

Calpain and caspase proteolytic markers co-localize with rat cortical neurons after exposure to methamphetamine and MDMA

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Abstract Abuse of the club drugs Methamphetamine (Meth) and Ecstasy (MDMA) is an international problem. The seriousness of this problem is the result of what appears to be programmed cell death (PCD) occurring within the brain following their use. This follow up study focused on determining which cell types, neurons and/or glial cells, were affected in the brains of drug-injected rats. Two proteolytic enzyme families involved in PCD, calpains and caspases, were previously shown to be activated and to degrade the brain cytoskeletal associated protein α II-spectrin. Using methods employed and confirmed in traumatic brain injury (TBI) studies, rat brain tissues were examined, 24 and 48 h after Meth and MDMA exposure, for the activation of calpain-1 and caspase-3, and their subsequent α II-spectrin cleavage breakdown products (SBDPs), SBDP145, and SBDP120, respectively. Based upon our previous studies we know that activated calpain-1 and caspase-3 were

up-regulated after drug use as were the levels of their cleaved SBDPs, SBDP145, and SBDP120, respectively, which is indicative of PCD. Here we show that activated calpain-1 and caspase-3 increases could be localized to neurons in the cortex where the products of their cleaved targets were found to be concentrated, particularly, to the axonal regions. These findings support the hypothesis that calpains and caspases mediate PCD in cortical neurons following club drug abuse and, more importantly, appear to contribute to the neuropathology suffered by abusers.

Keywords Calpain · Caspase · MDMA · Methamphetamine · Spectrin

Introduction

Abuse of club drugs is a serious problem internationally. Drugs such as methamphetamine (Speed, or Meth) and 3, 4-methylenedioxymethamphetamine (Ecstasy, or MDMA) are used by all socio-economic classes and, in particular, youths in the underground club and rave scenes. The United Nations International Drug Control (UNIDC) has estimated that over 250 million people have abused all forms of psychoactive drugs [29]. In the United States, by 2003 ~8.7% of all Americans had experimented with stimulants [36] and although some research suggests Meth and MDMA usage is decreasing [24] it is still a major problem. An important consequence of club drug abuse is neurotoxicity.

Although neurodegeneration has not yet been confirmed for MDMA, MDMA neurotoxicity has been reported in rats (limbic area [31]), as well as in primates [30] and humans [18]. The suggested mechanisms for this toxicity, without considering the effects of hyperthermia and metabolites, include inhibition of tryptophan hydroxylase [31], release

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of 5-HT and its derivatives [31], release of dopamine [21], decreased glutamate efflux [47], and a strong possibility of increased nitric oxide concentrations [33].

Similar results have been observed for Meth as it has been noted to cause neurodegeneration and even apoptosis of various regions in the brain [4, 7, 11]. A number of animal studies have shown that Meth decreases multiple dopaminergic and serotonergic markers such as tyrosine and tryptophan hydroxylase, and tissue dopamine and serotonin [8, 14, 41]. Meth induces dopamine release from cytosolic vesicles into the extracellular space which can lead to its oxidation into quinones and peroxynitrites [2, 49] which are important determinants in Meth induced neurotoxicity [9].

A study by Wallace et al. [40] using an acute regimen of Meth treatment (10 mg/kg \times 4), showed a marked depletion of dopamine in the caudate nucleus and nucleus accumbens. Interestingly, an earlier work by Pu et al. [28] demonstrated that degeneration of the dopaminergic nerve terminals along with astrogliosis with marked hypertrophy could be detected in the neostriatum after acute regimen of Meth treatment.

There have also been several studies, including the one by Yuan et al. [49] suggesting Meth could cause neurodegeneration independent of dopamine. Meth has been shown to cause both down-regulation and up-regulation of anti- and pro-apoptotic members, respectively, of the bcl-2 family of genes in non-dopaminergic cells in cell culture [35] and in vivo [12]. A study by Cadet et al. [3] demonstrated that an immortalized neuronal cell line treated with Meth exhibited an apoptotic cell death phenotype. Similarly, neocortical and striatum cells of rodents were shown to express a cell death phenotype and showed a positive expression of apoptotic markers and apoptotic family proteins after Meth exposure [4, 5, 35]. Additionally, Meth was discovered to cause serotonergic and dopaminergic neurite and nerve terminal degeneration [17] and to up-regulate pro-apoptotic genes such as c-Jun, c-myc, and L-myc [17, 35, 38].

We previously reported that acute Meth and MDMA use up-regulated and activated members of two proteolytic enzyme families, calpains and caspases [44–46]. The results were similar to those seen in traumatic brain injury (TBI) [27] and ischemia [26]. These enzymes cleave the neuronal cytoskeletal protein α II-spectrin, releasing breakdown products with molecular weights that profile the activity induced by calpains and caspases. Specifically, cleavage of α II-spectrin by calpains results in breakdown products of 150 and 145 kDa [23, 42, 43] while caspases generate breakdown products of 150 and 120 kDa [22, 23, 43]. We hypothesized that this type of breakdown occurred in both neurons and glial cells in the brain tissues of rats injected with the drugs and, thus, may be a contributor to related clinical pathologies.

