

Microglial activation precedes dopamine terminal pathology in methamphetamine-induced neurotoxicity

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Abstract

Previous studies have demonstrated methamphetamine (METH)-induced toxicity to dopaminergic and serotonergic axons in rat striatum. Although several studies have identified the nature of reactive astrogliosis in this lesion model, the response of microglia has not been examined in detail. In this investigation, we characterized the temporal relationship of reactive microgliosis to neuropathological alterations of dopaminergic axons in striatum following exposure to methamphetamine. Adult male Sprague–Dawley rats were administered a neurotoxic regimen of methamphetamine and survived 12 h, or 1, 2, 4, and 6 days after treatment. Immunohistochemical methods were used to evaluate reactive changes in microglia throughout the brain of methamphetamine-treated rats, with a particular focus upon striatum. Pronounced morphological changes, indicative of reactive microgliosis, were evident in the brains of all methamphetamine-treated animals and were absent in saline-treated control animals. These included hyperplastic changes in cell morphology that substantially increased the size and staining intensity of reactive microglia. Quantitative analysis of reactive microglial changes in striatum demonstrated that these changes were most robust within the ventrolateral region and were maximal 2 days after methamphetamine administration. Analysis of tissue also revealed that microglial activation preceded the appearance of pathological changes in striatal dopamine fibers. Reactive microgliosis was also observed in extra-striatal regions (somatosensory and piriform cortices, and periaqueductal gray). These data demonstrate a consistent, robust, and selective activation of microglia in response to methamphetamine administration that, at least in striatum, precedes the appearance of morphological indicators of axon pathology. These observations raise the possibility that activated microglia may contribute to methamphetamine-induced neurotoxicity.

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Introduction

Repeated administration of methamphetamine (METH) to rodents has been shown to induce the degeneration of dopamine and serotonin terminals of the striatum, serotonin terminals of the hippocampus (Hotchkiss and Gibb, 1980; Ricaurte et al., 1982; Seiden et al., 1976; Wagner et al., 1980) as well as cortical and striatal neurons (Deng et al., 2001; Eisch et al., 1998; Ryan et al., 1990). Although the

mechanism of METH-induced toxicity is unknown, several factors appear to be involved (Cadet et al., 2003). For example, it is known that the neurotransmitter dopamine plays a significant role in METH-induced toxicity (Gibb and Kogan, 1979; LaVoie and Hastings, 1999; Schmidt et al., 1985). There is also considerable evidence of a role for oxidative stress (De Vito and Wagner, 1989; Gibb and Kogan, 1979; Giovanni et al., 1995; Hirata et al., 1995; LaVoie and Hastings, 1999; Yamamoto and Zhu, 1998), although METH-induced increases in extracellular levels of glutamate suggest that excitotoxicity may also contribute to the resulting pathology (Abekawa et al., 1994; Nash and Yamamoto, 1992). Finally, it has been established that the toxicity of many substituted amphetamine analogs is dependent upon an acute drug-induced hyperthermia (Albers and Sonsalla, 1995; Ali et al., 1994; Colado et al., 1995;

Abbreviations: GFAP, glial acidic fibrillar protein; METH, methamphetamine; TH-IR, tyrosine-hydroxylase immunoreactive.

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Malberg and Seiden, 1998; Schmidt et al., 1990). How these many factors interact to produce the selective degeneration of monoaminergic terminals of the rodent striatum is unknown.

Many of the hypotheses regarding the mechanism of METH-induced toxicity have focused on intra-neuronal events such as dopamine oxidation, oxidative stress, and excitotoxicity. However, available evidence suggests that METH-induced neuropathology may result from a multicellular response in which glial cells may play a prominent role. Several previous studies have demonstrated the appearance of astrocyte activation in METH-induced toxicity (Capon et al., 1997; Fukumura et al., 1998; O'Callaghan and Miller, 1994; Pu and Vorhees, 1993). For example, Pu and Vorhees (1993) demonstrated that METH-induced toxicity was associated with dramatic increases in the astrocyte marker glial fibrillar acidic protein (GFAP) that was most pronounced in the ventrolateral striatum. This striatal subregion has been shown to be most vulnerable to the toxic effects of METH and exhibits the greatest loss of tyrosine-hydroxylase immunoreactivity (Pu and Vorhees, 1993) and dopamine-transporter binding sites (Eisch et al., 1992) following neurotoxic administration of METH. A temporal analysis of astrogliosis in METH-treated mice demonstrated that the astroglial response peaked 2 days after administration and remained elevated for at least 7 days following METH treatment (O'Callaghan and Miller, 1994). In addition, the activation of astrocytes in response to METH was correlated with toxicity (Pu and Vorhees, 1993). Therefore, the astrocyte response occurs rapidly following METH treatment and is relatively prolonged. Nevertheless, the extent to which this response contributes to pathological changes in the striatum is unknown.

Although several studies have investigated changes in GFAP immunoreactivity or protein levels in METH-induced toxicity, the microglial response to METH administration has not been reported. Microglia are classically recognized for their phagocytic capabilities and are considered the resident immune cells of the brain (Streit et al., 1999). However, they also exhibit dynamic response properties and phenotypic changes that can exert an important influence upon the outcome of CNS disease or injury (Aschner et al., 1999). This raises the possibility that METH-induced microglial responses may contribute to the neuropathological changes that result from neurotoxic levels of this amphetamine. As a first step in testing this hypothesis, we defined the spatial and temporal relations of reactive microglia responses to METH-induced pathology.

