Contrasting Effects of the Neuropeptides Substance P, Somatostatin, and Neuropeptide Y on the Methamphetamine-Induced Production of Striatal Nitric Oxide in Mice

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Received 21 June 2012; Accepted 27 July 2012

Abstract Several laboratories have shown that methamphetamine (METH) neurotoxicity is associated with increases of nitric oxide (NO) production in striatal tissue and blockade of NO production protects from METH. Because substance P modulates NO production, we tested the hypothesis that intrinsic striatal neuropeptides such as somatostatin and neuropeptide Y (NPY) modulate striatal NO production in the presence of METH. To that end, METH (30 mg/kg, IP) was injected into adult male mice alone or in combination with pharmacological agonists or antagonists of the neurokinin-1 (substance P), somatostatin or NPY receptors and 3-nitrotyrosine (an indirect index of NO production) was assessed utilizing HPLC or a histological method. Pre-treatment with the systemic neurokinin-1 receptor antagonist WIN-51,708 significantly attenuated the METH-induced production of striatal 3-NT measured at two hours post-METH. Conversely, intrastriatal injection of NPY1 or 2 receptor agonists inhibited the METH-induced production of striatal 3-NT. Similarly, intrastriatal infusion of the somatostatin receptor agonist octreotide attenuated the METH-induced striatal production of 3-NT. Taken together, our results suggest the hypothesis that the neuropeptide substance P is pro-damage while the neuropeptides somatostatin and NPY are anti-damage in the presence of METH by targeting the production of NO.

Keywords methamphetamine; substance P; somatostatin; neuropeptide Y; nitric oxide; striatum

1 Introduction

Methamphetamine (METH) is a highly addictive drug that induces excessive overflow of dopamine and other catecholamines especially in the striatum [27,47]. Studies performed in the 1970s demonstrated that METH causes damage in the brains of cats and rhesus monkeys [14,45]. In rodents, exposure to METH causes reduction of dopamine transporter sites [16,55], decreased vesicular transporter function [15], decreased tissue dopamine content [55], and degeneration of striatal dopamine terminals [43]. Additionally, METH induces the apoptosis of neurons in the striatum, cortex, and olfactory bulb [11,10,13,59]. Imaging studies of the human brain demonstrate that METH induces deficits of striatal dopamine transporter [54] microglial activation [46] and grey matter deficits [51]. These studies unequivocally establish the severity of the METH-induced damage to the human brain. Nitric oxide (NO) is a diffusible gaseous messenger that has been shown to participate in various physiological aspects of synaptic transmission in the central nervous system [48]. NO is synthesized by nitric oxide synthase (NOS) of which three isoforms have been identified: neuronal, inducible, and endothelial [5,35]. The neuronal isoform is expressed throughout the brain with moderate levels of expression found in the basal ganglia, namely the striatum [7,53]. In the striatum, NOS is expressed by GABAergic interneurons while astrocytes express all three isoforms of the enzyme [5,35]. The neuronal isoform is expressed throughout the brain with moderate levels of expression found in the basal ganglia, namely the striatum [7,53]. In the striatum, NOS is expressed by GABAergic interneurons comprising less than 1% of all striatal neurons and co-expressing the neuropeptides somatostatin and NPY [28,30,49]. Excessive signaling by NO may contribute to neurodegeneration in the brain by exacerbating oxidative stress via the generation of reactive free radicals and other harmful nitrosative derivatives [4,8,25]. A causal relationship between METH neurotoxicity and NO has come from pharmacological and genetic studies demonstrating that inhibition of NOS or deletion of the gene for this enzyme in mice protects the striatum from METH [23,26]. Moreover, transgenic mice that overexpress copper/zinc superoxide dismutase, an enzyme that neutralizes superoxide radicals due to NO production, show attenuated responses to the neurotoxic actions of METH [9]. Striatal neuropeptides are in an ideal
position to modulate the METH-induced production of NO and some reports suggest that the neuropeptide substance P modulates NO production in peripheral tissues [38,40]. In the present study, we investigated the hypothesis that the striatal neuropeptides substance P, somatostatin, and NPY modulate the METH-induced production of NO.

2 Materials and methods

2.1 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 Hunter College animal facility is certified by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Institute for Cancer Research (ICR) male mice (Taconic, Germantown, NY, USA) between 11 to 12 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.

