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Potential of *N*-Methyl-D-Aspartate receptor-mediated neuronal injury during methamphetamine withdrawal in vitro requires co-activation of IP₃ receptors

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Abstract

Recent findings suggest that methamphetamine (METH) functions acutely to inhibit *N*-methyl-D-aspartate (NMDA) receptor function. Protracted withdrawal from METH exposure may increase the sensitivity of NMDA receptors to agonist exposure, promoting neuronal excitability. However, the relevance of METH effects on NMDA receptor activity with regard to neuronal viability has not been studied. The present studies examined the effects of protracted METH exposure (6 or 7 days; 1.0–100 μ M) and withdrawal (1 or 7 days) on NMDA receptor-dependent neurotoxicity, determined with use of the non-vital fluorescent marker propidium iodide, in organotypic slice cultures of male and female rats. Prolonged exposure to METH (100 μ M) produced only modest toxicity in the granule cell layer of the dentate gyrus. Withdrawal from METH exposure (1 or 7 days) did not produce overt neuronal injury in any region of slice cultures. Exposure to NMDA (5 μ M) produced marked neurotoxicity in the CA1 pyramidal cell layer. Neither co-exposure to METH nor 1 day of METH withdrawal in combination with NMDA exposure altered NMDA-induced neurotoxicity. In contrast, protracted withdrawal from METH exposure (7 days) was associated with a marked (~400%) increase in NMDA-induced neurotoxicity in CA1 region pyramidal cells. This potentiation of neurotoxicity was prevented by co-exposure to the selective NMDA receptor antagonist 5-2-amino-5-phosphonovaleric acid (20 μ M) and was markedly attenuated by co-exposure of slices to xestospongin C (1 μ M), an antagonist of IP₃ receptors. The results of the present studies suggest that long-term METH withdrawal functionally sensitizes the NMDA receptor to agonist exposure and requires the co-activation of NMDA and IP₃ receptors.

Keywords

stimulants; drug abuse; amphetamine; glutamate; brain injury

1. Introduction

Methamphetamine (METH) use represents a significant public health concern in the United States. In 2005, 4.3% of Americans 12 years or older reported using METH for nonmedical purposes at least once their lifetime (Substance Abuse and Mental Health Services Administration, 2005). METH use has been associated with several significant health risks,

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including cardiac arrhythmia, stroke, increased blood pressure, hyperthermia, and CNS abnormalities. (Davidson et al., 2001, Ricaurte et al., 1982). CNS abnormalities associated with METH use, including volumetric deficits, have been reported in a variety of brain regions (Ernst et al., 2000; Thompson et al., 2004; Volkow et al., 2001) and are thought to reflect changes in the signaling and metabolism of multiple neurotransmitters, including dopamine, serotonin and glutamate (Davidson et al., 2001, Spina & Cohen, 1989, Rocher and Gardier, 2001; Yamamoto & Zhu, 1998; Zhang et al., 2006, reviewed by Cadet et al. 2007; Tat and Yamamoto 2007). In some individuals, these abnormalities correlate with cognitive deficits during both active METH use and early abstinence (Kalechstein et al., 2003, Simon et al., 2000).

It has become increasingly evident that *N*-Methyl-D-Aspartate-type glutamate receptors are targets of METH actions (Moriguchi et al., 2002; Smith et al., 2007; Yeh et al., 2002). Previous literature has suggested that METH may directly or indirectly influence the NMDA receptor in a biphasic manner (Moriguchi et al., 2002; Smith et al., 2007; Yeh et al., 2002). Short-term, repeated administration of METH to rodents has been shown to increase hippocampal and striatal glutamate content (Mark et al. 2007; Rocher and Gardier, 2001) while long-term METH administration was shown to deplete hippocampal glutamate content (Kaiya et al. 1982). Amphetamines have been shown to displace [³H]N-[1-(2-thienyl)cyclohexyl] piperidine (TCP) binding to the NMDA receptor ion channel, in addition to, decreasing NMDA-induced intracellular ⁴⁵Ca accumulation in rat cortical neurons (Yeh et al., 2002). A recent report similarly demonstrated that short-term METH (≥1.0 μM) exposure antagonizes NMDA-induced neurotoxicity in organotypic hippocampal slice cultures, suggesting direct or indirect modulation of NMDA receptor activity (Smith et al., 2007). However, METH antagonism of the NMDA receptor does not appear to occur at the MK-801 sensitive channel site. Evidence that METH may act as a functional antagonist of the NMDA receptor, either by direct interactions or subsequent to METH-induced glutamate release (ie. desensitization, after Krupp et al. 1996), suggest that long-term METH exposure may result in adaptive changes of NMDA receptor abundance or function during withdrawal. In fact, Moriguchi et al. (2002) have reported that long-term METH exposure followed by long-term withdrawal may reduce Mg²⁺ blockade of the NMDA receptor resulting in increased EPSPs.

