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Role of Neurokinin-1 and Dopamine Receptors on the Striatal Methamphetamine-Induced Proliferation of New Cells in Mice

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Abstract

A neurotoxic dose of methamphetamine (METH) induces the loss of some striatal neurons. Interestingly, the METH-induced apoptosis in the striatum is immediately followed by the generation of new cells (cytogenesis). In the present study, we investigated the role of the neurokinin-1, dopamine D1 and D2 receptors on the METH-induced cytogenesis. To that end, male mice were given a single injection (30 mg/kg, ip) or a binge of METH (10 mg/kg, 4X at two-hour intervals, ip). BrdU (100 mg/kg, ip) was given 36 hours after the last injection of METH. Newly generated cells were detected by immunohistochemistry and cell counts were performed using unbiased computerized stereology. Either single or binge exposure to METH resulted in the generation of new cells. The single optimized dose was used for subsequent mechanistic studies. Pretreatment with the dopamine D1 receptor antagonist SCH23390 (0.1 mg/kg, ip) 30 minutes prior to METH abrogated the METH-induced striatal cytogenesis. Pretreatment with the dopamine D2 receptor antagonist raclopride (1 mg/kg, ip) failed to affect this phenomenon. Finally, pretreatment with the neurokinin-1 receptor antagonist WIN 51,708 (5 mg/kg, ip) 30 minutes prior to METH abrogated the METH-induced cytogenesis. In conclusion, neurokinin-1 and dopamine D1 receptors are required for the METH-induced striatal cytogenesis while the D2 receptor is without effect.

Keywords

methamphetamine; binge; cytogenesis; neurokinin-1 receptor; dopamine D1 receptor; dopamine D2 receptor

1. INTRODUCTION

Methamphetamine (METH) is a highly addictive psychostimulant that has increased in popularity in recent years world wide due to the inexpensive method of synthesis and the low street cost of the drug (United Nations Office on Drugs and Crime, 2007). In humans, METH use is associated with toxicity, cognitive and neurological damage, and excessive ingestion with cardiac complications and death (Darke et al., 2008; Scott et al., 2007; Barr et al., 2006). Chronic consumption of METH produces reductions of dopamine transporters in

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cortex, caudate and putamen (McCann et al., 1998, 2008; Volkow et al., 2001) as well as serotonin transporters (Sekine et al., 2006). METH abuse is also associated with microglial activation in various brain regions (Sekine et al., 2008) and morphological changes such as loss of gray matter and white matter hypertrophy (Thompson et al., 2004). These studies and many others provide compelling evidence in support of the hypothesis that METH abuse induces damage to various brain regions including cell loss.

Several studies employing animal models of METH toxicity demonstrate that METH is highly damaging to some brain regions. For example, METH induces reductions in dopamine synthesis, tissue dopamine content and dopamine transporters in non-human primates (Melega et al., 1997, 2000; Villemagne et al., 1998). In rodents, METH causes depletion of the dopamine terminal markers tyrosine hydroxylase and dopamine transporters (Ricaurte et al., 1980; Schmidt et al., 1985), inactivation and oligomerization of dopamine transporters (Fleckenstein et al., 1997; Baucum et al., 2004), activation of striatal microglia (Thomas et al., 2004, 2008), accumulation of intracellular inclusions in some striatal neurons (Fornai et al., 2004), degeneration of dopamine terminals (Ricaurte et al., 1982; O'Callaghan and Miller, 1994) and the loss of some striatal and cortical neurons (Pu et al., 1996; Eisch and Marshall, 1998; Deng et al., 2001; Bowyer et al., 2008; Zhu et al., 2006a, 2009). Our laboratory has found that between 24–48 hours after an acute exposure to a neurotoxic dose of METH, new cells are spontaneously generated throughout the striatum of mice (manuscript submitted). In the present study we assessed the role of dopamine D1, D2, and neurokinin-1 receptors on this METH-induced proliferation of new cells in the striatum by employing selective antagonists for these receptors that cross the blood-brain barrier.

