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9-cis retinoic acid protects against methamphetamine-induced neurotoxicity in nigrostriatal dopamine neurons

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Abstract

Methamphetamine (MA) is a drug of abuse as well as a dopaminergic neurotoxin. 9-cis retinoic acid (9cRA), a biologically active derivative of vitamin A, has protective effects against damage caused by H₂O₂ and oxygen-glucose deprivation in vitro as well as infarction and TUNEL labeling in ischemic brain. The purpose of this study was to examine if there was a protective role for 9cRA against MA toxicity in nigrostriatal dopaminergic neurons. Primary dopaminergic neurons, prepared from rat embryonic ventral mesencephalic tissue, were treated with MA. High doses of MA decreased tyrosine hydroxylase (TH) immunoreactivity while increasing TUNEL labeling. These toxicities were significantly reduced by 9cRA. 9cRA also inhibited the export of Nur77 from nucleus to cytosol, a response that activates apoptosis. The interaction of 9cRA and MA in vivo was next examined in adult rats. 9cRA was delivered intracerebroventricularly; MA was given (5 mg/kg, 4x) one day later. Locomotor behavior was measured two days after surgery for a period of 48 hours. High doses of MA significantly reduced locomotor activity and TH immunoreactivity in striatum. Administration of 9cRA antagonized these changes. Previous studies have shown that 9cRA can induce bone morphogenetic protein-7 (BMP7) expression and that administration of BMP7 attenuates MA toxicity. We demonstrated that MA treatment significantly reduced BMP7 mRNA expression in nigra. Noggin (a BMP antagonist) antagonized 9cRA-induced behavioral recovery and 9cRA-induced normalization of striatal TH levels. Our data suggest that 9cRA has a protective effect against MA-mediated neurodegeneration in dopaminergic neurons via upregulation of BMP.

Keywords

BMP7; Methamphetamine; retinoic acid; Nur77; neurotoxicity; dopamine

Introduction

Methamphetamine (MA) is a dopaminergic neurotoxin and a common drug of abuse that easily crosses the blood brain barrier. According to a report by the National Survey on Drug Use (<http://www.samhsa.gov/data/NSDUH.aspx>), 1.2 million Americans ages 12 or above abused MA at least once in 2008. High doses of MA activate caspase-3, up-regulate p53, and produce DNA fragmentation and cell death in dopaminergic neurons (Cadet et al., 2003; Choi et al., 2002; Deng et al., 2001). MA-mediated neurodegeneration in DA neurons can be antagonized by various proteins or chemicals through the inhibition of apoptosis (Chou et

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al., 2008b; Deng and Cadet, 2000; Harvey et al., 2009). Taken together, these data suggested that MA-mediated degeneration of dopaminergic neurons involves the activation of programmed cell death.

Retinoic acid (RA) is a biologically active derivative of vitamin A. Two major isoforms of RA, all-trans RA (atRA) and 9 cis RA (9cRA), have been identified. atRA is normally present at high levels in the developing spinal cord and at low levels in the forebrain of mouse embryos (Horton and Maden, 1995). On the other hand, 9cRA is not present in mouse embryos (Horton and Maden, 1995; Mic et al., 2003; Ulven et al., 2001) or tissue extracts from adult rats (Werner and Deluca, 2001). RAs interact with two major groups of nuclear receptors: retinoic acid receptors (RAR) and retinoid X receptors (RXR). 9cRA binds with high affinity to RXR (Levin et al., 1992).

RA has been reported to have protective effects after injury. In cultured cells, RA inhibited H₂O₂-induced apoptosis via suppression of c-fos/c-jun expression and JNK activation (Kitamura et al., 2002) and increased survival during anoxia/glucose deprivation (Boniece and Wagner, 1995). *In vivo* studies have demonstrated that 9cRA is more potent than atRA in reducing ischemia-mediated brain infarction (Harvey et al., 2004). Pretreatment with 9cRA attenuated DNA fragmentation in the lesioned cerebral cortex of stroke rats (Shen et al., 2009b). Taken together, these data suggest that 9cRA is neuroprotective against free radical and ischemic brain injury both *in vitro* and *in vivo*.

RA has been shown to reduce dopaminergic neurotoxin-mediated degeneration. RA attenuates 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium-mediated neurotoxicity in rats (Yin et al., 2012) and in SY5Y neuroblastoma cells (Cheung et al., 2009). The protective effect of RA in these cells involves the Akt pathway, increasing BCL2 while reducing p53 levels (Cheung et al., 2009; Tieu et al., 1999). One recent study has shown that systemic administration of a high dose of RA (1mg/kg, i.p.) for 15 days reversed rotenone-induced impairment of locomotor activity without significantly reversing the reduction of dopamine (DA) in striatum (Ulusoy et al., 2011). The protective role of 9cRA in dopaminergic neurons against MA-mediated neurodegeneration has not been reported.

