Concurrent calpain and caspase-3 mediated proteolysis of αII-spectrin and tau in rat brain after methamphetamine exposure: A similar profile to traumatic brain injury

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Abstract

Neurotoxicity in rat cortex and hippocampus following acute methamphetamine administration was characterized and compared to changes following traumatic brain injury. Doses of 10, 20, and 40 mg/kg of methamphetamine produced significant increases in calpain- and caspase-cleaved αII-spectrin and tau protein fragments, suggesting cell injury or death. Changes in proteolytic products were significantly increased over vehicle controls. Use of fragment specific biomarkers detected prominent calpain-mediated protein fragments in the cortex and hippocampus while caspase-mediated protein fragments were also detected in the hippocampus. Remarkably, proteolytic product increases at the 40 mg/kg dose after 24 h were as high as those observed in experimental traumatic brain injury. Use of calpain and caspase proteolytic inhibitors may be useful in preventing methamphetamine-induced neurotoxicity.

Keywords: Methamphetamine; Drug abuse; Traumatic brain injury; Caspases; Calpain; Alpha-spectrin; Tau protein; Cerebral cortex; Hippocampus

Introduction

Methamphetamine (Meth, or Speed) is now among the most popular drugs of abuse. Meth mentions in emergency departments have been increasing each year since 1999, with a 19% jump in mentions between 2001 and 2002 (DAWN, 2003). A single dose of Meth can cause adverse reactions, such as clinical hyperthermia (NIDA, 1997). Meth has been shown to cause dopaminergic and, to a lesser extent, serotonergic neurite and nerve terminal degeneration of neurons in the striatum and hippocampus (Larsen et al., 2002). This striatal cell damage leads to clinical psychosis which is currently the main end point in clinical evaluation (Martin et al., 2003). It has been proposed that Meth-induced redistribution of dopamine from synaptic vesicles to cytoplasmic compartments, where dopamine can oxidize to produce quinones and additional reactive oxygen species, may account for this selective neurotoxicity (De Vito and Wagner, 1989; Gluck et al., 2001). Furthermore, it has been reported that Meth exposure, by producing reactive oxygen species, can upregulate pro-apoptosis genes such as c-Jun, c-myc and L-myc and thus induce neuronal apoptosis directly (Stumm et al., 1999; Deng et al., 2002b; Thiriet et al., 2001). In support of those findings, Meth also induces apoptosis in both a CNS-derived catecholaminergic cell line

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Currently, no diagnostic or pharmacological therapy exists to diagnose or treat acute Meth neurotoxicity (Huber et al., 1997; Martin et al., 2003). The brain derived neurochemical bioamines, dopamine, serotonin and their metabolites (DOPAC and 5-HIAA) are used as neurochemical blood biomarkers for Ecstasy neurotoxicity, however such an approach is nonspecific and does not correlate well with the severity of the brain neurotoxicity (Ali et al., 1991). Similarly, in the case of Meth abuse, blood derived myoglobin is used as a biomarker, which is also a non-specific indicator of toxicity and it is more indicative of Meth induced renal failure (Ishigami et al., 2003). Finally, employing traditional biochemical and immunological methods to identify specific biomarkers represents a major challenge due to cost and the need for prior knowledge of the insult mechanisms involved.

Recently, however, there has been increasing research into developing markers and treatments for traumatic brain injury (TBI) (Pineda et al., 2004). Significant progress has been made in assessing acute neuronal damage following TBI. In brief, TBI results in the over activation of two major protease systems: the pro-necrotic calpain 1,2 (calpain) and the pro-apoptotic caspase-3 (caspase) (Yuen and Wang, 1998; Nicholson and Thomberry, 1997; Wang, 2000). These proteases, when dysregulated, attack neuronal proteins such as cytoskeletal protein II-spectrin and microtubule associated protein tau, resulting in distinct proteolytic products that become readily detectable in injured brain tissues. Importantly, calpain overactivation and the presence of calpain-produced II-spectrin breakdown products (SBDPs) at 150 and 145 kDa are associated with oncotic/necrotic neuronal cell damage and to a lesser extent apoptosis (Nath et al., 1996a;b; Wang, 1998, 2000). Recently, we have produced antibodies specific to the 150 kDa calpain-cleaved II-spectrin protein (Dutta et al., 2002). Alternatively, caspase-3 overactivation and the presence of caspase-3 products at 149 and 120 kDa are exclusively associated with apoptotic neuronal death (Nath et al., 1996a;b; Wang, 2000). We have also produced antibodies specific to the 120 kDa caspase-cleaved II-spectrin protein (Nath et al., 2000).

