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THE EFFECT OF AMPHETAMINE ANALOGS ON CLEAVED MAP-TAU FORMATION IN THE RAT BRAIN

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Abstract

The present study quantified the cleaved form of the microtubule associated protein tau (cleaved MAP-tau, C-tau), a previously demonstrated marker of CNS toxicity, following the administration of monoamine-depleting regimens of the psychostimulant drugs amphetamine (AMPH), methamphetamine (METH), $\pm$ 3,4-methylenedioxymethamphetamine (MDMA), or para-methoxyamphetamine (PMA) in an attempt to further characterize psychostimulant-induced toxicity. A dopamine (DA)-depleting regimen of AMPH produced an increase in C-tau immunoreactivity in the striatum, while a DA- and serotonin (5-HT)-depleting regimen of METH produced an increase in the number of C-tau immunoreactive cells in the striatum and CA2/CA3 and dentate gyrus regions of the hippocampus. MDMA and PMA, two psychostimulant drugs that produce selective 5-HT depletion in the striatum, had no effect on C-tau immunoreactivity in the striatum or hippocampus. Furthermore, 5,7-dihydroxytryptamine (5,7-DHT), an established 5-HT selective neurotoxin, did not produce an increase in C-tau immunoreactivity. Dual fluorescent immunocytochemistry with antibodies to GFAP and C-tau indicated that C-tau immunoreactivity was present in astrocytes, not neurons, suggesting that increased C-tau may be an alternative indicator of reactive gliosis. The present results are consistent with previous findings that the DA-depleting psychostimulants AMPH and METH produce reactive gliosis whereas the 5-HT-depleting drugs MDMA and PMA, as well as the known 5-HT selective neurotoxin 5,7-DHT, do not produce an appreciable glial response.

Keywords

methamphetamine; 3,4-methylenedioxymethamphetamine; amphetamine; para-methoxyamphetamine; 5,7-dihydroxytryptamine; toxicity

Methamphetamine (METH) and $\pm$ 3,4-methylenedioxymethamphetamine (MDMA) are substituted phenylethylamines whose popularity among young adults continues to be a public

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health concern. Results from studies in humans, non-human primates and rodents support the view that these compounds cause structural damage to monoamine nerve terminals and are thereby neurotoxic (O'Callaghan, 2002a, Lyles and Cadet, 2003, Itzhak and Achat-Mendes, 2004). Such data include the finding that repeated high-dose administration of METH results in a reduction in striatal dopamine (DA) and serotonin (5-HT) concentrations (Kogan et al., 1976, Ricaurte et al., 1980, Wagner et al., 1980), a decrease in the activity of tyrosine and tryptophan hydroxylase, the rate-limiting enzymes responsible for the production of DA and 5-HT (Hotchkiss and Gibb, 1980), and reduced number of amine uptake sites (Wagner et al., 1980). Likewise, repeated high-dose administration of MDMA produces selective, long-term reductions in striatal 5-HT concentration (Schmidt, 1987, Battaglia et al., 1988), tryptophan hydroxylase activity (Stone et al., 1986, Schmidt and Taylor, 1987, Stone et al., 1987) and the number of 5-HT reuptake sites (Battaglia et al., 1987).

Additional biomarkers support the assertion that METH produces neurotoxicity; these include increased glial fibrillary acidic protein (GFAP) expression and immunoreactivity (O'Callaghan and Miller, 1993, Bowyer et al., 1994) and increased silver staining for degenerating neurons (Ricaurte et al., 1982, Bowyer et al., 1994). Newer markers, including Fluoro-Jade staining (Schmued and Bowyer, 1997, Eisch and Marshall, 1998, Eisch et al., 1998), as well as microglial activation (Thomas et al., 2004, Thomas and Kuhn, 2005), increased density of peripheral benzodiazepine receptors and increased expression of heat shock protein 27 (both markers of gliosis) (Pubill et al., 2003), are also in support of METH-induced neurotoxicity.

