Regulation of Glutamate Release by Alpha 7 Nicotinic Receptors: Differential Role in Methamphetamine-Induced Damage to Dopaminergic and Serotonergic Terminals

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ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; DA, dopamine; 5-HT, serotonin; Meth, methamphetamine; MLA, methyllycaconitine; DAT, dopamine reuptake
transporter; SERT, serotonin reuptake transporter; VMAT-2, vesicular monoamine transporter-2; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; ip, intraperitoneally; im, intramuscular; HPLC, high performance liquid chromatography; NOS, nitric oxide synthase; NO, nitric oxide; ONOO·, peroxynitrite; NMDA, N-methyl D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; MDMA, 3,4-methylenedioxymethamphetamine

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Regulation of glutamate release is an important underlying mechanism in mediating excitotoxic events such as damage to dopamine (DA) and serotonin (5-HT) neurons observed after exposure to methamphetamine (Meth). One way to regulate glutamate release may be through the modulation of \( \alpha_7 \) nicotinic acetylcholine (nACh) receptors. Meth administration is known to increase acetylcholine release; however, it is unknown if Meth increases glutamate release and causes long-term damage to both DA and 5-HT terminals through the activation of \( \alpha_7 \) nACh receptors. To test this hypothesis, the \( \alpha_7 \) nACh receptor antagonist, methyllycaconitine (MLA), was administered prior to the administration of repeated doses of Meth while simultaneously monitoring extracellular striatal glutamate with \textit{in vivo} microdialysis. In addition, the subsequent long-term decreases in markers of dopaminergic and serotonergic terminals, including DAT, SERT, VMAT-2, vesicular DA and vesicular 5-HT content in the rat striatum were measured. The results show that MLA pretreatment prevented Meth-induced increases in striatal glutamate and protected against the subsequent long-term decreases in striatal DAT and vesicular DA content without affecting the hyperthermia produced by Meth. In contrast, the Meth-induced decreases in striatal SERT immunoreactivity and vesicular 5-HT content was not affected by MLA. This suggests that the \( \alpha_7 \) nACh receptor differentially mediates glutamate dependent damage to DA but not 5-HT terminals in a manner that is independent of hyperthermia. Furthermore, antagonism of \( \alpha_7 \) nACh receptors may be a possible therapeutic strategy for decreasing extracellular glutamate and preventing the excitotoxic damage observed in other DA-related neurodegenerative disorders.
INTRODUCTION

Glutamate is commonly accepted as the most abundant excitatory neurotransmitter in the brain and plays a major role in normal brain functions, including development, cognition, learning and memory. However, it is also well known that excess glutamate can be damaging to neurons and has been implicated in many diseases of the central nervous system (CNS) including the addiction and damage produced by drugs of abuse (Choi, 1988; Wolf, 1998). Upon activation of calcium permeable NMDA and AMPA receptors, glutamate produces an increase in intracellular calcium, which in excess activates calcium dependent enzymes and generates reactive oxygen and nitrogen species, ultimately leading to excitotoxic neuronal damage. (Siman and Noszek, 1988; Staszewski and Yamamoto, 2006). Therefore, the concentrations of extracellular glutamate in the brain must be tightly regulated.

Regulation of glutamate release can be mediated by acetylcholine. More specifically, acetylcholine can modulate glutamate release through presynaptic nicotinic receptors. Although the presence of α7 nicotinic acetylcholine (nACh) receptors on presynaptic glutamate terminals is controversial, there is substantial evidence for the ability of α7 nACh receptors to modulate glutamate release from isolated striatal synaptosomes (Marchi et al., 2002) and in the prefrontal cortex of freely moving rats (Konradsson-Geuken et al., 2009). Thus, the modulation of extracellular levels of glutamate through α7 nACh receptors may be one way to protect the brain from excitotoxic damage.

Excitotoxicity is known to play a role in damage to dopamine (DA) and serotonin (5-HT) neurons of the striatum (Storey et al., 1992) and is involved in the long-term...
striatal damage after exposure to methamphetamine (Meth) (Staszewski and Yamamoto, 2006). The damage observed in response to Meth is marked by long-term decreases in dopamine and serotonin transporters (DAT and SERT), vesicular monoamine transporter-2 (VMAT-2), tyrosine hydroxylase (TH), and tryptophan hydroxylase (TPH) immunoreactivity. In addition, there are long-term decreases in DA and 5-HT tissue content (Hotchkiss and Gibb, 1980; Ricaurte et al., 1980; Frey et al., 1997) and increases in markers of excitotoxicity evidenced by increases in calcium mediated spectrin proteolysis (Staszewski and Yamamoto, 2006).

