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Modulation of Methamphetamine-induced Nitric Oxide Production by Neuropeptide Y in the Murine Striatum

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Abstract

Methamphetamine (METH) is a potent stimulant that induces both acute and long-lasting neurochemical changes in the brain including neuronal cell loss. Our laboratory demonstrated that the neuropeptide substance P enhances the striatal METH-induced production of nitric oxide (NO). In order to better understand the role of the striatal neuropeptides on the METH-induced production of NO, we used agonists and antagonists of the NPY (Y1R and Y2R) receptors infused via intrastriatal microinjection followed by a bolus of METH (30 mg/kg, ip) and measured 3-NT immunofluorescence, an indirect index of NO production. One striatum received pharmacological agent while the contralateral striatum received aCSF and served as control. NPY receptor agonists dose dependently attenuated the METH-induced production of striatal 3-NT. Conversely, NPY receptor antagonists had the opposite effect. Moreover, METH induced the accumulation of cyclic GMP and activated caspase-3 in approximately 18% of striatal neurons, a phenomenon that was attenuated by pre-treatment with NPY2 receptor agonist. Lastly, METH increased the levels of striatal preproneuropeptide Y mRNA nearly five-fold 16 hours after injection as determined by RT-PCR, suggesting increased utilization of the neuropeptide. In conclusion, NPY inhibits the METH-induced production of NO in striatal tissue. Consequently, production of this second messenger induces the accumulation of cyclic GMP and activated caspase-3 in some striatal neurons, an event that may precede the apoptosis of some striatal neurons.

Keywords

methamphetamine; striatum; neuropeptide Y; nitric oxide; cyclic GMP

1. INTRODUCTION

The psychostimulant methamphetamine (METH) produces long-term behavioral and biological effects in the brain of its users. Methamphetamine use remains a public health concern, and is a multi-million dollar cost to governments via crime prevention and

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addiction recovery (United Nations Office on Drugs and Crime, 2011). Over the last 40 years, research has demonstrated that METH induces dopamine and glutamate overflow in the striatum (Fibiger and McGeer, 1971; Stephans and Yamamoto, 1994; Jones et al., 1998) leading to excitotoxicity in dopaminergic terminals and GABA-producing neurons (Hotchkiss et al., 1979; Krasnova and Cadet, 2009). We observed that approximately 25% of striatal neurons are apoptotic 24 hours post-METH treatment (Zhu et al., 2005) and that METH triggers the overproduction of nitric oxide (NO) and reactive oxidative species consequently inducing neurodegeneration (Dawson and Dawson, 1996; Cadet and Brannock, 1998). There is a correlation between NO production and METH-induced cell death. For example, pharmacological inhibition of neuronal NOS attenuates the METH-induced neural toxicity and cell damage (Itzhak et al. 1996) and mice lacking the gene coding this enzyme show partial resistance to METH (Imam et al., 2001c). Moreover, behavioral studies demonstrate attenuation of locomotion stereotypy when neuronal NOS (Abekawa et al, 1997) or expression is inhibited (Itzhak et al., 1998). In addition, inhibition of neuronal NOS activity is neuroprotective for markers of striatal dopamine terminals such as reduction of tissue dopamine content, dopamine transporters and tyrosine hydroxylase (Wang and Angulo, 2011; Di Monte et al., 1996). Despite a wealth of information on the role of dopamine and glutamate on the neurotoxic effects of METH, there exists a dearth of information on the potential contributions of neuropeptides.

Our laboratory has shown that neurokinin-1 receptor signaling by the neuropeptide substance P contributes to the overproduction of striatal NO induced by METH (Wang et al., 2008). Interestingly, striatal NO production is significantly attenuated by pre-treatment with a neurokinin-1 receptor antagonist, and ablation of neurokinin-1 receptors abolishes the METH-induced apoptosis of some striatal neurons (Zhu et al., 2009). Striatal neurokinin-1 receptors are expressed by cholinergic and SST/NPY/NOS interneurons, the latter a subset of striatal interneurons that comprise less than 1% of all striatal neurons (Kawaguchi et al., 1995). Neuropeptide Y is of interest because it has been shown to be a neuromodulator and is co-expressed with transmitters such as glutamate, GABA and somatostatin in various brain regions (Silva et al., 2003). Moreover, in a model of potassium chloride-evoked glutamate release, NPY attenuated glutamate release in the hippocampus (Silva et al., 2005) and also NPY receptor activation confers protection against AMPA and kainate-induced neurodegeneration (Silva et al., 2003).