9cRA can regulate the expression of certain protective trophic factors. 9cRA time-dependently upregulates BMP7 mRNA expression in rodent brain (Shen et al., 2009b). On the other hand, high doses of MA suppressed bone morphogenetic protein-7 (BMP7) mRNA expression in cerebral cortex (Shen et al., 2008). We previously reported that BMP7 antagonized MA-induced DA cell loss in cultured dopaminergic neurons and striatal TH fiber density reduction *in vivo* (Chou et al., 2008b). Knocking down BMP7 or BMPRII receptor expression in mice increased sensitivity to MA in nigrostriatal dopaminergic neurons (Chou et al., 2008a; Chou et al., 2008b). These data suggest that BMP7 has protective effects against MA-mediated degeneration in dopaminergic neurons. Since 9cRA enhances BMP7 expression, we reasoned that 9cRA may also induce protection against MA lesioning through the BMP7 pathway.

Nur77, also known as nerve growth factor IB (NGFI-B), has been described as an orphan nuclear receptor and a potent proapoptotic molecule. Acute administration of dopaminergic neurotoxins, such as 6-OHDA or MA, upregulated Nur77 expression (Akiyama et al., 2008; Backman and Morales, 2002; No et al., 2010; Shilling et al., 2006). The increase in Nur77 expression preceded changes in Fas-mediated cell death induced by MA (Jayanthi et al., 2005). In response to injury, Nur77 translocates from the nucleus to the cytoplasm and targets mitochondria to induce cytochrome C release and apoptosis (Li et al., 2000). Blockade of the nuclear export of Nur77 attenuated 6-OHDA-induced cell death, suggesting that 6-OHDA-mediated cell death is dependent on Nur77 nuclear export (No et al., 2010).

Interestingly, the export of Nur77 from the nucleus is regulated by the RXR. Mitochondrial targeting of the RXRa/Nur77 heterodimer and induction of apoptosis are potentially inhibited by RXR ligands, such as 9cRA (Cao et al., 2004). It is thus possible that 9cRA can also modulate MA-mediated neurodegeneration through Nur77 translocation.

In this study, we examined the protective effect of 9cRA in cellular and rodent animal models of MA toxicity. Previous studies have shown that high doses of MA are required to induce programmed cell death, suppression of DA release, and reduction in tyrosine hydroxylase (TH) immunoreactivity in neuronal cultures and rodent brain (Jayanthi et al., 2005; Zhu et al., 2006). We found that treatment with 9cRA significantly reduced high dose MA-mediated behavioral bradykinesia *in vivo* while it increased the survival of dopamine cells *in vitro*. Our data suggest that 9cRA is neuroprotective against neurodegeneration induced by high doses of MA.

1. Experimental procedures

Primary cultures of rat ventral mesencephalon

Primary cultures were prepared from embryonic (E14-15) ventral mesencephalon (VM) tissues obtained from fetuses of timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), according to published procedures with some modification. The whole brain was removed aseptically and a small piece of tissue comprising the VM was dissected. After removing the blood vessels and meninges, pooled VM tissues were trypsinized (0.25%; Invitrogen, Carlsbad, CA) with gentle mixing for 15 min at 37°C. After rinsing off trypsin with pre-warmed DMEM/F-12 (Invitrogen), cells were dissociated by trituration, counted and plated into 96-well (6.0×10^4 /well) cell culture plates pre-coated with poly-D-lysine (Sigma-Aldrich). The culture plating medium consisted of Dulbecco's modified Eagle medium/F12 supplemented with 10% heat-inactivated fetal bovine serum, 1 mM L-glutamine and 2% B27 (Invitrogen). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The cultures were fed by exchanging 50% of media with feed media (Neurobasal medium, Invitrogen) with 0.5 mM l-glutamate and 2% B27 with antioxidants supplement on DIV (days *in vitro*) 3 and 5. On DIV7, cultures were fed with media containing B27 supplement without antioxidants (Invitrogen). Freshly made (+) MA or saline was added to the wells about 5-min after administration of RA or vehicle on DIV 10. Cells were returned to a 37°C incubator for 48 hours then fixed with 4 % paraformaldehyde (PFA) for immunoreactivity.

In Cell Western

Cells were prepared and incubated in TH antibody as described previously (Chou et al., 2008b). The cells were then rinsed three times in PBS and incubated in secondary antibody (1:500, IRDye800, Rockland, Gilbertsville, PA) diluted in PBS containing 0.1% Triton X-100 and 5% goat serum for 1 hour at RT. Cells were washed again with PBS twice and incubated in TOPRO-3 Iodide (1:2500, Invitrogen) diluted in PBS for 1 hour at RT. Cells were washed in PBS for three times more and then imaged using a Licor Odyssey IR scanner. All In Cell Western analyses were done using Licor Odyssey software v 3.0.

In vitro Terminal deoxynucleotidyl transferase (TdT)-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). Briefly, 4% PFA fixed cells were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 2 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 using a Nikon TE2000 inverted microscope equipped with fluorescence. TUNEL (+) cells were manually counted in 20x images (4 fields per well of 96 well plate).

