Published in final edited form as:

Brain Res. 2007 July 9; 1157: 74–80. doi:10.1016/j.brainres.2007.04.056.

Methamphetamine exposure antagonizes *N*-methyl-D-aspartate receptor-mediated neurotoxicity in organotypic hippocampal slice cultures

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Abstract

Glutamatergic systems have been increasingly recognized as mediators of methamphetamine's (METH) pharmacological effects though little is known about the means by which METH interacts with glutamate receptors. The present studies examined effects of METH (0.1–100 μ M) on [³H] MK-801 binding to membranes prepared from adult rat cortex, hippocampus and cerebellum, as well as the neurotoxicity produced by 24-h exposure to *N*-methyl-D-aspartate (5–10 μ M; NMDA) employing organotypic hippocampal slice cultures of neonatal rat. Co-incubation of [³H]MK-801 with METH (0.1–100 μ M) did not reduce dextromethorphan (1 mM)-displaceable ligand binding. Exposure of slice cultures to NMDA for 24-h produced increases in uptake of the non-vital fluorescent marker propidium iodide (PI) of 150–500% above control levels, most notably, in the CA1 region pyramidal cell layer. Co-exposure to METH (>1.0 μ M) with NMDA (5 μ M) reduced PI uptake by approximately 50% in each subregion, though the CA1 pyramidal cell layer was markedly more sensitive to the protective effects of METH exposure. In contrast, METH exposure did not reduce PI uptake stimulated by 24-h exposure to 10 μ M NMDA. Co-exposure to the NMDA receptor antagonist D-2-amino-5-phosphonovaleric acid (20 μ M) prevented toxicity produced by exposure to 5 or 10 μ M NMDA. These findings indicate that the pharmacological effects of short-term METH exposure involve inhibition of NMDA receptor-mediated neuronal signaling, not reflective of direct channel inhibition at an MK-801-sensitive site.

Keywords

Stimulants; Glutamate; Hippocampus; Excitotoxicity; Amphetamine

1. Introduction

Methamphetamine (METH) use throughout the United States has markedly increased during recent decades (Office of National Drug Control Policy, 2003) and represents a significant public health concern, particularly given evidence that METH use has been reported to induce abnormalities in neuronal function and neuronal injury (Davidson et al., 2001; Gehrke et al., 2006; Rau et al., 2006; Ricaurte et al., 1982). These neurotoxic effects of METH abuse are widely thought to reflect changes in monoamines, glutamate and γ -amino-butyric acid

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signaling, the initiation of multiple pro-oxidant pathways, and to possibly correlate with volumetric changes in many cortical and subcortical brain regions (Ernst et al., 2000; Jernigan et al., 2005; Stephans and Yamamoto, 1994; Thompson et al., 2004, for a review, see Yamamoto and Bankson, 2005).

The hippocampus has been increasingly recognized as a brain region that is highly vulnerable to METH-induced changes in neuronal signaling. Short-term, repeated METH injection (3× at 2-h intervals) was reported to increase ventral hippocampal glutamate content (Rocher and Gardier, 2001), while chronic (16 days) exposure depleted whole hippocampal glutamate content (Kaiya et al., 1983). Others have reported depletions of serotonin (5-HT; Friedman et al., 1998); the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA; Ohmori et al., 1993); and dopamine (Anderson and Itzhak, 2006) with the use of similar, short-term METH exposure regimens. Notably, Ohmori et al. (1993) reported that pretreatment with antagonists of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors prevented METH-induced reductions in 5-HT and 5-HIAA content in the hippocampus. This suggests that NMDA receptors may mediate many of METH's pharmacological effects and that brain regions expressing high levels of these receptors, such as the hippocampus, may be particularly susceptible to METH-induced abnormalities in neuronal function.

Recent literature has suggested that METH may have direct actions on NMDA receptors. Castro et al. (1999) reported that exposure to high concentrations of dopamine (DA) produced voltage-dependent inhibition of NMDA-induced currents in native tissue. Yeh et al. (2002) reported that the structurally similar amphetamines displaced binding of [³H]*N*-[1-(2-thienyl)cyclohexyl] piperidine (TCP) to the NMDA receptor ion channel with low and high affinity, as well as, decreased NMDA receptor-mediated intracellular ⁴⁵Ca accumulation in rat cortical neurons. Further, Moriguchi et al. (2002) reported that prolonged METH exposure produced resistance of striatal NMDA receptors to Mg²⁺ blockade during METH withdrawal, suggesting a possible compensatory response to prolonged NMDA receptor inhibition involving concurrent activation of protein kinases C and A with subsequent phosphorylation of NMDA receptors.