Tau, which is a major microtubule-binding protein highly enriched in the dendrites, like II-spectrin, is also vulnerable to truncation by both calpain and caspase-3 (Wallace et al., 2003). Following ischemic brain injury, tau is partially fragmented (Pettigrew et al., 1996). Tau is also degraded by calpain in PC-12 cells challenged by ceramide (Xie and Johnson, 1997). Tau digestion with calpain yields multiple fragments of about 30–40 kDa (Yang and Ksiezak-Reding, 1995; Yen et al., 1999). On the other hand, tau cleavage by caspase-3 has also been identified in neuronal cells undergoing apoptosis in cerebellar granule neurons (Canu et al., 1998) in culture and in Alzheimer’s diseased brains (Roh et al., 2002). Thus, by examining the presence of various II-spectrin and tau fragments in the brain, one can associate the presence of neuronal toxicity and also identify the mechanisms leading to neuronal loss, that is, calpain- and caspase-mediated proteolysis.

Our previous work has shown that these same proteolytic markers are present in the rat cortex, hippocampus and cerebrospinal fluid following acute overdose of Meth (Warren et al., 2004). These dosages of Meth known to be neurotoxic in animals fall squarely in the range of dosages typically used by recreational Meth users via interspecies scaling (Deng et al., 2002b). Though research literature thus far has strongly supported an apoptosis-like cell injury mechanism with Meth neurotoxicity (Deng et al., 2002b), this is in contrast to the mode of cell death in TBI, which has a mixture of oncosis and apoptosis (Pike et al., 1998; Raghupathi et al., 2000), the former being the predominant mode of cell death. Consistent with that, necrosis- or oncosis-linked, calpain-generated SBDP150 and SBDP145 appear to be dominantly observed in brain tissues after TBI in rats and in humans (Pike et al., 1998, 2001). Smaller amounts of apoptosis-linked, caspase-generated SBDP120 have been reported in certain brain regions (e.g. thalamus) after TBI (Raghupathi et al., 2000). We hypothesize that in the case of Meth exposure, brain tissue such as cortex or hippocampus will have detectable levels of SBDPs and TBDPs comparable to that of TBI and that the predominant form(s) of cell death can be examined given cell death specific biomarkers. This will allow us to characterize the Meth induced cell toxicity as calpain- or caspase-induced and make a direct comparison to that of blunt trauma.

Materials and methods

In vivo model of Meth neurotoxicity

All experiments were carried out in male Sprague Dawley rats. Rats were divided into groups consisting of n=6. Each group received either Meth, at a dosage of 10, 20 or 40 mg/kg, or physiological saline (vehicle group). Rats were intraperitoneally injected with either one 10 mg/kg dose of Meth at a time, which was repeated as necessary every hour to achieve the desired dose, or saline in a bolus of 0.3 cc. The rats ranged in weight from 250 to 275 g. Animals were 90 days old at the time of sacrifice. All experiments were performed in compliance with NIH guidelines on animal care.

