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Evaluating the Role of Neuronal Nitric Oxide Synthase-Containing Striatal Interneurons in Methamphetamine-Induced Dopamine Neurotoxicity

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Abstract

Production of nitric oxide (NO) has been implicated in methamphetamine (METH)-induced dopamine (DA) neurotoxicity. The source of this NO has not been clearly delineated, but recent evidence suggests that it arises from activation of neuronal nitric oxide synthase (nNOS), which is selectively expressed in a subpopulation of striatal interneurons. Our objective was to determine whether inhibiting activation of nNOS-containing interneurons in the striatum blocks METH-induced neurotoxicity. These interneurons selectively express the neurokinin-1 (NK-1) receptor, which is activated by substance P. One particular toxin, a conjugate of substance P to the ribosome-inactivating protein saporin (SSP–SAP), selectively destroys neurons expressing the NK-1 receptor. Thus, we examined the extent to which depletion of the nNOS-containing interneurons alters production of NO and attenuates METH-induced neurotoxicity. The SSP–SAP lesions resulted in significant loss of nNOS-containing interneurons throughout striatum. Surprisingly, this marked deletion did not confer resistance to METH-induced DA neurotoxicity, even in areas devoid of nNOS-positive cells. Furthermore, these lesions did not attenuate NO production, even in areas lacking nNOS. These data suggest that nNOS-containing interneurons either are not necessary for METH-induced DA neurotoxicity or produce NO that can diffuse extensively through striatal tissue and thereby still mediate neurotoxicity.

Keywords

Methamphetamine; Neurotoxicity; Nitric oxide synthase; Dopamine

Introduction

The abuse of methamphetamine (METH) continues to be a major public health concern. As many as 60 million people worldwide report abusing amphetamine-type stimulants, especially METH (Maxwell 2005). The use of METH is highly problematic, not only because of the acute effects of the drug which can include psychosis and aggressive

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behavior, but also because of the now-documented, long-term consequences on the structure and function of the central nervous system, and concomitant cognitive deficits.

It is well established that exposure to multiple high doses of METH produces damage to central monoamine systems, and this toxicity has been modeled in numerous species. In nonhuman primates, decreases in markers of dopamine (DA) innervation have been reported to exist for up to 4 years (Woolverton et al. 1989). In rodents, partial monoamine losses have been reported to persist for up to 6 months, although there may be some recovery over time (Bittner et al. 1981; Cass 2000; Cass and Manning 1999). A number of post-synaptic events in the striatum, including the production of nitric oxide (NO), have been implicated in this toxicity. Specifically, peroxynitrite inhibits complex II of the electron transport chain (ETC), as does METH, and peroxynitrite decomposition catalysts block METH-induced inhibition of complex II of the ETC (Brown et al. 2005), as well as METH-induced neurotoxicity (Imam et al. 1999).

While it is relatively clear that NO contributes to METH-induced toxicity to the DA nerve terminal, the source of this NO has not been clearly delineated. However, there is considerable evidence suggesting that the generation of NO may arise as a consequence of the activation of neuronal nitric oxide synthase (nNOS). For example, a neurotoxic regimen of METH fails to induce striatal DA loss in mice with a targeted deletion of nNOS (Imam et al. 2001; Itzhak et al. 2004; Itzhak et al. 1998). Unfortunately, this blockade of toxicity is confounded by the fact that the mice thus treated do not show the METH-induced hyperthermia known to critically contribute to METH-induced neurotoxicity. However, pretreatment of mice with nNOS inhibitors does not attenuate METH-induced hyperthermia, but does prevent METH-induced neurotoxicity to DA neurons (Itzhak et al. 2000; Abekawa et al. 1996; Di Monte et al. 1996; Itzhak and Ali 1996; Sanchez et al. 2003). It is important to note here that nNOS is not expressed in DA nerve terminals (Fujiyama and Masuko 1996; Hwang et al. 1998). Rather, in the striatum, nNOS is expressed post-synaptic to the DA nerve terminals in a population of striatal interneurons that also express DA D1-family receptors (Dawson et al. 1991; Figueredo-Cardenas et al. 1996; Rushlow et al. 1995; Vincent et al. 1983). Thus, activation of nNOS is likely to be downstream of DA D1-family receptor signaling. Activation of nNOS, and the subsequent production of NO, has been shown to be sensitive to phasic DA signaling via DA D1 receptors (Sammur et al. 2006), and phasic DA signaling has been shown to be impaired in rats with partial DA depletions (Bergstrom and Garis 2003), including those induced by METH (Howard et al. 2011). Thus, we have hypothesized that DA-mediated activation of the nNOS-containing striatal interneurons is necessary for METH-induced neurotoxicity.

These interneurons, along with the cholinergic interneurons of striatum, selectively express the neurokinin-1 (NK-1) receptor which is activated by the neuropeptide substance P (Gerfen 1991; Li et al. 2001; Li et al. 2000). Consequently, toxins targeted to NK-1 receptor-containing neurons can be used to lesion these populations of striatal interneurons (Saka et al. 2002; Zhu et al. 2006a). One such toxin, a conjugate of substance P to the ribosome-inactivating protein saporin (SSP-SAP), has been shown to be effective in selectively destroying neurons expressing the NK-1 receptor in striatum (Wiley and Lappi 1999). One research group has previously examined the effect of single injection of this toxin on METH-induced damage in the striata of mice (Zhu et al. 2006a), and no effect on METH-induced DA depletion was observed. While this finding might suggest a lack of involvement of this interneuron population in METH-induced neurotoxicity, several issues remain to be examined regarding this point. First, as reviewed above, several lines of evidence do implicate nNOS in METH-induced neurotoxicity to striatal DA. Second, in the study by Zhu and colleagues, the effects of these SSP-SAP-induced lesions were examined in mice 3 days after exposure to a single high dose of METH. It is clear that this single-dose

exposure to METH in mice produces effects in striatum that are somewhat distinct from those long-lasting depletions of DA produced by a binge paradigm (Hotchkiss et al. 1979; Yu et al. 2002; Zhu et al. 2006b). In light of these issues, we felt it was critical to examine the involvement of this interneuron population in the persistent DA deficits induced by the binge regimen of METH administration in rats. Therefore, we used targeted deletion of the nNOS-containing interneurons via SSP-SAP to examine the involvement of nNOS-containing striatal interneurons in METH-induced neurotoxicity to the DA innervation of the striatum.

Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) were individually housed on a 12-h light/dark cycle with ad libitum access to food and water. The housing conditions and care of the animals were in accordance with those specified in the *Guide for the Care and Use of Laboratory Animals* (8th Ed., National Research Council) and were approved by the Institutional Animal Care and Use Committee at the University of Utah. All procedures were performed during the light part of the diurnal cycle. Rats undergoing surgery weighed 275–300 g and were handled for 2 days before surgery.

SSP-SAP Lesions

Animals were anesthetized with ketamine/xylazine (90/10 mg/kg, i.p.) and placed in a stereotaxic instrument. SSP-SAP, or Blank-SAP as a control, (1 μ l of 3 ng/ μ l; Advanced Targeting Systems, San Diego, CA) was unilaterally injected into four separate locations in the right striatum [AP: +1.0 mm, relative to bregma, ML: –2.2 mm, relative to the midsagittal suture, DV: –4.5 mm from skull; AP: –0.6 mm, ML: –2.8 mm, DV: –4.5 mm; AP: –0.6 mm, ML: –3.8 mm, DV: –6.2 mm; AP: +1.0 mm, ML: –3.2 mm, DV: –6.0 mm] (Paxinos and Watson 2005). The toxin was dissolved in 0.1 M phosphate buffered saline, pH 7.4 (PBS) and injected via a 33-gauge metal cannula (Plastics One, Roanoke, VA) attached to a 10- μ l Hamilton syringe (Hamilton Company, Reno, NV) driven by an infusion pump (Harvard Apparatus, Holliston, MA). Each 1- μ l infusion was given over 6 min to minimize mechanical damage, and the cannula was left in place for an additional 3 min to allow for diffusion of the toxin. After completion of the four infusions, the scalp wound was stapled shut, and the animals were monitored until fully recovered from the anesthesia.

METH Injections

Three weeks later, the animals were housed in groups of four in plastic tub cages (33 cm $\times$ 28 cm $\times$ 17 cm) with corncob bedding. Animals were treated with either a neurotoxic regimen of ($\pm$)-METH-HCl (National Institute on Drug Abuse, Bethesda, MD; 4 $\times$ 7.5 mg/kg, s.c. at 2-hr intervals) or saline (4 $\times$ 1 ml/kg, s.c.), creating four groups of animals: Blank-SAP/Saline, Blank-SAP/METH, SSP-SAP/Saline, and SSP-SAP/METH. To monitor METH-induced hyperthermia, a response that has previously been shown to correlate with subsequent neurotoxicity (Bowyer et al. 1994; Tata et al. 2007), rectal temperatures were recorded using a digital thermometer (BAT-12, Physitemp Instruments, Clifton, NJ). Baseline temperatures for each animal were recorded 30 min before the first injection, and subsequent temperatures were recorded 1 h after each injection. Animals whose core temperature exceeded 40.5 $^{\circ}$ C were cooled by placement in a plastic tub cage resting on ice and wetting with cold water until their core temperature fell below 39 $^{\circ}$ C. The next day, animals to be carried out to the 7-day survival time point were returned to their home cages in the vivarium.

Tissue Preparation

Either 1 h or 7 days following the last injection of METH or saline, animals were sacrificed via exposure to CO₂ and decapitated. Their brains were rapidly removed and submerged in 4 % paraformaldehyde with 0.9 % NaCl for 24 h at 4 °C, then cryoprotected in 30 % sucrose in 0.1 M PBS and stored at 4 °C. Thirty-six sections (30 µm each) were collected at the level of the striatum (beginning rostrally at ~1.5-mm anterior to bregma) using a freezing-stage microtome and stored at 4 °C in 0.1 M PBS containing 1 mg/ml sodium azide. Serial sections were used for histochemical and immunohistochemical labeling.

NADPH Diaphorase Histochemistry

Free-floating sections were washed in 0.1 M Tris–HCl (pH 8.0) followed by preincubation in 0.1 M Tris–HCl containing 0.04 % Tween-80 and 0.05 % TritonX-100. The tissue was then incubated in 0.1 M Tris–HCl with 0.8 mg/ml NADP, 0.16 mg/ml NBT, 0.04 % Tween-80, 0.05 % TritonX-100, 1 mM MgCl₂, and 15 mM malate for 2 h at 37 °C, rinsed in 0.1 M Tris–HCl, mounted onto slides, allowed to dry, dehydrated in escalating EtOH concentrations (70, 95, 100 % for 3 min each), and coverslipped using Vectamount (Vector Labs, Burlingame, CA).

NeuN Immunohistochemistry (IHC)

Free-floating sections were dehydrated in 50 % EtOH then rinsed in 0.1 M PBS. All subsequent solutions were made in 0.1 M PBS. The tissue was incubated with 10 % bovine serum albumin (BSA) and 0.02 % TritonX-100 for 2 h, then in 5 % BSA containing biotinylated anti-NeuN mouse monoclonal antibody (1:600, Millipore, MAB377B) for 1 h at 37 °C, and for an additional 2 h at room temperature. Sections were then rinsed and incubated with avidin-biotinylated peroxidase complex (ABC Elite kit, Vector) for 45 min. The reaction was terminated by rinsing three times in 0.1 M PBS. The tissue was then incubated in a 1:1 dilution of nickel-enhanced diaminobenzidine tetrahydrochloride (Ni-DAB, black reaction product, Vector) for 3 min before being rinsed in 0.1 M PBS. Sections were mounted onto slides, allowed to dry, dehydrated, and coverslipped as above.