Material and methods

Adult male Sprague–Dawley rats (Zivic Miller, Zelienople, PA) weighing 300–350 g were used in this study and

were provided food and water ad libitum. All experimental protocols conformed to regulations stipulated in the *NIH Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Rats were administered METH (15 mg/kg, sc) or saline every 2 h for a total of four injections. Core body temperature was measured in all animals at 1-h intervals throughout drug administration. Experimental animals were treated with METH ($n = 23$) or saline ($n = 6$) at either room temperature (23°C; $n = 19$) or at a reduced ambient temperature of 5°C ($n = 4$). Animals treated at 5°C were maintained in the reduced temperature environment 30 min before the first injection until 1 h after the last METH injection (total of 7.5 h). This regimen has previously been demonstrated to block METH-induced toxicity to monoaminergic terminals (LaVoie and Hastings, 1999). Twelve hours, or 1, 2, 4, or 6 days after the last injection of METH, animals were anesthetized with a mixture of ketamine and xylazine (60 mg/kg ketamine, 7 mg/kg xylazine, ip) and perfused transcardially with saline followed by 4% paraformaldehyde containing lysine and sodium metaperiodate fixative (McLean and Nakane, 1974). Brains were removed, postfixed for 1 h, and cryoprotected in several changes of 20% sucrose. Coronal tissue sections (35 μ m) were then cut throughout the entire brain using a freezing microtome and stored in cryoprotectant at –20°C before immunohistochemical processing. Immunoperoxidase localizations were conducted with mouse monoclonal antibodies generated against GFAP (1:20,000; Chemicon, Temecula, CA), specific for astrocytes, and the rat CD11b receptor (OX42, 1:1,000; Serotec, Raleigh, NC), specific for microglia. Free-floating sections were incubated in primary antibody at 4°C for 24 h, rinsed with several changes of phosphate buffer (0.1 M phosphate-buffered saline, pH 7.4), and incubated in donkey anti-mouse biotin-conjugated secondary antibody for 2 h at room temperature (1:500; Jackson ImmunoResearch; West Grove, PA). Following a second wash in phosphate buffer, sections were processed using the Vectastain Elite[®] horseradish peroxidase-avidin kit (1:250, Vector Laboratories, Burlingame, CA) and diaminobenzadine (DAB). Sections were then mounted on Fisher Superfrost slides and coverslipped using Cytoseal 60 (Stephens Scientific; Kalamazoo, MI). Dopaminergic fibers were visualized using a rabbit polyclonal anti-tyrosine-hydroxylase antibody (1:500; Protos Biotech, New York, NY) and donkey anti-rabbit CY3 conjugated secondary antibody (1:2000; Jackson ImmunoResearch Labs). Sections were mounted and coverslipped as described above.

Qualitative analysis of the reactive microglial changes in the brains of METH-treated rats was accomplished using a Simple 32 image analysis system (Compix, Cranberry, PA) and an MTI 3CCD video camera. Regions of striatum were sampled at 20 \times (area = 592 \times 222 μ m) using a Zeiss Axioplan photomicroscope equipped for bright field and

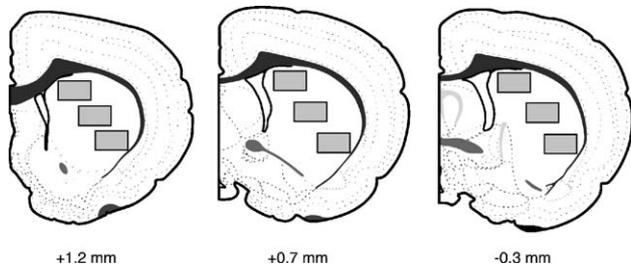


Fig. 1. Schematic diagram defining the nine regions of interest analyzed in this study. We chose three striatal regions at three rostral–caudal planes within each brain for analysis of microglial changes following METH administration. These nine fields-of-interest are shown as the shaded rectangular boxes. Distance from Bregma (in mm) is noted below each coronal plane (images adapted from Swanson, 1992).

fluorescence microscopy and interfaced with the image analysis system. Regions in the dorsomedial, central, and ventrolateral right striatum were analyzed at three rostral–caudal levels, corresponding to +1.2 mm, +0.7 mm, and –0.3 mm to Bregma (Fig. 1). The image analysis software allows for quantification of the percentage of each field that is occupied by immunoperoxidase label. Use of this quantitative approach was validated in a prior study using a different model (Rinaman et al., 1999). Control data were compared to METH-treated time points by two-way repeated-measures ANOVA. When significant differences for main effects (control vs. METH-treated) were obtained ($P < 0.05$), post hoc pairwise comparisons were made for within-subjects differences (dorsomedial vs. ventrolateral) at each rostral–caudal plane using layered Bonferroni correction.

Results

Microglial morphology

The morphology of microglia in the resting state and following METH-induced activation is illustrated in Fig. 2.

In control animals, microglia displayed a sessile morphology in which thin, finely branching processes extended radially from small, oblong somata (Fig. 2A). After METH administration, microglia in the ventrolateral striatum exhibited marked hyperplastic changes that were most pronounced at 2 days (Fig. 2B). These reactive changes were characterized by large increases in somal size and staining intensity and the presence of thicker, branched processes (Fig. 2B). METH administration also induced focal reactive microglial responses in areas outside of striatum that were more robust compared to reactive microglia in striatum (Fig. 2C). These reactive responses were consistent across all experimental animals and were absent in controls.