After 24 h, animals were briefly anaesthetized and immediately killed by decapitation. Brains were immediately removed, rinsed with ice cold PBS and entire brain regions (cerebral cortex and hippocampus) were rapidly dissected, snap-frozen in liquid nitrogen, and frozen at −80 °C until used. For Western blot analysis, the brain samples were pulverized with a small motor-pastel set over dry ice to a fine powder. The pulverized brain tissue powder was then lysed for 90 min at 4 °C with 50 mM Tris (pH 7.4), 5 mM EDTA, 1% (v/v) Triton x-100, 1 mM DTT, 1× protease inhibitor cocktail. The brain lysates were then centrifuged at 8000 ×g for 5 min at 4 °C to clear and remove insoluble debris, snap-frozen and stored at −85 °C until used. Previously prepared tissue lysates were used as controls, including naive negative controls and TBI (1.6 mm) positive controls (Pineda et al., 2004). Cell culture
positive controls were also used including cortical and hippocampal neurons treated with necrosis-inducing maitotoxin (MTX) (Wang et al., 1996) or apoptosis-inducing staurosporine (STS) (Nath et al., 1996a,b).

SDS-polyacrylamide gel electrophoresis and electrotransfer (for tissue lysate analysis)

Protein concentrations of tissue lysates were determined by bicinchoninic acid microprotein assays (Pierce Inc., Rockford, IL, USA) with albumin standards. Protein balanced samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in two-fold loading buffer containing 0.25 M Tris (pH 6.8), 0.2 M DTT, 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled H2O. Samples were heated for 10 min at 100 °C and centrifuged for 2 min at 8000 rpm in a microcentrifuge at ambient temperature. Twenty micrograms of protein per lane were routinely resolved by SDS-PAGE on 6.5% Tris/glycine gels for 2 h at 200 V. Following electrophoresis, separated proteins were laterally transferred to polyvinylidene fluoride (PVDF) membranes in a transfer buffer containing 0.192 M glycine and 0.025 M Tris (pH 8.3) 10% methanol at a constant voltage of 100 V for 1 h at 4 °C. Blots were blocked for 1 h at ambient temperature in 5% non-fat milk in TBS and 0.05% Tween-20. Gels were stained with Ponceau Red (Sigma, St Louis, MO, USA) to confirm successful transfer of proteins and to insure that an equal amount of protein was loaded in each lane.

Immunoblot analyses of brain tissue samples

Blotting membranes containing tissue protein were probed with a primary antibody (mouse monoclonal or rabbit polyclonal) as needed by incubating blots overnight at 4 °C in TBST (20 mM Tris–HCl, pH 7.4, 150 mM NaCl+0.05% Tween 20) with 5% non-fat milk. Primary antibodies included monoclonal anti-II-spectrin antibody (Amersham), polyclonal anti-III-spectrin antibody at 145 kDa, as detected by fragment-length specific antibody (II-spectrin antibody). Data similar to II-spectrin and breakdown products analysis using total II-spectrin antibody

Analysis of the Western blots for II-spectrin and its breakdown products in the rat cortex and hippocampus (Fig. 1A and B, respectively) showed a strong presence of II-spectrin parent protein in both vehicle and Meth-treated animals. Additionally, in Meth-treated animals, there were notable increases in specific breakdown products at 150, 145, 120 kDa in both brain regions. A dose-dependent increase of BDPs seemed evident in both brain regions from the Meth administration and the band intensity at the highest doses appeared to be comparable to that of the TBI positive controls. β-actin levels were measured as internal standard and indicated loading equivalence.

II-spectrin breakdown products from calpain cleavage

The calpain specific II-spectrin breakdown product at 150 kDa, as detected by fragment-length specific antibody SBDP150, was increased after Meth administration. In the rat cortex (Fig. 2A), a one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different (p < 0.001). A Bonferroni post hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups (p < 0.05). In the hippocampus (Fig. 2B), similar results were seen. A one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different (p < 0.001). A Bonferroni post hoc pairwise comparison test revealed that the 40 mg/kg Meth group is significantly greater than all other groups (p < 0.05). For both brain regions, the levels of SBDP150 were greatest for the 40 mg/kg Meth group, more so than the TBI.