Dopamine Transporter (DAT) IHC

Free-floating sections were processed for antigen retrieval in citrate buffer (10 mM sodium citrate in 0.9 % saline with 0.05 % Tween-20, pH 6.0) at 92 °C for 20 min, and then cooled for 40 min. All subsequent solutions were made in 0.1 M PBS. The tissue was rinsed, incubated in 3 % H₂O₂ to block endogenous peroxidases, and rinsed again. Nonspecific binding in the tissue was blocked with 5 % milk containing 0.2 % TritonX-100. The tissue was then incubated overnight at 4 °C in 2 % milk containing 0.2 % TritonX-100 and anti-DAT rat monoclonal antibody (1:5,000, Millipore, MAB369). Sections were then rinsed, incubated for 1 h in 2 % milk containing 0.2 % TritonX-100 and biotinylated rabbit anti-rat IgG (1:200, Vector, BA-4000), rinsed again, and then incubated with avidin-biotinylated peroxidase complex (ABC Elite kit, Vector) for 30 min. The reaction was terminated by rinsing three times in 0.1 M PBS. The tissue was then incubated in a 1:1 dilution of Ni-DAB for 5 min before being rinsed in 0.1 M PBS. Sections were mounted onto slides, dried, dehydrated, and coverslipped as above.

Nitrotyrosine (NT) IHC

Tissue was processed as it was for DAT IHC with the following exceptions. No antigen retrieval step was necessary. Nonspecific binding in the tissue was blocked for 1 h with 10 % normal horse serum (NHS), and the tissue was then incubated overnight at 4 °C in 5 % NHS with anti-NT mouse monoclonal antibody (1:100, Abcam, ab78163). The secondary

antibody used was a rat-adsorbed, biotinylated horse anti-mouse IgG (1:200, Vector, BA-2001).

Image Analysis

For DAT and NT IHC, images were captured on a lightbox and digitized. Densitometric analysis was performed using NIH ImageJ software, yielding average gray values in the striatum. Four striatal sections, covering the rostral–caudal extent of the lesion were analyzed per rat. For NADPH, sections were imaged at 100× using a Leica DM 4000B microscope to assess the extent of the SSP–SAP-induced lesions. Areas devoid of NADPH-positive cell bodies or processes were considered lesioned and were recorded on diagrams from the rat brain atlas (Paxinos and Watson 2005). For detailed analysis of the NADPH diaphorase histochemical staining, 3 × 3 montages were captured at 400× magnification. In the infused striatum, these montages were centered over regions completely devoid of NADPH-positive cell bodies. In the noninfused striatum, the montages were positioned to match the corresponding position in the infused striatum. The montaged images were analyzed using ImageJ. Image analysis was standardized by thresholding the images to include only the NADPH-positive cell bodies and processes (minimum value = 0, maximum value = 16 points above the lowest edge of the threshold histogram). The percent area of the field with signal was then measured for each image. As above, four striatal sections were analyzed per rat. NeuN immunohistochemical images were inspected at 100× to ensure the specificity of the SSP–SAP lesions and to check for mechanical damage due to the infusions.

Statistics

The experimenter was blinded to treatment groups during the collection of all dependent measures. Repeated measures MANOVAs (pretreatment × treatment × time) were conducted to determine differences in body temperature during the METH injections. Because there were no significant effects of the SSP–SAP pretreatment on the temperature responses to the binge-regimen of METH, post hoc analyses of significant treatment × time interactions were accomplished by post hoc, two-tailed t-tests at each time point. Likewise, a repeated measures MANOVA (pretreatment × treatment × hemisphere) was used to examine the effects of the toxin on METH-induced loss of DAT, NADPH, and NT staining in the striatum. Post-hoc analyses via two-way ANOVA (pretreatment × treatment) followed by the Tukey's HSD test were conducted to determine significant group differences within hemispheres, as appropriate. Statistical significance was set at $p = 0.05$.

Results

METH-Induced Hyperthermia

In all cases, the METH-treated groups exhibited the METH-induced hyperthermia known to be a critical predictor of future neurotoxicity. In the animals sacrificed at 7 days post-METH, there were significant main effects of treatment (MANOVA, $F_{(1,6)} = 181.6$, $p < 0.0001$) and time (MANOVA, $F_{(4,3)} = 12.4$, $p < 0.05$) on body temperature with METH animals showing higher temperatures relative to saline. A significant time × treatment interaction (MANOVA, $F_{(4,3)} = 15.3$, $p < 0.05$) was obtained such that animals treated with METH were significantly hyperthermic at the first post-METH time point (60 min; $t = 9.4$, $p < 0.0001$) and remained so for the duration of the monitoring period (p values < 0.0001 ; Fig. 1a). Similar effects were seen in the animals sacrificed at 1 h post-METH. There were significant main effects of treatment (MANOVA, $F_{(1,25)} = 254.8$, $p < 0.0001$) and time (MANOVA, $F_{(3,23)} = 100.1$, $p < 0.0001$) on body temperature, as well as a significant time × treatment interaction (MANOVA, $F_{(3,23)} = 105.6$, $p < 0.0001$). METH-treated rats were significantly hyperthermic at the first post-METH time point (60 min; $t = 7.6$, $p < 0.0001$)

and remained so for the duration of the monitoring period (p values < 0.0001 ; Fig. 1b). In no case was a main effect of pretreatment or pretreatment $\times$ treatment, pretreatment $\times$ time, or pretreatment $\times$ treatment $\times$ time interactions observed (p values > 0.05), confirming that the prior intrastratial infusion with SSP-SAP or blank-SAP did not alter METH-induced hyperthermia.

