate Meth self-administration and Meth-associated cue-induced relapsing behaviour and that the AAV-mediated Gdnf gene transfer in the brain may be a valuable gene therapy against drug dependence and protracted relapse in clinical settings.

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Key words: Adeno-associated virus, cue-induced reinstatement, gene therapy, glial cell line-derived neurotrophic factor, methamphetamine, self-administration.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) has been widely tested as a potential therapeutic agent for the treatment of Parkinson's disease (Tomac et al., 1995; Choi-Lundberg et al., 1997; Mandel et al., 1997; Kordower et al., 2000; Wang et al., 2002; Kirik et al., 2004), since GDNF was originally purified from a rat glioma cell-line supernatant as a trophic factor for embryonic midbrain dopamine neurons (Lin et al., 1993). Dopaminergic transmission from the ventral tegmental area to nucleus accumbens and prefrontal cortex plays an important role in the development of drug addiction and striatal dopaminergic transmission is critical for the conversion from drug use to drug abuse or habit formation (Everitt and Robbins, 2005; Di Ciano et al., 2008). Therefore, it is reasonable to postulate that GDNF may be involved in the development of drug addiction (Pierce and Bari, 2001). Indeed, GDNF has been identified as a critical modulator in the development of drug dependence in animal models (Messer et al., 2000; He et al., 2005; Niwa et al., 2007a, b; Lu et al., 2009). The manipulations that increase contents of GDNF in the striatum and nucleus accumbens attenuate acquisition of cocaine self-administration in rats (Green-Sadan et al., 2003, 2005). In contrast, the manipulations that decrease contents of GDNF in the brain facilitate drug-induced conditioned place preference and drug self-administration in rodent animals (Messer et al., 2000; Niwa et al., 2007a, b; Yan et al., 2007b). Previously, we have reported that a reduction of endogenous GDNF protein in heterozygous GDNF knockout mice (GDNF$^{+/-}$ mice) not only facilitates the acquisition of methamphetamine (Meth) self-administration, results in an upward shift in the dose–response curve and increases

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motivation to take Meth, but also leads to increased vulnerability to Meth-primed reinstatement and enduring vulnerability to cue-induced relapsing behaviour (Yan et al., 2007b). In a clinical setting, GDNF itself cannot be orally administered for the treatment of brain diseases. The next challenge we had was to investigate safe and permanent potentiation of GDNF expression only in the critical local brain areas. Adeno-associated viral (AAV) vector is one of the most useful tools for the delivery of therapeutic genes into the brain as a potential therapeutic strategy against brain diseases, because of its safety and sustainable expression in the dopaminergic transmission pathways in the brain (Wang et al., 2002; Eberling et al., 2009; Su et al., 2009). In aged rats or Parkinsonian non-human primates, AAV-Gdnf-treated animals show clinical improvement and functional recovery in the nigrostriatal pathway without adverse effects (Eberling et al., 2009; Johnston et al., 2009; Kells et al., 2010). Recently, we used the AAV vector as a vehicle of the aromatic L-amino acid decarboxylase gene into the putamen of Parkinson's disease patients for a clinical phase I study (Muramatsu et al., 2010). In this study, we determine effects of an intrastriatal microinjection of the AAV-Gdnf on Meth self-administration, extinction and reinstatement of Meth-seeking behaviour in mice.

Materials and method

Subjects and drugs

Male C57BL/6J mice were aged 8 wk and weighed 20–25 g at the beginning of the experiments. They were kept in a regulated environment (23 ± 0.5 °C; 50 ± 0.5% humidity) with a 12-h light/dark cycle (lights on 09:00 hours). Water and food were available *ad libitum*. To minimize the number of animals in the experiments, a within-subjects design was used in Meth self-administration, extinction and reinstatement of Meth-seeking behaviour induced by either Meth-priming injection or presentation of Meth-associated cues in our experiments. All procedures followed the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Nagoya University Animal Care and Use Committee.

Meth hydrochloride (Dainippon Pharmaceutical Ltd, 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; Yan et al., 2006, 2007a).

The AAV vectors expressing GDNF

The AAV-Gdnf or AAV-EGFP was constructed and prepared as previously described (Wang et al., 2002). In the present study, the final particle titre for the intrastriatal microinjection of the AAV-Gdnf and AAV-EGFP was 8.4×10^{13} and 6.4×10^{13} vector genome copies/ml, respectively.

Surgery for intravenous implantation of catheter and bilateral intrastriatal injection of the AAV-Gdnf

Catheter implantation for Meth infusion

Naive mice were anaesthetized with pentobarbital sodium (50 mg/kg i.p.). Indwelling catheters were constructed of micro-silicone tubing (inner diameter, 0.50 mm; outer diameter, 0.7 mm; Imamura Co., Ltd, 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 and was temporarily closed with a clamp.

Bilateral intrastriatal microinjection of the AAV-Gdnf and AAV-EGFP vectors

Once the intravenous was successfully implanted as described above, the animals received bilateral microinjection of the AAV vectors into the striatum, according to the standard mouse brain coordinates (Franklin and Paxinos, 2007). Based on previous reports (Tomac et al., 1995; Chen et al., 2008), the AAV-Gdnf or AAV-EGFP vectors were bilaterally injected into the striatum ($+0.9$ mm anteroposterior, ± 1.5 mm mediolateral, -3.0 and -2.0 mm dorsoventral) at two different depths through a 10 μl Hamilton syringe (Hamilton Company, USA) with a 33 gauge blunt hypodermic needle. At a lower site, the vectors were bilaterally injected in a volume of 1.0 μl/site over 2 min and the syringe needle was left in place for an additional 3 min. After the needle was pulled upward 1.0 mm (upper site), the vectors were bilaterally injected in a volume of 1.0 μl/site over 2 min and an additional 3 min for the needle in place. The burr hole was sealed with quick self-curing acrylic resin (Shofu Inc., Japan) after each injection.

Measurement of motility in a novel environment

Motility in a novel environment was measured in a transparent acrylic cage with a black Plexiglas floor ($45 \times 45 \times 40$ cm) using infrared counters (Scanet SV-40; MELQUEST, Japan). Two weeks after recovering from the bilateral intrastriatal microinjection of the AAV-Gdnf or AAV-EGFP vectors, the two groups of mice (AAV-Gdnf and AAV-EGFP) were placed in the centre of the cage and allowed to move freely for 60 min. Locomotion and rearing were analysed to examine effects of the intrastriatal microinjection of the AAV-Gdnf or AAV-EGFP vector on motility in general in mice.

Immunohistochemical staining for GDNF

Brain sections (16 μM) were cut on a cryostat, thaw-mounted on Silane-coated slides and stored at -80 °C

until used. The brain sections were permeabilized with 0.1% Triton X-100, blocked with 1% bovine serum albumin and incubated with the mouse monoclonal antibody against GDNF (R&D Systems, USA) at 4 °C overnight. To detect specific signals, the brain sections were incubated with CF594-conjugated secondary antibodies (Biotium, USA) for 2 h at room temperature. Images were captured and the density was evaluated with fluorescence microscopy (BZ9000; Keyence, Japan).

