Published in final edited form as:

Ann N Y Acad Sci. 2008 October ; 1139: 164–171. doi:10.1196/annals.1432.001.

Connection Between the Striatal Neurokinin-1 Receptor and Nitric Oxide Formation During Methamphetamine Exposure

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Abstract

Methamphetamine (METH) is a widely used club drug that produces neural damage in the brain including the loss of some neurons. The METH-induced striatal neuronal loss was attenuated by pre-treatment with the neurokinin-1 receptor antagonist WIN-51,708 in mice. We have observed using a histological method the internalization of the neurokinin-1 receptor into endosomes in the striatal somatostatin/NPY/nitric oxide synthase interneurons. To investigate the role of this interneuron in the striatal cell death induced by METH, we assessed by immunohistochemistry the number of striatal nitric oxide synthase-positive neurons in the presence of METH at 8 and 16 hours after systemic injection of a bolus of METH (30 mg/kg, i.p.). We found the number of striatal nitric oxide synthase-positive neurons unchanged at these time points after METH. In a separate experiment we measured the levels of striatal 3-nitrotyrosine (3-NT) by HPLC (high-pressure liquid chromatography) as an indirect index of nitric oxide synthesis. METH increased the levels of 3-nitrotyrosine in the striatum and this increase was significantly attenuated by pre-treatment with a selective neurokinin-1 receptor antagonist. These observations suggest a causal relationship between the neurokinin-1 receptor and the activation of neuronal nitric oxide synthase that warrants further investigation.

Keywords

methamphetamine; apoptosis; 3-nitrotyrosine; nitric oxide synthase; neurokinin-1 receptor; striatum

Introduction

Methamphetamine (METH) is a widely used drug of abuse in the USA. For over 30 years it has been recognized that METH induces excessive release of dopamine which becomes depleted with time¹. METH-induced neurotoxicity has also been shown to include considerable loss of dopaminergic terminals in the striatum, as measured by loss of the terminal markers tyrosine hydroxylase and dopamine transporters^{2,3}. More recent studies have reported striatal and cortical neuronal cell death in animals given METH^{4,5,6,7}. An important concern stems from what appears from studies on animals and humans to be permanent METH-induced changes. Even after 3 years of abstinence, PET scans reveal dopamine transporter loss in METH users^{8,9,10,11}, and MRI suggests METH-induced cell

death¹². Taken together, these findings strongly point to permanent brain damage with METH use, leaving a profound burden on both the user and society at large.

A large body of evidence demonstrates a causal connection between METH-induced neural damage and oxidative stress¹³. METH-induced increases of nitric oxide are damaging to striatal dopaminergic terminals because pharmacological inhibition of neuronal nitric oxide synthase or deletion of the gene for this enzyme in mice protects the striatum from METH^{14,15}. Moreover, agents that block the synthesis of nitric oxide also attenuate METH-induced loss of mesencephalic neurons *in vitro*, suggesting that nitric oxide synthesis may be causally related to the neurotoxic effects of METH¹⁶. Further evidence implicating a role for nitric oxide comes from experiments with transgenic mice that over express copper/zinc superoxide dismutase (CuZnSOD). Nitric oxide synthesis leads to accumulation of superoxide radicals that are neutralized by CuZnSOD. Homozygous transgenic mice over expressing CuZnSOD have 5.7-fold and heterozygous mice have 2.5-fold greater activity of this enzyme than do wild type mice. Heterozygous mice are less sensitive to a dose of 2.5 mg/kg of METH than wild type and the homozygous are nearly resistant to METH^{17,18,19}.

Our laboratory has demonstrated that METH-induced striatal injury can be attenuated by pre-treatment with pharmacological agents that block the neurokinin-1 receptor^{20,21}. In order to extend this observation, in the present study we have investigated the connection between the striatal neurokinin-1 receptor (substance P is the natural ligand) and the synthesis of nitric oxide in the striatum of mice. Our results demonstrate a connection between METH-induced nitric oxide production and the substance P receptor in the striatum.