METH-induced activation of microglia within the striatum

Qualitative observations

Microglial morphology was investigated at several time points following neurotoxic administration of METH. We chose three rostral–caudal levels through the striatum from each brain and identified a dorsomedial, central, and ventrolateral region-of-interest at each level for analysis (see Fig. 1). These nine fields were analyzed from animals 12 h, or 1, 2, 4, and 6 days following administration of METH or saline. Striatal microglia of saline-treated rats ($n = 6$) demonstrated a sessile morphology characterized by ramified processes of very fine caliber extending from a small cell soma (Figs. 2A and 3A). Twelve hours following administration of METH, no significant changes in microglial morphology were observed in the striatum or any other brain region ($n = 3$) (data not shown). At 1 day following treatment, activated microglia appeared throughout striatum, with the greatest response in the ventrolateral region ($n = 4$; Fig. 4A). The soma and processes of these cells were larger and the staining intensity of individual cells was more pronounced than that observed in control animals. By 2 days post-METH, reactive microglia were observed throughout the entire striatum (Fig. 3B; $n = 6$), although regional differences in the magnitude of the

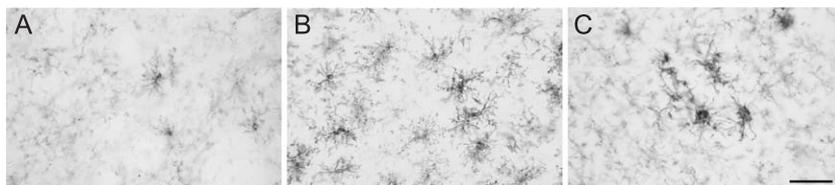


Fig. 2. Activated microglia of the striatum and non-striatal brain regions showed remarkably different morphologies. Rats were treated with saline or a neurotoxic regimen of METH and brains were analyzed for OX42 immunoreactivity at various time points to determine microglial morphology. (A) Resting state microglia within the ventrolateral striatum of a saline-injected rat are illustrated. These cells demonstrate a classic sessile morphology consisting of numerous fine caliber processes, a very small cytoplasmic volume primarily containing the cell nucleus, and moderate OX42 immunoreactivity. (B) Reactive microglia within the ventrolateral striatum 2 days following administration of METH are illustrated. These microglia show pronounced hyperplastic changes with shortened, thickened processes, an increase in cytoplasmic volume and intense OX42 immunoreactivity. (C) Reactive microglia from extra-striatal brain regions that develop METH-induced pathology are illustrated. Note the dramatic reduction in the number of microglial processes and the significant swelling of the cytoplasmic volume of activated microglia found within the ventromedial column of the periaqueductal gray. Scale bar = 20 μm .

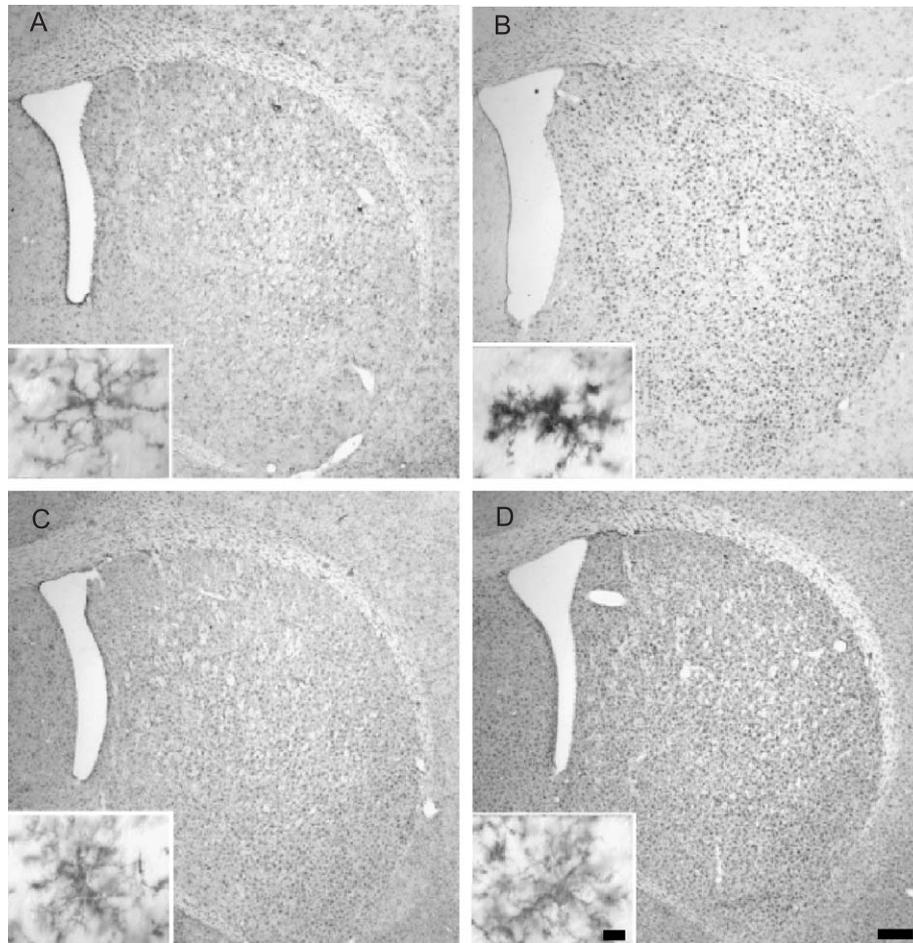


Fig. 3. Administration of METH resulted in the activation of microglia throughout the striatum. The microglial response to METH was most pronounced 2 days following treatment. Thereafter, the staining intensity of cells was reduced but never reach control levels. (A) Resting microglia exhibited a homogeneous distribution throughout the entire striatum in saline-treated rats. (B) Reactive microglia were present throughout the striatum 2 days after METH treatment. This time point corresponds to the maximal observed changes in microglial morphology. (C) Four days post-METH, reactive microgliosis was reduced compared to 2 days and is further attenuated at 6 days (D). Scale bar for figures = 300 μm , scale bar for inserts = 10 μm .

response were observed. The reactive microglia at this time point showed shortening and thickening of their processes and stained much more intensely than those at earlier survival intervals (Fig. 2B). It also appeared as if the density of microglial cells per unit area was increased over controls. However, the small size of the cell bodies combined with the pronounced hyperplastic changes in the proximal processes of individual cells made it impossible to make reliable cell counts.

