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DISTINCT MORPHOLOGIC CLASSES OF SEROTONERGIC AXONS IN PRIMATES EXHIBIT DIFFERENTIAL VULNERABILITY TO THE PSYCHOTROPIC DRUG 3,4-METHYLENEDIOXYMETHAMPHETAMINE

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Abstract—Immunohistochemical methods were used to analyse the distribution and morphology of serotonergic axons in normal macaque monkeys and in monkeys given (±)3,4-methylenedioxymethamphetamine. In untreated monkeys, we observed two morphologic classes of serotonergic axon terminals, which differ in regional and laminar distribution. These two axon types, fine and beaded, correspond to the serotonergic axon types which have been described in the rat. In 3,4-methylenedioxymethamphetamine-treated monkeys, there is a profound loss of serotonergic axon terminals, yet some are consistently spared. The surviving axon terminals are nearly all of the beaded type; in contrast, fine serotonergic axons are markedly reduced in density. There are regional differences in the magnitude of denervation, which reflect differences in the distribution of these two types of serotonergic axons in controls.

The present study demonstrates that 3,4-methylenedioxymethamphetamine has differential neurotoxic effects on fine and beaded serotonergic axons. These results indicate that in the primate there are two distinct classes of serotonergic axon terminals, which differ in morphology, distribution, and vulnerability to psychotropic drugs. We hypothesize that in the primate, as demonstrated in the rat, these two classes of serotonergic axon terminals may arise from different raphe nuclei. In both rodent and primate, the dorsal and median raphe nuclei give rise to parallel ascending serotonergic projections, which are likely to have different pharmacologic properties and functions.

The psychotropic amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA) has been illicitly used for recreational purposes by humans and has been advocated for clinical use as an adjunct to psychotherapy. However, concern has arisen that MDMA and other substituted amphetamines may be neurotoxic.[24] In the rat, MDMA causes widespread degeneration of serotonergic (5-HT) axon terminals in the forebrain.[3,21,22,27] Furthermore, two different morphologic types of 5-HT axon terminals which have been identified in the rat brain[14] are differentially vulnerable to 3,4-methylenedioxyamphetamine (MDA) and MDMA.[18,21] The restricted neurotoxic effect of MDMA on one class of 5-HT axons may prove useful for analysing the organization of serotonergic projections.

MDMA is highly neurotoxic in the primate, where preliminary studies indicate that it selectively damages 5-HT neurons.[25,26] The present study is a more detailed anatomic analysis of the neurotoxicity of MDMA in subhuman primates. The primary goal of this study is to determine whether in primate cerebral cortex, as in the rat, there are anatomically distinct classes of 5-HT axons that differ in their vulnerability to MDMA. We have utilized immunocytochemical methods to examine the morphology of 5-HT axons and to characterize the regional pattern of cortical denervation caused by MDMA. A preliminary report of the present study appeared in abstract form.[34]

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Abbreviations: ABC, avidin–biotin–peroxidase complex; 5-HT, serotonin (5-hydroxytryptamine); MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; PBS, phosphate-buffered saline; PCA, *p*-chloroamphetamine; PHA-L, *Phaseolus vulgaris* leukoagglutinin; TH, tyrosine hydroxylase.

EXPERIMENTAL PROCEDURES

Adult macaque monkeys (three *Macaca fascicularis* and one *Macaca mulatta*) received (±)MDMA (5 mg/kg free base, s.c.) every 12 hours for 4 days. A systematic analysis of the behavioral effects of MDMA was not conducted in this study; however, it should be noted that after receiving MDMA, the animals did not appear significantly impaired, and exhibited no signs of distress or discomfort. There was no evidence of the increased body temperature or motor hyperactivity characteristic of the 5-HT syndrome seen in rats given MDMA. After each of the initial injections, the animals appeared to be somewhat calmer than usual for one to two hours, and were less responsive to food or threatening stimuli. Two of the animals moved to the front of the cage, approaching the investigators in an unthreatening manner, quite uncharacteristic of macaques. These acute behavioral effects were attenuated after the third or fourth injection in the series. Two weeks after drug administration was completed, treated monkeys ($n = 4$) or untreated controls ($n = 4$) were given the monoamine oxidase inhibitor

tranylcypromine (10 mg/kg, i.p.) and killed by intracardiac perfusion under deep sodium pentobarbital anesthesia. After the vascular tree was cleared with ice-cold phosphate-buffered saline (PBS), perfusion was continued with buffered 4% paraformaldehyde, pH 6.5, followed by buffered 4% paraformaldehyde and 0.12% glutaraldehyde, pH 9.5. Tissue blocks were placed in buffered 4% paraformaldehyde for seven hours and then in 10% dimethyl sulfoxide in PBS overnight. Frozen sections (30 μm) or Vibratome sections (50 μm) were incubated in rabbit antisera directed against 5-HT, diluted 1:5000 (or in rabbit antityrosine hydroxylase (TH) antisera, diluted 1 unit:48 ml) in PBS with 1% normal goat serum and 0.2% Triton X-100 at 4°C for three days. The antibody was visualized with the avidin–biotin–peroxidase complex (ABC) method[13] (Vector Laboratories, Inc., Burlingame, CA), and staining enhanced with the osmiophilic reaction sequence of C. Gerfen.[10] The boundaries of cytoarchitectural areas and laminae were determined in adjacent sections stained with Cresyl Violet.

Materials

(±)MDMA–HCl (M.W. 229.71) was generously provided by the National Institute of Drug Abuse. Tranylcypromine (*trans*-2-phenylcyclopropylamine) was purchased from Regis Chemical Co., Morton Grove, Il. The rabbit anti-5-HT antisera was generated against 5-HT conjugated to bovine serum albumin with formaldehyde, and has been characterized previously.[15] Rabbit anti-TH antisera was purchased from Eugene Tech, Allendale, N.J.

RESULTS

Two weeks after treatment, MDMA consistently produces a marked reduction in the density of 5-HT immunoreactive axons throughout the cerebral cortex of monkeys (Figs 1, 2). The overall degree of denervation was observed to vary somewhat from animal to animal. The magnitude of axon loss was not determined quantitatively in this study; however, based on a qualitative comparison with controls, we estimate that 60–90 percent of the 5-HT axons are lost in different forebrain regions of MDMA-treated animals. No difference was noted between treated and control monkeys in the density of catecholaminergic axons observed in sections processed for TH immunocytochemistry (Fig. 3). There is no apparent loss of cell bodies or change in 5-HT immunoreactivity in the raphe nuclei, nor are there obvious cytopathologic changes, such as swelling, pyknosis, or chromatolysis. However, due to the fixation requirements for sensitive immunocytochemical staining of 5-HT axon terminals in cerebral cortex, the morphologic preservation in this material is not optimal for detection of subtle cytologic changes in raphe cell bodies.

While many of the 5-HT axons in the MDMA-treated animals are lost, some axons are spared; furthermore, the regional distribution of spared axons is highly consistent from animal to animal. Relatively few 5-HT axons survive in the areas of frontal and parietal cortex examined, whereas more axons remain in several other cortical areas. For example, in somatosensory cortex (Areas 3, 1, and 2), where a very high density of 5-HT axon terminals is found in control animals, few 5-HT axons remain after MDMA treatment (Fig. 1). In other regions, such as striate cortex, the hippocampus, dentate gyrus, entorhinal cortex, and amygdala, despite a moderate denervation, numerous 5-HT axons survive in the treated animals (Figs 4–6).

Surviving 5-HT axons not only have a consistent regional distribution, but also exhibit a characteristic laminar distribution. In frontal and parietal cortex (e.g. dorsolateral prefrontal cortex, primary somatosensory cortex, Figs 2, 1) spared axon terminals are found predominantly in layer I, fewer are found in layer II, and very few, scattered axons remain in deeper layers. A different distribution is found in primary visual cortex (Area 17, Fig. 4), where few axons remain in layers I and II; while some spared axons in this area are found in all of the remaining layers, they are especially numerous in layers IVCα and VA. There is a profound denervation in layers III–IVCα, where the control animals have an extremely high density of fine, highly-arborized axon terminals.

The morphology of 5-HT axons in primate cerebral cortex is heterogeneous. In all regions, there is a small population of large diameter 5-HT axons which lack varicosities; these relatively straight fibers are considered to be preterminal axons and are spared by MDMA (Fig. 7). In addition, finer axons that are varicose and highly arborized are considered to be axon terminals, and can generally be divided into two classes, based on the size and shape of their varicosities: (i) *fine axons*, which have small fusiform or granular varicosities with fine intervaricose segments (Fig. 8), and (ii) *beaded axons*, which have large spherical varicosities separated by intervaricose segments of varying diameter (Fig. 9). Both types of axon terminals are present in all areas of the forebrain examined; however, their regional and laminar distributions differ. In control animals, the majority of 5-HT axons in all regions of cerebral cortex examined are of the fine type.