Methods

Animals

Male ICR mice (Taconic, Germantown, NY) between 8 to 9 weeks of age were housed individually on a 12-h light/dark cycle with food and water available *ad libitum*. The mice were habituated for two weeks prior to commencement of intraperitoneal (i.p.) drug administration. 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 at Hunter College of the City University of New York.

Drug administration

METH (Sigma) was dissolved in saline and injected i.p. at a dose of 30 mg/kg (injection volume of 0.2 mL). The neurokinin-1 receptor antagonist WIN-51,708 (Sigma) was dissolved in vehicle (45% w/v 2-hydroxypropyl- β -cyclodextrin from Sigma) and injected (5 mg/kg, i.p.) 30 minutes prior to the injection of METH.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling)

Histochemistry

The method used was as previously described by our laboratory²². Freshly frozen 20 μ m coronal sections were taken between bregma 0.38 ± 0.1 mm and fixed in 4% paraformaldehyde for 30 minutes. After washing with phosphate-buffered saline, pH 7.4 (PBS), sections were immersed in 0.4% Triton-X-100 in PBS for five to ten minutes at 70 °C. Sections were then washed and TUNEL reactions (Roche Applied Science, Indianapolis, IN) were applied directly onto sections and incubated for one hour in a humidified chamber. After TUNEL staining, sections were counterstained with DAPI. Stained sections were then

washed in PBS and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA). Images were taken using the Leica TCS SP2 spectral scanning confocal microscope.

Immunohistochemistry

The animals were fully anesthetized with a mixture of Ketamine (100 mg/kg)/Acepromazine (1 mg/kg) and were perfused intracardially with 20 mL of 0.01M PBS followed by an equivalent volume of 4% paraformaldehyde. The brains were post-fixed overnight in the same fixative at 4°C followed by 20% sucrose over 24 hours at 4°C for cryoprotection. 20 µm sections were cut in the cryostat at -20°C and stored in a solution of 30% glycerin in ethylene glycol. The sections were washed 3× for 5 minutes each in 0.1M PBS and incubated in a solution of 3% H₂O₂ for 30 minutes. The sections were then blocked with blocking buffer (0.1M PBS with 0.3% Triton X-100, 10% rabbit serum) for one hour. The sections were incubated with rabbit anti-neuronal nitric oxide synthase (1:1000, Chemicon) in blocking buffer at 4°C overnight. The tissue was then treated with VECTASTAIN ABC Kit (Vector Labs) and 3,3'-diaminobenzidine substrate kit according to the manufacturer's instructions. For immunohistochemical detection of the neurokinin-1 receptor, the tissue was incubated with a rabbit anti-neurokinin-1 receptor antibody (1:500, Chemicon) followed by sheep anti-rabbit secondary antibody conjugated to FITC (1:1000, Chemicon).

HPLC-EC detection of 3-NT and tyrosine concentration

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 passed 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 (930mV)]. The analytical column was a Luna C18 column (3 micron, 2.1× 150mm, Phenomenex Co., Torrance, CA). 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.

Cell counts

The method of cell counting was as described²³. All sections were taken from Bregma 0.38 ± 0.1mm²⁴. Nitric oxide synthase-positive cells were counted from 20-µm thick coronal sections within the area of 0.26 mm² in each of four quadrants in the striatum (dorsomedial, dorsolateral, ventromedial, ventrolateral). Cell counts for the four quadrants were pooled from each of four animals.

Statistical analysis

Analysis was performed from mean ± SEM. The differences between groups were analyzed first by ANOVA and then followed by post hoc comparison using Fisher's protected least significance test. The significance criterion was set at $p \leq 0.05$.

Results

One bolus injection of METH (30 mg/kg, i.p.) caused the loss of approximately 25% of striatal neurons in the mouse (Figure 1). This METH-induced loss of some striatal neurons was significantly attenuated when the animals were injected with the neurokinin-1 receptor antagonist WIN-51,708 (5 mg/kg, i.p.) 30 minutes prior to the METH injection (Figure 1). This observation suggests that METH induces excessive signaling through the striatal neurokinin-1 receptors.