Although the entire striatum exhibited a pronounced activation of microglia at 2 days, the intensity of staining in the ventrolateral striatum was consistently greater than that observed in the central or dorsomedial regions. Furthermore, there appeared to be a rostral–caudal gradient of microglial activation, with intermediate and caudal striatum showing greater reactive changes than at rostral levels. The reactive response decreased progressively after 2 days but never returned to control levels (Figs. 3C and D). The principal change in morphology at these longer

survival times was a decrease in the staining intensity of processes, but there was no obvious change in relative density of microglia within the striatum.

Quantitative analysis

Quantitative densitometric analysis revealed statistically significant increases in the percentage of field occupied by reactive microglia, as compared to control animals beginning at day 1. These changes correlated with the qualitative morphological analysis, demonstrating a peak at 2 days post-METH administration (Fig. 4). Further, these increases were more pronounced in the ventrolateral quadrant at caudal levels of the striatum.

The apparent attenuation in the microglial response observed at 4 and 6 days in the morphological analysis was not apparent in the densitometric measures. This is due in large part to the subtle changes in morphology that characterized the morphological attenuation at these longer survival times. Specifically, the processes of the

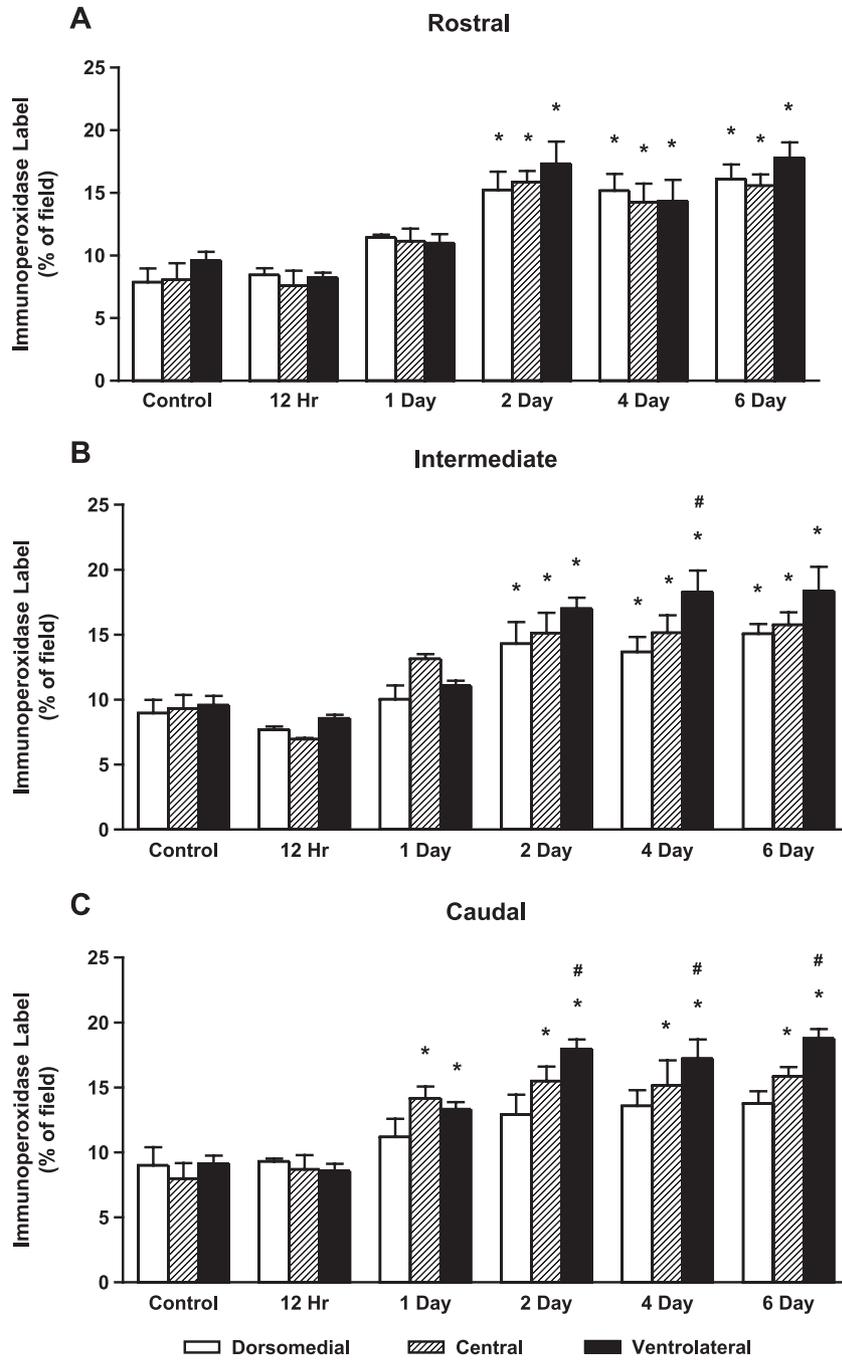


Fig. 4. Quantitative densitometric analysis of reactive microgliosis in subregions of striatum revealed differences in the temporal and morphological aspects of microglial responses. For these analyses, saline-treated rats were combined from all time points, as there was no effect of the vehicle in any group. Data are represented as the percentage of each field that was occupied by OX42 immunoreactivity. Images A–C represent the three rostral–caudal planes examined in this study. Examination of tissue sections with light microscopy suggested increases in OX42 immunoreactivity were first observed at intermediate and caudal levels of striatum at 1 day post-METH, and were increased in all regions at subsequent time points. There was also a consistent trend of increased OX42 immunoreactivity in the central and ventrolateral vs. dorsomedial striatum. Statistical analysis of the densitometric measures revealed that 1 day was the first significant increase in microglial immunoreactivity, and that several rostral–caudal planes showed a significant increase in microgliosis in the ventrolateral field as compared to the dorsomedial striatum (* denotes statistically significant from control; # denotes statistically significant from dorsomedial region within the same tissue section; $P < 0.05$).