Although previous work suggests that METH either directly or indirectly influences function of some NMDA receptors, little is known of the consequences of this influence with regard to METH withdrawal. Therefore, the present studies examined the ability of long-term METH exposure or withdrawal to influence NMDA receptor mediated neurotoxicity in organotypic hippocampal slice cultures.

2. Results

2.1: Continuous Methamphetamine Exposure & NMDA Toxicity

Organotypic hippocampal slice cultures were initially exposed to METH (1.0-100 μM) for 7 days. During the final day of METH exposure, one-half of cultures were also exposed to NMDA (5 μM). Twenty-four hours later, no significant increases in propidium iodide (PI) uptake were observed in control (CTRL) or METH treated tissue in the CA1 and CA3 pyramidal cell layers. PI is a highly polar nucleic acid marker that only labels cells with marked membrane compromise (Zimmer et al. 2000). However, 7 days of exposure to the highest concentration of METH did produce a modest but significant increase in PI uptake in the granule cell layer of the DG (Table 1). Exposure to NMDA for 24 hr produced a marked increase in PI uptake (~200%) in the CA1 pyramidal cell region ($F(7,136)=6.711$, $P<0.001$; Fig. 1), an effect that was prevented by co-exposure to the selective NMDA receptor antagonist 5-2-amino-5-phosphonovaleric acid (20 μM). METH co-exposure did not affect NMDA induced toxicity.

2.2: Methamphetamine Withdrawal & NMDA Toxicity

Organotypic hippocampal slice cultures were exposed to 6 days of METH treatment, followed by 1 or 7 days of withdrawal. On the final day of withdrawal, cultures were then exposed to 5 μ M NMDA. After 1 day of withdrawal, there were no significant increases in PI uptake in control and METH withdrawn tissue (Table 1). Tissue treated with NMDA displayed significant increases in PI uptake, in the CA1 pyramidal cell layers, regardless of pretreatment ($F(7,204)=9.897, P<0.001$; Fig. 2; $F(7,204)=9.897, P<0.001$). The DG and CA3 did not display significant increases in PI uptake.

After 7 days of withdrawal from METH, there were no significant increases in PI uptake in NMDA naïve tissue. The CA1 pyramidal cell layer, displayed significant PI uptake in NMDA treated tissue, an effect that was significantly increased by prior METH exposure, at all concentrations ($F(9,150)=25.652, P<0.001$; 100 μ M shown in Fig. 3). PI uptake in METH withdrawn tissue (100 μ M) treated with NMDA was approximately 80% more than control tissue treated with NMDA. All increases in PI uptake were blocked by exposure to 5-2-amino-5-phosphonovaleric acid, a selective NMDA receptor antagonist (20 μ M; APV) (Fig. 3). The DG and CA3 regions did not display significant increases in PI uptake.

Exposure to the inositol triphosphate (IP_3) receptor antagonist xestospongine C (1 μ M) with NMDA did not alter PI uptake in control or METH withdrawn tissue. Additionally, xestospongine C treatment did not significantly influence PI uptake in CTRL tissue treated with NMDA, suggesting the lack of involvement of Ca^{2+} release from IP_3 -sensitive stores in the observed toxicity. However, xestospongine C co-exposure did significantly reduced METH-potentiated (100 μ M) NMDA-induced neurotoxicity ($F(7,139)=24.049, P<0.001$; Fig. 3) in the CA1 pyramidal cell layer, by approximately 23%. Representative images of PI uptake are included in Fig. 4.