One mechanism by which Meth might produce neurotoxicity is through the release of acetylcholine or increase in α7 nACh receptor binding sites. In fact, there is evidence that systemic Meth administration causes an acute increase in extracellular acetylcholine in the rat striatum (Taguchi et al., 1998) and exposure to Meth increases α7 nACh receptor binding sites in differentiated PC-12 cells (Garcia-Rates et al., 2007). In addition, activation of the α7 nACh receptor mediates the Meth-induced production of reactive oxygen species in isolated rat striatal synaptosomes (Pubill et al., 2005). Moreover, antagonism of the α7 nACh receptor with methyllycaconitine (MLA) protects against Meth-induced decreases in DAT binding (Escubedo et al., 2005); however, the mechanisms underlying this neuroprotective effect have not been investigated.

The current study tested the hypothesis that the specific α7 nACh receptor antagonist, MLA, will block Meth-induced increases in extracellular glutamate and protect against the long-term decreases in DAT, SERT, VMAT-2 and vesicular DA and 5-HT content in the rat striatum. The results indicate that MLA protects against Meth-induced long-term damage to striatal DA terminals, but not 5-HT neurons, through the
attenuation of Meth-induced increases in extracellular glutamate.
MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (180-250 g, Harlan, Indianapolis, IN) were used in all experiments. Rats were group housed in clear plastic containers (45 x 24 x 20 cm) and allowed 4-5 days to acclimate to the animal colony after arrival. Rats were housed in a temperature (23 ± 1°C), and humidity (40 ± 5%) controlled environment on a 12 hour light/dark cycle (lights on at 7:00 am and off at 7:00 pm). Rats had ad libitum access to food and water. All treatments were administered during the light cycle. Efforts were made to minimize suffering and reduce the number of animals used in the experiments. All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Boston University and University of Toledo Institutional Animal Care and Use Committees.

Drugs and Drug Treatment

(+) Methamphetamine-hydrochloride (Meth) was purchased from Sigma (St. Louis, MO, Cat. M-8750). Meth was dissolved in 0.9% NaCl (saline) and administered intraperitoneally (ip), at a dose of 10 mg/kg, for 4 injections, every 2 hours. This dosing paradigm was used because it has been shown that it produces neurotoxicity in rats. Controls for Meth treatment were rats treated with 4 ip injections of saline (1 mL/kg) every 2 hours.

Methyllycaconitine citrate (MLA) was purchased from Tocris (Ellisville, MO, Cat. 1029). MLA was dissolved in saline and MLA pretreatments consisted of 5.5 mg/kg ip injections 15 minutes prior to each Meth or saline injection. MLA is a potent α7 nACh
receptor antagonist (Ki = 1.4 nM), however, at concentrations greater than 40 nM, it can also interact with α4β2 and α6β2 nACh receptors. A dose of 5.5 mg/kg was chosen for our experiments based on previous studies indicating that similar doses produce brain concentrations, which would selectively antagonize the nACh receptors containing α7 subunits (Turek et al., 1995; Escubedo et al., 2005). In order to control for MLA pretreatment, rats were treated with 1 mL/kg saline 15 minutes prior to each Meth or saline injection. These treatments resulted in a total of 4 experimental groups; Saline+Saline, MLA+Saline, Saline+Meth, and MLA+Meth, for each experiment. Separate groups of animals were used for in vivo microdialysis experiments and experiments investigating the neurotoxic effects of Meth. Those animals that were used in the in vivo microdialysis experiments were killed 24 hours after treatment, while those used for the neurotoxicity studies were killed 7 days following treatment.

During all treatments, rectal temperatures were measured via a digital thermometer with probe (Thermalert TH-8; Physitemp Instruments Inc., Clifton, NJ, USA) prior to the first injection and an hour after each injection. To minimize animal death, rats that reached temperatures greater than 41°C were cooled briefly by placing ice packs on top of their cages while their temperatures were closely monitored.

**In Vivo Microdialysis**

For in vivo microdialysis experiments, rats underwent intracranial surgery for probe implantation into the striatum and the following day rats were treated and brain dialysate was collected.
Rats were anesthetized with a solution of xylazine (5 mg/kg) and ketamine (75 mg/kg) via intramuscular (im) injection and placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The skull was exposed and a small hole was drilled through the skull above the striatum. A microdialysis probe, constructed as previously described (Yamamoto and Pehek, 1990) with an active membrane (13,000 molecular weight cutoff; 216 µm outer diameter) (Spectrum Laboratories, Rancho Dominguez, CA) length of 4mm, was slowly lowered into the striatum (AP +1.2; ML ± 3.0) using the stereotaxic frame, so that the tip of the probe was 6.25 mm ventral to the surface of the brain. This resulted in the actively dialyzed region of the striatum to be 2.25 to 6.25 mm from the brain surface. Probes were secured in the appropriate position with cranioplastic cement (CO-ORAL-ITE Dental MFG. CO., Diamond Springs, CA).