The present observations indicate that MDMA has differential effects on the two types of axon terminals. In MDMA-treated animals, there is a profound, extensive loss of fine axons in all areas examined.

Fig. 1. Somatosensory cortex (Area 3b, cynomolgus). A high density of 5-HT immunoreactive axons is present in all cortical layers of the control. MDMA produces a marked reduction in 5-HT axons in SI, particularly in the deeper layers. Some 5-HT axons are spared in layer I and the outer part of layer II. This distribution of spared fibers is similar to that observed in most areas of frontal and parietal cortex examined. Serotonin immunocytochemistry, dark-field photomicrograph, bar = 100 μm.

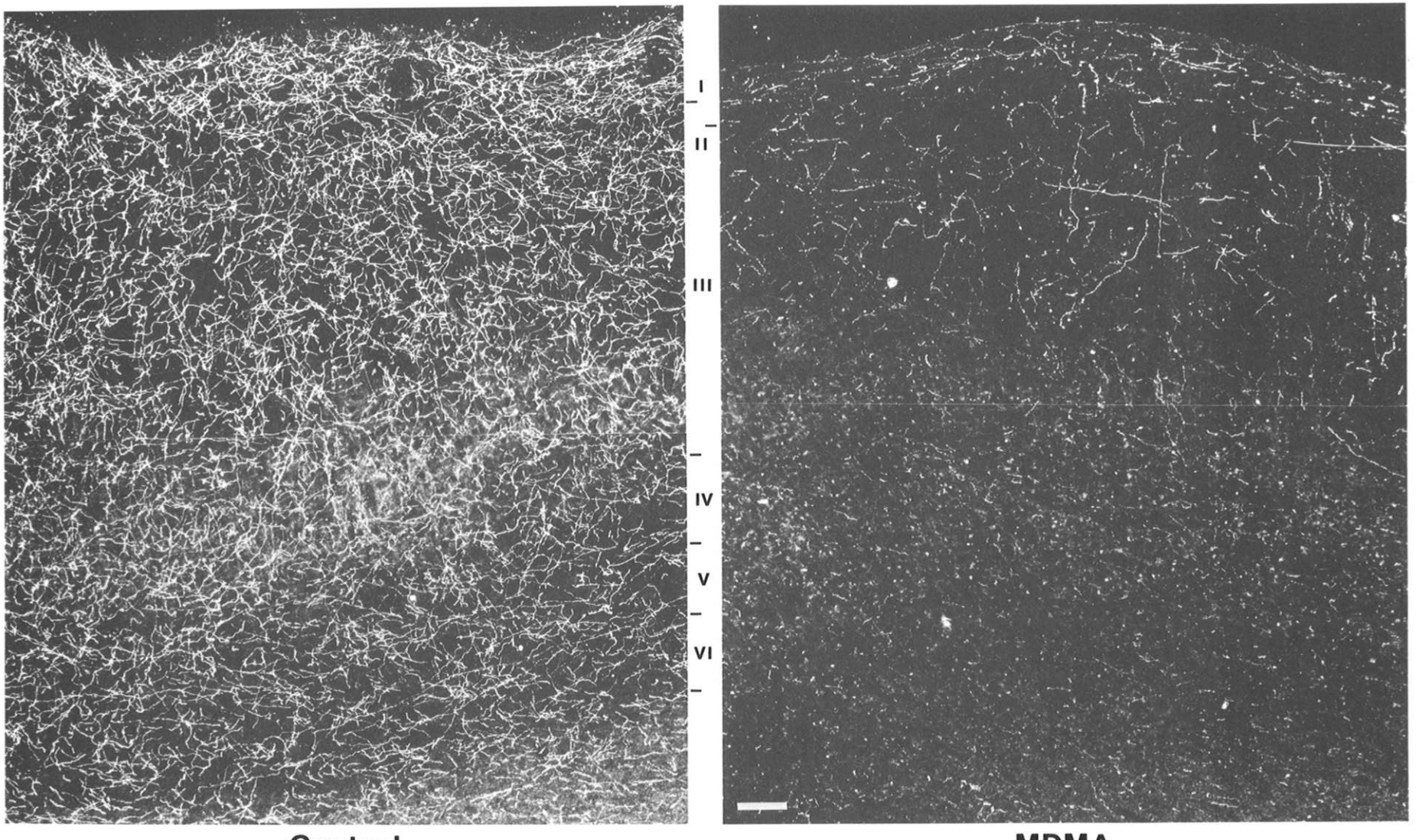


Fig. 1

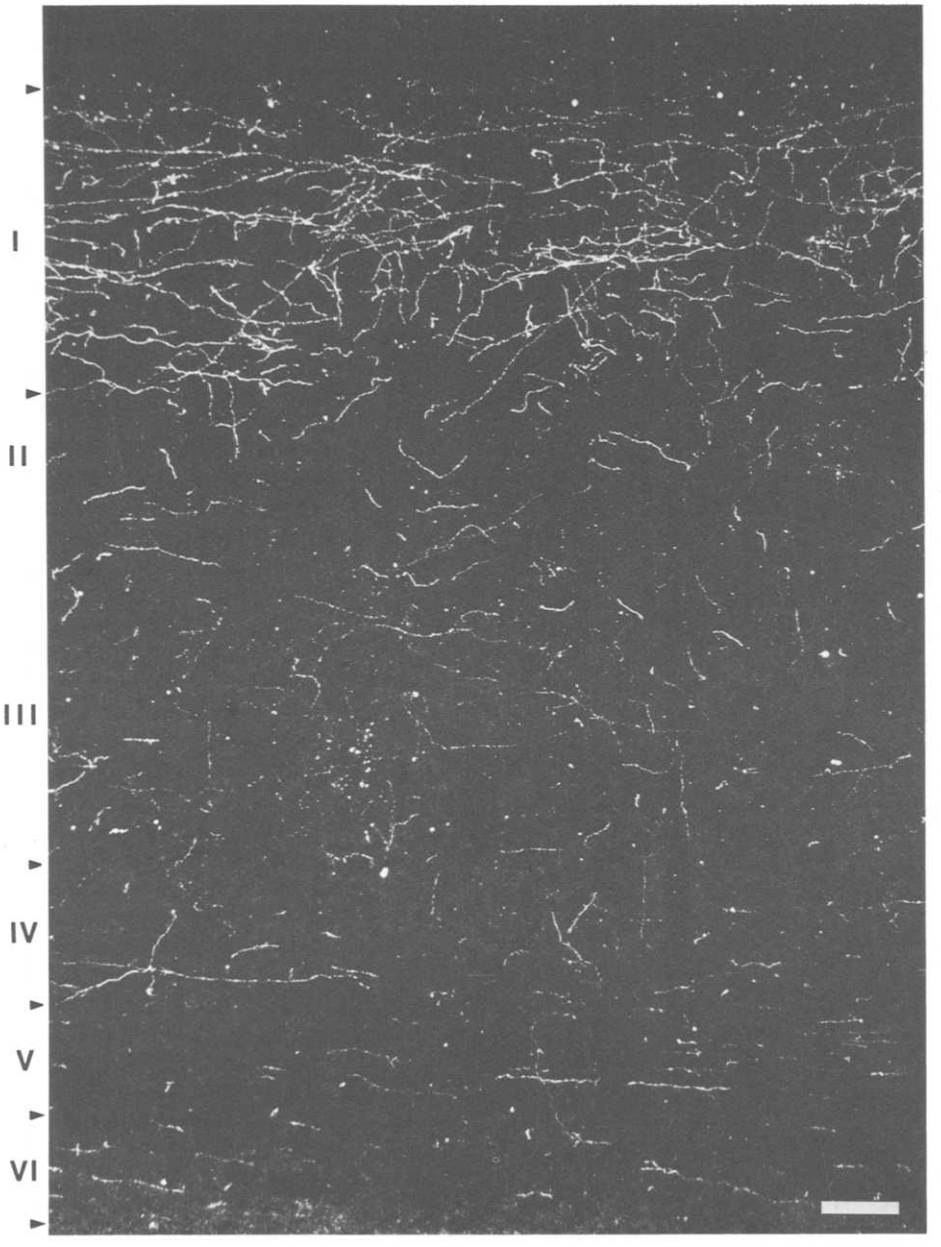


Fig. 2. Dorsolateral prefrontal cortex, MDMA-treated monkey (ventral bank of principal sulcus, Area 46, cynomolgus). The density of 5-HT axons in controls (not shown) is somewhat less than that present in SI. A striking denervation is observed in prefrontal cortex of MDMA-treated monkeys. Spared 5-HT fibers in this area are found predominantly in layer I, a distribution which is similar to that observed in SI. Bar = 100 μm.

