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Research report

The involvement of brain-derived neurotrophic factor in 3,4-methylenedioxymethamphetamine-induced place preference and behavioral sensitization

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ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA) is known to induce dependence and psychosis in humans. Brain-derived neurotrophic factor (BDNF) is involved in the synaptic plasticity and neurotrophy in midbrain dopaminergic neurons. This study aimed to investigate the role of BDNF in MDMA-induced dependence and psychosis. A single dose of MDMA (10 mg/kg) induced BDNF mRNA expression in the prefrontal cortex, nucleus accumbens, and amygdala, but not in the striatum or the hippocampus. However, repeated MDMA administration for 7 days induced BDNF mRNA expression in the striatum and hippocampus. Both precursor and mature BDNF protein expression increased in the nucleus accumbens, mainly in the neurons. Additionally, rapidly increased extracellular serotonin levels and gradually and modestly increased extracellular dopamine levels were noted within the nucleus accumbens of mice after repeated MDMA administration. Dopamine receptor antagonists attenuated the effect of repeated MDMA administration on BDNF mRNA expression in the nucleus accumbens. To examine the role of endogenous BDNF in the behavioral and neurochemical effects of MDMA, we used mice with heterozygous deletions of the BDNF gene. MDMA-induced place preference, behavioral sensitization, and an increase in the levels of extracellular serotonin and dopamine within the nucleus accumbens, were attenuated in BDNF heterozygous knockout mice. These results suggest that BDNF is implicated in MDMA-induced dependence and psychosis by activating the midbrain serotonergic and dopaminergic neurons.

1. Introduction

An amphetamine derivative, 3,4-methylenedioxymethamphetamine (MDMA, also known as “ecstasy”), is a widely used as recreational drug that has both stimulant and hallucinogenic properties [1]. Like other amphetamine-type psychostimulants, some ecstasy users develop dependence and psychosis [2,3]. In animal models, the effects of MDMA are modeled by using behavioral parameters of conditioned place preference [4,5]. This allows us, to assess drug dependence based on the rewarding effect of the drug, by associating of environmental stimuli with the drug [4,5]. Additionally, we can analyze behavioral sensitization after repeated drug application to assess psychosis induced by a long-term drug intake [6], or self-administration to assess dependence based on voluntary drug-seeking and drug-taking behaviors, using operant conditioning [7]. Several studies have shown that serotonin and dopamine are involved in the dependence and psychotic

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effects of MDMA [8]. MDMA stimulates serotonin release from the neurons after uptake by the serotonin transporter as a substrate. However, it interacts to a lesser extent with the dopaminergic system, indirectly *via* serotonergic activation, and directly *via* dopamine transporters [9,10]. Psychostimulant-induced neuronal plasticity *via* gene expression is thought to be responsible for the development of dependence and psychosis [11]. However, the molecular mechanisms of MDMA-induced dependence and psychosis occurring after these serotonergic and dopaminergic events have not yet been elucidated.

Brain-derived neurotrophic factor (BDNF) is a member of secreted proteins known as neurotrophins, which are related to the canonical nerve growth factor [12]. BDNF has neurotrophic properties that regulate the survival of existing neurons and encourage the growth and differentiation of new neurons and synapses [13,14]. In addition to these functions, BDNF has been shown to contribute to synaptic plasticity [15]. BDNF is released from postsynaptic neurons in an activity-dependent manner [16], and acts as a target-derived messenger in synaptic plasticity and synaptic development, both during development and in the adult central nervous system [17]. The mesocorticolimbic dopaminergic system is known for its reward pathway, and is highly involved in drug-induced psychosis and dependence [18,19]. Since it regulates neuronal plasticity of the reward pathway, BDNF is widely implicated in the dependence of drugs, such as psychomotor stimulants, morphine, nicotine and alcohol [20]. MDMA modulates BDNF expression in the rat brain [21] and in the serum of addicted humans [22].

In this study, we investigate whether BDNF is involved in MDMA-induced dependence and psychosis, using conditioned place preference and behavioral sensitization, respectively, in mice. Here, we show that the expression of BDNF is induced by single and repeated MDMA administrations in the brain, and that BDNF expression is involved in the development of MDMA-induced place preference and behavioral sensitization, by regulating MDMA-induced serotonin and dopamine release in the nucleus accumbens.

2. Materials and methods

2.1. Animals and drugs

Mice were housed under a standard 12:12-hours light/dark cycle (lights on at 9:00 am), at a constant temperature of 23 ± 1 °C, with *ad libitum* access to food and water throughout experiments. Animals were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Meijo University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6J BDNF heterozygous knockout ($BDNF^{+/-}$) mice were obtained from Jackson Laboratory (Bar Harbor, ME), and raised in our laboratory. Male $BDNF^{+/-}$ mice, aged 8–12 weeks, were used for our experiments because BDNF homozygous knockout ($BDNF^{-/-}$) mice die a few days after birth (postnatal 7 days). Thus, $BDNF^{+/-}$ mice are a viable option [23]. Littermate $BDNF^{+/+}$ mice were used as wild-type mice in all experiments. (+/−)-3,4-Methylenedioxymethamphetamine HCl (MDMA; synthesized by Dr Tatsunori Iwamura, Matsuyama University), dopamine D_1 receptor antagonist, R-(+)-SCH23390 (Sigma-Aldrich, St. Louis, MO), and dopamine D_2 receptor antagonist, S-(−)-raclopride (Sigma-Aldrich), were dissolved in saline. Subcutaneous (s.c.) doses of MDMA (10 mg/kg), SCH23390 (0.1 mg/kg), and raclopride (2 mg/kg), were selected based on previous reports [24–26].

