

Glial Cell Response to 3,4-(±)-Methylenedioxymethamphetamine and Its Metabolites

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3,4-(±)-Methylenedioxymethamphetamine (MDMA) and 3,4-(±)-methylenedioxyamphetamine (MDA), a primary metabolite of MDMA, are phenylethylamine derivatives that cause serotonergic neurotoxicity. Although several phenylethylamine derivatives activate microglia, little is known about the effects of MDMA on glial cells, and evidence of MDMA-induced microglial activation remains ambiguous. We initially determined microglial occupancy status of the parietal cortex in rats at various time points following a single neurotoxic dose of MDMA (20 mg/kg, SC). A biphasic microglial response to MDMA was observed, with peak microglial occupancy occurring 12- and 72-h post-MDMA administration. Because direct injection of MDMA into the brain does not produce neurotoxicity, the glial response to MDMA metabolites was subsequently examined *in vivo* and *in vitro*. Rats were treated with MDA (20 mg/kg, SC) followed by *ex vivo* biopsy culture to determine the activation of quiescent microglia. A reactive microglial response was observed 72 h after MDA administration that subsided by 7 days. In contrast, intracerebroventricular (ICV) administration of MDA failed to produce a microglial response. However, thioether metabolites of MDA derived from α -methyl dopamine (α -MeDA) elicited a robust microglial response following ICV injection. We subsequently determined the direct effects of various MDMA metabolites on primary cultures of E18 hippocampal mixed glial and neuronal cells. 5-(Glutathion-S-yl)- α -MeDA, 2,5-bis-(glutathion-S-yl)- α -MeDA, and 5-(N-acetylcystein-S-yl)- α -MeDA all stimulated the proliferation of glial fibrillary acidic protein-positive astrocytes at a dose of 10 μ M. The findings indicate that glial cells are activated in response to MDMA/MDA and support a role for thioether metabolites of α -MeDA in the neurotoxicity.

Key Words: 3,4-(±)-methylenedioxymethamphetamine; metabolites; microglial activation.

3,4-(±)-Methylenedioxymethamphetamine (MDMA) and its primary metabolite, 3,4-(±)-methylenedioxyamphetamine (MDA), are widely abused illicit amphetamine derivatives. The neurotoxic effects of MDMA/MDA on central dopaminergic (dopamine [DA]) and serotonergic (serotonin [5-HT])

neurons have been well studied (O'Hearn *et al.*, 1988; Stone *et al.*, 1986). 5-HT axons are widely distributed in the forebrain, where 2 morphologically distinct classes (fine and bead) of 5-HT axon terminals exist (Kosofsky and Molliver, 1987; Törk, 1990). Fine axons, the predominant 5-HT axons in most areas of the forebrain, are vulnerable to the neurotoxic effects of MDMA (Mamounas *et al.*, 1991; Molliver, 1987). However, although the serotonergic neurotoxicity of MDMA/MDA is well established, the mechanisms by which MDMA/MDA cause neurotoxicity remain to be elucidated. Moreover, controversy still remains over whether the axonal damage caused by this class of drugs is sufficient to trigger a glial response.

Neuroglia (astrocytes, oligodendrocytes, and microglia) provide physical and trophic support for neurons and respond to local or remote tissue injury, which may either be protective against or potentiate tissue injury (Aschner *et al.*, 1999). Reactive gliosis is a response to various central nervous system (CNS) injuries (Norton *et al.*, 1992; Streit *et al.*, 1999). Astrocytes and microglia are 2 major populations of reactive glial cells, while oligodendrocytes typically do not show reactive changes after CNS injury (Norton, 1999). Reactive microglial cells undergo transformation from ramified resting microglia to phagocytic microglia and alter their immunophenotype, as well as the pattern of cytokine and growth factor secretion (Streit *et al.*, 1999). Reactive astroglia manifests as cell swelling, hypertrophy of cellular process and hyperplasia, and changes associated with increased expression of glial fibrillary acidic protein (GFAP) (Norton *et al.*, 1992).

Although reactive gliosis is a universal response to many types of brain injury, little work has been conducted to thoroughly study the characteristics and dynamics of the glial response to amphetamine analogs. In addition, there remains debate over whether the degenerating 5-HT axons following exposure to amphetamine analogs is sufficient to trigger a glial response. *para*-Chloroamphetamine (PCA) causes selective ablation of serotonergic axons in the forebrain of rats and evokes a mild and delayed microglial response characterized

by microglial cell hyper-ramification, an intermediate stage of microglia between the resting and the reactive forms, without affecting astrocytic GFAP expression (Wilson and Molliver, 1994). These data suggest that sensitive microglial markers can detect a subtle axonal lesion that provokes no detectable increase in GFAP expression by astrocytes. MDMA (20 mg/kg, IP) increased hippocampal GFAP expression in rats, an effect prevented by prior administration of the antioxidant, α -lipoic acid (Aguirre *et al.*, 1999). In contrast, Pubill *et al.* (2003) reported that MDMA (20 mg/kg, SC, 2 times a day, for 4 days) failed to induce GFAP or Hsp27 immunoreactivity, or microglial activation (determined by assessing [3 H]PK11195 binding and OX-6 immunostaining). In C57BL/6J mice, (\pm)-MDA and (\pm)-MDMA (20 mg/kg \times 4, SC, at 2-h intervals) produce a large increase in GFAP, associated with damage to DA projections in the striatum (Johnson *et al.*, 2002; Miller and O'Callaghan, 1995; O'Callaghan and Miller, 1994). This increase of GFAP is robust 3 days after drug administration and is resolved by 3 weeks (Miller and O'Callaghan, 1995; O'Callaghan and Miller, 1994). In addition, (\pm)-MDA and (\pm)-MDMA (20 mg/kg \times 4, SC, at 2-h intervals) also increase cortical GFAP in C57BL/6J mice, an effect, which parallels the transient decreases in cortical 5-HT (O'Callaghan and Miller, 1994). However, fenfluramine produces a prolonged decrease in cortical 5-HT in rats and mice, without corresponding glial reactions (O'Callaghan and Miller, 1994; Rowland *et al.*, 1993).