Nearly all of the surviving axons are of the beaded type, with large spherical varicosities. A small proportion of the axons which survive are morphologically abnormal, having markedly swollen varicosities which contain lightly-stained, irregular patches (Fig. 11). However, the morphology of most of the surviving axons (Fig. 10) is indistinguishable from that of beaded axons in control animals. Moreover, the regional and laminar distribution of spared axon terminals in MDMA-treated animals described above corresponds to the distribution of beaded axons in untreated animals. This correspondence between the distribution of spared axons in treated animals and the distribution of beaded axons in controls is observed in all areas examined, and is particularly evident in visual cortex (layer IVCα), frontal and parietal cortex (layer I), and the hippocampus (molecular layer). In those locations where fine axons predominate in controls, very few axons are spared in MDMA-treated animals. Thus, a comparison of treated and control animals with respect to the morphology and the distribution of 5-HT axons indicates that MDMA preferentially ablates fine 5-HT axon terminals.

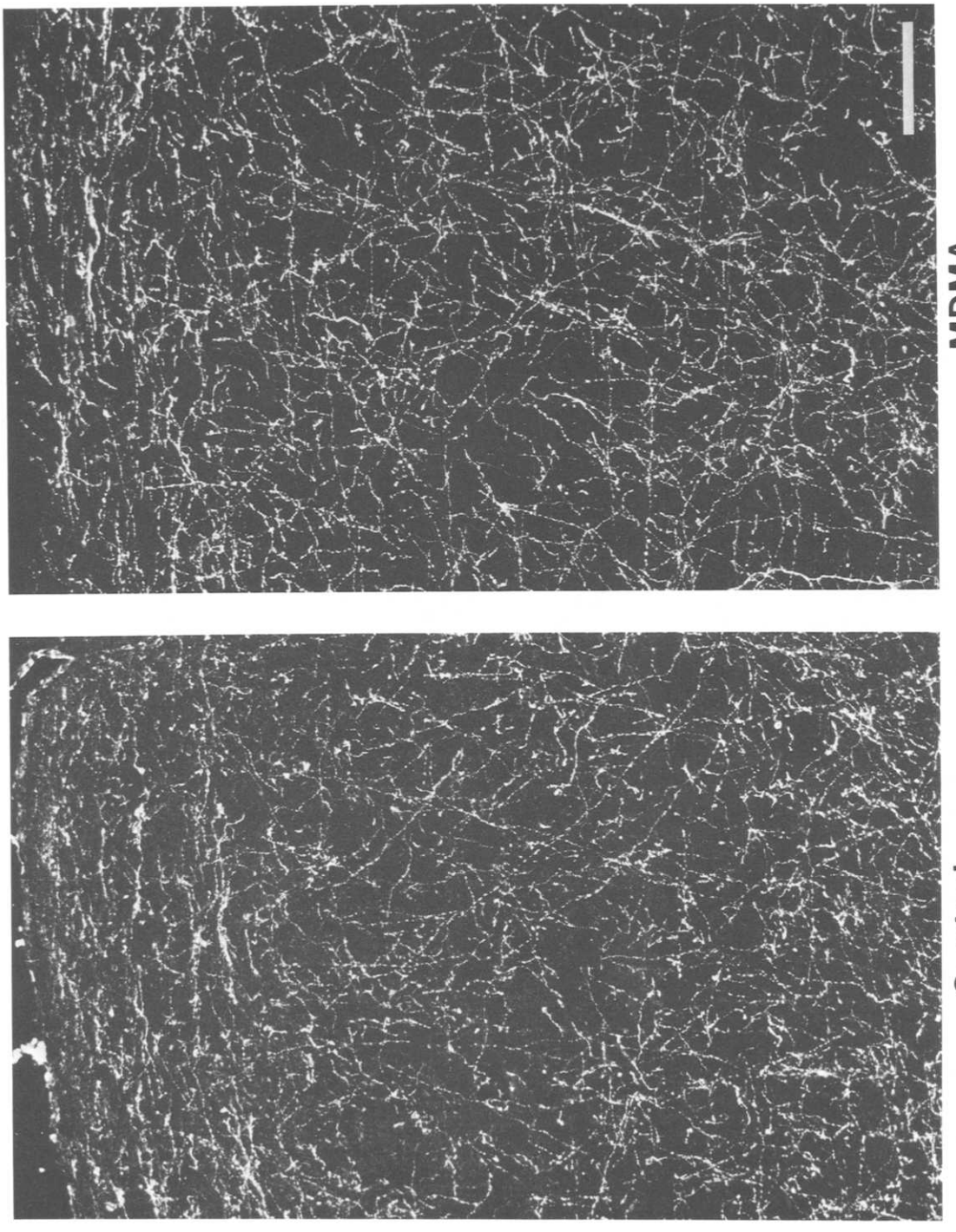


Fig. 3. Tyrosine hydroxylase immunoreactive axons (motor cortex, cynomolgus). The density of catecholaminergic axons in MDMA-treated monkeys does not differ from that in controls. Since both dopaminergic and noradrenergic axons contain TH, both kinds of catecholaminergic axons are visualized with this antiserum. The sparing of catecholaminergic axons provides evidence for a specific neurotoxic action of MDMA upon 5-HT axons. Bar = 100 μm.