However, MDMA appears to be less effective when compared to METH in producing non-serotonergic markers of neurotoxicity. For example, MDMA appears to be relatively ineffective in producing reactive gliosis, as indicated by a lack of a pronounced increase in expression or immunoreactivity of GFAP (O'Callaghan and Miller, 1993, Pubill et al., 2003, Wang et al., 2004b), although in a single exception, Aguirre and colleagues report an increase in GFAP immunoreactivity in the hippocampus 7 days after MDMA administration (Aguirre et al., 1999). MDMA also does not produce an appreciable increase in the expression of heat shock protein 27, particularly when compared to responses evoked by METH (Pubill et al., 2003). MDMA does not consistently produce increases in markers of gliosis, as Orio et al. (2004) demonstrate early increases in IL-1 β and peripheral benzodiazepine receptor (PBZ) binding in the per hypothalamus and cortex, yet Pubill et al. (2003) indicate that MDMA does not produce increased microglial activation as indicated by [3 H] PK11195 binding or OX-6 immunostaining in the striatum and cortex. Wang et al (2004b) have reported that SERT expression remains unchanged two weeks after a multiple-dose regimen of MDMA despite studies that have decreased radioligand binding to SERT. However, these results are contradicted by a recent report of Xie et al. (Xie et al., 2006) in which a MDMA-induced reduction in SERT protein expression was demonstrated, suggesting a loss of 5-HT axons. Additionally, studies have indicated a lack of Fluoro-Jade-B positive neurons in the hippocampus and striatum following administration of MDMA, although a limited number of degenerating neurons have been noted in other brain regions (Schmued, 2003). Furthermore, although some investigators have reported silver staining of degenerating axon terminals in the brains of rats treated with MDMA (Commins et al., 1987, Molliver et al., 1990), others have reported inconsistent results or patterns of staining that are not in agreement with other indicators of altered 5-HT innervation (Jensen et al., 1993). Given the mixed data regarding loss of 5-HT nerve terminals following administration of MDMA, investigation of additional markers of CNS injury are warranted.

Recently, proteolytically cleaved MAP-tau has been reported to be a novel biomarker of CNS toxicity (Zemlan et al., 1999, Irazuzta et al., 2001). MAP-tau is primarily a cytoskeletal protein which functions to stabilize microtubules. Upon CNS damage by a variety of insults, MAP-tau appears to be proteolytically cleaved (Irazuzta et al., 2001, Zemlan et al., 2002). Cleaved

tau (C-tau) concentrations have been shown to be increased following administration of the prototypic neurotoxins kainic acid (Zemlan et al., 2003) and trimethyltin (Straiko et al., 2005 (submitted)) or following traumatic brain injury in rodents (Gabbita et al., 2005) and humans (Zemlan et al., 2002). Notably, METH also has been reported to produce a time-dependent increase in C-tau in the striatum, hippocampus and, to a lesser extent, frontal cortex (Wallace et al., 2003). The purpose of the present study was to compare the extent of C-tau formation produced by monoamine-depleting regimens of METH, MDMA, amphetamine (AMPH) and para-methoxyamphetamine (PMA).

Experimental Procedures

Animals and Treatment Procedures

Adult male (270–300 g) Sprague-Dawley rats (Harlan Laboratories; Indianapolis, IN, USA) were group-housed, maintained on a 12 hour light/dark cycle and allowed food and water *ad libitum*. Dosing regimens for drugs were as follows: AMPH (iprindole, 10 mg/kg, i.p. followed 60 minutes later with AMPH, 9 mg/kg, i.p.); METH (a total of 4 injections consisting of 2 i.p. injections of 10 mg/kg, and 1 i.p. injection each of 7.5 mg/kg and 5 mg/kg spaced 2 hours apart); MDMA (10 mg/kg, i.p., given every 2 hours for 4 injections) or PMA (10 mg/kg, i.p., given every 2 hours for 4 injections). All drugs were injected in a volume of 1 ml/kg. Vehicle injections consisted of 0.9% NaCl. Two hours prior to and throughout drug treatment, rats were maintained at an elevated room temperature of 24–25 °C.

In separate experiments, rats received i.c.v. injections (coordinates: +0.8 mm A/P; 1.4 mm L from Bregma, 3.5 mm ventral to the surface of the brain) of 10 µl of vehicle (0.1% ascorbic acid in 0.9% NaCl) or 5,7-dihydroxytryptamine (5,7-DHT) (150 µg) 60 minutes following the administration of mazindol (5 mg/kg, i.p.).