2.2. Real-time reverse transcription-PCR

Two hours after a single or the last repeat of MDMA administration, the mice were sacrificed by decapitation, and the brains were immediately removed (Fig. 1A). The prefrontal cortex, nucleus accumbens, striatum, hippocampus, and amygdala were dissected out in accordance with the atlas on an ice-cold plate, frozen, and stored at −80 °C until further use. Each frozen brain sample was homogenized, all the RNA was extracted using an RNeasy® total RNA isolation kit (Qiagen, Valencia, CA), and converted into cDNA using a SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen Life Technologies). The primers used were as follows: BDNF (GenBank accession number BC034862), forward primer, 5′-GCAAACATGTCTATGAGGGTTCG-3′, and reverse primer, 5′-ACTCGCTAATACTGTCACACACG-3′; TaqMan probe, 5′-ACTCCGACCCTGCCCGCCGT-3′, and β-actin (GenBank accession number NM007393), forward primer, 5′-GGGCTATGCTCTCCCTC-ACG-3′, and reverse primer, 5′-GTCACGCACGATTTCCCTCTC-3′; TaqMan probe, 5′-CCTGCGTCTGGACCTGGCTGGC-3′. PCRs were performed using the Platinum Taq DNA polymerase (Invitrogen). The reaction profile consisted of the first round at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 34 s, and extension at 72 °C for 1 min, with the final extension reaction carried out at 72 °C for 10 min in an iCycle iQ Detection System (Bio-Rad Laboratories, Hercules, CA). To standardize the quantification, β-actin was amplified simultaneously. Expression levels were calculated by the delta-delta Ct method.

2.3. Western blotting

Three hours after the last repeated MDMA administration for 7days, the mice were sacrificed by decapitation, and the brains were immediately removed (Fig. 1A). Tissue extracts were prepared by homogenizing brain tissue by sonication in an ice-cold lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1% NP-40, 1 mM sodium orthovanadate, pepstatin 20 μg/mL, aprotinin 20 μg/mL, and leupeptin 20 μg/mL]. The homogenate was centrifuged at 16,000 × g for 20 min and the supernatant was used. The protein concentration was determined using a DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Samples (10–100 μg of protein) were boiled in the Laemmli sample buffer [125 mM Tris–HCl (pH 6.8), 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, and 0.004% bromophenol blue], separated on a polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Billerica, MA). The membranes were blocked with a Detector Block Kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and probed with a primary antibody. Membranes were washed with the washing buffer [50 mM Tris–HCl (pH 7.4), 0.05% Tween 20, and 150 mM NaCl] and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody. Immune complexes were detected by ChemiDoc XRS (Bio-rad) based on chemiluminescence (ECL kit, GE Healthcare, Buckinghamshire, UK). Band intensities were analyzed by densitometry using the ATTO Densitograph Software Library Lane Analyzer (ATTO, Tokyo, Japan). To normalize each sample, membranes were stripped with stripping buffer [100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl (pH 6.7)] at 50 °C for 30 min, and the amount of β-actin protein was determined using the antibody and chemiluminescence. The primary antibodies were rabbit anti-BDNF (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-β-actin (1: 500; Santa Cruz Biotechnology). The secondary antibodies, used at a dilution of 1:2000, were horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (Kirkegaard and Perry Laboratories).

2.4. Immunofluorescence

Histological procedures were performed as previously described [27] with a minor modification. Three hours after the last repeat of the 7-day MDMA administration, the mice were deeply anesthetized with sodium pentobarbital [40 mg/kg, intraperitoneal (i.p.)], and perfused transcardially with ice-cold phosphate-buffered saline (PBS), followed

