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BONE MORPHOGENETIC PROTEIN-7 REDUCES TOXICITY INDUCED BY HIGH DOSES OF METHAMPHETAMINE IN RODENTS

J. CHOU, **Y. LUO**, **C.-C. KUO**, **K. POWERS**, **H. SHEN**, **B. K. HARVEY**, **B. J. HOFFER**, and **Y. WANG***

National Institute on Drug Abuse, Intramural Research Program, 5500 Nathan Shock Drive, Baltimore, MD 21224, USA

Abstract

Methamphetamine (MA) is a drug of abuse as well as a dopaminergic neurotoxin. We have previously demonstrated that pretreatment with bone morphogenetic protein 7 (BMP7) reduced 6-hydroxydopamine-mediated neurodegeneration in a rodent model of Parkinson's disease. In this study, we examined the neuroprotective effects of BMP7 against MA-mediated toxicity in dopaminergic neurons. Primary dopaminergic neurons, prepared from rat embryonic ventral mesencephalic tissue, were treated with MA. High doses of MA decreased tyrosine hydroxylase immunoreactivity (THir) while increasing terminal deoxynucleotidyl transferase–mediated dNTP nick end labeling. These toxicities were significantly antagonized by BMP7. Interaction of BMP7 and MA *in vivo* was first examined in CD1 mice. High doses of MA (10 mg/kg ×4 s.c.) significantly reduced locomotor activity and THir in striatum. I.c.v. administration of BMP7 antagonized these changes. In BMP7 +/− mice, MA suppressed locomotor activity and reduced TH immunoreactivity in nigra reticulata to a greater degree than in wild type BMP7 +/+ mice, suggesting that deficiency in BMP7 expression increases vulnerability to MA insults. Since BMP7 +/− mice also carry a LacZ-expressing reporter allele at the BMP7 locus, the expression of BMP7 was indirectly measured through the enzymatic activity of β-galactosidase (β-gal) in BMP7 +/− mice. High doses of MA significantly suppressed β-gal activity in striatum, suggesting that MA may inhibit BMP7 expression at the terminals of the nigrostriatal pathway. A similar effect was also found in CD1 mice in that high doses of MA suppressed BMP7 mRNA expression in nigra. In conclusion, our data indicate that MA can cause lesioning in the nigrostriatal dopaminergic terminals and that BMP7 is protective against MA-mediated neurotoxicity in central dopaminergic neurons. Published by Elsevier Ltd on behalf of IBRO.

Keywords

methamphetamine; bone morphogenetic protein-7; tyrosine hydroxylase; protection; degeneration; gene expression

*Corresponding author. Tel: +1-410-550-1753; fax: +1-410-550-1425. ywang@intra.nida.nih.gov (Y. Wang).

Bone morphogenetic protein-7 (BMP7) is a trophic factor in the transforming growth factor (TGF)-β superfamily. Both BMP7 and its receptor are found in the nigrostriatal pathway in the CNS (Chen et al., 2003). BMP7 mRNA is expressed in nigral area of adult rats. Its receptor, bone morphogenetic protein receptor type II (BMPR-II), mRNA is found in neurons of the cortex, dentate gyrus, hippocampus, habenula and substantia nigra in the adult brain (Soderstrom et al., 1996).

The physiological function of BMP7 in nigrostriatal DA neurons has been examined in different laboratories. In rat mesencephalic cell cultures, BMP7 increased the number of tyrosine hydroxylase (TH) cells (Lee et al., 2003) and dopamine (DA) uptake (Jordan et al., 1997). Intranigral delivery of BMP7 reduced motor deficits, prevented the loss of TH immunoreactivity in nigra, and restored DA release in striatum in 6-hydroxydopamine (6-OHDA) -lesioned rats (Harvey et al., 2004). These data suggest that BMP7 has a protective effect against 6-OHDA lesioning in the nigrostriatal DA pathway. Its protective effect against other dopaminergic neurotoxins with more clinical relevance has not been reported.

The protective effect of BMP7 has also been demonstrated in other brain regions. BMP7 reduces H_2O_2-mediated toxicity in primary cortical cultures through activation of the mitogen-activated protein kinase pathway (Cox et al., 2004). Intracerebral administration of BMP7 reduces cerebral infarction after a transient middle cerebral artery occlusion in adult rats (Lin et al., 1999). Similarly, intracerebral transplantation of fetal kidney tissue, which contains high levels of BMP7 protein, reduces caspase-3 activity and cerebral infarction in stroke rats (Chang et al., 2002). These data suggest that an anti-apoptotic mechanism may be involved in BMP7-mediated protection from ischemic injury.

Methamphetamine (MA), at high concentrations, induces degeneration of dopaminergic neurons through the activation of programmed cell death. Acute administration of high doses of MA activates caspase-3 and PARS, up-regulates p53, and produces DNA fragmentation and cell death in dopaminergic neurons (Cadet et al., 2003). Since BMP7 has anti-apoptotic effects against acute brain injury after ischemia and, furthermore, reduces toxicity of the dopaminergic neurotoxin 6-OHDA in the nigrostriatal pathway, we studied whether BMP7 may also reduce MA-mediated damage in dopaminergic neurons through a similar mechanism.

In this communication, we examined the interaction of MA and BMP7 in dopaminergic neurons *in vivo* and *in vitro.* We found that exogenous application of BMP7 reduced MA-mediated neurotoxicity in ventromesencephalic (VM) cultures and in nigrostriatal dopaminergic neurons in adult mice. Reduced BMP7 expression in transgenic mice increased MA toxicity. Our data suggest that BMP7 is neuroprotective against MA-mediated neurotoxicity in dopaminergic neurons both *in vivo* and *in vitro.*

EXPERIMENTAL PROCEDURES

In vitro studies

Primary cultures of rat ventral mesencephalon.—Cultures were prepared from the VM of gestation day 14–15 embryos (E14-15) from Sprague–Dawley rats, obtained from

Charles River Laboratories. Pooled VM tissues were trypsinized (×1; Invitrogen, Carlsbad, CA, USA) for 20 min. After rinsing with cold DMEM media (Invitrogen), cells were resuspended and plated into 96-well cell culture plates pre-coated with poly-lysine at the density of 6×10^4/ml/well. The plating medium consisted of DMEM/F12 (Invitrogen) with 10% heat-inactivated fetal bovine serum and 2% B27 supplement (×1; Invitrogen). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Two days later the cultures were replenished with 0.1 ml of neurobasal medium with B27 supplement (1%) and 0.25 mM L-glutamine. On DIV (days *in vitro*) 9, BMP7 was added to the medium containing B27 supplement without antioxidants. MA was added approximately 20 min later. On DIV 12, cells were fixed with 4% paraformaldehyde (PFA) for DAPI, TUNEL or TH-immunoreactivity.

TH immunocytochemistry in cell cultures.—Cell cultures were fixed for 1 h at room temperature in 4% PFA in PBS. After washing (3×) with PBS, the fixed cultures were treated for 1 h with blocking solution (2% BSA, 0.01% Triton X-100 and 5% goat serum in PBS). The cultures were then incubated 2 days at 4 °C with a monoclonal antibody against TH (1:500; Chemicon, Temecula, CA, USA). The cells were then rinsed three times in PBS. The bound primary antibody was visualized using the Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen). Optical density of TH immunoreactivity per well was analyzed using MetaMorph software (Universal Imaging Corporation, Dowington, PA, USA). TH cell density was counted per field and extrapolated to total area of a well. Averages over four to six wells were used for comparison between groups.

Terminal deoxynucleotidyl transferase–mediated dNTP nick end labeling (TUNEL).—Cultures were assayed for DNA fragmentation using a TUNEL-based method (In Situ Cell Death Detection Kit; Roche, Indianapolis, IN, USA). Briefly, 4% PFA fixed cells were permeabilized in 0.5% Triton X-100 in 0.01 M PBS for 5 min on ice. To label damaged nuclei, 50 μl of the TUNEL reaction mixture was added to each sample and kept at 37 °C in a humidified chamber for 60 min. Procedures for positive and negative controls were carried out as described in the manufacturer's manual (Roche). Controls consisted of not adding the label solution (terminal deoxynucleotidyl transferase) to the TUNEL reaction mixture. Material was examined under a fluorescence microscope after rinsing with 1 × PBS twice.