Meth self-administration, extinction and reinstatement of Meth-seeking behaviour

Apparatus

The standard mouse operant conditioning chambers (ENV-307A; Med Associates, USA) used in the current study were described previously (Yan et al., 2006, 2007a; Yan and Nabeshima, 2009).

Meth self-administration

After a 2-wk interval from the microinjection of the AAV-Gdnf or AAV-EGFP vectors into the striatum, the mice were subjected to daily 3-h sessions of Meth self-administration under a fixed ratio (FR) schedule of reinforcement. Throughout each session of self-administration, the house lights were illuminated and cue- and hole-lamps indicated the availability of Meth. Once the mice made nose-poke responses in the active hole, the cue- and hole-lamps were turned off and Meth (0.1 mg/kg.infusion) was delivered over 5 s followed by a 5-s time-out period. Responses in the active hole during the time-out period and in the inactive hole had no programmed consequences but were recorded. Self-administration was initially under an FR1 schedule of Meth reinforcement. Once the mice made 60% active nose pokes on average, an FR2 schedule of Meth reinforcement was introduced until the mice acquired stable Meth self-administration behaviour (deviations of $<15\%$ of the mean of active responses in three consecutive training sessions).

Extinction

The mice were then subjected to 8–16 daily 3-h sessions of extinction before the Meth-primed reinstatement test and then 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 two consecutive sessions). The number of extinction training sessions for each mouse largely varied in the same treatment of group. 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 animals were first subjected to a 3-h session of the operant test 30 min after the injection (i.p.) of saline as a control for the Meth-primed reinstatement. From the next day, the mice were subjected to daily 3-h tests for Meth-primed reinstatement 30 min after the i.p. injection with increasing doses of Meth (0.2, 0.4, 1.0 or 2.0 mg/kg, each dose for one daily 3-h session). All of mice were tested with each dose of Meth for drug-primed reinstatement on different days, but there was no extinction training between the tests. This is because: (1) different to drug-primed instatement in rats, drug-primed reinstatement in mice is transient; (2) it takes a much longer time for mice to be extinguished from drug self-administration than that in rats. 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

After testing Meth-primed reinstatement, the same group of animals was subjected to extinction training once again. Once the extinction criterion was met, the animals were subjected to cue-induced reinstatement for the first time (the first test). Two months later, the same group of animals was subjected to cue-induced reinstatement for the second time (the second test). 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 were expressed as the mean ± S.E.M. The data of GDNF densities, locomotor activities and total Meth intake between AAV-Gdnf and AAV-EGFP-treated mice were analysed with Student's t test. A two-way analysis of variance (ANOVA) with (or without) repeated measures was performed for the difference in either active or inactive nose-poke responses between the AAV-Gdnf and AAV-EGFP-treated mice during Meth self-administration, extinction training, Meth-primed and cue-induced reinstatement of Meth-seeking behaviour, followed *post hoc* by Bonferroni's multiple

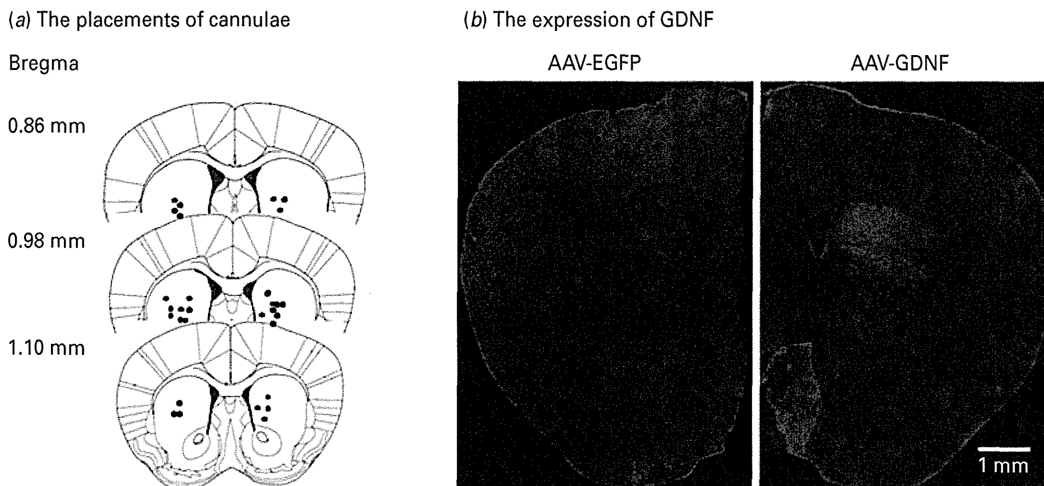

Fig. 1. Expression of GDNF in adeno-associated virus-mediated glial cell line-derived neurotrophic factor (AAV-GDNF)-treated mice. (*a*) Illustrates the placements of injectors within the mouse brain; (*b*) indicates GDNF protein expression in the dorsal striatum of AAV-GDNF- and adeno-associated virus-mediated enhanced green fluorescent protein (AAV-EGFP)-treated mice.

comparison test. In all cases, a significant difference was set at $p<0.05$.

Results

Enhancement of GDNF expression in the dorsal striatum of the AAV-Gdnf-treated mice

Figure 1*a* indicates the placement of cannulae for the intrastriatal microinjection of the AAV-Gdnf or AAV-EGFP vectors into the mouse brain. Figure 1*b* shows that the expression level of GDNF protein was clearly enhanced in the striatum 2 wk after the intrastriatal microinjection of the AAV-Gdnf vectors as compared to that after the microinjection of AAV-EGFP vectors. The densities of GDNF expression were 47.1 ± 0.35, 137.1 ± 5.06 in the striatum of AAV-EGFP and AAV-Gdnf, respectively, indicating that GDNF content increased significantly in the striatum by microinjection of AAV-Gdnf (Student's t test, $p<0.001$, d.f.$=41$, $t=4.08$). To investigate the effects of the intrastriatal microinjection of AAV-Gdnf and AAV-EGFP vectors on behavioural performance in general, the motility in a novel environment was measured for locomotion and rearing, as the motor issue and exploratory motivation, respectively. Neither locomotion nor rearing during a 60-min period of observation differed significantly between the AAV-Gdnf (locomotion: $28\,223.7 \pm 1978.0$ counts; rearing: 526.5 ± 52.9 counts, $N=6$) and AAV-EGFP-treated mice (locomotion: $24\,539.6 \pm 976.5$ counts; rearing: 542.4 ± 49.9 and 526.5 ± 130.0 counts, $N=6$; Student's t test, $p=0.94$, d.f.$=10$, $t=-1.67$ for locomotion; $p=0.415$, d.f.$=10$, $t=0.220$ for rearing). These results indicate that microinjection of AAV-Gdnf has no significant influence on the general locomotion and exploratory motivation system in mice.