Extent of the SSP-SAP Lesions

The four unilateral infusions of SSP-SAP resulted in a significant loss of nNOS-containing interneurons throughout the striatum, although it was not possible to completely eliminate all of the neurons, even when using multiple sites of injection, because of nonspecific toxicity associated with higher concentrations of the SSP-SAP toxin. The extent of the SSP-SAP-induced lesions was assessed by labeling the nNOS-containing interneurons using NADPH diaphorase histochemistry. There was a significant loss of NADPH diaphorase-positive cell bodies and processes in the SSP-SAP-infused striatum, but no loss in the noninfused striatum (Fig. 2a). This observation was quantified by counting the number of NADPH diaphorase-positive cell bodies in the areas analyzed for all subsequent dependent measures. MANOVA revealed a significant main effect of pretreatment ($F_{(1,24)} = 4.8$, $p < 0.0001$) and a significant pretreatment $\times$ hemisphere interaction ($F_{(1,24)} = 73.6$, $p < 0.0001$). Subsequent two-way ANOVAs on the data from each hemisphere revealed no effect of pretreatment in the noninfused striatum ($F_{(1,24)} = 1.5$, $p > 0.05$), but a highly significant effect of pretreatment in the infused hemisphere ($F_{(1,24)} = 365.9$, $p < 0.0001$). Thus, in the infused striatum, SSP-SAP induced a highly significant loss of NADPH diaphorase-positive cell bodies (Fig. 2c). To verify the selectivity of the lesion, serial sections were stained for NeuN, a marker of neuronal nuclei. In sharp contrast to the NADPH diaphorase-labeled sections, no gross loss of NeuN-positive cells was observed in the infused striatum (Fig. 2b), although in areas corresponding to the cannulae tracks, minimal loss of NeuN-positive cells was observed, presumably reflecting unavoidable mechanical damage due to insertion of the cannulae.

METH-Induced DAT Depletions

Quantification of DAT levels in the animals sacrificed 7 days post-METH revealed that the marked loss of nNOS-containing interneurons of striatum did not confer resistance to METH-induced DA neurotoxicity. MANOVA revealed a significant main effect of treatment ($F_{(1,6)} = 91.3$, $p < 0.0001$) and a significant treatment $\times$ hemisphere interaction ($F_{(1,6)} = 6.5$, $p < 0.05$). The significant interaction appeared to reflect the fact that DAT staining was slightly higher in the noninfused than in the infused hemisphere in saline-pretreated rats, but slightly lower in the noninfused than in the infused hemisphere in the METH-pretreated rats. Importantly, post hoc analysis revealed that the effect of METH on DAT staining was not different between the infused vs. the non-infused striatum ($t = 1.7$, $p > 0.05$). In addition, there was no significant main effect of pretreatment ($F_{(1,6)} = 3.1$, $p > 0.05$) and no significant pretreatment $\times$ treatment ($F_{(1,6)} = 3.6$, $p > 0.05$), pretreatment $\times$ hemisphere ($F_{(1,6)} = 0.004$, $p > 0.05$), or pretreatment $\times$ treatment $\times$ hemisphere ($F_{(1,6)} = 5.1$, $p > 0.05$) interactions. Thus, infusion of SSP-SAP had no effect on either basal DAT levels or the magnitude of the METH-induced depletion in DAT (Fig. 3).

METH-Induced NO Production

Because significant ablation of the nNOS-containing interneurons did not confer resistance to METH-induced toxicity, we chose to examine whether these lesions impaired METH-induced NO production. Previous work by the present author group has shown increases in NADPH diaphorase histochemical staining, reflecting increases in nNOS activity, as well as increases in NT immunoreactivity, an indirect marker of NO production, induced by exposure to a binge regimen of METH regardless of whether that regimen induced an acute

DA neuron toxicity (Friend et al. 2013). The present study confirms the increase in these measures by the binge-regimen of METH, revealing a significant hemisphere $\times$ pretreatment $\times$ treatment interaction ($F_{(1,24)} = 6.5, p < 0.02$). Subsequent two-way ANOVAs on the data from each hemisphere revealed that in the noninfused striatum, there was a main effect of METH treatment ($F_{(1,24)} = 12.5, p < 0.002$), but no significant effect of pretreatment ($F_{(1,24)} = 0.56, p > 0.05$) and no significant pretreatment $\times$ treatment interaction ($F_{(1,24)} = 0.43, p > 0.05$). Thus, in the non-infused striatum, METH produced an increase in NADPH diaphorase histochemical staining. In the infused striatum, there was no main effect of METH treatment (ANOVA, $F_{(1,24)} = 3.41, p > 0.05$), but there was a main effect of pretreatment (ANOVA, $F_{(1,24)} = 55.56, p < 0.0001$) and a significant pretreatment $\times$ treatment interaction (ANOVA, $F_{(1,24)} = 4.96, p < 0.05$). Post-hoc analysis revealed that both SSP–SAP groups were significantly different from their respective Blank–SAP groups (Tukey's HSD, $p < 0.05$), consistent with the extensive loss of NADPH-diaphorase-positive cell bodies shown in Fig. 2. Post-hoc analysis further revealed that the Blank–SAP/METH group showed the expected (Friend et al. 2013) increase in NADPH diaphorase staining relative to the Blank–SAP/Saline group (Tukey's HSD, $p < 0.05$), but that the SSP–SAP/METH group did not show such an increase relative to the SSP–SAP/Saline group (Tukey's HSD, $p > 0.05$; Fig. 4): the SSP–SAP-induced depletion of nNOS-containing interneurons completely abolished the METH-induced increase in NADPH diaphorase staining.

Interestingly, the NT IHC results do not follow this same pattern (Fig. 5). The overall MANOVA revealed a significant main effect of treatment ($F_{(1,25)} = 6.4, p < 0.02$) and a significant main effect of hemisphere ($F_{(1,25)} = 13.6, p < 0.002$), but no main effect of pretreatment ($F_{(1,25)} = 0.13, p > 0.05$) and no significant interactions between the factors (all p values > 0.05). Thus, although the METH-induced increase in NADPH-diaphorase histochemical staining was abolished in the SSP–SAP-infused hemisphere, the METH-induced increase in NT immunohistochemical staining was not.