In vivo studies

Animals and drug administration.—Adult male CD1 were purchased from the Charles River Laboratories. BMP7-lacZ heterozygous with a null BMP7 allele and β-galactosidase insertion (BMP7 +/−) as well as wild-type (+/+) mice were originally generated by Dr. Elizabeth Robertson (Godin et al., 1998). CD1 mice were anesthetized with chloral hydrate (400 mg/kg, i.p.). BMP7 (5 μg/5 μl) or vehicle (5 μl) was injected using a Hamilton syringe into the lateral cerebral ventricle. All animals were injected s.c. with four doses (2 h apart) of MA (10 mg/kg s.c.) or saline (0.01 ml/10 g, s.c.). During the injection period, animals were housed individually without bedding. Animals were used for histological or behavioral study after MA injection as described below.

Behavioral measurements.—Mice were placed in an Accuscan activity monitor (Accuscan Instruments, Columbus, OH, USA) for 72 h of continuous monitoring starting from 1 day after MA or saline injections. The monitor contained 16 horizontal and eight vertical infrared sensors spaced 2.5 cm apart. The vertical sensors were situated 10 cm from the floor of the chamber. Each animal was individually placed in a 42×42×31 cm Plexiglas open box for 3 days. Motor activity was calculated using the number of beams broken by the animals.

TH immunostaining.—Mice were killed on the 4th day after MA application for TH immunoreactivity. Animals were anesthetized and perfused transcardially with saline followed by 4% PFA in phosphate buffer (PB; 0.1 M; pH 7.2). The brains were dissected, postfixed in PFA for 16 h, and transferred to 18% sucrose in 0.1 M PB for at least 16 h. Serial sections of the entire brain were cut at 25 μm thickness in a cryostat. One series from every 4th section was stained for each antibody used. In order to control for staining variability, specimens from all experimental groups were included in every batch and reacted together in a net well tray under the same conditions. Sections were rinsed in 0.1 M PB, and blocked with 4% bovine serum albumin (BSA) and 0.3% Triton X-100 in 0.1 M PB. Sections were then incubated in a primary antibody solution rabbit anti-TH (Chemicon) diluted (1:500) in 4% BSA and 0.3% Triton X-100 in 0.1 M PB for 18 h at 4 °C. Sections were rinsed in 0.1 M PB and incubated in biotinylated goat anti-rabbit IgG in the buffer (1:200; Vector Laboratories, Burlingame, CA, USA) for 1 h, followed by incubation for 1 h with avidin–biotin–horseradish peroxidase complex. Staining was developed with 2,3' diaminobenzidine tetrahydrochloride (0.5 mg/mL in 50 mM Tris–HCl buffer 7.4). Control sections were incubated without primary antibody. Sections were mounted on slides, and coverslipped. Histological images were acquired using an Infinity3 camera and NIKON 80i microscope. TH immunoreactivity in striatum was visualized with the use of a Nikon super-coolscan 9000 scanner.

The optical density of TH immunoreactivity in striatum was analyzed using Scion Image (version 4.02) and averaged from three sections with a visualized anterior commissure (AP: +0.26 mm, +0.14 mm, +0.02 mm to bregma). TH fiber optical density in substantia nigra pars reticulata (SNpr) and TH neuronal density in substantia nigra pars compacta (SNpc) were quantified and averaged from three sections (AP: −3.28 mm, −3.40 mm, −3.52 mm to bregma). TH optical density and TH neuron counts from right and left hemispheres were averaged in each mouse for statistical analysis. All immunocytochemical measurements were done by blinded observers.

Quantitative reverse transcription–PCR (qRT-PCR).—Mice were killed and the brains were immediately harvested and chilled on ice. The SN or whole brain was dissected out and total RNA was extracted following the instruction from the kit (RNAqueous, Ambion, Austin, TX, USA). Total RNA (1 μg) was treated with RQ-1 RNase-free DNase I and reverse transcribed into cDNA using random hexamers by AMV reverse transcriptase (Roche). cDNA levels for HPRT, PGK1, BMP7 and DAT were determined by specific universal probe Library primer probe sets (Roche) by quantitative RT-PCR. For each sample, duplicates were measured in real-time PCR and the results were repeated at least once with

similar results. Primers and FAM-labeled probes used in the quantitative RT-PCR for each gene are as follows:

HPRT: forward primer (5′-tgatagatccattcctatgactgtaga); reverse primer (5′-aagacattctttccagttaaagttgag); probe (5′-tggtggag, mouse universal probe library #22, Roche)

PGK1: forward primer (5′-tacctgctggctggatgg); reverse primer (5′-cacagcctcggcatatttct); probe (5′-gagagcag, mouse universal probe library #108, Roche)

BMP7: forward primer (5′-cgagaccttccagatcacagt); reverse primer (5′-cagcaagaagaggtccgact); probe (5′-gctccagg, mouse universal probe library #1, Roche)

DAT: forward primer (5′-caacctgtactggcggctat); reverse primer (5′-atgctgaccacgaccacata); probe (5′-ctgcttcc, mouse universal probe library #38, Roche)

Measurement of β-galactosidase enzymatic activity.—Two days post-MA or saline injection, BMP7 +/− and +/+ mice were killed by cervical dislocation. The brains were immediately harvested and chilled on ice. The striatum and cortex were dissected out and homogenized in 100 μl of lysis solution (100 mM potassium phosphate (pH=7.8), 0.2% Triton X-100, 1 mM DTT, 1× proteinase inhibitor cocktail, Sigma, St. Louis, MO, USA). The lysate was centrifuged for 10 min at 12,600×*g* at 4 °C and supernatant was heated to 48 °C for 60 min. Two microliter aliquots of the heated supernatant were added to 70 μl Galacto-light™ reaction buffer (Applied Biosystems, Foster City, CA, USA) and incubated for 60 min at room temperature. A 100 μl light emission accelerator was added to each sample before the samples were read in a luminometer (FLX 800, Bio-Tek Instruments, Winooski, VT, USA). Small aliquots were subjected to protein assay (Bio-Rad Protein Assay kit, Bio-Rad, Hercules, CA, USA) before heating to 48 °C and the β-galactosidase activity was normalized to total protein concentration for each sample.

RESULTS

In vitro VM cultures

TH-immunoreactivity and nuclei counts.—Primary VM cultured cells were treated on DIV 9 with BMP7 (1.5 nM) or vehicle and 20 min later, with saline or MA (1 mM). Cells were fixed for TH immunostaining 3 days after MA application. MA reduced THir fiber and neuronal density. A typical MA-induced degeneration of TH positive neurons is shown in Fig. 1A. The density of THir neurons and fibers was quantified by optical density analysis. MA significantly reduced THir fiber density, which was antagonized by the pretreatment with BMP7 (Fig. 1B-1, $P<0.001$, $F_{(3,140)} = 15.899$, one-way ANOVA; $P<0.05$, post hoc Student-Newman-Keuls test). MA also significantly reduced number of TH positive cells and treatment with BMP-7 significantly antagonized the loss of TH positive cells by MA (Fig. 1B-2, $P<0.001$, Kruskal-Wallis one-way ANOVA on ranks; $P<0.05$, post hoc Student-Newman-Keuls test). Overall density of cells was analyzed using MetaMorph software after

DAPI staining. Treatment of VM cells with either 1 mM MA (P=0.456, $F_{(1,140)}$=0.559, two-way ANOVA) or 1.5 nM BMP7 (P=0.666, $F_{(1,140)}$=0.187, two-way ANOVA) did not alter the overall density of DAPI-stained nuclei in the wells.