Immunocytochemistry and quantitation

After removing PFA solution, cells were washed with PBS and the fixed cultures were treated for 1 hour with blocking solution (2% BSA, 0.1% Triton X-100 and 5% goat serum in PBS). The cells were then incubated for 2 days at 4°C with a mouse monoclonal antibody against TH (1:500; Millipore, Billerica, MA) or rabbit polyclonal antibody against Nur77 (1:400; Abcam, Cambridge, MA). Nuclei were stained with DAPI (10 min) at room temperature. The cells were then rinsed three times in PBS. The bound primary antibody was visualized using AlexaFluor 488 goat anti-mouse secondary (Invitrogen) and AlexaFluor 568 goat anti-rabbit secondary (Invitrogen). Images were acquired using a SPOT RT camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a NIKON TE2000 inverted microscope and a confocal Laser-scanning microscope with 100 × oil-immersion objective and Nikon Eclipse 80i with EZ-C1 3.9 software. TH+ cells were manually counted in 10x images (4 fields per well of 96 well plate). All immunoreactive counts and quantitation were expressed as a percentage of untreated cells. Experiments were repeated 2-3 times with n=3-9 wells per group per experiment.

Western blot/cell culture

VM cells from rat embryos were seeded at 1×10^6 /well on 6-well plates. On DIV 10, cells were treated with MA and/or 9cRA and were harvested at 10 to 15 min after drug treatment. Cytoplasmic and nuclear proteins of VM were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo, #78835) with Halt Protease Inhibitor Cocktail (Thermo, 87786) according to the manufacturer's instruction. Following measurement of protein concentrations using DC™ Protein Assay Kit (Bio-Rad, #500-0116), proteins were reduced using NuPAGE® Sample Reducing Agent (10X) (Life technologies, #NP0009) in NuPAGE® LDS Sample Buffer (4X) (Life technologies, #NP0007) then loaded onto NuPAGE® Novex® 4-12% Bis-Tris Gels (Life Technologies, #NP0322BOX), separated for 2h at 100V and transferred to Immobilon-FL PVDF Membrane (Millipore, IPFL00010) for 45min at 160V. Proteins were probed with specific primary antibodies (anti-Nur77 antibody, Abcam, #ab109180, 1:1000; anti-Actin, Sigma, #A3853, 1:1000; anti-TATA binding protein TBP antibody, Abcam, #ab818) diluted in Odyssey Blocking Buffer (Li-cor, 927-40000, 1:1000), then incubated at room temperature for 2 hours. The secondary antibodies (ti-mouse IRDye800® conjugated antibody, Rockland, 610-132-121; anti-rabbit IRDye® 700DX conjugated antibody, Rockland, 611-130-122) were diluted in Odyssey Blocking Buffer (1:2500) and incubated with blots for 1h at ambient temperature. Bands were visualized using the Odyssey® SA Infrared Imaging System (LI-COR) and images were captured and quantified through ImageJ (v1.46) software.

Animals and surgery

Adult male Sprague-Dawley rats (Charles River Labs, 200-250 g upon arrival) were used for the intracerebroventricular injections. The use of animals was approved by Animal Care and Use Committee, NIDA, IRP. Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and injected i.c.v. with 9-cis retinoic acid (Sigma-Aldrich, $1 \mu\text{g}/\mu\text{L} \times 20 \mu\text{L}$, pH 7.4) or vehicle (10% DMSO, $20 \mu\text{L}$) using a $25 \mu\text{L}$ Hamilton syringe. The coordinates for the i.c.v. injection were 0.8 mm posterior to bregma, 1.2 mm lateral (Left) to midline, and 3.7 mm ventral to the dural surface. Using MALDIOTOFMS, we previously demonstrated that unilaterally i.c.v. administered astaxthin, a carotenoid structurally similar to retinoic acid,

can be transported to bilateral hemispheres (Shen et al., 2009a). The noggin-treated rats were injected i.c.v. with either noggin ($1\mu\text{g}/\mu\text{l} \times 20\mu\text{L}$) or noggin ($2\mu\text{g}/\mu\text{l} \times 10\mu\text{L}$) combined with 9cRA ($2\mu\text{g}/\mu\text{L} \times 10\mu\text{L}$). The rate of infusion ($4\mu\text{L}/\text{min}$) was adjusted by a microprocessor controlled injector mounted to the stereotaxic frame (UMP4; World Precision Instruments, Sarasota, FL, USA). The needle was slowly removed after 2 minutes upon completion of the injection. The following day animals were injected with MA (5 mg/kg , s.c., 4 times/ 2 hr) or 0.9% saline (s.c., 4 times/ 2 hr). Body temperature was measured by placing a thermometer in the ear canal near the tympanic membrane prior to MA or saline administration and just after the last administration of MA or saline. The animals were then kept in cages without bedding and food overnight to avoid suffocation and returned to their home cages the next morning.