Neuropeptide Y is 36 amino acids long and is expressed throughout the central nervous system, with five known corresponding receptors in mice (Y_1 , Y_2 , Y_4 , Y_5 , Y_6) (Kask et al., 2002). In recent years, studies have described the participation of NPY and its receptors in a variety of pathologies including major depression, bipolar disorders, schizophrenia, anxiety, obesity, Huntington's disease and epilepsy (Furtinger et al., 2001; Thorsell et al., 2002; Okahisa et al., 2009; Zambello et al., 2011). The messenger RNA for both the NPY-1 (Jacques et al., 1996; Caberlotto et al., 1997) and NPY-2 (Gehlert et al., 1996; Caberlotto et al., 1998) receptors is expressed in the striatum. We hypothesized that striatal NPY participates in the production of NO induced by METH and that this property may account for the neuroprotective effects of NPY in the presence of METH in the mouse striatum (Thiriet et al., 2005). To that end, we assessed the impact of NPY1 and NPY2 receptor agonists and antagonists on the METH-induced striatal production of 3-nitrotyrosine (3-NT)

and the co-localization of cyclic GMP and activated caspase-3 in striatal neurons. We also measured the impact of METH on striatal preproneuropeptide Y mRNA. 3-NT is a reliable index of NO-derived oxidative stress in brain tissue (Imam et al., 2001a & b; Zhu et al., 2009).

2. RESULTS

2.1. NPY1 and NPY2 receptor agonists and antagonists

We hypothesized that NPY receptor agonists protect from METH by attenuating the METH-induced production of striatal NO. To that end, we infused 1 µl of NPY1 or NPY2 receptor agonist (5, 10 & 20 µmol of each agonist) into one striatum and an equivalent volume of aCSF into the contralateral striatum (n=6). The mice received a bolus of METH (30 mg/kg, ip) 30 minutes after the intrastriatal infusions and were sacrificed 4 hours after METH. 3-NT (indirect index of NO production) was detected with immunofluorescence in soma and neuropil using the confocal microscope. Both NPY1 and NPY2 receptor agonists dose dependently attenuated the METH-induced production of 3-NT in the striatum. The 20 µmol dose showed the largest effect (Figure 1). Data were analyzed by Two-Way ANOVA and Bonferroni's post-hoc test (NPY1 receptor agonist $p < 0.001$, $F = 11.58$; NPY2 receptor agonist $F = 55.34$). Conversely, NPY1 (BIBP3226) or NPY2 (BIIE0246) receptor antagonists should enhance the METH-induced production of 3-NT. We infused three concentrations of NPY1 receptor antagonist (BIBP3226: 340, 680 and 1360 µmol) or NPY2 receptor antagonist (BIIE0246: 1, 2 and 4 nmol) followed by METH as described above for the agonists. The concentrations used were derived from published work (Thorsell et al., 2002). The NPY receptor antagonists augmented the METH-induced production of striatal 3-NT in a dose dependent fashion (Figure 2).

2.2. Cyclic-GMP in striatal neuronal populations

Because NO is known to activate guanylyl cyclase, we investigated the question of whether all striatal neuronal populations respond equally to NO by accumulating cyclic GMP. We characterized the cellular response to the METH-induced NO production by fluorescent co-label involving cyclic GMP and select markers for striatal neurons. The cells of the striatum can be divided into two major subgroups (projection neurons and interneurons) and further individualized by their receptor and protein expression. We labeled projection neurons with DARPP-32 and interneurons with cholineacetyl transferase (ChAT), parvalbumin and somatostatin. Animals were sacrificed 4 or 8 hours after injection of METH (30 mg/kg, ip, n=6). METH-treated animals displayed increased cyclic GMP expression in all cell types after both 4 and 8 hours by histological assessment. The results are expressed as mean percent above control levels (Figure 3A-D). While cyclic GMP response persisted until 8 hours in 3 cell types, cyclic GMP immunoreactivity decreased in SOM/NPY/NOS interneurons by 8 hours (Figure 3C). In the latter population of interneuron cyclic GMP staining decreased from 20% at four 4 hours to 5.5% at 8 hours after METH (Figure 3C). It is interesting to note that this population of striatal neurons is refractory to the METH-induced apoptosis (Zhu et al., 2006).