2. RESULTS

In the present study we assessed the effect of the neurokinin-1 and dopamine receptors on the METH-induced striatal cytogenesis at 36 hours post-METH because in previous studies performed in our laboratory this time point was found to be optimal to study this phenomenon (manuscript submitted). Previous studies in our laboratory have routinely used the single high dose of METH (30 mg/kg), however, several laboratories investigating the mechanisms of METH-induced toxicity employ the binge schedule of drug delivery consisting of four injections of METH (10 mg/kg) at two-hour intervals delivering a cumulative dose of 40 mg/kg of body weight (for review see Krasnova and Cadet, 2009). Here we demonstrate that the binge of METH (10 mg/kg, 4X, 2-hour intervals) is as effective as the single high dose injection for the induction of new cells in the striatum (Figure 1C). New cells were detected by injecting the animals with the mitotic marker BrdU that becomes stably incorporated into nascent DNA chains. BrdU-positive nuclei were visualized with immunohistochemistry (Figure 1 A&B). BrdU-positive cells were counted using computerized unbiased stereology. Two-way ANOVA revealed no significant interaction effect [$F(1,16) = 0.22$, $p = 0.64$] or difference between binge and bolus treatment regimen [$F(1, 16) = 0.10$, $p = 0.76$]. However, a significant effect of drug treatment was revealed [$F(1,16) = 43.3$, $p < 0.0001$]. Bonferroni post-hoc analysis showed that METH-treated mice had significantly more BrdU-positive nuclei compared to saline treated mice for both the binge ($p < 0.01$) and the bolus ($p < 0.001$) regimen. For the mechanistic receptor studies described below we utilized the single high dose of METH.

To assess the role of dopamine D1 or D2 receptors on the striatal METH-induced cell proliferation, we injected animals with the selective D1 receptor antagonist SCH-23390 (0.1 mg/kg, ip) or the D2 receptor antagonist raclopride (1.0 mg/kg, ip) 30 minutes prior to METH. Published work from our laboratory determined these doses to be optimal for pharmacological blockade of D1 or D2 dopamine receptors (Xu et al., 2005). Pretreatment with SCH-23390 abrogated the METH-induced striatal cytogenesis (Figure 2). One-way

ANOVA revealed significant differences between treatment groups [$F(3, 20) = 37.3$, $p < 0.001$], accounting for 85% of the variance. Bonferroni post-hoc test showed that mice receiving only METH had significantly more BrdU-positive nuclei than those receiving saline alone ($p < 0.001$) or SCH-23390 alone ($p < 0.001$). Mice receiving METH alone also had significantly more BrdU-positive nuclei than mice pretreated with SCH-23390 before METH ($p < 0.001$) (Figure 2). In contrast, pretreatment with the D2 receptor antagonist raclopride had no effect on the METH-induced cytogenesis (Figure 3). One-way ANOVA revealed significant differences between treatment groups [$F(3, 20) = 9.6$, $p < 0.001$] that accounted for 59% of the variance. Bonferroni post-hoc test demonstrated that mice treated with METH had significantly greater BrdU-positive cells compared to mice treated with saline alone ($p < 0.001$). Mice pretreated with raclopride before METH injections also had significantly more BrdU-positive nuclei compared to mice treated with saline ($p < 0.01$) alone but were not different from mice that received METH only. Note that raclopride alone induces modest proliferation of new cells (Figure 3) but this effect was not statistically significant.

Finally, we assessed the role of the neurokinin-1 receptor by injecting the non-peptide selective antagonist WIN-51,708 (5 mg/kg, ip) 30 minutes prior to METH. Published work from our laboratory determined this dose to be optimal for pharmacological studies (Zhu et al., 2009). Pretreatment with WIN-51,708 abrogated the METH-induced striatal cytogenesis (Figure 4). One-way ANOVA revealed significant differences in the number of BrdU-positive nuclei between treatment groups [$F(3, 20) = 31.0$, $p < 0.001$]. METH-treated mice had significantly more BrdU-positive nuclei compared to mice treated with saline alone ($p < 0.001$), WIN-51,708 alone ($p < 0.001$) or pretreated with WIN-51,708 before METH ($p < 0.001$). Note that WIN-51,708 blocked the METH-induced proliferation of new cells, but when given alone it induces modest cell proliferation (Figure 4). However, this result did not reach statistical significance.

