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Light and electron microscopic immunocytochemical analysis of the noradrenaline innervation of the rat visual cortex

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Summary

Immunocytochemistry with an antiserum against noradrenaline was used to examine the organization and morphology of noradrenergic axons in the rat visual cortex. Observations with the light microscope confirmed earlier reports concerning the distribution pattern of noradrenergic fibres, and provided some further clues about their intracortical organization. Particularly striking was the finding of fibres which followed an oscillating course within the boundaries of layers II–IV as they ran in the mediolateral direction. Examination of the morphological characteristics of noradrenaline-containing axon terminals in serial ultrathin sections has provided further evidence that the vast majority (87.6%) form conventional synapses in the visual and frontoparietal cortex, and has given clues about the postsynaptic elements involved in these synaptic contacts; they are, in decreasing frequency, spines, dendritic shafts of various diameters, and pyramidal and non-pyramidal somata. In addition, a few labelled terminals were visualized in close association with intracerebral capillaries.

Introduction

The noradrenergic afferents of the rat cerebral cortex, deriving exclusively from a relatively small number of neurons in the locus coeruleus, have been studied extensively by a variety of methods (see Molliver *et al.*, 1982; Parnavelas & McDonald, 1983; Levitt *et al.*, 1984b; Lindvall & Björklund, 1984 for reviews). The original Falck–Hillarp method provided the first detailed, although incomplete, description of the noradrenergic innervation pattern of the rat cerebral cortex (Fuxe *et al.*, 1968). The introduction of more sensitive methods, such as glyoxylic acid fluorescence histochemistry and dopamine-β-hydroxylase (DBH) immunofluorescence, significantly improved the originally described picture and revealed that noradrenaline (NA)-containing afferents form a rich network throughout all cortical layers (Levitt & Moore, 1978; Morrison *et al.*, 1978). In addition, the presence of a fairly consistent pattern of NA innervation in all regions of the rat neocortex and the elucidation of some principles concerning the trajectory and distribution of the intracortical NA axons (Morrison *et al.*, 1979, 1981) suggested that the NA cortical system is an organized, although widely projecting, transmitter specific system. Studies in higher mammals, which revealed greater regional and laminar variations in the density of NA axons compared to the rather uniform innervation of the less differentiated rodent neocortex, also favour this notion (Morrison *et al.*, 1982; Levitt *et al.*, 1984a).

The mode of termination of the NA-labelled axons has been examined at the ultrastructural level. Descarries and coworkers, using high-resolution radioautography following topical application of [^{3}H]NA onto the rat frontoparietal cortex, reported that NA-containing axon terminals rarely formed synaptic junctions (Descarries & Lapierre, 1973; Descarries *et al.*, 1977; Beaudet & Descarries, 1978). These observations, which were later supported by studies in the kitten visual cortex (Itakura *et al.*, 1981) and the turtle dorsal cortex (Ouimet *et al.*, 1981), led Descarries and colleagues to propose that NA released from terminals diffuses beyond adjacent postsynaptic elements to exert its influence on a large number of neurons. However, the observations of other investigators have challenged this hypothesis. Use of the false neurotransmitter 5-hydroxydopamine in the newborn rat (Molliver & Kristt, 1975; Zecevic & Molliver, 1978), DBH-immunohistochemistry (Olschowka *et al.*, 1981)

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and radioautography following topical and intraventricular application of [^{3}H]NA (Parnavelas *et al.*, 1985) have demonstrated the frequent incidence of noradrenergic terminals with specialized junctional appositions. The recent development of a specific antiserum against NA has prompted us to study immunocytochemically the precise organization and ultrastructural features of the NA afferents in the rat visual cortex.