Tunel.—Cultures were analyzed by TUNEL staining at 3 days after exposure to MA. MA (1 mM) greatly increased the number of TUNEL (+) cells (P<0.05, $F_{(1,32)}$=53.264, two-way ANOVA). There was a statistically significant interaction between MA and BMP7 (P=0.006, $F_{(1,32)}$=8.863, two-way ANOVA). Pretreatment with 1.5 nM BMP7 significantly reduced the MA-related TUNEL staining (Fig. 1B-3, P<0.05, $F_{(1,32)}$=19.288, two-way ANOVA).

Neuroprotection *in vivo*

Decreased BMP7 mRNA level in BMP7 +/− mice.—BMP-7 mRNA levels in the brain of +/+ mice (n=5) and +/− mice (n=5) were measured by qRT-PCR. HPRT1 mRNA showed no differences between +/+ and +/− mice and thus was chosen as a reference gene. After normalization to HPRT1 mRNA levels in each sample, BMP7 mRNA levels were found to be significantly decreased in +/− mice compared with +/+ mice (Fig. 2B). The BMP7 mRNA level in +/− mice was also directly compared with the mean of +/+ mice. The results show that there was a significant decrease (about 50%) in +/− mice (Fig. 2C, P<0.005, Student's t-test). In contrast, another housekeeping gene PGK1 showed no difference between the mRNA level in +/+ and +/− mice (Fig. 2A). These data suggest that BMP7 +/− mice have a decreased BMP7 mRNA level in the brain.

Locomotor activity in CD1 mice.—Locomotor activity was first examined in animals receiving parenteral saline after i.c.v. administration of BMP7 or vehicle. Eight mice were injected with BMP7 (1 μg/μl×5 μl, i.c.v.) and then four doses of saline (0.01 ml/10 g, s.c., every 2 h) and another eight mice were treated with vehicle (5 μl, i.c.v.) and then saline (0.01 ml/10 g, s.c., ×4, every 2 h). Locomotor activity, such as total distance traveled, movement time, and horizontal activity was monitored daily for 3 days starting from 24 h after the first dose of saline. We found that i.c.v. administration of BMP7 did not alter any locomotor parameter as compared with those receiving i.c.v. vehicle (Fig. 3A1-3, P>0.05, two-way ANOVA).

A total of 10 mice were treated with BMP7 (1 μg/μl×5 μl, i.c.v., n=5) or vehicle (5 μl, i.c.v.) and then four doses of MA (10 mg/kg, s.c., every 2 h). The daily locomotor activity after MA injection was normalized by comparison with the average activity on day 1 from the control mice receiving i.c.v. vehicle and parenteral saline injection (Fig. 4A). In all parameters measured, MA greatly reduced locomotor activity to 40–60% of control (Fig. 4A). MA-mediated bradykinesia was antagonized by pretreatment with BMP7. Total distance traveled (P=0.004, $F_{(1,24)}$ = 10.080, two-way ANOVA), movement time (P=0.011, $F_{(1,24)}$=7.686, two-way ANOVA), and horizontal activity (P=0.013, $F_{(1,24)}$=7.176, two-way ANOVA) were all significantly increased toward control levels by BMP7 pretreatment in animals receiving MA.

Locomotor activity in BMP7 −/+ and +/+ mice.—Sixteen mice (BMP7 +/−, n=8 and BMP7 +/+, n=8) were used to measure basal locomotor activity for 3 days. In the absence of

MA, BMP7 +/− mice had a significant increase in total distance traveled (P<0.001, $F_{(1,42)}$=12.988, two-way ANOVA), movement time (P=0.004, $F_{(1,42)}$=9.182, two-way ANOVA), and horizontal activity (P=0.002, $F_{(1,42)}$=10.421, two-way ANOVA, compared with the +/+ mice (Fig. 3B1-3).

The change of locomotor activity after MA was examined in BMP7 +/− (n=8) and BMP7 +/+ (n=8) mice. The daily locomotor activity after MA injection was normalized by comparison to the mean activity on day 1 in heterozygous and wild-type mice without MA injection (Fig. 4B1-3). High doses of MA greatly suppressed the locomotor activity in both BMP7 +/− and +/+ animals. Importantly, a greater reduction in total distance traveled (P=0.003, $F_{(1,42)}$=10.037, two-way ANOVA) and movement time (P=0.002, $F_{(1,42)}$=11.456, two-way ANOVA) was found in the BMP7 +/− mice (Fig. 4B1-3). There was a marginal difference in horizontal activity (P=0.087, $F_{(1,42)}$=3.061, two-way ANOVA) between the BMP7 +/− and +/+ mice after MA injection. These data suggest that BMP7 +/− mice are more sensitive than +/+ animals to reduction in locomotor activity after high doses of MA.

TH immunoreactivity in CD1 mice.—Twenty-six CD1 mice were used for TH immunoreactivity analysis in three regions: THir optical density in striatum; THir fiber density in SNpr; and THir neuron density in SNpc. In animals receiving parenteral saline injection, BMP7 did not alter THir density in striatum (P=0.144, Fig. 5A-1 vs. Fig. 5A-3, Fig. 5B) and SNpr (P=0.851) as well as THir neuronal density in the SNpc (P=0.516, $F_{(3,22)}$=0.783, one-way ANOVA). Parenteral administration of MA significantly reduced striatal THir fiber density in mice pretreated with i.c.v. vehicle (P=0.002, $F_{(3,22)}$=6.966, one-way ANOVA; P=0.001, post hoc Newman-Keuls test; Fig. 5A-1 vs. Fig. 5A-2,Fig. 5B). Administration of BMP7 significantly reduced the loss of THir fiber density in striatum in MA-treated mice (Fig. 5A-2 vs. Fig. 5A-4, Fig. 5B, P=0.002, $F_{(3,22)}$=6.966, one-way ANOVA; P=0.029, post hoc Newman-Keuls test). No difference was found in THir neuron density in SNpc or THir fiber density in SNpr among animals treated with vehicle +/− MA or BMP7 +/− MA (P>0.05, one-way ANOVA). These data suggest that i.c.v. administration of BMP7 has protective effects on the TH terminals in striatum, reducing MA toxicity in CD1 mice.

TH immunoreactivity in BMP7 +/− mice.—A total of 18 mice were used to analyze the effects of MA on TH immunoreactivity. Animals were killed on the 4th day after MA or saline administration. No difference in THir optical density in striatum, TH neuronal density in SNpc, and TH fiber density in SNpr was found between BMP +/− and +/+ mice after saline injection (Figs. 6-1,6–3 and 7). MA did not alter the density of THir neurons in SNpc (P>0.05, $F_{(3,14)}$=2.699, one-way ANOVA), but significantly reduced THir density in striatum (P<0.001, $F_{(3,14)}$=20.789, one-way ANOVA; P=0.001, post hoc Newman-Keuls test, Fig. 6 A-1, 2; 6B) and SNpr (P<0.001, $F_{(3,14)}$=12.010, one-way ANOVA; P=0.006, post hoc Newman-Keuls test; Fig. 7A1-3; 7B), in BMP7 +/+ mice. MA caused a greater reduction of TH immunoreactivity in SNpr of BMP7 +/− mice, compared with the +/+ mice (P=0.044, $F_{(3,14)}$=12.010, one-way ANOVA+Newman-Keuls test; Fig. 7A2-4; Fig. 7B). There was a marginal difference in THir fiber density in striatum between BMP7 +/− and +/+ after MA injection (P=0.086, one-way ANOVA+Newman-Keuls test; Fig. 6B). No

difference was found in the density of THir neurons in SNpc after MA injection in the BMP7 +/− mice ($P>0.05$, $F_{(3,14)}=2.699$, one-way ANOVA). These histological findings suggest that BMP7 +/− mice are more sensitive than +/+ animals to MA-mediated damage of dopaminergic nerve terminals.