3. DISCUSSION

A single exposure to a neurotoxic dose of METH (30 mg/kg) induces toxicity of striatal dopamine terminals (Zhu et al., 2005) and the loss of approximately 25% of striatal neurons (Deng et al., 2001; Zhu et al., 2005). In a previous study we showed that a binge of METH (10 mg/kg, 4X at two-hour intervals) also induces striatal apoptosis (Zhu et al., 2006b). Here we report that the same binge of METH also induces striatal cytogenesis in the striatum. The apoptosis of striatal neurons occurs within the first 24 hours after METH as assessed using TUNEL (Zhu et al., 2005) or Fluoro-Jade C (Bowyer et al., 2008). Curiously, the phase of cytogenesis generating new cells throughout the striatum occurs within a narrow window lasting between 24–48 hours after METH (manuscript submitted). Approximately 30% of the newly generated cells survive up to 12 weeks after METH (manuscript submitted). Two previous studies demonstrated that agents affecting dopaminergic homeostasis induce contrasting effects on cytogenesis in rats. For example, acute exposure to d-amphetamine (10 mg/kg) inhibited spontaneous striatal cytogenesis (Mao and Wang, 2001). We observed extremely low levels of spontaneous cytogenesis in the striatum of mice. In another study, rats were exposed to the dopaminergic neurotoxin MPTP via systemic injections (25 mg/kg/day for five days). This treatment resulted in the generation of new cells in one plane of the striatum as measured by BrdU incorporation into nascent DNA strands (Mao et al., 2001). The MPTP-induced striatal cytogenesis resulted in new cells expressing a glial phenotype and surviving up to 60 days (Mao et al., 2001).

In contrast to the above studies, the METH-induced striatal cytogenesis reported in this study is robust and about 30% of the new cells survive up to 12 weeks post-METH and differentiate into striatal neurons (manuscript in preparation). In the light of the above, it is

of interest to determine the involvement of dopamine and neurokinin-1 receptors on the METH-induced striatal cytogenesis because pharmacological agents that antagonize these receptors protect the striatum from the METH-induced pre- and postsynaptic damage (Yu et al., 2002; Xu et al., 2005; Zhu et al., 2009).

Our results demonstrate that pretreatment with the selective dopamine D1 receptor antagonist SCH-23390 abrogated the METH-induced generation of new cells throughout the striatum. Several studies demonstrate that dopaminergic transmission affects striatal neurons. For example, the dopamine D1 receptor agonist SKF 38393 induced the expression of the immediate early genes *zif268* and *c-fos* in the dopamine-depleted striatum of rats (Keefe and Gerfen, 1996). Similarly, intrastriatal infusion of dopamine receptor antagonists modulated the d-amphetamine-induced expression of *c-fos* (Gross and Marshall, 2009). Moreover, administration of the dopamine D1 receptor antagonist SCH-23390 to rats prevented the METH-induced inhibition of striatal tyrosine hydroxylase activity and the depletion of tissue dopamine content (Sonsalla et al., 1986). A more recent study demonstrated that pretreatment with SCH-23390 30 minutes prior to METH (30 mg/kg) attenuated the METH-induced deficits of striatal tyrosine hydroxylase and dopamine transporters and the apoptosis of some striatal neurons (Xu et al., 2005). The data reported here show that D1 receptor activity is prerequisite for the induction of cytogenesis in the aftermath of METH. Interestingly, the D2 receptor antagonist raclopride failed to prevent the METH-induced cytogenesis. The dopamine neurotoxicity induced by acute METH involves the increase of striatal glutamate via a dopamine D1 receptor mechanism (Mark et al., 2004).

