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SYNAPSE 23:328–334 (1996)

Effect of Methamphetamine on Glutamate-Positive Neurons in the Adult and Developing Rat Somatosensory Cortex

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KEY WORDS Methamphetamine, Glutamate, Astrocyte, Neurotoxicity, Somatosensory cortex, GFAP, Rats

ABSTRACT The neurotoxic effects of methamphetamine (MA) on dopaminergic and serotonergic terminals have been well-documented. Another neurotoxic effect of MA is neuronal degeneration in the somatosensory cortex, as seen by silver staining. The neurochemical characteristics of these degenerating neurons are unknown. Using glutamate and glial fibrillary acid protein (GFAP) immunohistochemistry, it was found that MA exposure in adult rats (10 mg/kg given 4 times intraperotoneally (i.p.) at 2-h intervals) causes localized depletion of glutamate-positive neurons and astrogliosis in the somatosensory cortex 3 days following treatment. The affected region covered the middle one-third portion from the longitudinal fissure to the rhinal sulcus and was predominately seen in layers II–III of the cortex. This pattern of depletion is consistent with that demonstrated previously with silver staining following MA, d-amphetamine, and 3,4-methylenedioxymethamphetmine (MDMA) exposures. Comparable effects were not found in developing animals at ages previously shown to also be resistant to MA-induced effects on dopaminergic terminals (age 20 and 40 days). Results suggest that MA exposure induces degeneration of glutamatergic neurons in the somatosensory cortex of adult rats.

INTRODUCTION

The neurotoxic effects of methamphetamine (MA) on the dopaminergic and serotonergic systems have been well-documented. It is clear that MA induces degeneration of dopaminergic terminals in the striatum (Ricaurte et al., 1980, 1982; Seiden et al., 1975/1976) and depletion of serotonin in most forebrain regions (Axt and Molliver, 1991; Ricaurte et al., 1980). Another neurotoxic effect of MA is neuronal degeneration (argyrophilia) in the somatosensory cortex, as demonstrated by silver staining (Commins and Seiden, 1986; Ricaurte et al., 1980). However, the neurochemical characteristics of these neurons are unknown.

Two lines of evidence suggest that MA may induce degeneration of a corticostriatal pathway. First, d-amphetamine has been demonstrated to induce argyrophilia in neurons of the somatosensory cortex and myelinated fibers in the striatum and corpus callosum (Ryan et al., 1990). Myelinated fibers are characteristic of corticostriatal projection pathways but not of dopaminergic or serotonergic fibers. Second, astrogliosis, which is a reliable indicator of neuronal damage, was found in the striatum without accompanying depletion of dopaminergic terminals in developing rats (Pu [illegible]d Vorhees, 1993), and in amfonelic acid-pretreated adult rats following MA treatment (Pu et al., 1994). It may be that MA induces degeneration of corticostriatal projections, and this in turn causes the localized striatal astrogliosis described above. Biochemical (McGeer et al., 1977; Perschak and Cuenod, 1990; Roberts et al., 1982; Spencer, 1976) and histochemical (Dinopoulos et al., 1989) evidence supports the view that glutamate is the principal neurotransmitter in corticostriatal pa[illegible]ways. Therefore, it was hypothesized that MA-induced

Received May 24, 1995; accepted in revised form December 27, 1995.

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degenerated neurons in the somatosensory cortex are glutamatergic.

Accordingly, glutamate and glial fibrillary acid protein (GFAP) immunohistochemistry were used to evaluate MA-induced damage in the somatosensory cortex. Results show that MA reduces glutamate-immunoreactivity and increases GFAP-positive cells in the somatosensory cortex of rats.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Charles River, Raleigh, NC) were used. They were age-matched, fed ad libitum, and housed at 22 ± 2°C with controlled lighting. A total of 16 animals was used in the adult study. Eight animals were treated intraperotoneally (i.p.) with 10 mg/kg d-MA-HCl (Sigma Chemical Co., St. Louis, MO), expressed as free base, given 4 times in 1 day at 2-h intervals. Eight control animals were treated with saline. All animals were sacrificed 3 days after the last injection. To investigate the effect of MA treatment on developing animals, two additional group of rats (age 20 and 40 days) were treated with MA using the same dosing regimen as above. Each group consisted of 5 MA-treated and 5 saline-treated controls.