Immunohistochemical (IHC) techniques were used in this study to determine the cell-types targeted by the club drugs by examining rat cortical tissue for activated calpain-1 and caspase-3 and their respective α II-spectrin breakdown products (SBDPs). One previous study found an increase in caspase-3 in the rostral forebrain and the hippocampus of rats after 5 days following 20 mg/kg of MDMA [20]. None of these studies, however, attempted to determine if any proteolytic enzymes were also activated within neurons or glial cells after drug abuse. NeuN, a well established marker for neurons within the brain, although it does not label all neurons, was used to co-localize the increased signals for active caspases and calpains and the breakdown products of their targeted substrate, spectrin, to neurons. For astrocytes, a number of studies have suggested that when there is increased pathological activity such as may occur after drug use there is an increase in the levels of the glial fibrillary acidic protein (GFAP). For example 30 mg/kg of Meth was shown to induce GFAP after 3 days in the cortex [50] and striatum [48]. Induction of GFAP, as well as microglial activation, was also observed, after 48 h, with 20 mg/kg of MDMA [39]. Thus, this biomarker was used for this study. Our laboratory has been successful with these types of studies after TBI with activated caspase family members in the rat cortex and hippocampus [15, 16].

In this study the proteolytic enzymes, calpain-1 and caspase-3, known to be activated in programmed cell death (PCD), were found to be activated in cortical neurons. Their respective and specific SBDPs 145 kDa (calpain) and 120 kDa (caspase), particularly the latter, were found to be elevated in neurons chiefly in the axonal regions. Similar studies were done for astrocytes with limited success (data not reported).

Materials and methods

Animals

All experiments were performed using male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) that were aged to 60 days and weighing between 240 and 270 g. Animals were housed in groups of two per cage and maintained on a 12 h light/dark cycle. Food and water were available ad libitum. Rats were divided into groups receiving either drug or saline ($n = 2$ per treatment group). Rats received either 40 mg/kg Meth or MDMA and sacrificed after 24 or 48 h, or a 0.3 cm³ bolus of physiological saline and sacrificed after 48 h. The dose of 40 mg/kg falls squarely in the range of a typical human single use overdose via intra-species scaling (24). All experiments were approved by the University of Florida International Animal Care and Use Committees (IACUC).

Drug administration

Pharmacologic agents (\pm)-3,4-Methylenedioxymethamphetamine hydrochloride (MDMA) and (\pm)-Methamphetamine hydrochloride were obtained from the Sigma-Aldrich, St. Louis, MO, USA. The drugs were dissolved in 0.9% saline and injected intra-peritoneally. For the dosages of MDMA or Meth, 10 mg/kg of drug was administered at once and repeated each subsequent hour to achieve a 40 mg/kg dose over 3 h to prevent lethal hyperthermia, as previously described [44].

Immunohistochemistry analysis

At the appropriate time points, the animals were anesthetized using 4% isoflurane in a carrier gas of O₂ (6 min), subsequently perfused with 0.9% saline (pH 7.4) followed by 400 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (fix) over 10 min, killed by decapitation and then, the brains were removed. The brains were left in fixative for an additional 72 h and then stored in cryoprotection buffer at -20°C until ready to use. Vibratome-cut 40 μM sections were fluorescently immunolabeled with a cell type-specific monoclonal antibody, proteolytic specific polyclonal antibodies, and a nuclear counterstain. Briefly, tissue sections were rinsed in PBS then incubated for 1 h at room temperature in 10% goat serum/10% horse serum/2% Triton-X100 in Tris-buffered saline (TBS; block) to decrease non-specific labeling. The sections were incubated with a cell-type specific primary antibody in block either: the mouse anti-neuron specific nuclear protein antibody (neuronal nuclei-anti-NeuN) at a concentration of 1:1,000 (Chemicon, Temecula, CA, USA), the mouse anti-gial acidic fibrillary protein antibody for astrocytes (anti-GFAP) at a concentration of 1:1,000 (Sternberger Monoclonals, Lutherville, MD, USA), the mouse anti-myelin basic protein antibody for oligodendrocytes (anti-MBP) at a concentration of 1:1,000 (Chemicon), the mouse anti-microglia-specific antibody for resting/activated microglia (anti-OX42) at a concentration of 1:1,000 (Serotec Inc., Raleigh, NC, USA) or the mouse anti-neurofilament-200-specific protein antibody (anti-NF-200) at a concentration of 1:1,000 (Chemicon). The sections were simultaneously incubated in block with either (a) the rabbit anti-activated-calpain-1 at a concentration of 1:100 and the rabbit anti-SBDP145 at a concentration of 1:50 or (b) the rabbit anti-activated-caspase-3 at a concentration of 1:500 and the rabbit anti-SBDP120 at a concentration of 1:1,000, for 2 days in block at 4°C . After being rinsed in PBS, the tissue sections were incubated with species-specific Alexa Fluor (Molecular Probes Inc., Eugene, OR, USA) secondary antibodies, Alexa Fluor 485 and 594, at a concentration of 1:3,000 in block for 1 h at room temperature. The

sections were then washed with PBS, cover-slipped in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA), viewed and digitally captured with a Zeiss Axiovert 200 microscope equipped with a Spot Real Time (RT) Slider high-resolution color CCD digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Sections without secondary antibodies were similarly processed to control for non-specific binding of the primary antibodies, as were sections with secondary but no primary antibodies, and, also, sections with neither primary nor secondary antibodies. No specific immunoreactivity was detected in these control sections.