Studies from our laboratory have implicated the neurokinin-1 receptor in the mechanism by which METH induces striatal injury. Pretreatment with the non-peptide neurokinin-1 receptor antagonist WIN-51,708 attenuated the METH-induced deficits of the presynaptic markers tyrosine hydroxylase, dopamine transporters and tissue dopamine content (Yu et al., 2002, 2004), as well as the apoptosis of some striatal neurons (Zhu et al., 2009). Interestingly, acute METH induces signaling through the neurokinin-1 receptors of the striatum as demonstrated by the formation of endosomes in the somatostatin/NPY/NOS interneurons of the striatum (Wang and Angulo, 2011a). Moreover, pretreatment with WIN-51,708 prevented the METH-induced production of nitric oxide in the striatum (Wang and Angulo, 2011b). In the present study we demonstrate that pretreatment with WIN-51,708 abrogated the striatal METH-induced production of new cells. More studies are needed to determine if these effects of WIN-51,708 and SCH-23390 are mediated directly or indirectly by preventing the accumulation of cellular debris associated with the METH-induced apoptosis in the striatum.

In summary, a single injection or a binge of METH induces the generation of new cells throughout the mouse striatum. Pretreatment with selective dopamine D1 (SCH-23390) or the neurokinin-1 receptor (WIN-51,708) antagonists abrogated the METH-induced generation of new cells. The dopamine D2 receptor antagonist raclopride failed to abrogate this phenomenon. Work in our laboratory is investigating the mechanism by which cytogenesis occurs in the striatum in the aftermath of METH.

4. EXPERIMENTAL PROCEDURES

4.1 Animal Care and Use

All procedures regarding animal use were performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of Hunter College of the City University of New York. The animal facility at Hunter College is certified by The American

Association for Accreditation of Laboratory Animal Care (AAALAC). The work described in this article was carried out in accordance with EC Directive 86/609/EEC for animal experiments. Male ICR mice (Taconic, Germantown, NY) between 11 to weeks of age were housed individually on a 12-h light/dark cycle with food and water available *ad libitum*. Mice were habituated to the housing environment and the experimenters for two weeks prior to commencement of intraperitoneal (ip) drug administration.

4.2. Drug preparation and treatment

(+)-Methamphetamine hydrochloride (Sigma, St. Louis, MO) was dissolved in 10 mM PBS and groups of animals were injected ip with either saline or one bolus of METH (30 mg/kg of body weight) in a volume of 200 μ L. A different set of animals received a binge of METH (10 mg/kg, ip) 4X at two-hour intervals. To assess the generation of new cell, 100 mg/kg of the mitotic marker BrdU (Sigma, St. Louis, MO) was injected at 36 hours after the last injection of METH. The non-peptide neurokinin-1 receptor antagonist WIN-51,708 (17-hydroxy-17-ethynyl-5 α -androstano [3,2-b] pyrimido [1,2-a] benzimidazole) (RBI/Sigma, Natick, MA) was dissolved in vehicle (45% (w/v) 2-Hydroxypropyl- β -Cyclodextrin) (Sigma, St. Louis, MO). Vehicle or WIN-51,708 (5mg/kg, ip) was given 30 minutes prior to the injection of METH. The selective dopamine D1 receptor antagonist R-(+)-SCH-23390 hydrochloride (Sigma, St. Louis, MO) or the D2 receptor antagonist S-(+)-raclopride (+)-tartrate salt (Sigma, St. Louis, MO) were dissolved in distilled water and administered (ip) at doses of 0.1 mg/kg and 1.0 mg/kg, respectively, 30 minutes before METH treatment. The mitotic marker BrdU (Sigma, St. Louis, MO) was injected (ip) 36 hours after the last injection of METH. Animals were sacrificed at 12 hours after BrdU injection. The 12-hour time point was chosen to allow for cells currently undergoing mitosis to incorporate BrdU into the DNA. Sacrifice and striatal tissue collection was done by anesthetizing each animal with a 1:3 mixture of ketamine/acepromazine (100mg/kg of body weight) then transcardial perfusion with 25ml of PBS followed by 25ml of 4% paraformaldehyde in PBS. Brains were dissected out and immediately post-fixed for 12 hours in 4% paraformaldehyde at 4 $^{\circ}$ C, followed by cryoprotection in 30% sucrose in PBS solution at 4 $^{\circ}$ C for 48 hrs. Tissue sections were then stored at -80° C until used for immunohistochemical assays.