Effort was made to minimize the number of animals used in this study. Animal procedures adhered to the policies set forth in the NRC *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee.

Dopamine and Serotonin Analyses

Three days following the administration of drug or vehicle, rats were killed by decapitation and brains removed. Striatum was dissected from 1 mm coronal sections of the brain and maintained at –80 °C until analysis for dopamine (DA) and serotonin (5-HT). For animals receiving i.c.v. injections, the striatum ipsilateral to the injection site was analyzed. DA and 5-HT concentrations were determined by high performance liquid chromatography with electrochemical detection (Gudelsky et al., 1994). The mobile phase consisted of 35 mM citric acid, 54 mM sodium acetate, 60 mg/L octanesulfonic acid, 50 mg/L EDTA, 5% (volume/volume) methanol at pH 4.2. The mobile phase was pumped at 0.3 mL/min onto a C18 column connected to an LC-4C amperometric detector (BAS, Inc., West Lafayette, IN, USA). Peak heights were quantified with a Hewlett-Packard integrator.

Tissue Preparation for C-tau Analysis

Rats were treated with drug or vehicle and, 3 days later, deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and perfused transcardially with 200 ml 0.9% NaCl followed by 500 ml 4% paraformaldehyde made in PB (0.1 M, pH 7.3). Following perfusion, brains were removed and postfixed in 4% paraformaldehyde for 1 hour, then placed in 20% sucrose and stored at 4 °C until the time of sectioning. Coronal sections were cut at 35 µm thickness in 4 parallel series on a freezing microtome from the olfactory region through the rostral brainstem and stored as floating sections in cryoprotectant (30% sucrose, 30% ethylene glycol in 0.1 M PB; (Watson et al., 1986)) at –20 °C until the time of staining.

C-tau Immunocytochemistry

All staining was performed at room temperature in the light. Free-floating sections were rinsed in 0.1 M PBS for 4 hours, changing buffer every 10 minutes, to remove cryoprotectant and then in TBST (TBS containing 1% Tween, 3 rinses of 10 minutes each) and then blocked in 0.5% H₂O₂ in PBS for 20 minutes. Sections were rinsed in TBST and blocked a second time in 4% normal horse serum for 1 hour and incubated overnight in PBS containing the primary antibody C-tau 7 (1:120,000). Following washes in TBST, sections were incubated in secondary antibody (biotinylated anti-mouse IgG; Vector Laboratories, Burlingame, CA, USA) for 1 hour, followed by rinsing in TBST. Sections were incubated in Vectastain ABC-HRP reagent (Vector Laboratories) for 30 minutes and bound antibody visualized with nickel sulfate enhanced diaminobenzidine (DAB, Sigma, St. Louis, MO, USA). Sections were mounted on glass slides with 0.3% gelatin, air-dried overnight, rehydrated in ddH₂O and then dehydrated in graded alcohol and coverslipped with DPX (Electron Microscopy Sciences, Fort Washington, PA, USA) (Balfour et al., 2006).

C-tau and GFAP Dual Fluorescent Immunocytochemistry

All staining was performed at room temperature in the light unless indicated otherwise. Free-floating sections were thoroughly washed in 10 minute rinses of 0.1 M PBS containing 1% Tween for 4 hours to remove cryoprotectant, then washed in TBST and blocked in 4% normal horse serum in TBS for 1 hour. Sections were incubated overnight in TBS containing the primary antibodies C-tau7 (1:1000) and rabbit anti-GFAP (1:250; Sigma). Following washes in TBST, tissue sections were covered with aluminum foil to prevent exposure to the light. Sections were incubated in CY3-conjugated donkey anti-mouse (1:100; Jackson ImmunoResearch, Westgrove, PA, USA) for 30 minutes, rinsed with TBS and incubated in Alexa 488-conjugated goat anti-rabbit (1:100; Molecular Probes, Eugene, OR, USA) for 30 minutes. Sections were rinsed in phosphate buffer, mounted on glass slides and coverslipped with Gelvatol (Harlow and Lane, 1988) containing an anti-fading agent (1,4-diazabicyclo(2,2)octane; Sigma).