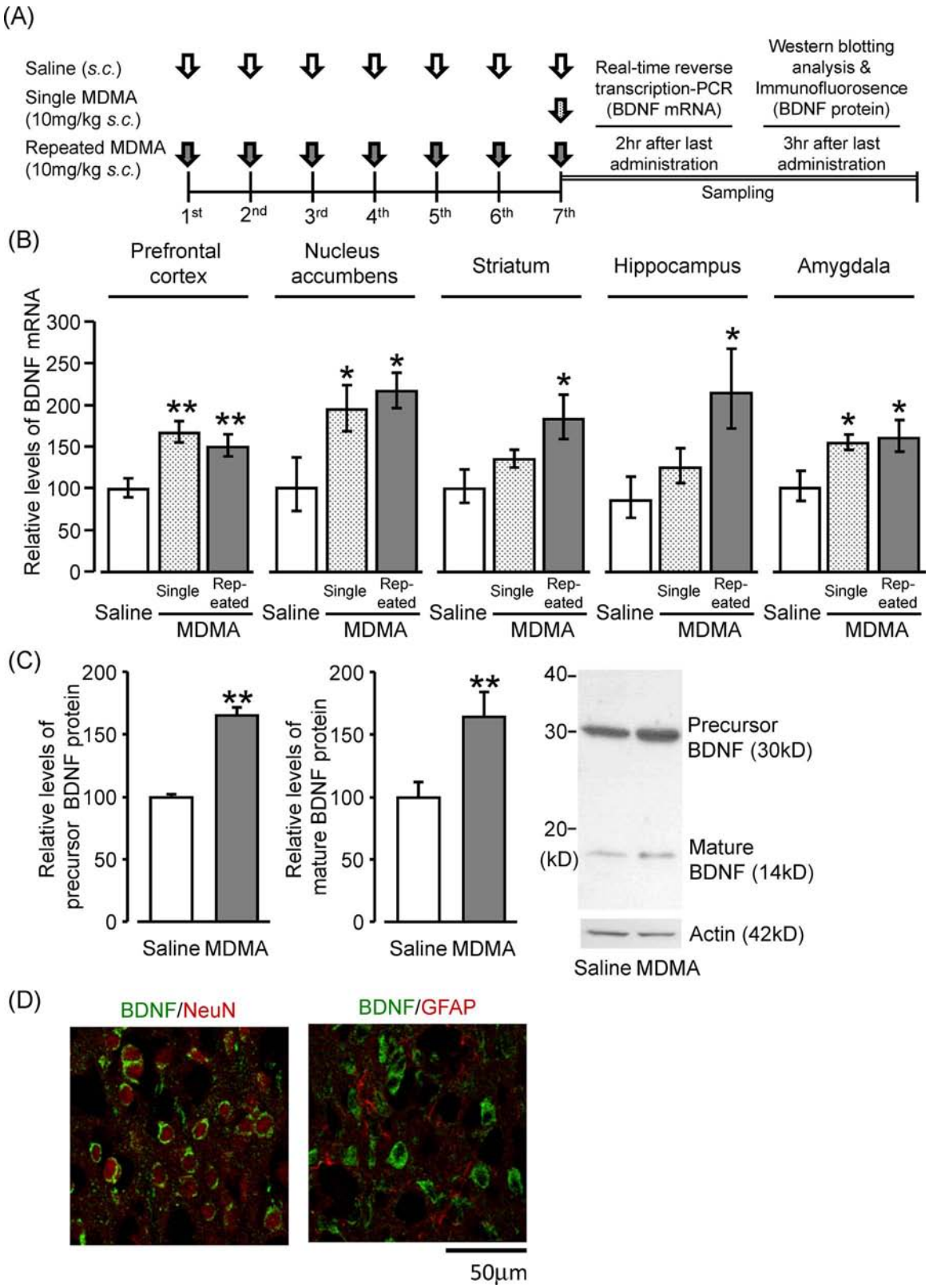

Fig. 1. BDNF mRNA and protein expression induced by MDMA, and its localization in nucleus accumbens. (A) Experimental schedule for real-time reverse transcription-PCR, Western blot analysis and immunofluorescence. (B) Changes in BDNF mRNA expression in the mouse brain after single (1st) and repeated (7th) MDMA administration: Brain samples were collected 2 h after single or the last repeat of MDMA administration (10 mg/kg, s.c., for 7 days). (C) Changes in BDNF protein expression in the nucleus accumbens after the last repeat of MDMA administration: Mice were administrated with MDMA (10 mg/kg, s.c.) for 7 days. The nucleus accumbens was dissected out 3 h after the last MDMA administration. (D) Immunohistochemical analysis of MDMA-induced BDNF expression in the nucleus accumbens of mice: Mice were administrated with MDMA (10 mg/kg, s.c.) for 7 days. Mice were anesthetized and perfused transcardially with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS 3 h after the last MDMA administration. Values indicate mean ± SEM. $*p < 0.05$ and $**p < 0.01$ compared to saline/saline-administrated mice.

by 4% paraformaldehyde in PBS (Fig. 1A). The brains were removed, postfixed in the same fixative for 2 hours, and then soaked in 20% sucrose in PBS. Coronal sections, 15-μm-thick, were cut with a Cryostar HM560 cryostat (Microm International, GmbH, Walldorf, Germany). For immunocytochemistry, cells were plated at low density on Lab-Tek® Chamber Slides (BD Bioscience, San Jose, CA). Forty-eight hours after transfection, cells were washed with PBS and fixed with 4% paraformaldehyde prepared in PBS. For immunostaining, the samples were treated with 4%-BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) dissolved in 0.3% Triton-TBS (TTBS). Primary antibodies, such as a rabbit anti-BDNF (1:500; Santa Cruz Biotechnology), a mouse anti-neuron-specific nuclear antigen (NeuN) (1:500; Millipore), and a mouse anti-GFAP (1:500; Millipore), were applied to brain slices. Fluorescently conjugated secondary antibodies (Alexa 488, 546, Invitrogen) were used for detecting chromogen. Images were acquired with a confocal microscope (LSM510, Carl Zeiss, Jena, Germany).

2.5. *In vivo* microdialysis

In vivo microdialysis was performed as previously described [28]. Six hours after the repeated MDMA administration for 6 days, mice were anesthetized with sodium pentobarbital (40 mg/kg i.p.) before stereotaxic implantation of a guide cannula (AG-6, Eicom) into the nucleus accumbens (1.7 mm anteroposterior, ± 0.8 mm mediolateral from the bregma, −4.0 mm dorsoventral from the skull) (Figs. 2A, 5A). One day after the operation, a dialysis probe (AI-6-1; 1 mm membrane length, Eicom) was inserted through the guide cannula and perfused with artificial cerebrospinal fluid (CSF: 147 mM NaCl, 4 mM KCl and 2.3 mM $CaCl_2$) at a flow rate of 1 μL/min. The dialysate was collected every 10 min. Dialysates were analyzed by High Performance Liquid Chromatography (HPLC) with an electrochemical detector (HTEC-500, Eicom). Three time points were chosen for measurements, to establish baseline levels of the extracellular neurotransmitter.

2.6. Conditioned place preference test

The conditioned place preference test was performed in accordance with a previous report [29], with minor modifications. The apparatus consisted of two compartments: a transparent Plexiglas box and a black Plexiglas box (both 15 × 15 × 15 cm). To enable the mice to distinguish the transparent box from the black box, the floors of the transparent and black boxes were covered with a white plastic mesh and a frosting Plexiglas, respectively. In the pre-conditioning period, the target mouse was allowed to move freely between both the boxes for 15 min, once a day, for 3 days (Fig. 3A). On day 3, the time that the mouse spent in the transparent and black boxes was measured using a SCANET SV-10 LD (Brainscience idea, Osaka, Japan). The conditioning was performed for 6 successive days. On days 4, 6, and 8, the mouse was administrated MDMA and confined in the non-preferred box where the animal had spent less time than the other (preferred) box during the pre-conditioning test, for 30 min. On days 5, 7, and 9, the mouse was administered saline and confined in the preferred box for 30 min. On day 10, the post-conditioning test was performed without drug administration, and the time that the mouse spent in the transparent and black boxes for 15 min was measured. MDMA (10 mg/kg, s.c.) was injected 30 min before the conditioning test. lace conditioning behavior was expressed by “post” and “pre” values calculated as follows: [(post value) − (pre value)], where post and pre values were the difference in time spent at the drug-conditioning, and the saline-conditioning sites in the post-conditioning and pre-conditioning, respectively.

2.7. Measurement of locomotor activity

Locomotor activity was measured in accordance with a previous report [29], with minor modifications. Mice were individually placed in a transparent acrylic cage with a black frosting Plexiglas floor (30 × 47.5 × 35 cm), and locomotor activity was measured every 5 min for 60 min using digital counters with an infrared sensor, SCANET SV-10 LD (Brainscience Idea). Each mouse was habituated to