To investigate the hypothesis that METH induces excessive signaling through the striatal neurokinin-1 receptors, we utilized a histological assay described by Mantyh *et al*25. The neuropeptide substance P forms a complex with its receptor that becomes internalized into endosomes. The endosomes can be visualized immunohistochemically using an antibody against the neurokinin-1 receptor. In the absence of substance P, or during basal levels of signaling when extracellular levels of the peptide are very low, the neurokinin-1 receptor was found located on the plasma membrane of the neuron (Figure 2, left panel). However, during excessive signaling the peptide-receptor complexes are internalized into endosomes that accumulate in the perinuclear region of the neuron (Figure 2, right panel). Exposure to METH caused the accumulation of endosomes staining positive for the neurokinin-1 receptor in the perinuclear space as early as 30 minutes after exposure to METH (Figure 2, right panel). This state of excessive signaling of the neurokinin-1 receptor induced by METH occurs predominantly in the somatostatin/NPY/NOS interneurons of the striatum (unpublished results). This observation suggests the hypothesis that signaling through the neurokinin-1 receptor in this interneuron may be linked to the activation of nitric oxide synthase.

We first investigated the possibility that METH would increase the number of striatal neurons expressing the enzyme nitric oxide synthase. To that end, we visualized the neurons expressing this enzyme by immunohistochemistry utilizing an antibody against neuronal nitric oxide synthase. We found that the number of striatal neurons expressing nitric oxide synthase remained unchanged up to 16 hours after METH (Figure 3A & B). In a separate experiment, we measured the levels of 3-NT (an indirect index of nitric oxide synthesis) at one and eight hours after METH by high-pressure liquid chromatography. At the latter time point, the levels of striatal 3-NT are approximately 7-fold higher after METH (Figure 4). Interestingly, pre-treatment with WIN-51,708 significantly attenuated the METH-induced elevation of 3-NT in the striatum (Figure 4), suggesting a relationship between the neurokinin-1 receptor and the production of nitric oxide in the presence of METH.

Discussion

Our data demonstrate that pharmacological blockade of the neurokinin-1 receptors with an antagonist that crosses the blood-brain barrier conferred protection from METH to some striatal neurons. This observation suggests that METH causes excessive signaling through the striatal neurokinin-1 receptors presumably due to excessive release of the neuropeptide substance P. The striatal neurokinin-1 receptors are expressed by the cholinergic and somatostatin/NPY/nitric oxide synthase interneurons26·27·28·29. Our results demonstrate the endocytosis of the neurokinin-1 receptors 30 minutes after the injection of METH suggesting the METH-induced release and signaling of substance P through its receptor. Moreover, our results demonstrate that METH induces endocytosis of the neurokinin-1 receptors primarily in the somatostatin/NPY/nitric oxide synthase interneurons (unpublished results). This METH-induced internalization of the neurokinin-1 receptors can be prevented by pre-treatment with an antagonist of this receptor (unpublished results). Overall, these

observations suggest the hypothesis that activation of the neurokinin-1 receptors in these interneurons may be connected to the activation of the enzyme nitric oxide synthase.

Although our results demonstrate that exposure to METH for up to 16 hours after the injection failed to increase the number of striatal neurons expressing nitric oxide synthase, this enzyme must be activated in the presence of METH since our results show that the levels of 3-NT, an indirect index of nitric oxide synthesis, were elevated approximately 7-fold eight hours after METH. Moreover, the METH-induced increase of 3-NT was significantly attenuated by pre-treatment with the neurokinin-1 receptor antagonist WIN-51,708. The somatostatin/NPY/nitric oxide synthase interneuron is the only striatal neuron refractory to the METH-induced apoptosis⁷. These observations suggest that the substance P receptor may contribute to the METH-induced injury of the striatum. Substance P may modulate the METH-induced release of glutamate in the striatum.