This recent evidence suggests that METH may directly or indirectly influence NMDA receptor function, though the means by which it may do so has not been adequately elucidated. The present studies examined the ability of METH to interact with the NMDA receptor channel binding domain identified by the use of [³H]MK-801. Further, these studies examined the ability of brief (24 h) METH exposure to influence neuronal viability and NMDA-induced neurotoxicity employing an organotypic hippocampal slice preparation.

2. Results

2.1. [³H]MK801 binding

Binding of [³H]MK-801 to crude membranes was not significantly affected by co-incubation with METH at any concentration (0.1–100 μM; Table 1). In contrast, co-incubation of membranes with the NMDA receptor channel blocker dextromethorphan (1 mM) produced an approximately 75% inhibition of [³H]MK-801 binding [$F(4,19)=163.19$, $P<0.001$].

2.2. METH and NMDA effects on propidium iodide uptake

Twenty-four hours of exposure to METH (0.1–100 μM) in NMDA-naïve slice cultures failed to alter propidium iodide uptake, in any region of the hippocampal slice cultures (data not shown). Exposure of additional slices to NMDA (5 μM) resulted in a marked increase in uptake of propidium iodide, to approximately 230% of control values in the pyramidal cell layer of the CA1 region [$F(13, 337)=19.917$, $P<0.001$] and 120% of control values in the pyramidal

cell layer of the CA3 hippocampal region [$F(13, 340)=4.555, P<0.001$] (Fig. 1). This increase in propidium iodide uptake was prevented in both regions by co-exposure of slices to the NMDA receptor antagonist D-2-amino-5-phosphonovaleric acid (20 μM). This antagonist did not alter propidium iodide uptake in NMDA-naïve slice cultures. NMDA exposure did not produce cytotoxicity in the granule cell layer of the dentate gyrus. Post hoc analysis (Fisher's LSD test) further indicated that co-exposure to METH ($\geq 1.0 \mu\text{M}$) with NMDA for the 24-h incubation period produced significant reductions of approximately 25–40% in propidium iodide uptake, as compared to NMDA-treated controls, in the CA1 region. A similar reduction in NMDA-induced propidium iodide uptake was observed in the CA3 region, although only after exposure to the highest concentration of METH (100 μM ; all post hoc analyses, $P<0.05$). Exposure to METH at concentrations below 1 μM did not significantly reduce NMDA-induced neurotoxicity. Representative images of propidium iodide uptake are illustrated in Fig. 3.

A subsequent experiment was conducted to examine the ability of METH (1.0–100 μM) to attenuate the cytotoxicity produced by 24 h of exposure to 10 μM NMDA. Exposure of slices to this higher concentration of NMDA produced marked increases in propidium iodide uptake in each region of the slice cultures. In the pyramidal cell layer of the CA1 region, cytotoxicity was increased to nearly 500% above control values [$F(7, 109)=88.435, P<0.001$], whereas in the pyramidal cell layer of CA3 [$F(7, 109)=6.817, P<0.001$] and the granule cell layer of the dentate gyrus [$F(7, 117)=9.203, P<0.001$], it was elevated by approximately 230% and 200%, respectively (all post hoc analyses, $P<0.05$). Co-exposure to METH (1.0–100 μM) failed to attenuate NMDA-induced increases in propidium iodide uptake in any region of slice cultures (Fig. 2). Representative images of propidium iodide uptake are illustrated in Fig. 3.

3. Discussion

The present studies were designed to examine the ability of METH to alter binding of [^3H] MK-801 to an NMDA receptor channel binding site and to influence NMDA receptor-mediated neurotoxicity in hippocampal slice cultures. Previous work has suggested that glutamatergic systems, including the NMDA receptor system, may mediate METH-induced changes in monoamine content in several brain regions (Anderson and Itzhak, 2006; Friedman et al., 1998; Ricaurte et al., 1982). Further, others have demonstrated that METH, at low μM concentrations, partially inhibits NMDA receptor-mediated accumulation of intracellular Ca^{2+} in primary neuronal cell cultures of rat brain and reduces non-equilibrated, glycine and NMDA-stimulated [^3H]TCP binding in rat cortical membranes with $\text{IC}_{50}\text{'s}$ at low μM concentrations (Yeh et al., 2002). Notably, equilibrated [^3H]TCP binding was not altered by METH exposure below mM concentrations, suggesting a low-affinity binding site for METH. These findings were interpreted to suggest that METH may inhibit NMDA receptors via a high-affinity antagonism at an agonist site and a low-affinity channel blockade. The present studies employed a crude membrane preparation derived from rat cortex, hippocampus and cerebellum to examine effects of METH on [^3H] MK-801 binding. Incubation of ligand with METH (0.1–100 μM) did not result in a diminution of [^3H]MK-801 binding at any concentration of METH. In contrast, incubation of crude membranes with non-labeled NMDA receptor channel blocker dextromethorphan reduced [^3H]MK-801 by approximately 75%. These findings are consistent with those of Yeh et al. (2002) in suggesting that exposure to abuse-relevant concentrations of METH exposure does not produce high-affinity blockade of the NMDA receptor channel.