reactive microglia were less pronounced at days 4 and 6 and the staining intensity of cells appeared less intense. However, as noted above, there was no apparent decrease in the density of microglia in the striatum and the

overlapping distribution of individual cells in different planes of the tissue sections reduced the ability of the image analysis system to detect these subtle changes in morphology.

Temporal relations of striatal microglial activation and dopaminergic axonal pathology

Dual localization of tyrosine hydroxylase and microglial markers was used to determine the temporal relationship between microglial responses and morphologically distinct dopamine axonal pathology. In control ventrolateral striatum, tyrosine hydroxylase-immunoreactive fibers (TH-IR;

red fluorochromes) were of a very fine caliber and of moderate immunofluorescence intensity (Fig. 5A) coextensive with uniformly distributed resting microglia (Fig. 5B). At 1 day, there was little or no evidence of swollen varicose TH-IR fibers in the central and dorsomedial striatal regions, yet there were significant reactive microglial changes observed (Fig. 5C). Reactive microgliosis at this time point was observed throughout the striatum but

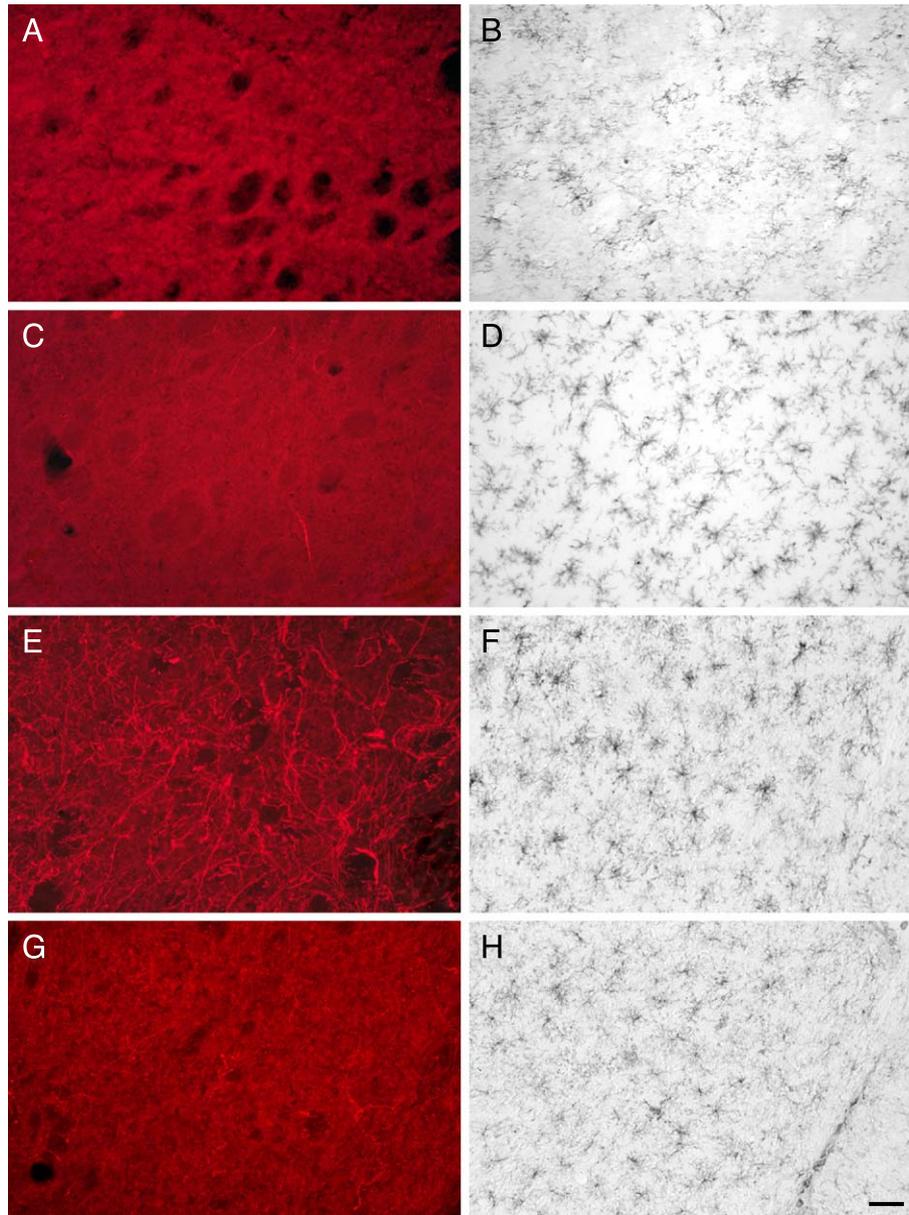


Fig. 5. Robust microglial activation preceded the appearance of severe dopamine axonal pathology following METH administration. Regions of the ventrolateral striatum at the intermediate rostral–caudal plane (see Fig. 1) were examined with immunofluorescence to assess TH-IR fiber morphology and immunoperoxidase localization of microglia to visualize reactive microgliosis. (A) The characteristic fine caliber TH-IR fibers (red) of the striatum are shown from a control subject. (B) Microglia of the ventrolateral striatum of a control rat exhibit a classic resting state morphology with thin processes and a small cell soma. (C) One day following METH treatment, only scattered TH-IR axons exhibit varicose pathology but there is a prominent reactive microgliosis. (D) At this time point, microglia throughout the ventrolateral striatum show a reactive phenotype with thickened processes and increased cytoplasmic volumes. At 2 days post-METH, many swollen, varicose and twisted TH-IR axons (E) are apparent and microglia (F) demonstrate the peak of their response to METH. By 4 days following administration of METH, pathologic TH-IR fibers (G) are absent, and microglia (H) appear to be returning toward a resting state. Scale bar = 50 μ m.