On the day of sacrifice, rats were deeply anesthetized with sodium pentobarbital (30 mg/kg) and perfused transcardially with oxygenated calcium-free Tyrodes followed by 400–500 ml 4% paraformaldehyde (PFA) and 0.3% glutaraldehyde, in 0.1 M phosphate buffer, pH 7.4, at 4°C. After perfusion, brains were removed and immersed in the PFA-glutaraldehyde for 90 min at 4°C. Coronal sections (40 μm) of these brains were cut on a vibratome, and sections between Bregma levels 1.0 to −2.3 (Paxinos and Watson, 1986) were collected in cold phosphate-buffered saline (PBS), pH 7.4.

Free-floating sections were incubated with continual agitation at room temperature for 20 min in PBS containing 10% normal goat serum (NGS), and then for 12–18 h in mouse antiserum directed against glutamate-glutaradehyde (Incstar, Inc., Stillwater, MN) at 1:3,000 dilution. The antisera were diluted in 0.1 M PBS containing 0.2% NGS and 0.3% Triton X-100. Subsequently, biotinylated goat anti-mouse Ig-G antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1:200, and avidin-biotinylated peroxidase complex (Vector Laboratories, Inc., Burlingame, CA), were conjugated to the primary antibodies at room temperature for 1 h each. Immunolabeled peroxidase was visualized by incubation at room temperature for 5 min with 0.05% diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in 0.1 M phosphate buffer, pH 7.6. Sections were mounted on gelatin-coated slides, dehydrated, coverslipped with DPX®, and examined by bright- and dark-field microscopy.

Two control experiments were performed in additional animals to determine specificity of the glutamate antibody. First, glutamate antibody was omitted. Second, glutamate antibody was preabsorbed by incubating with 0.1 mg/ml glutamate-BSA conjugate at 4°C overnight. The conjugate was synthesized using glutaraldehyde based upon a protocol obtained from Incstar, Inc.

For GFAP immunohistochemistry, 10 animals were treated with MA and 8 animals with saline at age 77–80 days. Treatment regimen was the same as above. The staining procedure was similar to the glutamate immunohistochemistry, except that saline followed by 4% PFA in 0.1 M phosphate buffer was used for perfusion; for details, see Pu and Vorhees (1993). GFAP staining could not be performed in the same animals used for glutamate immunohistochemistry because the glutaraldehyde in the perfusing solution was not compatible across the two methods. The neuroanatomical distribution of the stained structures and boundaries of the cortical cell layers were described with the assistance of the atlas of Paxinos and Watson (1986) in separate hematoxylin and eosin (H&E)-stained sections.

RESULTS

In control animals, glutamate immunoreactivity was found throughout the six layers of the somatosensory cortex (Fig. 1a). Positive staining occurred in cell bodies which were located in layers I–VI and fibers in layers I–V (Figs. 1a, 2a). Layer I stained with the greatest intensity, apparently because it contains the apical dendrites of neurons from deeper layers. However, very few positive cell bodies were found in this layer. Numerous positively-labeled cell bodies were found in layers II–III, and most were densely stained. The boundary between layers II and III was difficult to define. In layer IV, positively-stained cells were less densely distributed and showed lighter staining compared to layers II–III. Neurons of layer V were the most intensely stained, showing clear apical dendrites projecting from the cell body toward the surface of the cortex. Although there were many glutamatergic cells in layer VI, they were distributed sparsely and stained lightly. Some very lightly stained cell bodies and fibers were also seen among the more heavily stained cells in layer II–VI, which may represent cells containing less glutamate or cells located more distal to the cut surface. Due to the high background of the staining, no clear glutamate-positive staining was visible in the white matter or striatum.