Methamphetamine, a phenylethylamine derivative that shares structural similarities with MDMA/MDA, is neurotoxic (Carvalho *et al.*, 2012) and activates microglia (Sekine *et al.*, 2008; Thomas *et al.*, 2004b). Microglial responses have been detected after treatment with a number of substituted amphetamine derivatives, leading some to posit that microglial activation could be used as a marker of amphetamine neurotoxicity (Thomas *et al.*, 2004a). Given the contrasting reports on the ability of MDMA to activate microglia, and the advent of more selective microglial markers (eg, ionized calcium-binding adapter molecule 1 [Iba1]), we initiated an investigation of the degree of microglial activation in response to MDMA administration. Moreover, because direct injection of MDMA into brain does not produce neurotoxicity, we focused our studies on the ability of MDMA metabolites to activate a microglial response *in vivo* and *in vitro*.

MATERIALS AND METHODS

Chemicals. (\pm)-MDMA-HCl and (\pm)-MDA-HCl (both listed at >95% purity) were kindly provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, Maryland), and identity of these compounds was verified by tandem mass spectrometry. α -Methyldopamine (α -MeDA) was a generous gift from Anthony Y. H. Lu (Merck Research Laboratories, Rahway, New Jersey). Glutathione and mushroom tyrosinase (5600 U/mg) were obtained from Sigma Chemical Co (St Louis, Missouri). Acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-ac-LDL) was acquired from Biomedical Technologies, Inc (Cambridge, Massachusetts). Primary antibody against Iba1 was purchased

from Wako Chemicals (Richmond, Virginia). MACH4 detection system and blocking reagent were purchased from BioCare Medical (Concord, California). Antibody diluent was purchased from MP Biomedicals (Solon, Ohio). Protease inhibitor cocktail and monoclonal antibody against GFAP were obtained from Roche Molecular Biochemicals (Germany). Hyperfilms used for the Western Blot were purchased from Amersham Life Science (United Kingdom). Rabbit monoclonal antibody against neuron-specific enolase (NSE) and mouse monoclonal antibody against tryptophan hydroxylase (TPH) were obtained from Chemicon International and Sigma Chemical Co, respectively. 5-(Glutathion-S-yl)- α -MeDA, 2,5-bis-(glutathion-S-yl)- α -MeDA, and 5-(N-acetylcystein-S-yl)- α -MeDA were synthesized and purified as previously described (Miller *et al.*, 1995). For primary cell cultures, trypsin, DNase, and Ca $^{2+}$ /Mg $^{2+}$ -free Hank's buffer were purchased from Sigma Chemical Co. Dulbecco's modified eagle medium-F12 and minimum essential medium and N2 supplies were obtained from Gibco.

Animals. Adult male Sprague Dawley rats (~200 g) were group housed and maintained on a 12-h light/dark cycle. Food and water were provided *ad libitum*. Rats were allowed 1 week to acclimate to their surroundings before initiation of experiments. All procedures were approved by the Institutional Animal Care and Use Committee either at the University of Arizona or the University of Texas. Animals were kept at 22°C \pm 1°C for housing and all experiments. Embryos from pregnant female Holzman rats (Harlan Sprague Dawley) were obtained at the 18th day of pregnancy and were used for the isolation of cells from the hippocampus.

Drug administration. Animals were injected with either MDMA (20 mg/kg [104 μ mol/kg], 1.4 ml/kg, SC), MDA (20 mg/kg [93 μ mol/kg], 1.2 ml/kg, SC), or saline. Animals were anesthetized with 3.5 ml/kg of a 0.9% saline solution containing 9.4 mg/ml sodium pentobarbital and 37.5 mg/ml chloral hydrate and surgically implanted with intracerebroventricular (ICV) cannulae as previously described (Miller *et al.*, 1995). Rats were allowed to recover from the surgery for 2 weeks before initiation of drug treatment. Animals were dosed every 12 h for a total of 4 consecutive doses. 5-(Glutathion-S-yl)- α -MeDA (720 nmol \times 1, 360 nmol \times 3), 2,5-bis-(glutathion-S-yl)- α -MeDA (450 nmol \times 4), or MDA (2 μ mol \times 1, 1 μ mol \times 3) were infused by ICV injection in 10- μ l artificial cerebrospinal fluid into the left ventricle of awake, freely moving rats at a rate of 0.2 μ l/min, 4 times at 12-h intervals, 14 days after implantation of the cannulae. MDA was administered SC (93 μ mol/kg) as a positive control. Control animals experienced the same surgical and recovery procedures and received the same volume of vehicle. Because stab wounding of the rodent cerebrum is a common and well-established model of reactive gliosis (Fujita *et al.*, 1998; Miyake *et al.*, 1992), it is important to minimize the complications of such physical injury following the implantation of cannulae. Reactive astrogliosis can be detected within hours, and reaches a maximum three-seven days postoperation (Miyake *et al.*, 1992). In the present study, all the rats were allowed to recover for 2 weeks from the cannula implantation surgery before drug administration, in order to avoid the reactive gliotic reaction due to the trauma of surgery. Because no phagocytic microglial cells were detected in the control rats following this protocol (Fig. 2A), the microglial response due to the surgery must have subsided by the time of drug administration. In addition, no changes in GFAP expression were observed in the striatum, cortex, and hippocampus of controls at all the sampling time points, indicating that astrocytes were also in a relatively stable status. Thus, we can assume that the changes in GFAP expression and the presence of phagocytic microglial cells in this study were due to toxicant exposure rather than physical damage arising as a consequence of surgery.