Data Analysis

Camera lucida drawings were made of each analyzed section from each rat using a Leica microscope with an attached drawing tube (Leica Microsystems; Wetzlar, Germany). In the striatum of each vehicle- or drug-treated rat, cell counts were made in standardized areas (0.36 mm²) in 4 bilateral sections approximately 280 μ m apart representative of 4 rostral to caudal levels ranging from Bregma +1.20 to Bregma +0.48. In the hippocampus of METH-, MDMA- or VEH-treated rats, cell counts were made in standardized areas of the CA1 (0.4 mm²), CA2/CA3 (0.36 mm²) and dentate gyrus (0.4 mm²), in four bilateral sections approximately 280 μ m apart, representative of 4 rostral to caudal levels of the dorsal hippocampus ranging from Bregma -2.56 to Bregma -3.30. The average cell count of the four striatal and hippocampal sections were determined for each animal. In rats treated with VEH or 5,7-DHT, cell counts were determined in standard areas (0.36 mm²) in four sections of striatum ipsilateral to the injection site approximately 280 μ m apart. For each area of analysis, the average of the four sections was determined per animal and group averages were calculated.

Digital images of immunostained sections were captured using a digital camera (MagnaFire, Optonics) attached to a Leica microscope (Leica Microsystems; Wetzlar, Germany). To demonstrate the location of C-tau and GFAP, fluorescent-stained sections were examined using a Zeiss laser-scanning confocal microscope system (Zeiss LSM-510; Heidelberg, Germany). CY3 fluorescence was imaged with a 567 nm emission filter and a HeNe laser, and Alexa 488 with a 505 nm emission filter and Argon laser. Stacks of 5 μ m optical sections along the z-axis were obtained at 25x magnification. Images were imported into Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) to compose figures and were only adjusted for brightness.

Statistical Analyses

Analysis of the number of C-tau positive cells and tissue concentrations of DA and 5-HT were evaluated with a one-way analysis of variance. Individual post-hoc group comparisons were made using the Student-Newman-Keuls test. Treatment differences were considered significant at $P < 0.05$.

Results

Concentrations of DA and/or 5-HT in the striatum of rats 3 days following the regimens of METH, AMPH, MDMA, and PMA are documented in Table 1. Treatment with either AMPH or METH produced a significant [$F(4,44) = 45.039$; $P < 0.001$] decrease in striatal DA concentrations, whereas the striatal DA concentration was not significantly affected by treatment with MDMA ($P = 0.190$) or PMA ($P = 0.240$) compared to VEH-treated controls. In contrast, treatment with METH, MDMA or PMA produced a significant [$F(4,44) = 6.982$; $P < 0.001$] decrease in striatal concentrations of 5-HT compared to VEH-treated controls. The striatal concentration of 5-HT was not significantly affected by AMPH ($P = 0.203$). Treatment with 5,7-DHT produced a significant [$F(1,8) = 12.822$; $P < 0.01$] decrease in striatal 5-HT concentrations compared to VEH-injected controls but was without effect on striatal DA concentrations ($P = 0.144$) (Table 3).

The numbers of C-tau immunoreactive cells in the striatum and CA1, CA2/CA3 and dentate gyrus regions of the hippocampus of rats treated with VEH, METH or MDMA are depicted in Figure 1. Treatment with METH resulted in a number of C-tau immunoreactive cells in the striatum that was 19 times greater than that in VEH-treated controls [$F(2,17) = 64.607$; $P < 0.001$]. METH also produced a significant increase in the number of C-tau immunoreactive cells in the CA2/CA3 [$F(2,17) = 7.180$; $P < 0.05$] and dentate gyrus [$F(2,17) = 5.646$; $P < 0.05$] regions of the hippocampus. The number of C-tau positive cells in the CA2/CA3 and dentate gyrus regions of the hippocampus of METH-treated rats was almost twice that in the same brain region of VEH-treated rats. The number of C-tau immunoreactive cells in the CA1 region of the hippocampus was not significantly different between VEH- and METH-treated rats, although there was a trend for a METH-induced increase [$F(2,17) = 6.155$; $P = 0.069$]. In contrast to the significant METH-induced increase in C-tau formation, MDMA treatment did not significantly alter the number of C-tau positive cells in the striatum ($P = 0.922$), CA1 ($P = 0.247$), CA2/CA3 ($P = 0.329$) or dentate gyrus ($P = 0.696$) regions of the hippocampus (Figure 1). Photomicrographs of sections of striatum (Figure 2A, C, E) and dentate gyrus (Figure 2B, D, F) of rats treated with VEH (Figure 2A, B) or monoamine-depleting regimens of METH (Figure 2C, D) or MDMA (Figure 2E, F) further illustrate the increase in C-tau immunoreactive cells present in the brain regions in METH-, but not MDMA-, treated rats.