was particularly prominent in the ventrolateral striatum (Fig. 5D). At 2 days post-METH, there was a dramatic increase in the number of swollen, varicose TH-IR axons (Fig. 5E). This time point (2 days) correlated with the maximal response of the microglia, with the most prominent activated microglia found in the ventrolateral striatum (Fig. 5F). By 4 days post-METH, the appearance of TH-IR swollen fibers was greatly reduced, suggesting that the fibers had degenerated or lost TH-expression phenotype (Fig. 5G). Analysis of the microglial morphology at 4 days also revealed a reduction in the hyperplasia of the striatal microglia, consistent with that observed in the single localization studies (Fig. 5H).

The effect of hypothermia on methamphetamine-induced reactive microgliosis

It is well documented that administration of METH at reduced ambient temperature can block both METH-induced hyperthermia and METH-induced toxicity (Bowyer et al., 1992; LaVoie and Hastings, 1999). Therefore, we administered METH at both room temperature (23°C) and at 5°C, and examined microglial activation 2 days following the METH treatment. Although the microglial response appeared attenuated at 2 days when METH was administered at 5°C as compared to 23°C, the response was clearly above control levels (Fig. 6).

Extra-striatal brain regions show intense, focal microglial activation

There is recent evidence that METH damages more brain areas than previously documented. For example, cortical (Eisch et al., 1998; Ryan et al., 1990) and hippocampal (Schmued and Bowyer, 1997) neurons have been shown to be vulnerable to amphetamine toxicity. Upon examination of the entire brain in sections at a frequency of 210 μm , we observed focal hyperplastic microglial activation in the parietal and piriform cortices and in the ventromedial column of the periaqueductal gray (Fig. 7). The morphology of reactive microglia in these areas varied greatly from that observed in the striatum. Specifically, the reactive microglia in these regions were substantially larger than those in the striatum and their processes were thicker and restricted to the immediate vicinity of the somata (Fig. 2C). Often, the processes appeared to ensconce to individual neurons. The appearance of these cells was reminiscent of an amoeboid/phagocytic state of activation (Streit et al., 1999). It is important to note that the activated microglia within these brain regions demonstrated their hyperplastic changes over a greater period of time than the activated microglia within the striatum; there was no attenuation in reactive microgliosis at 6 days within these extra-striatal brain regions, whereas the striatal microglia consistently exhibited mor-

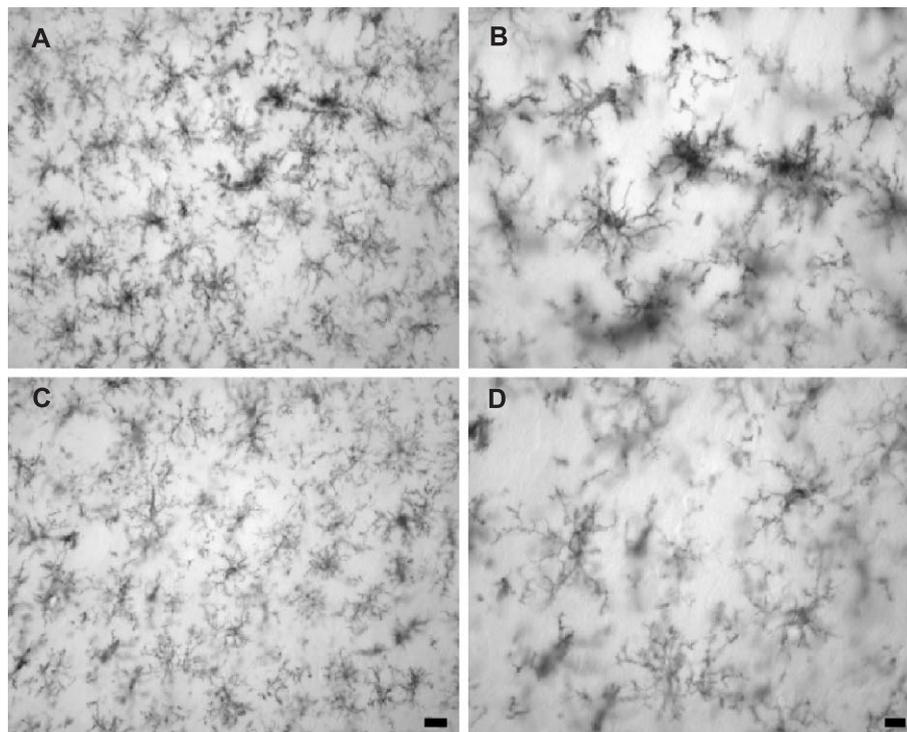


Fig. 6. Hypothermic neuroprotection attenuates the activation of microglia in response to METH. Rats were treated with METH or saline at room temperature (23°C) or 5°C ($n = 4$). Examination of the ventrolateral striatum at 2 days post-METH (23°C) revealed microglial activation (A and B) that was somewhat attenuated when rats were treated with METH at 5°C (C and D). Panels B and D are a higher magnification (scale bar = 10 μm) of a subfield of panels A and C (scale bar = 20 μm), respectively.