Glutamate causes cell death and METH has been shown to induce the overflow of glutamate in the striatum^{30,31}. The neuropeptide SP has been associated with cell death in the hippocampus. For example, the release of SP induces glutamate release in the hippocampus of the rodent brain³². Several laboratories have demonstrated that the release of glutamate triggers excitatory activities in neurons that ultimately lead to toxicity and cell death in various brain regions³³. The link between glutamate excitotoxicity and SP in the hippocampus is demonstrated by studies with genetic mutants lacking the preprotachykinin-A gene. Exposure to the excitotoxin kainate in wild type mice results in the death of neurons in the hippocampus, but mice lacking the preprotachykinin-A gene treated with kainate do not display hippocampal cell death³⁴. Our results are consistent with the hypothesis that excessive signaling by the neurokinin-1 receptors of the striatum represents one mechanism among others that contributes to the neural damage caused by METH in this brain region.

In summary, we demonstrate that pharmacological antagonism of the neurokinin-1 receptors conferred protection from the METH-induced apoptosis of some striatal neurons. METH induces excessive trafficking of the striatal neurokinin-1 receptors and augments the synthesis of nitric oxide, both of which can be attenuated by pharmacological blockade of the neurokinin-1 receptors. Work in progress in our laboratory is shedding light on the mechanism by which the neurokinin-1 receptors of the striatum help mediate the METH-induced injury of this brain region.

Acknowledgments

This work was supported by grant R01 DA020142 from the *National Institute on Drug Abuse* to JAA. Support for infrastructure came from the Research Centers in Minority Institutions grant awarded to Hunter College.

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Figure 1.

Pre-treatment with the neurokinin-1 receptor antagonist WIN-51,708 attenuated the METH-induced apoptosis of some striatal neurons. The mice (n=6) were given METH (30 mg/kg, i.p.) and were sacrificed 24 hours later. Apoptosis was visualized with the TUNEL assay. Note that pre-treatment with WIN-51,708 (5 mg/kg, i.p.) 30 minutes prior to METH significantly attenuated the METH-induced apoptosis. Symbols over the bars represent standard error of the mean values. * $p < 0.05$ (Student's t-test). V, vehicle; M, METH; W, WIN-51,708.

Figure 2.

METH induced the endocytosis of the striatal neurokinin-1 receptors. METH (30 mg/kg, i.p.) was injected and the mouse was sacrificed 30 minutes later. The neurokinin-1 receptor was visualized in dark field with confocal microscopy for greater contrast. Note that in the absence of METH the neurokinin-1 receptor was associated with the plasma membrane. Arrows show the location of two prominent endosomes. The METH-induced endocytosis of the neurokinin-1 receptor was observed in six out of six mice.

Figure 3. METH failed to increase the number of striatal neurons expressing nitric oxide synthase. The mice (n=6) received an injection of METH (30 mg/kg, i.p.) and were sacrificed at 8 or 16 hours after the injection. Nitric oxide synthase was visualized by immunohistochemistry. A) Representative micrographs from an animal treated with vehicle (left panel) or METH (right panel). Arrows indicate the position of representative neurons expressing nitric oxide synthase. B) The number of nitric oxide synthase-positive neurons was counted in four quadrants of the striatum as described in the “Methods.” The values from each quadrant were pooled. No difference was observed between control and METH groups at either 8 or 16 hours after METH. Symbols over the bars represent standard error of the mean values.

Figure 4.

The neurokinin-1 receptor antagonist WIN-51,708 attenuated the METH-induced production of striatal 3-NT. WIN-51,708 (5 mg/kg, i.p.) was given 30 minutes prior to METH (30 mg/kg, i.p.) and the mice (n=6) were sacrificed at 1 or 8 hours after the injection of METH. 3-NT levels were determined by high-pressure liquid chromatography. Symbols over the bars represent standard error of the mean values. *p<0.001, **p<0.005, !p<0.05 (Student's t-test).