Attenuation of Meth self-administration behaviour in AAV-Gdnf-treated mice

Active and inactive nose-poke responses of AAV-Gdnf and AAV-EGFP-treated mice during Meth self-administration training are shown in Fig. 2*a*. Repeated two-way ANOVA analysis for active nose-poke responses (AAV vectors are between-subjects factors and training sessions are within-subjects factors) revealed that there was no significant difference in active nose-poke responses in the early phase of Meth self-administration (day 1–11) between the AAV-Gdnf and AAV-EGFP-treated mice. In the late phase of Meth self-administration, however, the active nose-poke responses to take Meth were lower in the AAV-Gdnf-treated mice than in the AAV-EGFP-treated mice (main effect of AAV vectors: $F_{1,14}=3.94$, $p<0.05$; main effect of training sessions: $F_{15,210}=28.39$, $p<0.001$; AAV vector $\times$ training session interaction: $F_{15,210}=2.31$, $p<0.01$). There was no significant difference in inactive nose-poke responses between the AAV-Gdnf and AAV-EGFP-treated mice throughout Meth self-administration training (day 1–16). We have previously reported that total intake of Meth during drug self-administration affects the subsequent Meth-primed reinstatement (Yan et al., 2007a). After both groups of animals acquired stable Meth self-administration, the AAV-Gdnf-treated mice continued to be subject to Meth self-administration for four additional sessions to make two groups of animals with an equivalent total intake of Meth during drug self-administration training (Fig. 2*a*, day 17–20). As shown in Fig. 2*b*, the total intake of Meth during drug self-administration was 23.03 ± 3.09 mg/kg in AAV-Gdnf-treated mice for 20 d and 22.86 ± 3.22 mg/kg in AAV-EGFP-treated mice for 16 d (Student's t test, $p=0.97$, d.f.$=14$, $t=0.04$). These observations suggest that the intrastriatal microinjection

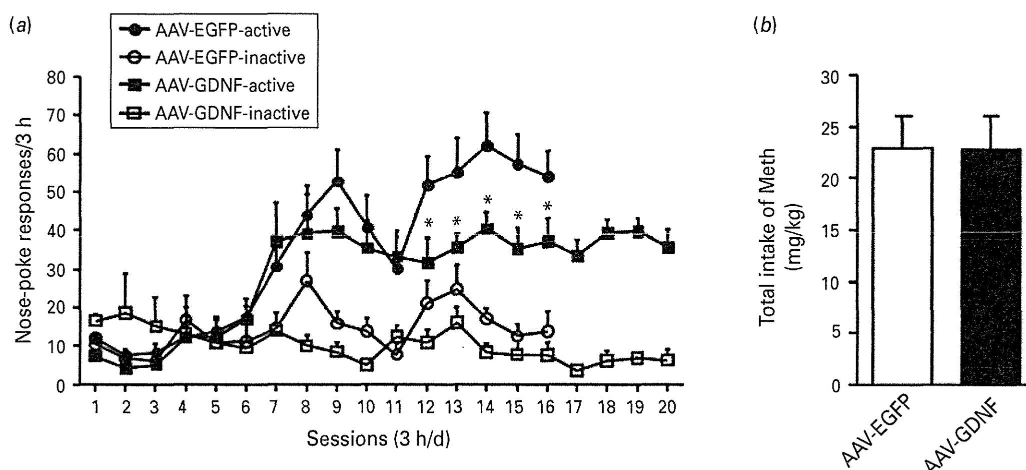

Fig. 2. Acquisition of methamphetamine (Meth) self-administration behaviour and total intake of Meth in adeno-associated virus-mediated glial cell line-derived neurotrophic factor (AAV-GDNF) and adeno-associated virus-mediated enhanced green fluorescent protein (AAV-EGFP)-treated mice. (*a*) Indicates the number of active and inactive nose-poke responses during Meth self-administration. * $p<0.05$ *vs.* active nose-poke responses of AAV-EGFP-treated mice on the same training day. (*b*) Indicates the total intake of Meth during Meth self-administration training in AAV-GDNF and AAV-EGFP-treated mice (23.03±3.09 mg/kg for 20 d and 22.86±3.22 mg/kg for 16 d, respectively). Data are presented as the mean±S.E.M. and $N=7$–8 for each group.

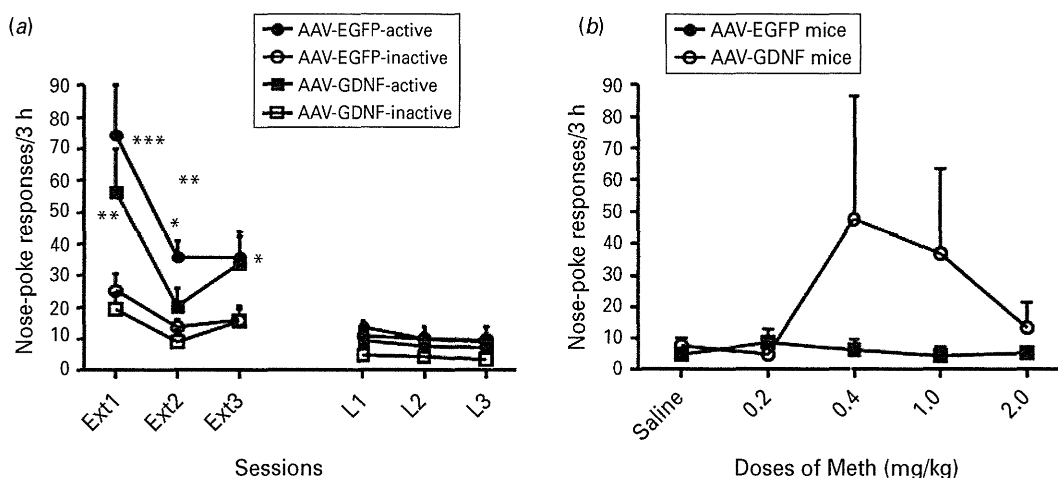

Fig. 3. Extinction performance and methamphetamine (Meth)-primed reinstatement of drug-seeking behaviour in adeno-associated virus-mediated glial cell line-derived neurotrophic factor (AAV-GDNF) and adeno-associated virus-mediated enhanced green fluorescent protein (AAV-EGFP)-treated mice. (*a*) Indicates nose-poke responses during the extinction training. The data are from the first three daily 3-h sessions (indicated by Ext1–3) and the last three daily 3-h sessions (indicated by L1–L3) during 8–16 extinction training sessions before the test for Meth-primed reinstatement. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* the corresponding inactive nose-poke responses in the same group. (*b*) Indicates the active nose-poke responses during Meth-primed reinstatement between AAV-Gdnf and AAV-EGFP-treated mice. Data are presented as the mean±S.E.M. and $N=7$–8 for each group.

of the AAV-Gdnf vectors is effective to attenuate the late phase of Meth self-administration behaviour in mice.