Effects of MA on BMP7 mRNA levels in mice.—The effect of MA on BMP7 gene regulation was directly measured by qRT-PCR. Animals were treated with MA ($n=9$) or saline ($n=9$). One day after injection, SN tissue was harvested and subjected to qRT-PCR. For each sample, gene expression was normalized to the housekeeping gene HPRT1 (hypoxanthine guanine phosphoribosyl transferase 1). The expression level of another housekeeping gene PGK1 (phosphoglycerate kinase 1) was not altered by MA injection (Fig. 8A). MA significantly decreased BMP7 mRNA levels, compared with animals receiving saline (Fig. 8B). The BMP7 mRNA level in MA-treated mice normalized to the mean of saline-treated mice also showed a significant decrease (50%) in MA-treated mice (Fig. 8C, $P<0.05$, Student's *t*-test). In contrast, DAT mRNA levels were not different between MA- and saline-injected groups (data not shown), suggesting that alteration in BMP7 mRNA levels was not due to the loss of dopaminergic neurons or to a general suppression of gene expression.

The effect of MA on BMP-7 expression was also indirectly confirmed using β-galactosidase assays in BMP7 +/− mice. BMP7 +/− ($n=16$) mice were treated with MA or saline. Striatal and cortical tissue was collected 2 days after injection. β-Gal activity was quantitatively analyzed using an enzymatic assay. MA significantly reduced β-gal activity in striatum (Fig. 8D, $P<0.05$, Student's *t*-test), but not in cortex in BMP7 +/− mice. These data suggest that high doses of MA selectively suppress BMP7 expression in the nigrostriatal pathway.

DISCUSSION

Previous studies have indicated that the nigrostriatal DA pathway is particularly vulnerable to the toxic effect of MA. In this study, we demonstrated that high doses of MA cause damage to dopaminergic neurons *in vitro* and *in vivo.* In primary VM culture cells, MA decreased the density of THir fiber density without affecting number of DAPI cells, suggesting that the neurodegeneration mainly occurs in DA neurons. MA-mediated damage is also found *in vivo.* MA suppressed locomotor activity and reduced TH fiber density in striatum in CD1 mice. Unlike the *in vitro* findings, less damage to the dopaminergic cell bodies was found in the SNpc *in vivo.* Previous studies have indicated that high doses of MA increase extracellular concentration of DA, DA metabolites, and glutamate in the nigrostriatal terminals *in vivo* (LaVoie and Hastings, 1999; Mark et al., 2004). These toxic metabolites/compounds may contribute the selective toxicity of MA at the dopaminergic terminals *in vivo.*

BMP7 has been shown to reduce 6-OHDA-mediated injury in VM cells (Harvey et al., 2004). Since 6-OHDA shares several neurodegenerative mechanisms with MA in DA neurons, such as apoptosis (Zuch et al., 2000), it is possible that BMP7 can reduce MA toxicity by mechanisms similar to that seen with 6-OHDA. In this study, we found that pretreatment with BMP7 attenuated the MA-mediated decrease in the density of THir fiber

density in VM cultures. BMP7 also antagonized the MA-mediated decrease in TH immunoreactivity in striatum in CD1 mice. The change in TH immunoreactivity correlates with the changes in behavior as BMP7 restored locomotor activity toward control levels in MA-treated CD1 mice. Taken together, these data suggest BMP7, given exogenously, can reduce MA-mediated toxicity in dopaminergic circuits *in vivo* and *in vitro.*

There is evidence that MA induces neuronal degeneration through activation of programmed cell death (Deng et al., 2001; Koike et al., 2005). MA-mediated neuronal injury is associated with over-expression of BAX, decreases in mitochondrial membrane potential, release of cytochrome C from mitochondria, activation of caspase-9, cleavage of caspase-3, and DNA fragmentation-related factor 45 (Deng et al., 2002). High doses of MA increased TUNEL labeling in several brain areas including the striatum, cortex, indusium griseum, medial habenular nucleus, and hippocampus in mice (Deng et al., 2001). We also found that high doses of MA enhance TUNEL activity in VM cultures. However, few co-localizations of TUNEL and TH activity were found after MA treatment. It is possible that the TH phenotype was suppressed in injured cells. Pretreatment with BMP7 reduced MA-mediated increases in TUNEL labeling. Taken together, our data suggest that exogenous administration of BMP7 antagonizes MA-induced apoptosis *in vitro.*

Since BMP7 null mutants (−/−) die within 24 h of birth because of uremia secondary to renal agenesis, the function of endogenous BMP7 could only be examined in BMP7 +/− and +/+ mice *in vivo.* We found that BMP7 +/− mice had about a 50% decrease in BMP7 mRNA levels in brain, as compared with +/+ mice. The BMP7 +/− mice are more sensitive to MA. Increased bradykinesia was found after MA injection in the BMP7 +/−, compared with the BMP7 +/+, mice. There was also a significantly greater decrease in TH immunoreactivity in SNpr and marginal reduction in striatum in the BMP7 +/− compared with +/+ animals, suggesting that MA-mediated dysfunction in locomotor activity may be related to the degeneration of TH nerve terminals in SNpr and striatum. These data also suggest that deficiency in endogenous BMP7 expression increases sensitivity to toxic doses of MA. In preliminary studies, we also found a shift of the dose-response relationship between MA and bradykinesia to the left in the +/− mice.

Previous studies indicate that the expression of BMP7 mRNA in striatum was significantly suppressed after unilateral 6-OHDA lesioning (Chen et al., 2003). In this study, the expression of BMP7 was first indirectly measured through the expression of β-gal in BMP7 +/− mice, since the LacZ reporter gene was inserted in the BMP7 +/− mice. The β-gal enzymatic activity was greatly suppressed by MA in striatum, but not in cortex, suggesting that high doses of MA selectively inhibit endogenous BMP7 expression in terminals of the nigrostriatal pathway. Using qRT-PCR, we also confirmed that MA down-regulated the expression of BMP7, but not the housekeeping gene HPRT1 or PGK1.

CONCLUSION

In conclusion, we found that endogenous and exogenous BMP7 has protective effects against MA toxicity. The protective effect of BMP7 may have clinical significance. There is a high incidence of MA abuse (the NSDUH report, Sept 16, 2005, National Surveys on Drug

Use and Health) in the United States and significant MA-related secondary neurodegenerative changes. Our data suggest that lack of endogenous BMP signaling increases vulnerability to MA injury and exogenous BMP7 can reduce MA toxicity. Further studies on BMP7 mechanisms of action may provide the basis for novel therapeutic strategies for MA-induced damage in humans.

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Abbreviations:

BMP7	Bone morphogenetic protein-7
BSA	bovine serum albumin
DA	dopamine
DIV	days *in vitro*
MA	methamphetamine
PB	phosphate buffer
PFA	paraformaldehyde
qRT-PCR	quantitative reverse transcription–PCR
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
TH	tyrosine hydroxylase
TUNEL	terminal deoxynucleotidyl transferase–mediated dNTP nick end labeling
VM	ventromesencephalic
6-OHDA	6-hydroxydopamine

