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Research Article

Methamphetamine Injections Cause Widespread Increases in Caspase-8 Expression in the Mouse Brain

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Abstract Methamphetamine administration can cause neuronal apoptotic death in the rat brain via activation of the extrinsic cell death pathway (Fas ligand/Fas/caspase-8 cascade). In the present study, we injected mice with a toxic regimen of methamphetamine (10 mg/kg × 4 given at 2-h intervals) to assess the role of the Fas ligand/Fas/caspase-8 death pathway in their brain. Methamphetamine did cause the appearance of TUNEL-positive cells in the frontal cortex, striatum, and hippocampus of these animals. The number of TUNEL-positive cells was higher in the striatum than in the frontal cortex and hippocampus. Immunohistochemical and immunoblot techniques revealed significant methamphetamine-induced increases in Fas ligand expression and diffuse appearance of the active caspase-8 in these brain regions. Taken together, our data suggest that methamphetamine can cause marked activation of the caspase-8-dependent death pathway in the brain.

Keywords METH toxicity; apoptosis; extrinsic death pathway; Fas ligand; TUNEL

1. Introduction

METH is a stimulant drug whose abuse has reached epidemic proportion in the world [16]. Long-term METH abuse is associated with cognitive deficits, behavioral disorders, and psychiatric symptoms [17]. Although the mechanisms underlying METH-induced neuropsychiatric impairments are not clear, METH-induced neurodegeneration in the brain is considered, at least partially, the pathological substrate for these clinical syndromes [3]. In humans, METH-induced neurodegeneration has been reported in several brain regions including the caudate-putamen, hippocampus, and limbic cortices [4, 25, 33, 39]. In animals, METH administration can cause depletion of monoamines, destruction of monoaminergic terminals, and death of non-monoaminergic neurons [1, 3, 22, 40].

In addition to its neurodegenerative effects, METH administration is associated with increased expression of genes that participate in a number of cell death pathways [2, 18, 19]. For example, we have shown that METH administration can cause neuronal death in the rat via up-regulated expression of Fas ligand (FasL) which participates in the extrinsic apoptotic death pathway [18]. Fas ligand (FasL) is a 40-kDa protein which is stored in cytosolic vesicles or expressed as a transmembrane protein [20]. Binding of FasL to the Fas receptor triggers formation of the death-inducing signaling complex (DISC) by recruiting an adaptor molecule Fas-associating protein with death domain (FADD) to Fas [36]. This is accompanied by recruitment of pro-caspase-8 which undergoes auto-proteolytic processing followed by release of the active form of the enzyme into the cytosol [36]. Activation of caspase-8 initiates downstream apoptotic processes that include the activation of caspases-3, -6, -7, and mitochondrial damage [32]. The role of Fas-FasL pathway in the death of lymphocytes has been studied extensively [20], while a few reports have discussed its potential participation in degenerative disorders of the brain [5, 10, 13].

Caspase-8 has been implicated to play a non-redundant role in the extrinsic apoptotic pathway in normal tissues, solid tumors, and leukemias. Loss of caspase-8 expression has been linked with survival in the paediatric tumor medulloblastoma [30]. The Fas-linked death receptor pathway appears to also be involved in GDNF- or BDNF-deprived dopaminergic neurons, and blockade of caspase-8 provides protection against death of these neurons [42].

METH-induced cell death has also been shown to involve activation of the FasL/Fas pathway and increased expression of active caspase-8 in the rat striatum [18]. Although these observations had provided evidence that the Fas/FasL-caspase-8 pathway may be involved in METH-induced neuronal death in the rat striatum, it is, nevertheless, not clear to what extent these results could be generalized to other species. Also because that study had used a single

injection of METH (40 mg/kg), it was also not clear if these findings could be reproduced with different patterns of METH injections. We also wanted to know if METH injections would also cause activation of the Fas/FasL-caspase-8 pathway in other brain regions. Therefore, the present study was conducted to answer these questions. We thus used multiple METH injections that are known to be toxic to monoaminergic systems in the mouse and measured the expression of FasL and caspase-8, two key components in the extrinsic death pathway. Herein, we report that METH caused increased expression of FasL and of active caspase-8 in the striatum, cortex, and hippocampus of the mouse.