Discussion

The abuse of METH in the United States has increased markedly in the past 15 years, due in large measure to the ease with which the drug can be synthesized illicitly and the long-lasting, intense euphoria that its administration produces. Use is endemic in the Western states, while growing notably in both the Midwest and the Atlantic states. Psychostimulant abuse carries with it several potential health risks, and METH abuse carries the additional danger of permanent brain injury. It is well established that exposure to multiple high doses of METH produces damage to central monoamine systems. Long-lasting decreases in markers of DA innervation of the striatum have been reported in both rodent models of binge METH use (Bittner et al. 1981; Cass 2000; Cass and Manning 1999; Friedman et al. 1998) and human METH abusers (McCann et al. 1998; Volkow et al. 2001a; Volkow et al. 2001b). Furthermore, human METH abusers are more likely to develop Parkinson's disease (Callaghan et al. 2010; Callaghan et al. 2012), suggesting long-lasting and possibly progressive DA loss as a consequence. Thus, understanding the basis for such DA neurotoxicity induced by METH will be critical for the development of therapies to mitigate these long-term consequences.

As reviewed in the Introduction, the production of NO by nNOS has been implicated in METH-induced neurotoxicity to DA nerve terminals (Abekawa et al. 1996; Brown et al. 2005; Di Monte et al. 1996; Imam et al. 1999; Imam et al. 2001; Itzhak and Ali 1996; Itzhak et al. 2004; Itzhak et al. 2000; Sanchez et al. 2003). Consequently, we hypothesized that ablation of these nNOS-containing striatal interneurons by the targeted toxin SSP–SAP would attenuate METH-induced neurotoxicity. The SSP–SAP lesions resulted in a significant loss of nNOS-containing interneurons throughout the striatum without notable

impact on overall NeuN labeling of neurons (Fig. 2). Surprisingly, this marked ablation of nNOS-containing interneurons did not confer resistance to METH-induced DA neurotoxicity (Fig. 3). This lack of protection was observed even in areas completely devoid of nNOS-positive cell bodies and histochemical detection of NOS activity with NADPH diaphorase histochemistry. These findings thus are consistent with and extend the previous observations of Angulo and colleagues (Zhu et al. 2006a) who reported that a more restricted loss of the nNOS-containing interneurons did not confer resistance to more transient DA loss induced in mice by a single injection of a high dose of METH. Given the known differences in neurotoxic effects of the single bolus dose regimen and the repeated dose binge regimen used herein (Hotchkiss et al. 1979; Yu et al. 2002; Zhu et al. 2006b), the results presented here provide confirmation of a similar lack of protection with a greater degree of interneuron loss and in the rat binge model. It is important to note here that, while our current results support a lack of protection against DA terminal neurotoxicity, ablation of the NK-1 receptor expressing interneurons has been shown to protect against METH-induced striatal apoptosis (Zhu et al. 2009). Thus, we cannot exclude the potential role of NO in the striatal efferent neuron toxicity observed in some models.

Because SSP-SAP lesions of the nNOS-containing striatal interneurons did not attenuate METH-induced DA neurotoxicity, it was prudent to investigate whether such lesions inhibited NO production. Remarkably, despite generating significant loss of nNOS-containing striatal interneurons and abolishing METH-induced increases in nNOS activity (Fig. 4), these lesions did not attenuate METH-induced NO production as assessed via NT IHC (Fig. 5). These data suggest that although nNOS activation by METH is lost, NO is likely still being formed in response to the neurotoxic regimen of METH.

Taken together, these data suggest one of two possible interpretations. One possibility is that the nNOS-containing interneurons are not necessary for METH-induced DA neurotoxicity, leaving open the potential contribution of other sources of NO such as inducible NOS (iNOS) or endothelial NOS (eNOS). Studies from our lab and others have shown no induction of iNOS mRNA (Friend et al. 2013) or protein expression (Deng and Cadet 1999) following a neurotoxic regimen of METH. Thus, a contribution of iNOS to the METH-induced NO production seen in this study is unlikely. The potential involvement of eNOS cannot be explicitly ruled out. Data from our lab demonstrate a lack of METH-induced eNOS mRNA expression using a similar dosing paradigm (Friend et al. 2013), which might suggest a limited role for eNOS in METH-induced DA neurotoxicity. However, like nNOS, eNOS is constitutively expressed. Thus, activation of eNOS may provide a source of NO in response to METH.

The other possible interpretation capitalizes on the fact that the nNOS-containing striatal interneurons, while markedly ablated, were not completely eliminated in this study. Therefore, it is possible that the remaining nNOS-containing interneurons are still producing NO and reactive nitrogen species (RNS) in response to METH. NO has been shown to diffuse rapidly and extensively through rat neuronal tissue (Santos et al. 2011), which could explain the METH-induced increases in NT immunohistochemical staining seen in this study, even in regions of the striatum devoid of NADPH diaphorase-positive cell bodies and METH-induced increases in NADPH diaphorase histochemical staining. By means of rapid and extensive diffusion, the NO produced by residual nNOS-containing striatal interneurons may thus participate in the METH-induced neurotoxicity shown here. Given that systemic administration of nNOS inhibitors has been reported to block METH-induced toxicity to striatal DA systems in the absence of effects on METH-induced hyperthermia (Itzhak et al. 2000; Abekawa et al. 1996; Di Monte et al. 1996; Itzhak and Ali 1996; Sanchez et al. 2003), this latter interpretation seems possible. However, other work from our group suggests that even if NO is necessary for METH-induced neurotoxicity, it is not sufficient, as METH

increases nNOS activity and NT immunohistochemical staining even in rats resistant to the acute neurotoxic effects of METH on DA neurons (Friend et al. 2013).