No difference in the process of extinction, but decrease of Meth-primed reinstatement in the AAV-Gdnf-treated mice

After the above-mentioned Meth self-administration, the same two groups of mice were subjected to 8–16 daily 3-h sessions of extinction training. As shown in Fig. 3*a*, repeated two-way ANOVA for active *vs.* inactive nose-poke holes in the same AAV vector treatment revealed that both groups of mice exhibit higher active than inactive nose-poke responses at the early phase of extinction training (for AAV-Gdnf-treated mice, main effect of within-subjects factor nose-poke holes: $F_{1,14}=9.64$, $p<0.01$; main effect of within-subjects factor training sessions: $F_{5,70}=14.03$, $p<0.001$; nose-poke hole × training session interaction: $F_{5,70}=3.44$, $p<0.01$. For AAV-EGFP-treated mice, main effect of within-subjects factor

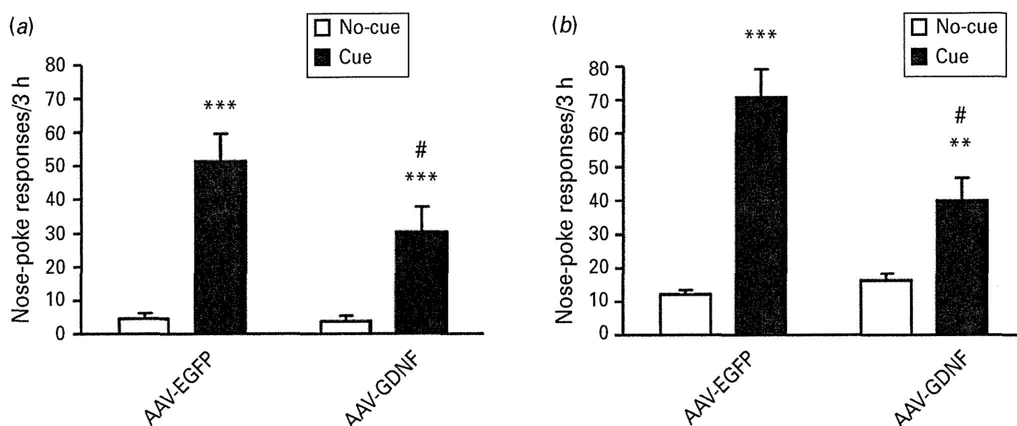

Fig. 4. Cue-induced reinstatement of methamphetamine (Meth)-seeking behaviour in adeno-associated virus-mediated glial cell line-derived neurotrophic factor (AAV-GDNF) and adeno-associated virus-mediated enhanced green fluorescent protein (AAV-EGFP)-treated mice. (*a*) Indicates cue-induced relapsing behaviour for the first test (the extinction criteria met after the test for Meth-primed reinstatement). *** $p<0.001$ *vs.* the no-cue condition, # $p<0.05$ *vs.* AAV-EGFP-treated mice. (*b*) Indicates the second test for cue-induced relapsing behaviour (2 months after the first test for cue-induced reinstatement). *** $p<0.001$ *vs.* the no-cue condition, # $p<0.05$ *vs.* AAV-EGFP-treated mice. Data are presented as the mean ± S.E.M. and $N=7$–8 for each group. No-cue, Control for the reinstatement test (neither Meth-associated cues nor Meth infusion); Cue, Meth-associated cue-induced reinstatement (with Meth-associated cues but no Meth infusion).

nose-poke holes: $F_{1,14}=8.45$, $p<0.01$; main effect of within-subjects factor training sessions: $F_{5,70}=17.95$, $p<0.001$; nose-poke hole × training session interaction: $F_{5,70}=6.99$, $p<0.001$). However, there was no significant difference in active nose-poke responses between the AAV-Gdnf and AAV-EGFP-treated mice throughout extinction training (main effect of between-subjects factor AAV vectors: $F_{1,14}=1.82$, $p=0.20$; main effect of within-subjects factor training sessions: $F_{5,70}=21.99$, $p<0.001$; AAV vector × training session interaction: $F_{5,70}=0.61$, $p=0.69$). There was no significant difference in the number of sessions (d) taken for extinction training between the AAV-Gdnf and AAV-EGFP-treated mice (data not shown). When the extinction criteria met, the two groups of mice were subjected to testing for Meth-primed reinstatement of drug-seeking behaviour. As shown in Fig. 3*b*, the AAV-EGFP-treated mice showed a clear dose-dependent tendency for drug-seeking behaviour induced by the i.p. priming injection of Meth. In contrast, the AAV-Gdnf-treated mice failed to show Meth-seeking behaviour after the priming injection of Meth at all doses examined (0.2–2.0 mg/kg i.p.). These data indicate that the intrastriatal microinjection of the AAV-Gdnf vectors may also be effective to attenuate Meth-primed reinstatement of Meth-seeking behaviour in mice.

Long-lasting inhibition of cue-induced relapsing behaviour in the AAV-Gdnf-treated mice

After testing for Meth-primed reinstatement, the same two groups of mice were subjected to 3–6 daily 3-h sessions of extinction training until the extinction criteria were met. When exposed to previous Meth-associated cues, both groups of mice showed cue-induced relapsing behaviour (Fig. 4*a*, two-way ANOVA, main effect of within-subjects factor cue and no-cue factors: $F_{1,26}=38.82$, $p<0.001$). Importantly, the number of active nose-poke responses was significantly reduced in the AAV-Gdnf-treated mice as compared to those in the AAV-EGFP-treated mice (Fig. 4*a*, two-way ANOVA, main effect of between-subjects factor AAV-Gdnf and AAV-EGFP treatments: $F_{1,26}=3.44$, $p<0.05$; cue × treatments interaction: $F_{1,26}=2.97$, $p=0.10$). Two months after this testing, the same two groups of mice were subjected to extinction training once again until the criteria were met. As shown in Fig. 4*b*, cue-induced reinstatement was significantly attenuated in the AAV-Gdnf-treated mice as compared to that in the AAV-EGFP-treated mice (Fig. 4*b*, two-way ANOVA, main effect of between-subjects factor AAV-Gdnf and AAV-EGFP treatments: $F_{1,26}=5.55$, $p<0.05$; cue × treatments interaction: $F_{1,26}=10.11$, $p<0.01$), although both groups of mice still showed cue-induced reinstatement of Meth-seeking behaviour (Fig. 4*b*, two-way ANOVA, main effect of within-subjects factor, cue and no-cue factors: $F_{1,26}=56.01$, $p<0.001$). These findings suggest that the inhibitory effects of intrastriatal AAV-Gdnf vectors on cue-induced reinstatement of Meth-seeking behaviour are long-lasting.

Discussion

GDNF has been considered as a potential therapeutic molecule to treat drug addiction (Ron and Janak, 2005; Niwa et al., 2008) and the AAV vectors are one of the most attractive gene delivery vehicles into the brain for the treatment of neurological diseases (Miyazaki et al., 2012). In our study, AAV-mediated delivery of a *Gdnf* gene into the striatum increased GDNF protein

expression without activation of spontaneous locomotion (Fig. 1*b*). The increased GDNF significantly attenuated Meth self-administration. Moreover, the AAV-Gdnf vectors in the striatum persistently reduced cue-induced relapsing behaviour. In addition, this manipulation also showed a clear tendency to block Meth-primed reinstatement in mice. These findings suggest that the manipulation of GDNF expression via the AAV vectors may be valuable in a clinical setting for the treatment of drug addiction and relapse.