After surgery, all rats were housed individually in round buckets and attached to a spring tether and swivel, which allowed the rats to move freely. The probes were attached to polyethylene 50 (PE50) tubing, connected to a Harvard Apparatus syringe perfusion pump 22 (Holliston, MA). Dulbecco’s phosphate-buffered saline (138 mM NaCl, 2.1 mM KCl, 0.5 mM MgCl₂, 1.5 mM KH₂PO₄, 8.1 mm NaHPO₄, 1.2 mm CaCl₂, and 0.5 mM D-glucose, pH 7.4) (Sigma, St. Louis, MO) was perfused at a flow rate of 0.25 µL/min overnight. Approximately 15 hours after surgery, the pump flow rate was increased to 1.5 µL/min. After a 2 hour equilibration period, baseline dialysate samples were collected every hour for 2 hours, before the first Meth or saline injection, and all subsequent samples collected every hour during treatment through 2 hours after the last Meth or saline injection. Rats were killed by rapid decapitation 24 hours after treatment and their brains were removed and frozen on dry ice. A cryostat (Microm HM
Thermo Scientific, Waltham, MA) was used to cut coronal slices in order to verify probe placement.

**High-Performance Liquid Chromatography (HPLC) for Measurement of Extracellular Glutamate**

Extracellular concentrations of striatal glutamate were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ED). Each dialysate sample was derivatized with an o-pthalaldialdehyde (OPA) (Cat. P0657, Sigma, St. Louis, MO) solution, comprised of (OPA, methanol, β-mercaptoethanol and sodium tetraborate, pH 9.3) prior to injection onto the column. An ESA model 542 autosampler (ESA, Chelmsford, MA) was used to add 10 µL of the OPA solution to 20 µL of the dialysate sample. The autosampler mixed the solution and allowed it to incubate for 90 seconds, before injecting the mixture onto a Hyperclone 3 µm C18 column, 150 x 2.00 mm (Phenomenex, Torrance, CA). The mobile phase used for detection of glutamate consisted of 0.1 M Na₂HPO₄ and 0.1 mM EDTA in 12% methanol, at a pH of 6.6. Electrochemical detection of glutamate was performed with an LC-4C amperometric detector (Bioanalytical Systems, Inc., Lafayette, IN) using a 6 mm glassy working electrode maintained at a potential of 0.7 V relative to an Ag-AgCl reference electrode. Data was collected and analyzed using EZ Chrome software (Scientific Software, Pleasanton, CA).

Striatal extracellular glutamate levels were represented as a percent of baseline. To obtain these values, the concentration of glutamate at each time point for an animal
was calculated as a percent of that animal’s baseline concentration. For each group, the mean and standard error of the mean was calculated and plotted.

**Western Blot for VMAT-2, DAT and SERT**

Detection of vesicular monoamine transporter-2 (VMAT-2), dopamine reuptake transporter (DAT) and serotonin reuptake transporter (SERT) protein were measured in the striatum 7 days following treatment using Western Blot. Rats were rapidly decapitated 7 days following treatment, brains were dissected and tissue was rapidly frozen on dry ice and stored at -80°C until use.

DAT and SERT were measured in the synaptosomal fraction of the striatal tissue, while VMAT-2 was measured in the vesicular fraction. The following procedure was used to obtain subcellular fractions. Striatal tissue was homogenized in ice cold 0.32 M sucrose (20 µL per 1mg of tissue) and centrifuged for 24 minutes at 800 x g, at 4°C to pellet insoluble material. The supernatant (S1) was collected and centrifuged for 17 minutes at 22,000 x g, at 4°C. The supernatant from the second spin (S2), was discarded and the pellet (P2), containing the synaptosomes, was resuspended in ice cold ddH2O. A portion of this synaptosomal preparation was retained for later use and the remaining sample was centrifuged at 22,000 x g for 17 minutes, at 4°C. The resulting supernatant (S3) contained the vesicular fraction and the pellet (P3) contained the membrane fraction.

The Bradford assay (BioRad, Hercules, CA) was used to determine total protein content in the synaptosomal and vesicular fractions. Samples were then diluted with 2x
sodium dodecyl sulfate (SDS) loading buffer (Invitrogen, Grand Island, NY, USA), boiled at 85°C for 5 minutes and stored at -80°C until use.

Equal amounts of protein were loaded per well for each sample (10 ug of synaptosomal protein for DAT and SERT, 5 ug of vesicular protein for VMAT-2), onto a 10% Tris-Glycine SDS-PAGE gel (Invitrogen, Grand Island, NY, USA). Following electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl), containing 0.5% Tween-20 and 5% non-fat powdered milk, for 1 hour at room temperature. Membranes were incubated with primary antibodies (anti-DAT, 1:2500, (Santa Cruz, sc-1433); anti-SERT, 1:1000, (Santa Cruz, sc-1458); anti-VMAT-2, 1:5000, (Chemicon, AB1767); anti-α tubulin, 1:2000, (Sigma, T6074)) in blocking buffer for about 17 hours at 4°C. Following 3, 5 minute, washes with TBS containing 0.5% Tween-20 (TBS-T), membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit anti-goat, 1:2500, (Santa Cruz; goat anti-rabbit), 1:2500, (Chemicon); goat anti-mouse IgG, 1:2500, (Santa Cruz)) in blocking buffer for 1 hour at room temperature. Membranes were washed again, 3 times for 5 minutes, with TBS-T.