4.3. Immunohistochemistry for BrdU-positive nuclei

Coronal sections from each striatal hemisphere were cut at 40 μ m thickness and serially collected from the striatum between bregma 0.02 and 1.4 mm with every sixth sample per striatum collected into one of six adjacent sample wells per animal. We collected approximately 36 striatal samples in six wells per hemisphere. Immunostained BrdU-positive cells were visualized using the Vectastain[®]ABC peroxidase method with DAB. Sections were first washed in PBS and antigen unmasking was accomplished by incubation in a 1:1 solution of formamide and 4X SSC for 2 hours at 65 $^{\circ}$ C, followed by 30 minutes in 2N hydrochloric acid at 37 $^{\circ}$ C. Samples were then rinsed with 0.1 M boric acid, pH 8.5, at room temperature for 10 minutes. Endogenous peroxidases were quenched in 0.3% H₂O₂ for 5 minutes. Non-specific binding was blocked according to instructions from the Vectastain[®]ABC elite sheep kit (Vector Labs, Burlingame CA), followed by overnight incubation in primary antibody, 1:500 sheep-anti-BrdU (Novus Biologicals, Littleton, CO) at 4 $^{\circ}$ C and staining visualized with Impact DAB[®] substrate reaction (Vector Labs, Burlingame, CA). Sections were then washed and mounted on coded superfrost glass slides. Dehydration and clearing was done with 2-minute incubations in a 50% solution of xylene in ethanol followed by 100% xylene. Slides were cover slipped with VectorMount[™] permanent mounting medium (Vector laboratories, Burlingame CA) and stored for unbiased computerized stereological analysis.

Fluorescent immunostaining was visualized as follows: PBS wash, followed by incubation in 65°C solution of 1:1 formamide in 4X SSC for two hours, then incubation in 2N HCL at 37°C for 30 minutes. After a 10-minute rinse in 0.1M boric acid at pH 8.5, non-specific binding sites were blocked with 5% donkey serum in 0.2% triton X100 in PBS at room temperature for an hour. Sections were then incubated in primary antibody 1:500 sheep anti-BrdU (Novus Biologicals, Littleton, CO) in 1% normal donkey serum overnight at 4°C. After 2 PBS washes, sections were incubated for 1 hour in secondary antibody, 1:500 FITC donkey anti-sheep (Novus Biologicals, Littleton, CO) at room temperature. After two PBS washes, tissue sections were mounted on superfrost glass slides. They were then sealed and cover slipped with Vectorshield hard set™ mounting medium for fluorescence (Vector laboratories, Burlingame CA). Each slide was then coded and images taken via Leica TCS SP2 confocal microscope (Leica Microsystems, Germany) for comparison with light microscopy images.

4.4. Quantification

All slides were coded and two different individuals blind to the treatment conditions performed counts of BrdU-positive nuclei using Stereologer™ (Stereology Resource Center; Chester, MD) unbiased stereology software for MAC. Hardware component of the stereology system included a Leica DM 2500 microscope (Leica Microsystems, Germany) attached to a Prior optiscan II motorized XYZ stage system (Prior Scientific, MA), a Sony CCD video camera and a MAC pro computer with the settings described below. A cross section of the striatum from one hemisphere was outlined in 5X magnification for each tissue sample to get an estimate of the structure, but nuclei counts were done at 100 X magnification. The dissector probe in Stereologer™ was used with a Cavalieri estimator for separate counts of the mean number of BrdU-positive nuclei. Sampling frame area was 50 m² and the frame moved in steps automatically set at (x-step · y-step = 100 μm²) in a raster pattern for approximately 200 frames of striatal tissue per animal. For all groups, Stereologer™ estimated sampling error (CE) was well within acceptable range and less than 0.1.