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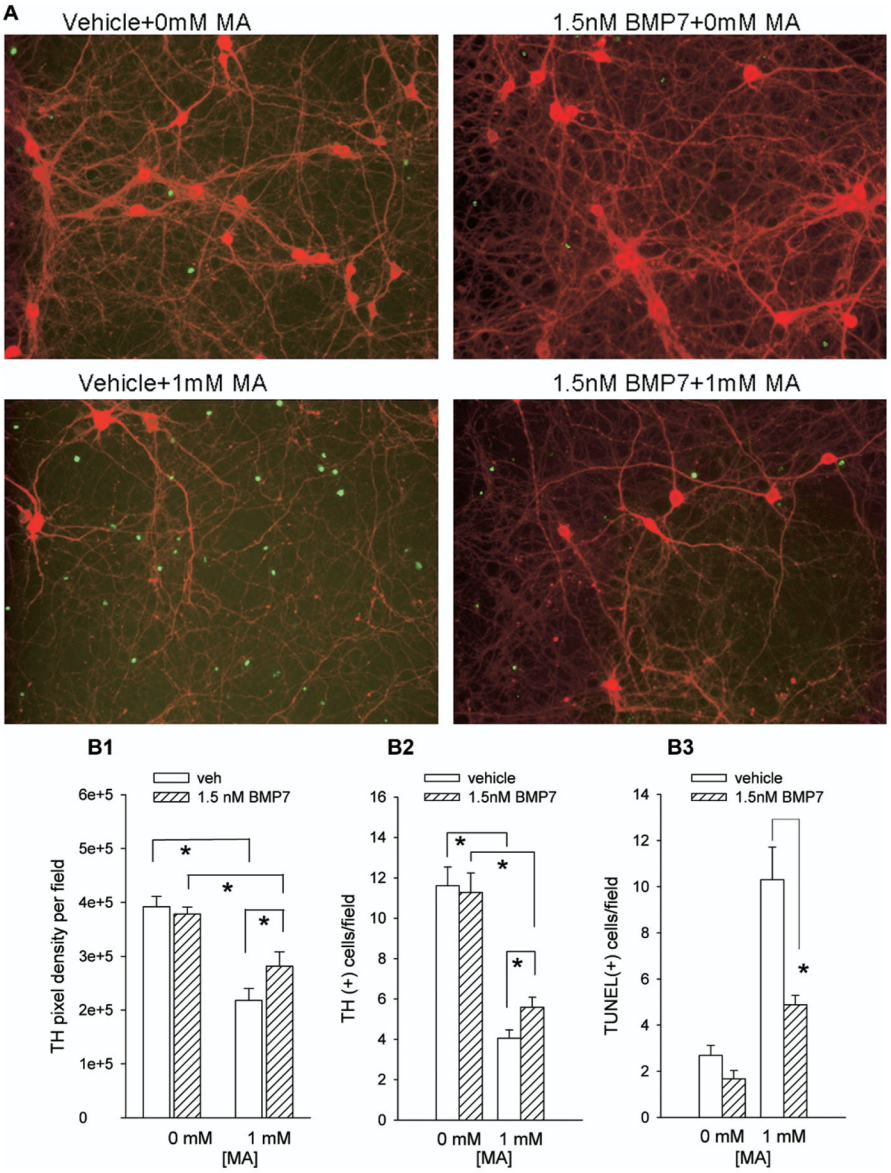

Fig. 1.

BMP7 reduces MA-induced toxicity in ventral mesencephalic cultures. (A) Primary cultures prepared from rat VM (E14) were treated with 1.5 nM BMP7 for 20 min then exposed to 1 mM MA for 72 h. Cells were fixed and assayed using TH immunostaining (red) and TUNEL (green). MA increased TUNEL and decreased TH cell numbers and fiber density. (B1) BMP7 alone did not alter the THir fiber density; however, BMP7 antagonized the MA-mediated decrease in THir. (B2) BMP7 also antagonized the MA-induced decrease in number of TH positive neurons in culture. (B3) Pretreatment with BMP7 reduced the MA-mediated increase in TUNEL (* P<0.05).

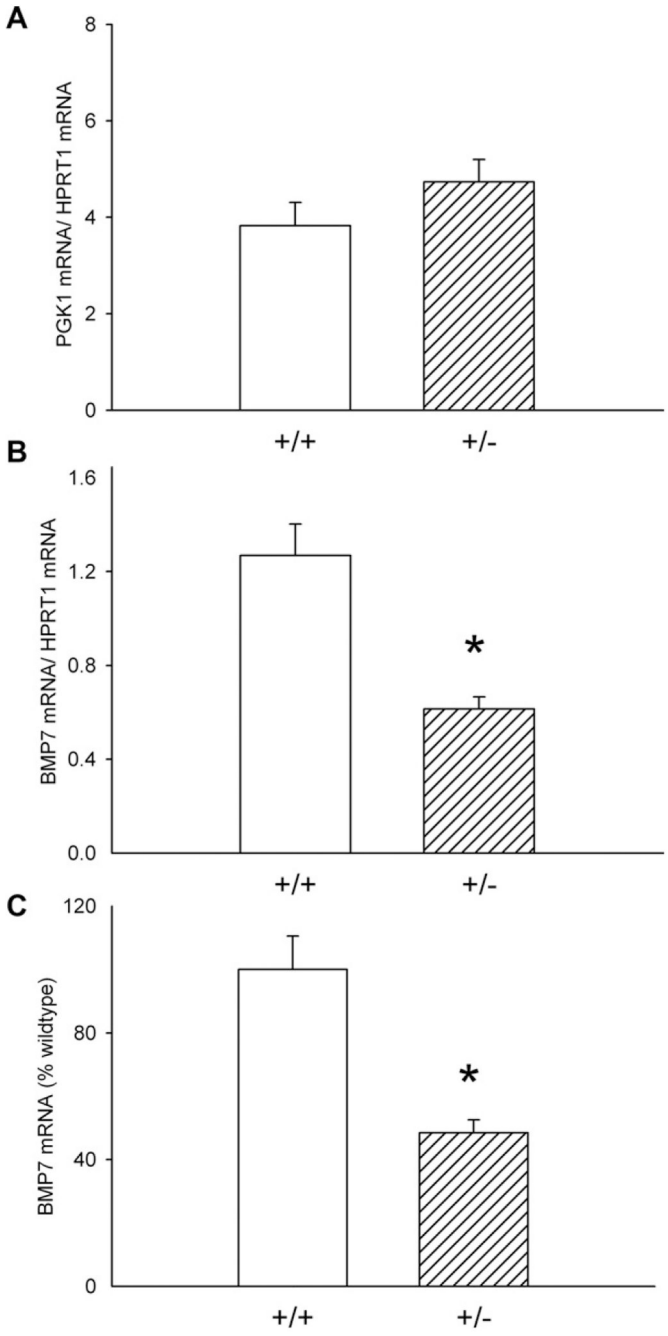

Fig. 2.

BMP7 mRNA levels are decreased in the brains of BMP7 heterozygous mice. Brain mRNA levels for BMP7, PGK1 and HPRT1 were measured in adult BMP7 +/+ (n=5) and +/− mice (n=5) by qRT-PCR. For each sample, mRNA levels for genes of interest were normalized to the housekeeping gene HPRT1 mRNA level. Another housekeeping gene PGK1 mRNA level did not differ between +/+ and +/− mice. BMP7 mRNA level is significantly decreased (50%) in +/− mice compared with the level in +/+ mice (* P<0.005, Student's t-test).