2. Methods

2.1. Materials

Triton X-100 and monoclonal α-tubulin antibody was obtained from Sigma-Aldrich, St. Louis, MO, USA. The following antibodies were used in immunohistochemical analysis: FasL was from Oncogene Research Products, CA, USA; and cleaved caspase-8 was from Cell Signaling, Beverly, MA, USA. Polyclonal anti-caspase-8 antibody used in Western blot analysis was from BioVision, Mountain View, CA, USA. ABC kit was obtained from Vector Laboratories, Burlingame, CA, USA; and BioRad Dc Protein assay reagent was obtained from BioRad Laboratories, Hercules, CA, USA. Hybond-P membrane was from Amersham Pharmacia Biotech, Piscataway, NJ, USA. Other standard reagents were from Sigma, and Western blot reagents were from BioRad Laboratories.

2.2. Animals and METH injections

Male ICR mice, 10–15 weeks old and weighing 33–42 g, were obtained from Taconic Labs (Germantown, NY, USA). The animals were given four intraperitoneal injections of 10 mg/kg METH or saline at 2-h intervals [8]. Mice were then sacrificed at various times after the treatments and used in the experiments described below. All animal use procedures were according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

2.3. Terminal deoxynucleotidyl transferase-mediated deoxyribonucleotide triphosphate nick-end labeling histochemistry (TUNEL)

Brain section preparations were performed according to previously reported methods [9]. Briefly, 2 days after METH injections, mice were killed by cervical dislocation. The brains were dissected and cut into 30 μm thickness using a cryostat (Microm, Walldorf, Germany). To expose free DNA 3-OH ends in apoptotic cells, slide-mounted sections were rinsed in 0.5% Triton X-100 (Sigma-Aldrich) in 0.01 mol/L phosphate-buffered saline for 20 min at 80 °C. To label damaged nuclei, 50 μL of the TUNEL reaction mixture was added to each sample in a humidified chamber followed by 60-min incubation at 37 °C. TUNEL-positive cells in mouse brain were counted using a Zeiss fluorescent microscope. The numbers of TUNEL-positive cells from three sections obtained from each animal were counted and added for statistical analysis. For the frontal cortex, we selected three coronal sections at Bregma 1.98, 1.80, and 1.70 mm; for the striatum, we used sections from Bregma 1.34, 0.50, and -0.34 mm; and for the hippocampus, we used Bregma -2.06, -3.08, and -3.80.

2.4. Immunohistochemistry

Immunohistochemical techniques were used to measure the expression of FasL and active caspase-8 in the mouse brain after METH injections. The antibodies used were FasL (Oncogene Research Products, CA) and cleaved caspase-8 (Cell Signaling, Beverly, MA, USA). Mice were sacrificed at various time-points after the last METH or saline injection and processed according to previously published protocols [6, 7]. Briefly, mice were perfused transcardially, under deep pentobarbital anesthesia, first with saline followed by 20 mL of 4% paraformaldehyde in 0.1 M phosphate buffer at 4 °C. The brains were removed, post-fixed overnight in 4% paraformaldehyde, and then allowed to equilibrate in 30% sucrose for 24 h; 30 μm coronal sections were then cut using a cryostat. Free-floating sections were exposed to 1% hydrogen peroxide for 20 min and then incubated for 30 min in 1% bovine serum albumin and 0.3% Triton X-100, followed by incubation with primary antibodies. Subsequent processing with biotinylated secondary antibody and ABC complex was performed according to the manufacturer's procedures described in the ABC kit (Vector Laboratories). The free-floating sections were then reacted with 3, 3′-diaminobenzidine (DAB) and hydrogen peroxide to visualize the peroxidase reaction. At the end of the reaction, the sections were mounted on microscope slides for further visualization and analysis. Numbers of positive cells were counted under light microscopy (20X objective lens). Four to six animals per group were used for quantification.