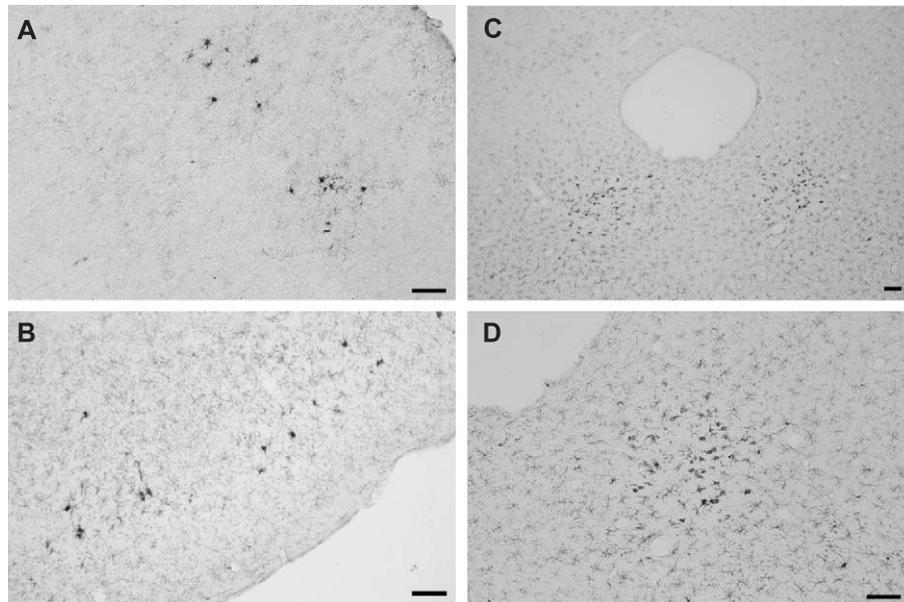


Fig. 7. Robust microglial activation was observed in the parietal and piriform cortices, and ventromedial column of the periaqueductal gray. (A) Activated microglia were consistently found in the parietal cortex 2–6 days following METH treatment. (B) Piriform cortex showed robust microglial activation that persisted throughout the time points analyzed. (C) The ventromedial column of the periaqueductal gray also showed intense reactive microgliosis. Note the bilateral zones containing activated microglia adjacent to the third ventricle. (D) A higher magnification of the right hemisphere shows the significant hyperplasia of the reactive microglia in the periaqueductal gray. Scale bar = 100 μ m.

phology suggestive of a reduction in reactive state at this time point.

Discussion

Although the mechanism of METH-induced toxicity remains unknown, it has been hypothesized to involve the neurotransmitter DA and oxidative stress (Cadet et al., 1994; De Vito and Wagner, 1989; Gibb and Kogan, 1979; Giovanni et al., 1995; Hirata et al., 1995; LaVoie and Hastings, 1999; Wu et al., 2002). In addition, the drug-induced hyperthermia that accompanies high doses of many amphetamine compounds is a necessary component of the toxicity of these drugs (Bowyer et al., 1992; Miller and O'Callaghan, 1994, 1995), suggesting a complex, yet undefined mechanism of cellular injury. In an effort to understand more completely the events that accompany the loss of monoaminergic terminals of the striatum, we have investigated the response of microglia to METH administration. Previous studies have reported both the temporal and anatomical features of astrocyte activation in METH-induced toxicity, and those studies provided the framework for the present analysis (O'Callaghan and Miller, 1994; Pu and Vorhees, 1993; Pu et al., 1994). Our data demonstrate that reactive microglia are present in the striatum as early as 1 day following administration of METH, that morphological and immunohistochemical indicators of the reactive response peak by 2 days, and that there are regional differences in the magnitude of the response both in the striatum and in other regions that suffer METH-induced neurotoxic-

ity. Additionally, our data suggest that a robust microglia response is temporally advanced compared to the axonal pathology observed in the striatum. Collectively, the data suggest that reactive microgliosis may contribute to neuropathogenesis rather than being a secondary consequence of neuropathology.

Previous studies have suggested that the ventrolateral striatum is more vulnerable to the neurotoxic effects of METH than other striatal subregions. It has been demonstrated that this area shows the greatest loss of dopamine-transporter binding sites (Eisch et al., 1992) and the greatest loss of tyrosine-hydroxylase immunoreactivity (Pu and Vorhees, 1993). Our data are consistent with those findings. A small number of swollen TH-IR axons were restricted to the ventrolateral striatum 1 day following METH administration. At 2 days post-METH, varicose changes in TH-IR axons were pronounced in the ventrolateral striatum and were only rarely observed in the dorsomedial region. Pathologic TH-IR fibers were decreased by 4 and 6 days due to either a degeneration of dopaminergic axons and terminals or a loss of TH immunoreactivity. Further, the rostral striatum showed little evidence of dopamine terminal pathology, whereas the intermediate and caudal regions of the striatum were dramatically affected. Reactive microglial changes paralleled the distribution of TH axon pathology. However, the apparent magnitude of reactive microgliosis was more pronounced at earlier survival times compared to the pathology of TH-IR axons, raising the possibility that this glial response contributed to neuronal pathology rather than being simply a secondary consequence of neuropathogenesis.