The AAV vectors have unique characteristics, including the lack of any disease associated with the wild-type virus, an ability to infect non-dividing cells, long-term transgene expression with a minimal inflammatory or immune response and the physical stability of viral particles (Miyazaki et al., 2012). Using the AAV vectors, several research groups have constructed different versions of the AAV vectors for the Gdnf gene transfer, most of which express functional GDNF protein in a sustained manner after local injections and produce a functional recovery of the impaired dopaminergic system in the brain (Mandel et al., 1997; Wang et al., 2002; Eberling et al., 2009; Kells et al., 2010). Consistently, our previous studies have demonstrated that the expression of GDNF protein driven by an AAV-mediated Gdnf vector could be detected in the striatum from week 2 after local injection to lifetime. Furthermore, the GDNF protein could be retrogradely transported to the dopaminergic neuron cell bodies in substantial nigra from the terminals in the striatum 4 wk after the injection. The nigral dopaminergic neurons are prevented from progressive degeneration, thereby contributing to behavioural improvement in a rat model of Parkinson's disease (Wang et al., 2002). In the current study, the inhibitory effects of the AAV-Gdnf vectors into the striatum on cue-induced relapsing behaviour were sustained for at least 2 months in mice. Such persistently inhibitory effects of the AAV-mediated delivery of a *Gdnf* gene on cue-induced relapsing behaviour may result from a sustained expression of the AAV-Gdnf vectors in the nigra-striatal circuit after bilateral intrastriatal microinjection. This observation is consistent with our previous findings that $Gdnf^{+/-}$ mice show an enduring vulnerability to cue-induced reinstatement of Meth-seeking behaviour (Yan et al., 2007b). In the present study, the bilateral intrastriatal injection of the AAV-Gdnf vectors also decreased the late phase of Meth self-administration in mice (Fig. 2*a*).

It is unlikely that persistent inhibitory effects of bilateral intrastriatal injection of the vector-mediated delivery of a *GDNF* gene on Meth self-administration and cue-induced reinstatement result from non-specific procedures of microinjection. First, there was no significant difference in locomotion and rearing after the bilateral intrastriatal injection between AAV-Gdnf- and AAV-EGFP-treated mice. Second, there was no difference in extinction after the bilateral intrastriatal injection between AAV-Gdnf- and AAV-EGFP-treated mice. This observation, however, seems to be in discrepancy with one previous report in which microinjection of an AAV-Gdnf vector into the ventral tegmental area potentiates extinction responding in Long Evens rats (Lu et al., 2009). One parsimonious explanation is that the difference may reflect a distinct role of the nigrostriatal pathway or ventral tegmental area–nucleus accumbens in extinction responding (injection into the striatum in our study *vs.* injection into the ventral tegmental area in the report of Lu et al. 2009). Chen et al. (2008) have recently reported that the expression of GDNF protein via an AAV-Gdnf vector in the dorsal striatum prevents neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced deficits in the striatal synaptic plasticity. These findings may provide a potential molecular mechanism by which bilateral intrastriatal injection of the AAV-Gdnf vectors attenuated cue-induced reinstatement of Meth-seeking behaviour in our study, since it is well known that neurotoxic effects of Meth in the brain play an important role in the development of Meth addiction.

It has been well established that the nucleus accumbens and striatum have a distinct role in the development of drug addiction. The nucleus accumbens is well known to mediate the reinforcing effects of addictive drugs, whereas the striatum is critical to the transition from initial drug use to habitual drug abuse to compulsion (Everitt and Robbins, 2005). It has been postulated that, during the development of drug self-administration, neutral drug-conditioned environmental cues acquire a reinforcing property, which evokes drug craving and relapse. Previous studies have shown that the striatum is critical for cue-induced reinstatement of drug-seeking behaviour in animals (Di Ciano et al., 2008). Consistently, bilateral intrastriatal injection of the AAV-Gdnf vectors persistently attenuated cue-induced reinstatement of Meth-seeking behaviour in mice. This phenomenon may reflect a specific role of the nigra-striatal dopaminergic transmission pathway in the cue-induced relapse or the late stage of drug dependence/addiction. In addition, previous studies have shown that over-expression of GDNF in the striatum and nucleus accumbens attenuates cocaine self-administration behaviour in rats (Green-Sadan et al., 2005). In agreement with these findings, in our current study, bilateral intrastriatal injection of the AAV-Gdnf vectors significantly reduced the late phase of Meth self-administration or potentially blocked Meth-primed reinstatement.

Taken together, the bilateral intrastriatal microinjection of the AAV-Gdnf vectors in the brain significantly attenuated Meth self-administration and cue-induced reinstatement of Meth-seeking behaviour in mice, without affecting either general locomotor activity or extinction. This suggests that increased expression of exogenous GDNF protein through the microinjection of AAV-Gdnf vectors in the brain may be a gene therapeutic strategy to treat drug dependence and relapse in a clinical setting.

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Statement of Interest

None.

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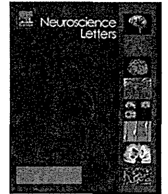

Absence of SHATI/Nat8l reduces social interaction in mice

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HIGHLIGHTS

- ► We succeeded in producing mice that are deficient of the *Shati* gene.
- ► Deficiency of SHATI may affect behavior.
- ► Deficiency of SHATI reduces social interaction with novel mice.
- ► Deficiency of SHATI changes the mRNA levels of BDNF, GDNF and LITAF.

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ABSTRACT

We previously identified a novel molecule "Shati/Nat8l" from the nucleus accumbens of mice. However, the physiological roles of the SHATI protein are not clear. To investigate the effect of SHATI on the central nervous system and behavior, we studied knockout mice of this protein. We carried out various behavior tests using *Shati*-knockout mice. *Shati*-knockout mice did not differ from wild type mice in learning and memory. In the open field test, *Shati*-knockout mice did not differ from wild-type mice in time of stay in the outer, middle and center areas. On the other hand, *Shati*-knockout mice showed increases in rearing and grooming time in the open field test, and exploration time of novel objects. These results suggested that knockout of the *Shati* gene may increase exploration in specific circumstances. Interestingly, the *Shati*-knockout mice avoided social interaction with unfamiliar mice out of their home cage, although there was no difference in social interaction in their home cage compared with wild type mice. Lack of the *Shati* gene increased brain-derived neurotrophic factor (BDNF) mRNA in the prefrontal cortex and hippocampus, and decreased glial cell line-derived neurotrophic factor (GDNF) mRNA in the striatum and hippocampus, and lipopolysaccharides-induced TNF-α factor (LITAF) mRNA in the striatum. Since these factors play important roles in behavior, alteration of expression of these factors may be related to the induction of exploration and reduction of social interaction in *Shati*-knockout mice.