Locomotor behavior

Locomotion was measured using an infrared activity monitor (Accuscan, Columbus, OH) two days after surgery for a period of 48 h. Rats were individually placed in a $42 \times 42 \times 21$ cm closed plexiglass box which contained horizontal infrared sensors placed 2.5 cm apart. Food and water was available *ad libitum*. The following variables were measured: (i) horizontal activity (HACTV, the total number of beam interruptions that occurred in the horizontal sensors), (ii) movements time (MOVNO), and (iii) total distance traveled (TOTDIST, the distance, in centimeters, traveled by the animals), (iv) No of movements (MOVNO). (v) Rest time (RESTIME), (vi) Vertical activity (VACTV, total number of beam interruptions that occurred in the vertical sensors), (vii) number of vertical movements (VMOVNO), (viii) vertical movement time (VTIME).

Real-Time Quantitative Reverse Transcription PCR

Total RNAs were isolated from substantia nigra of adult rats using TRIZOL Reagent (Life Technologies, #15596-026) and cDNAs were synthesized from 2.5ug total RNA using SuperScript VILO™ cDNA Synthesis Kit (Life Technologies, #11754-050). The TaqMan® Gene Expression Assays (primer and probe set) for specifically detecting rat Bmp7 (#Rn01528889_m1), Noggin (#Rn01467399_s1) and Beta-actin (Rn00667869_m1) were purchased from Life Technologies. Quantitative Real-Time PCR (qRT-PCR) was carried out using TaqMan Fast Advanced Master Mix (Life Technologies, #4444557) and Applied Biosystems 7500 Fast Real-Time PCR System following the reaction condition recommended in manufacturer's protocol. Expression and normalization of the target genes, Bmp7 and Noggin, were calculated relative to the endogenous reference gene (Beta-actin) by the use of qBase v 1.3.5, software for automated analysis of qRT-PCR data (Hellemans et al., 2007), with a modified delta-delta-Ct algorithm that takes specific gene specific amplification efficiency into account for accurate calculation.

Western blotting/brain tissue

Animals were humanely sacrificed by decapitation. The brains were removed, placed in a brain matrix. The right and left striatum and substantia nigra (SN) were collected and frozen. The left striatum was used for Western blotting. For the 9cRA neuroprotection experiment, 30 animals were used in Western analysis, while in the noggin experiment, 43 animals were used in Western analysis. For Western blotting, the tissue was homogenized in RIPA lysis buffer (Cell Signal). The homogenate was centrifuged at 14000 rpm for 5 min at 4°C and the supernatant was collected. A DC assay was performed using bovine serum albumin to determine protein concentrations. The samples were diluted with RIPA buffer according to the DC assay. Gels were transferred to a PVDF membrane after electrophoresis. The membranes were blocked overnight in blocking buffer (Odyssey, Li-Cor) at 4°C and blocked an additional hour at room temperature the next day. The blots were then probed

with mouse anti-TH antibody (1:2000, Millipore), or actin (1:500, Sigma) at 4 °C for 2 hours. The amount of TH was normalized with actin on the same membrane. Western analysis was performed by probing with IR 680 dye goat anti-rabbit (1:2500, Odyssey) and IR 800 goat anti-mouse antibody (1:2500, Odyssey) for 1 hour at room temperature and then scanned by infrared imaging system (Odyssey, LI-COR). Band intensity was quantified using Image J.

Statistical analysis

All data were expressed as means $\pm$ SEM. Data were analyzed using two tailed Student's t test, two way ANOVA and post-hoc Newman-Keuls or Dunnett's test. All analyses were calculated by SigmaStat software ver 3.0. Statistical significance was determined at $p < 0.05$.

Results

In vitro VM cell cultures

We first tested for protective effects of 9cRA against MA toxicity in ventromesencephalic (VM) neuronal cultures. Cells were fixed at 48 hours after treatment. Typical TH immunoreactivity of VM culture cells is demonstrated in Fig 1A. MA dose-dependently reduced TH (+) cell density (Fig 1A & D, $p < 0.001$, $F_{2,62} = 366.902$, 2-Way ANOVA). Similar to our previous study (Yin et al., 2012), we found that treatment with 9cRA (50 nM) did not alter TH cell density in non-lesioned cells (Fig 1A & D, $p = 0.414$, lower left panel). However, 9cRA significantly attenuated MA-induced loss of TH cells (Fig 1A lower right panel, and 1D, $p = 0.003$ at 1 mM MA; $p = 0.007$ at 2 mM MA, two way ANOVA + Newman-Keuls test). TH immunoreactivity of dendrites and cell bodies in culture wells were analyzed using in-Cell Western through a Licor Odyssey system. Similar to TH cell density, administration of MA suppressed TH pixel density in each well (Fig 1B). Treatment with 9cRA significantly antagonized the MA-mediated reduction in TH activity (Fig 1B & E, $p = 0.006$, $F_{1,38} = 8.624$, two way ANOVA). Post-hoc analysis indicates that 9cRA significantly antagonized the reduction of TH pixel density induced by 1 mM MA ($p = 0.017$). No difference was found at 2 mM MA ($p = 0.568$). It has been well documented that MA induces neurodegeneration through apoptosis in dopaminergic neurons. MA – mediated DNA fragmentation was examined using TUNEL labeling; treatment with 9cRA significantly reduced MA – mediated TUNEL activity (Fig 1C & F, $p < 0.001$, $F_{1,42} = 0.024$). Post-hoc analysis indicates that 9cRA significantly reduces TUNEL at 1 mM MA ($p = 0.006$).