In summary, the data presented here show that a significant lesion of the nNOS-containing interneuron population of the striatum abolished METH-induced increases in NADPH diaphorase histochemical staining, but did not inhibit the METH-induced increase in NT immunohistochemical staining or protect against METH-induced DA depletion in these same regions of striatum. Future studies investigating the role of eNOS in METH-induced NO production and the diffusion of NO in striatal tissue will be necessary to fully elucidate the contribution of NO in METH-induced DA neurotoxicity.

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References

- Abekawa T, Ohmori T, Koyama T. Effects of nitric oxide synthesis inhibition on methamphetamine-induced dopaminergic and serotonergic neurotoxicity in the rat brain. *J Neural Transm.* 1996; 103(6):671–680. [PubMed: 8836929]
- Bergstrom BP, Garris PA. “Passive stabilization” of striatal extracellular dopamine across the lesion spectrum encompassing the presymptomatic phase of Parkinson’s disease: a voltammetric study in the 6-OHDA-lesioned rat. *J Neurochem.* 2003; 87(5):1224–1236. [PubMed: 14622102]
- Bittner SE, Wagner GC, Aigner TG, Seiden LS. Effects of a high-dose treatment of methamphetamine on caudate dopamine and anorexia in rats. *Pharmacol Biochem Behav.* 1981; 14(4):481–486. [PubMed: 6940202]
- Bowyer JF, Davies DL, Schmued L, Broening HW, Newport GD, Slikker W Jr, Holson RR. Further studies of the role of hyperthermia in methamphetamine neurotoxicity. *J Pharmacol Exp Ther.* 1994; 268(3):1571–1580. [PubMed: 8138969]
- Brown JM, Quinton MS, Yamamoto BK. Methamphetamine-induced inhibition of mitochondrial complex II: roles of glutamate and peroxynitrite. *J Neurochem.* 2005; 95(2):429–436. doi:10.1111/j.1471-4159.2005.03379.x. [PubMed: 16086684]
- Callaghan RC, Cunningham JK, Sajeev G, Kish SJ. Incidence of Parkinson’s disease among hospital patients with methamphetamine-use disorders. *Mov Disord.* 2010; 25(14):2333–2339. doi:10.1002/mds.23263. [PubMed: 20737543]
- Callaghan RC, Cunningham JK, Sykes J, Kish SJ. Increased risk of Parkinson’s disease in individuals hospitalized with conditions related to the use of methamphetamine or other amphetamine-type drugs. *Drug Alcohol Depend.* 2012; 120(1-3):35–40. doi:10.1016/j.drugalcdep.2011.06.013. [PubMed: 21794992]
- Cass WA. Attenuation and recovery of evoked overflow of striatal serotonin in rats treated with neurotoxic doses of methamphetamine. *J Neurochem.* 2000; 74(3):1079–1085. [PubMed: 10693939]
- Cass WA, Manning MW. Recovery of presynaptic dopaminergic functioning in rats treated with neurotoxic doses of methamphetamine. *J Neurosci.* 1999; 19(17):7653–7660. [PubMed: 10460271]
- Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH. Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc Natl Acad Sci U S A.* 1991; 88(17):7797–7801. [PubMed: 1715581]
- Deng X, Cadet JL. Methamphetamine administration causes overexpression of nNOS in the mouse striatum. *Brain Res.* 1999; 851(1-2):254–257. [PubMed: 10642852]
- Di Monte DA, Royland JE, Jakowec MW, Langston JW. Role of nitric oxide in methamphetamine neurotoxicity: protection by 7-nitroindazole, an inhibitor of neuronal nitric oxide synthase. *J Neurochem.* 1996; 67(6):2443–2450. [PubMed: 8931477]