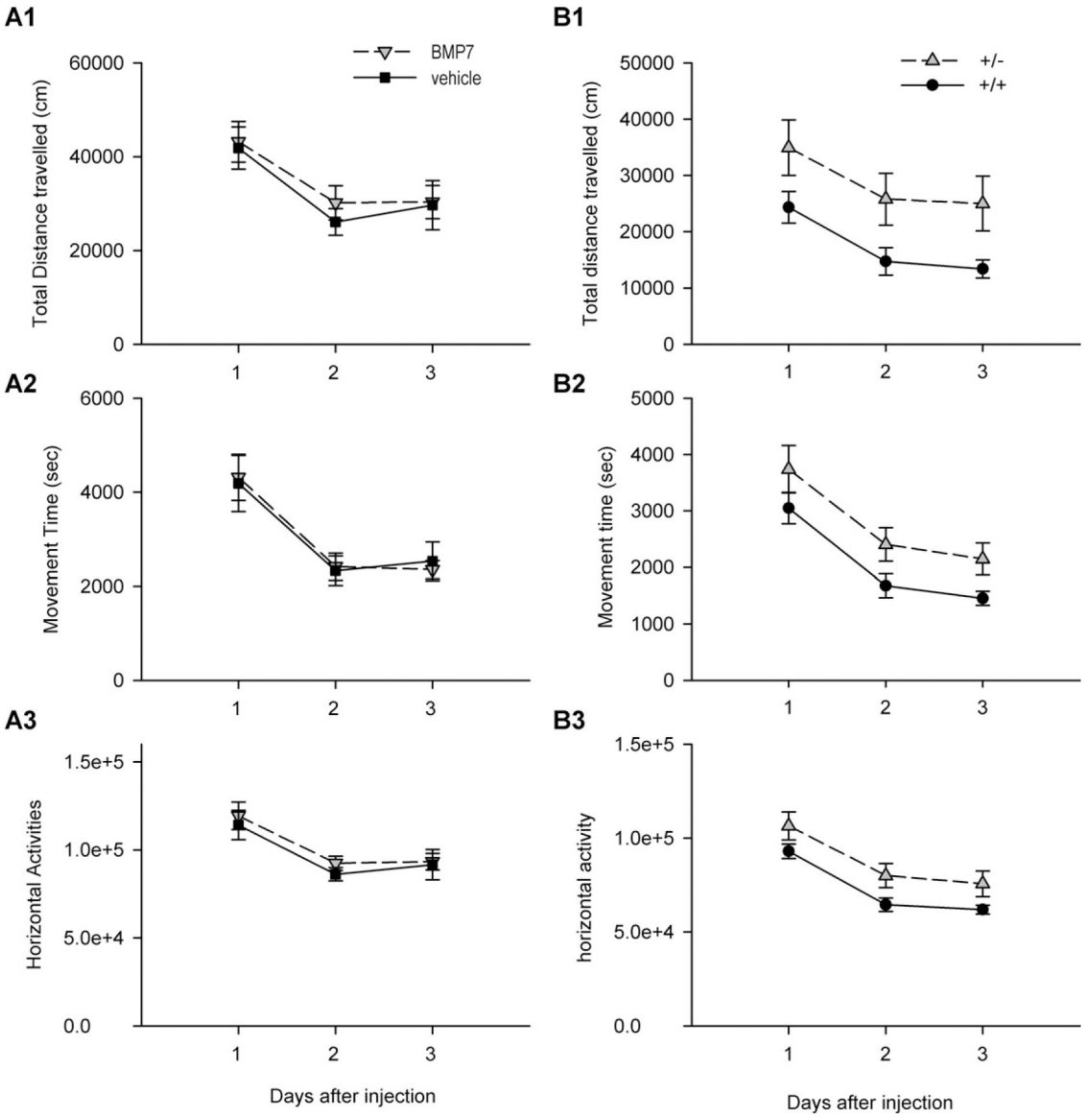

Fig. 3.

Basal locomotor activity of CD1 and BMP7 transgenic mice. (A) In CD1 mice, BMP7 (1 μg/μl×5 μl, i.c.v., –∇–) did not alter (A1) total distance traveled, (A2) movement time, and (A3) horizontal activity, compared with animals receiving i.c.v. vehicle (—■—). (B) BMP7 +/− mice (—▲—) had a significant increase in all basal locomotor activities compared with the +/+ mice (—●—).

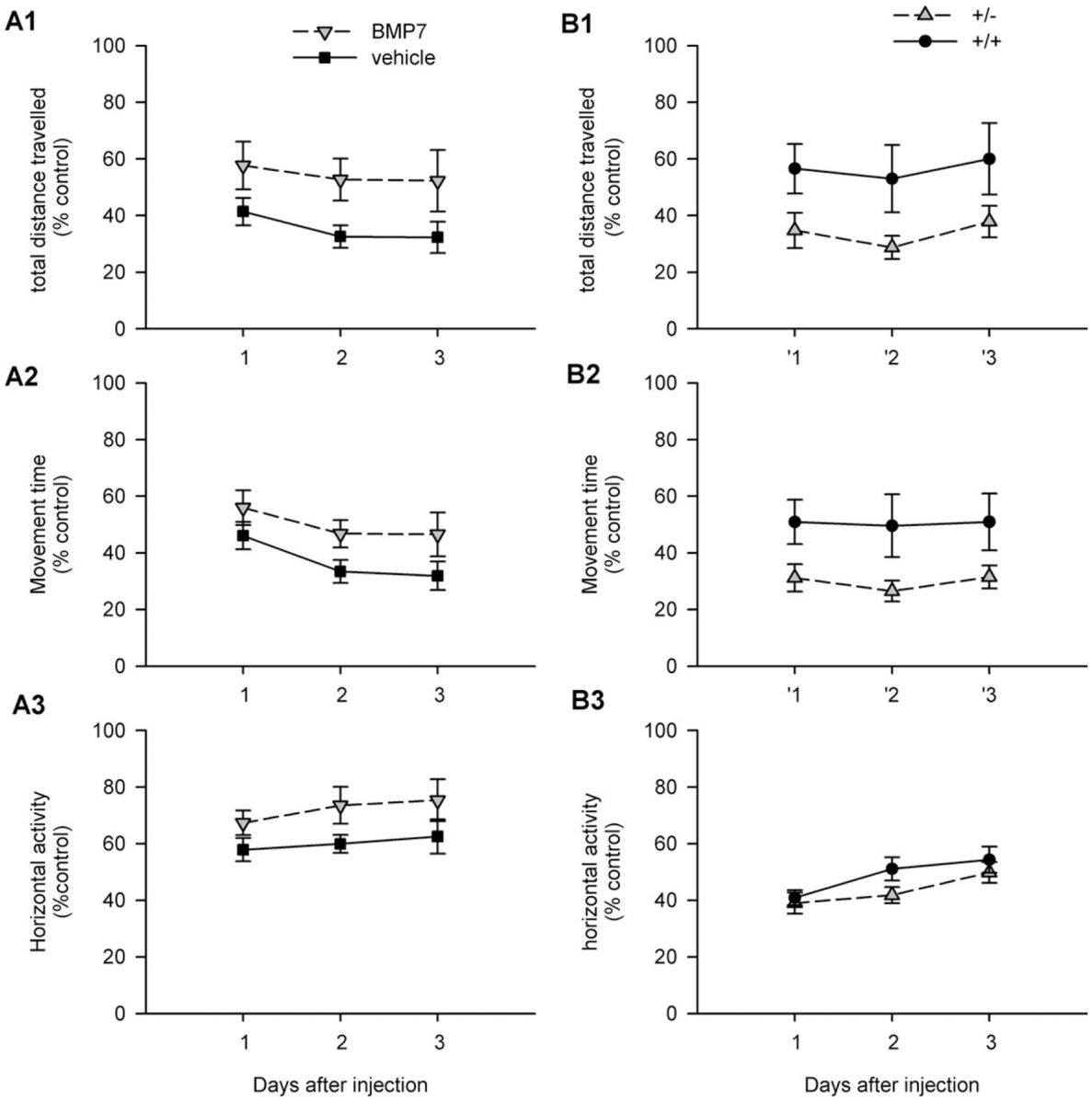

Fig. 4.

Behavioral interactions of BMP7 and MA in CD1 and BMP7 transgenic mice. Daily locomotor activity after MA injection was normalized by comparison to the average activity on day 1 from the control mice. (A) In CD1 mice, MA alone (—■—) reduced locomotor activity. Administration of BMP7 (—▽—) significantly antagonized the MA-mediated decrease in (A1) total distance traveled, (A2) movement time, and (A3) horizontal activity (P<0.05). (B) MA also suppressed the locomotor activity in BMP7 +/− (—▲—) and +/+ (—●—) mice. There is a significant difference (P<0.05) in total distance traveled and movement time and marginal difference (P=0.087) in horizontal activity between BMP7 +/− and BMP7 +/+ mice, with the +/− mice showing a greater MA effect.