Previous studies have indicated that neurotoxins induce translocation of Nur77 from nucleus to cytosol and results in activation of apoptosis while blockade of nuclear export attenuates cell death (No et al., 2010). To evaluate if MA induces translocation of Nur77 protein to cytosol, Western blot assay was carried out before as well as 4, 8 and 24 hours after treatments of MA in primary VM cultures. Immunoreactivity of Nur77 was normalized to actin (for cytosol) or TBP (for nucleus, Fig 2). A significant elevation of cytoplasmic Nur77 immunoreactivity was found at 8h of incubation with MA ($p = 0.030$, $F_{3,35} = 3.210$, one way ANOVA + post-hoc Dunnett's test). In contrast, there was no difference in nuclear Nur77 expression at 4-24 hours after MA treatment ($p = 0.756$). The data suggests that MA increases redistribution of Nur77 protein to cytosol in primary VM cells.

Morphological distribution of Nur77 in cultured VM dopaminergic cells was examined using con-focal microscopy. Nur77 immunoreactivity mainly resided in the cytosol of dopaminergic neurons in culture after MA (1mM) treatment (Fig 3A). This reaction was attenuated by co-treatment with 9cRA. Nur77 immunoreactivity was mainly localized in the nucleus of TH (+) neurons after 9cRA and MA treatment (Fig 3B). In non-MA -treated neurons, either pre-exposed to 9cRA (Fig 3C) or vehicle (Fig 3D), Nur77 immunoreactivity

was found in the cytosol and nuclei. These data suggests that 9cRA suppressed MA-mediated translocation of Nur77, which may contribute to the attenuation of MA-mediated apoptosis and cell death in dopaminergic neurons.

MA –treatment reduces locomotor activity in adult rats

Thirty one rats received intracerebroventricular injection of vehicle one day prior to MA or saline injection to control for the 9cRA injection in the following section (see section 3.3). MA (5 mg/kg, n=18) or saline (n=13) was given subcutaneously 4 times, once every 2 hours. Tympanic temperature was measured before the first injection and immediately after the last injection. Similar to previously studies, administration of MA significantly increased temperature and there was no difference in hyperthermia between vehicle and 9cRA treatment (Supplement Fig S1).

At 24 hours after the first injection, animals were individually placed in the activity chambers for 2 days. Using a two way ANOVA and a post-hoc Newman-Keuls test (Fig 4), we found that high doses of MA significantly ($p<0.05$) reduced horizontal activity (HACTV), total distance traveled (TOTDIST), movement number (MOVNO), movement time (MOVTIME), and vertical movement time (VTIME) while it significantly increased rest time (RESTIME). There is a marginal reduction in MA-mediated suppression of vertical movement number (VMOVNO) and vertical activity (VACTV). These data suggest that repeated high doses of MA treatment reduce locomotor activity in rats.

Treatment with 9cRA antagonizes MA-mediated behavioral response

Animals were pretreated with 9cRA (n=18) or vehicle (n=18) one day before MA injection. Locomotor activity was monitored for 2 days after MA injection. All behavior data were normalized to the mean levels in vehicle –treated animals with saline. Pretreatment with 9cRA significantly enhanced vertical and horizontal activity in MA -treated rats (Fig 5, $p<0.05$, 2-way ANOVA + Newman-Keuls test). The rest time was also reduced in 9cRA –treated rats after MA treatment. In another set of animals, we found that i.c.v. 9cRA did not alter locomotor activity in animals receiving saline injection (Supplement Fig S2).

9cRA attenuates the MA –mediated reduction in TH expression in striatum

Striatal tissue was harvested from 30 rats at 3 days after MA or saline injection for TH Western blotting. In the animals pretreated with i.c.v. vehicle, administration of MA significantly decreased TH immunoreactivity (Fig 6, $p=0.006$, 2-way ANOVA + Newman-Keuls test). In contrast, such a difference was not found in animals pretreated with 9cRA (Fig 6, $p=0.815$). These results suggest that 9cRA antagonized the MA-induced loss in dopaminergic innervation to striatum.