Rationale for dosage selection. The single MDMA/MDA dose was selected based on work by Schmued (2003), where a single dose of MDMA was sufficient to elicit positive Fluoro-Jade B staining in regions of the rat brain including the parietal cortex, indicative of neurodegeneration. We therefore sought to determine whether a single dose of MDMA/MDA would be sufficient to elicit a microglial response on the assumption that if so, then a multiple-dosing regimen of MDMA/MDA would also elicit a similar response. However, with the ICV studies, our prior work revealed that multiple intracranial doses of MDMA/MDA metabolites are necessary to produce neurotoxicity

without lethality (Bai *et al.*, 1999; Jones *et al.*, 2005; Miller *et al.*, 1996, 1997). Therefore, we determined that intracranially administered MDA should also be delivered in a multiple-dose regimen.

Immunohistochemistry. Rats were transcardially perfused under sodium pentobarbital anesthesia with approximately 200ml of 10mM PBS pH 7.2 followed by 200ml of 4% paraformaldehyde in 10mM PBS pH 7.2. Whole brains were then immersed in 4% paraformaldehyde and placed at 4°C for 48h for post-fixation. Brains were then rinsed with Milli-Q water and immersed in 70% ethanol before submission to the Histology Service Laboratory at the University of Arizona for paraffin embedding and sectioning. Coronal tissue sections of 7 μ m were taken beginning 5mm from the tip of the frontal cortex and mounted on slides for immunohistochemical analysis. The immunohistochemistry method was adapted from Hardwick *et al.* (2010). Tissue sections were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was achieved using citrate buffer (10mM citric acid, 0.05% vol/vol Tween 20, pH 6.0) at 100°C for 6min. Endogenous peroxidase activity was blocked by immersing slides in 0.3% vol/vol H₂O₂ in methanol, for 20min. Sections were incubated in a 1:1000 dilution of Iba1 antibody for 2h at room temperature and then overnight at 4°C. Antibody binding was detected using the MACH4 detection system following manufacturer recommendations. Color development was achieved by immersion in a 3,3'-diaminobenzidine (DAB) solution (2.5-g nickel (II) sulfate and 20-mg DAB in 100ml 0.175M sodium acetate) for 15min. For quantitation of Iba1 stained area, slides were imaged using an Olympus IMT-2 microscope (Olympus America Inc, Center Valley, Pennsylvania) and a Hamamatsu Orca-100 camera (Hamamatsu Corp, Bridgewater, New Jersey). The digital images were analyzed with SimplePCI v6.5 software (Hamamatsu Corp, Sewickley, Pennsylvania) using a calibration file matched to the magnification used ($\times 200$). Images were digitally captured in a systematic fashion, and an intensity threshold was set to select all the stained cells and processes in the field. To discriminate in-plane objects from out-of-plane objects, a second step removed all threshold objects smaller than 2 μ m², as well as any objects that touched the edge of the image. The software then measured the area of the remaining threshold objects. Twelve images were captured per animal, and the average of the 12 images was considered the stained area from a single animal. Two-way ANOVA followed by Bonferroni post hoc tests were employed to determine statistically significant differences over both treatment group and time (GraphPad Prism Software version 5, La Jolla, CA).

Biopsy culture. Animals were anesthetized with 200mg/kg sodium pentobarbital (IP) and subsequently euthanized by decapitation 72h and 7 days after MDMA single administration (SC) or 3 days after multiple dosing with MDA, 5-(glutathion-S-yl)- α -MeDA, or 2,5-bis-(glutathion-S-yl)- α -MeDA (ICV). Tissue samples (2.0 \times 1.0mm) from both experimental and control rats were isolated by punch microdissection from frontal cortex, hippocampus, striatum, cerebellum, and hypothalamus. These biopsies were incubated for 24h in chemically defined N2 medium (Bottenstein and Sato, 1979) with 10% fetal bovine serum (FBS). Samples were then incubated with DiI-ac-LDL for 6h and fixed in 3% formaldehyde in PBS. To determine the number of DiI-ac-LDL(+) microglia present, fixed tissue was pressed under glass coverslips and viewed by fluorescence microscopy ($\times 200$ magnification) (Giulian and Ingeman, 1988).

Cell isolation and identification. Mixed glial and neuronal primary cultures were prepared from E18 embryos. Briefly, the hippocampus was dissociated by trituration with Pasteur pipettes after 15 min incubation with 0.2% trypsin (Sigma) and 96U/ml DNase (Sigma) in Ca²⁺/Mg²⁺-free Hank's buffer (Hank's). Cells were then washed twice with Hank's, containing 5% FBS, and once with N2 culture media containing 5% FBS and 30mM KCl, followed by centrifugation at 800 \times g for 10 min. Cells were then resuspended in N2 media supplemented with 5% FBS and 30mM KCl and seeded on poly-L-lysine-coated glass coverslips in 24-well plates (0.25 \times 10⁶/ml/well). After 7 days in culture, half of the media was replaced with fresh N2 media supplemented with 5% FBS and 30mM KCl once a day for 3 days. The final media contained 0.6% FBS. 5-HT-containing neurons were identified in mixed cell preparations by indirect immunohistochemical double staining for NSE and TPH using rabbit monoclonal anti-NSE (1:150; Chemicon International Inc) and mouse monoclonal anti-TPH (1:1000; Sigma). Astroglia were stained to identify GFAP

using mouse monoclonal anti-GFAP (1:40, 0.5 mg/ml; Boehringer Mannheim Biochemicals).