C-tau immunoreactive cells also were quantified in the striatum of rats treated with VEH or dosing regimens of AMPH or PMA demonstrated to produce depletion of DA alone (AMPH) or 5-HT alone (PMA) (Table 1). Although AMPH treatment resulted in a significant increase in C-tau immunoreactive cells [$F(2,11) = 6.859$; $P < 0.05$] in the striatum compared to VEH-treated controls, PMA treatment was without effect in this regard ($P = 0.461$) (Table 2).

Additionally, C-tau immunoreactive cells were quantified in the striatum of rats that received i.c.v. injections of VEH or 5,7-DHT, a selective 5-HT neurotoxin. Although treatment with 5,7-DHT produced a 70% reduction in the striatal concentration of 5-HT, 5,7-DHT did not significantly increase the number of C-tau immunoreactive cells in the striatum ($P = 0.727$) (Table 3).

Additional studies employing dual fluorescent immunocytochemistry with antibodies for C-tau and GFAP (an astrocytic marker) and confocal microscopy were performed to examine the

potential localization of C-tau within astrocytes. Cells in the striatum (Figure 3A, C and E) and dentate gyrus (Figure 3B, D and F) of VEH- (Figure 3A, B), METH- (Figure 3C, D) and MDMA- (Figure 3E, F) treated rats that were C-tau immunoreactive were also immunoreactive for GFAP. As indicated in Figure 3, there was essentially complete overlap of C-tau and GFAP immunoreactivity, indicating that C-tau positive cells were astrocytes.

Discussion

The key findings of this study are that dosing regimens of METH or AMPH, psychostimulant drugs demonstrated to produce significant long-term depletions of striatal 5-HT and DA (METH) or DA alone (AMPH) produced a significant increase in the number of C-tau immunoreactive cells in the striatum and the CA2/CA3 and dentate gyrus regions of the hippocampus. In contrast, the psychostimulants MDMA and PMA, and 5,7-DHT, a known 5-HT neurotoxin, which produce a selective depletion of 5-HT, did not increase the number of C-tau immunoreactive cells in either the striatum or hippocampus. Additionally, colocalization of C-tau and GFAP, as demonstrated with dual fluorescent immunocytochemistry and confocal microscopy, indicated that C-tau positive cells in the striatum and hippocampus of both VEH- and METH-treated animals were astrocytes.

It has been well documented that AMPH and METH produce selective depletions of striatal DA and/or 5-HT in the rat (Kogan et al., 1976, Ricaurte et al., 1980, Wagner et al., 1980) and that MDMA produces a selective long-term depletion of 5-HT (Schmidt, 1987, Battaglia et al., 1988). In the present study, repeated administration of PMA also resulted in a selective depletion of striatal 5-HT. To our knowledge, this is the first demonstration of PMA-induced 5-HT depletion.

Increased C-tau formation has been demonstrated following induction of experimental bacterial meningitis (Irazuzta et al., 2000, Irazuzta et al., 2001) and traumatic brain injury (Gabbita et al., 2005), as well as following the administration of the known neurotoxins kainic acid (Zemlan et al., 2003) and trimethyltin (Straiko et al., 2005 (submitted)). Additionally, Wallace et al. (2003) have reported that C-tau concentrations determined by ELISA are increased in the striatum and hippocampus following administration of a monoamine-depleting regimen of METH. The results of the present study, in which the number of C-tau immunoreactive cells in the striatum and hippocampus was increased following METH treatment, are in accord with the findings of Wallace et al (2003).