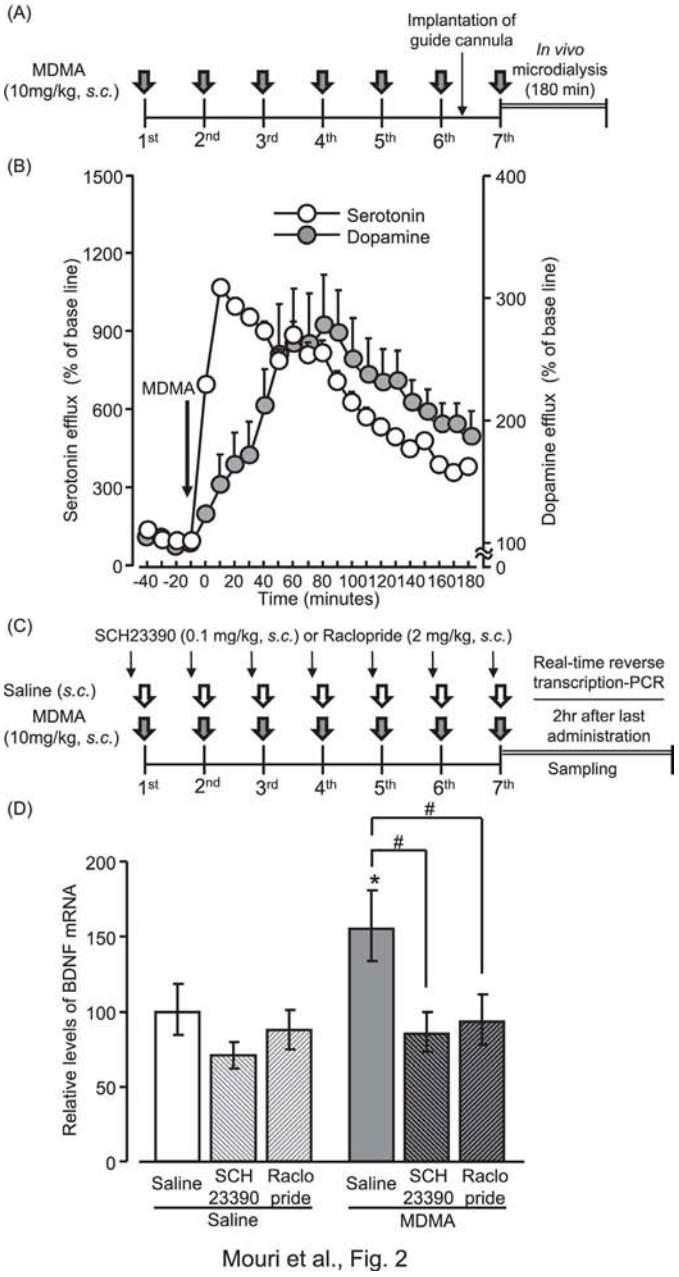

Fig. 2. Involvement of the mesolimbic dopaminergic system in MDMA-induced BDNF mRNA expression. (A) Experimental schedule for *in vivo* microdialysis. (B) The levels of extracellular serotonin and dopamine release induced by MDMA challenge were measured in the nucleus accumbens of mice with repeated MDMA administration (10 mg/kg, s.c.) for 7 days. Six hours after the repeat of MDMA administration at the 6th day, guide cannula was implanted into the nucleus accumbens of the target mice. One day after the implantation of guide cannula, a dialysis probe was inserted through the guide cannula and perfused continuously with CSF. Baseline extracellular levels of serotonin and dopamine were 0.020 ± 0.003 pmol/10 μL/10 min and 0.201 ± 0.071 pmol/10 μL/10 min, respectively. The arrows indicate the last repeat of the 7-day MDMA administration. (C) Experimental schedule for real-time reverse transcription-PCR. (D) Effects of the dopamine D_1 receptor antagonist, SCH23390, or D_2 receptor antagonist, raclopride, on MDMA-induced BDNF mRNA expression in the nucleus accumbens. Mice were administrated SCH23390 (0.1 mg/kg, s.c.) or raclopride (2 mg/kg, s.c.), 30 min before daily MDMA (10 mg/kg, s.c. for 7 days) administration. Brain samples were collected 2 h after the last MDMA administration. Values indicate mean ± SEM. $^*p < 0.05$ compared to saline/saline-administrated mice, $\#p < 0.05$ compared to saline/MDMA-administrated mice.

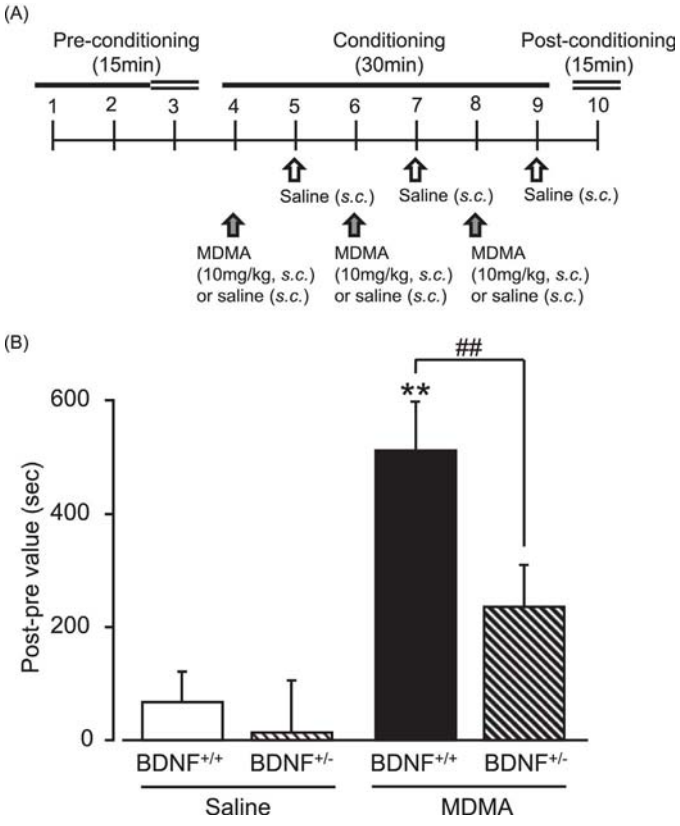

Fig. 3. Role of BDNF in MDMA-induced place preference. (A) Experimental schedule for the conditioned place preference test. (B) $BDNF^{+/+}$ and $BDNF^{+/-}$ mice were trained for place preference under MDMA administration (10 mg/kg, s.c.). Values indicate means ± SEM. $^{**}p < 0.01$ compared to saline-administrated $BDNF^{+/+}$ mice. $\#\#p < 0.01$ compared to MDMA-administrated $BDNF^{+/+}$ mice. $BDNF^{+/+}$: wild-type. $BDNF^{+/-}$: BDNF heterozygous knockout.