RESULTS

MDMA Produces a Modest Increase in Microglial Occupancy in the Parietal Cortex

To initially determine the ability of systemically administered MDMA to induce a microglial response *in vivo*, we determined microglial occupancy, specifically of the parietal cortex, based on Schmued (2003), whose work indicated this region of the brain showed evidence of degenerating neurons following a systemic MDMA dose of 104 μ mol/kg. Microglia from the parietal cortex of rats treated with either saline or MDMA, from 12h to 1 week after drug administration, revealed no readily apparent uniform phenotypic changes by this technique. However, there was a 1.73-fold increase in microglial occupancy (μ m²/field) when the mean of all fields from MDMA-treated rats was compared against the mean of all fields from saline-treated rats (Fig. 1). Moreover, it appears there exists a biphasic increase in microglial occupancy after MDMA administration, with peak values reached at 12 and 72h after dosing. Two-way ANOVA analysis revealed that drug treatment effect ($p < .001$) was more significant than either the effect of time ($p = .093$) or the combined drug and time effect ($p = .472$). Post hoc Bonferroni tests revealed that only the elevations in stained area at the 12 and 72h time points were significant ($p < .05$).

MDA, 5-(Glutathion-S-yl)- α -MeDA, and 2,5-bis-(Glutathion-S-yl)- α -MeDA Stimulate a Glial Cell Response Following In Vivo Administration

Because MDMA fails to induce neurotoxicity when injected directly into the brain, we subsequently examined the ability of various MDMA metabolites to activate microglial cells using a coupled *in vivo/ex vivo* assay. Using biopsy culture, a reactive

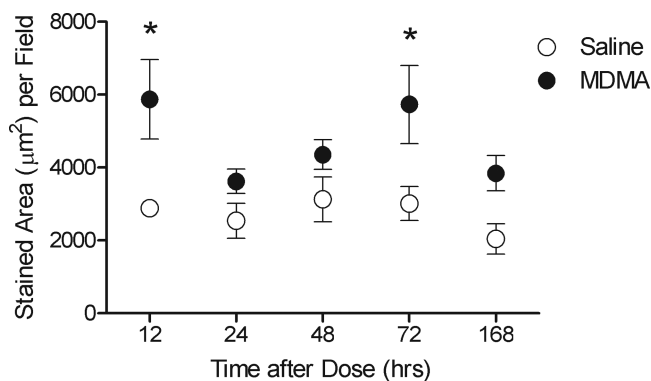


FIG. 1. MDMA increases microglial occupancy in the parietal cortex. Closed circles represent those animals treated with MDMA (104 μ mol/kg, SC) and open circles represent those animals treated with saline. Each symbol represents the mean \pm SEM of 4 animals. The value for each animal was the mean of stained area from 12 fields. * $p < .05$ compared with saline-treated animals of identical time point. Abbreviation: MDMA, 3,4-(\pm)-methylenedioxymethamphetamine.

microglial response was observed 72h after a single systemic administration of MDA (93 $\mu\text{mol}/\text{kg}$, SC; Fig. 2C), which subsided by 7 days (data not shown). Activated microglial cells exhibited a rounded, amoeboid morphology. These amoeboid microglia were phagocytic and expressed acetylated low-density lipoprotein (ac-LDL) receptors, as evidenced by the ability of these cells to phagocytize fluorescent DiI-ac-LDL (Fig. 2). However, direct injection of MDA (2 $\mu\text{mol} \times 1$, 1 $\mu\text{mol} \times 3$) into brain failed to reproduce the microglial response observed following systemic administration of MDA (Fig. 2B).

Both 5-(glutathion-S-yl)- α -MeDA and 2,5-bis-(glutathion-S-yl)- α -MeDA also provoked reactive microgliosis, 3 days after direct ICV administration. The morphology of the DiI-ac-LDL(+) microglial cells present in rats treated with 5-(glutathion-S-yl)- α -MeDA (Fig. 2D and E) or 2,5-bis-(glutathion-S-yl)- α -MeDA (Fig. 2F and G) was identical to that seen in the rats treated peripherally with MDA (Fig. 2C). However, the degree of the microglial response differed between brain regions and was dependent upon the compound administered. In 5-(glutathion-S-yl)- α -MeDA-treated rats, large numbers of DiI-ac-LDL(+) microglia were present in the cortex and striatum, whereas fewer DiI-ac-LDL(+) microglia were observed in the hippocampus. In contrast, the numbers of DiI-ac-LDL(+) microglia were highest in the hippocampus of 2,5-bis-(glutathion-S-yl)- α -MeDA-treated rats, followed by the cortex. Only a few DiI-ac-LDL(+) microglia were observed in the striatum of 2,5-bis-(glutathion-S-yl)- α -MeDA-treated rats.