Upon quantifying C-tau immunoreactive cells, it was noted further that the C-tau positive cells exhibited a stellate morphology and distribution that was consistent with that of astrocytes. Although a similar morphology of C-tau immunoreactive cells was evident in an earlier study (Zemlan et al., 2003), dual immunohistochemistry was not employed in that study to distinguish neuronal vs. astrocytic localization of C-tau. Fluorescence immunocytochemistry and confocal analysis in the present study revealed the co-localization of C-tau and GFAP immunoreactivity. Furthermore, in a separate experiment, there was no overlap in the localization of immunoreactivity for C-tau and the neuronal marker NeuN (unpublished observations). These results further support the conclusion that C-tau positive cells were astrocytes. However, additional studies are necessary to determine whether C-tau immunoreactivity in astrocytes results either from C-tau of neuronal origin that is scavenged by astrocytes, leading to its localization within these cells or from C-tau isoforms that always reside within astrocytes. Although tau is usually associated with the axons of neurons, tau has also been shown to be present in glial cells (Bloom et al., 1984, LoPresti et al., 1995, Yoshiyama et al., 2003). Tau accumulation in glia is the result of increased synthesis or reduced clearance of the molecule that results in tau fibrillization and degeneration of the affected cells (Yoshiyama et al., 2003). Thus, it is possible that C-tau is a novel marker for reactive astrogliosis in response to

CNS insult. In this regard, it is noteworthy that numerous CNS insults (e.g. kainic acid, trimethyltin, traumatic brain injury) that have been shown to increase C-tau formation (Irazuzta et al., 2001, Zemlan et al., 2003, Gabbita et al., 2005) also increase GFAP, a marker of reactive gliosis (Brock and O'Callaghan, 1987, O'Callaghan and Miller, 1993, McCann et al., 1996, Koczyk and Oderfeld-Nowak, 2000, Little et al., 2002, Wang et al., 2004a, Guo et al., 2006).

Furthermore, the results of the present experiment, in which the number of C-tau immunoreactive cells has been shown to be increased in the striatum following METH or AMPH treatment, but not MDMA or PMA treatment, are in agreement with previous studies that have examined GFAP immunoreactivity or protein expression. Treatment of rodents with a METH regimen similar to that used in this study or single high-dose METH administration has been reported to produce an increase in GFAP in the striatum as measured by semi-quantitative immunoblot (Fukumura et al., 1998) or immunoreactivity (Bowyer et al., 1994, Miller and O'Callaghan, 1994, O'Callaghan and Miller, 1994, Broening et al., 1997) 3 days after drug treatment. Various dosing regimens of AMPH also have been reported to increase striatal GFAP immunoreactivity (Bowyer, 2000, Armstrong et al., 2004, Bowyer et al., 2004). According to O'Callaghan and Miller (1993), reactive gliosis may be elicited by damage to any neuronal or glial cell type. Although most studies have focused on neuronal toxicity following administration of psychostimulant drugs, studies with primary astrocyte cultures suggest that METH also may act directly on astrocytes to produce astrogliosis and oxidative stress (Stadlin et al., 1998). Thus, the increase in C-tau in response to METH or AMPH may be reflective of damage to either neurons or astrocytes in these brain regions.

In contrast to the pronounced effect of METH and AMPH on the number of C-tau immunoreactive cells, treatment of rats with a 5-HT depleting regimen of MDMA failed to significantly increase the number of C-tau positive cells in the striatum and hippocampus; similarly, PMA was without effect on C-tau immunoreactivity in the striatum. Although only a limited number of amphetamine analogs was examined in the present study, it is intriguing that an increase in the number of C-tau immunoreactive cells was produced by psychostimulants (e.g., METH and AMPH) that produce DA depletion but not by those (e.g., MDMA, PMA) that produce selective 5-HT depletion.