The calpain specific II-spectrin breakdown product at 145 kDa was detected by total II-spectrin antibody. Data similar to the 150 kDa level were produced. In the rat cortex (Fig. 3A), a...
A one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different (p<0.001). A Bonferroni post hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups (p<0.05).

In the hippocampus (Fig. 3B), a one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different (p<0.001). A Bonferroni post hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups (p<0.05).

In the cortex, the level of neurotoxicity markers after 40 mg/kg of Meth were indistinguishable from those of TBI while they were increased over TBI in the hippocampus.

The caspase specific αII-spectrin breakdown product at 120 kDa, as detected by fragment-length specific antibody SBDP120, was increased after Meth administration. In the rat cortex (Fig. 4A), evidence was insufficient to show that levels of SBDPs differed, although some difference was suggested. For the hippocampus (Fig. 4B), a one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different (p<0.001). A Bonferroni post hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups (p<0.05). In the hippocampus, the level of neuro-
toxicity markers after 40 mg/kg of Meth were indistinguishable from those of TBI.

Intact tau and breakdown products analysis using total tau antibody

Western blots for the tau parent protein and its breakdown products in the rat cortex and hippocampus (Fig. 5A and B, respectively) showed presence of TBDPs at 36, 32, 26, and 12 kDa. A dose-dependent increase of BDP band intensity seemed evident and the band intensity at the highest doses appeared to be comparable to, if not greater than, that of the TBI counterparts. These TBDPs resulted from calpain-related events, as was demonstrated by cortical neuron culture treated with MTX (Fig. 6). Total tau antibody in the rat cortex (Fig. 7) were studied with one-way ANOVA tests and revealed that means for accumulation of breakdown products among Meth treatment groups were different ($p < 0.001$). A Bonferroni post hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups ($p < 0.05$).

Fig. 3. Accumulation of αII-spectrin breakdown products increased at 145 kDa from calpain activation in rat brain 24 h following Meth administration. For saline and Meth treatments, $n = 6$. For naïve and TBI (1.6 mm), $n = 3$. (A) Rat cortex. A one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different ($p < 0.001$). A Bonferroni post hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups ($p < 0.05$). (B) Rat hippocampus. A one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were different ($p < 0.001$). A Bonferroni post hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups ($p < 0.05$).

Western blots for the tau parent protein and its breakdown products in the rat cortex and hippocampus (Fig. 5A and B, respectively) showed presence of TBDPs at 36, 32, 26, and 12 kDa. A dose-dependent increase of BDP band intensity seemed evident and the band intensity at the highest doses appeared to be comparable to, if not greater than, that of the TBI counterparts. These TBDPs resulted from calpain-related events, as was demonstrated by cortical neuron culture treated with MTX (Fig. 6). Total tau antibody in the rat cortex (Fig. 7) were studied with one-way ANOVA tests and revealed that means for accumulation of breakdown products among Meth treatment groups were different ($p < 0.001$). A Bonferroni post hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups ($p < 0.05$).

Fig. 4. Accumulation of αII-spectrin breakdown products increased at 120 kDa from caspase activation in rat hippocampus 24 h following Meth administration. For saline and Meth treatments, $n = 6$. For naïve and TBI (1.6 mm), $n = 3$. A positive control of cells treated with staurosporine (STS, a caspase activator) was used for comparison in the caspase specific experiment. (A) Rat cortex. A one-way ANOVA test revealed that means for accumulation of breakdown products among Meth treatment groups were not different. (B) Rat hippocampus. A one-way ANOVA tests revealed that means for accumulation of breakdown products among Meth treatment groups were different ($p < 0.001$). A Bonferroni post hoc pairwise comparison test revealed that the 40 mg/kg Meth group was significantly greater than all other groups ($p < 0.05$).
accumulation of breakdown products among Meth treatment groups were different for three breakdown products ($p = 0.003$ for 36 kDa, $p = 0.001$ for 32 kDa and $p = 0.007$ for 26 kDa).