2.3. Co-localization of cyclic GMP and activated caspase-3

Various studies have demonstrated apoptotic cell loss induced by METH in the striatum and other brain regions. In order to establish a potential causative connection between excessive NO production and striatal apoptosis, we assessed the number of striatal cells co-expressing cyclic GMP and activated caspase-3 at 8 hours after a bolus of METH (30 mg/kg, ip). Approximately 50% of striatal cells expressed high levels of cyclic GMP and about 65% expressed activated caspase-3 8 hours after METH (data not shown). However, approximately 18% of cells co-expressed both markers and pre-treatment with the NPY2 receptor agonist (20 μ M) reduced this double-labeled population to approximately 4% (Figure 4).

2.4. Impact of METH on preproneuropeptide Y mRNA

In order to determine the effect of METH on striatal NPY, we measured the level of preproneuropeptide Y mRNA at 4 and 16 hours after METH (30 mg/kg, ip). Taqman RT-PCR technique was employed to detect NPY mRNA levels in the mouse striatum after methamphetamine administration. Preproneuropeptide Y mRNA production was elevated at 4 and 16 hours post-drug treatment. A mean fold increase of 0.36 was observed at 4 hours and a mean fold increase of 1.65 at 16 hours, that is, a 4.6-fold increase from 4–16 hours after METH (Figure 5). GAPDH was utilized as endogenous control due to its ubiquitous and stable expression. Statistical analysis was performed by ANOVA followed by Bonferroni's multiple comparison test. One-way ANOVA shows significant differences between 4 and 16-hour time points ($***p<0.0001$). Bonferroni's multiple comparison post-hoc test confirmed statistically significant differences in treatment from control ($**p<0.001$, $***p<0.0001$) as well as between cohorts ($***p<0.0001$) (Figure 5).

3. DISCUSSION

The process by which METH induces the toxicity of the striatal dopamine terminals and the apoptotic loss of some striatal neurons involves several mechanisms converging on the phenotypic outcome of neural damage. Key biochemical molecules that function normally under homeostatic conditions become excessively utilized in the presence of METH resulting in tissue damage. Striatal neuropeptides represent one such type of regulatory molecule that in response to METH-induced neurotransmitter changes either exacerbate the impact of METH or attempt to restore the homeostatic balance of the striatum. NPY is a 36-amino acid peptide variously expressed in the brain. In the striatum, it is synthesized and released from a neuronal population comprising approximately 1% of all striatal neurons and co-expressing somatostatin and nitric oxide synthase (Kawaguchi, 1997; Adrian et al., 1983). NPY signals through G-protein coupled receptors affecting the intracellular concentration of the second messenger cyclic AMP and calcium (Pedrazzini et al., 2003). This neuropeptide has been implicated in various brain functions like memory and appetite regulation (Grundemar and Hakanson, 1994) as well as mood disorders such as anxiety and depression (Heilig et al., 1988, 1989).

Our results demonstrate that NPY1 and 2 receptor agonists dose dependently attenuate the METH-induced progressive accumulation of 3-NT, an indirect index of NO production. This

effect is selective for the NPY1 and 2 receptors because selective antagonists had the opposite effect on the METH-induced production of striatal 3-NT. In a previous study we reported that the same dose of METH (30 mg/kg) induced the progressive accumulation of striatal 3-NT up to 24 hours after METH (Zhu et al., 2009). Moreover, a different group reported that NPY receptor agonists protected the striatum from the apoptotic loss of neurons (Thiriet et al., 2005). We postulate that NPY receptor activation protects from METH by attenuating the build-up of striatal NO consequently reducing oxidative stress. Published work implicates NO in the METH-induced striatal injury. For example, pharmacological inhibition of neuronal nitric oxide synthase or deletion of the gene for this enzyme in mice protects the striatum from METH (Itzhak and Ali, 1996; Imam et al., 2001c). Moreover, agents that block the synthesis of NO also attenuate METH-induced loss of mesencephalic neurons *in vitro*, suggesting that NO synthesis may be causally related to the neurotoxic effects of METH (Cadet and Brannock, 1998). Further evidence implicating a role for NO comes from experiments with transgenic mice that over-express copper/zinc superoxide dismutase (CuZnSOD). NO 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 wildtype and the homozygous are nearly resistant to METH (Cadet et al., 1994; Hirata et al., 1996; Epstein et al., 1987). In the light of the above, it is clear that NO plays a role in the METH-induced striatal cell loss. But do all striatal neurons respond to NO?