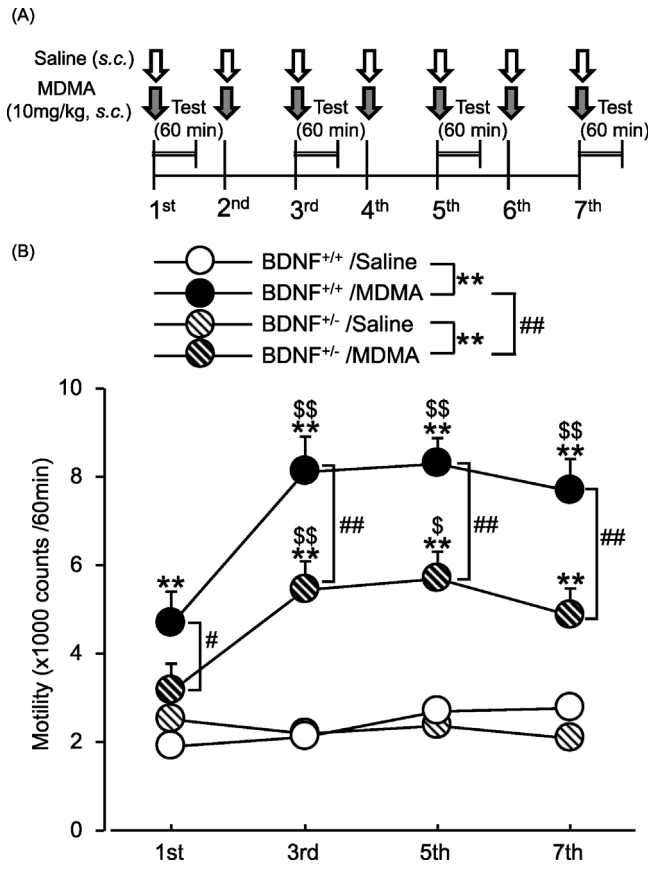

Fig. 4. Role of BDNF in MDMA-induced behavioral sensitization. (A) Experimental schedule for locomotor activity. (B) $BDNF^{+/+}$ and $BDNF^{+/-}$ mice were administered MDMA (10 mg/kg, s.c.) or saline once a day for 7 days. Locomotor activity for 60 min was measured on the 1st, 3rd, 5th, and 7th day after MDMA administration. The mice were habituated to the test cage for 30 min before MDMA administration. Locomotor activity was measured for 60 min. Values indicate mean ± SEM. $^{**}p < 0.01$ compared to corresponding saline-administrated mice. $\#p < 0.05$ and $\#\#p < 0.01$ compared to MDMA-administrated $BDNF^{+/+}$ mice. $\$p < 0.05$ and $\$\$p < 0.01$ compared to the 1st day. $BDNF^{+/+}$: wild-type. $BDNF^{+/-}$: BDNF heterozygous knockout.

the test environment for 60 min before the measurement of locomotor activity. $BDNF^{+/+}$ and $BDNF^{+/-}$ mice were injected with saline or MDMA (10 mg/kg, s.c.), and locomotor activity was measured on the 1st, 3rd, 5th and 7th day after administration (Fig. 4A).

2.8. Statistical analysis

All results were expressed as the mean ± SEM for each group. The difference between groups was analyzed using one-way or Two-way analysis of variance (ANOVA), followed by the Fisher's PLSD test. Student's *t*-test was used to compare two sets of data. *P*-values less than 0.05 ($p < 0.05$) indicated statistically significant differences.

3. Results

3.1. Effect of MDMA on BDNF mRNA expression in the mouse brain:

Single MDMA administration remarkably increased BDNF mRNA expression in the prefrontal cortex, nucleus accumbens, and amygdala, but not in the striatum or hippocampus, compared to saline administration (Fig. 1B, prefrontal cortex; one-way ANOVA: $F_{(2,23)} = 8.27$, $p < 0.01$, nucleus accumbens; one-way ANOVA: $F_{(2,23)} = 4.35$, $p < 0.05$, amygdala; One-way ANOVA: $F_{(2,23)} = 4.16$, $p < 0.05$). However, repeated administration of MDMA enhanced BDNF mRNA expression significantly in the aforementioned brain regions and in the striatum and hippocampus of mice, compared to expression in mice administered with saline (Fig. 1B, striatum; one-way ANOVA: $F_{(2,23)} = 3.38$, $p < 0.05$, hippocampus; one-way ANOVA: $F_{(2,23)} = 3.26$, $p < 0.05$).

3.2. BDNF protein expression induced by repeated MDMA administration and its localization in the nucleus accumbens

We focused on the nucleus accumbens in the following experiments, since it plays very important roles in drug dependence [11]. The protein expression levels of both precursor and mature BDNF in the nucleus accumbens were determined because BDNF is synthesized as a precursor BDNF protein that is further cleaved into a mature BDNF protein [30]. Both precursor and mature protein expression levels in mice, with repeated MDMA administration, were significantly higher than those in mice administered saline (Fig. 1C, precursor BDNF: Student's *t*-test, $t_{12} = -10.50$, $p < 0.01$, mature BDNF: Student's *t*-test, $t_{12} = -3.10$, $p < 0.01$). To determine the cell types that expressed BDNF upon repeated MDMA administration, double immunostaining of BDNF with NeuN, a neuronal marker, or GFAP, a glial marker, was performed because BDNF is synthesized and released from neurons and glia in the brain [31,32]. BDNF immunoreactivity merged with NeuN, and also with GFAP, although sparsely, in the nucleus accumbens (Fig. 1D).

3.3. Involvement of the mesolimbic dopaminergic system in MDMA-induced BDNF mRNA expression

MDMA primarily induces serotonin release by the inhibition of the serotonin transporters (SERT) and the activation of the postsynaptic serotonin 5-HT_{2A} receptors located on the mesolimbic dopaminergic neurons, projecting to the nucleus accumbens [33]. *In vivo* microdia-