2.5. Western blot

Expression of cleaved caspase-8 was measured using Western blot analysis with polyclonal anti-caspase-8 antibody (BioVision) as detailed previously [18]. Mice were killed at various time points up to a week after the last METH injection. Briefly, brain tissues were lysed on ice in a buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L ethylene glycol-bis (beta-aminoethyl ether)-N,N,N′, N′-tetraacetic acid (EGTA), 1 mmol/L phenylmethylsulphonylfluoride (PMSF), 0.5% NP-40, 0.25% sodium dodecyl sulfate (SDS), 5 μg/mL leupeptin, and 5 μg/mL aprotinin (all chemicals

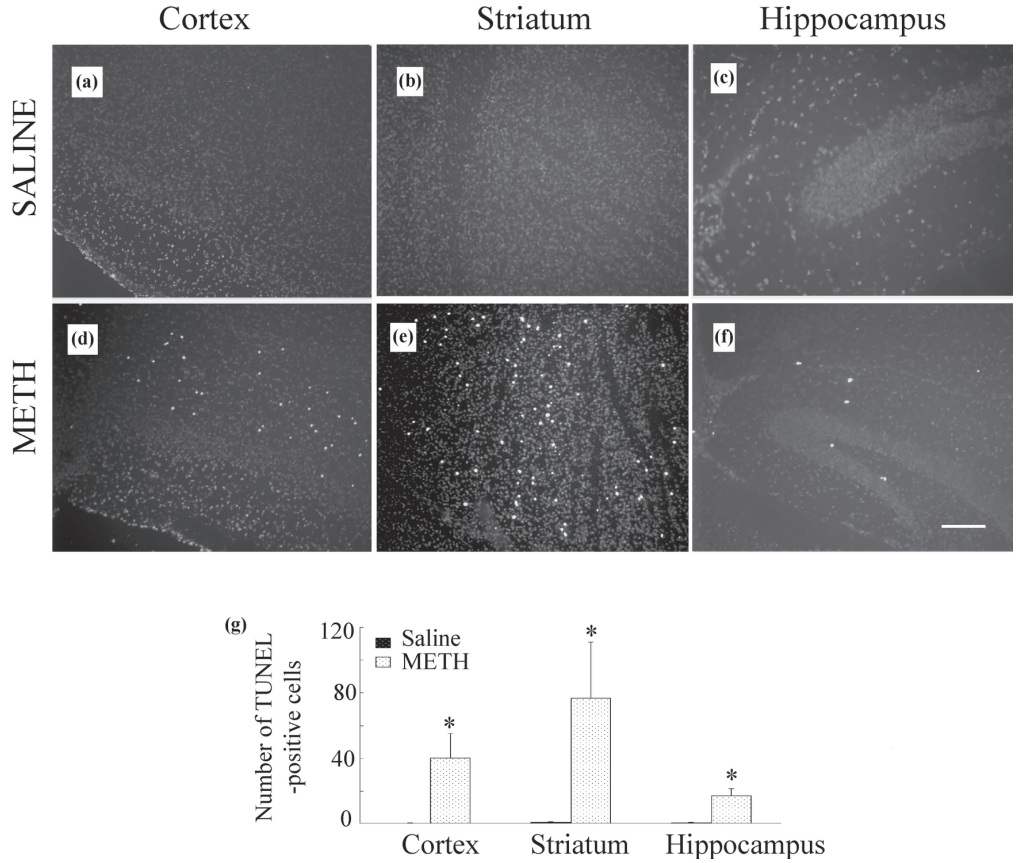

Figure 1: METH administration causes cell death in the mouse brain. There were almost no TUNEL-positive cells observed in the frontal cortex (a), striatum (b), and hippocampus (c) of saline-treated mice. Apoptotic cell death was detected in the mouse brain at 2 days after METH injections (d), (e), and (f). Photomicrographs were generated by using a Carl Zeiss Laser Scanning Confocal System with Axiovert 153-inverted microscopy. The objective lens was 40X. Scale bar $= 200\,\mu$m. Quantitative data and statistical analysis are shown in (g). Key to statistics: $^{*}P < .01$ in comparison with the same brain region of saline-treated mice.