HyGLO enhanced chemiluminescence (ECL) (Denville Scientific Inc., Metuchen, NJ) was used for antibody detection. Chemiluminescence was imaged using the Fuji LAS-4000 mini system (FujiFilm Corp. Life Science Division, Tokyo, Japan) and analyzed using Multi Gauge software (FujiFilm Corp. Life Science Division, Tokyo, Japan). Optical densities were measured for bands of all proteins. DAT and SERT, in the synaptosomal fractions, were normalized to an internal α-tubulin control. The VMAT-
2 was not normalized to α-tubulin, since there was little to no α-tubulin in the isolated vesicular fraction. Each sample was calculated as a percent of the mean of all Saline+Saline samples on the same gel in order to compare across gels. VMAT-2, DAT and SERT immunoreactivity were presented as percent of Saline+Saline treated animals.

**Measurement of Vesicular DA and 5-HT Content via HPLC**

Decreases in vesicular DA and 5-HT content are indicative of Meth-induced striatal damage (Sandoval et al., 2003); therefore, vesicular DA and 5-HT content was measured in a pure vesicular fraction. The vesicular fraction was prepared as described by Sandoval et al. (2003). Briefly, the crude vesicular preparation from the subcellular fractionation above was centrifuged at 100,000 x g for 45 minutes at 4°C to pellet the vesicles from the cytosolic fraction. The supernatant was discarded and the pellet was resuspended and sonicated, using a Fisher Scientific Model 150E ultrasonic dismembrator (Pittsburgh, PA) at 30% amplitude in 0.1 N perchloric acid. Samples were then centrifuged at 14,000 x g for 20 minutes to pellet proteins. Supernatants, containing DA and 5-HT, were injected onto the HPLC column and pellets, containing protein were resuspended in 1 N NaOH for protein quantification using the Bradford Assay (BioRad, Hercules, CA).

An ESA model 542 autosampler (ESA, Chelmsford, MA) was used inject 20 µL of each sample onto a Varian Microsorb-MV 100, 5 µm C-18 column, 250 x 4.6 mm (Varian Inc., Lake Forest, CA). The mobile phase used for detection of monoamines consisted of 11 mM citric acid, 0.81 mM 1-octanesulfonic acid sodium salt, 75 mM
sodium phosphate dibasic, and 11% methanol, at pH 4.4. Electrochemical detection of DA and 5-HT was performed with an LC-4C amperometric detector (Bioanalytical Systems, Inc., Lafayette, IN) using a 6 mm glassy working electrode maintained at a potential of 0.7 V relative to an Ag-AgCl reference electrode. Data was collected and analyzed using EZ Chrome software (Scientific Software, Pleasanton, CA). DA and 5-HT HPLC values were normalized to protein levels and presented as percent of Saline+Saline controls.

**Statistical Analyses**

Body temperatures and striatal extracellular glutamate concentrations were analyzed with a two-way analysis of variance (ANOVA) with repeated measures, followed by Tukey’s post hoc analyses. A two-way ANOVA was used to compare the effects of Meth or saline treatment and MLA or saline pretreatments on VMAT-2, DAT and SERT immunoreactivity and vesicular DA and 5-HT content. In order to compare the effects of MLA pretreatment in Meth treated rats, a post hoc t-test was used. In all cases where a two-way interaction was revealed, Tukey’s post hoc analyses were used to identify significant differences between treatment groups. For all experiments, statistical significance was set at p < 0.05.
RESULTS

Effects of MLA Pretreatment on Meth-Induced Hyperthermia

The effects of drug treatment on body temperatures are illustrated in Figure 1. Meth (10 mg/kg ip x 4 every 2 hr) administration significantly increased body temperature by approximately 3° C during the treatment period compared to those rats receiving saline treatment (F_{12,239} = 14.493, p < 0.001). Furthermore, MLA (5.5 mg/kg) pretreatment, administered 15 minutes before each Meth or saline injection, had no effect on Meth or saline-induced body temperatures. It is important to note anecdotally that MLA pretreatment did not appear to have any behavioral effects on its own and did not alter Meth-induced hyperlocomotion.