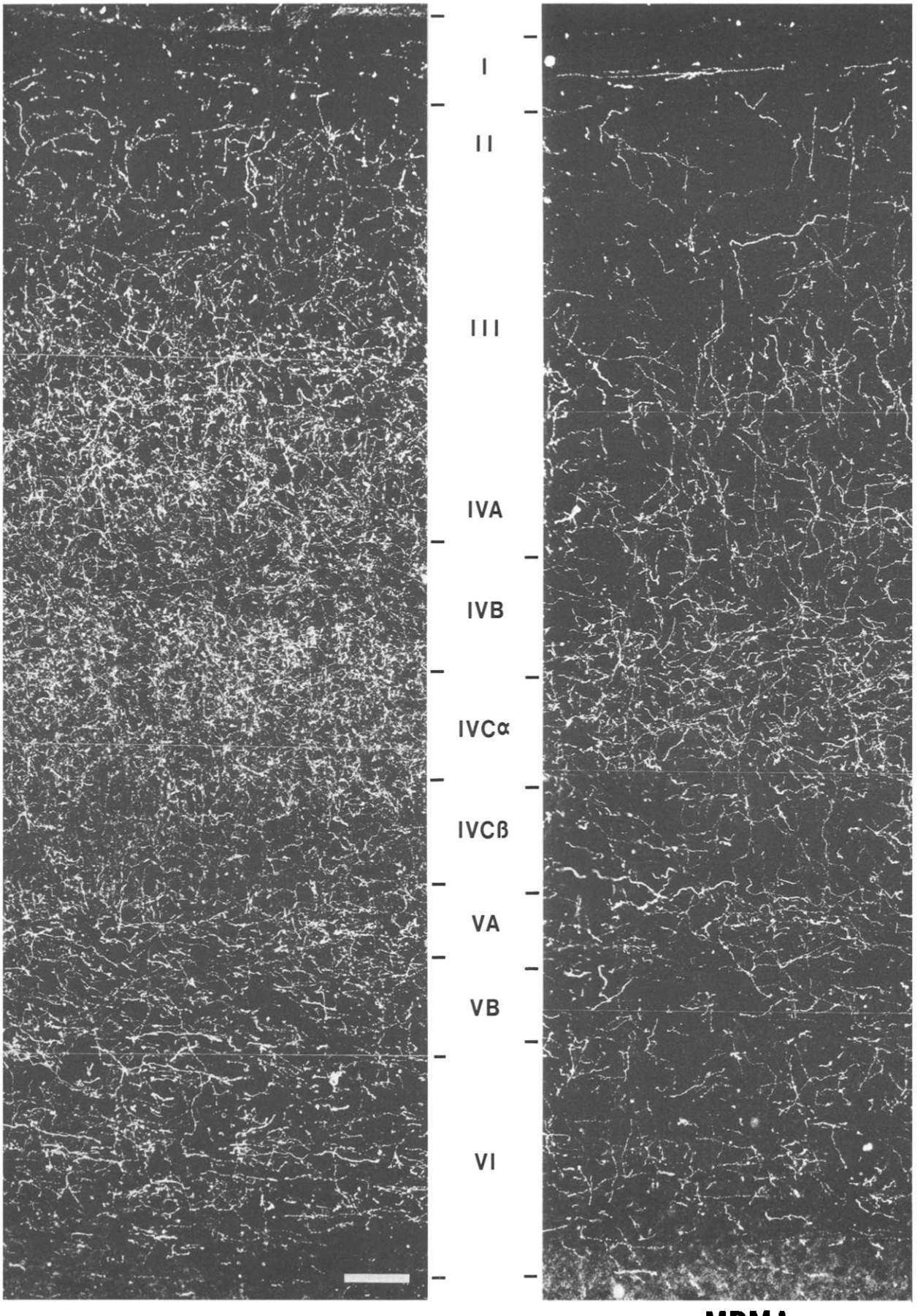


Fig. 4

DISCUSSION

Neurotoxicity of 3,4-methylenedioxymethamphetamine

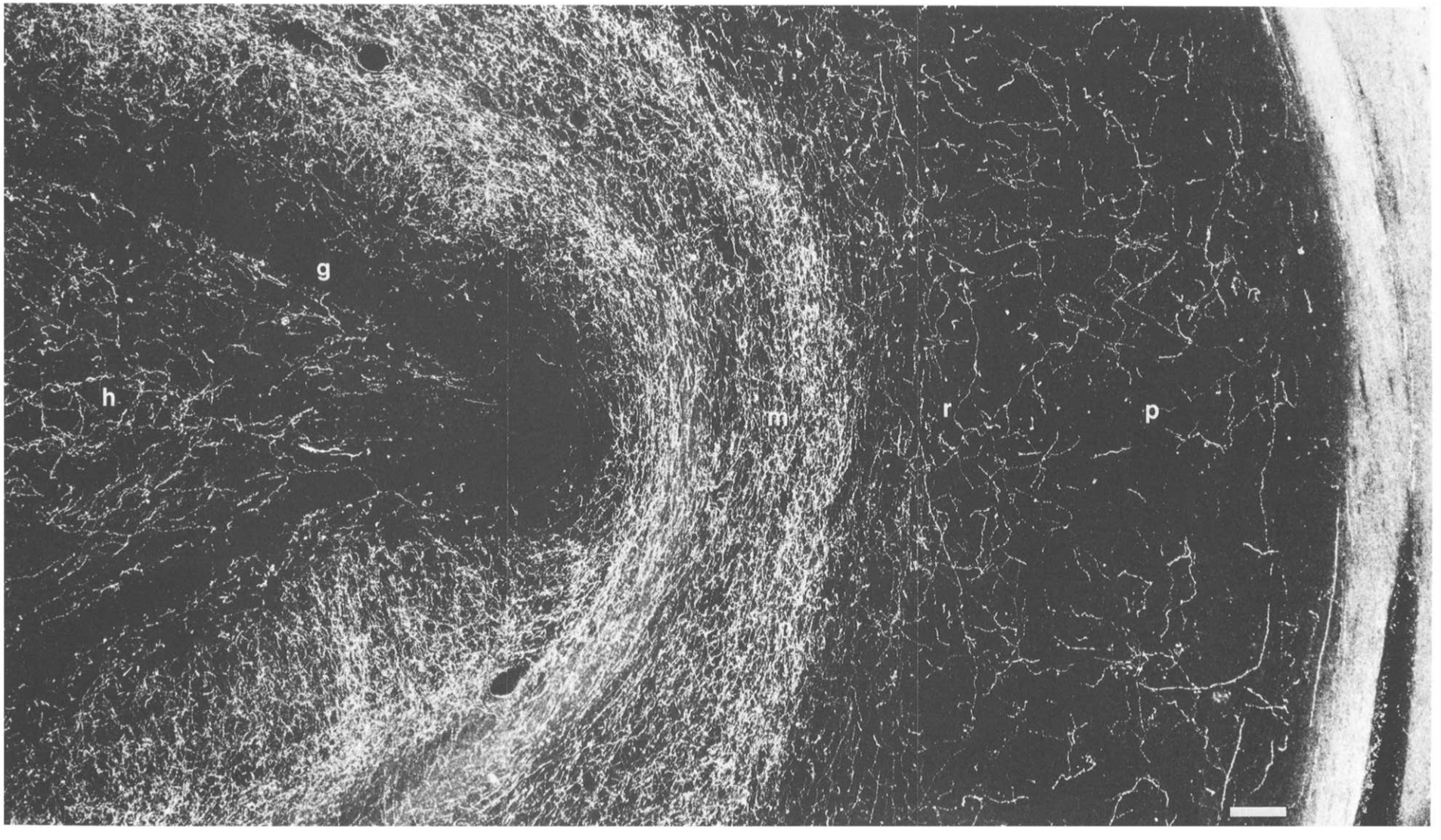
These findings extend a previous report that in primates,[25,26] as in the rat,[21,22] MDMA produces widespread, selective loss of 5-HT axon terminals in the forebrain. Ricaurte *et al.*[26] report that two weeks after the same regimen of MDMA as that used in the present experiments, neocortical levels of 5-HT were reduced by 90% in squirrel monkeys. In the present study, immunocytochemical methods reveal a reduction of comparable magnitude in 5-HT axon terminal density in MDMA-treated macaque monkeys. These data indicate that 5-HT axon terminals in primates are highly vulnerable to the neurotoxic effects of MDMA, while 5-HT cell bodies and preterminal axons appear to be spared. The loss of 5-HT axons in monkeys is greater than that observed in rats given a four-fold higher dose of MDMA and is comparable to that obtained in rats given high doses of MDA.[22] The latter substituted amphetamine, the *N*-desmethyl derivative of MDMA, is more neurotoxic than MDMA in the rat.[22,29,30] Thus, the neurotoxic potency of MDMA in the monkey appears to simulate that of MDA in the rat. With regard to selectivity of drug action, the neurotoxic effect of MDMA appears to be specific for 5-HT axons, since we did not observe any evidence of damage to catecholaminergic axons in sections processed for TH immunocytochemistry. Thus, as reported previously in rats[3,21,22,30] and in primates,[25,26] MDMA does not damage other (i.e. non-serotonergic) monoaminergic neurons.

The loss of 5-HT immunoreactive axons observed in MDMA-treated monkeys is likely to reflect degeneration of 5-HT axon terminals. Alternatively, the terminals may be intact but undetected due to neurotransmitter depletion. Several lines of evidence support the former interpretation. (i) Serotonin immunoreactivity is present (and appears to be enhanced) in cell bodies of the dorsal and median raphe nuclei and in preterminal axons, indicating that synthesis and storage of 5-HT is intact in those compartments. (ii) The time-course of changes in 5-HT markers in the forebrain has been studied in rats given a single dose of MDMA.[27] An initial, transient depletion of 5-HT is followed by a gradual loss of both the neurotransmitter and its uptake sites over the following 1–2 weeks. The loss of uptake sites accompanied by reduced neurotransmitter levels is indicative of axon terminal damage.[3,22] Thus, the response to MDMA is biphasic, with a rapid, transient depletion of 5-HT followed by axon degeneration. In the present study, the loss of 5-HT immunoreactive fibers persisting two weeks after treatment is likely to reflect axon terminal damage, rather than depletion. (iii) Two different methods have been utilized to demonstrate damage to 5-HT axon terminals in rats treated with MDA or MDMA: the Fink–Heimer method was used by Ricaurte *et al.*[24] to stain degenerating axon terminals, which were associated with reduced 5-HT levels. However, silver-staining methods do not permit the direct identification of the neurotransmitter contained in degenerating terminals. Immunocytochemical methods used by O'Hearn *et al.*[21,22] demonstrated swollen, fragmented 5-HT immunoreactive axons in MDMA-treated rats. In the present study, similar abnormalities of 5-HT immunoreactive axons were observed in MDMA-treated monkeys. Therefore, we conclude that 5-HT axon terminals in MDMA-treated monkeys, like those in the rat, suffer significant structural damage and degenerate.