3. Discussion

Previous literature has suggested that both short-term and long-term METH exposure may result in changes to glutamatergic signaling (Kaiya et al., 1983; Rocher & Gardier, 2001). Furthermore, some studies suggest that METH may act as an NMDA receptor antagonist (Smith et al., 2007; Yeh et al., 2002). Yeh et al. (2002) reported that METH reduced NMDA-induced intracellular Ca^{2+} accumulation as well as displaced [H^3]TCP binding. Antagonism of NMDA-induced neurotoxicity by METH was reported in organotypic hippocampal slice cultures (Smith et al., 2007), further suggesting that METH may act as a NMDA receptor antagonist. Long-term NMDA receptor antagonism by METH may result in neuronal excitability during withdrawal. In fact, Moriguchi et al. (2002) reported increased amplitudes of NMDA mediated excitatory postsynaptic potentials (EPSPs) in neostriatal slices following withdrawal from METH. Evidence of neuronal excitability during protracted METH withdrawal suggests that compensatory responses to chronic METH administration may be similar to other NMDA antagonists. For example, compensatory responses to chronic ethanol exposure, such as increased cell surface expression of NR subunits, contribute to increased excitability and a loss of GABAergic tone during ethanol withdrawal (Alele & Devaud et al., 2005; Davidson et al., 1995; Maler et al., 2005; Prendergast et al., 2000). Such effects are likely to contribute to neurotoxicity, hippocampal deficits, cognitive problems, and possibly cue-induced craving during withdrawal (Bartels et al., 2007; Prendergast et al., 2004; Schneider et al., 2001).

The present studies examined the effects of long-term METH exposure, followed by withdrawal, on NMDA-induced neurotoxicity in organotypic hippocampal slice cultures. Long-term exposure (7 days) to METH did not produce overt neurotoxicity in pyramidal cells of the CA1 or CA3 regions, though a small but significant increase in toxicity was observed

in granule cells of the DG with exposure to 100 μ M METH. This finding is potentially of interest in that an isolated tissue model was employed that was devoid of METH effects on body temperature. However, the relevance of this high concentration of METH to the study of recreational METH use has not been fully addressed. Chung et al. (2004) did report that plasma levels of METH reached high μ M to low mM in a sample of South Korean individuals suffering acute lethality associated with METH use (ie. from traumatic injury or cardiac arrhythmia). Further, Melega et al. (1995) demonstrated that brain concentrations of METH were approximately 10-fold greater than were plasma concentrations following a single METH injection in rodents, raising the possibility that METH may be sequestered in brain lipids.

Withdrawal (1 or 7 days) from METH did not result in any observable toxicity. However, long-term withdrawal (7 days) did result in a markedly potentiated neurotoxic response to NMDA exposure, a finding that is consistent with the electrophysiological findings of Moriguchi et al. (2002). Long-term withdrawal does appear to be an essential component of the observed neurotoxicity, since short-term withdrawal and chronic exposure without withdrawal did not result in a potentiated NMDA effect. It is unclear as to why evidence of sensitized NMDA receptor-mediated response was not evident within 24 hr of the start of METH withdrawal, though it is possible that METH may be sequestered in brain lipid (after Melega et al. 1995), requiring a protracted time period to achieve complete clearance of METH. Furthermore, CA1 pyramidal cells appear more susceptible to NMDA-induced neurotoxicity. This effect may reflect the higher receptor composition of the CA1 region, since NMDA receptors are extremely dense in the basal and apical dendritic fields of the CA1 (Martens et al, 1998; Mulholland et al, 2003; Prendergast et al, 2004; Shin & Lee, 1999). The observed potentiation was reduced by exposure to APV, an NMDA receptor antagonist. Furthermore, inhibition of IP₃ mediated Ca²⁺ stores blocked the potentiated neurotoxicity to NMDA. These latter data, in particular, suggest that following NMDA receptor activation, Ca²⁺-induced Ca²⁺ release from endoplasmic reticulum (ER) is a key mediator of this METH withdrawal-potentiated NMDA response.