Effects of MLA Pretreatment on Meth-Induced Acute Increases in Extracellular Glutamate

Extracellular glutamate levels were measured prior to, during and after treatments, and are illustrated as percent of baseline glutamate (Fig. 2). The average baseline concentration of glutamate in the dialysate was 1359.89 ± 201.80 pg/20uL. Meth administration significantly increased striatal extracellular concentrations of glutamate over all other treatment groups, as revealed by a significant two-way interaction between drug treatment and time (F_{24,236} = 2.149, p < 0.001). Tukey’s post hoc analyses indicated that the Saline+Meth treatment group produced a significant 2.5 fold increase, compared to all the other treatment groups at hours 7 and 8 (p < 0.05). MLA pretreatment significantly blocked Meth-induced increases in extracellular glutamate, as the MLA+Meth group was significantly different from Saline+Meth at
hours 6, 7 and 8 (p < 0.05). MLA+Meth was not different from saline treated controls at any time.

Effects of MLA Pretreatment on Long-Term Meth-Induced Decreases in Striatal Monoaminergic Markers

Rats were treated with Meth (10 mg/kg i.p. every 2 hr x 4) or saline (1 mL/kg i.p. every 2 hr x 4) and pretreated with MLA (5.5 mg/kg) or saline, 15 minutes prior to each Meth or saline injection. Seven days after treatment, markers of dopaminergic and serotonergic terminals were assessed in the striatum.

Figure 3 illustrates striatal vesicular VMAT-2 immunoreactivity 7 days following treatment. Meth treatment significantly decreased VMAT-2 immunoreactivity, as revealed by a significant main effect of Meth treatment (F1,47 = 27.358, p < 0.001). MLA pretreatment had no effect in saline treated rats. MLA+Meth treated rats showed a partial protection in VMAT-2 compared to Saline+Meth rats (27±7 % depletion in the MLA+Meth versus a 65±7 % depletion in the Saline+Meth), as illustrated by a significant two-way interaction between Meth treatment and MLA pretreatment (F1,47 = 4.046, p < 0.05). Tukey’s post hoc analyses revealed that Saline+Meth treatment produced a significant 65±7 % depletion of VMAT-2 immunoreactivity as compared to Saline+Saline treated rats (p < 0.05). MLA+Meth treatment produced a slight but significant 27±7 % depletion as compared to the MLA+Saline treated group (p < 0.05).

Vesicular DA content was measured in the striatum 7 days after treatment (Fig. 4). Meth significantly depleted vesicular DA content and MLA pretreatment completely blocked Meth-induced DA depletions (13±10 % depletion in the MLA+Meth versus a
54±6 % depletion in the Saline+Meth), as revealed by a two-way interaction between
drug treatment and pretreatment (F_{1,62} = 5.699, p < 0.05). Tukey’s post hoc analyses
indicated that while Saline+Meth treated rats had a significant 54±6 % vesicular DA
depletion when compared to Saline+Saline and MLA+Saline treatments (p < 0.05),
MLA+Meth treatment resulted in a non-significant, 13±10 % depletion from
Saline+Saline treated rats, and no depletion when compared to MLA+Saline treated
rats.

Figure 5 illustrates DAT immunoreactivity in striatal synaptosomes, 7 days after
treatment. Meth produced a significant depletion of striatal DAT immunoreactivity, as
revealed by a main effect of Meth treatment (F_{1,39} = 14.785, p < 0.001). Post hoc t-tests
revealed that Saline+Meth treatment produced a 58±9 % depletion of DAT compared to
Saline+Saline controls (p < 0.05) and MLA+Meth treatment was significantly (p < 0.05)
different from Saline+Meth treatment (a 27±10 % depletion in the MLA+Meth versus a
58±9 % depletion in the Saline+Meth), but did not differ from Saline+Saline or
MLA+Saline treatment. Furthermore, MLA pretreatment had no effect on saline treated
rats.

Vesicular serotonin content was measured in the striatum 7 days after treatment
(Fig.6). Meth treatment significantly depleted vesicular serotonin content, as indicated
by a significant main effect of Meth treatment (F_{1,17} = 9.348, p < 0.05). Saline+Meth
treated rats had a significant 54±10 % depletion of vesicular serotonin content as
compared to Saline+Saline treated rats (p < 0.05), as indicated by a Tukey’s post hoc
analysis. MLA pretreatment had no effect on saline or Meth-induced depletions of
vesicular serotonin, indicated by the 56±5 % depletion in the MLA+Meth treated group, as compared to the MLA+Saline treated group.