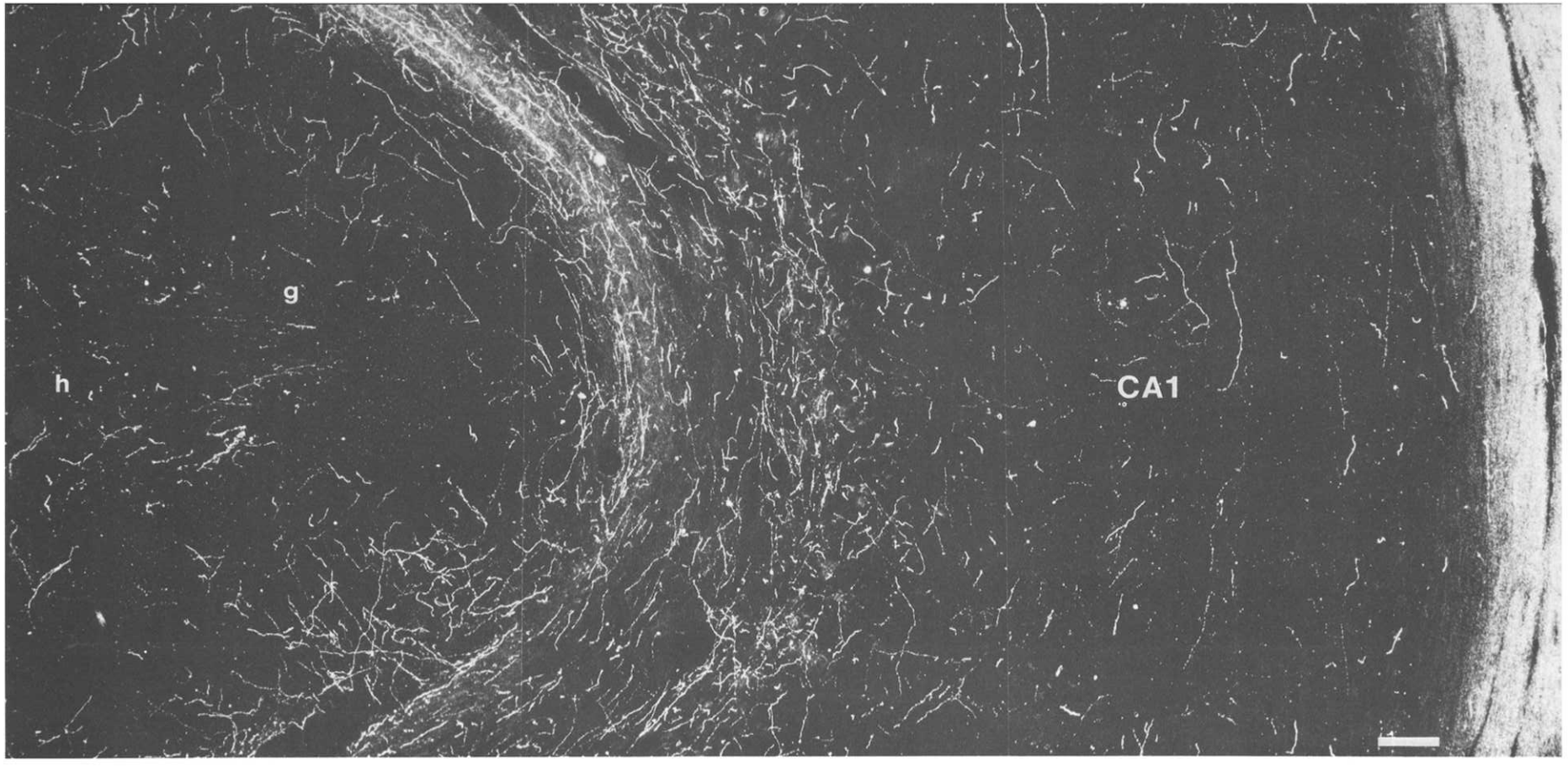
Morphology of serotonergic axon terminals

Previous studies in cerebral cortex of the rat reveal two classes of 5-HT axon terminals which differ in (i) morphology, (ii) distribution, (iii) nucleus of origin, and (iv) sensitivity to substituted amphetamine neurotoxins such as MDA, MDMA, and *p*-chloroamphetamine (PCA).[14,16,18,21,22] Anterograde transport of *Phaseolus vulgaris* leukoagglutinin (PHA-L) was combined with 5-HT immunocytochemistry to study raphe–cortical projections in the rat.[14] This method demonstrated two classes of ascending 5-HT axons which have different origins within the raphe nuclei: (i) fine axons with small pleomorphic varicosities, which arise from the dorsal raphe nucleus, and (ii) axons of varying diameter with large spherical varicosities, which arise from the median raphe nucleus. One objective of the present study was to determine whether multiple classes of 5-HT axon terminals can be differentiated in primates, as in rodents. We report here that, while the morphology of 5-HT axon terminals in the primate is somewhat more heterogeneous than in the rat, we can distinguish two morphologic classes of axons, fine and beaded, which differ in regional and laminar distribution. The regional distribution of the two axon types is similar to that reported in the rat, particularly in the predominance of beaded axons in certain regions of limbic cortex, such as the hippocampus, dentate gyrus, entorhinal cortex, and amygdala. The generality of these subsets of 5-HT axon terminals is further supported by a recent report

Fig. 4. Visual cortex (Area 17, rhesus). Numerous 5-HT axons in Area 17 survive MDMA treatment. At higher magnification, one can observe that spared fibers are predominantly of the beaded type (cf. Fig. 9), whereas most fine axons are absent. The spared axons are particularly evident in layers IVCα and VA. The laminar distribution of spared fibers in this area differs from that in frontal and parietal neocortex; however, it corresponds to the distribution of beaded 5-HT axon terminals in Area 17 of control monkeys. Bar = 100 μm.



Control



MDMA

Fig. 5. Hippocampus (CA1) and dentate gyrus. In the hippocampal formation, a striking laminar pattern of 5-HT innervation is evident in normal cynomolgus monkeys (cf. control). At higher power (not shown), one can observe the distribution of fine and beaded axons in different laminae. In CA1, a high density of both fine and beaded 5-HT axons is present in the molecular layer (m), increasing to a higher density of both axon types in the stratum lacunosum. The stratum radiatum (r) receives a moderate density of fine 5-HT axon terminals, with occasional clusters of beaded axons. A low density of fine axon terminals is present in the stratum pyramidale (p), with rare clusters of beaded axon terminals. The stratum oriens receives a low density of fine and beaded axons. In the dentate gyrus, a very high density of both fine and beaded 5-HT axon terminals is present in the outer half of the molecular layer, decreasing to a low density in the inner half of the molecular layer. Only rare 5-HT axons are observed traversing the granule cell layer (g). In the polymorphic layer of the dentate hilus (h), a moderate density of 5-HT axons is present; these axons are predominantly beaded, with some fine axons. The density of fine axons increases near the center of the hilus. In MDMA-treated monkeys, a substantial denervation is evident. Spared axons are particularly evident in the molecular layer of both the hippocampus and dentate gyrus and in the stratum lacunosum of CA1. These axons are nearly all of the beaded type, and their laminar distribution corresponds to that of beaded axons in controls. Bar = 100 μm.