lysis revealed that the extracellular serotonin level rapidly and explosively increased in the nucleus accumbens of mice with repeated MDMA administration (Fig. 2B, two-way ANOVA, $F_{\text{transmitter (1, 190)}} = 144.70$, $p < 0.01$, $F_{\text{time (18, 190)}} = 1.87$, $p < 0.05$, $F_{\text{transmitter} \times \text{time (18, 190)}} = 1.97$, $p < 0.05$). Followed by an increase in the extracellular serotonin level, extracellular dopamine level also gradually, yet modestly, increased in the nucleus accumbens of mice with repeated MDMA administration (Fig. 2B). Serotonin 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2A} antagonists fail to alter MDMA self-administration in rats [7]. However, dopamine D_1 and D_2 receptors in the nucleus accumbens play a critical role in psychosis and drug dependence [34]. To determine whether the activation of dopamine D_1/D_2 receptors, followed by an increase in the extracellular dopamine level in the nucleus accumbens, is involved in MDMA-induced BDNF mRNA expression, the dopamine D_1 receptor antagonist, SCH23390, or dopamine D_2 receptor antagonist, raclopride, was administrated 30 min prior to each MDMA administration for 7 consecutive days. The MDMA-induced BDNF mRNA expression in the nucleus accumbens was significantly and completely prevented by the pre-administration of either SCH23390, or raclopride (Fig. 2D, two-way ANOVA, $F_{\text{MDMA (1, 48)}} = 4.63$, $p < 0.05$, $F_{\text{dopamine antagonists (2, 48)}} = 4.62$, $p < 0.05$, $F_{\text{MDMA} \times \text{dopamine antagonists (2, 48)}} = 0.65$, $p = 0.52$).

3.4. Involvement of BDNF in MDMA-induced place preference

We focused on the involvement of BDNF in the rewarding effects of MDMA, which can be assessed by using the conditioned place preference test. In $BDNF^{+/+}$ mice, MDMA administration induced place preference, indicating the induction of the rewarding effect. However, saline administration had no effect on place preference (Fig. 3B, two-way ANOVA, $F_{\text{MDMA (1, 40)}} = 4.76$, $p < 0.05$, $F_{\text{BDNF KO (1, 40)}} = 18.93$, $p < 0.05$, $F_{\text{MDMA} \times \text{BDNF KO (1, 40)}} = 2.05$, $p = 0.15$). Interestingly, the MDMA-induced place preference in $BDNF^{+/-}$ mice was significantly lower than that in $BDNF^{+/+}$ mice (Fig. 3B). Furthermore, the place preference in $BDNF^{+/-}$ mice was not significant, compared to saline-treated $BDNF^{+/-}$ mice (Fig. 3B).

3.5. Involvement of BDNF in MDMA-induced behavioral sensitization

The nucleus accumbens is involved not only in place preference but also in behavioral sensitization to psychostimulants [35]. Therefore, we measured the locomotor-stimulating effects of MDMA in $BDNF^{+/-}$ mice. Single MDMA administration induced a hyperlocomotion in $BDNF^{+/+}$ mice but not in $BDNF^{+/-}$ mice, and the magnitude was significantly less compared to that in $BDNF^{+/+}$ mice (Fig. 4B, two-way ANOVA, $F_{\text{group (3, 156)}} = 95.07$, $p < 0.01$, $F_{\text{day (3, 156)}} = 8.76$, $p < 0.01$, $F_{\text{group} \times \text{day (9, 156)}} = 3.16$, $p < 0.01$). MDMA-induced hyperlocomotion was augmented by repeated a MDMA administration in the $BDNF^{+/+}$ mice and $BDNF^{+/-}$ mice, but the magnitude in $BDNF^{+/-}$ mice was significantly lesser, compared to that in $BDNF^{+/+}$ mice (Fig. 4B).

3.6. Role of BDNF in MDMA-induced serotonin and dopamine release in the nucleus accumbens

To determine the BDNF-mediated mechanisms of MDMA-induced place preference and behavioral sensitization, we measured levels of extracellular serotonin and dopamine in the nucleus accumbens of $BDNF^{+/-}$ mice after repeated MDMA administration. *In vivo* microdialysis revealed that basal levels of serotonin and dopamine in the nucleus accumbens were not different between $BDNF^{+/+}$ and $BDNF^{+/-}$ mice (serotonin; $BDNF^{+/+}$, 0.023 ± 0.004 pmol/10 μL/10 min, $n = 4$; $BDNF^{+/-}$, 0.029 ± 0.006 pmol/10 μL/10 min, $n = 4$, dopamine; $BDNF^{+/+}$, 0.180 ± 0.043 pmol/10 μL/10 min, $n = 4$; $BDNF^{+/-}$, 0.159 ± 0.027 pmol/10 μL/10 min, $n = 4$). The levels of extracellular serotonin and dopamine in the nucleus accumbens were markedly