The receptor for NO is the soluble form of guanylyl cyclase, an enzyme that when activated by NO converts guanosine triphosphate to cyclic GMP, a second messenger that affects the state of cyclic nucleotide-gated ion channels (Kaupp and Seifert, 2002) and the catalytic activation of protein kinase G (Garthwaite, 2008). Cyclic GMP (Ariano and Matus, 1981) and guanylyl cyclase (Ariano et al., 1982) have been localized within striatal neurons by immunohistochemical methods. In the present study, we used immunohistochemistry to co-localize cyclic GMP with selective markers of striatal neurons. Low levels of cyclic GMP are expressed by some striatal neurons but in the presence of METH a significant population of projection neurons and interneurons (cholinergic, parvalbumin and SST/NPY/NOS) become intensely labeled for cyclic GMP between 4 and 8 hours after METH. Interestingly, the SST/NPY/NOS interneurons show a decrease to near control levels between 4 and 8 hours after METH. This observation is interesting in the light of a previous report from our laboratory demonstrating that this type of striatal interneuron is refractory to the METH-induced apoptosis (Zhu et al., 2006). This decrease of cyclic GMP may be due to desensitization of guanylyl cyclase and/or activation of phosphodiesterase-2 (Wykes et al., 2002).

METH induces the loss of approximately 20% of striatal neurons in mice (Zhu et al., 2006). In an attempt to establish a causal relationship between cyclic GMP accumulation and cell death, we co-labeled cyclic GMP with activated caspase-3, an early marker of apoptosis (Jayanthi et al., 2004). Interestingly, approximately 18% of striatal neurons labeled positive for these two markers and this number decreased to about 5% when an NPY2 receptor

agonist was infused into the striatum 30 minutes prior to METH. It is tempting to speculate that there might be a causal relationship between elevation of cyclic GMP, activation of caspase-3 and apoptosis. However, a previous study showed that partial lesions of the dopaminergic neurons of the substantia nigra pars compacta with 6-hydroxydopamine resulted in long-term expression of activated caspase-3 without apoptosis in striatopallidal projection neurons (Ariano et al., 2005).

We observed a five-fold increase in preproneuropeptide Y mRNA at 16 hours post-METH by RT-PCR. Similar increases of striatal preproneuropeptide Y mRNA in response to METH have been observed by other groups (Thiriet et al., 2005; Horner et al., 2006). One group observed an increase in the number of striatal cells expressing preproneuropeptide Y mRNA by *in situ* hybridization histochemistry (Horner et al., 2006). We hypothesize that the increased levels of preproneuropeptide Y mRNA represent a homeostatic adaptation to replenish the intracellular pool of NPY due to METH-induced release and degradation of this neuropeptide. Exposure to METH has been shown to decrease striatal levels of NPY-like immunoreactivity (Westwood and Hanson, 1999) consistent with the hypothesis that METH increases the utilization of striatal NPY.

In conclusion, our results show that activation of the NPY1 and 2 receptors by selective pharmacological agonists attenuated the METH-induced striatal NO production. NO induces the accumulation of cyclic GMP in nearly half of all striatal neurons, 18% of which also co-expressed activated caspase-3. Interestingly, the SST/NPY/NOS interneurons appear to activate a mechanism to degrade cyclic GMP between 4 to 8 hours after METH. Moreover, the mRNA for preproneuropeptide Y increased 5-fold 16 hours after METH suggesting a high rate of utilization of this neuropeptide in the presence of METH. Experiments in progress are evaluating the involvement of other striatal neuropeptides on the METH-induced production of NO.