Bonferroni post hoc pairwise comparison tests revealed that the 40 mg/kg Meth group was significantly greater than the saline group for the 36, 32 and 26 kDa BDP ($p < 0.05$) and it was also significantly greater than the 10 mg/kg Meth group for the 26 kDa BDP ($p < 0.05$). Bonferroni post hoc pairwise comparison tests revealed that the 20 mg/kg Meth group was significantly greater than the saline group for the 36 and 32 kDa BDP ($p < 0.05$). In the hippocampus, the level of neurotoxicity markers after 40 mg/kg of Meth for TBDPs 26 and 12 kDa are indistinguishable from those of TBI.

Discussion

In this study, the level of neurotoxicity in the cortex and hippocampus, as suggested by proteolytic markers, was at least equivalent to that of our model of traumatic brain injury. In both the cortex and the hippocampus, and with both $\alpha$II-spectrin and tau proteolytic markers, the levels of quantified neurotoxicity from Meth exposure either equaled or exceed that of TBI. Calpain-associated proteolysis was increased in both the rat cortex and hippocampus as indicated by SBDP150 and SBDP145 (Figs. 2 and 3). At 10 and 20 mg/kg of METH, the levels of breakdown products mimicked that of TBI, while levels for 40 mg/kg of METH exceeded them. Caspase-associated $\alpha$II-spectrin proteolysis was clearly established in the hippocampus (Fig. 4). In the studies of tau, the total tau antibody used did not differentiate the fragments generated by caspase versus calpain. However, caspase-3 tends to produce high molecular weight fragments about 40 kDa whereas calpain produces tau fragments of lower molecular weight (Fig. 9). Indeed, several of these lower molecular weight tau fragments were observed in rat brain after both Meth administration or TBI suggesting calpain is actively attacking tau proteins (Figs. 7 and 8). Again, the increase of TBDP after 40 mg/kg of Meth approached or exceeded that of TBI. A dose somewhat higher than typical for recreational users is likely to manifest clinically. Given the impurities and unknown concentration of amphetamines in club drugs bought on the streets, some users tend to consume more than one pill in attempts to reach a satisfactory response (Makino et al., 2003). The results from the $\alpha$II-spectrin were consistent with a number of studies concerning TBI and other injuries using it as the biomarker of choice. Traumatic brain injury in rats resulted in increased SBDPs 150 and 145 in the cortex (Pineda et al., 2004). More specifically, it has been shown in TBI after 24 h that the calpain system was active in the cortex but not necessarily the pro-apoptotic caspase system (Pike et al., 1998). It is worth noting that calpain is overactivated in both necrotic and apoptotic cell injury or death (Wang, 2000). While our data was not strong enough to show unequivocal evidence of caspase activation in rat cortex after just 24 h, other studies have suggested evidence of apoptosis in cortex and neocortical neurons following Meth exposure (Dent et al., 2002; Summ et al., 1999). There has been, however, evidence of pro-apoptotic caspase activity in the rat hippocampus, thalamus, and striatum.
Fig. 7. Tau breakdown products increased in rat cortex 24 h following Meth administration. For saline and Meth treatments, \( n = 6 \). For naïve and TBI (1.6 mm), \( n = 3 \). One-way ANOVA tests revealed that means for accumulation of breakdown products among Meth treatment groups were different for three breakdown products (\( p = 0.057 \) for 36 kDa, \( p = 0.010 \) for 26 kDa, and \( p = 0.008 \) for 12 kDa). Bonferroni post hoc pairwise comparison tests revealed that the 40 mg/kg Meth group was significantly greater than the saline group for the 36 and 12 kDa BDP (\( p < 0.05 \)) and that it was significantly greater than all other groups for the 26 kDa BDP (\( p < 0.05 \)).