2.2 Drug preparation and treatment

(+) -Methamphetamine hydrochloride (Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS), pH 7.4, and injected intraperitoneally (IP) at a dose of 30 mg/kg of body weight in a volume of 200 μL. The non-peptide neurokinin-1 receptor antagonist WIN-51,708 (17-hydroxy-17-ethynyl-5-androstano[3,2,β]pyrimido[1,2-α]benzimidazole) (RBI/Sigma, Natick, MA, USA) was dissolved in PBS. The mobile phase was 50 mM NaAc, pH 4.8. HPLC was performed under isocratic conditions.

2.3 HPLC-EC detection of 3-NT and tyrosine concentration

Determination of 3-NT and tyrosine in mouse striatum was performed by HPLC-Coularrly electrochemical detection method [24]. Briefly, each striatal tissue was dissected out and sonicated in 400 μL of 10 mM sodium acetate NaOAc, pH 6.5. A 25 μL aliquot of the homogenate was used to determine protein concentration (BCA method).

The remaining homogenate was centrifuged at 14,000 rpm (Eppendorf 5403 centrifuge) for 10 minutes at 4 °C. The supernatant was removed and treated with 100 mL of 1 mg/mL pronase for 18 hours at 50 °C. Enzymatic digests were then treated with 0.5 mL of 10% TCA and centrifuged at 14,000 rpm for 10 minutes at 4 °C. Supernatants were then washed through a 0.2 μm PVDF filter before injection onto the HPLC instrument. Samples were analyzed on an ESA (Cambridge, MA, USA) CoulArray HPLC equipped with 8 electrochemical channels using platinum electrodes arranged in line and set to increasing specified potentials [channel (potential): 1 (320 mV); 2 (450 mV); 3 (490 mV); 4 (610 mV); 5 (670 mV); 6 (870 mV); 7 (900 mV); 8 (930 mV)]. The analytical column was a Luna C18 column (3 micron, 2.1 × 150 mm, Phenomenex Co., Torrance, CA, USA). The mobile phase was 50 mM NaAc, 5% (v/v) methanol, pH 4.8. HPLC was performed under isocratic conditions. 3-NT and tyrosine were quantified relative to known standards. 3-NT values were represented as 3-NT per 100 tyrosines.

2.4 3-nitrotyrosine immunohistochemistry

For sacrifice and perfusion, animals were fully anesthetized with ketamine (100 mg/kg) and acepromazine (3 mg/kg), and perfused through the heart with 20 mL of PBS followed by 20 mL of 4% paraformaldehyde. The brains were post-fixed overnight in the fixative at 4 °C followed by 30% sucrose solution over 24 hours at 4 °C for cryo-protection. The brains were frozen at −80 °C until used. Sectioning and staining was carried out by the free-floating method. Striatal 30 μm coronal sections were cut on a cryostat at −20 °C. The sections were collected serially between Bregma 0.02 mm and 1.4 mm, with each tissue sample separated from the proceeding sample in the series by 180 μm. Thus, each sample well represents an entire striatum. They were then stored in a solution of 30% glycerin, 30% ethylene glycol, 40% PBS at −20 °C until used. The sections were then rinsed in PBS and incubated 3X for 10 minutes in 10 mM citric acid at 65 °C. They were washed with PBS 3X for 5 minutes each followed by incubation in the M.O.M (mouse on mouse) kit blocking reagent (Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. Then they were incubated for 10 minutes at room temperature in M.O.M kit diluent solution, proceeded by incubation overnight at 4 °C in mouse monoclonal anti-3-NT (1:200, Santa Cruz Biotech, Santa Cruz, CA, USA) in M.O.M diluent buffer. The next day they were washed with PBS 3X for 5 minutes each. Sections were incubated in a solution of 5% goat serum in 0.2% triton PBS for 1 hour at room temperature. Then another 1 hour at room temperature in Cy3 goat anti-mouse (1:500, Millipore, Temecula, CA, USA) in 1% goat serum and 0.2% triton PBS. They were then washed an additional 3X with PBS for 5 minutes each,
mounted and coverslipped using Vectashield fluorescent hardset mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The slides were imaged with a Leica TCS SP2 scanning confocal microscope (Leica Microsystems, Germany) and quantified using the Leica imaging software.