4. MATERIALS AND METHODS

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 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). ICR Male Mice (12–13 weeks old, Taconic, Germantown, NY) weighing approximately 40 grams were housed in a temperature-controlled environment with a 12h light/dark cycle. The animals had food and water available ad libitum. Mice were habituated for two weeks prior to commencement of drug administration. *The work described in this article was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments.*

4.2. Drug Preparation and Administration

The following NPY receptor compounds were dissolved in aCSF and infused intrastrially in a volume of 1µl: NPY Y1 agonist Leu31–Pro34 NPY, H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-

Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr- Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Leu-Thr-Arg-Pro-Arg-Tyr-NH₂, (H-8575, Bachem, Torrance, CA), NPY Y2 agonist NPY (3–36), H-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro- Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH₂, (H-8570, Bachem, Torrance, CA), NPY Y1 antagonist (BIBP3226 (Bachem, Torrance, CA) or NPY Y2 antagonist BIIE0246 (Tocris Biosciences, Ellisville, MO). Agonists and antagonists were infused into one striatum and the contralateral striatum received an equivalent volume of aCSF (n=6). (+)-Methamphetamine hydrochloride (Sigma, St. Louis, MO) was dissolved in 10mM phosphate-buffered saline, pH 7.4 (PBS) and injected intraperitoneally at a dose of 30 mg/kg of body weight immediately following stereotaxic surgery. A matching volume of saline was given for control animals. Intrastratial microinjections were given in the striatum (bregma 0.5 mm, lateral 2mm, dorsal 2.5mm; Franklin and Paxinos, 1997) under isofluorane gas anesthesia. Intraperitoneal injections were given of either methamphetamine or saline at doses listed above.

4.3. Sacrifice and Cryostat Sectioning

All animals were anesthetized and perfused with PBS, followed by 4% paraformaldehyde in PBS at 4, 8, or 16 hours after treatment. For mRNA study, animals were sacrificed by cervical dislocation at 4 and 16 hours post-treatment (n=5 per group). Coronal sections were cut at 30µm thickness and collected serially from the striatum between bregma 0.02 and 1.4 mm into cryoprotectant solution. Every sixth sample per striata was collected into one of six adjacent sample wells per animal so that 36 sections were processed using the free-floating method. Brains were nicked in the left dorsal cortex for orientation.

4.4. Immunofluorescence

3-Nitrotyrosine, cyclic GMP, active caspase-3, NPY receptors and neuronal cell types were labeled by immunofluorescent technique. For each immunohistochemical assay, we used 1 well of tissue (6 sections) per animal. Free-floating sections were washed in PBS with 0.3% Triton X-100 (Tx-PBS) and blocked for non-specific binding using 10% Normal Donkey Serum (NDS) (Y1R, Y2R, cGMP, DARPP-32, ChAT, Parvalbumin staining) or Mouse-on-Mouse IgG (BMK-2202, Vector laboratories, Burlingame, CA) (3-NT, NOS1 staining) at room temperature for 1 hr. Primary antibodies were administered in 5% NDS 0.2%Tx-PBS.

3-Nitrotyrosine—Mouse-on-Mouse blocking was followed by incubation in working solution of M.O.M. diluents buffer (80µl/ml in Tx-PBS) for 10 minutes. These sections were incubated with a monoclonal anti-mouse antibody against 3-nitrotyrosine at 4 °C (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). These sections were rinsed with PBS 3 times 24–36 hours later for 10 minutes each and stained with donkey anti-mouse conjugated to Cy3 (Chemicon, Temecula, CA) for 1 hour while protected from light at room temperature.

NPY Receptors—The sections were then incubated in primary antibody for for 24–36 hours at 4°C. For receptor label, polyclonal rabbit anti-Y1R (1:250, Novus Biologicals, Littleton, CO) or rabbit anti-Y2R (1:100, Novus Biologicals, Littleton, CO) was used. Mouse anti-NOS1 (1:250. Santa Cruz Biotechnology, Inc., Santa Cruz, CA), goat anti-ChAT

(1:500, Millipore, Billerica, CA), goat anti-DARPP-32 (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or mouse anti-Parvalbumin (1:500, Millipore, Billerica, CA) were used to label cell-type. After primary incubation, sections were rinsed 3 times (10 minutes each) and stained with two secondary antibodies, each for one hour. These included donkey anti-goat FITC, donkey anti-mouse FITC, donkey anti-rabbit FITC, rabbit anti-mouse FITC and donkey anti-rabbit Cy3 (Chemicon, Temecula, CA) while protected from light at room temperature.

cGMP colabel—Rabbit anti-cGMP (1:500, Millipore, Billerica, CA) was used as a co-label with phenotype markers used as described in the above assay for NPY receptors. Sections were rinsed (PBS, 3 times, 5 minutes) before applying donkey anti-rabbit cy3 (1:500, Chemicon, Temecula, CA) was used for secondary antibody incubation for 1 hour while protected from light at room temperature.