Reactive microglia may influence the outcome of tissue injury or cell death. Through trophic support, microglia can aid in cell survival and axon regrowth. In MPTP-induced toxicity, another dopamine-selective lesion, it has been shown that expression of IL-1 by reactive astrocytes is associated with terminal sprouting and re-innervation of the striatum by the VTA (Ho and Blum, 1998). Evidence of re-innervation of the striatum by both dopamine and serotonin terminals following METH administration has also been reported (Cass and Manning, 1999; Friedman et al., 1998). Therefore, the activation of astrocytes and/or microglia may result in the secretion of cytokine or trophic signals that aid in the recovery-of-function suggested to occur in METH-induced toxicity. In fact, it has been shown that the neurotrophic factors GDNF and BDNF can protect dopamine terminals from METH-induced toxicity (Cass, 1996; Maggio et al., 1998). Reactive glia within the striatum have been shown to express these neurotrophins in response to other types of tissue injury (Batchelor et al., 1999), and these events may be triggered in METH-induced toxicity as well. Therefore, we cannot exclude the possibility that the reactive microgliosis observed in our analysis reflects a proactive response to protect cells and axons from injury.

Activated microglia may contribute to neurotoxicity through the expression of various cytokines and reactive oxygen and nitrogen species. Microglia can express cytokines such as IL-1 β , IL-6, and TNF α that can initiate and promote inflammation in brain tissue (for review, see Kreutzberg, 1996; Stollg and Jander, 1999; Streit et al., 1999). Depending on the species studied, activated microglia can also express inducible nitric oxide synthase, NADPH oxidase, or myeloperoxidase, resulting in the secretion of large amounts of nitric oxide, superoxide, or hydrogen peroxide and hypochlorous acid, respectively (Orr et al., 2002; Teismann et al., 2003). Thus, one explanation for our findings is that the METH-induced changes in microglial phenotype that characterize the reactive response may contribute to the drug-induced pathology. In support of this conclusion is the robust reactive microgliosis that precedes the appearance of the majority of pathology in TH-IR axons in the striatum. Nevertheless, definitive test of this hypothesis awaits studies in which the microglia response to METH is prevented by administration of drugs such as minocycline, which have been shown to selectively reduce reactive microgliosis (Tikka et al., 2001).

METH-induced toxicity is known to affect not only dopamine and serotonin terminals of the striatum, but also cause the loss of serotonin terminals in the hippocampus (Hotchkiss and Gibb, 1980). We found no evidence of microglial activation in the hippocampus of METH-treated rats at any of the time points investigated. Although there is evidence of serotonergic terminal loss in the hippocampus, other factors of METH-induced toxicity, such as glutamate release and oxidative stress, have been less well characterized in this brain region. Therefore, it is likely that some aspects of striatal vs. hippocampal toxicity differ in this

model, and these differences may explain the lack of microglial responses in the hippocampus. Since the projections of the substantia nigra and the raphe nuclei are greatly affected by METH, we also analyzed these brain regions and found no evidence of reactive microgliosis. Although we did not find reactive microgliosis in the hippocampus, substantia nigra, nucleus accumbens, or raphe nuclei, there were several brain areas that exhibited a distinctive focal microgliosis similar to the amoeboid or phagocytic phenotype (Streit and Graeber, 1996). In the parietal cortex, these amoeboid/phagocytic microglia were confined to intermediate layers of the somatosensory cortex in a region that correlates with METH-induced cortical damage revealed by the neurodegeneration-specific stain, Fluoro-Jade (Eisch et al., 1998). This finding is consistent with the ultrastructural evidence of cortical cell loss resulting from neurotoxic amphetamine administration (Ryan et al., 1990). Microglia of the amoeboid/phagocytic phenotype were also found in the piriform cortex and piriform–amygdaloid transition zone. One study has previously suggested that this brain region may also be vulnerable to the effects of METH (Schmued and Bowyer, 1997). Lastly, the ventromedial column of the periaqueductal gray also showed intense activation of microglia, and this brain region has not been previously identified as vulnerable to METH. Thus, it appears that neurotoxicity resulting from METH administration also occurs in other regions of the CNS. This conclusion is consistent with prior suggestions (Kreutzberg, 1996) that reactive microgliosis is characterized by distinctive morphologies (e.g., ramified vs. amoeboid/phagocytic phenotypes) that reflect the magnitude of injury. Thus, we interpret the more pronounced and protracted reactive microgliosis in extra-striatal areas as being indicative of neurotoxic cell loss, although the more subtle ramified microgliosis apparent in the striatum is reflective of axonal damage.

METH toxicity has been shown to require an acute but severe hyperthermia and that administration of METH at reduced ambient temperatures protects against amphetamine-mediated toxicity (Bowyer et al., 1992; LaVoie and Hastings, 1999; Malberg and Seiden, 1998; Miller and O'Callaghan, 1995). Therefore, we also investigated whether administration of METH at 5°C would prevent the METH-induced activation of microglia. We observed an attenuation of the microglial response in striatum when METH was administered at 5°C as compared to rats treated with METH at room temperature. Although the response was still above control levels, it is possible that the hyperthermia interferes with the ability of microglia to respond, which is reflected in the reduced toxicity (Kreutzberg, 1996). Additionally, treatment at 5°C prevented the activation of microglia within extra-striatal regions, the piriform cortex, parietal cortex, and periaqueductal gray (data not shown).

In this study, we have shown that METH-induced toxicity is associated with the activation of microglia within

several distinct regions of the affected brain. In the striatum, the changes in microglial morphology preceded the robust expression of dopaminergic axonal pathology, suggesting the possibility of a link between these processes. Recent studies have demonstrated where activated microglia have contributed to the loss of dopaminergic neurons in substantia nigra (Gao et al., 2002; Wu et al., 2002). The contribution of these glial responses in METH-induced toxicity is unknown; however, further work may provide critical information on the effects of neuronal–glial interactions in this lesion model.

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