The lack of C-tau response following MDMA treatment is generally consistent with the results from several studies that have assessed the effects of MDMA on GFAP. Repeated high-dose administration of MDMA that results in significant 5-HT depletion has been reported to have no effect on GFAP immunoreactivity (O'Callaghan, 2002b, Pubill et al., 2003, Wang et al., 2004b), although Aguirre et al. (1999) have reported a GFAP response to MDMA. O'Callaghan and Miller (1993) also have reported that multiple injections of exceptionally high doses of MDMA produce an increase in GFAP that is, nevertheless, relatively modest when compared to the response to METH. However, MDMA does produce a marked increase in GFAP in the mouse (O'Callaghan and Miller, 1994), but it should be noted that MDMA produces DA, not 5-HT, depletion in the mouse. This pattern of response in the mouse also appears to be consistent with the differential effects of DA and 5-HT depleting amphetamine analogs on C-tau and GFAP expression.

The lack of GFAP response and inconsistencies in silver staining following a 5-HT-depleting regimen of MDMA have led to debate as to whether MDMA is neurotoxic to 5-HT axon terminals (Commins et al., 1987, Jensen et al., 1993, O'Callaghan and Miller, 1993, Pubill et al., 2003). In the present study, the lack of an increase in the number of C-tau positive astrocytes in response to MDMA and PMA also could be viewed as indirect evidence that these amphetamine analogs are not neurotoxic. However, the i.c.v. administration of the prototypic 5-HT neurotoxin 5,7-DHT produced a 70% reduction in striatal 5-HT concentrations but did not increase the number of C-tau immunoreactive cells. Thus, it would appear that C-tau

expression is not a reliable marker of degenerating 5-HT axon terminals. It could be suggested that the extent of 5-HT depletion produced by MDMA or 5,7-DHT may not have been sufficient to elicit a C-tau response. However, the magnitude of DA depletion produced by METH administration was no greater than the magnitude of 5-HT depletion produced by MDMA or 5,7-DHT, yet METH did produce an increase in C-tau positive cells. Alternatively, degenerating 5-HT axon terminals may not trigger an astrocytic response. The i.c.v. administration of 5,7-DHT not only fails to increase C-tau formation, as shown in the present study, but also produces little or no increase in GFAP message or protein expression (O'Callaghan and Miller, 1993, Rowland et al., 1993, Bendotti et al., 1994, Fages et al., 1994, Dugar et al., 1998, Wang et al., 2004b). Reasons for the failure of 5-HT neurotoxins to produce robust histological or immunocytochemical markers of toxicity are unknown.

In summary, treatment of rats with DA-depleting regimens of METH or AMPH resulted in an increase in the number of C-tau immunoreactive cells in the striatum and hippocampus. In contrast, the psychostimulants MDMA and PMA, as well as 5,7-DHT, which produce selective depletions of brain 5-HT, did not increase C-tau immunoreactivity. Furthermore, C-tau formation was localized exclusively to astrocytes. These data are consistent with previous findings that treatment with METH, but not MDMA, produces a response of reactive gliosis.

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Abbreviations

AMPH	amphetamine
CNS	central nervous system
C-tau	cleaved microtubule associated protein tau
DA	dopamine
DG	dentate gyrus
5	7-DHT, 5, 7-dihydroxytryptamine
EDTA	ethylenediaminetetraacetic acid
ELISA	

	enzyme-linked immunosorbant assay
5-HT	5-hydroxytryptamine
GFAP	glial fibrillary acidic protein
MAP-tau	microtubule associated protein tau
MDA	methylenedioxyamphetamine
MDMA	$\pm$ 3, 4-methylenedioxymethamphetamine
METH	methamphetamine
PBS	phosphate buffered saline
PMA	para-methoxyamphetamine
SERT	serotonin transporter
STR	striatum
TBS	tris buffered saline
TBST	tris buffered saline containing 1% Tween
VEH	vehicle

Figure 1.

Quantification of C-tau labeling in the striatum and hippocampus of rats treated with methamphetamine or $\pm$ 3,4 methylenedioxymethamphetamine. C-tau positive cells were counted in defined regions of the striatum and CA1, CA2/CA3 and dentate gyrus regions of the hippocampus of rats killed 3 days after treatment with METH, MDMA or VEH, administered as described in the legend of Table 1. Bars represent mean $\pm$ SEM of 5–9 rats. *** $P < 0.001$ and * $P < 0.05$ compared to VEH-treated rats.

Figure 2.