from Sigma-Aldrich). After removal of cell debris by centrifugation, the protein concentration of the cell lysate was determined with the BioRad Dc Protein assay reagent (BioRad Laboratories), and the lysates were denatured with sample buffer (62.5 mmol/L Tris-HCl, 10% glycerol, 2% SDS, .1% bromophenol blue, and 50 mmol/L dithiothreitol) at 100 °C for 5 min and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to Hybond-P membrane (Amersham Pharmacia Biotech). Membrane blocking, caspase-8 primary antibody and secondary antibody incubations, and chemiluminescence reactions were carried out according to the protocol described by individual antibody suppliers. To confirm equal protein loading, blots were reprobed with α-tubulin antibody (1:2,000; Sigma-Aldrich). Signal intensity was measured using densitometric analysis (IS-1000 Digital Imaging System, Alpha Innotech Corp., San Leandro, CA, USA) and quantified using Fluorochem version 2.

2.6. Statistics

For statistical analysis, we used one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test to determine differences between the different time points and the saline-treated animals. All analyses were done using Statview 4.02 (SAS Institute, Cary, NC, USA). The null hypothesis was rejected at $P < .05$.

3. Results

3.1. METH administration causes the appearance of TUNEL-positive cells in the mouse brain

There was almost no TUNEL-positive cell in the frontal cortex (Figure 1(a)), striatum (Figure 1(b)), and hippocampus (Figure 1(c)) of saline-treated mice. However, TUNEL-positive cells were observed in these brain regions (Figures 1(d), 1(e), and 1(f)) at 2 days after METH injections. These results confirmed observations reported by us and others [6,7,9,43].