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1. Introduction

SHATI has been identified as N-acetyltransferase 8-like protein (NAT8L), which contains a conserved sequence in the superfamily of N-acetyltransferases. Ariyannur et al. [2] demonstrated that SHATI/NAT8L has biosynthetic activity for N-acetylaspartate. We previously found that administration of methamphetamine (METH) increased the mRNA level of *Shati/Nat8l* in the nuclear accumbens (NAc) of mice using the PCR-select complementary DNA subtraction method [13]. We also suggested that SHATI suppresses METH-induced abnormal behavior via interaction with dopamine uptake [12]. Dopamine uptake is activated by the proinflammatory cytokine, TNF-α [11]. Exogenous TNF-α attenuated the METH-induced increase in extracellular striatal dopamine as demonstrated by in vivo microdialysis studies, and potentiated striatal dopamine uptake into synaptosomes in vitro and in vivo [11]. Moreover, it was suggested that proinflammatory cytokines, for example TNF-α and IL-6, induce behavioral

Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; LITAF, lipopolysaccharides-induced tumor necrosis factor-α factor; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor-α.

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alterations [1], including impaired cognition, altered social behavior and reduced locomotor activity and exploratory behavior [8]. Transcription of these proinflammatory cytokines is increased by lipopolysaccharides-induced TNF-α factor (LITAF), which is a transcription-related protein [16]. Since SHATI induces the expression of LITAF [12], a decreased level of SHATI may affect the LITAF expression level and change behavior. Brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) also play important roles in various behaviors, including learning and memory, social behavior and exploratory behavior. In the present study, we examined the behavior of a knockout mouse of SHATI, and evaluated the effect of absence of SHATI on the expression levels of BDNF, GDNF and LITAF.

2. Materials and methods

2.1. Animals and environments

Eight- to twelve-week-old *Shati*-knockout ($n = 10$) and wild type ($n = 10$) mice were used in behavioral tests, and *Shati*-knockout (homozygous ($n = 4$) and heterozygous ($n = 4$)) and wild type ($n = 6$) mice were used to determine mRNAs level. These mice were littermates from intercrosses of *Shati* heterozygous mice. They were housed in a controlled environment (23 ± 1 °C, 50 ± 5% humidity), maintained on a 9:00 a.m. to 9:00 p.m. light cycle and allowed access to food and water ad libitum. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Nagoya University Graduate School of Medicine (ID: 21256, 22158). All animal procedures and care conformed to the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006)

2.2. Materials

The total RNA extraction kit was purchased from QIAGEN (Tokyo, Japan). Reverse transcriptase and reagents of real-time reverse transcription polymerase chain reaction (RT-PCR) were purchased from Invitrogen (Carlsbad, CA, USA).

2.3. Production of Shati-knockout mice

We entrusted Unitech (Chiba, Japan) to produce knockout mice in which *Shati/Nat8l* DNA (NM_001001985.3) [13] was absent in the entire body. Briefly, the entire exons coding SHATI were deleted by a targeting vector including neomycin resistance sequence. The *Shati*-knockout mice were created via homologous recombinant ES cells of C57BL/6J mice. The primers used for the genotyping were as follows: 5′-CTATGAGTCACTGGGCTTCAGAC-3′ (forward for wild type), 5′-CGGAGAACCTGCGTGCAATCCATCTTGTTC-3′ (forward for knockout) and 5′-TGTTGTAGAGGTTCTGTGCTTGAT-3′ (reverse). The genetic background of the *Shati*-KO mouse was C57BL/6J. The absence of SHATI protein in the knockout mice could not be confirmed by Western blotting because of legal restrictions on the importation of anti-SHATI antibodies to Japan.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

The level of *Shati* mRNA was determined by real-time RT-PCR using a Fast Real-Time PCR System (Life Technologies Japan, Tokyo, Japan). *Shati*-knockout ($n = 4$), and heterogeneous type ($n = 4$) and wild type ($n = 6$) mice were used to check of mRNA level. Total RNA was isolated from the prefrontal cortex, striatum, nuclear accumbens, hippocampus and cerebellum of 8-week-old *Shati*-knockout mice and wild type mice using an RNeasy Mini Kit. One microgram RNA was reverse transcribed using Prime Script Reverse Transcription. Total cDNA (1 μl) was amplified in a 25 μl reaction mixture using 0.1 μM each of forward and reverse primers and Power SYBR-Green kit (Life Technologies Japan). The following primers for amplification of mouse *Shati* were used: 5′-CCTGGCCCTGCGCTACTA-3′ (forward) and 5′-CACACTCCAGGTAGGCCAGAA-3′ (reverse). The following primers for amplification of mouse BDNF were used: 5′-GCAAACATGTCTATGAGGGTTCG-3′ (forward) and 5′-ACTCGCTAATACTGTCACACACG-3′ (reverse). The following primers for amplification of mouse GDNF were used: 5′-GAAGAGAGAGGAATCGGCAGG-3′ (forward) and 5′-TGGCCTCTGCGACCTTTC-3′ (reverse). The following primers for amplification of mouse LITAF were used: 5′-CCCTTGCCCAGCCAGAA-3′ (forward) and 5′-CCCCCTAAAAGACACGAAGATG-3′ (reverse). For the internal control, the following primers for amplification of mouse glyceraldehyde-3-phosphate dehydrogenase were used: 5′-CATGGCCTTCCGTGTTCCTA-3′ (forward) and 5′-ATGCCTGCTTCACCACCTTCT-3′ (reverse).

2.5. Order of behavior tests

We administered the behavior tests in the following order: (1) open field test, (2) Y-maze test, (3) novel object recognition test, (4) elevated plus maze test, and (5) social interaction test. The time interval between successive tests was 2–3 days. For behavioral experiments, the same mice were used in each test.

2.6. Open field test

The open field consisted of a round area with gray walls (60 cm diameter, 30 cm high) and was set in a dark, sound-attenuated room [9]. The floor of the field was divided into outer, middle and center areas so that the animals' place preference could be measured. Each mouse was placed in the center of the open field. The mouse was allowed to freely explore the environment for 5 min. During this time, the ambulation of the mice was measured by EthoVision (Noldus, Leesburg, VA, USA). The number of rearing and grooming events was also recorded.

2.7. Y-maze test

Immediate working memory was assessed by recording spontaneous alternation behavior during a single session in a Y-maze made of black painted wood [4]. Each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom, 10 cm wide at the top, and converged in an equilateral triangular central area. Each mouse, naïve to the maze, was placed at the end of one arm and allowed to move freely through the maze during an 8 min session, and the number of arm entries was counted [15]. Each series of arm entries was recorded visually, and an arm entry was defined as when the hind paws of the mouse were completely within the arm. Alternation was defined as successive entries into the three arms on the overlapping triplet sets. The percentage alternation was calculated using the following formula: (number of alternations)/(total number of arm entries-2) × 100 (%).