2.5 Measurement of 3-NT staining intensity

From each slide at least 4 out of 6 sections were selected for imaging with the Leica scanning confocal (Leica Microsystems, Germany). All sections selected must have a visible needle tract to ensure that the effect observed is due to the injected solution. Per section, 4 areas were chosen within each striatum. The regions chosen were adjacent to each side of the needle tip but avoiding the visible needle damage. They were then scanned only once to prevent quenching at 63X with preset parameters that give the most resolved image in the baseline condition. Hardware and software settings were maintained using the same settings for all images scanned thereafter. The overall settings were as follows: area scanned was 56889.33 mm², frame average of 12, line average of 1, pinhole of 149.91 μm, zoom factor of 1.00, and a scan speed of 400 Hz. The digitized image is 512 × 512 pixels and an 8 bit grey resolution with a range in intensity of 0–255. Analysis of intensity was done using the Leica confocal software. The background produced by nonspecific binding was removed using the baseline correction feature (eliminate autofluorescence) in the image process option. Then using the histogram feature under the quantify tab we were able to get the mean energy of each image, which represents the intensity of the image. The average of the mean energy of the animal was obtained and then statistically analyzed.

2.6 Statistical analysis

Statistical comparisons were performed from mean ± SEM. Differences between groups were analyzed by ANOVA followed by post hoc comparison using Fisher’s protected least significance test. Differences between two groups were analyzed by Student’s t-test. The significance criterion was set at $P = .05$.

3 Results

We investigated the participation of select striatal neuropeptides on the METH-induced production of NO by combining METH treatment (30 mg/kg, IP) with specific agonists or antagonists of neuropeptide receptors given via systemic injection or intrastriatal infusion. HPLC was utilized to assess NO production by measuring 3-NT levels in striatal tissue extracts. In addition, 3-NT fluorescence intensity was quantified in striatal tissue sections by confocal microscopy.

A systemic injection of METH (30 mg/kg, IP) increased striatal 3-NT levels 3-fold relative to vehicle injected control male mice (Figure 1). Interestingly, a systemic injection of the non-peptide neurokinin-1 receptor antagonist WIN-51,708 (5 mg/kg, IP) that crosses the blood-brain barrier 30 minutes prior to METH significantly attenuated the METH-induced augmentation of striatal 3-NT (an indirect index of NO synthesis). The data were analyzed by ANOVA. Data were normalized to control group (vehicle/saline). *$P < .0001$.

Figure 1: Pretreatment with the neurokinin-1 receptor antagonist WIN-51,708 attenuated the METH-induced increase of striatal 3-nitrotyrosine (3-NT). The mice ($n = 8$) received an injection of WIN-51,708 (WIN, 5 mg/kg, IP) 30 minutes prior to METH (30 mg/kg, IP) and the animals were sacrificed two hours post-METH. 3-NT levels were determined by HPLC from one striatum. Note that the neurokinin-1 receptor antagonist significantly attenuated the METH-induced augmentation of striatal 3-NT (an indirect index of NO synthesis). The data were analyzed by ANOVA. Data were normalized to control group (vehicle/saline). *$P < .0001$.

In contrast to substance P and in concordance with neuropeptide Y, the intrastratal infusion of the somatostatin...
agonist octreotide significantly inhibited the METH-induced production of striatal 3-NT at six hours post-METH (30 mg/kg, IP) injection (Figure 3). The contralateral striatum served as control and received 1.0 μL of vehicle. In a separate group of mice it was determined that the intrastriatal infusion of octreotide alone failed to affect basal levels of striatal 3-NT as assessed by confocal microscopy (Figure 3).

4 Discussion

Our data demonstrate that the striatal neuropeptides substance P, somatostatin, and NPY modulate the production of NO in the presence of METH. The striatal interneurons expressing NOS comprise approximately 1% of all striatal neurons [44]. However, their axons arborize and course for as long as 1 mm and their dendrites give rise to an arborization of up to 600 μm in diameter [12]. Excessive NO signaling can be detrimental because most electrophysiological studies demonstrate that NO signaling facilitates glutamate transmission at corticostriatal synapses [37]. Below we discuss the role of striatal neuropeptides in modulating NO production in the presence of METH.