Figure 7 illustrates SERT immunoreactivity in striatal synaptosomes, 7 days after treatment. Meth treatment significantly decreased SERT immunoreactivity in striatal synaptosomes, as revealed by a significant main effect of drug treatment ($F_{1,15} = 6.01, p < 0.05$). Tukey’s post hoc analyses indicated a significant 51±20 % depletion of SERT in the Saline+Meth group compared to the Saline+Saline group ($p < 0.05$) and the MLA+Meth treatment resulted in a significant 45±21 % decrease in SERT as compared to MLA+Saline treatment ($p < 0.05$). Thus, MLA had no effect on SERT in saline or Meth treated rats.
DISCUSSION

The role of the α7 nACh receptor in Meth-induced increases in extracellular glutamate and long-term damage to DA and 5-HT terminals in the rat striatum was investigated. Antagonism of the α7 nACh receptor with MLA blocked Meth-induced increases in striatal extracellular glutamate. In addition, MLA blocked Meth-induced decreases in markers of striatal DA terminals including DAT and vesicular dopamine content without affecting markers of 5-HT terminals or Meth-induced hyperthermia.

Meth significantly increased body temperature but MLA pretreatment did not affect Meth-induced hyperthermia (Fig 1). Hyperthermia is known to play an important role in the toxic effects of Meth (Bowyer et al., 1992). Meth may cause hyperthermia through an increase in acetylcholine (ACh) release (Taguchi et al., 1998), as there is evidence that ACh is involved in thermoregulation (Crawshaw, 1973). Because MLA did not alter Meth-induced hyperthermia, the effects of MLA on the acute increases in extracellular glutamate and the long-term decreases in DA and 5-HT terminal markers after Meth are likely due to its pharmacological properties that are distinct from the role of ACh in thermoregulation. Therefore, the α7 nACh receptor does not appear to be involved in Meth-induced hyperthermia.

The current results confirm that Meth administration increases extracellular glutamate in the rat striatum (Nash and Yamamoto, 1992; Abekawa et al., 1994) and support previous in vitro studies which demonstrated that the α7 nACh receptor modulates glutamate release. Nicotinic receptor agonists did not affect basal release of [3H]-d-aspartate from isolated rat striatal synaptosomes; however, the nicotinic receptor agonist, anatoxin-a, enhanced [3H]-d-aspartate overflow when synaptosomes were
depolarized with potassium chloride (Marchi et al., 2002). This effect was inhibited by the α7 nACh receptor antagonists, alpha-bungarotoxin and MLA, suggesting that α7 nACh receptors regulate glutamate release from striatal presynaptic glutamatergic terminals. More recently, nicotine or choline was found to stimulate glutamate release in the prefrontal cortex in a manner that was attenuated or blocked, respectively, by α-bungarotoxin (Konradsson-Geuken et al., 2009). Regardless, the present study is the first report that antagonism of the α7 nACh receptor can block Meth-induced increases in striatal extracellular glutamate.

As high extracellular concentrations of glutamate have been implicated in excitotoxic damage to striatal DA and 5-HT neurons (Storey et al., 1992; Staszewski and Yamamoto, 2006) and MLA blocks Meth-induced increases in extracellular striatal glutamate, we hypothesized that MLA would also block Meth-induced long-term damage to striatal dopaminergic and serotonergic terminals. Pretreatment with MLA, 15 minutes prior to each Meth injection, blocked Meth-induced decreases in vesicular DA content (Fig 4) and DAT (Fig 5) immunoreactivity, while having no effect on Meth-induced decreases in markers of 5-HT terminals (Fig 6 & 7). MLA only partially protected against Meth-induced decreases in VMAT-2 immunoreactivity (Fig 3), which most likely reflects the selective protection of DA terminals despite the localization of VMAT-2 to both DA and 5-HT terminals and the long-term damage to both DA and 5-HT terminals produced by Meth.

These data appear to be in opposition to the protective effects of nicotine against Meth-induced striatal dopaminergic damage. Nicotine binds non-selectively to all nicotinic receptors and its protective effects are likely due to activation of nACh
receptors other than α7 nACh receptors. In fact, while acute nicotine pretreatment protected against Meth-induced decreases in [3H]mazindol binding sites in wild-type mice, nicotine had no effect in α4 nACh receptor subunit knockout mice (Ryan et al., 2001). In addition, there is evidence that nicotine has a greater affinity for nACh receptors containing the α4 subunits (Xiao et al., 2009) suggesting that nicotine affords protection through activation of nACh receptors containing α4 subunits whereas ACh affects glutamate release and excitotoxicity through the α7 receptor.

Escubedo and colleagues suggested that MLA protects against Meth-induced striatal dopaminergic damage in mice through the attenuation of Meth-induced oxidative stress (Escubedo et al., 2005); however the initiation of a pro-oxidant process was not identified. The ability of MLA to attenuate oxidative stress may be related to the blockade of Meth-induced increases in extracellular glutamate (Fig 2). It is well known that glutamate, via activation of its ionotropic receptors, activates nitric oxide synthase (NOS) and produces nitric oxide (NO) and peroxynitrite (ONOO⁻) (Bhardwaj et al., 1997). Furthermore, NO and ONOO⁻ can damage proteins of monoaminergic terminals. For example, tyrosine hydroxylase and tryptophan hydroxylase are inactivated after nitration by NO and ONOO⁻ in rat tissue (Kuhn and Geddes, 1999; Sadidi et al., 2005) and DAT function in humans is inhibited by ONOO⁻ (Park et al., 2002). In addition, nNOS inhibition prevents Meth-induced nitrosylation of VMAT-2 1 hour after treatment, and prevents long-term decreases in VMAT-2 and DAT immunoreactivity and vesicular DA content in the rat striatum (Eyerman and Yamamoto, 2007). Serotonin terminals are also vulnerable to oxidative stress as evidenced by the attenuation of Meth-induced...
SERT depletions in copper-zinc superoxide dismutase (CuZnSOD) transgenic mice (Hirata et al., 1995).