2.8. Novel object recognition test

A plastic chamber (35 cm × 35 cm × 35 cm) was used in low light conditions during the light phase of the light/dark cycle. The mouse was allowed to explore two different floor-fixed objects (A and B) in the open field for 10 min (acquisition phase). Twenty-four hours after the acquisition phase, the mouse was allowed to explore the open field in the presence of two objects, the familiar object A and a novel object C, for 10 min. The time spent on exploring each individual object was recorded in a blinded manner. The recognition index, calculated for each mouse, was expressed as the ratio

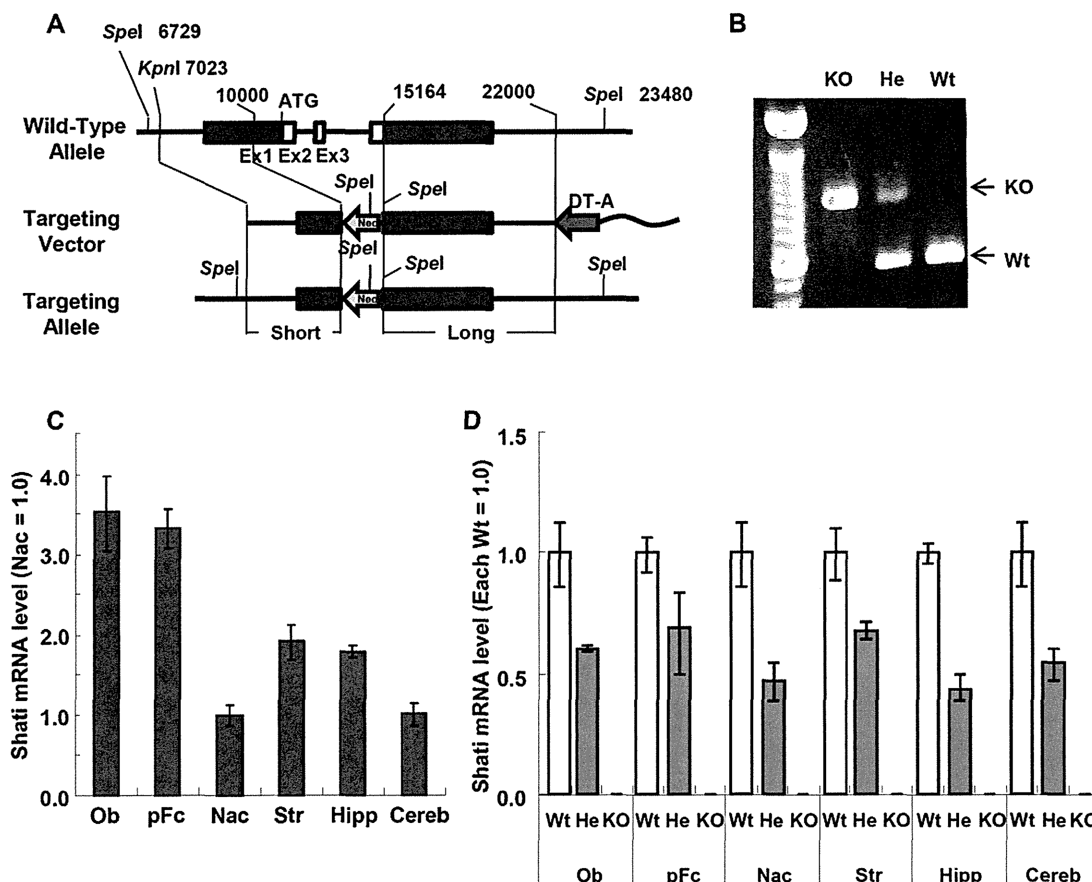

Fig. 1. Production of knockout mice of *Shati* gene. (A) Targeting vector that was used to produce *Shati*-knockout mice. The three exons of the *Shati* gene are shown as Ex1, Ex2 and Ex3. (B) Genotyping pattern of heterozygous and homozygous *Shati*-knockout mice compared with wild type mice. (C) Transcription level of the *Shati* gene in various structures of the brain of wild-type mice. The *Shati* mRNA level in the nuclear accumbens was used as the standard. Ob, olfactory bulb; pFc, prefrontal cortex; Nac, nuclear accumbens; Str, striatum; Hipp, hippocampus; Cereb, cerebellum. (D) Transcription level of the *Shati* gene in heterozygous (He) and homozygous knockout (KO) mice. The *Shati* mRNA level in each area of the brain of wild type mice (Wt) was used as the standard.

(TC × 100)/(TA + TC), where TA and TC are the times spent during the retention phase on object A and object C, respectively [3].

2.9. Elevated plus maze test

The elevated plus maze consisted of two open arms (25 cm × 8 cm × 0.5 cm) and two closed arms (25 cm × 8 cm × 20 cm) emanating from a common central platform (8 cm × 8 cm) to form a plus shape [6]. The entire apparatus was elevated to a height of 50 cm above floor level. Testing commenced by placing a mouse on the central platform of the maze facing an open arm, and the standard 5 min test duration was employed. The time spent in the open arms was measured by Etho Vision.

2.10. Social interaction test

The apparatus for the social interaction test was made of a gray polycarbonate (30 cm × 25 cm × 25 cm high) [14]. After habituation for 2 days, the mouse was randomly assigned to an unfamiliar partner of the same sex. The pair of unfamiliar mice was placed in the apparatus for 10 min and the total amount of time spent in active social interaction, such as sniffing, grooming, following and mounting as well as crawling over or under the stranger mouse in direct contact, was recorded by a blind investigator. For the intruder test, each test mouse was housed in the individual test cage for 2 days. Then, the novel intruder mouse was placed in the test cage for 10 min and the total amount of time spent in active social interaction was recorded.

2.11. Statistical analysis

The results are expressed as the mean ± S.E. statistical significance was determined with one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni's multiple comparisons test. Significance was set at $p < 0.05$.

3. Results

3.1. Construction of Shati knockout mouse

The entire exons coding *Shati* were deleted in the whole body of mice by a targeting vector including the neomycin resistance sequence (Fig. 1A). The genotype of deletion of the *Shati* coding region was determined by PCR (Fig. 1B). Expression levels of *Shati* mRNA in the brain were determined by real-time reverse transcription PCR (Fig. 1C). In wild-type mice, the expression levels of *Shati* mRNA in various areas of the brain were compared with the level of *Shati* mRNA in the nuclear accumbens (Nac) (Fig. 1C). Compared with the expression level of *Shati* mRNA in the NAc, the expression level of *Shati* mRNA was 3.5 times higher in the olfactory bulb, 2 times higher in the striatum and in the hippocampus, and the same in the cerebellum (Fig. 1C). *Shati* mRNA levels in the heterozygous knockout mice were approximately half of those in the wild