Active Caspase-3—Goat anti-cleaved caspase-3 (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as a co-label with cGMP as described above. 24–36 hours later sections were rinsed in PBS 3 times (10 minutes each) before application of donkey anti-goat FITC (1:500, Chemicon, Temecula, CA) at room temperature without light for 1 hour. All immunohistochemical sections were washed following three times with PBS (5 minutes each) and mounted onto superfrost glass slides, sealed and coverslipped with Vectorshield hard set™ mounting medium for fluorescence (Vector Laboratories, Burlingame CA).

4.5. Confocal Imaging

Fluorescent immunostaining was visualized and staining intensity was quantified. 3-NT images were taken with Hamamatsu 1394 ORCA-ERA Spinning Disk Confocal Microscope using a 60x objective lens. Data from 6 control animals and 6 animals for each drug concentration were analyzed. Images were taken against the striatal border (<1mm from injection site) to avoid counting cells expressing 3-NT as a result of needle damage. The remaining area of the striatum was divided into 4 quadrants for image capture. 4 images (dorsal lateral, dorsal medial, ventral lateral, ventral medial) were taken against the striatal border. Cells were quantified by quadrant and summed for each hemisphere. The aCSF hemisphere was nicked prior to acquiring slices in order to differentiate the treatments. Fluorescence intensity was measured using Volocity 5.2.0 (2008, PerkinElmer, Waltham, MA). The Voxel Spy feature was used for unbiased measurement of fluorescent intensity and averaged for each tissue. Background fluorescence was subtracted manually from each image. Distinct borders, morphology and significant stain throughout cell shape defined positive cells. Cyclic GMP, NPY receptors, and caspase-3 colabels were taken with a Leica SP2 confocal microscope and corresponding Leica Lite LCS software system (Leica Microsystems, Heidelberg, Germany) using a 63X objective lens. FITC and Cy3 signals corresponded to single wavelength laser line 488 (green) and 588 (red), respectively. The striatum was again divided into four regions (see above) and z-stack images from each striatal region were taken in 4–8 animals and analyzed for cyclic GMP study, NPY receptors study, and active caspase-3 colocalization. Data from 6 animals were analyzed for active caspase-3 study with striatal microinjection. To avoid cross detection between the signals, the pinhole setting was less than 2µm and z-stacks 10µm thick were recorded sequentially

between frames in a raster pattern series. 3-NT staining intensity was measured in striatal areas that included cells and neuropil.

4.6. Quantitative RT-PCR

Striata were dissected from fresh frozen tissue and total RNA was extracted using Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). RNA integrity and quantification was performed using Thermo Scientific Nano Drop 1000 Spectrophotometer. The purity and integrity of the samples were determined using the ratio A_{260}/A_{280} . All samples used fell between 1.8 and 2.2. RNA was normalized to 100ng for PCR. Total RNA was reverse-transcribed to cDNA and amplified using Taqman One-Step PCR kit with TAMRA Taqman probe and custom primers (NPY: 5'-GTG TTT GGG CAT TCT G-3'/5'-TTC TGT GCT TTC CTT CAT-3') (Applied Biosystems CITY STATE). Real-time (RT)-PCR approach (Applied Biosystems Universal Thermal cycling, 7500 Real Time PCR System) measured levels of preproneuropeptide Y mRNA in mice striata. Samples and GAPDH internal control were run in triplicate with each assay for 6 animals. $33C_T$ values were calculated for paired drug and control animals.

4.7. Statistics

Statistical analysis was performed by ANOVA followed by Bonferroni's multiple comparison tests utilizing Prism software (GraphPad inc. La Jolla, CA).