MDMA Metabolites Stimulate GFAP Expression in Primary Hippocampal Mixed Neuronal/Glial Cell Cultures

5-(Glutathion-S-yl)- α -MeDA, 5-(N-acetylcystein-S-yl)- α -MeDA, and 2,5-bis-(glutathion-S-yl)- α -MeDA stimulated the dose-dependent proliferation of GFAP(+) astrocytes in E18 primary cell cultures 24h following drug treatment (Fig. 3). Each

of the MDMA metabolites produced significant increases in the number of GFAP(+) cells at the lowest dose tested (10 μM). The number of GFAP(+) astrocytes increased 2.4-fold in the presence of 100 μM 5-(glutathion-S-yl)- α -MeDA compared with controls. Astrocytes in 5-(glutathion-S-yl)- α -MeDA-, 5-(N-acetylcystein-S-yl)- α -MeDA-, or 2,5-bis-(glutathion-S-yl)- α -MeDA-treated mixed primary cell cultures became hyperplastic and hypertrophic (Fig. 4B). Cells labeled with antibodies against GFAP showed darker staining (Fig. 4B) compared with controls (Fig. 4A). GFAP(+) cells were labeled with monoclonal antibody against GFAP and quantified by counting GFAP(+) cells in 10 random selected fields under the bright field microscope. A one-way ANOVA followed by Student Newman-Keuls tests were conducted on the data. GFAP(+) cells were significantly increased following 5-(glutathion-S-yl)- α -MeDA [$F(4,45) = 124.695$, $p = .0001$], 5-(N-acetylcystein-S-yl)- α -MeDA [$F(4,45) = 86.782$, $p = .0001$], and 2,5-bis-(glutathion-S-yl)- α -MeDA [$F(4,45) = 106.686$, $p = .0001$] treatment. GFAP(+) cells were also significantly increased following MDA (100 and 200 μM) treatment [$F(4,45) = 7.147$, $p = .0002$].

Immunohistochemical double labeling of E18-derived primary hippocampal mixed neuronal/glial cell cultures revealed a clear enrichment in 5-HT-positive staining cells (Fig. 5A). Exposure of the mixed neuronal/glial cell cultures to 5-(glutathion-S-yl)- α -MeDA (100 μM) for 24h caused a loss of axons in 5-HT-positive neurons, increased the caliber of 5-HT-positive axons, and induced the formation of large varicosities in these same neurons (Fig. 5B).

DISCUSSION

Whether or not degenerating 5-HT axons trigger gliosis following exposure to analogs of amphetamine is the subject

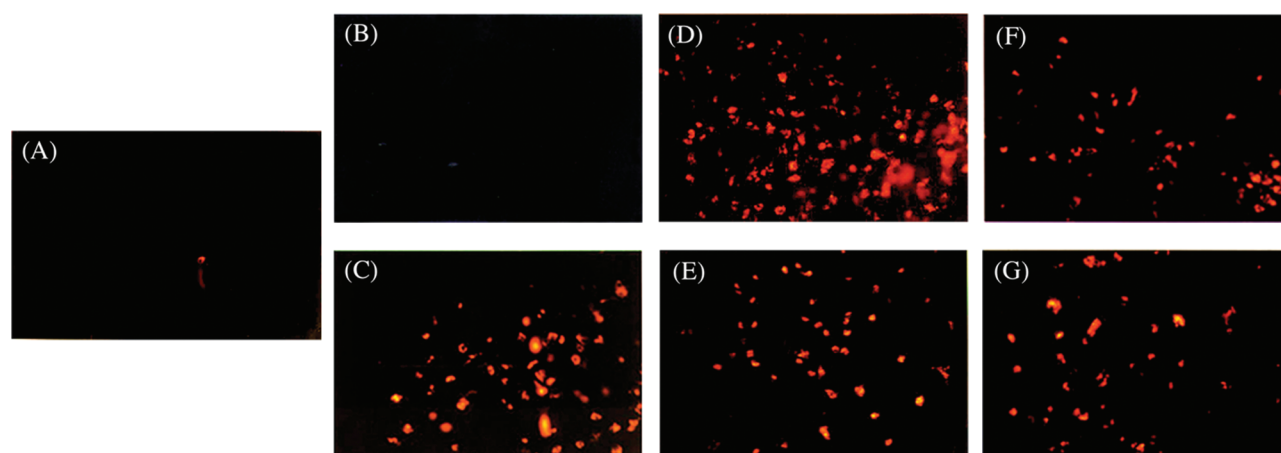


FIG. 2. Transformation of quiescent microglia to phagocytic microglia following 3,4-(\pm)-methylenedioxyamphetamine (MDA) (SC), 5-(glutathion-S-yl)- α -MeDA (ICV), or 2,5-bis-(glutathion-S-yl)- α -MeDA (ICV) administration. Activated microglial cells were detected 72h after drug treatment using biopsy cultures coupled with the phagocytosis of fluorescent DiI-ac-LDL, shown as red phagocytic cells. A, control; B, MDA ICV; C, the cortex of MDA (SC)-treated rats; D, the cortex of 5-(glutathion-S-yl)- α -MeDA (ICV)-treated rats; E, the striatum of 5-(glutathion-S-yl)- α -MeDA (ICV)-treated rats; F, the striatum of 2,5-bis-(glutathion-S-yl)- α -MeDA (ICV)-treated rats; and G, the cortex of 2,5-bis-(glutathion-S-yl)- α -MeDA (ICV)-treated rats. Abbreviations: α -MeDA, α -methyl dopamine; DiI-ac-LDL, acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindolyl-carbocyanine perchlorate.