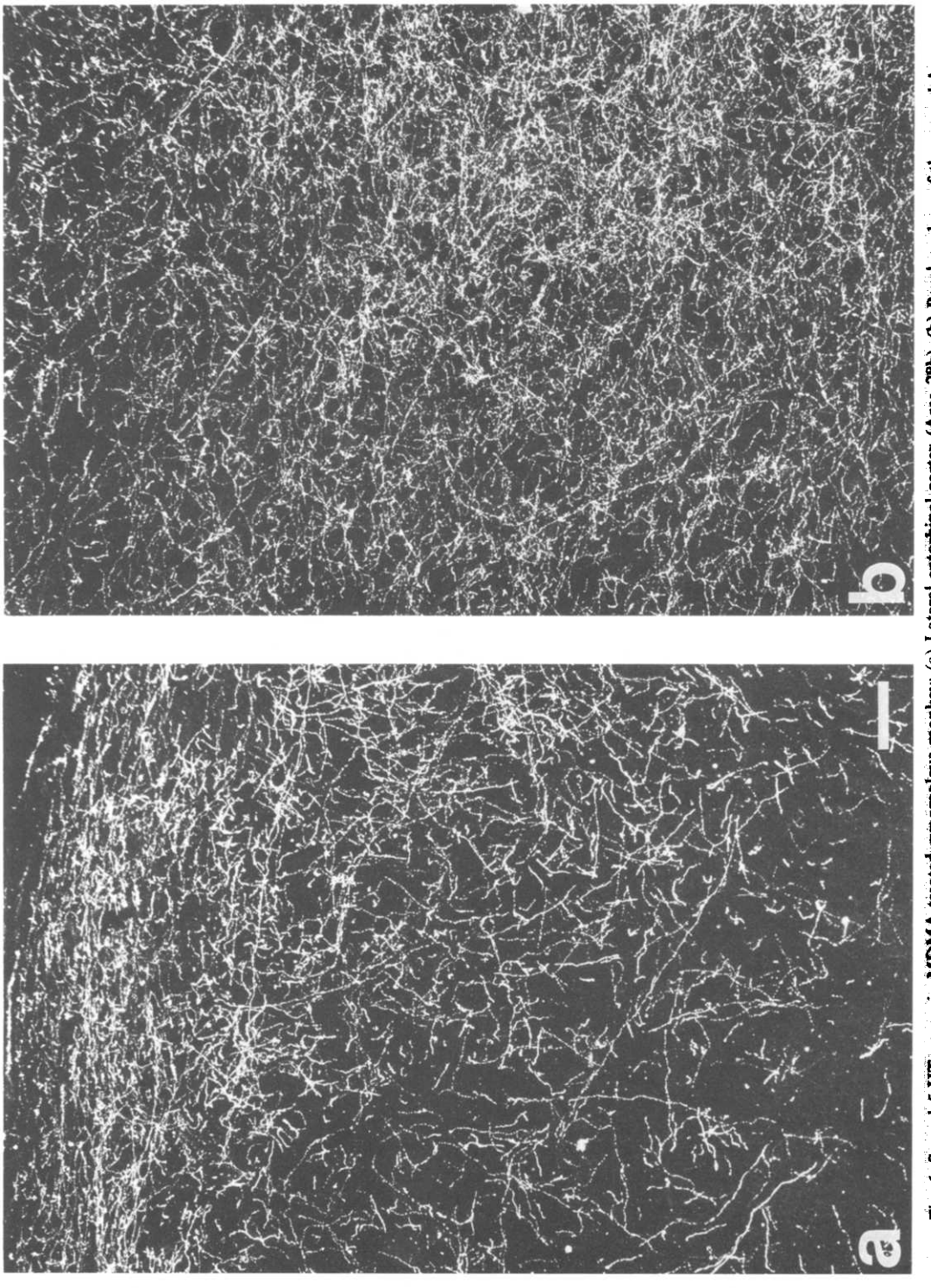


Fig. 6. Spared 5-HT axons in MDMA-treated cynomolgus monkey: (a) **Lateral entorhinal cortex (Area 28b), (b) Basal nucleus of the amygdala (magnocellular division). Despite a moderate denervation, many 5-HT axon terminals survive in these two areas. Spared 5-HT axons in entorhinal cortex are found predominantly in the supragranular layers. In the amygdala, a high density of spared axons is found in the magnocellular division of the basal nucleus. In control animals (not shown) a high density of beaded axons is found in these areas, interspersed with fine axons. Bar = 100 μm.**

of two morphologically-distinct classes of 5-HT axon terminals in cats. Mulligan and Tork[19] observed "large varicose axons" which form pericellular "baskets" in the supragranular layers, predominantly layer I; these are similar in morphology and laminar distribution to the beaded, drug-resistant axons we have observed in the primate. The frequency with which the beaded axons in primates form pericellular baskets is uncertain, since we have observed only rare examples of beaded axons surrounding the soma of neurons (cf. Fig. 9).

Differential effects of 3,4-methylenedioxymethamphetamine

O'Hearn and colleagues[21,22] report that MDA and MDMA selectively ablate fine 5-HT axon terminals in the rat; our observations indicate that, at the dose used in this study, MDMA preferentially ablates fine 5-HT axon terminals in the monkey, as well. In both species, beaded 5-HT axon terminals are spared, and apparently undamaged. In order to determine whether the large varicosities of the spared axons are normal or are dilatations induced by MDMA, the morphology and distribution of 5-HT axons were compared in treated and control animals. As noted above, a small proportion of the axons remaining after MDMA treatment are abnormally swollen. However, the majority of the spared 5-HT axon terminals are not morphologically abnormal, rather, they are identical in appearance to beaded axons in controls. Moreover, we observed a consistent regional and laminar pattern in the distribution of spared axons; this pattern corresponds to the distribution of beaded axons in control monkeys. These observations indicate that beaded axon terminals are resistant to the toxic effects of MDMA, leading us to conclude that the two classes of 5-HT axon terminals in the primate, which differ in morphology and distribution, are differentially vulnerable to MDMA. Since only one dose regimen was employed in this study, a dose–response series is needed to determine whether the differential susceptibility of fine and beaded axons is dose-related. At higher doses of MDMA, beaded axons may degenerate.

The mechanism of MDMA neurotoxicity is not known, making it difficult to predict what structural

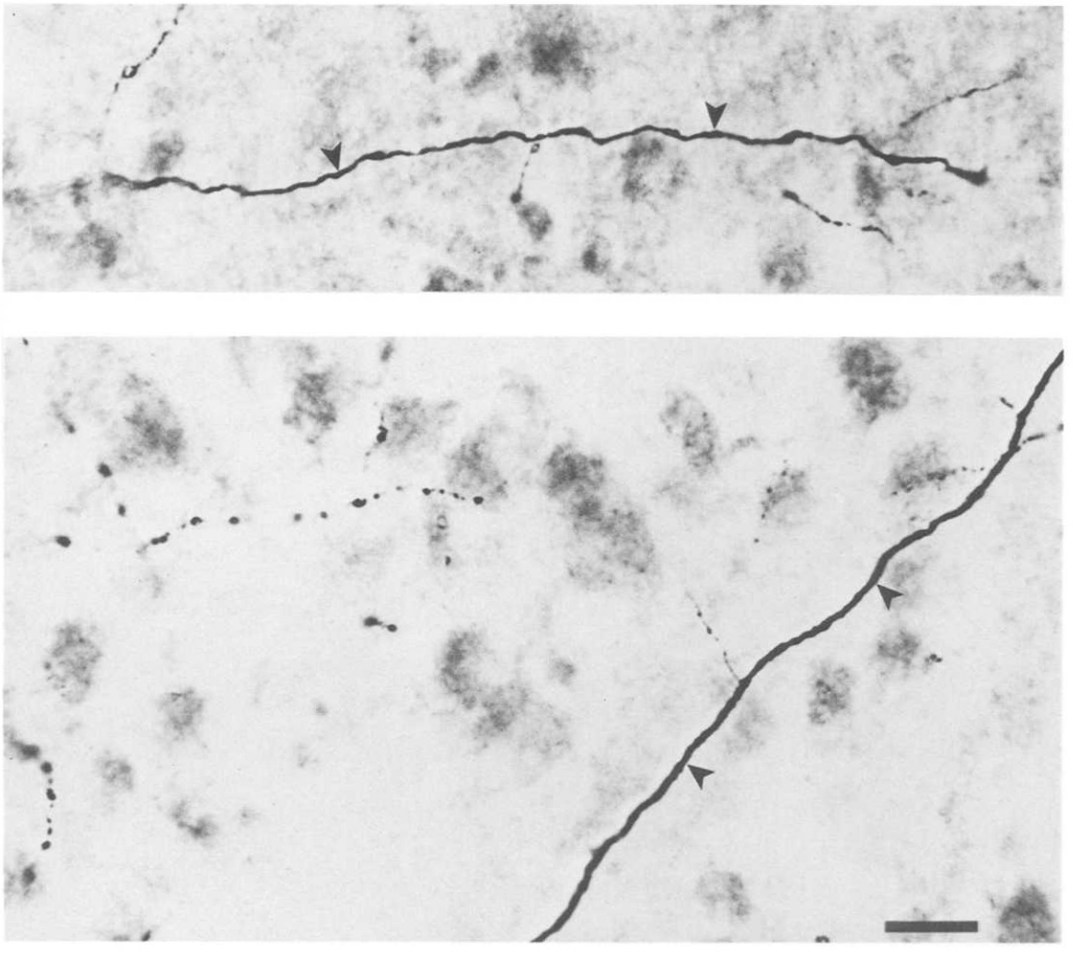


Fig. 7. Preterminal 5-HT axons, MDMA-treated monkeys. Large diameter axons which lack varicosities (arrowheads) are considered to be preterminal. These axons are spared by MDMA. Bright-field photomicrograph, bar = 10 μm.