Western blot analysis of NF-200

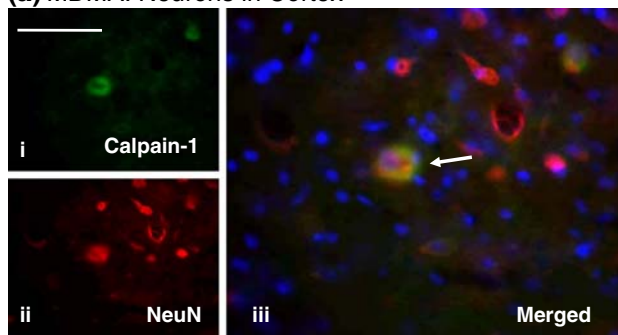
Brain tissue lysates from saline treated animals ($n = 5$) and 40 mg/kg Meth treated animals ($n = 3$) were collected from previously performed experiments [44]. Protein balanced samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in twofold loading buffer containing 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 8,000 rpm in a microcentrifuge at ambient temperatures. Twenty micrograms of protein per lane were routinely resolved by SDS-PAGE on 6% 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, 0.025 M Tris (pH 8.3), and 10% methanol at a constant voltage of 100 V for 1 h at 4°C . After electrotransfer, blotting membranes were blocked for 1 h at room temperature in 10% non-fat milk in TBS and 0.05% Tween-20 (TBST), then incubated with primary monoclonal anti-NF-200 antibody in TBST with 10% milk at 4°C overnight. Following three washes with TBST and 2 h incubation at room temperature with a biotinylated secondary antibody (Amersham, Piscataway, NJ, USA), the membrane was incubated for 1 h with Streptavidin-conjugated alkaline phosphatase. Finally, colorimetric development was performed with the one-step BCIP-reagent (Sigma). Quantitative evaluation of protein levels on immunoblots were performed via computer-assisted one-dimensional densitometric scanning (Hewlett Packard Scan-Jet 6300) coupled with ImageJ software (NIH). Data were acquired as integrated densitometric values and transformed to percentages of the densitometric levels obtained on scans from saline administered animals visualized in the same blot. All values are given as mean \pm SEM. Differences among means of treatment groups were compared using a Student's *t*-test at an alpha level of 0.05. β -actin obtained from the same samples is displayed to show loading equivalence.

Results

Immunohistochemical analysis of calpain-1 expression after club drug exposure

The cortex of drug exposed rats showed strong immunoreactive expression for calpain-1, as compared to brain tissue of saline treated controls (Figs. 1, 2). Brain tissue of MDMA exposed rats showed visible immunopositive expressions for calpain-1 in neurons labeled with NeuN (Fig. 1a), a neuronal cell marker, but not in astrocytes labeled with GFAP (data not shown). In comparison to MDMA, brain tissue of Meth exposed rats revealed a much stronger immunopositive signal for calpain-1 (Fig. 1b). NeuN stained neuronal cells also showed evidence of PCD morphopathology. Where the more abundant levels of calpain-1 induction were located the tissue morphology of the drug exposed rats had a highly granular appearance, typically seen after TBI, as compared to saline controls typical of cells undergoing PCD. Calpain-1 was not detected in

(a) MDMA: Neurons in Cortex



(b) Meth: Neurons in Cortex

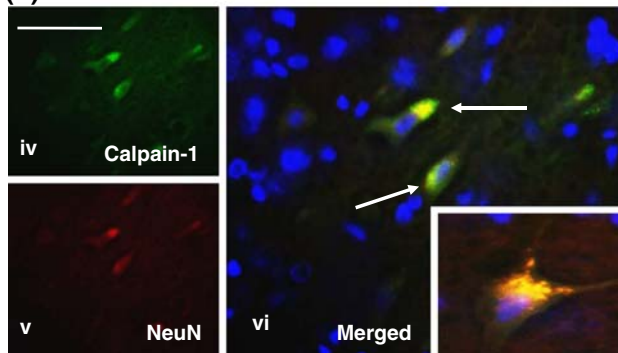


Fig. 1 Club drug induced activation of calpain-1 in neurons in the cortex. **a** Cortex of rats treated with 40 mg/kg MDMA after 24 h. Brain tissue with activated calpain-1 staining (i). NeuN stained neuronal cells (ii). Activated calpain-1 co-localizes in neurons with NeuN (iii, arrow). **b** Cortex of rats treated with 40 mg/kg Meth after 48 h. Brain tissue with activated calpain-1 staining (iv). NeuN stained neuronal cells (v). Activated calpain-1 co-localizes in neurons with NeuN with some granulation (\times arrows; inset shows granulation more specifically). DAPI staining for chromatin (blue). Photomicrographs are at $\times 640$; scale bar 100 μ m