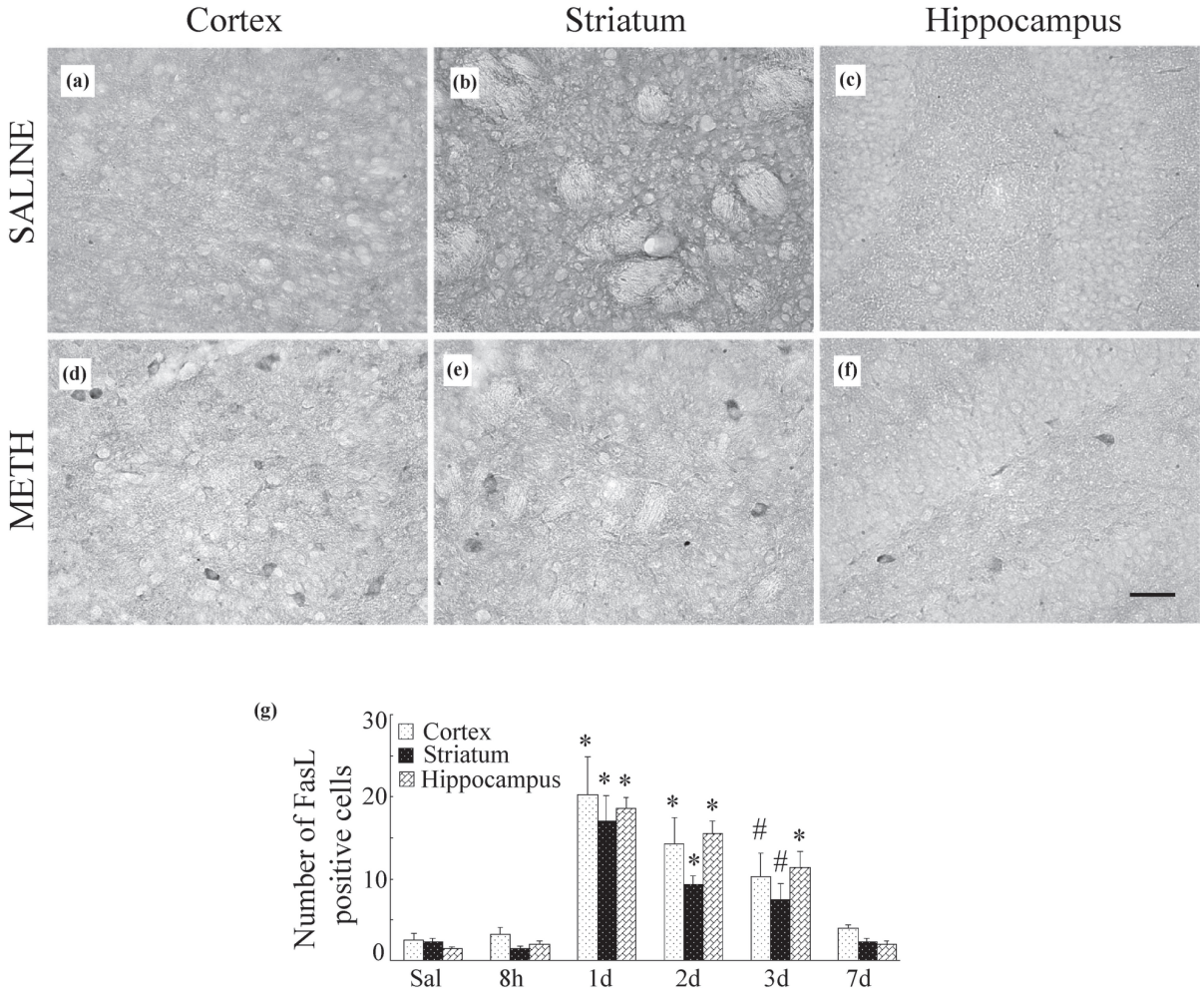

Figure 2: METH administration causes increases of FasL-positive cells in the mouse brain. There were no observable FasL positive cells in the frontal cortex (a), striatum (b), and hippocampus (c) in saline-treated mice. METH administration caused the appearance of FasL-positive cells in the frontal cortex (d), striatum (e), and hippocampus (f) measured at 1 day after the injections. Quantitative data and statistical analysis for FasL-positive cells are shown in (g). Key to statistics: $^{*}P < .01$ and $^{\#}P < .05$ in comparison with the same brain region of saline-treated mice. Scale bar $= 100\,\mu$m.

3.2. Toxic doses of METH cause increases in FasL-positive cells in the mouse brain

We used immunohistochemical techniques to assess the effects of injections of a toxic METH regimen on the expression of FasL protein in the brain. As shown in Figure 2, there were very few FasL-positive cells in the cortex (Figure 2(a)), striatum (Figure 2(b)), and hippocampus (Figure 2(c)) of the control mice. In contrast, METH injections caused the appearance of FasL-positive cells in these brain areas (Figures 2(d), 2(e), and 2(f)). The quantification of the data is shown in Figure 2G and revealed that METH caused peaked increases in FasL expression at 1 day post drug which gradually reverted to normal by day 7 after drug injections. We also attempted to measure the expression of the Fas receptor in these mice, but we were unsuccessful with the antibodies used.

3.3. METH administration causes increased expression of active caspase-8 in the mouse brain

In order to test if the appearance of TUNEL-positive cells and the increased expression of FasL were associated with activation of the FasL/Fas/caspase-8 death receptor pathway, we used an antibody against active caspase-8 and carried immunohistochemical studies of caspase-8 expression after METH injections. There was almost no activated caspase-8 expression in the cortex (Figure 3(a)), striatum (Figure 3(b)), or hippocampus (Figure 3(c)) of saline-treated mice. However, METH injections cause the appearance of activated caspase-8 in those brain areas (Figures 3(d), 3(e), and 3(f)). The quantitative data are provided in Figure 3(g).

The changes in cleaved caspase-8 induced by METH injections were confirmed by Western blot analysis which revealed time-dependent increases in the 43-kDa cleaved