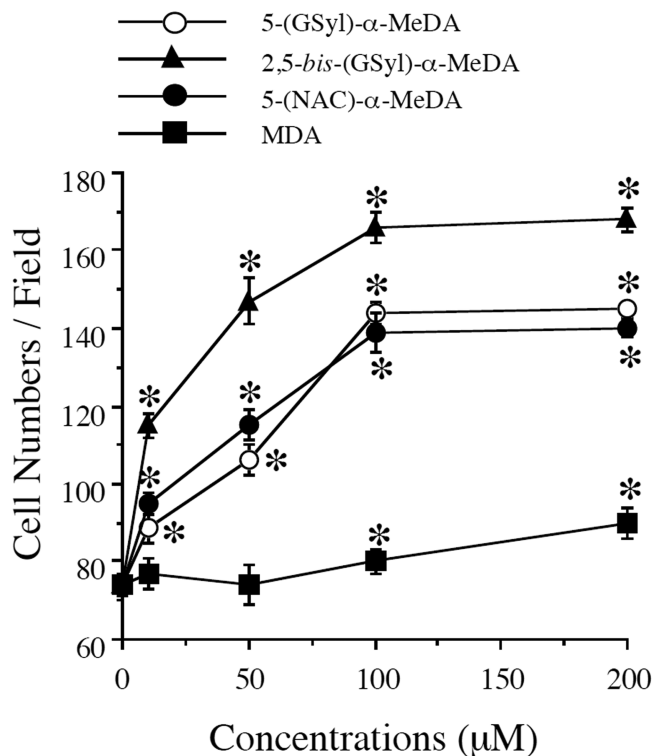


FIG. 3. Increases in the number of glial fibrillary acidic protein (GFAP)(+) cells following α -MeDA-thioethers or MDA treatment in E18 mixed glial and neuronal primary cultures. E18 mixed glial and neuronal primary cells were treated with 5-(glutathion-S-yl)- α -MeDA, 5-(N-acetylcystein-S-yl)- α -MeDA, 2,5-bis-(glutathion-S-yl)- α -MeDA, or MDA for 24 h. The number of GFAP(+) cells was expressed as the mean \pm SEM ($n = 10$ for each treatment). * $p < .05$ compared with control. Abbreviations: α -MeDA, α -methyldopamine; MDA, 3,4-(\pm)-methylenedioxyamphetamine.

of debate. (\pm)- or (+)-Fenfluramine produces a dose-dependent loss of 5-HT uptake and swelling of 5-HT axons, in the absence of concomitant astrocytic reaction *in vivo* (Rowland *et al.*, 1993). We now show that systemic MDMA administration produces a significant increase in stained microglial area in the parietal cortex (Fig. 1). However, metabolism of MDMA is required to produce neurotoxicity, and although SC injection of MDA, a primary metabolite of MDMA, also produced microglial activation (Fig. 2C), direct injection of MDA into rat brain failed to reproduce this effect (Fig. 2B). The onset of the microglial response to MDMA/MDA (SC) started as early as 12 h after administration (Fig. 1), reached a maximum by 3 days (Figs. 1 and 2), and subsided by 7 days (Fig. 1). We speculate that the initial pharmacologic effects of MDMA (hyperthermia) produce the first wave of the microglial response, with the subsequent developing neurodegeneration accounting for the second wave of microglial occupation. Hyperthermia is likely the most important acute MDMA-induced physiological event, because microglial activation is minimal in mice administered methamphetamine in the absence of hyperthermia (Bowyer *et al.*, 2008). As nerve terminals begin to degenerate between 1 and 3 days (Molliver *et al.*, 1990), a secondary microglial

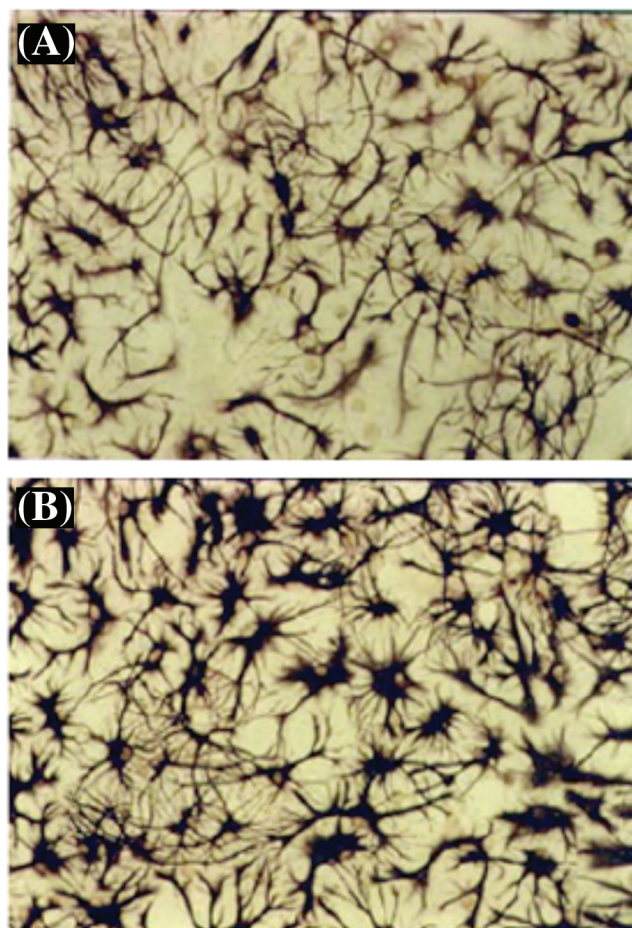


FIG. 4. *In vitro* activation of glia following 5-(glutathion-S-yl)- α -MeDA MDA treatment in E18 mixed glial and neuronal primary cell cultures. Astrocytes in E18 mixed glial and neuronal primary cell cultures are evident as purple colored, star-shaped cells (A). Hypertrophy and overexpression of glial fibrillary acidic protein (GFAP) occurs in GFAP(+) cells when the primary cells are treated with 5-(glutathion-S-yl)- α -MeDA (100 μ M) (B) for 24 h. Similar morphological changes of astrocytes were also observed in primary cells treated with 2,5-bis-(glutathion-S-yl)- α -MeDA (100 μ M) or 5-(N-acetylcystein-S-yl)- α -MeDA (100 μ M). Abbreviations: α -MeDA, α -methyldopamine; MDA, 3,4-(\pm)-methylenedioxyamphetamine.

response to this neuronal damage is observed. In another study of acute methamphetamine exposure, a significant increase in active microglia was observed 72 h following administration (Bowyer *et al.*, 2008).