- Figueredo-Cardenas G, Morello M, Sancesario G, Bernardi G, Reiner A. Colocalization of somatostatin, neuropeptide Y, neuronal nitric oxide synthase and NADPH-diaphorase in striatal interneurons in rats. *Brain Res.* 1996; 735(2):317–324. 0006-8993(96) 00801-3 [pii]. [PubMed: 8911672]
- Friedman SD, Castaneda E, Hodge GK. Long-term monoamine depletion, differential recovery, and subtle behavioral impairment following methamphetamine-induced neurotoxicity. *Pharmacol Biochem Behav.* 1998; 61(1):35–44. [PubMed: 9715805]
- Friend DM, Son JH, Keefe KA, Fricks-Gleason AN. Expression and activity of nitric oxide synthase isoforms in methamphetamine-induced striatal dopamine toxicity. *J Pharmacol Exp Ther.* 2013; 344(2):511–521. doi:10.1124/jpet.112.199745. [PubMed: 23230214]
- Fujiyama F, Masuko S. Association of dopaminergic terminals and neurons releasing nitric oxide in the rat striatum: an electron microscopic study using NADPH-diaphorase histochemistry and tyrosine hydroxylase immunohistochemistry. *Brain Res Bull.* 1996; 40(2):121–127. [PubMed: 8724430]
- Gerfen CR. Substance P (neurokinin-1) receptor mRNA is selectively expressed in cholinergic neurons in the striatum and basal forebrain. *Brain Res.* 1991; 556(1):165–170. [PubMed: 1718557]
- Hotchkiss AJ, Morgan ME, Gibb JW. The long-term effects of multiple doses of methamphetamine on neostriatal tryptophan hydroxylase, tyrosine hydroxylase, choline acetyltransferase and glutamate decarboxylase activities. *Life Sci.* 1979; 25(16):1373–1378. [PubMed: 42834]
- Howard CD, Keefe KA, Garriss PA, Daberkow DP. Methamphetamine neurotoxicity decreases phasic, but not tonic, dopaminergic signaling in the rat striatum. *J Neurochem.* 2011; 118(4):668–676. doi:10.1111/j.1471-4159.2011.07342.x. [PubMed: 21668447]
- Hwang O, Baker H, Gross S, Joh TH. Localization of GTP cyclohydrolase in monoaminergic but not nitric oxide-producing cells. *Synapse.* 1998; 28(2):140–153. doi:10.1002/(SICI)1098-2396(199802)28:2<140::AID-SYN4>3.0.CO;2-B. [PubMed: 9450514]
- Imam SZ, Crow JP, Newport GD, Islam F, Slikker W Jr, Ali SF. Methamphetamine generates peroxynitrite and produces dopaminergic neurotoxicity in mice: protective effects of peroxynitrite decomposition catalyst. *Brain Res.* 1999; 837(1-2):15–21. [PubMed: 10433983]
- Imam SZ, Newport GD, Itzhak Y, Cadet JL, Islam F, Slikker W Jr, Ali SF. Peroxynitrite plays a role in methamphetamine-induced dopaminergic neurotoxicity: evidence from mice lacking neuronal nitric oxide synthase gene or overexpressing copper-zinc superoxide dismutase. *J Neurochem.* 2001; 76(3):745–749. [PubMed: 11158245]
- Itzhak Y, Ali SF. The neuronal nitric oxide synthase inhibitor, 7-nitroindazole, protects against methamphetamine-induced neurotoxicity in vivo. *J Neurochem.* 1996; 67(4):1770–1773. [PubMed: 8858965]
- Itzhak Y, Gandia C, Huang PL, Ali SF. Resistance of neuronal nitric oxide synthase-deficient mice to methamphetamine-induced dopaminergic neurotoxicity. *J Pharmacol Exp Ther.* 1998; 284(3):1040–1047. [PubMed: 9495865]
- Itzhak Y, Martin JL, Ali SF. nNOS inhibitors attenuate methamphetamine-induced dopaminergic neurotoxicity but not hyperthermia in mice. *NeuroReport.* 2000; 11(13):2943–2946. [PubMed: 11006970]
- Itzhak Y, Anderson KL, Ali SF. Differential response of nNOS knockout mice to MDMA (“ecstasy”) and methamphetamine-induced psychomotor sensitization and neurotoxicity. *Ann N Y Acad Sci.* 2004; 1025:119–128. doi:10.1196/annals.1316.015. [PubMed: 15542708]
- Li JL, Wang D, Kaneko T, Shigemoto R, Nomura S, Mizuno N. Relationship between neurokinin-1 receptor and substance P in the striatum: light and electron microscopic immunohistochemical study in the rat. *J Comp Neurol.* 2000; 418(2):156–163. doi:10.1002/(SICI)1096-9861(20000306)418:2<156::AID-CNE3>3.0.CO;2-Z. [PubMed: 10701441]
- Li JL, Kaneko T, Mizuno N. Colocalization of neuronal nitric oxide synthase and neurokinin-1 receptor in striatal interneurons in the rat. *Neurosci Lett.* 2001; 310(2-3):109–112. [PubMed: 11585579]
- Maxwell JC. Emerging research on methamphetamine. *Curr Opin Psychiatry.* 2005; 18(3):235–242. doi:10.1097/01.yco.0000165592.52811.8400001504-200505000-00004. [PubMed: 16639146]

- McCann UD, Wong DF, Yokoi F, Villemagne V, Dannals RF, Ricaurte GA. Reduced striatal dopamine transporter density in abstinent methamphetamine and methcathinone users: evidence from positron emission tomography studies with [¹¹C]WIN-35,428. *J Neurosci*. 1998; 18(20): 8417–8422. [PubMed: 9763484]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. 5th edn.. Elsevier Academic; Burlington: 2005.
- Rushlow W, Flumerfelt BA, Naus CC. Colocalization of somatostatin, neuropeptide Y, and NADPH-diaphorase in the caudate-putamen of the rat. *J Comp Neurol*. 1995; 351(4):499–508. doi:10.1002/cne.903510403. [PubMed: 7721980]
- Saka E, Iadarola M, Fitzgerald DJ, Graybiel AM. Local circuit neurons in the striatum regulate neural and behavioral responses to dopaminergic stimulation. *Proc Natl Acad Sci U S A*. 2002; 99(13): 9004–9009. doi:10.1073/pnas.132212499132212499. [PubMed: 12060713]
- Sammur S, Dec A, Mitchell D, Linardakis J, Ortiguera M, West AR. Phasic dopaminergic transmission increases NO efflux in the rat dorsal striatum via a neuronal NOS and a dopamine D(1/5) receptor-dependent mechanism. *Neuropsychopharmacology*. 2006; 31(3):493–505. doi:10.1038/sj.npp.1300826. [PubMed: 16012530]
- Sanchez V, Zeini M, Camarero J, O'Shea E, Bosca L, Green AR, Colado MI. The nNOS inhibitor, AR-R17477AR, prevents the loss of NF68 immunoreactivity induced by methamphetamine in the mouse striatum. *J Neurochem*. 2003; 85(2):515–524. [PubMed: 12675928]
- Santos RM, Lourenco CF, Gerhardt GA, Cadenas E, Laranjinha J, Barbosa RM. Evidence for a pathway that facilitates nitric oxide diffusion in the brain. *Neurochem Int*. 2011; 59(1):90–96. doi: 10.1016/j.neuint.2011.05.016. [PubMed: 21672575]
- Tata DA, Raudensky J, Yamamoto BK. Augmentation of methamphetamine-induced toxicity in the rat striatum by unpredictable stress: contribution of enhanced hyperthermia. *The European journal of neuroscience*. 2007; 26(3):739–748. doi:10.1111/j.1460-9568.2007.05688.x. [PubMed: 17686046]
- Vincent SR, Johansson O, Hokfelt T, Skirboll L, Elde RP, Terenius L, Kimmel J, Goldstein M. NADPH-diaphorase: a selective histochemical marker for striatal neurons containing both somatostatin- and avian pancreatic polypeptide (APP)-like immunoreactivities. *J Comp Neurol*. 1983; 217(3):252–263. doi:10.1002/cne.902170303. [PubMed: 6136531]
- Volkow ND, Chang L, Wang GJ, Fowler JS, Franceschi D, Sedler M, Gatley SJ, Miller E, Hitzemann R, Ding YS, Logan J. Loss of dopamine transporters in methamphetamine abusers recovers with protracted abstinence. *J Neurosci*. 2001a; 21(23):9414–9418. [PubMed: 11717374]
- Volkow ND, Chang L, Wang GJ, Fowler JS, Leonido-Yee M, Franceschi D, Sedler MJ, Gatley SJ, Hitzemann R, Ding YS, Logan J, Wong C, Miller EN. Association of dopamine transporter reduction with psychomotor impairment in methamphetamine abusers. *Am J Psychiatry*. 2001b; 158(3):377–382. [PubMed: 11229977]
- Wiley RG, Lappi DA. Targeting neurokinin-1 receptor-expressing neurons with [Sar⁹, Met^(O2)]¹¹ substance P-saporin. *Neurosci Lett*. 1999; 277(1):1–4. [PubMed: 10643883]
- Woolverton WL, Ricaurte GA, Forno LS, Seiden LS. Long-term effects of chronic methamphetamine administration in rhesus monkeys. *Brain Res*. 1989; 486(1):73–78. [PubMed: 2720435]
- Yu J, Cadet JL, Angulo JA. Neurokinin-1 (NK-1) receptor antagonists abrogate methamphetamine-induced striatal dopaminergic neurotoxicity in the murine brain. *J Neurochem*. 2002; 83(3):613–622. [PubMed: 12390523]
- Zhu JP, Xu W, Angulo JA. Distinct mechanisms mediating methamphetamine-induced neuronal apoptosis and dopamine terminal damage share the neuropeptide substance p in the striatum of mice. *Ann N Y Acad Sci*. 2006a; 1074:135–148. doi:10.1196/annals.1369.013. [PubMed: 17105911]
- Zhu JP, Xu W, Angulo N, Angulo JA. Methamphetamine-induced striatal apoptosis in the mouse brain: comparison of a binge to an acute bolus drug administration. *Neurotoxicology*. 2006b; 27(1):131–136. doi:10.1016/j.neuro.2005.05.014. [PubMed: 16165214]
- Zhu J, Xu W, Wang J, Ali SF, Angulo JA. The neurokinin-1 receptor modulates the methamphetamine-induced striatal apoptosis and nitric oxide formation in mice. *J Neurochem*. 2009; 111(3):656–668. doi:10.1111/j.1471-4159.2009.06330.x. [PubMed: 19682209]