It is clear, though, that METH acts as a function antagonist of NMDA receptor activity or as a mediator of downstream effectors stimulated by NMDA receptor activity. As noted above, Yeh et al. (2002) demonstrated that METH attenuated NMDA-induced ^{45}Ca accumulation in primary cortical cultures of rodents. In the present studies, exposure to 5 μM NMDA produced a nearly 150% increase in cytotoxicity in CA1 region pyramidal cells and a more modest increase in the CA3 pyramidal cell layer. These effects were completely blocked by co-

exposure to the NMDA receptor channel blocker APV and were, most interestingly, reduced by approximately 50% by co-exposure to METH ($\geq 1.0 \mu\text{M}$). In contrast, exposure to $10 \mu\text{M}$ NMDA produced 150–600% increases in cytotoxicity in the primary neuronal layers of each slice culture subregion that was blocked by APV, but not METH, co-exposure. These data demonstrate functional antagonism of NMDA receptor-mediated toxicity at concentrations of METH 100-fold less than that previously reported (Yeh et al., 2002). Competitive pharmacologic binding studies including METH and [^3H]-NMDA would be of use in identifying the extent to which METH may interact at an NMDA binding site. Additionally, METH may influence the activity of signaling effectors downstream of NMDA receptor activation (i.e. Ca^{2+} -binding proteins including calbindin-D 28K) in a reversible manner.

The concentrations of METH chosen for use in these studies are similar to those achieved both during single and repeated injection of moderate doses of the drug in both humans and rats (Cho et al., 2001; Melega et al., 2007). Further, Chung et al. (2004) reported that lethality associated with METH use (including cardiac death and traumatic injury) was associated with plasma levels of approximately $3 \mu\text{M}$ – 1 mM in a sample of South Korean individuals. Perhaps most significantly, Melega et al. (1995) demonstrated that rat brain METH concentrations were approximately ten times greater than were plasma levels following a single METH injection suggesting that it may be sequestered in lipids in the CNS. This may suggest that CNS levels of METH approach high μM concentrations particularly with repeated use.

Regarding the marked topographical differences in NMDA toxicity observed, a large number of studies have demonstrated that pyramidal cells of the CA1 hippocampal region are more highly sensitive to the neurotoxic effects of NMDA receptor activation than are those of the CA3 region or neurons of the dentate gyrus (Mulholland and Prendergast, 2003; Prendergast et al., 2004; Sakaguchi et al., 1997). Our findings are fully consistent with this literature in demonstrating that exposure to a low concentration of NMDA ($5 \mu\text{M}$) produced cytotoxicity only in the pyramidal cell layer of the CA1 region but that exposure to a higher concentration ($10 \mu\text{M}$) of NMDA produced toxicity in other subregions of the slice cultures most proximal to CA1 pyramidal cell projection fields. It may be suggested that the heightened sensitivity of CA1 pyramidal cells to excitotoxic insult, as compared to other regions of the hippocampal formation, may represent the greater density of NMDA receptors (Martens and Wree, 2001); the resistance of the subtype of NMDA receptors in this region to Mg^{2+} block (Sakaguchi et al., 1997); and/or the relatively low level of expression of the Ca^{2+} buffering protein calbindin-D28K (Prendergast et al., 2002). It is interesting to note that the modest increase in propidium iodide uptake in the CA3 region produced by exposure to NMDA ($5 \mu\text{M}$) was not attenuated by the lowest two concentrations of METH, whereas exposure to each concentration of METH reduced toxicity in the CA1 region. While the reason for this discrepancy is not clear, we have observed that the CA1 soma and projection layers possess a greater concentration of polyamine-sensitive NR2 subunits of the NMDA receptor than do the CA3 and dentate regions. Thus, this differential sensitivity to METH antagonism of NMDA toxicity may be related to differences in patterns of NR subunit expression in the CA1 and CA3 region.