Controls: Saline Treated Brains

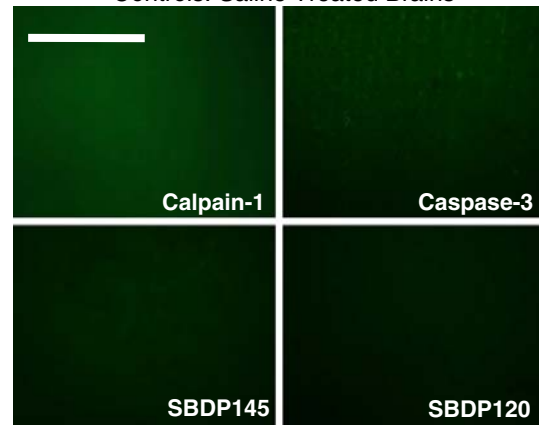


Fig. 2 Brain tissue of saline treated rats (controls) showed no reaction to calpain-1, caspase-3, SBDP145, or SBDP120. Photomicrographs $\times 400$ x; scale bar 62.5 μ m. Also appropriate controls (primary/no secondary and secondary/no primary) were performed, no reaction was noted (data not shown)

oligodendrocytes (MBP) or microglial (OX42) (data not shown).

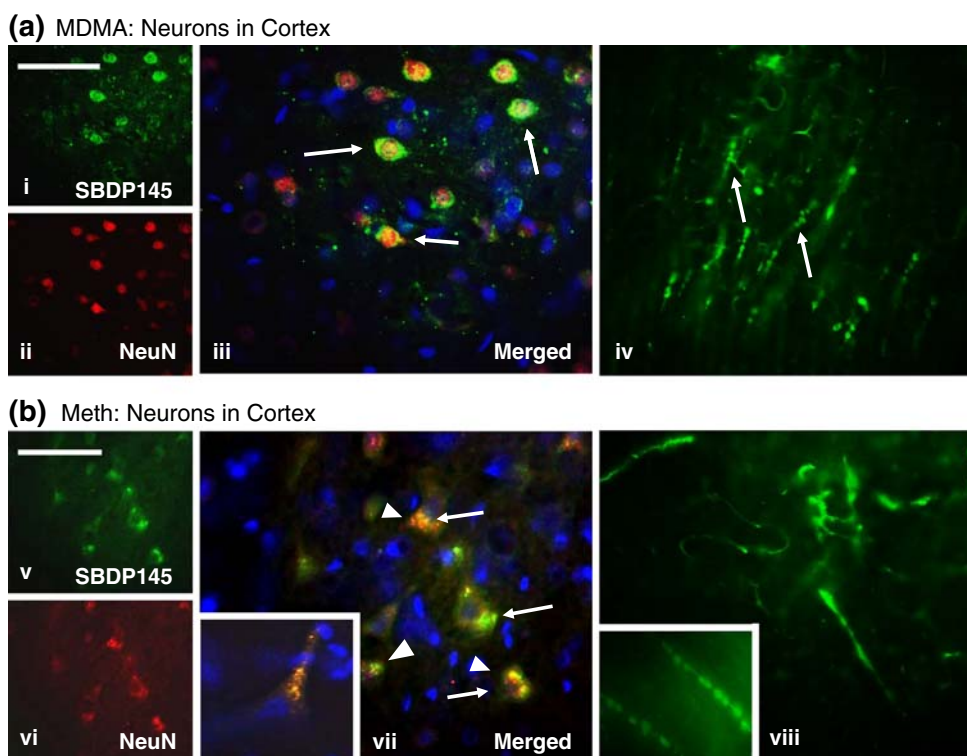
Immunohistochemical analysis of SBDP145 expression after club drug exposure

To confirm calpain-1 activation, the cortex of the same drug exposed rats revealed marked increases in immunoreactive expression for calpain-specific SBDP145 cleavage product, evident in both the MDMA and Meth exposed rats, as compared to brain tissue of saline treated controls (Figs. 2, 3). The increase in immunopositive expression for SBDP145, primarily localized in the axons, in NeuN stained neuronal cells showed evidence of morphopathology including apoptotic bodies (Fig. 3a-vii). Similar to the calpain-1 results, the morphology of the drug exposed brains, where the more abundant levels of SBDP145 were discovered, appeared to have a pathomorphology typically seen in TBI tissues as compared to saline controls. For example, in these brain regions the axons visualized using SBDP145 had a “beads on a string” effect, a morphological earmark of cells undergoing cell death. SBDP145, however, was not found within GFAP labeled astrocytes (data not shown). SBDP145 was, also, not detected oligodendrocytes (MBP), or microglial (OX42) (data not shown).