Methamphetamine suppresses BMP7 mRNA expression in rat substantia nigra

A total of 18 rats were used to examine the expression of BMP7 and its antagonist noggin by qRT-PCR. Nigral tissues were harvested at 24 hours after the first dose of MA or saline. MA treatment caused a significant reduction in BMP7 mRNA levels compared to the saline group (Fig 7, $p=0.0162$, Student's t test). Noggin was slightly, but not significantly, up-regulated by MA ($p=0.0563$, two-tailed Student's t-test). The ratio of BMP7 to noggin was also significantly enhanced by MA ($p=0.021$, t test). These data suggest that high dose MA suppressed the expression of BMP7 in nigra.

Noggin antagonizes 9cRA mediated protection in behavior

Previous studies have shown that 9cRA can induce neuroprotection through the activation of BMP7 in cerebral cortex (Shen et al., 2009b). We next studied if noggin can alter 9cRA-

mediated protection in MA –treated animals. Adult rats were pretreated (i.c.v.) with 9cRA +noggin (n=18) or vehicle + noggin (n=18) one day before MA administration. Behavior was examined for two days as described in 3.2 and 3.3. In all parameters measured, co-treatment with 9cRA and noggin, compared to vehicle and noggin, did not enhance locomotion in MA treated rats. Furthermore, noggin and 9cRA treatment led to a decrease in locomotor behavior, lower than that of the noggin and vehicle treatment group (Fig 8, $p<0.05$). These results indicate that noggin not only antagonized the 9cRA-induced behavior effect, but also reversed it.

Noggin antagonizes 9cRA mediated protection in striatal TH

Animals were treated with vehicle+noggin (n=21) or 9cRA+noggin (n=22) one day prior to MA or saline injection. Striatal tissues were harvested for TH Western blot analysis at 3 days after MA/saline injection. Administration of MA, compared to saline, significantly reduced TH immunoreactivity in both groups (Fig 9. $p<0.001$, two way ANOVA). No difference in TH expression was found between vehicle +noggin and 9cRA+noggin animals after MA treatment ($p=0.288$). Striatal TH immunoreactivity was also analyzed in animals treated 9cRA, 9cRA+MA (from Result 3.4, Fig 6A, right two columns), noggin+9cRA, and noggin+9cRA+MA (Fig 9A, right two columns). A significant reduction in TH immunoreactivity was found in animals receiving noggin+9cRA+MA, compared to 9cRA +MA ($p<0.001$, two way ANOVA+ Newman-Keuls test). No difference was found between 9cRA and 9cRA+noggin.

Discussion

Previous reports have indicated that the nigrostriatal DA pathway is particularly vulnerable to the toxic effect of MA. In agreement with these studies (Chou et al., 2008b; Jayanthi et al., 2005; Shen et al., 2011), we demonstrated that high doses of MA causes damage to dopaminergic neurons *in vitro* and *in vivo*. We demonstrated that high doses of MA suppressed the density of TH (+) neurons and TH immunoreactivity, examined by in primary VM culture cells. Similarly, high doses of MA cause bradykinesia and reduction of striatal TH immunoreactivity in adult rats. We found that treatment with 9cRA significantly antagonized the MA -mediated reduction of TH cell density *in vitro* and TH immunoreactivity both *in vivo* and *in vitro*, suggesting that 9cRA is neuroprotective against MA -mediated neurodegeneration in dopaminergic neurons.

High doses of MA activate apoptotic pathways that lead to the death of VM dopaminergic neurons (Chou et al., 2008b; Deng et al., 2001). In our *in vitro* study, we also reported that high doses of MA enhance DNA fragmentation as seen in the TUNEL assay. MA-induced TUNEL labeling was significantly attenuated by 9cRA. Similarly, we and others have reported that 9cRA can induce protection through the inhibition of apoptosis in ischemic brain (Shen et al., 2009b) and other systems (Bissonnette et al., 1995). It is likely that the protective effect of 9cRA against MA seen here involves an anti-apoptotic mechanism.

High doses of MA enhance Nur77 expression (Akiyama et al., 2008; Backman and Morales, 2002; No et al., 2010; Shilling et al., 2006). Translocation of Nur77 from the nucleus to the cytoplasm, a reaction regulated by RXR in the absence of an RXR agonist (Cao et al., 2004), activates apoptotic responses in mitochondria (Li et al., 2000) after injury, such as with 6-OHDA lesioning (No et al., 2010). 9cRA, a potent agonist of RXR, modulates the RXR/ Nur77 interaction to favor DNA binding and nuclear localization, activates neuroprotective genes and inhibits apoptosis (Cao et al., 2004). In this study, we demonstrated that MA enhanced Nur77 translocation to cytosol. Furthermore, administration of 9cRA suppressed Nur77 translocation to cytosol in MA –treated dopaminergic neurons. It is likely that the

redistribution of Nur77 after 9cRA treatment results in suppression of MA-mediated TUNEL labeling or apoptosis in our culture study.