In MA-treated animals, the number of glutamate-positive neurons in the somatosensory cortex was decreased (Figs. 1a, 2b). This effect occurred primarily in layers II–III, with minor effects in layer IV. The effect was confined to the middle one third of the cortical region, spanning the longitudinal fissure to the rhinal sulcus, which corresponds to somatosensory cortex region I. The effect was most apparent at Bregma level −0.8 to −1.3 (Paxinos and Watson, 1986), and covered total anterior-posterior distance of about 1 mm. No depletion was found in other cortical regions on the

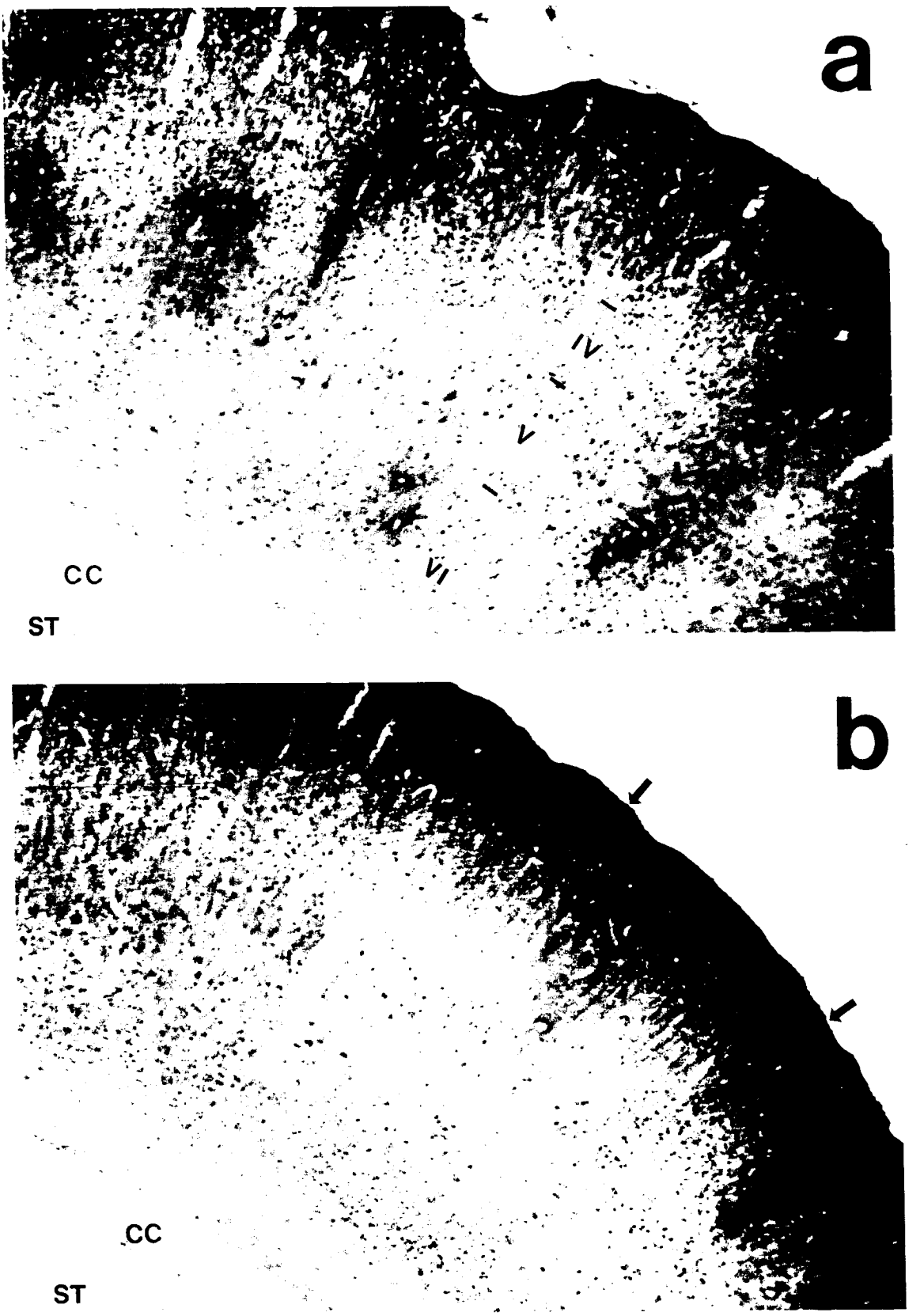

Fig. 1. Effect of MA exposure on glutamate-positive neurons in a coronal section of somatosensory cortex. **a:** Normal distribution pattern of glutamate-positive neurons in a saline-treated control animal, showing glutamate-positive neurons and fibers in layers I–VI. **b:** Depletion of glutamate-positive neurons in MA-treated animal, showing reduction of glutamate-positive neurons in layers II–IV (region between small arrows). Large arrows indicate region shown in Figure 2b under higher magnification. CC, corpus callosum; ST, striatum. Bar, 300 μm (a and b).