4.5. Statistical analysis

In all statistical analyses, $p < 0.05$ was considered significantly different from chance. Analysis was done with Graphpad Prism software (San Diego, CA). In all experiments the dependent variable was the mean number of BrdU-positive nuclei. For the experiment that compared the binge versus bolus treatment regimen, a two-way ANOVA was done. Significant effects were followed by Bonferroni corrected post-hoc test to determine drug treatment differences. One-way ANOVAs were used to analyze results from the D1, D2 and neurokinin-1 receptor experiments. Each significant one-way ANOVA was followed by Bonferroni corrected post-hoc test for differences between treatment groups for a given receptor experiment.

Abbreviations used

BrdU	5-Bromo-2'-deoxyuridine
DAB	+3,3'-diaminobenzidine
ICR	Institute for Cancer Research
ip	intraperitoneal
METH	(+)-methamphetamine hydrochloride
PBS	phosphate-buffered saline, pH 7.4

RAC	raclopride
SCH	SCH-23390
WIN-51,708	17- β -Hydroxy-17- α -ethynyl-5- α -androstan[3,2- β]pyrimido[1,2- a]benzimidazole
SSC, SSC	0.15M sodium chloride/0.015M sodium citrate buffer, pH 7.0

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Figure 1.

A single injection or a binge of METH is equally effective inducing striatal cell proliferation. A & B, coronal sections of the striatum of control and METH-treated mice, respectively. BrdU-positive cells appear green. Male mice (n=8) received a single injection (30 mg/kg) or a binge (10 mg/kg, 4X at two-hour intervals). The animals received BrdU (100 mg/kg) 36 hours after the last injection of METH. Newly generated cells were visualized by immunohistochemistry. (C) BrdU-positive cells were counted using computerized unbiased stereology. Note that the single high dose would be more convenient for mechanistic studies. **p<0.01; ***p<0.001 (one-way ANOVA, Bonferroni post-hoc). CC, corpus callosum; SVZ, subventricular zone.

Figure 2.

The dopamine D1 receptor antagonist SCH-23390 (SCH) abrogated the METH-induced striatal cell proliferation. SCH-23390 (0.1 mg/kg) was given 30 minutes before METH (30 mg/kg, n=8). BrdU (100 mg/kg) was given 36 hours after METH. BrdU-positive cells were visualized by immunohistochemistry and counted using unbiased stereology. Mice receiving METH alone had significantly more BrdU-positive nuclei than mice pretreated with SCH-23390 before METH ($p < 0.001$). Note that SCH-23390 alone was without effect on the striatal cell proliferation. *** $p < 0.001$ (one-way ANOVA, Bonferroni post-hoc).

Figure 3.

The dopamine D2 receptor antagonist raclopride (RAC) attenuates the METH-induced striatal generation of new cells. Raclopride (1 mg/kg) was given 30 minutes prior to METH (30 mg/kg, n=8). The mitotic marker BrdU (100 mg/kg) was given 36 hours after METH. BrdU-positive cells were counted using unbiased stereology. Note that raclopride alone (RAC + Saline) induces modest proliferation of new cells but this result did not approach statistical significance. *** $p < 0.001$ (one-way ANOVA, Bonferroni post-hoc).

Figure 4.

The neurokinin-1 receptor antagonist WIN-51,708 (WIN) abrogated the striatal METH-induced proliferation of new cells. WIN-51,708 (5 mg/kg) was given 30 minutes before METH (30 mg/kg, $n=8$). BrdU (100 mg/kg) was injected 36 hours after METH. BrdU-positive cells were counted using unbiased stereology. The modest cell proliferation induced by WIN-51,708 alone did not reach statistical significance. *** $p<0.001$ (one-way ANOVA, Bonferroni post-hoc).