We previously demonstrated that MA treatment (10 mg/kg×4) did not lead to loss of dopaminergic neurons in substantia nigra (Luo et al., 2010; Yu et al., 2012), however, significantly suppressed TH immunoreactivity in striatum (Chou et al., 2008b), suggesting that MA produced mainly DA terminal lesions *in vivo*. In this study, we demonstrated that MA (5 mg/kg×4) attenuated TH immunoreactivity in striatum, suppressed the expression of BMP7, and resulted in bradykinesia in adult rats. Suppression of BMP7 signaling enhanced MA neurotoxicity. For example, deficiency in BMP7 (Chou et al., 2008b) or its receptor BMPRII expression (Chou et al., 2008a) increased MA-mediated dopaminergic neurodegeneration in transgenic animals. Taken together, these data suggest that MA suppresses BMP7 expression, which facilitates dopaminergic terminals degeneration. In contrast, 9cRA upregulated BMP7 mRNA expression in brain cells (Shen et al., 2009). 9cRA antagonized the MA-mediated decrease in TH immunoreactivity in striatum and bradykinesia in current *in vivo* study. Treatment with BMP7 protected against the MA-mediated reduction in TH immunoreactivity both *in vivo* and in dopaminergic neuronal culture *in vivo* (Chou et al., 2008b). To further characterize the role of BMP in 9cRA-mediated protection, we administered BMP antagonist noggin before MA administration. Noggin antagonized 9cRA-mediated normalization in locomotor behavior and TH immunoreactivity in striatum. These data suggest that the protective effects of 9cRA against MA is indirectly mediated through a BMP signaling mechanism.

Although 9cRA has a high affinity for RXR, it is not detectable in adult brain tissue and, thus, may not be a candidate endogenous ligand for this receptor. Studies have indicated that the polyunsaturated fatty acids, such as linolenic and docosahexaenoic acid, activate RXR (de Urquiza et al., 2000; Goldstein et al., 2003). These polyunsaturated fatty acids have also been reported to reduce neurodegeneration (Bazan et al., 2011; Calandria et al., 2012). Future experiments are required to investigate the mechanism of protection induced by these polyunsaturated fatty acids against MA toxicity and their interaction with RXR.

In summary, our data suggest that 9cRA reduced MA toxicity in rat brains and primary VM cultures through BMP, via anti-apoptotic and Nur77 pathways. We found that either pre or co-administered 9cRA with MA reduces the toxicity of MA *in vivo* and *in vitro*. Pretreatment with 9cRA may have clinical significance in patients addicted to MA. Administration of 9cRA may prophylactically reduce MA-mediated neurodegeneration in MA abusers. Further studies on 9cRA mechanisms of action may provide the basis for novel therapeutic strategies for human neurodegenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

6-OHDA	6-hydroxydopamine
9cRA	9-cis retinoic acid
atRA	all-trans RA

BMP7	bone morphogenetic protein-7
DA	dopamine
DIV	day in vitro
HACTV	horizontal activity
MA	Methamphetamine
MOVNO	movement number
MOVTIME	movement time
NGFI-B	nerve growth factor IB
PFA	paraformaldehyde
qRT-PCR	Quantitative Real-Time PCR
RA	Retinoic acid
RAR	retinoic acid receptors
RESTIME	rest time
RXR	retinoid X receptors
SN	substantia nigra
TH	tyrosine hydroxylase
TOTDIST	total distance traveled
TUNEL	Terminal deoxynucleotidyl transferase -mediated dNTP nick -end labeling
VACTV	vertical activity
VM	ventral mesencephalon
VMOVNO	vertical movement number
VTIME	vertical movement time

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Fig 1. 9cRA antagonized MA –mediated neurodegeneration of TH neurons in VM dopaminergic neuronal culture

Primary VM neuronal cultured cells were treated with 0, 1, or 2 mM MA and either vehicle or 50 nM 9cRA on DIV10 and fixed 48 hours later. (A,D) Treatment with MA dose-dependently reduced TH (+) cell density ($p < 0.001$). (D) MA dose-dependently reduced TH cell density ($p < 0.001$). 9cRA treatment did not alter TH cell density in non MA-treated cells (A, lower left panel), but (D) significantly attenuated MA-induced loss of TH cells ($p = 0.003$ at 1 mM MA; $p = 0.007$ at 2 mM MA). Typical 9cRA –mediated protection against MA is demonstrated in A (lower right panel vs. Lower upper panel). (B) MA treatment reduced TH pixel density in culture wells (0.64 cm in diameter) as measured by in cell Western. (B,E) Addition of 9cRA significantly antagonized the MA-mediated reduction in TH pixel density ($p = 0.006$, two way ANOVA). (C,F) MA –mediated DNA fragmentation was examined using TUNEL labeling; treatment with 9cRA significantly reduced MA –mediated TUNEL activity ($p < 0.001$). Concentration of MA in (A-C) = 1mM. (D) TH cell density and (E) TH pixel density were normalized to the averaged density in culture wells treated with vehicle/saline. (F) TUNEL density was normalized by comparison to the activity in the presence of 2mM MA/vehicle. * indicates $p < 0.05$, 2-way ANOVA + Newman-Keuls test. Error bars indicate $\pm$ SEM.