Immunohistochemical analysis of caspase-3 expression after club drug exposure

The neurons of the cortex of rats exposed to MDMA and Meth showed evidence of increased immunoreactive expression for caspase-3 as compared to saline treated controls which showed no caspase-3 immunoreactivity

Fig. 3 Club drug induced activation of SBDP145 in neurons in the cortex. **a** Cortex of rats treated with 40 mg/kg MDMA after 24 h. Brain tissue with SBDP145 staining (i). NeuN stained neuronal cells (ii). SBDP145 co-localizes in neurons with NeuN (iii, *arrows*). SBDP145 in axons; displaying a “beads on a string” effect (iv, *arrows*, $\times 200$). **b** Cortex of rats treated with 40 mg/kg Meth after 48 h. Brain tissue with SBDP145 staining (v). NeuN stained neuronal cells (vi). SBDP145 co-localizes in neurons with NeuN (vii, *arrows*; inset). Apoptotic bodies are clearly visible (*arrowheads*). SBDP145 in axons; displaying a “beads on a string” effect (viii; inset, $\times 200$). DAPI staining for chromatin (*blue*). Photomicrographs are at $\times 640$ unless otherwise specified; *scale bar* 100 μm



(Figs. 2, 4). The brain tissue of MDMA exposed rats with immunopositive expression for caspase-3 in NeuN stained neuronal cells showed evidence of morphopathology (Fig. 4a). Likewise brain tissue of rats exposed to Meth showed increases in immunopositive expression for caspase-3 in NeuN stained neuronal cells (Fig. 4b). Axon visualization with caspase-3 antibodies, due to its increased expression, was possible after Meth treatment (Fig. 4b-vi). Caspase-3 appeared co-localize with GFAP labeled astrocytes in the hippocampi of rats exposed to Meth but not MDMA but due its limited expression data was not shown. Caspase-3 was not seen to co-localize with oligodendrocytes (MBP) or microglial (OX42) (data not shown).

Immunohistochemical analysis of SBDP120 expression after club drug exposure

Confirming the increased expression of activated caspase-3 is the marked increase in immunoreactive expression of the caspase-specific SBDP120 cleavage product in the neurons of the cortex of MDMA and Meth exposed rats as compared to saline treated controls (Figs. 2, 5). The immunopositive SBDP120 expression in NeuN stained neuronal cells, particularly in the axons of the cortex and hippocampus, also showed evidence of morphopathology highlighted by some granulation as noted in axons displaying the “beads on a string” effect, a morphological earmark of cells undergoing cell death (Fig. 5a-iv, b-viii). Similar to SBDP145, the morphology of the drugged exposed rats’ brain tissue, for the

more abundant expressed SBDP120 regions, appeared to have lost its normal coherent expression when compared to saline controls, a morphology typically seen in TBI as tissues lose cohesion of cells undergoing PCD when compared to saline controls. These two morphologies are typical of cells undergoing cell death such as apoptosis. SBDP120 was not detected in astrocytes, oligodendrocytes (MBP), or microglial (OX42) (data not shown).

Immunohistochemical analysis of SBDP145 co-localization with NF-200

Since the SBDPs appeared to profile the axons, we examined whether SBDPs co-localized with neurofilament-200 (NF-200), a protein present in axons. SBDP145, for both MDMA (Fig. 6a) and Meth (Fig. 6b) treated rat cortices, co-localized with NF-200. The results of the Western blot analyses of control saline rats ($n = 5$) versus Meth (40 mg/kg; $n = 3$) treated rats suggested that NF-200 was also degraded following Meth treatment (Fig. 6c). β -actin was used to show equivalent protein loading. Student’s *t*-test confirmed a significant decrease in NF-200 for Meth treatment ($P < 0.001$). Similar results were found for SBDP120 (data not shown).

Discussion

The brain is populated by a vast variety of neuronal cell types. They may be classified by function (unipolar, bipolar,