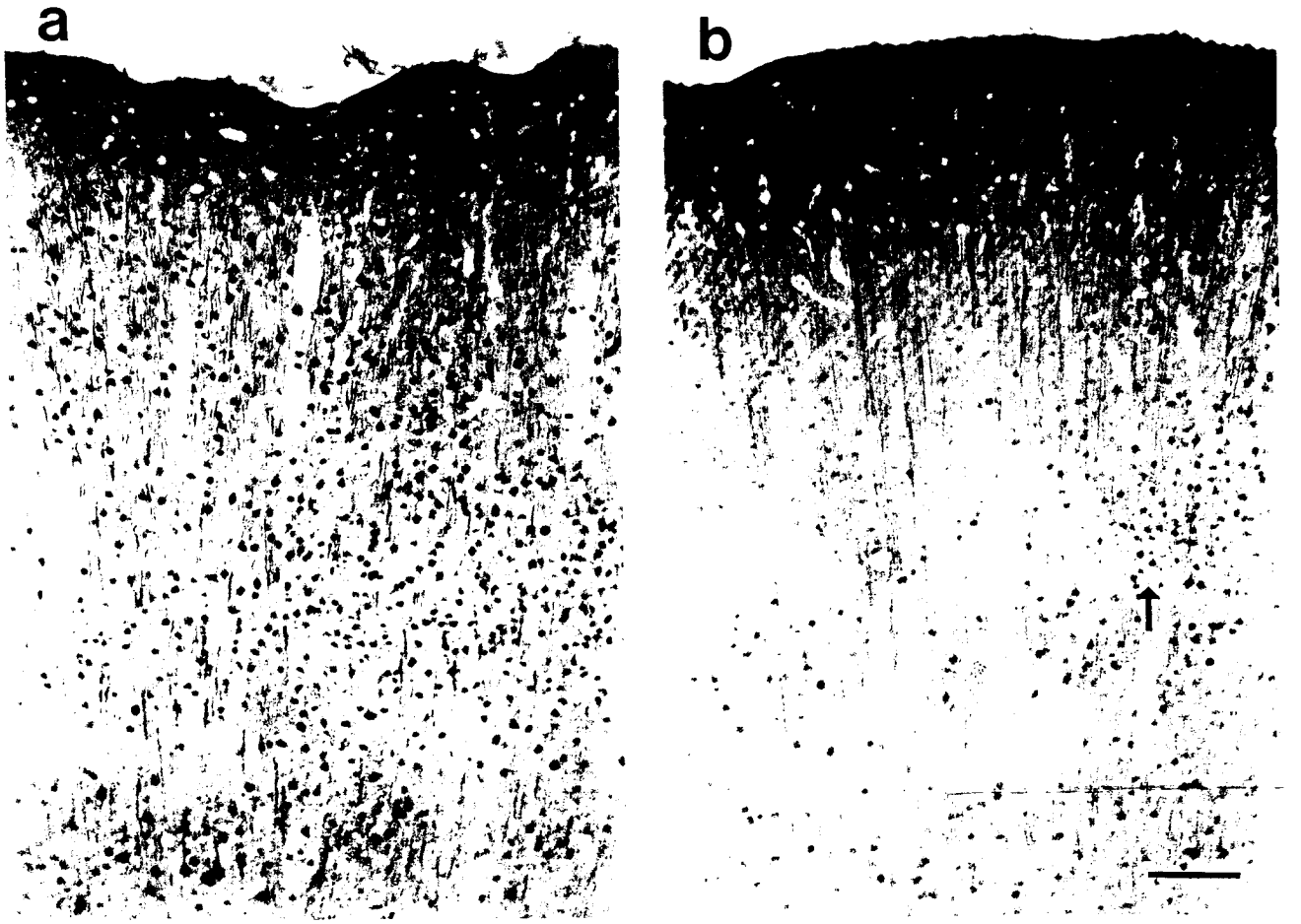

Fig. 2. Effect of MA exposure on glutamate-positive neurons in a coronal section of somatosensory cortex under higher magnification. **a:** From adjacent section of Figure 1a. Glutamate-positive neurons and fibers in layers I–V in control animal. **b:** From same section of Figure 1b. Reduction of glutamate-positive neurons in MA-treated animal, and boundary (arrow) between depleted (left of arrow) and unaffected (right of arrow) regions. Bar, 100 μm (a and b).