Fig. 8. Tau breakdown products increased in rat hippocampus 24 h following Meth administration. For saline and Meth treatments, \( n = 6 \). For naïve and TBI (1.6 mm), \( n = 3 \). One-way ANOVA tests revealed that means for accumulation of breakdown products among Meth treatment groups were different for three breakdown products (\( p = 0.003 \) for 36 kDa, \( p = 0.001 \) for 32 kDa and \( p = 0.007 \) for 26 kDa). Bonferroni post hoc pairwise comparison tests revealed that the 40 mg/kg Meth group was significantly greater than the saline group for the 36, 32 and 26 kDa BDP (\( p < 0.05 \)) and it was also significantly greater than the 10 mg/kg Meth group for the 26 kDa BDP (\( p < 0.05 \)). Bonferroni post hoc pairwise comparison tests revealed that the 20 mg/kg Meth group was significantly greater than the saline group for the 36 and 32 kDa BDP (\( p < 0.05 \)).
following TBI (Pike et al., 2001). Studies on age related cell death in rats also suggests calpain activity in the cortex without caspase (Bernath et al., in preparation). Following middle cerebral artery occlusion (MCAO) in rats, both calpain and caspase were evident after 24 h (Pike et al., 2003). Similar clubbing cerebral artery occlusion (MCAO) in rats, both calpain and caspase (Bernath et al., in preparation). Following middle cerebral artery occlusion (MCAO) in rats, both calpain and caspase were evident after 24 h (Pike et al., 2003). Similar clubbing cerebral artery occlusion (MCAO) in rats, both calpain and caspase (Bernath et al., in preparation). Following middle cerebral artery occlusion (MCAO) in rats, both calpain and caspase were evident after 24 h (Pike et al., 2003).

The implication of this study is that a single use of methamphetamine can be as neurotoxic in the cortex and hippocampus as traumatic brain injury. Given the effects of the drug on the cortex and hippocampus, cortical- and memory-related clinical outcomes should be considered when treating a patient with methamphetamine toxicity. Finally, use of calpain and caspase inhibitors as neuroprotectants has been suggested (Wang, 2000). Further understanding of the mechanism of cell death and brain localization could further the effort of clinical treatment for prevent of neurotoxicity with drug use.

A number of future studies must be performed to further data. First, these data should be confirmed by use of immunohistochemistry to study morphological and anatomical changes. TUNEL assays, as well as double-labelling immuno-histochemical studies with known markers of cell death could further characterize the neurotoxicity in brain regions as necrotic or apoptotic. A time course should be studied to determine the levels of biomarkers present at varying points after administration, especially since changes in proteolytic expression is suggested to differ between 24 and 72 h. Furthermore, investigation into other brain regions such as cerebellum, striatum, and thalamus should be attempted to explain clinical symptomology outside of the cortex and hippocampus. Also, a low-dose, multiple administration chronic model should be examined to discover if similar neurotoxicity is seen when compared to this acute, single-use model. In addition to studying rats, post-mortem studies could be performed in human brain tissues following deaths involving methamphetamine presence after identification in the blood.

Since we have shown the utility of a neurotrauma approach to characterizing neurotoxicity following drug exposure, other drugs could be studied under the same mechanism. Also, the combination of drug use with traumatic brain injury could be studied as a model of a motor vehicle accident while under the influence of drugs. Studies should be performed to see if such drug use acts as a “priming dose” which exacerbates neurotoxicity with subsequent TBI. Arguably the most important study would be the use of calpain and caspase inhibitors to prevent proteolysis induced by drug exposure. Such work has already begun with some success in TBI (Buki et al., 2003; Knoblauch et al., 2004).

**Conclusion**

This study indicated that cell toxicity in the cortex from calpain-induced proteolysis and in the hippocampus from calpain- and caspase-induced proteolysis was evident after single administration of methamphetamine in rats. The level of neurotoxicity approached and exceeded that of our model of traumatic brain injury. A dose of 40 mg/kg Meth was not significantly different in effect as TBI. The impact of Meth on cell degeneration has important prevention and clinical implications. Calpain and caspase inhibitors should be explored for the possible reduction of proteolytic activity. Further studies should be done to examine similar effects of other drugs in the brain.

**Uncited reference**

Hirata et al., 1995

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