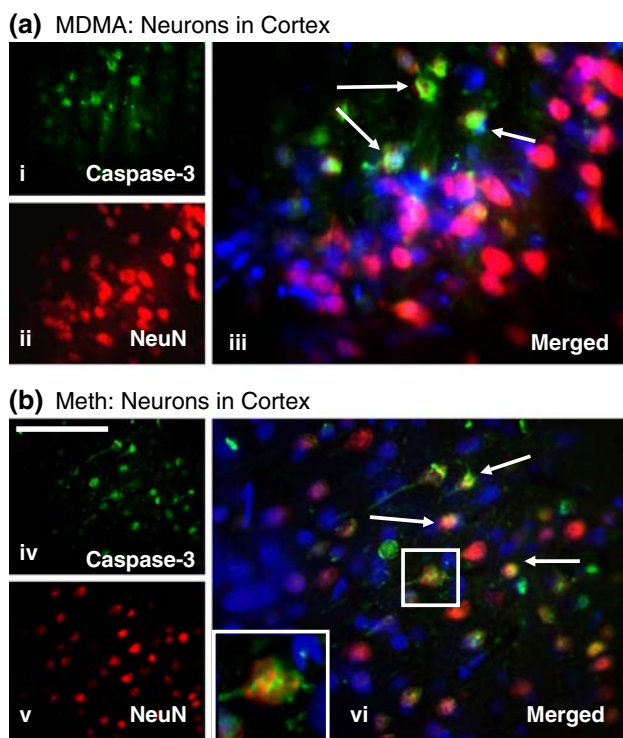


Fig. 4 Club drug induced activation of caspase-3 in neurons in the cortex. **a** Cortex of rats treated with 40 mg/kg MDMA after 24 h. Brain tissue with activated caspase-3 (i). NeuN stained neuronal cells (ii). Activated caspase-3 co-localizes in neurons with NeuN (iii, arrows). **b** Cortex of rats treated with 40 mg/kg Meth after 48 h. Brain tissue with activated caspase-3 (iv). NeuN stained neuronal cells (v). Activated caspase-3 co-localizes in neurons with NeuN (vi, arrows; inset). DAPI staining for chromatin (blue). Photomicrographs are at $\times 640$; scale bar 100 μm

and multipolar), by action (excitatory, inhibitory, and modulatory), by discharge patterns (tonic or regular, phasic or bursting, fast spiking, and thin spiking) or by neurotransmitter released (e.g., cholinergic, GABAergic, glutamatergic, and dopaminergic). Although most studies do not venture to guess on the number (e.g. [19]), Stufflebeam [34] speculated that there may be as many as 10,000 different types of neurons. In this study the focus was limited to determining which cell type (glial or neuronal) and what brain region was affected by the drugs at selected time points and because of the number of neuronal subtypes determination of the vulnerability of selected neurons was not attempted.

The results of these IHC studies demonstrated that the activated proteolytic enzymes calpain-1 and caspase-3 were up-regulated in rat cortical neurons after MDMA and Meth exposure. The calpain and caspase specific expression of the spectrin breakdown products, SBDP145 kDa and SBDP120 kDa, respectively, were also found elevated and these were particularly noticeable in the axons. These data suggest that PCD, both necrotic and apoptotic,

was activated. To our knowledge, this is the first study to show that these proteolytic and PCD markers and their specific breakdown products could be found expressly in neurons of the rat cortex following drug exposure. The results support the earlier works of Eisch et al. [6] and O'Dell and Marshall [25]. These earlier studies showed marked neuronal damage in the parietal cortex regions and the somatosensory area following Meth treatment as measured by fluoro-Jade fluorescent staining. Our findings, also, parallel those that show club drugs induce serotonergic and dopaminergic nerve terminal degeneration [17].

Surprisingly, in contrast to the cortical neuronal results, we had very limited success in detecting the up-regulation of the activated forms of both proteolytic enzymes and their breakdown products in other brain regions and in glial cells including astrocytes, oligodendrocytes or microglia. There are a number of potential reasons for this including the possibility that glial cells respond more vigorously at later time points than those examined for this study. It could also be that the cells are not as sensitive to the drugs in terms of triggering PCD but may still cause other molecular changes not tested in this study. These data, however, does support the conclusion that there is a greater toxicity with Meth and that it more strongly targets neuronal cells and both drugs target neurons in the cortex activating PCD pathways. Our findings are consistent with and support the hypothesis that calpains and caspases mediate proteolysis of cortical neurons following club drug abuse thereby further emphasizing the dangerous nature of these drugs and their apparent neuropathology.

The time points at which we found that the proteases were most highly up-regulated and their cleavage products were most clearly evident occurred after 24 h of treatment for MDMA and 48 h for Meth. Activation of necrosis in the neurons is suggested by the calpain cleavage-specific 145 kDa SBDP [10] and the up-regulation of activated calpain-1 (a known necrotic as well as apoptotic activator) even though it was difficult to capture the necrotic morphology in our photomicrographs. SBDP145 was evident and most prominent in the axons. When the axons were more closely inspected, swelling could be seen within the axons, displaying a “beads on a string” effect which is morphological earmark of cells undergoing cell death. This observed profile mimics the phenotype observed in TBI as we have shown in previous studies [44, 45]. This supports our hypothesis that neurons of the cortex were undergoing PCD, and perhaps the necrotic form of cell death.

Moreover, neuronal apoptosis is suggested by the caspase cleavage-specific 120 kDa SBDP [22, 26] and the up-regulation and activation of caspase-3. The SBDP120 was likewise found to be most prominent in the axons and likewise displayed a “beads on a string” expression indicating that these cells were undergoing apoptotic cell death.