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Abbreviations used

aCSF	artificial cerebrospinal fluid
DARPP-32	dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein, 32 kDa
ICR	Institute for Cancer Research
ip	intraperitoneal
METH	(+)-methamphetamine hydrochloride
NPY	neuropeptide Y
3-NT	3-nitrotyrosine
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate-buffered saline, pH 7.4
RT-PCR	real time-polymerase chain reaction

SST

somatostatin

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RESEARCH HIGHLIGHTS

- We investigated the role of neuropeptide Y on the methamphetamine-induced production of striatal nitric oxide.
- Neuropeptide Y receptor agonists (Y1 & Y2) attenuate nitric oxide production stimulated by methamphetamine.
- Neuropeptide Y2 receptor agonist inhibits the co-expression of cyclic GMP and activated caspase-3 in some striatal neurons.
- Preproneuropeptide Y mRNA is increased by methamphetamine suggesting increased utilization of the peptide.

Figure 1.

NPY1 and 2 receptor agonists attenuate the METH-induced NO production in striatal neurons. All animals received aCSF injections in left striata and agonist in right striata 30 minutes prior to systemic injection of METH (30 mg/kg, ip). 3-NT was detected by immunohistofluorescence with the confocal microscope. Note that both agonists demonstrate a significant dose dependent effect on the production of 3-NT in the striatum at 4 hours post-drug treatment (n=6 per group). Analysis was performed from mean $\pm$ SEM. Differences between groups and concentrations were analyzed by Two-Way ANOVA. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ relative to aCSF + Saline).

Figure 2.

NPY1 and 2 receptor antagonists enhance the METH-induced 3-NT production in striatal neurons. Low, Mid and High doses under the x-axis refer to concentrations of NPY1 receptor antagonist (BIBP3226: 340, 680 and 1360 μ mol) or NPY2 receptor antagonist (BIIIE0246: 1, 2 and 4 nmol). 3-NT fluorescent intensity increases in a dose-dependent manner with NPY receptor antagonist administration at 4 hours post-METH (30 mg/kg, ip) treatment (n=6 per group). Analysis was performed from mean $\pm$ SEM. Differences between groups and concentrations were analyzed by Two-Way ANOVA and Bonferroni's post-hoc test.

p<0.01, *p<0.001 relative to aCSF plus saline. !!p<0.01, !!!p<0.001 relative to aCSF plus antagonist.

Figure 3.

Immunohistochemical co-localization of cyclic GMP with select markers of striatal projection (A) and interneurons (B-D). A), DARPP-32 in projection neurons; B), parvalbumin; C), nitric oxide synthase; and D) cholineacetyl transferase. Mice (n=6) were injected with METH (30 mg/kg, ip) and sacrificed at 4 and 8 hours post-injection. Stained neurons were counted using computerized unbiased stereology and the results normalized relative to saline-treated controls. Note that the percentage of striatal neurons staining positive for cyclic GMP increase between 4 and 8 hours in all neuronal populations except the SST/NPY/NOS interneurons where staining decreased between 4 and 8 hours. Differences between groups were analyzed by Two-Way ANOVA and Bonferroni's post-hoc test. *** $p < 0.001$; **** $p < 0.0001$.

Figure 4.

Exposure to METH significantly increases the number of striatal cells that co-localize cyclic GMP with activated caspase-3 and effect of NPY2R agonist. Control and experimental animals were given aCSF infusion into one striatum and 1.0 μ l of 20 μ M NPY2R agonist into the contralateral striatum. Mice (n=6) received an injection of METH (30 mg/kg, ip) 30 minutes after the intrastriatal infusions and were sacrificed 8 hours after the injection. Activated caspase-3 and cyclic GMP were visualized in the same section of striatal tissue by immunofluorescence in the confocal microscope. Note that the number of striatal cells co-expressing these two markers increases nearly five-fold in the group exposed to METH and the NPY2R agonist significantly attenuates this effect of METH. Differences between groups were analyzed by t-test and Mann Whitney's post-hoc test (** $p < 0.01$).

Figure 5.

Preproenkephalin mRNA production is elevated at 4 and 16 hours after METH treatment. Mice (n=5) received an injection of METH (30 mg/kg, ip) and were sacrificed 4 or 16 hours after the injection. Striatal preproenkephalin mRNA in control and METH-treated groups was measured by Taqman One-Step RT-PCR and normalized to GAPDH. Differences between groups were analyzed by Two-Way ANOVA and Bonferroni's post-hoc test. There is a significant effect from control and between time points (**p<0.01; ***p<0.001).