Despite the similarities in the oxidative mechanisms that mediate damage to both DA and 5-HT terminals, the reasons for the selective protection to DA terminals afforded by MLA are unclear. Key differences may reside in the specificity of interactions between neurotransmitter systems in the striatum. One possibility is that DA and 5-HT terminals differentially express calcium permeable α7 nACh receptors. Therefore, the effects of MLA would be relatively selective for the cholinergic modulation of calcium influx (Seguela et al., 1993) into dopaminergic terminals via α7 nACh receptors. However, there is no direct evidence for the localization of α7 nACh receptors to DA terminals and the effect of α7 nACh ligands on DA release in the striatum is most likely mediated through the modulation of glutamate release (Wonnacott et al., 2000; Marchi et al., 2002). Another possibility that could explain the relatively selective effect of MLA on the protection against the long-term decreases in markers of DA compared to 5-HT terminals is the differential expression of calcium permeable NMDA and AMPA receptors on dopaminergic and serotonergic terminals. There is evidence of calcium permeable NMDA and AMPA receptors on striatal DA terminals (Segovia et al., 1997; Hernandez et al., 2003) but less evidence of their expression on 5-HT terminals. However, there are studies indicating that both DA and 5-HT neurons are susceptible to excitotoxic damage in response to excess glutamate (Storey et al., 1992; Staszewski and Yamamoto, 2006). A third possibility is that glutamatergic terminals that synapse onto DA terminals express α7 nACh receptors, while those that synapse onto 5-HT terminals do not express α7 nACh receptors. Therefore, glutamatergic projections that
synapse onto 5-HT terminals would be refractory to the antagonist effects of MLA, continue to release glutamate in response to Meth despite the presence of MLA, and eventually damage 5-HT terminals. This too is unlikely because MLA completely blocked Meth-induced increases in striatal extracellular glutamate (Fig 2).

A more likely explanation for why MLA protects against Meth-induced DA terminal damage, and not 5-HT terminal damage, is that the damage to 5-HT terminals produced by Meth is not mediated by a glutamate dependent mechanism but occurs either through inflammation, mitochondrial dysfunction or hyperthermia. Pro-inflammatory mechanisms, such as microglial activation, are observed after Meth and MDMA administration (Thomas et al., 2004). Furthermore, MDMA-induced decreases in SERT binding are prevented by minocycline pretreatment, which also prevents MDMA-induced NFκB activation, IL-1β release and microglial activation (Orio et al., 2010). MLA, however, has been shown to prevent Meth-induced microglial activation (Escubedo et al., 2005), which may be dependent on the ability of MLA to prevent increases in glutamate and the subsequent activation of AMPA and kainate receptors on microglia (Noda et al., 2000).

Previous studies have shown that mitochondrial dysfunction or metabolic compromise is involved in serotonergic damage produced by Meth as evidenced by the ability of malonate, a mitochondrial complex II inhibitor, to synergize with Meth to cause serotonergic damage (Burrows et al., 2000). In addition, α7 nACh receptors have been implicated in ethanol-induced mitochondrial dysfunction (Li et al., 2002). However, there is little evidence for a differential role of mitochondrial dysfunction in mediating damage to 5-HT versus DA terminals that would explain the selective protection afforded by
MLA. Therefore, the most likely explanation for differential effects of MLA on DA and 5-HT terminals is the dependence on glutamate and the α7 nACh receptor for Meth-induced damage to DA terminals relative to the importance of hyperthermia in mediating damage to 5-HT terminals (LaVoie and Hastings, 1999; Haughey et al., 2000).

In conclusion, while both DA and 5-HT terminals are damaged by Meth, the α7 nACh receptor mediates damage to DA but not 5-HT terminals in a manner that is independent of hyperthermia and is dependent on the release of glutamate. Therefore, antagonism of α7 nACh receptors may be a plausible way of decreasing extracellular glutamate and preventing excitotoxic damage in other neurodegenerative disorders, such as Parkinson’s Disease.
Authorship Contributions

Participated in research design: Northrop, Smith, Yamamoto and Eyerman.

Conducted experiments: Northrop, Smith and Eyerman.