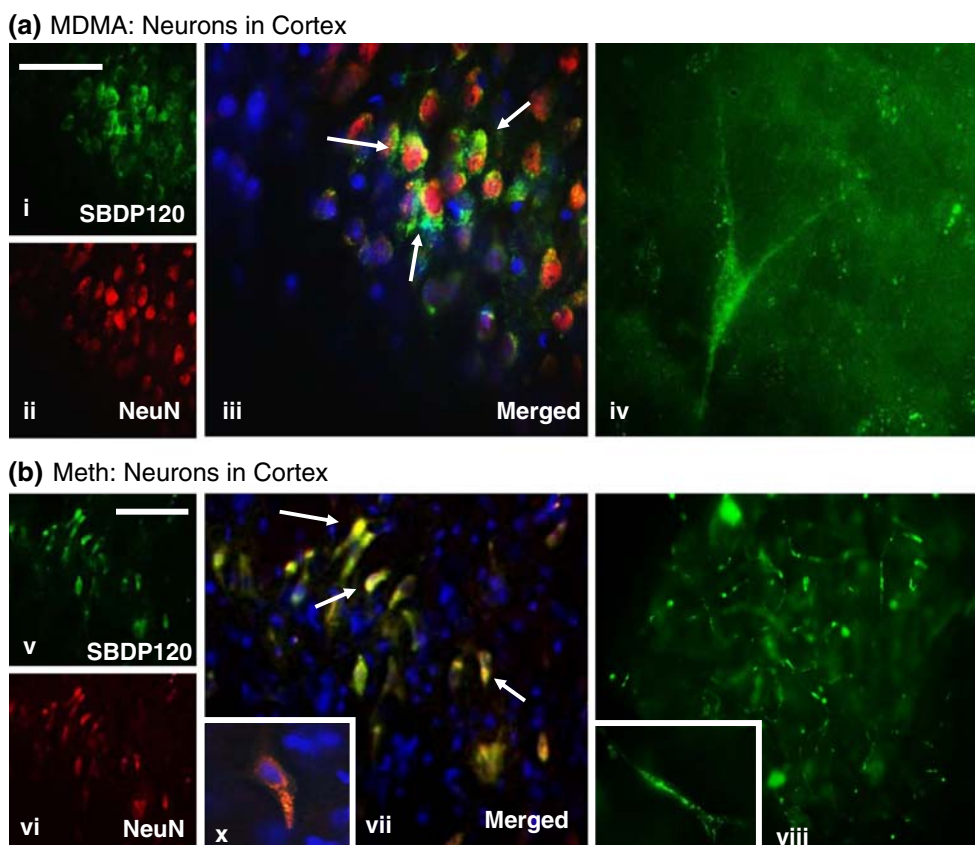


Fig. 5 Club drug induced activation of SBDP120 in neurons in the cortex. **a** Cortex of rats treated with 40 mg/kg MDMA after 24 h. Brain tissue with SBDP120 staining (i). NeuN stained neuronal cells (ii). SBDP120 co-localizes in neurons with NeuN (iii). SBDP120 in axons (iv, $\times 200$). **b** Cortex of rats treated with 40 mg/kg Meth after 48 h. Brain tissue with SBDP120 staining (v). NeuN stained neuronal cells

(vi). SBDP120 co-localizes in neurons with NeuN with granulation (vii, arrows; inset). SBDP120 in axons displaying a “beads on a string” expression (viii, $\times 200$, inset). DAPI staining for chromatin (blue). Photomicrographs are at $\times 640$ unless otherwise specified; scale bar 100 μm

Similar findings have been reported for mice treated with 30 mg/kg Meth. In that study 25% of the striatum neurons were found to be apoptotic after just 24 h of exposure [50]. In a follow up study by the same authors the neurons most vulnerable in this brain region were the striatal projection neurons and the GABA-parvalbumin and the cholinergic interneurons [51]. Taken together, these findings suggesting that because both proteolytic enzyme families were activated, a simultaneous necrotic and apoptotic assault is taking place in the cortical neurons aftermath of club drug use.

To confirm the presence of SBDPs in the axons of the cortex, SBDP145 and SBDP120 were counterstained with anti-neurofilament (NF-200), a more specific marker of vertebrate neuronal axons. Upon Western blot examination, NF-200 appeared to be extensively degraded after exposure to Meth when compared to saline controls. However, those neurofilaments that were still intact were found to co-localize with SBDP145 or SBDP120 in the axons following both Meth and MDMA exposure. Since α II-spectrin is mainly

located in the axonal membrane where it plays a crucial role in synaptic transmission, this localization of compartmental damage may provide additional explanation for why there is nerve terminal damage previously reported with club drug usage [32].

The concept of neuronal cell death has not been previously well-demonstrated in studies with MDMA, especially in vivo. What is known is that high doses of MDMA lead to the depletion of neuronal serotonin which has been interpreted as an indicator of neurotoxicity. However, whether 5-HT depletion reflects neuronal degeneration is still a matter of ongoing debate as illustrated very recently by Baumann et al. [1]. Although the earlier work by Meyer et al. [20] showed evidence of caspase-3 activation in neonatal rats which may reflect physiological processes rather than MDMA induced response, it is interesting that a novel study by the same group [37] indicated that MDMA induced caspase-3 activation in the limbic system in adult male C57 mice. It is worth noting that the regimen models (acute versus chronic) dosage, experimental animals and