In summary, the present studies demonstrate that METH, at clinically relevant concentrations, acts as a functional antagonist of NMDA receptor activation. The implications of this action for understanding the behavioral and/or neurotoxic effects of METH use are unclear, however. For example, acute blockade of hippocampal NMDA receptors, in particular, may contribute to the cognitive impairment observed with acute administration of moderate and high doses of METH (i.e. Shoblock et al., 2003). With repeated use, persistent antagonism of NMDA receptor function may produce a compensatory upregulation of NR subunits (as has been reported with exposure to other NMDA receptor antagonists; i.e. Harris et al., 2003), leading to neuronal hyperexcitability and possibly NMDA receptor-mediated excitotoxicity during abstinence from METH use. It will be of importance in further studying METH effects on

NMDA receptor function to examine these issues so as to clarify the relevance of this interaction to the clinical use of METH and related stimulants.

4. Experimental procedures

4.1. [³H]MK-801 binding assay

Sixty-day-old male Sprague–Dawley rats (Harlan Laboratories; Indianapolis, ID, USA) were sacrificed and the hippocampus, cortex, and cerebellum were rapidly removed and placed into a vial of buffer (0.32 M sucrose in 50 mM Tris buffer, pH 7.4; Fisher; St. Louis, MO, USA) for 10 min. All aspects of the binding protocols were performed on ice (4 °C). The tissue was then homogenized for three 30-s intervals and centrifuged at 1000×g for 10 min. The supernatant was removed and stored. The pellet was resuspended in buffer (0.32 M sucrose in 50 mM Tris buffer, pH 7.4) and then was centrifuged again at 1000×g for 10 min. The reserved supernatants were then combined and centrifuged at 49000×g for 20 min. The pellet was resuspended in buffer (10 ml of 50 mM Tris buffer) and washed three times at 49000×g for 20 min. The protein content was then estimated utilizing the Pierce Reagent assay. The tissue homogenates were then aliquoted and stored at –80 °C.

Binding assays were conducted to determine if +-meth (METH; Sigma; St. Louis, MO, USA) would influence the binding of [³H]MK801 to the NMDA ion channel under non-equilibrated binding conditions. Microtiter plates (Corning; NY, USA) were filled with 100 µl of [³H]MK801 at 2 nM (NEN Life Sciences; Boston, MA, USA) in Tris buffer (50 mM). Concentrations of METH (0.1–100 µM) in Tris buffer (50 mM) were also added to each well. To determine non-specific [³H]MK801 binding, additional membranes were co-incubated with 1 mM dextromethorphan hydrobromide monohydrate (Sigma). The addition of the tissue homogenate (100 µl; 0.15 mg protein/well) began the binding reaction. The binding solution was incubated for 45 min and terminated with the removal of the well contents. The filters were then washed three times with 350 µl of Tris buffer (50 mM). Filters were allowed to dry overnight and then were wetted with 35 µl of Microscint 20 scintillation cocktail (Packard Instruments; Meriden, CT, USA). Counting took place on a 96-well scintillation counter (Packard Instruments; Meriden, CT, USA). When appropriate, quench corrections were made based upon values from a standard nitromethane curve.

4.2. Hippocampal cell culture

Eight-day-old, male and female, Sprague–Dawley rat pups (Harlan Laboratories; Indianapolis, ID, USA) were sacrificed and brains were aseptically removed. After the brains were extracted, they were transferred to ice-cold dissecting medium, composed of Minimum Essential Medium (MEM) (Gibco BRL, Gaithersburg, MD), 25 mM HEPES, 200 mM glutamine and 50 µM streptomycin/penicillin. Bilateral hippocampi were then removed in whole. Hippocampi were then cleaned of extra tissue and placed in culture media (containing dissection medium with the addition of 36 mM glucose, 25% Hanks' balanced salt solution (HBSS) (Gibco BRL, Gaithersburg, MD) and 25% heat-inactivated horse serum (HIHS) (Sigma, St. Louis, MO). Afterward, unilateral hippocampi were then sectioned coronally at 200 µm using the McIlwain Tissue Chopper (Campden Instruments Ltd; Lafayette, ID, USA). Once sectioned three intact hippocampi slices were plated onto Millicell-CM biopore membrane inserts, with 1 ml of pre-incubated culture medium added to the bottom of each well. Plates were then placed in an incubator, at 37 °C with a gas composition of 5% CO₂, 21% oxygen and 74% nitrogen for 5 days to allow hippocampi to affix to the silicon membrane. Care of all animals was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23).