sections examined. It is noteworthy that even in the affected region, some glutamate-positive fibers and cell bodies remained in all animals. Although both sides of the brain were affected, the severity of depletion was not identical. The pattern of depletion was also not the same among animals; some showed a more restricted zone of effect than others. Two out of 8 MA-treated animals demonstrated marked depletion, and 3/8 showed moderate depletion. No depletion was identified in the rest of the MA-treated animals (3/8). No depletions were seen in any of the controls.

Experiments were conducted to determine the specificity glutamate immunoreaction under the conditions used in these investigations. Specifically, preabsorption with a glutamate-BSA conjugate eliminated all staining. Omission of the glutamate antibody from the immunoreaction protocol also eliminated all staining (not shown).

For GFAP immunohistochemistry, lightly-stained astrocytes were found in the somatosensory cortex, and they were predominately located in layers I–II and V–VI in control animals. Fewer labeled cells were located in layers III–IV (Fig. 3a). In 3/10 MA-treated animals there was a band of astrogliosis (increased size and staining intensity) observed in layers II–III of the somatosensory region (Fig. 3b). This GFAP-positive band was not seen in the remainder of the MA-treated animals (7/10). The region of astrogliosis was nearly identical to that seen in the depleted zone of glutamate-stained neurons in MA-treated rats. No reactive astrocytes were found in any of the control animals (0/10). The effect was not found in other cortical regions.

In the 20- and 40-day-old animals, no differences were observed in the somatosensory cortex following glutamate staining (not shown).

DISCUSSION

The results of this experiment demonstrate that MA exposure can induce a depletion of glutamate-positive neurons and produce astrogliosis in the somatosensory