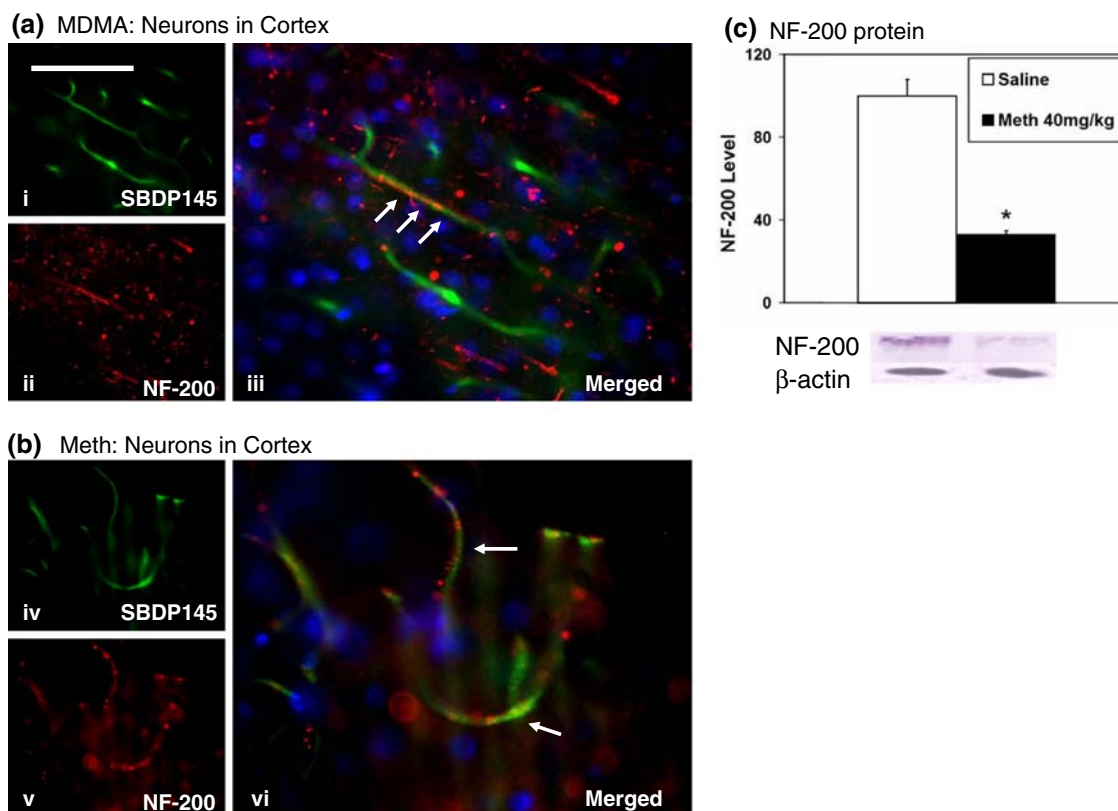


Fig. 6 Club drug induced co-localization of SBDP145 with NF-200 in the cortex. **a** Cortex of rats treated with 40 mg/kg MDMA after 48 h. Brain tissue with SBDP145 staining (i). NF-200 stained neuronal axons (ii). SBDP145 co-localizes in axons with NF-200 (iii, arrows). **b** Cortex of rats treated with 40 mg/kg Meth after 48 h. Brain tissue with SBDP145 staining (iv). NF-200 stained neuronal axons (v). SBDP145 co-localizes in axons with NF-200 (vi, arrows). DAPI

staining for chromatin (blue). Photomicrographs are at $\times 640$; scale bar 100 μm . **c** NF-200 protein in saline ($n = 5$) versus Meth 40 mg/kg ($n = 3$). Western blot shows degradation of NF-200 after Meth treatment; β -actin remains constant, suggesting equivalent loading. A Student's *t*-test reveals a significant decrease in NF-200 for Meth treatment ($*P < 0.001$). Similar results were seen for SBDP120 (not shown)

conditions (temperature) are all factors determining MDMA/Meth neurotoxicity. Recent *in vivo* studies from our laboratory and others have demonstrated that caspase activation can be indicative of MDMA and Meth mediated neuronal cell death [13, 46].

To summarize, the pro-necrotic calpain-1 and the pro-apoptotic caspase-3 were found to be up-regulated and activated in rat cortical neurons upon exposure to Meth and MDMA, but not in oligodendrocytes or microglia and only limited expression in astrocytes. Furthermore, elevated levels of SBDP145 and SBDP120 were found in cortical neurons, particularly in the axonal segments as further verified by co-localization with neurofilament 200. These results strongly suggest that MDMA and Meth may be selectively targeting neurons of the cortex thereby invoking a simultaneous proteolytic assault via necrotic and apoptotic processes, particularly in the axonal regions. Future studies need to be performed to more specifically target dopaminergic or serotonergic neurons to confirm that they are, in fact, affected by these club drugs. Other brain regions such as the hippocampus, striatum, and cerebellum will also

need to be examined to link clinical findings after drug use with histological evidence of cell death. Finally, other drugs that are abused should be examined using this same approach to determine whether they also may play a role in brain PCD.

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