Glial cell activation and/or proliferation occurs after CNS injury and involves both activated astroglia and microglia (Hatten *et al.*, 1991). Microglia of the normal brain are maintained in a "surveillance state," in which cells are characterized by multiple ramifications, cellular processes withdrawn and rebuilt to scan a clearly defined territory of brain parenchyma, low proliferative activity, and exhibit no phagocytic activity (Hanisch and Kettenmann, 2007). Ramified microglia, when activated, display an enhanced expression of certain cell surface markers, retract processes, migrate to areas of tissue damage, and engage in vigorous phagocytosis (Wilson and Molliver,

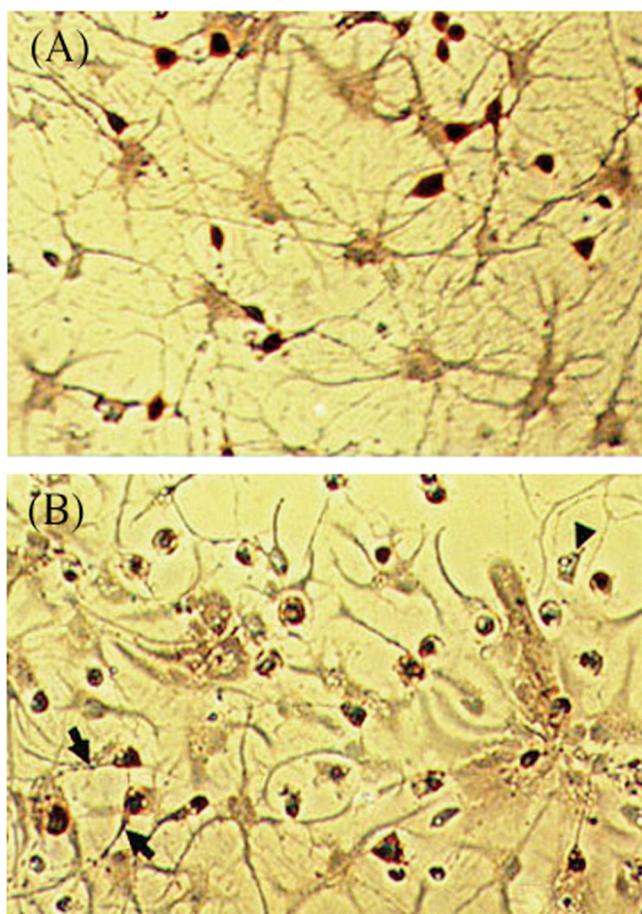


FIG. 5. Damage to serotonergic neurons following 5-(glutathion-S-yl)- α -MeDA treatment in E18 mixed glial and neuronal primary cultures. Serotonin (5-HT)-enriched neurons were labeled using immunohistochemistry double staining for neuron-specific enolase and TPH. A, 5-HT neuronal cell bodies in the control cells exhibit a dark orange color, with fine axonal projections. B, Swelling of the axons (arrows), loss of axons (arrowhead), and vacuolization in the cytoplasm (arrowhead) occurs in 5-HT neurons treated with 5-(GSyl)- α -MeDA (100 μ M) for 24h. Abbreviation: α -MeDA, α -methylidopamine.

1994). MDA elicited the transformation of quiescent microglia to fully activated phagocytic microglia (Fig. 2C), in contrast to the hyper-ramified intermediate stage present following PCA treatment (Streit *et al.*, 1999; Wilson and Molliver, 1994). It is noteworthy that ICV administration of MDA failed to trigger a microglial response (Fig. 2B), suggesting that MDMA metabolites further downstream of MDA are contributing to the microglial response. Consistent with this view, direct injection of MDA or MDMA into the brain fails to reproduce the acute or long-term neurotoxic effects observed after systemic administration (Esteban *et al.*, 2001; O'Shea *et al.*, 1998).

5-(Glutathion-S-yl)- α -MeDA and 2,5-bis-(glutathion-S-yl)- α -MeDA produce long-term depletions in 5-HT following intrastriatal or intracortical administration (Bai *et al.*, 1999), and potential pathways for their uptake into brain following systemic administration of MDMA/MDA have been described

(Monks and Lau, 1997). We have now demonstrated that these serotonergic neurotoxicants are also capable of eliciting reactive microgliosis. In CNS lesions, the presence of activated astrocytes and microglia are most pronounced at the site of injured neurons and their processes (Streit *et al.*, 1999). The microglial response to 5-(glutathion-S-yl)- α -MeDA and 2,5-bis-(glutathion-S-yl)- α -MeDA occurred in brain regions enriched in the 5-HT nerve terminals, the targets of MDMA neurotoxicity. The temporal, anatomical, and morphological characteristics of the reactive gliosis produced by 5-(glutathion-S-yl)- α -MeDA and 2,5-bis-(glutathion-S-yl)- α -MeDA were also comparable with those seen following systemic administration of MDA (Fig. 2C). Moreover, it is important to note that the doses of the various MDA metabolites used in these studies are within the range of amounts formed *in vivo* following systemic administration of MDA (Miller *et al.*, 1996).