Contributed new reagents or analytic tools: Yamamoto.

Performed data analysis: Northrop, Smith, Yamamoto and Eyerman.

Wrote or contributed to the writing of the manuscript: Northrop, Yamamoto and Eyerman.
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Footnotes

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b) This work was presented at the Society for Neurosciences Conference, 2010.

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FIGURE LEGENDS

Figure 1. Rectal temperatures after Meth or saline exposure. Rectal temperatures were significantly increased over the period of Meth treatment (***, p < 0.001). MLA pretreatment, administered 15 min before each Meth or saline injection, had no effect on saline or Meth-induced body temperatures. (n=10-14 for each group) Larger arrows indicate Meth or saline injections and smaller arrows indicate MLA or saline pretreatment injections.

Figure 2. Striatal extracellular glutamate concentrations during Meth or saline treatment. Meth significantly increased glutamate release over time and MLA completely blocked the Meth-induced increase in glutamate (*, p < 0.05, Saline+Meth compared to Saline+Saline; #, p < 0.05; ##, p < 0.01, MLA+Meth compared to Saline+Meth). (n=6-9 for each group) Larger arrows indicate Meth or saline injections and smaller arrows indicate MLA or saline pretreatment injections.

Figure 3. Striatal vesicular VMAT-2 immunoreactivity 7 days after Meth or saline treatment. A) Meth administration significantly decreased VMAT-2 immunoreactivity and MLA pretreatment significantly attenuated the Meth-induced decrease (***, p < 0.001, Saline+Meth compared to Saline+Saline; ##, p < 0.01, MLA+Meth compared to Saline+Meth; &, p < 0.05, MLA+Meth compared to MLA+Saline). (n=8-14 for each group) B) Representative VMAT-2 Western Blot image illustrating VMAT-2 at 68 kDa.
Figure 4. Striatal vesicular DA content 7 days after Meth or saline treatment. Meth administration significantly depleted vesicular DA content and MLA pretreatment significantly blocked the Meth-induced depletion. (***, p < 0.001, Saline+Meth compared to Saline+Saline; ##, p < 0.01, MLA+Meth compared to Saline+Meth) (n=8-22 for each group)

Figure 5. Striatal synaptosomal DAT immunoreactivity 7 days after Meth or saline treatment. A) Meth administration significantly decreased DAT immunoreactivity, and MLA pretreatment significantly blocked the Meth-induced decrease. (***, p < 0.001, Saline+Meth compared to Saline+Saline; #, p < 0.05, MLA+Meth compared to Saline+Meth) (n=8-12 for each group) B) Representative Western Blot image illustrating an immunoreactive band for DAT at 70 kDa and α-tubuin at 50 kDa.

Figure 6. Striatal vesicular 5-HT content 7 days after Meth or saline treatment. Meth treatment significantly depleted in vesicular 5-HT content, in both saline and MLA pretreated rats. (**, p < 0.01) (n=4-8 for each group)

Figure 7. Striatal synaptosomal SERT immunoreactivity 7 days after Meth or saline treatment. A) Meth treatment significantly depleted synaptosomal SERT immunoreactivity, in both saline and MLA pretreated rats. (**, p < 0.01) (n=4-8 for each group) B) Representative Western Blot image illustrating an immunoreactive band for SERT at 68 kDa and α-tubuin at 50 kDa.
Figure 1

- **Saline+Saline**
- **MLA+Saline**
- **Saline+Meth**
- **MLA+Meth**

**Body Temperature (°C)**

**Time After First Treatment Injection (hours)**

![Graph showing body temperature changes over time for different treatment groups.](image-url)
Figure 2
**Figure 4**

Bar graph showing vesicular DA content (% Saline+Saline) for different conditions:
- **Saline+Saline**
- **MLA+Saline**
- **Saline+Meth**
- **MLA+Meth**

Statistical symbols: ******* and **##** indicate significant differences.
Figure 5

A) Synapsosomal DAT Immunoreactivity (% Saline-Saline)

- Saline+Saline
- MLA+Saline
- Saline+Meth
- MLA+Meth

B) Western blot analysis

- Saline + Saline
- MLA + Saline
- Saline + Meth
- MLA + Meth

Markers:
- DAT 75 kDa
- α-tubulin 50 kDa
Figure 6

Vesicular 5-HT Content (% Saline+Saline)

Saline+Saline  MLA+Saline  Saline+Meth  MLA+Meth

**  **
Figure 7

A)

- A bar graph showing synaptic SERT immunoreactivity (%) normalized to Saline+Saline.
- Treatments: Saline+Saline, MLA+Saline, Saline+Meth, MLA+Meth.
- * indicates statistical significance.

B)

- A Western blot image comparing saline and MLA treatments in the presence or absence of methamphetamine.
- Marked bands at 68 kDa and 50 kDa correspond to SERT and α-tubulin, respectively.

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