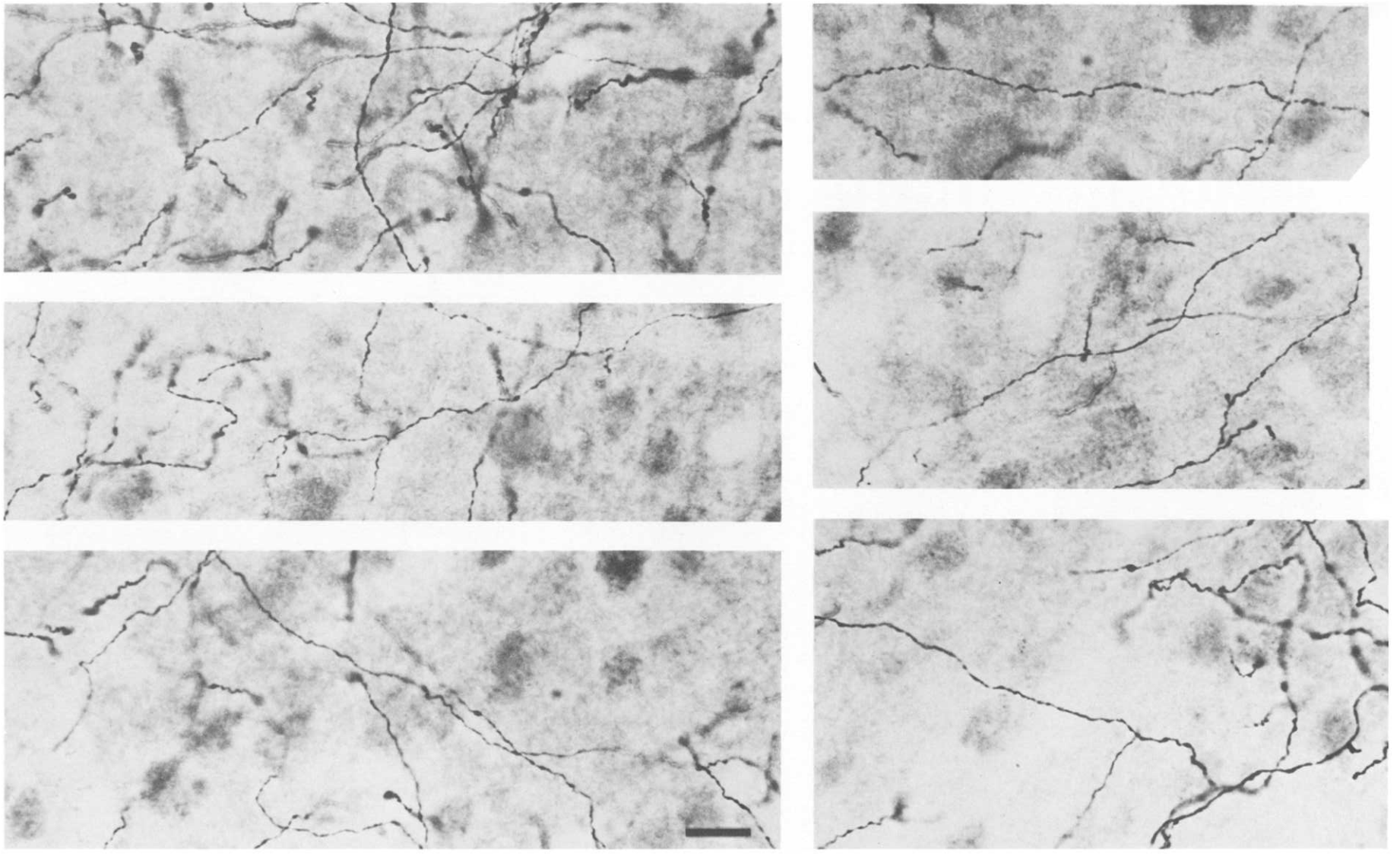


Fig. 8. Fine 5-HT axons, control monkeys. Fine axon terminals have small pleomorphic varicosities. Bar = 10 μm.

or metabolic differences may account for the differential vulnerability of fine and beaded axon terminals. However, fluoxetine and citalopram, which block the 5-HT uptake carrier, prevent the neurotoxic effect of MDMA or PCA, if administered simultaneously with, or several hours after, these amphetamine derivatives.[4,7 9,27] These studies indicate that MDMA neurotoxicity may be dependent upon binding to the 5-HT uptake carrier. The two axon types may have 5-HT uptake carriers which differ in affinity for the neurotoxin, or the axons may differ in the density or distribution of uptake sites. Alternatively, the differential effect of MDMA on fine and beaded axons may be due to differences in 5-HT content, in rates of activation or breakdown of the neurotoxin, or structural differences such as surface area to volume ratios, or extent of axonal arborization.

Origin of fine and beaded serotonergic axons

In the rat, fine and beaded 5-HT axon terminals have been shown to originate from different nuclei, the dorsal and median raphe.[14,16] Based on the anatomic and pharmacologic similarities of 5-HT axons primate and in rat, we propose that the fine 5-HT terminals in primate forebrain may arise from the dorsal raphe nucleus, while the beaded 5-HT terminals may arise from the median raphe nucleus. This possibility is consistent with previous reports showing a predominant median raphe projection to primate visual cortex[31,32] and hippocampus,[1] areas where we observe a high density of beaded axons. Moreover, more neurons in the dorsal raphe than in the median raphe project to primate prefrontal cortex,[23] where we see a predominance of fine 5-HT axons. Transport experiments will be needed to investigate the origin of the two 5-HT axon types in primates. MDMA, which provides a means of selectively ablating fine 5-HT axons, will be a helpful tool in such studies.

CONCLUSIONS

The present results indicate that in the primate, as in the rat, there are two distinct 5-HT projections which exhibit differential vulnerability to psychotropic drugs, and differ in axon morphology and in regional and laminar distribution. We propose that there may be two parallel ascending serotonergic projections with separate origins, subserving different functions. In addition, the selective neurotoxic effect of MDMA on fine 5-HT axon terminals indicates one of the sites of action of this mood-altering drug, lending support to the hypothesis that 5-HT projections are involved in the control of affective state.[2,6,17,28,33]

Note: Drugs like MDMA have been suggested to be of therapeutic value in psychiatry.[11,12,20,35] While the experimental approach utilized in the present study may be of value in screening such compounds for neurotoxicity, the present study was not designed to evaluate the toxic potential of MDMA in humans. The dose of MDMA administered subcutaneously to monkeys in this study (5 mg/kg) is approximately twice that commonly used orally for recreational purposes by humans,[5,11] and our multiple dose administration schedule may not be comparable to occasional human use. Nevertheless, the extensive destruction of 5-HT axon terminals observed in monkeys given this drug indicates that MDMA should be considered potentially hazardous.

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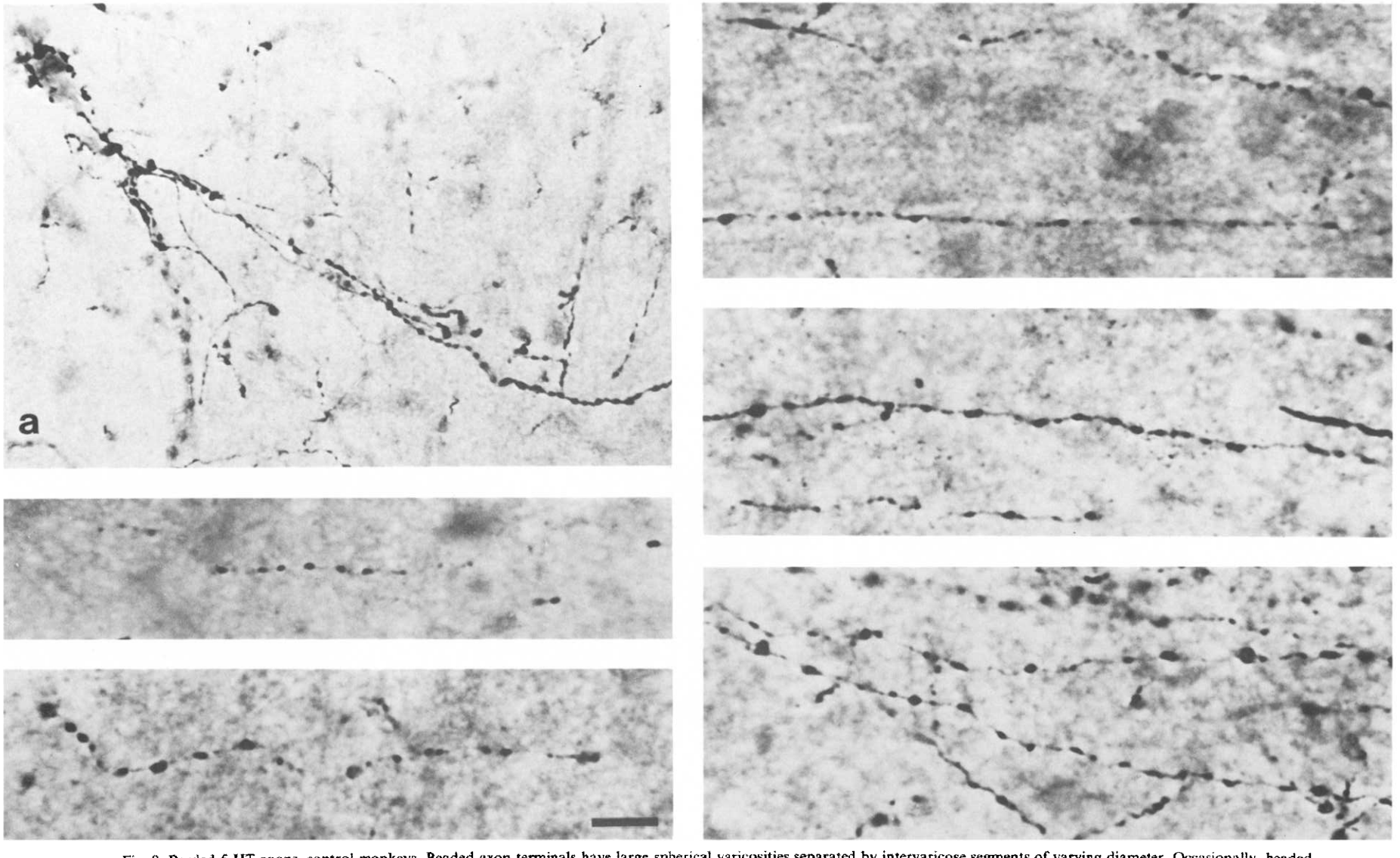