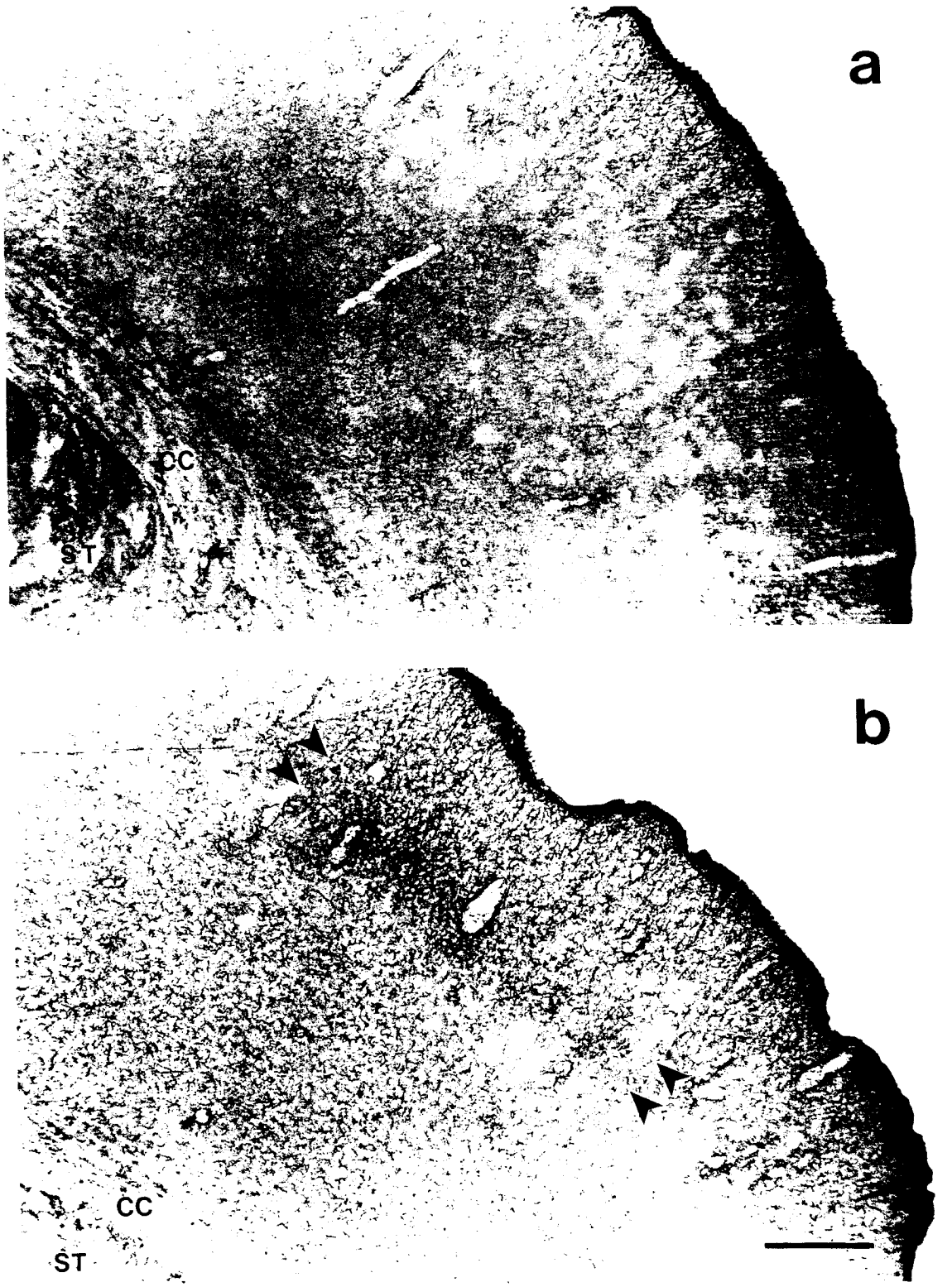

Fig. 3. Effect of MA exposure on GFAP-positive astrocytes in a coronal section of the somatosensory cortex. **a:** Normal distribution pattern of astrocytes in saline-treated control animal. **b:** Astrocytic response in MA-treated animal, showing a band of gliosis (between arrows). CC, corpus callosum; ST, striatum. Bar, 300 μm (A and B).

cortex of rats. This may be caused either by a depletion of glutamate in the neurons or by the degeneration of these neurons. The affected region was similar to that cortical region in which neuronal degeneration was demonstrated by silver staining following MA (Commins and Seiden, 1986; Ricaurte et al., 1980), amphetamine (Ryan et al., 1990), and MDMA treatment (Commins et al., 1987). Taken together, these data suggest that MA causes degeneration of glutamatergic neurons in the somatosensory cortex.

The mechanism of MA-induced degeneration of cortical neurons is unknown. It has been shown that blocking dopamine synthesis with α-methyl-*p*-tyrosine can prevent MA-induced neuronal degeneration in the somatosensory cortex (Commins and Seiden, 1986). This finding suggests that MA-induced dopamine (DA) release is related to the induction of cortical neuronal degeneration. However, it is difficult to explain how DA release could result in glutamatergic neuronal degeneration.

There are reports that MA may directly affect the function of glutamatergic neurons. MA can induce overrelease of glutamate in the striatum (Nash and Yamamoto, 1992) and alter the electrophysiological function of glutamatergic neurons of hippocampus in vitro (Chirwa and Clayton, 1994). Thus, MA may directly affect the function of glutamatergic neurons, causing excessive excitation and consequently cell death. Further investigation is needed to test this hypothesis.

Recently, it has been found that MA exposure causes an increase in N-methyl-D-aspartate (NMDA) receptor number in the same region of the somatosensory cortex where we have seen glutamate-positive neuron reduction (Eisch et al., 1995). This suggests a compensatory up regulation of NMDA receptors on adjacent dendrites following the death of vulnerable glutamatergic neurons.