C-tau immunolabeling in the striatum and dentate gyrus regions of the hippocampus. Immunocytochemistry was performed with an antibody specific to cleaved MAP-tau (C-tau7) and striatal (panels A, C, E) and hippocampal (panels B, D, F) tissue of rats killed 3 days after treatment with VEH (panels A and B), METH (panels C and D) or MDMA (panels E and F). Drugs were administered as described in the legend of Table 1. Magnification, 20x.

Figure 3.

Co-localization of C-tau and GFAP immunoreactivity in striatum and dentate gyrus regions of the hippocampus. Dual fluorescent immunocytochemistry was performed with antibodies to C-tau7 and GFAP and striatal (panels A, C, E) and hippocampal (panels B, D, F) tissue from rats killed 3 days after treatment with VEH (panels A and B), METH (panels C and D) or MDMA (panels E and F) according to the regimen described in detail in the legend of Figure 1. Presence of yellow color indicates co-localization of C-tau and GFAP antibodies. Magnification, 40x.

Table 1

Striatal dopamine and serotonin depletions produced by the psychostimulant drugs amphetamine, methamphetamine, $\pm$ 3,4-methylenedioxymethamphetamine, or para-methoxyamphetamine. Concentrations of dopamine (DA) and serotonin (5-HT) in the striatum of rats were determined 3 days after treatment with vehicle (saline, i.p.), amphetamine (AMPH; iprindole (10 mg/kg, i.p.) followed 1 hour later by AMPH (9.2 mg/kg, i.p.)) methamphetamine (METH; a total of 4 injections consisting of 2 i.p. injections of 10 mg/kg, and 1 i.p. injection each of 7.5 mg/kg and 5 mg/kg spaced 2 hours apart), $\pm$ 3,4-methylenedioxymethamphetamine (MDMA; 10 mg/kg, i.p., given every 2 hours for 4 injections), or para-methoxyamphetamine (PMA; 10 mg/kg, i.p. given every two hours for four injections). Values represent the mean $\pm$ SEM. Numbers in parentheses equals the number of determinations.

Drug	DA (ng/mg tissue $\pm$ SEM)	5-HT (ng/mg tissue $\pm$ SEM)
Vehicle	11.9 $\pm$ 0.43 (17)	0.41 $\pm$ 0.03 (17)
AMPH	2.2 $\pm$ 0.80 ^{***} (6)	0.36 $\pm$ 0.04 (6)
METH	4.2 $\pm$ 0.79 ^{***} (7)	0.26 $\pm$ 0.03 ^{***} (7)
MDMA	10.3 $\pm$ 0.83 (7)	0.26 $\pm$ 0.02 ^{**} (7)
PMA	10.8 $\pm$ 0.62 (7)	0.27 $\pm$ 0.03 ^{**} (7)

P < 0.001 and

**
P < 0.01 compared to vehicle-treated rats.

Table 2

Quantification of C-tau labeling in the striatum of rats treated with amphetamine or para-methoxyamphetamine. C-tau positive cells were counted in defined regions of the striatum of rats killed 3 days after injection with VEH, AMPH or PMA as described in the legend of Table 1. Values represent mean $\pm$ SEM of 4–5 rats.

Drug	Striatal C-tau (cell count $\pm$ SEM)
Vehicle	17 $\pm$ 6
AMPH	51 $\pm$ 7 *
PMA	24 $\pm$ 8

*
P < 0.05 compared to vehicle-treated rats.

Table 3

Dopamine and 5-HT concentrations and C-tau immunoreactive cells in the striatum of rats treated with 5,7-DHT. Concentrations of DA and 5-HT were determined and number of C-tau positive cells were counted in defined regions of the striatum of rats killed 3 days after i.c.v. administration of vehicle or 5,7-DHT (150 µg/10 µl). Values represent mean ± SEM of 3–6 rats.

Drug	DA (ng/mg tissue ± SEM)	5-HT	Striatal C-tau (cell count ± SEM)
Vehicle	16.5 ± 0.59	0.52 ± 0.12	2.58 ± 0.30
5,7-DHT	14.6 ± 0.84	0.15 ± 0.04 ^{**}	3.56 ± 2.23

^{**} P < 0.01 compared to vehicle-treated controls.