Fig. 9. Beaded 5-HT axons. control monkeys. Beaded axon terminals have large spherical varicosities separated by intervaricose segments of varying diameter. Occasionally, beaded 5-HT axons appear to form a basket[19] surrounding the soma and proximal dendrites of a neuron (a). Bar = 10 μm.

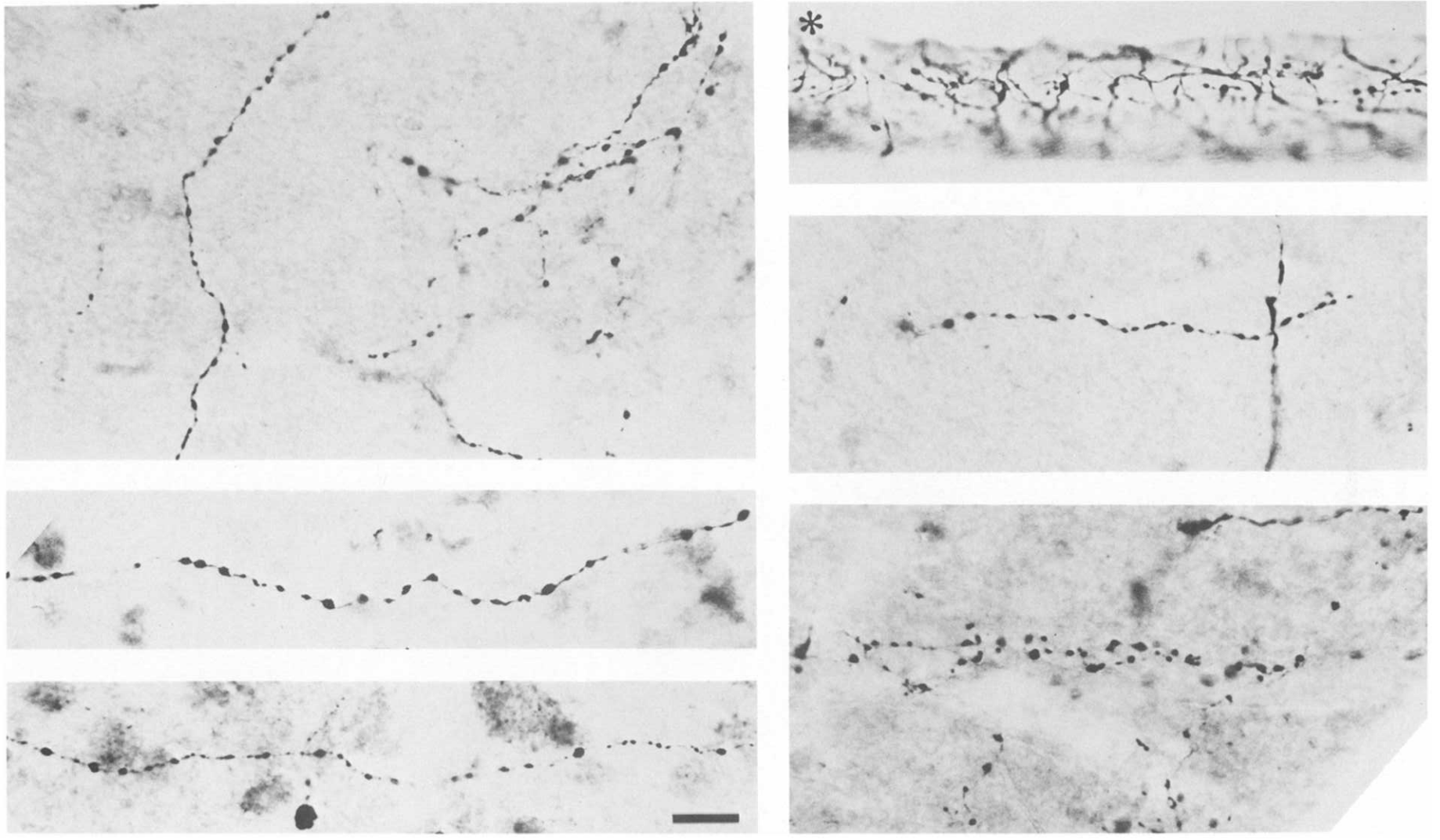

Fig. 10. 5-HT axon terminals spared by MDMA. Most of the spared axons in MDMA-treated monkeys have large spherical varicosities and appear identical to beaded axons in controls (cf. Fig. 9). In the ventricular plexus (*), beaded axons similar to those observed in cerebral cortex form a dense network spared by MDMA. Bar = 10 μm.

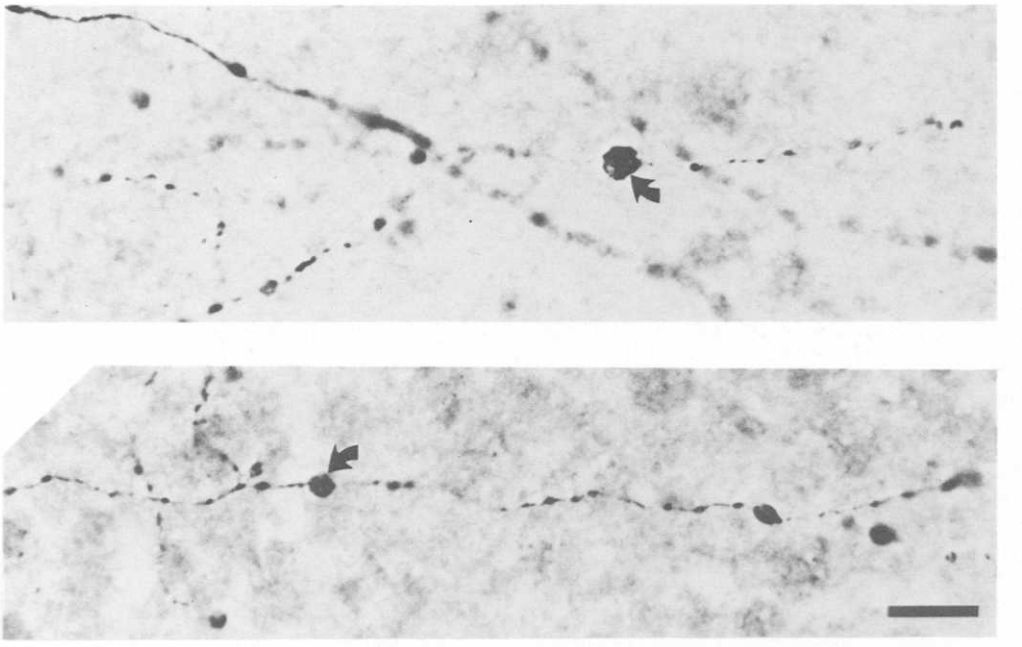

Fig. 11. Damaged 5-HT axons in MDMA-treated monkeys. A small portion of the surviving axons are morphologically abnormal, having extremely swollen varicosities (arrows). Bar = 10 μm.

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