Changes in intracellular Ca²⁺ signaling reflecting activation of IP₃ receptors may result from METH interactions with ER-bound sigma receptors (Hayashi et al., 2005). Related findings demonstrate that following long-term cocaine exposure, ER-bound sigma receptors promote dissociation of a cytoskeletal adaptor protein ankyrin from IP₃ receptors (Su & Hayashi, 2001). Dissociation of ankyrin leads to increases of IP₃ mediated intracellular Ca²⁺ accumulation. METH has been found to bind directly to sigma receptors (Su and Hayashi, 2003). Following METH self administration, sigma receptors were shown to be upregulated in the midbrain of rats (Stefanski et al., 2004). Furthermore, pharmacological antagonism of sigma receptors blocked the development of METH induced behavioral sensitization (Takahashi et al., 2000; Ujike et al., 1996). Thus, one possible interpretation of the present findings is that METH exposure produces a persisting increase in the sensitivity of ER Ca²⁺ stores to agonist-induced release into cytosol. Though the present studies did not examine the biochemical pathway(s) involved in the neuronal injury observed, METH administration has been shown to induce neuronal apoptosis, including that occurring after ER stress (Davidson et al., 2001; Jayanthi et al., 2004). For example, 30 min following METH administration (40mg/kg), calpain, a cytosolic cysteine protease associated with ER-dependent cell death, was activated and continued to remain activated for seven days (Jayanthi et al., 2004). It will be of interest in future studies to examine activation of calpain during protracted METH withdrawal.

While ER functioning does appear to be integral in the observed effects, other METH-induced signaling changes may also contribute to the initiation of neuronal excitability during withdrawal. Loss of GABAergic tone has been suggested to influence behavioral sensitization to METH (Zhang et al., 2006). Following long-term METH administration (25mg/kg/day) and withdrawal (28 days), GABA_A α 2 subunit protein levels were decreased in the nucleus

acumbens. Glutamic acid decarboxylase (GAD) protein levels were also decreased in the nucleus acumbens (Zhang et al., 2006). Downregulation of GABA receptors, as well as, decreases in GABAergic signaling could result in increased excitability and possibly explain the observed effects. Further, METH may directly interact with specific NR subunits of NMDA receptors, producing compensatory upregulation(s) in their expression or function, though one recent study did demonstrate that METH does not appear to interact with an MK-801 channel binding site (Smith et al. 2007).

It is intriguing that protracted METH withdrawal produces a hypersensitivity of glutamatergic systems in the hippocampus, as this may be related to drug craving. During METH withdrawal, increases in craving have been reported by regular METH users (McGregor et al, 2005). Cue-induced craving, in particular, is a significant risk factor for relapse in abstinent METH users (Chang and Haning, 2006; Newton et al., 2006) and has been suggested to involve activation of the hippocampus in stimulant and depressant users (Kilts et al., 2001; Schneider et al., 2001). Though the specific means by which METH may influence NMDA receptor-mediated responses has not been fully elucidated, this receptor system represents a molecular target of METH that should be further studied as it may represent a novel therapeutic target to be exploited in the maintenance of abstinence from METH use.

4. Methods

4.1 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 dissecting medium (containing Minimum Essential Medium (MEM) plus glutamine, 25 mM HEPES, 200 mM glutamine, and 50 μ M streptomycin/penicillin) (Gibco BRL, Gaithersburg, MD) and the bilateral hippocampi were dissected out. 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 μ m using the McIlwain Tissue Chopper (Campden Instruments Ltd; Lafayette, ID, USA). Once sectioned intact hippocampi were plated onto Millicell-CM biopore membrane inserts, with one ml of culture medium added to the bottom of each well. Plates were then placed in an incubator, at 37 °C and with a gas composition of 5% CO₂, 21% oxygen, and 74% nitrogen, for five days to allow hippocampi to affix to the teflon membrane.