The neuropeptide substance P is produced exclusively by the striatonigral projection neurons [18]. Substance P signals through the neurokinin-1 receptor, a G-protein coupled receptor that mobilizes intracellular calcium [33]. In the striatum, the neurokinin-1 receptor is expressed by cholinergic and somatostatin/NOS/NPY interneurons [29, 31]. Striatal somatostatin-expressing interneurons form synaptic contacts with projection neurons [34] and the latter, reciprocally, form synaptic contacts with this interneuron via intrastriatal axonal branches [31,32]. Studies from our laboratory suggest that substance P is released in the presence of METH in the striatum because minutes after a
systemic bolus injection of METH the neurokinin-1 receptor is internalized into endosomes primarily in the somatostatin/NOS/NPY interneurons [56]. When intracellular calcium levels increase, calmodulin is activated which in turn activates calcium-calmodulin-dependent kinase II, the enzyme that phosphorylates and activates the NOS [41]. We have recently shown that the neurokinin-1 receptor agonist GR-73632 increases striatal NO production via a mechanism requiring calmodulin [57]. Our results are consistent with the hypothesis that striatal substance P is released in the presence of METH resulting in the activation of NOS via a mechanism involving the neurokinin-1 receptor.

NPY is a 36-amino acid neuropeptide expressed in various brain regions and in the striatum by some interneurons that co-express somatostatin and NOS [1,28]. NPY signals via G-protein-coupled receptors (Y1–Y6) that inhibit adenylate cyclase and affect the mobilization of intracellular calcium [39]. This neuropeptide serves many physiological functions in the brain, most notably the regulation of appetite, memory formation, anxiety, and depression [19]. The central administration of NPY affects tests that are sensitive to anxiety and depression [20]. Moreover, various antidepressant treatments induce an augmentation of NPY concentration in different brain regions [21]. Recent reports suggest a role for NPY in the actions of METH in the striatum. For example, METH given acutely or multiple high dose injections increased the number of cells expressing the messenger RNA for NPY in all striatal regions via a mechanism requiring the dopamine D1 receptor [22]. Moreover, the intraventricular administration of NPY blocked the striatal METH-induced apoptosis of some striatal neurons. Additionally, the same group demonstrated that NPY knockout mice were more sensitive than wild type mice to the striatal cell loss induced by METH [50]. Our results demonstrating that NPY agonists attenuate the METH-induced elevation of NO suggest that the neuroprotective effects of NPY in the presence of METH are due to the reduction of NO signaling in the striatum. NPY may inhibit the synthesis of NO via activation of a phosphatase that interferes with the biochemical activation of NOS. It needs to be demonstrated how NPY inhibits NOS, that is, directly via an autocrine mechanism or via polysynaptic actions.

Somatostatin is a neuropeptide that was discovered as a factor that inhibited the release of growth hormone from the anterior lobe of the pituitary gland [6]. Somatostatin signals through six receptor subtypes utilizing the G-protein Gi/Go and inhibition of adenylate cyclase and calcium channels [36,42]. Somatostatin modulates classical neurotransmission, inhibits the excitability of hippocampal neurons, displays anti-convulsant properties in animal models of seizures [52,58]. In addition, it has been shown to attenuate the NMDA-induced necrotic cell death of cortical neurons, suggesting that somatostatin inhibits calcium-mediated neurotoxicity [17]. In the striatum, somatostatin is expressed by a medium aspiny interneuron that also expresses NPY and NOS [28]. In the present study, we microinjected into the striatum the somatostatin analog octreotide because it is a long-lasting somatostatin analog and is about 75 times more potent than somatostatin [3]. We observed that octreotide inhibited the METH-induced production of NO in a dose-dependent fashion. It is conceivable that somatostatin exerted this effect via the inhibition of calcium channels on the somatostatin/NOS/NPY interneurons through an autocrine mechanism. More work is needed to elucidate the mechanism by which somatostatin inhibits the METH-induced production of NO in the striatum.

Taken together, our results demonstrate that in the presence of METH there are two kinds of neuropeptides in the striatum: those that attenuate NO production (somatostatin and NPY) and those that enhance NO production (substance P). It is plausible to hypothesize that under normal conditions these neuropeptides modulate the induction of NO production to prevent excessive fluctuations in the levels of this second messenger. More work is needed to determine how these neuropeptides mediate their effects on NO production in the striatum and if other neuropeptides participate in this mode of regulation.

Acknowledgments This work was supported by R01 DA020142 from the National Institute on Drug Abuse to JAA. Support for infrastructure came from a grant from the National Center for Research Resources (G12 RR003037) and the National Institute on Minority Health Disparities (8 G12 MD007599) awarded to Hunter College by the NIH.

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