Fig 2. MA increases redistribution of Nur77 protein to cytosol in primary VM cells

Rat VM neuronal culture cells were treated with vehicle or 1mM MA for 4, 8 and 24h. (A) Cytoplasmic Nur77 immunoreactivity was enhanced at 4 and 8 hours after exposure to 1mM MA. Actin and TBP were used respectively as controls for cytoplasmic and nuclear proteins. (B) MA induces a significant increase in Nur77/actin ratio at 8 hours after drug treatment (* $p = 0.030$, One-Way ANOVA + Dunnett's Test). All data were normalized to the averaged cytoplasmic Nur77 immunoreactivity at 0h.

Fig 3. 9cRA antagonized MA-mediated translocation of Nur77 in cultured dopaminergic neurons
 All cells were fixed at 4 hours after MA (1mM) or saline treatment. Typical confocal images from TH neurons receiving (A) MA (1mM) + vehicle, (B) MA (1mM) + 9cRA, (C) saline + 9cRA, and (D) saline + vehicle. (A) Nur77 immunoreactivity was mainly found in cytosol of a TH (+) neuron after MA treatment. (B) Nur77 immunoreactivity is present in nucleus in a TH neuron exposed to 9cRA and MA. In contrast, Nur77 immunoreactivity was found in the cytosol and nuclei in non-MA -treated neurons, pre-exposed to (C) 9cRA or (D) vehicle.

Fig 4. Repeated administration of MA reduced locomotor activity in rats

Rats were pretreated i.c.v. with vehicle or 9cRA one day before MA administration. 24 hours after the first MA/saline injection, rats were individually placed in the activity chambers for 2 days. High doses of MA significantly ($p < 0.05$) reduced horizontal activity (HACTV), total distance traveled (TOTDIST), movement number (MOVNO), movement time (MOVTIME), and vertical movement time (VTIME) while it significantly increased rest time (RESTIME). There is a marginal reduction in MA-mediated suppression of vertical movement number (VMOVNO) and vertical activity (VACTV). All behavioral data were normalized to the mean in vehicle –treated with saline. * indicates significant post-hoc Newman Keuls test ($p < 0.05$) and # indicates significant effect of treatment by two-way ANOVA ($p < 0.05$).

Fig 5. 9cRA antagonizes MA-induced changes in locomotor activity

24 hours after the first MA/saline injection, rats were individually placed in the activity chambers for 2 days. Pretreatment with 9cRA significantly enhanced horizontal (left panels) and vertical activity in MA treated rats. The rest time was concomitantly reduced in 9cRA – treated rats after MA treatment. All behavior data were normalized to the mean in vehicle – treated with saline. * indicates significant post-hoc Newman Keuls test ($p < 0.05$) and # indicates significant effect of treatment by two-way ANOVA ($p < 0.05$).

Fig 6. 9cRA antagonizes MA-induced reduction in TH levels in the striatum

Striatal tissue was harvested from 30 rats at 3 days after MA/saline injection for TH Western blotting. (A) In the animals pre-treated with vehicle, administration of MA significantly decreased TH immunoreactivity. MA did not significantly reduce TH expression in animals pretreated with 9cRA. TH immunoreactivity was measured using Image J and was normalized to the samples treated with saline +vehicle. (B) Example of Western blotting. TH (55 kDa) is shown in green and actin (42 kDa) is shown in red. * indicates significance ($p=0.006$) by two way ANOVA and post-hoc Newman Keuls test.

Fig 7. Effect of MA on BMP7 and Noggin mRNA expression in rat substantia nigra
Real-time qPCR was utilized to evaluate the differential expression of (A) BMP7 and (B) Noggin mRNA levels in adult rats at 24 hours after receiving saline and MA treatment. The expression of BMP7 and Noggin was normalized to the mean of each gene in saline –treated animals. * $p=0.016$.

Fig 8. Treatment with noggin antagonizes 9cRA -induced normalization of locomotor activity in MA treated rats

Rats were pretreated i.c.v. with 9cRA+noggin (n=18) or vehicle + noggin (n=18) one day before MA administration. In all parameters measured, co-treatment with 9cRA and noggin, compared to vehicle and noggin, did not enhance locomotor in MA-treated rats. * indicates significant post-hoc Newman Keuls test ($p<0.05$) and # indicates significant effect of treatment by two-way ANOVA ($p<0.05$). Treatment with MA inhibited the expression of BMP7 in nigra.

Fig 9. Noggin prevents 9cRA -mediated neuroprotective responses against MA in striatal TH levels

(A) MA suppressed TH immunoreactivity equally in animals treated with noggin/vehicle and noggin/9cRA. * $p < 0.05$. (B) Example of Western blotting. TH (55 kDa) is in green and actin (42 kDa) is in red.