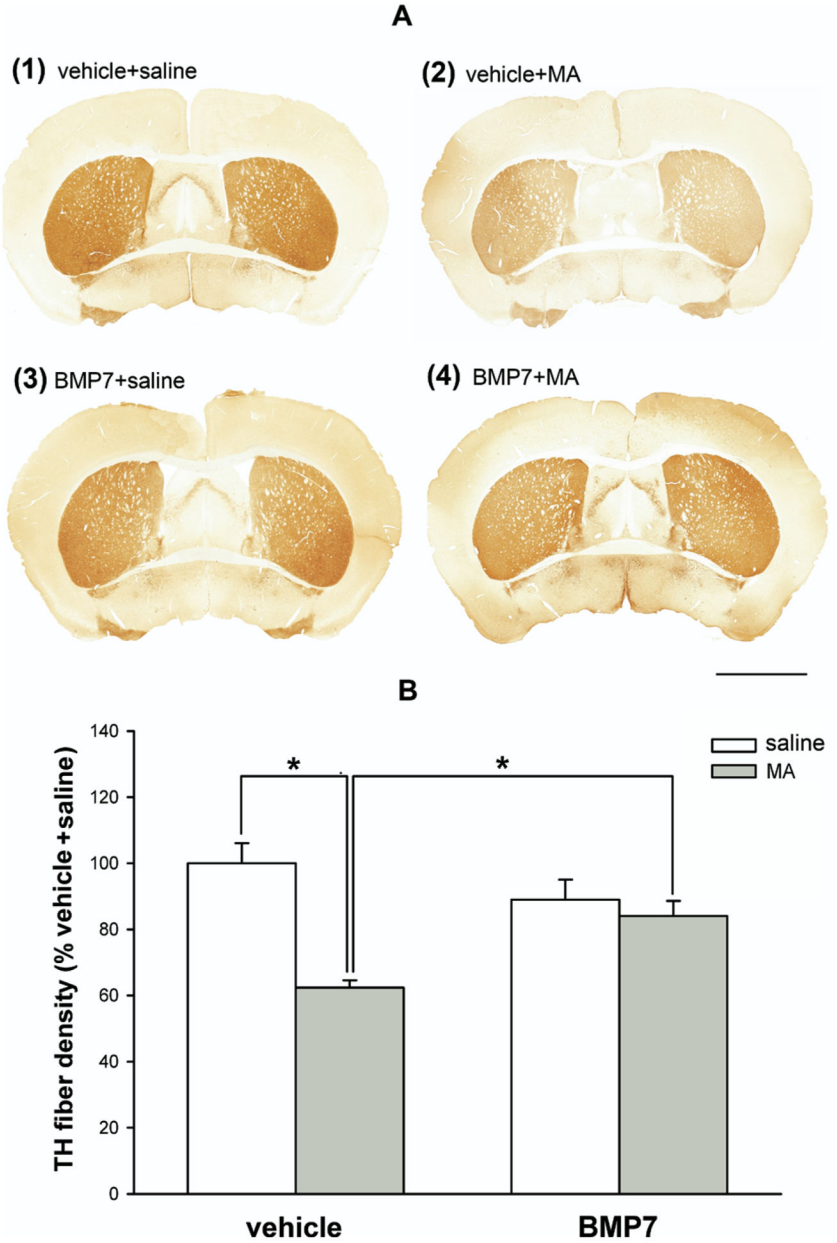

Fig. 5.

Exogenous BMP7 restores THir in striatum after MA administration in CD1 mice. Animals received 4×10 mg/kg doses of MA. Four days after MA exposure, animals were killed and brains analyzed for TH immunoreactivity (A) TH immunostaining indicates that MA administration suppressed striatal THir (1 vs. 2). I.c.v. administration of BMP7 restored THir (2 vs. 4). Scale bar=2 mm. (B) MA significantly reduced striatal THir fiber density in mice pretreated with i.c.v. vehicle. Administration of BMP7 antagonized the loss of TH fiber density in striatum in MA-treated mice (* $P<0.05$).

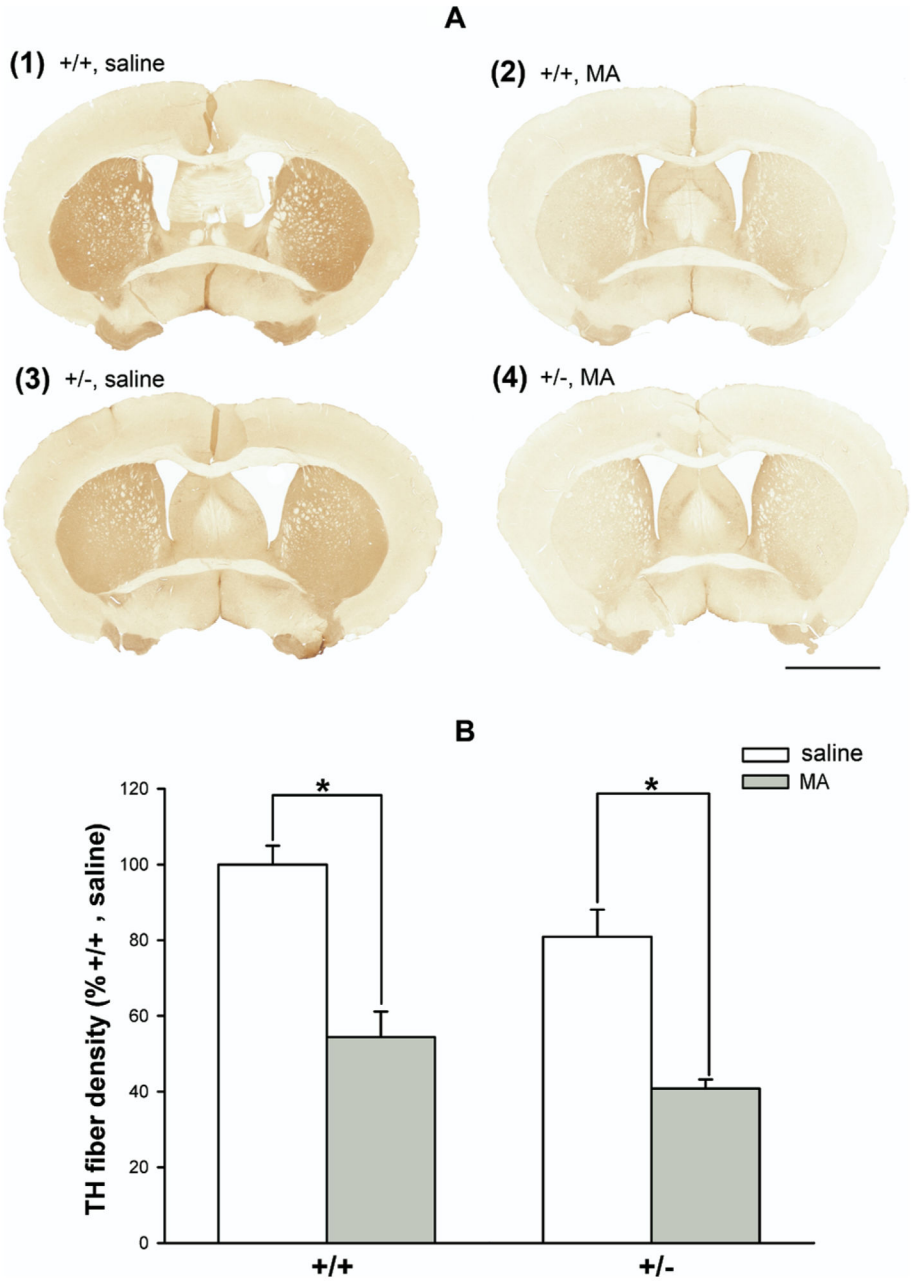

Fig. 6.

BMP7 heterozygous knockout mice exhibit decreased striatal TH-immunoreactivity following MA exposure. (A) MA administration suppressed THir in +/+ (1 vs. 2) and +/− mice (3 vs. 4). Scale bar=2 mm. (B) THir fiber density analysis indicated that MA suppressed THir in +/+ and +/− mice. There is a marginal difference in striatal THir (*P*=0.086) between +/+ and +/− mice after MA injection.