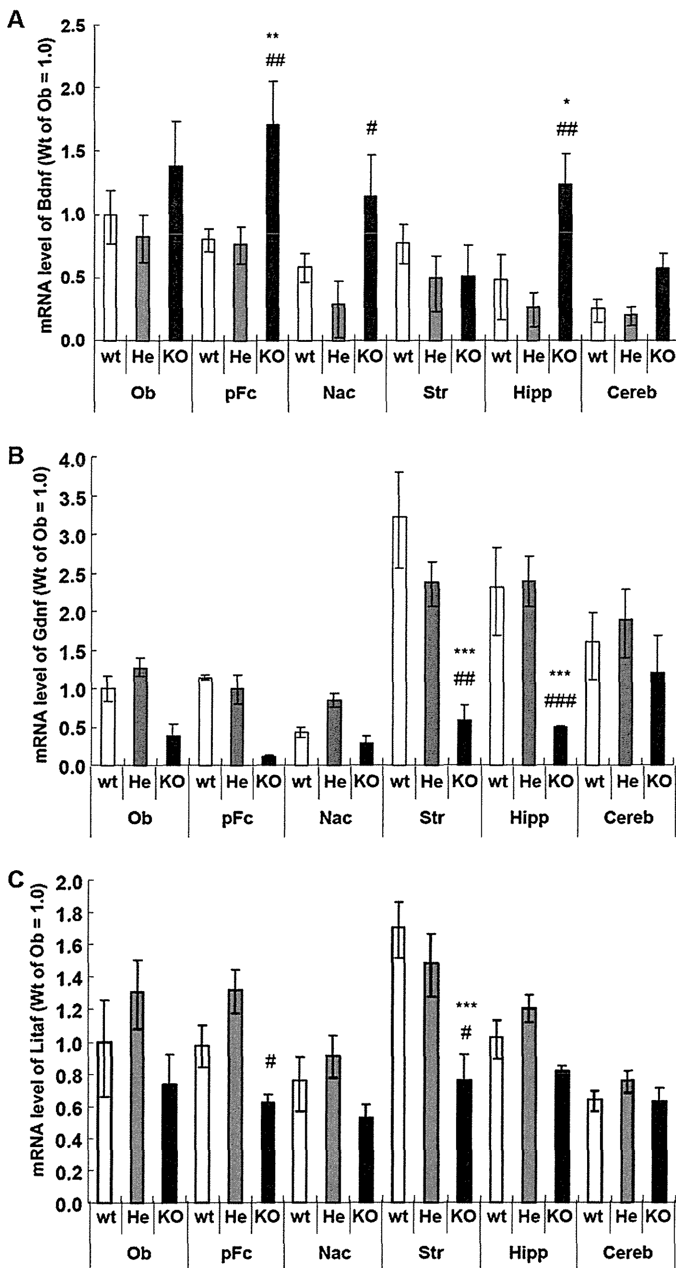

Fig. 2. Effect of lack of the *Shati* gene on the expression levels of BDNF, GDNF and LITAF. Levels of mRNA expression of BDNF (A), GDNF (B) and LITAF (C) in the brain of heterozygous (He) or homozygous *Shati*-knockout mice (KO) were compared with those in wild type mice (Wt). The mRNA level of each factor in the Ob of the respective mouse was used as the standard. Ob, olfactory bulb; pFc, prefrontal cortex; Nac, nuclear accumbens; Str, striatum; Hipp, hippocampus; Cereb, cerebellum. Results are presented as the mean ± S.E. $n = 6$ (Wt), $n = 4$ (each of He and KO). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ KO vs. Wt, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ KO vs. He by Bonferroni's post tests.

type mice, and *Shati* mRNA was not detected in the homozygous knockout mice (Fig. 1D).

3.2. Effects of absence of Shati on the expression of GDNF, BDNF and LITAF

Absence of the *Shati* gene remarkably increased the *Bdnf* mRNA level in the prefrontal cortex and hippocampus (brain area, $F(5, 66) = 6.98$, $p < 0.001$; genotype, $F(2, 66) = 16.76$, $p < 0.001$; interaction, $F(10, 66) = 1.52$, $p = 0.15$.) (Fig. 2A). On the other hand, the *Gdnf* mRNA level in the striatum and hippocampus of *Shati*-knockout mouse was significantly decreased compared with those in wild type and heterozygous mice (brain area, $F(5, 66) = 11.12$, $p < 0.001$; genotype, $F(2, 66) = 24.49$, $p < 0.001$; interaction, $F(10, 66) = 2.42$, $p = 0.016$) (Fig. 2B). The expression of *Litaf* mRNA was also decreased in the striatum of homozygous *Shati*-knockout mice (brain area, $F(5, 66) = 7.22$, $p < 0.001$; genotype, $F(2, 66) = 16.68$, $p < 0.001$; interaction, $F(10, 66) = 1.49$, $p = 0.163$) (Fig. 2C).

3.3. Absence of the Shati gene increased rearing and grooming behaviors

In the open field test, the distance traveled was lower in *Shati*-knockout mice compared with wild type mice during the 5 min measurement time (area $F(2, 45) = 658.98$, $p < 0.001$; genotype $F(1, 45) = 7.71$, $p < 0.01$; interaction $F(2, 45) = 3.39$, $p < 0.05$) (Fig. 3A). There was no difference between *Shati*-knockout and wild type mice in place preference among the outer, middle and center areas of the open field (Fig. 3B). The rearing time and grooming time were increased in the *Shati*-knockout mice (Fig. 3C and D). In the elevated plus maze test, there was no difference between *Shati*-knockout mice and wild type mice in the length of stay in the open arm (Fig. 3E).

3.4. Shati-knockout mice avoided social interaction with novel mice in unfamiliar environments

Shati-knockout mice actively investigated the novel mouse in their home cage, and there was no difference in the interaction time compared with wild type mice. However, the absence of SHATI in the knockout mice resulted in reduction of interaction time in an unfamiliar environment. *Shati*-knockout mice did not actively investigate but were largely submissive to the novel mouse out of their territory compared with wild type mice (environment, $F(1, 32) = 3.43$, $p = 0.074$; genotype, $F(1, 32) = 5.73$, $p = 0.023$; interaction, $F(1, 32) = 6.66$, $p = 0.015$) (Fig. 3F). Interestingly, the exploration time of novel objects in the acquisition phase of the novel object recognition test was significantly longer in the *Shati*-knockout mice compared with wild type mice (Fig. 3G).

3.5. Absence of SHATI did not affect memory

Shati-knockout mice demonstrated no significant differences in performance compared with wild type mice in the novel object recognition test (Fig. 3H). In the Y-maze test, the number of alternations and total number of arm entries of *Shati*-knockout mice were same as those of wild-type mice (Fig. 3I). These results suggest that deletion of the *Shati* gene had no effect on recognition memory and short-term memory.

3.6. Sex difference

Any sex difference was not observed in the behavioral experiments.

4. Discussion

In the present study, there were no differences between *Shati*-knockout and wild type mice in place preference among the outer, middle and center areas of the open field, or open arm and closed arm of the elevated plus maze. On the other hand, *Shati*-knockout mice spent longer periods of time with rearing and grooming in the open field test. An increase in time spent grooming can actually indicate greater anxiety, especially when the mouse has been placed into a stressful environment [5]. These results suggested that absence of *Shati* may increase anxiety-like behavior. Moreover, *Shati*-knockout mice showed significantly reduced interaction time