The morphology of activated microglia may be quite plastic and varies depending upon the pathological conditions. Activated microglia may appear as rod cells, perineuronal satellites, or foamy macrophages (Kreutzberg, 1996) and display an increase in expression of a number of cell surface receptors when responding to certain lesions. In particular, scavenger receptors responsible for endocytosis of modified low-density lipoprotein, such as LOX-1 and LRP-1, are among those that may be upregulated (Hendrickx *et al.*, 2013). The activated microglial cells present in the striatum, cortex, hippocampus, and hypothalamus following MDA, 5-(glutathion-S-yl)- α -MeDA and 2,5-bis-(glutathion-S-yl)- α -MeDA administration were rounded, ameboid, phagocytic microglia, expressing scavenger receptors, as evidenced by the ability of these cells to phagocytize the fluorescent probe, DiI-ac-LDL (Fig. 2). The axonal injury produced by MDA, 5-(glutathion-S-yl)- α -MeDA and 2,5-bis-(glutathion-S-yl)- α -MeDA may provide neuron-derived stress signals to glial cells, which subsequently attempt to clear potential deleterious cell debris through phagocytosis. Thus, in instances where mixed glial and neuronal cultures are exposed to either MDA or various thioether metabolites (Fig. 3), the metabolites increase the number of GFAP-positive cells without any observable adverse effects on the glial cells (Fig. 4) while simultaneously causing axonal swelling, cytosolic vacuolization, and axonal loss in 5-HT neurons (Fig. 5). Consistent with these observations, axotomy elicits microglial activation (Kalla *et al.*, 2001). However, signaling mechanisms facilitating this process remain to be established, and we cannot rule out the possibility that the metabolites also directly activate the microglial cells.

Microglia-derived cytokines and growth factors can play either beneficial or harmful roles in response to tissue injury (Giulian *et al.*, 1994; Streit *et al.*, 1999). Subtle, reversible neuronal injury tends to signal the production of neurotrophic factors such as nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor, all of which participate in both neuronal regeneration and the prevention of neuronal death (Elkabes *et al.*, 1996). In contrast, severe, "irreversible"

neuronal damage also signals the production of glial-derived neurotoxins that accelerate degeneration and the disposal of the dying neurons (Giulian *et al.*, 1993). For example, microglia-derived proinflammatory cytokines, reactive oxygen species, nitric oxide, and various proteases are either directly or indirectly capable of contributing to neuronal degeneration (Kraft and Harry, 2011). Elevations in both proinflammatory cytokines (Neri *et al.*, 2010; Torres *et al.*, 2011) and reactive oxygen species/reactive nitrogen species (Darvesh *et al.*, 2005) occur following MDMA treatment, providing a mechanism by which microglia may contribute to MDMA-induced neurotoxicity. In particular, incubation of DA quinones with cultured murine microglial cells proved sufficient to activate these cells (Kuhn *et al.*, 2006). Furthermore, dopaminergic cell membranes modified with DA quinones are also capable of activating cultured microglia that can subsequently damage dopaminergic cells (Le *et al.*, 2001). These findings are particularly relevant to the ability of MDMA metabolites to activate microglia, since the various thioether metabolites are capable of redox cycling (Felim *et al.*, 2007) and accumulate in the rat brain following systemic MDMA administration (Erives *et al.*, 2008).

Interestingly, microglial activation seems to play a larger role in methamphetamine-induced neurotoxicity than in MDMA-induced neurotoxicity, perhaps in part a consequence of the role of DA in the activation of microglia, as evidenced by the observation that elevations in extracellular DA concentrations were accompanied by greater microglial activation (Thomas *et al.*, 2008). Methamphetamine produces a greater magnitude of DA release than does MDMA (Rothman *et al.*, 2001), which may account for this observed difference in microglial response between these phenylethylamine derivatives. However, MDMA does stimulate DA release, albeit to a lesser extent than methamphetamine. Additionally, thioether metabolites of MDMA stimulate uptake of DA *via* serotonin reuptake transporter into serotonergic cells (Jones *et al.*, 2004). Thus, elevations in extracellular DA concentrations as well as neuronal injury due to DA oxidation within serotonergic terminals likely promote the activation of microglia following MDMA administration.

Serotonergic regeneration occurs 2–8 months after MDA, MDMA, and PCA treatment (Molliver *et al.*, 1990), likely in a process utilizing glia-derived neurotrophic factors. Intracortical infusion of brain-derived neurotrophic factor completely protects rats from PCA-induced loss of 5-HT axons, whereas neurotrophin-3 produces only a modest attenuation in the loss of 5-HT innervation in PCA-treated rats (Mamounas *et al.*, 1995). Microglia also communicate with astrocytes and coordinate response to the neuronal damage. For example, microglia-derived growth factor (interleukin-1) is an astroglial mitogen (Giulian and Lachman, 1985). In turn, microglial proliferation may be regulated *via* macrophage colony-stimulating factor released from astrocytes (Giulian and Ingeman, 1988; Kloss *et al.*, 1997). An increased presence of microglial cells is observed as early as 12h after MDMA administration but diminishes by 7 days. In contrast, GFAP expression was still

elevated 7 days after combined acivicin and MDA (10mg/kg, SC) treatment (Bai *et al.*, 2001). These temporal differences in the onset and recovery of the reactive microgliosis and reactive astrogliosis may reflect the sequential signal exchange between the neurons, microglia, and astrocytes.

In conclusion, MDMA and its metabolites, MDA, 5-(glutathion-S-yl)- α -MeDA, and 2,5-bis-(glutathion-S-yl)- α -MeDA, elicit a modest, but significant, microglial response that subsides 7 days post treatment. Detailed studies are needed to identify the complex signaling pathways involved in the intercellular cross-talk between microglia, astrocytes, and neurons during axonal degeneration and regeneration processed following amphetamine treatment. In particular, further studies are warranted to assess the activation state of the microglia, as well as chemical factors released by these cells following MDMA administration.

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