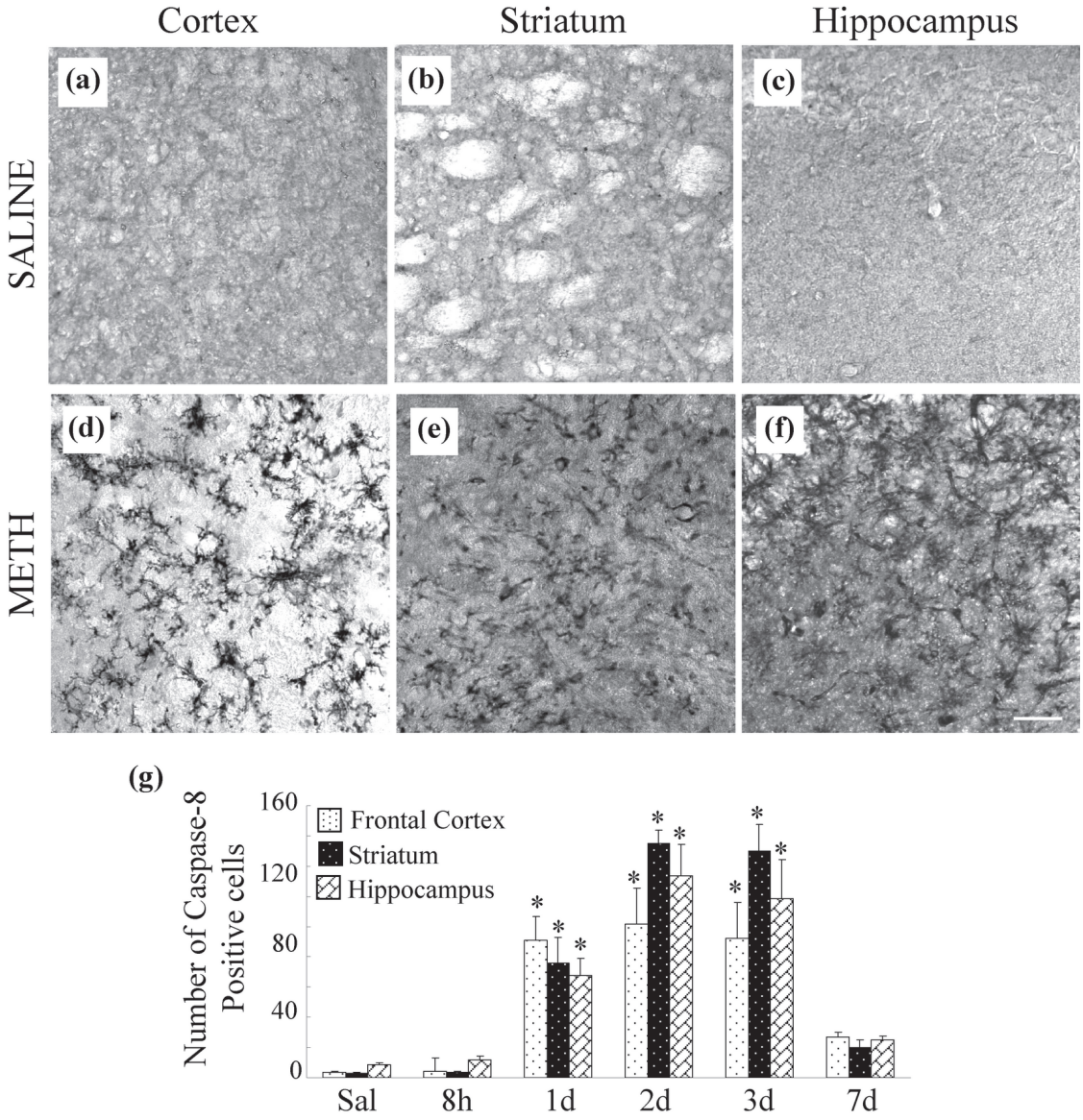

Figure 3: METH causes caspase-8 activation in the mouse brain. There was no evidence of any active caspase-8-like signals in the brains of saline-treated mice (a), (b), and (c). METH administration caused active caspase-8 expression in the frontal cortex (d), striatum (e), and hippocampus (f). Quantitative data and statistics are shown in (g). Key to statistics: $^{*}P < .01$ in comparison with the same brain region of saline-treated mice. Scale bar $= 100\,\mu$m.

fragment of caspase-8 starting at around 8 h and peaking at 3 days after the METH injections (Figure 4). In the cortex (Figure 4(a)) and striatum (Figure 4(b)), the protein started to revert toward control values at 7 days after METH, whereas the increases in caspase-8 expression were still around peak levels in the hippocampus (Figure 4(c)).