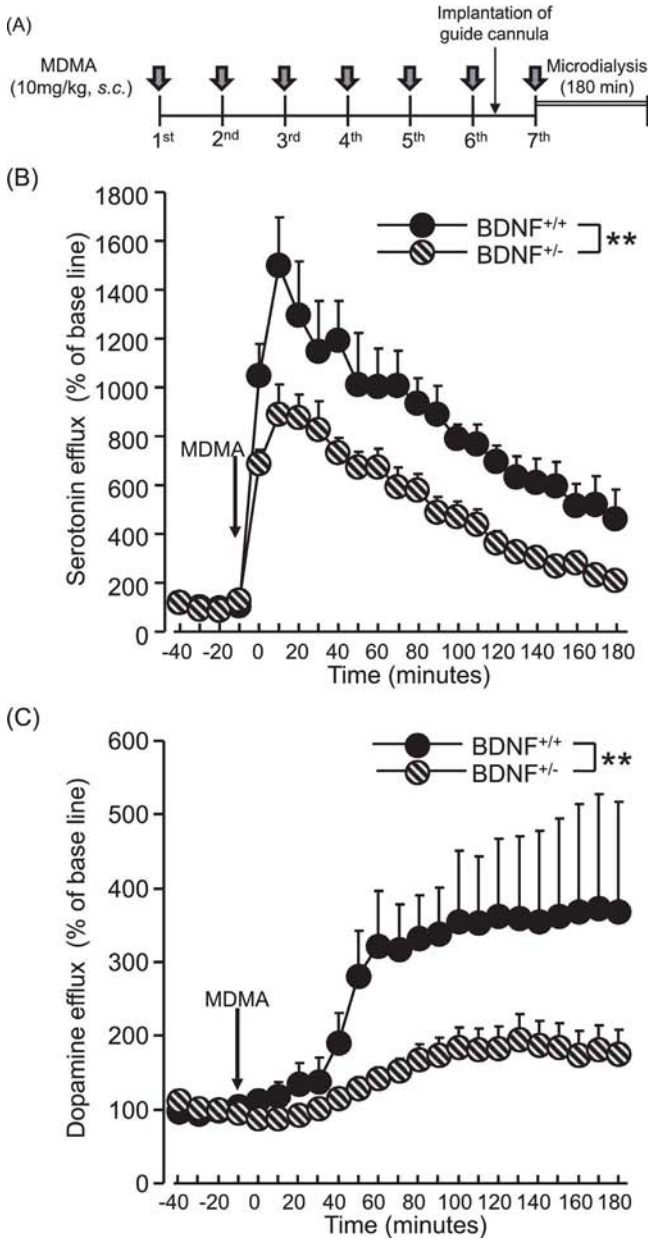

Fig. 5. Role of BDNF in MDMA-induced serotonin and dopamine release in the nucleus accumbens. (A) Experimental schedule for *in vivo* microdialysis. The levels of extracellular serotonin (B) and dopamine (C) release induced by MDMA challenge were measured in the nucleus accumbens of $BDNF^{+/+}$ and $BDNF^{+/-}$ mice, repeatedly administrated with MDMA (10 mg/kg, s.c.) for 7 days. Six hours after the repeated MDMA administration for 6 days, guide cannula was implanted into the nucleus accumbens of the mice. One day after the implantation of guide cannula, a dialysis probe was inserted through the guide cannula and perfused continuously with CSF. The arrows indicate the last repeat of the 7-day MDMA administration. Values are mean ± SEM. ** $p < 0.01$ compared to MDMA-administrated $BDNF^{+/+}$ mice. $BDNF^{+/+}$: wild-type. $BDNF^{+/-}$: BDNF heterozygous knockout.

increased by MDMA challenge at 10 mg/kg in $BDNF^{+/+}$ mice with repeated MDMA administration (Fig. 5B, C; 5B: two-way ANOVA, $F_{\text{BDNF KO (1, 114)}} = 40.78$, $p < 0.01$, $F_{\text{time (18, 114)}} = 2.00$, $p < 0.05$, $F_{\text{BDNF KO} \times \text{time (18, 114)}} = 0.42$, $p = 0.98$, 5C: two-way ANOVA, $F_{\text{BDNF KO (1, 114)}} = 79.72$, $p < 0.01$, $F_{\text{time (18, 114)}} = 9.76$, $p < 0.01$, $F_{\text{BDNF KO} \times \text{time (18, 114)}} = 0.29$, $p = 0.99$). Such MDMA-induced increases in the levels of extracellular serotonin and dopamine were inhibited in $BDNF^{+/-}$ mice after repeated MDMA administration (Fig. 5B, C).

4. Discussion

MDMA exhibits psychostimulant and hallucinogenic properties by increasing the synaptic serotonin and dopamine levels in the brain [1]. Neuronal plasticity of the serotonergic and dopaminergic system, in the response to repeated administration of MDMA, could be involved in long-lasting behavioral consequences, such as dependence and psychosis, and dysphoria, during drug withdrawal [36]. It has been demonstrated that BDNF takes part in the development, maturation, and maintenance of various neurons in the central nervous system and is involved in neuronal plasticity [17]. However, the involvement of BDNF in MDMA-induced neuronal plasticity, dependence, and psychosis, has not fully been explored. In the present study, we demonstrated that BDNF expression, in response to MDMA administration, is involved in MDMA-induced place preference and behavioral sensitization by regulating serotonin and dopamine release in the nucleus accumbens.

Psychostimulant-induced neuronal plasticity in areas of the reward pathway, such as the nucleus accumbens, prefrontal cortex, striatum, hippocampus, and amygdala, implicate drug rewards and sensitization [37]. Single MDMA administration induced BDNF mRNA expression in the prefrontal cortex, nucleus accumbens, and amygdala, but not in the striatum or the hippocampus. However, repeated MDMA administration increased the ability of MDMA to induce BDNF mRNA expression in the striatum and the hippocampus. Brain region specific BDNF mRNA expression, induced by a single MDMA administration, in the rat frontal cortex but not in the hippocampus, has been observed in previous studies [21,38]. Followed by the rapid and explosive increase of extracellular serotonin level, extracellular dopamine level gradually and modestly increased in the nucleus accumbens of mice with repeated MDMA administration. It is reported that the activation of serotonin and/or dopamine receptors results in the transcription of the BDNF gene *via* the activation of the cAMP response element-binding protein (CREB)[39]. Phoshphorylation of CREB in the prefrontal cortex but not in the hippocampus, is increased after a single MDMA administration [21]. The brain region specific expression of BDNF mRNA induced by a single MDMA administration may be, at least partially, due to the increase in activity of the CREB. Further, brain regions expressing BDNF mRNA, spread further, upon repeated administrations of MDMA.

The dopaminergic system is primarily involved in the pharmacological effects of MDMA [40]. Indeed, MDMA-induced hyperactivity is correlated with extracellular dopamine levels in the nucleus accumbens, striatum and the prefrontal cortex [41]. MDMA-induced BDNF mRNA expression in the nucleus accumbens was completely inhibited by pre-administration with the dopamine D_1 receptor antagonist, SCH23390, and the dopamine D_2 receptor antagonist, raclopride, suggesting that the activation of dopamine D_1 and D_2 receptors is attributable to MDMA-induced expression of BDNF in the nucleus accumbens. BDNF mRNA expression and the acceleration of morphological maturation and differentiation of striatal neurons may depend on the activation of PKA and CREB through Gs/olf in the dopamine D_1 receptor [42] and the activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIα) through Gq/11 in the dopamine D_1-D_2 receptor heteromer [43]. Although further experiments are needed to explore the mechanism of MDMA-induced BDNF mRNA expression in other brain regions, augmentation of BDNF mRNA expression in the nucleus accumbens might be associated with indirect functional stimulation of the dopamine D_1 receptor and the dopamine D_1-D_2 receptor heteromer pathway, following increases in extracellular dopamine levels induced by MDMA.

BDNF mRNA is translated to a precursor protein (30 kDa) that is proteolytically processed into a smaller (14 kDa), mature protein [44]. Western blot analysis revealed that repeated MDMA administration increased both precursor and mature BDNF protein expression in the nucleus accumbens. Double immunostaining of BDNF with NeuN showed that MDMA-induced BDNF protein was mainly expressed in the neurons of the nucleus accumbens. Thus, it is suggested that postsynaptic BDNF expression may participate in neuronal plasticity in the brain, following repeated exposure to MDMA.