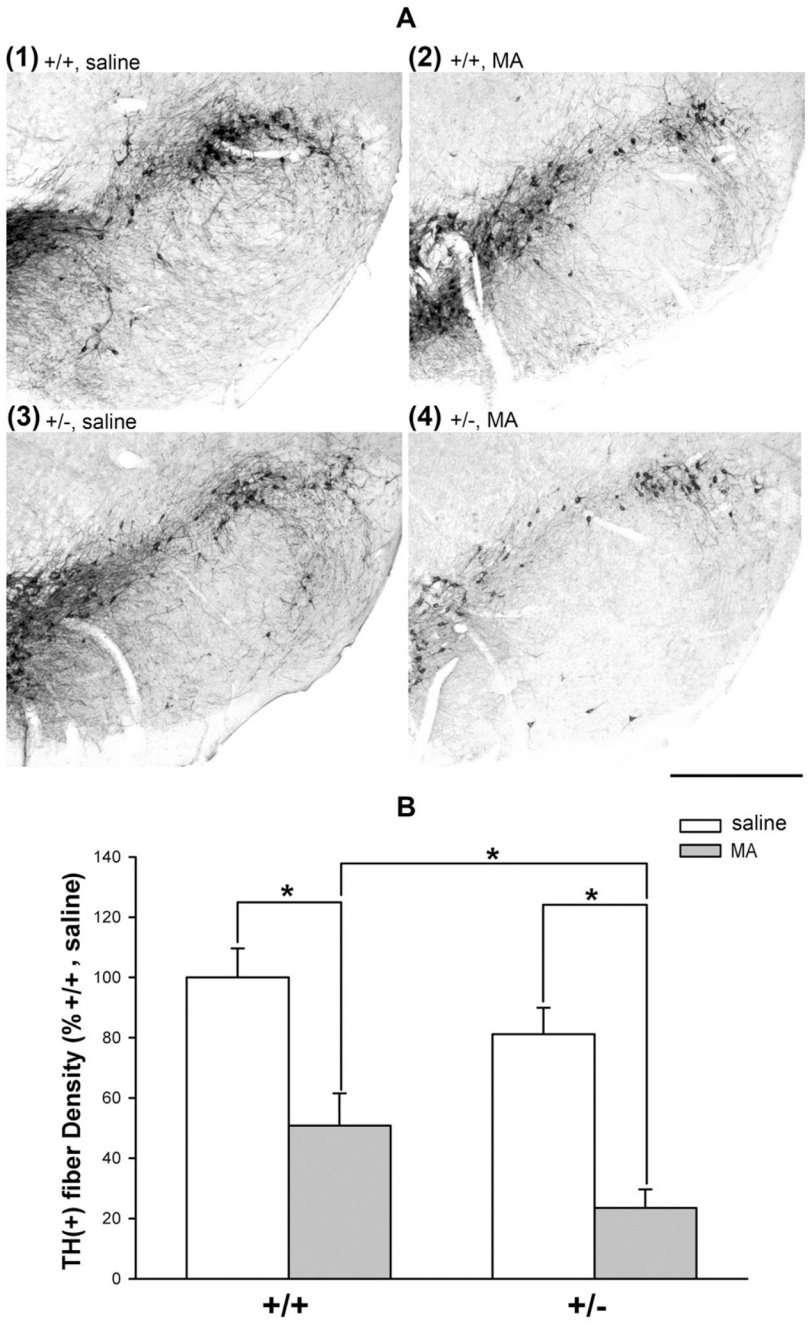

Fig. 7.

BMP7 heterozygous knockout mice exhibit decreased TH-immunoreactivity in SNpr following MA exposure. (A) TH immunostaining indicates that MA administration increases the reduction of THir in the SNpr of BMP7 +/−, compared with +/+, mice (2 vs. 4). Scale bar=500 μm. (B) THir fiber density analysis indicated MA reduced THir fiber density in SNpr in +/+ and +/− mice. A greater reduction in TH immunoreactivity was found in the BMP7 +/− mice.

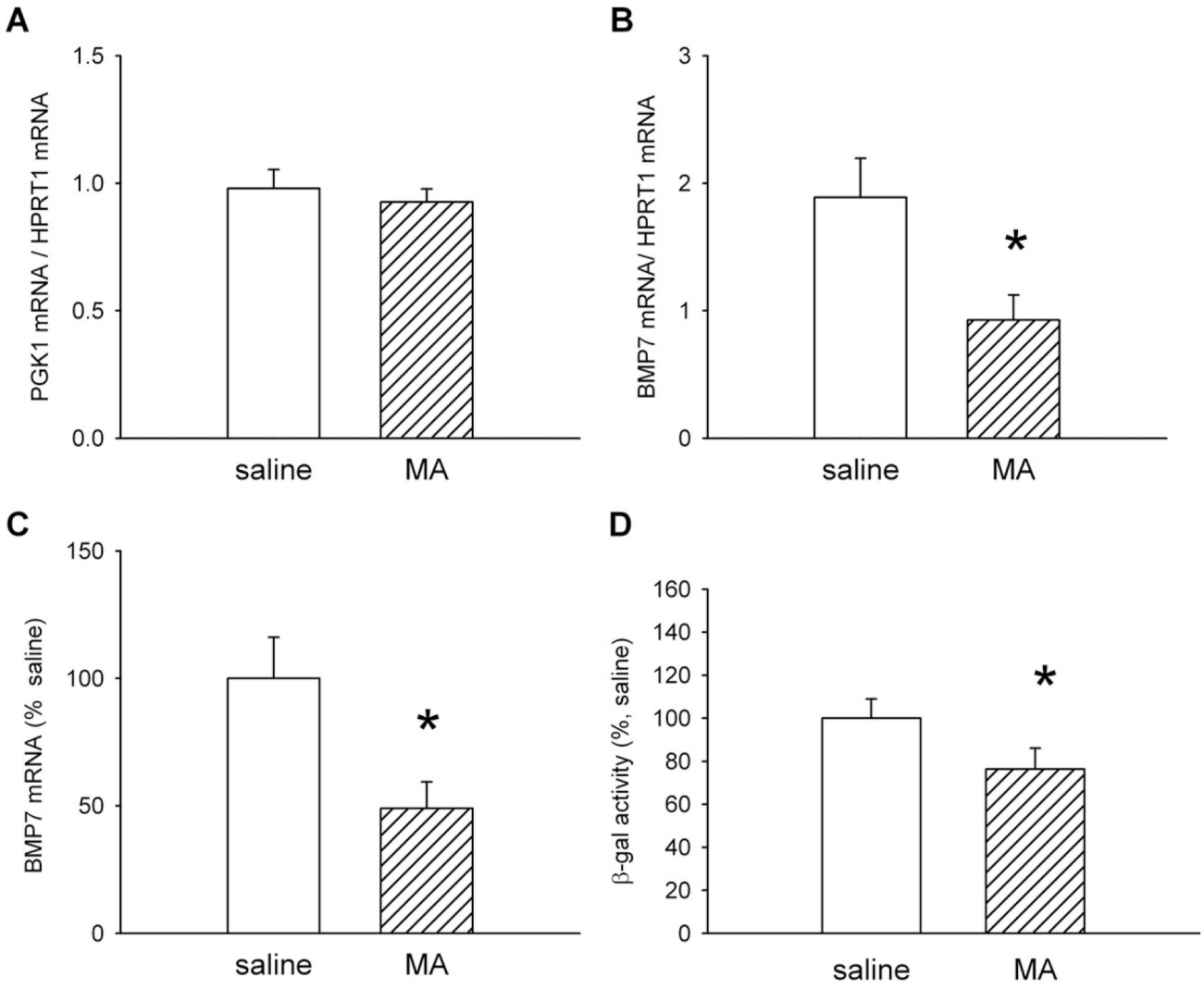

Fig. 8.

MA suppresses BMP7 mRNA levels in mouse SN. The effect of MA on BMP-7 gene regulation was directly measured by quantitative RT-PCR. mRNA levels for BMP7, PGK1 and HPRT were measured by qRT-PCR 1 day after injection of either MA or saline in CD1 mice. For each sample, mRNA levels for the specific gene were normalized to the housekeeping gene HPRT mRNA level. The housekeeping gene PGK1 mRNA level did not differ between saline and MA groups (A). Nigral BMP7 mRNA levels were significantly decreased in MA injected mice (n=9) compared with the levels in the saline injected group (n=9) (B, C; * P<0.05, Student's t-test). Downregulation of BMP7 mRNA by MA was indirectly measured by β-gal assays in BMP7 +/− mice (D). BMP7 +/− mice that received MA injection have significantly decreased striatal β-gal activity (76% of heterozygous +saline group), compared with BMP7 +/− mice receiving saline. (* P<0.05, Student's t-test).