Fig. 1.

Body temperatures during METH administration. **a** Rectal temperatures over time for each of the four treatment groups sacrificed 7 days after the last injection: Blank-SAP/Saline ($n = 2$), Blank-SAP/METH ($n = 2$), SSP-SAP/Saline ($n = 2$), SSP-SAP/METH ($n = 4$). Rats were given intrastratial infusions of Blank-SAP (control) or SSP-SAP (toxin) into one striatum followed by systemic administration of saline (4×1 ml/kg) or ($\pm$)-METH (4×7.5 mg/kg, s.c. at 2-hr intervals) 3 weeks later. * METH-treated rats significantly greater than saline-treated rats at each indicated time point, $p < 0.05$. **b** Rats were treated as described above for (a). Shown are rectal temperatures over time for each of the four treatment groups sacrificed 1 h after the last injection: Blank-SAP/Saline ($n = 6$), Blank-SAP/METH ($n = 8$), SSP-SAP/Saline ($n = 6$), SSP-SAP/METH ($n = 9$). * METH-treated rats significantly greater than saline-treated rats at each indicated time point, $p < 0.05$. Arrows indicate injection times

Fig. 2.

Representative photomicrographs from an animal that received SSP-SAP into the infused striatum followed by a systemic injection of saline three weeks later. **a** NADPH diaphorase histochemistry reveals a clear loss of nNOS-containing interneurons in the SSP-SAP-infused hemisphere. **b** NeuN IHC performed on an adjacent section confirms the specificity of the SSP-SAP-induced lesion. No gross loss of neurons was evident in the infused striatum other than mechanical damage from the cannulae. **c** Data are NADPH diaphorase histochemical staining in the striata of rats sacrificed 7 days after treatment with METH or saline (4 weeks after infusion of Blank-SAP or SSP-SAP into one striatum), and are represented as number of NADPH diaphorase-positive cell bodies per mm² (mean $\pm$ SEM). * Significant main effect of pretreatment, $p < 0.05$

Fig. 3. Effects of SSP-SAP infusions on METH-induced loss of DAT immunohistochemical staining in the striatum. Rats were treated as described in the legend to Fig. 1 and in the Methods. Data are DAT immunohistochemical staining in the striata of animals sacrificed 7 days after treatment with METH or saline (4 weeks after infusion of Blank-SAP or SSP-SAP into one striatum), and are represented as average gray values (mean $\pm$ SEM; arbitrary units). * Significant main effect of treatment, $p < 0.05$

Fig. 4. Effects of SSP-SAP infusions on NADPH diaphorase histochemical staining in the striatum. Rats were treated as described in the legend to Fig. 1 and in the Methods. Data are NADPH diaphorase histochemical staining in the striata of animals sacrificed 1 h after treatment with METH or saline, and are represented as percent area of the imaged field with NADPH diaphorase histochemical signal (mean $\pm$ SEM). * Significant differences, $p < 0.05$

Fig. 5.

Effects of SSP-SAP infusions on nitrotyrosine (NT) immunohistochemical staining in the striatum. Rats were treated as described in the legend to Fig. 1 and in the Methods. Data are NT immunohistochemical staining in the striata of animals sacrificed 1 h after treatment with METH or saline, and are represented as average gray values (mean $\pm$ SEM; arbitrary units). * Significant main effect of treatment, $p < 0.05$