4. Discussion

The main observations in the present paper are that multiple injections of METH can cause activation of FasL and cleavage of caspase-8 in the frontal cortex, striatum and hippocampus of mice. These findings extend earlier findings showing METH can also activate the FasL/Fas/caspase-8 death pathway in the rat striatum [18]. Increased FasL expression in the brain and in cultured neurons is thought to participate in the cellular demise observed in neurodegenerative processes [5, 14, 24]. FasL is thought to be involved in neuronal death observed after brain trauma [11, 14, 31], excitotoxic brain injuries [34], strokes [26, 35], ischemia [21], and Parkinson's disease [12, 15, 28]. FasL-induced clustering of Fas, FADD, and caspase-8 leads to autoproteolytic processing of caspase-8, the initiator caspase in the Fas signaling pathway which leads to activation of the executioner caspase-3 and consequent

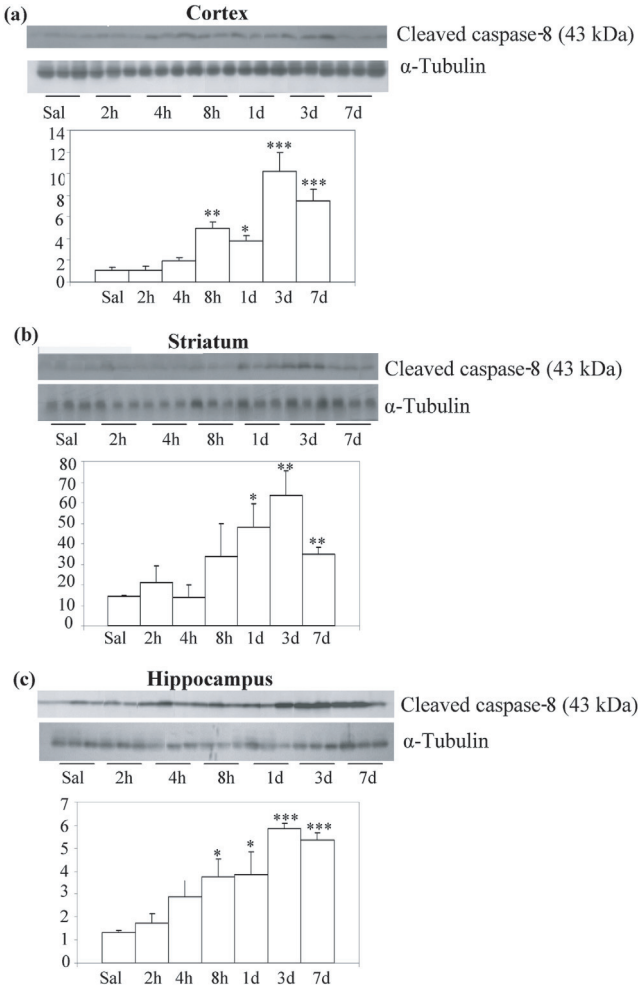

Figure 4: METH causes increases in the expression of active caspase-8 in the mouse brain. Western blot analysis shows time-dependent increases of cleaved caspase-8 (43 KDa) in the frontal cortex (a), striatum (b), and hippocampus (c) from 1 day to 7 days. The greatest changes occur at 3 days after METH treatment. Keys to statistics: $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ in comparison with the saline-treated groups.

cellular damage through apoptosis [23]. Studies using caspase-8-deficient mice indicate that this caspase is indeed required for Fas-mediated apoptosis [37].

Although caspase-8 is the most apical caspase component of the Fas death pathway because it is the primary caspase recruited to the death induced signaling complex (DISC) [27], the activation of caspase-8 appears to also be involved in processes of cellular proliferation, neutrophilic migration, cytokine expression, and cytokine release [29, 41]. In the brain, caspase-8 activation has been reported in both neurons and astroglial cells after excitotoxic damage to the brain [38]. Because METH toxicity is also known to involve glutamate-induced excitotoxic damage [3], the present observations suggest that excitatory amino acids might play an important role in the activation of the FasL/Fas/caspase-8 death pathway in the rodent brain after injections of toxic doses of this illicit neurotoxin. Future studies will need to assess to what extent blockade of specific glutamate receptors can modify the activation of the extrinsic death pathway in models of METH toxicity.

5. Conclusion

In conclusion, the present results confirm the observations that METH-induced neurodegeneration involves activation of multiple death pathways. These findings need to be taken into consideration in clinical settings that treat METH addicts who show cognitive deficits.

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