Behavioral sensitization is characterized by a progressive, enduring enhancement of locomotor response that develops following repeated MDMA administration [6]. Behavioral sensitization reflects neuroadaptive processes associated with drug-induced psychosis [45], and drug dependence [46]. We showed that MDMA-induced behavioral sensitization in $BDNF^{+/-}$ mice was blunted, compared to that in $BDNF^{+/+}$ mice. The development of sensitization, and dopamine release by methamphetamine, can be prevented by pre-administration with intranucleus accumbens injections of antibodies that neutralize BDNF and its specific receptor tyrosine kinase (TrkB) [47]. Conversely, infusion of BDNF into the nucleus accumbens enhances the stimulation of locomotor activity by cocaine in rats; whereas, the development of sensitization is delayed in $BDNF^{+/-}$ mice [48,49]. Conditioned place preference is widely used as a rodent animal model when measuring drug rewards [50,51]. MDMA-induced conditioned place preference was significantly attenuated in $BDNF^{+/-}$ mice as compared to $BDNF^{+/+}$ mice, suggesting that BDNF participates in the rewarding effects of MDMA. Thus, it is suggested that MDMA-induced BDNF expression in the brain plays a role in the development of MDMA-induced place preference and behavioral sensitization, which may be associated with neuronal plasticity. However, although BDNF may modulate the acquisition, retention or extinction of memory [52,53], we cannot exclude the possibility that $BDNF^{+/-}$ failed to associate MDMA-induced place preference with the context during conditioning.

It has been postulated that serotonergic as well as dopaminergic neurons in the nucleus accumbens play an important role in the rewarding effects and the locomotor-stimulating effects of psychostimulants [54]. MDMA has been found not only to competitively inhibit serotonin transport, but also to stimulate accumulated serotonin efflux by reversed membrane transport [9]. Following activation of serotonergic transmission, serotonin $5\text{-}HT_{1B}$ [55], $5\text{-}HT_{2A}$ [33] and $5\text{-}HT_{2B}$ [56,57] receptors are implicated in the rewarding and hyperlocomotion effects of MDMA *via* the regulation of the mesolimbic dopaminergic system. In the present study, MDMA-induced serotonin and dopamine release were markedly decreased in the nucleus accumbens of $BDNF^{+/-}$ mice with repeated MDMA administration. Thus, the attenuation of both MDMA-induced place preference and behavioral sensitization in $BDNF^{+/-}$ mice could be due to a decrease of serotonin and dopamine release in the nucleus accumbens.

5. Conclusion

MDMA administration induces BDNF expression in neurons of the nucleus accumbens. The increase in BDNF levels may be responsible, at least partially, for the development of MDMA-induced place preference and behavioral sensitization, and serotonin and dopamine release and uptake in the nucleus accumbens (Fig. 6). These results, together with the well-known function of BDNF in relation to neuronal plasticity, suggest that a repeated MDMA-induced overexpression of BDNF plays a crucial role in the functional, and possibly structural, changes of serotonergic and dopaminergic transmission in the nucleus accumbens, finally causing an MDMA-induced dependence and psychosis.

Conflict of interest

The authors declare that there is no conflict of interest.

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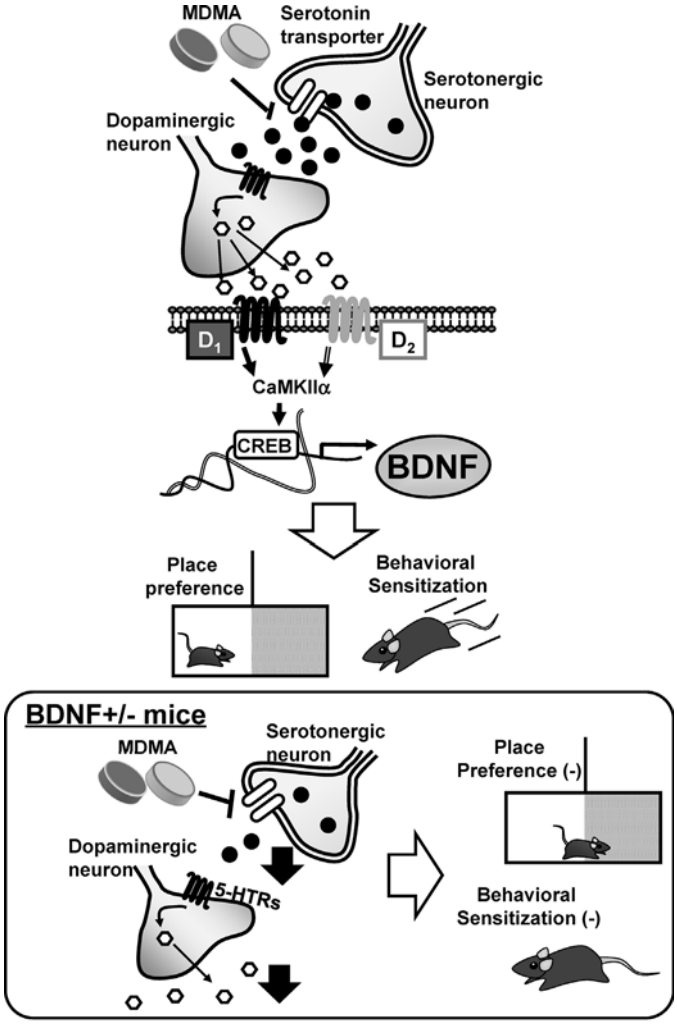

Fig. 6. Possible mechanisms of the involvement of BDNF in the MDMA-induced place preference and behavioral sensitization. MDMA primarily induces serotonin release by the inhibition of the serotonin transporters (SERT). Followed by the increase of extracellular serotonin level, extracellular dopamine level increases by the activation of the serotonin 5-HT receptors located on the presynaptic mesolimbic dopaminergic neurons. Stimulation of the dopamine D_1 receptor and the dopamine D_1-D_2 receptor heteromer induce BDNF mRNA expression *via* Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIα)-cAMP response element-binding protein (CREB) pathway. The increase in BDNF levels may be responsible for the development of MDMA-induced place preference and behavioral sensitization, and serotonin and dopamine release and uptake in the nucleus accumbens.

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