It has been suggested that amphetamine induces degeneration of corticostriatal projections based on the finding that there are degenerated myelinated fibers in the striatum and corpus collosum (Ryan et al., 1990). Antrograde (Webster, 1961) and retrograde (Akinsegun and Buxton, 1992; McGeorge and Faull, 1987) tracing studies have demonstrated that the neurons in the somatosensory area project to the striatum. Studies have shown that corticostriatal projections are predominately from layer V, with small amounts from layer III (Akinsegun and Buxton, 1992). Preliminary data collected using fluorogold as tracer injected into the ventral striatum also demonstrated that there are labeled neurons in the layers II–III and V (unpublished observations). These results support the idea that MA may induce degeneration of corticostriatal pathways, in addition to degeneration of dopaminergic terminals. Biochemical and histochemical data suggest that glutamate is a neurotransmitter in the corticostriatal pathway (Madl et al., 1986; McGeer et al., 1977; Ottersen and Storm-Mathisen, 1984; Perschak and Cuenod, 1990; Roberts et al., 1982; Spencer, 1976). Therefore, the results of this experiment suggest that MA may induce degeneration of portions of the corticostriatal glutamate pathway. This also implies that there may be degeneration of glutamatergic terminals in the striatum. If this is the case, it may be the cause of the residual astrogliosis seen in amfonelic acid (AFA)-pretreated animals following MA treatment (Pu, 1994). However, there is a topographic discrepancy between the zone of somatosensory Glu-immunoreactivity (IR) reduction and the residual zone of the astrogliosis in the striatum seen in AFA-pretreated rat. The majority of somatosensory projections have been mapped to the dorsal neostriatum (caudate-putamen), with only a few projecting to the ventral neostriatum (not including the nucleus accumbens) (McGeorge and Faull, 1989). Therefore, the area of Glu-IR reduction seen here and the ventral neostriatal astrogliosis observed previously do not correspond quantitatively with the majority of corticostriatal projections. Perhaps the corticostriatal cells responsive to MA represent a subset of those cells projecting to the neostriatum, a subset susceptible to MA-induced Glu reduction.

As mentioned previously, MA exposure induces glutamate overrelease in the striatum (Nash and Yamamoto, 1992), and this process may be involved in the effects of MA on dopaminergic (Green et al., 1992; Marshall et al., 1993; Ohmori et al., 1993; Pu and Vorhees, 1995; Sonsalla et al., 1989, 1991) and serotonergic (Farfel et al., 1992; Green et al., 1992; Johnson et al., 1989; Ohmori et al., 1993; Pu and Vorhees, 1995) terminals. The results of these experiments and those of Nash and Yamamoto (1992) suggest that MA may cause decreased innervation of a corticostriatal glutamate pathway, as well as MA-induced glutamate overrelease. There may be two explanations for this. First, MA-induced degeneration of glutamate neurons occurs in a very small region of the cortex. This region may only contribute a small portion of the corticostriatal input. The remaining corticostriatal input may function normally or may exhibit compensatory increases in activity. Second, MA-induced glutamate overrelease may be an initial response to MA exposure, while degeneration of the glutamate pathway may be a delayed response. It is known that MA-induced neurotoxicity to dopaminergic terminals is a multistep process, involving: 1) initial overrelease of DA, and 2) delayed degeneration of dopaminergic terminals (O'Dell et al., 1991; Schmidt et al., 1985).

The results also demonstrated that MA-induced depletion of glutamate-positive neurons occurred only in adult animals (70-day-old). No depletion was found in young animals at age 20 and 40 days. A similar developmental pattern was found for MA-induced loss of dopaminergic terminals (Pu and Vorhees, 1993). This indicates that the mechanism underlying both dopaminergic and glutamatergic neurotoxicity is not ac-

quired before age 40 days. Although the mechanism for this is unknown, the ontogeny of the dopaminergic and glutamatergic systems may be related. Studies of the ontogeny of the dopaminergic and glutamatergic systems in rats have shown that NMDA ion channels in the frontal cortex reach adult levels after age 45 days, and glutamate-stimulated striatal DA release in vitro remains significantly below adult levels even at age 45 days and increases several-fold by age 100 days (Ali et al., 1993). Using cyclic voltammetry it has been shown that there is a threefold increase in DA release between age 33–114 days in the striatum in response to electrical stimulation of the dopaminergic tracts in the medial forebrain bundle (Stamford, 1989). These findings indicate that maturation of the glutamatergic and dopaminergic systems is still occurring after age 40 days. This phenomenon may underlie the developmental resistance to MA-induced dopaminergic and glutamatergic neurotoxicity. Finally, the developmental profile of the Glu-IR reduction indicates that it does not contribute to the day 40 residual striatal astrogliosis. This finding does not support our original expectation, and suggests that there must be another mechanism responsible for the astrogliosis induced by MA at age 40 days that is neither dopaminergically or glutamatergically mediated.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant DA06733 and center grant ES06096 from the National Institutes of Health.

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