After the initial 5-day period during which slices affixed to membranes, tissue was maintained with culture media until 19 days in vitro (DIV) with medium changed every 5 days. At DIV 19 slice cultures were treated with METH (0.1–100 μ M) in cell culture medium with the addition of PI or were treated with the same media (METH and PI), but with the addition of NMDA (5 or 10 μ M) for 24 h. Additional cultures were exposed to NMDA with the NMDA receptor antagonist D-2-amino-5-phosphonovaleric acid (20 μ M). Following treatment exposure, all cultures were imaged and cytotoxicity was assessed as described below. METH (0.1–100 μ M) and NMDA (5 and 10 μ M) (Sigma, St. Louis, MO) were dissolved in cell culture medium and applied to the bottom of culture plate wells in one ml increments. Propidium iodide (PI; 3.74 μ M, Molecular Probes, Eugene, OR), a nucleic stain used to detect cell damage, was also utilized to detect cytotoxicity as described below.

4.3. Cytotoxicity assessment

PI only penetrates cell membranes of damaged or potentially dying cells, binding to DNA to produce a bright red fluorescence at 630 nm (Zimmer et al., 2000). Hippocampi were visualized with SPOT Advanced version 4.0.2 software for Windows (W. Nuhsbaum Inc.; McHenry, IL, USA) at a 5 $\times$ objective with a Leica DMIRB microscope (W. Nuhsbaum Inc.; McHenry, IL, USA) that is fitted for fluorescent detection (Mercury-arc lamp). The emission wavelength of PI is 620 nm in the visual range and has a peak excitation wavelength of 536 nm. PI was excited using a band-pass filter exciting wavelengths between 510 and 560 nm. Intensity of the PI fluorescence was then analyzed by densitometry using NIH Image in the pyramidal cell layers of the CA1 and CA3 regions, as well as, the granule cell layer of the dentate gyrus regions of the hippocampus. To minimize variability in PI uptake between replications, each replication was converted to percent control before analysis.

4.4. Statistics

To analyze effects of METH on [3 H]MK-801 binding, a one-way ANOVA was used (Systat Software, Inc., Point Richmond, CA) to compare the counts/minute generated by scintillation counting of membranes for each treatment group. For studies employing organotypic slice cultures, two-way ANOVAs were initially employed analyzing the effects of exposure to NMDA and/or METH in each slice culture subregion separately, based on the a priori hypothesis that the CA1 region will selectively demonstrate NMDA-induced neurotoxicity. Previous studies have shown that the CA1 region of the hippocampus is more sensitive to excitotoxic stimuli (Avignone et al., 2005; Gee et al., 2006; Self et al., 2005). As no sex differences in NMDA or METH effects were observed in any subregion of slice cultures, data were collapsed across sex and one-way ANOVAs of the different areas were performed to compare treatments within each individual area of the hippocampus. Statistical outliers were discovered using the Grubb's test for outliers (Grubbs, 1969). Post hoc comparisons were made using the Fisher LSD Method (Hayter, 1986). Significance was set at $P < 0.05$. All analyses were conducted on data converted to percent control, to compensate for variability observed across weeks.

Acknowledgements

Portions of this work were supported by DA016176.

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Fig. 1. Propidium iodide uptake following 24 h of exposure to NMDA (5 μ M) and methamphetamine (METH). Cytotoxicity observed in the CA1 and CA3 pyramidal cell layers following NMDA exposure was markedly reduced by APV (20 μ M) or METH co-exposure in both regions. * P <0.001 vs. control; ** P <0.001 vs. NMDA.

Fig. 2.

Propidium iodide uptake following 24 h of exposure to NMDA (10 μ M) and methamphetamine (METH; 1–100 μ M). Cytotoxicity observed in the CA1 and CA3 pyramidal cell layers following NMDA exposure was markedly reduced by APV (20 μ M) co-exposure in both regions, but not by METH co-exposure. * $P < 0.001$ vs. control; ** $P < 0.001$ vs. NMDA.

Fig. 3. Representative images of propidium iodide uptake in organotypic hippocampal slice cultures. (A) Control; (B) NMDA (5 μ M); (C) NMDA (5 μ M)+METH (100 μ M); (D) NMDA (10 μ M); (E) NMDA (10 μ M)+METH (100 μ M).

Table 1

Effects of co-exposure to dextromethorphan (dextro.) or (+)-methamphetamine (meth.) on [³H]MK-801 binding to crude membrane fractions

Drug	% control [³ H]MK-801 binding
Dextro. (1 mM)	25.74±2.19*
Meth. (0.1 μM)	101.41±3.43
Meth. (1 μM)	98.64±1.21
Meth. (10 μM)	109.37±3.4
Meth. (100 μM)	99.29±0.4

* $P < 0.05$ vs. [³H]MK-801 binding alone.