4.2 Drug Exposure

Slice cultures were exposed to METH (1.0-100 μ M; Sigma; St. Louis, MO, USA) for 6 or 7 days and uptake of propidium iodide (3.74 μ M; Molecular Probes), a highly polar nucleic acid stain used to detect cell damage, was assessed at several time points after METH exposure (0, 1, or 7 days later). Concentrations of METH employed were chosen to reflect the range of concentrations observed in both plasma and brain of rats following a single i.v. injection of METH (Melega et al. 1995), as well as, plasma and hair samples of humans who had ingested METH for recreational purposes (Nakashima et al. 2003). Additional cultures were exposed to NMDA (5 μ M; Sigma; St. Louis, MO, USA) at the end of the withdrawal period and were also labeled with PI. D,L,-2-amino-5-phosphovalerate, an NMDA antagonist, (APV) (20 μ M; Sigma; St. Louis, MO, USA) was utilized to confirm the role for NMDA receptor involvement in NMDA effects. Xestospongin C (1 μ M; EMD Biosciences; Darmstadt, Germany), an inositol triphosphate (IP₃) receptor antagonist, was also utilized on the final day of withdrawal to block IP₃ mediated intracellular Ca²⁺ accumulation. One ml increments of drugs were applied to the bottom of culture plates.

4.3 Cytotoxicity assessment

PI only penetrates cell membranes of damaged or potentially dying cells, binding to DNA to produce a bright, intensified red fluorescence at 630 nm (Zimmer et al, 2000). Hippocampi were visualized with SPOT Advanced version 4.0.2 software for Windows (W. Nuhsebaum Inc.; McHenry, IL, USA) at a 5X objective with a Leica DMIRB microscope (W. Nuhsebaum Inc.; McHenry, IL, USA) that is fitted for fluorescent detection (Mercury-arc lamp) using blue-green light, and connected to a personal computer through a SPOT 7.2 color mosaic camera (W. Nuhsebaum Inc.; McHenry, IL, USA). The emission wavelength of PI is 620 nm in the visual range, and has a peak excitation wavelength of 536 nm. PI is excited using a band-pass filter exciting wavelengths between 510 and 560 nm. Intensity of the PI fluorescence was then analyzed by densitometry using Image J. Pictures were then quantified by detecting optical intensity of the CA1 pyramidal cell layer, CA3 pyramidal cell layer, and dentate gyrus granule cell layer of the hippocampus following background subtraction. To minimize variability in PI uptake between replications, each replication was converted to percent control before analysis.

4.4 Statistics

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.

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Figure 1.

PI uptake in CA1 pyramidal cell layer following 7 days of METH (1-100 μ M) exposure with the addition of NMDA (5 μ M) on the seventh day. Treatment with NMDA, regardless of METH co-exposure, resulted in significant PI uptake. $\ast=P<0.05$ vs control.

Figure 2.

PI uptake in CA1 pyramidal cell layer following 6 days of METH exposure (100 μ M), and 24 hours of withdrawal with the addition of NMDA (5 μ M). Exposure to NMDA, regardless of pretreatment, resulted in significant PI uptake. *= P <0.05 vs control.

Figure 3.

PI uptake in CA1 pyramidal cell layer following 6 days of METH exposure (100 μ M), followed by 7 days of withdrawal and subsequent exposure to NMDA (5 μ M). METH withdrawal (1-100 μ M) resulted in a potentiated NMDA-induced response when compared to CTRL tissue exposed to NMDA. Potentiated response to NMDA is displayed as increases beyond the solid black line. APV (20 μ M) reduced all NMDA-induced PI uptake to CTRL levels. Xestospongine C (1 μ M) significantly reduced the observed potentiated effect following METH withdrawal.

* = $P < 0.001$ vs. CTRL + NMDA, ** = $p < 0.001$ vs. METH withdrawal + NMDA.

Figure 4. Representative images of PI uptake in control cultures and those exposed to Xestospongin C (20 M), NMDA (5 μ M) or a combination of both during protracted methamphetamine withdrawal.

Table 1

PI uptake in DG granule cell layer following 7 days of METH (100 μ M) exposure or following six days of METH exposure (100 μ M), and 24 hours of withdrawal. * = $P < 0.05$ vs. CTRL.

	CTRL	1 μ M METH	10 μ M METH	100 μ M METH
No Withdrawal	97.44 $\pm$ 2.82	95.46 $\pm$ 5.02	99.27 $\pm$ 4.44	120.38 $\pm$ 10.86*
24hr Withdrawal	103.39 $\pm$ 5.04	97.24 $\pm$ 3.89	98.16 $\pm$ 3.83	114.78 $\pm$ 5.82