Materials and methods

Twelve albino rats (eight Sprague–Dawley and four Wistar of approximately 200–250 g) of both sexes were used in this study. Animal and tissue preparation and immunocytochemical procedures applied were similar to those described previously (Papadopoulos *et al.*, 1987b). Briefly, animals were perfused under ether anaesthesia with a fixative solution containing 5% glutaraldehyde and 1% sodium metabisulphite in 0.05 M cacodylate buffer at pH 7.5. Coronal and parasagittal sections through the visual cortex (area 17) (Krieg, 1946) were cut with a Vibroslice at 50–60 μm and collected in 0.05 M Tris buffer saline containing 1% sodium metabisulphite, pH 7.5. They were then placed in sodium borohydride for 10 min to reduce the double bonds formed by glutaraldehyde with the amine and tissue protein. After several washes in Tris buffer saline, sections were processed with the peroxidase–antiperoxidase immunocytochemical technique using an antiserum directed against NA. Sections for electron microscopy were derived from four animals.

The preparation and characterization of the NA-antiserum (A46), which was used in a dilution of 1:2500, has been described elsewhere (Geffard *et al.*, 1986). Preabsorption of this antiserum with 10^{-5}M synthetic NA or omission of the link antiserum from the immunocytochemical procedure eliminated all positive staining in the visual cortex. However, occasionally a faintly stained cell body could be seen in the substantia nigra following preabsorption with synthetic NA, suggesting that the antiserum cross-reacts slightly with dopamine.

Results

Light microscopy

Immunocytochemical staining of the rat visual cortex with the NA antiserum revealed a dense plexus of NA-positive axons in all cortical layers (Fig. 1). Immunoreactive fibres were typically thin and varicose in appearance; their medium-sized varicosities were irregularly spaced along the fibres. Occasionally, a thicker fibre could be seen running either tangentially within layer I (Fig. 2, arrowheads) or radially across the other cortical layers.

The laminar distribution of noradrenergic afferents was studied in sections counterstained with Cresyl Violet after immunostaining. Their density and orientation within individual layers were similar to those previously described by using other methods (Levitt & Moore, 1978; Morrison *et al.*, 1978). Briefly, while tangential fibres running parallel to the pia predominated in layers I and VI (Figs 2, 3), relatively straight radial fibres traversed layers II and III (Fig. 1). Layer IV, which also contained a few radial axons, and particularly layer V, were characterized by short, tortuous fibres. Comparing the distribution pattern of NA axons in coronal and parasagittal sections, no differences could be observed, except that while long tangential fibres in layer I remained relatively constant in number irrespective of the plane of section, those present in layer VI were fewer in coronal sections.

Attempts to establish the continuity of individual fibres across cortical layers were mostly unsuccessful due to the high density of axons present at all levels of the section thickness. This was the case particularly for fibres running in the heavily innervated layer I and for the short tortuous axonal segments in layer V. However, careful examination of the radial axons traversing layers II and III gave some clues about their intracortical route, especially in the coronal plane. Tracing such axons revealed that many proceeded in the mediolateral direction following a meandrous course. In some cases, a given fibre showed several oscillations before leaving the plane of section (Fig. 4). Although the height of these oscillating fibres was not always the same, they were typically confined between the upper portion of layer II and deep layer IV. The parent axons of these sinuous fibres could not be established except in a few cases when they seemed to arise from horizontal axons in layers I and VI. Oscillating axons appeared to give collateral branches mainly from the outermost and innermost portions of the path. Branches arising from the outer portion of the loop coursed into layer I, while those emanating from the inner portion seemed at times to also follow an oscillating course.

Electron microscopy

Immunoreactive profiles, recognized by the presence of an electron-dense granular reaction product, were visualized throughout the thickness of the cortex. These profiles were either vesicle-containing varicosities (0.5–1 μm in diameter) or intervaricose segments (< 0.4 μm in diameter). In favourable planes of section, thin unmyelinated intervaricose segments were seen to interconnect varicosities of various shapes (Fig. 5). An estimate of the density of NA-positive varicosities in the cortex was obtained by counting such profiles in 110 μm wide strips of the visual cortex between the pia and subcortical white matter. On average, 25–30 NA-labelled varicosities were counted in each of 20 such cortical strips.

Description of the organelles contained within labelled profiles was usually hampered by the dense reaction product. However, in more lightly stained profiles, intervaricose segments were seen to contain

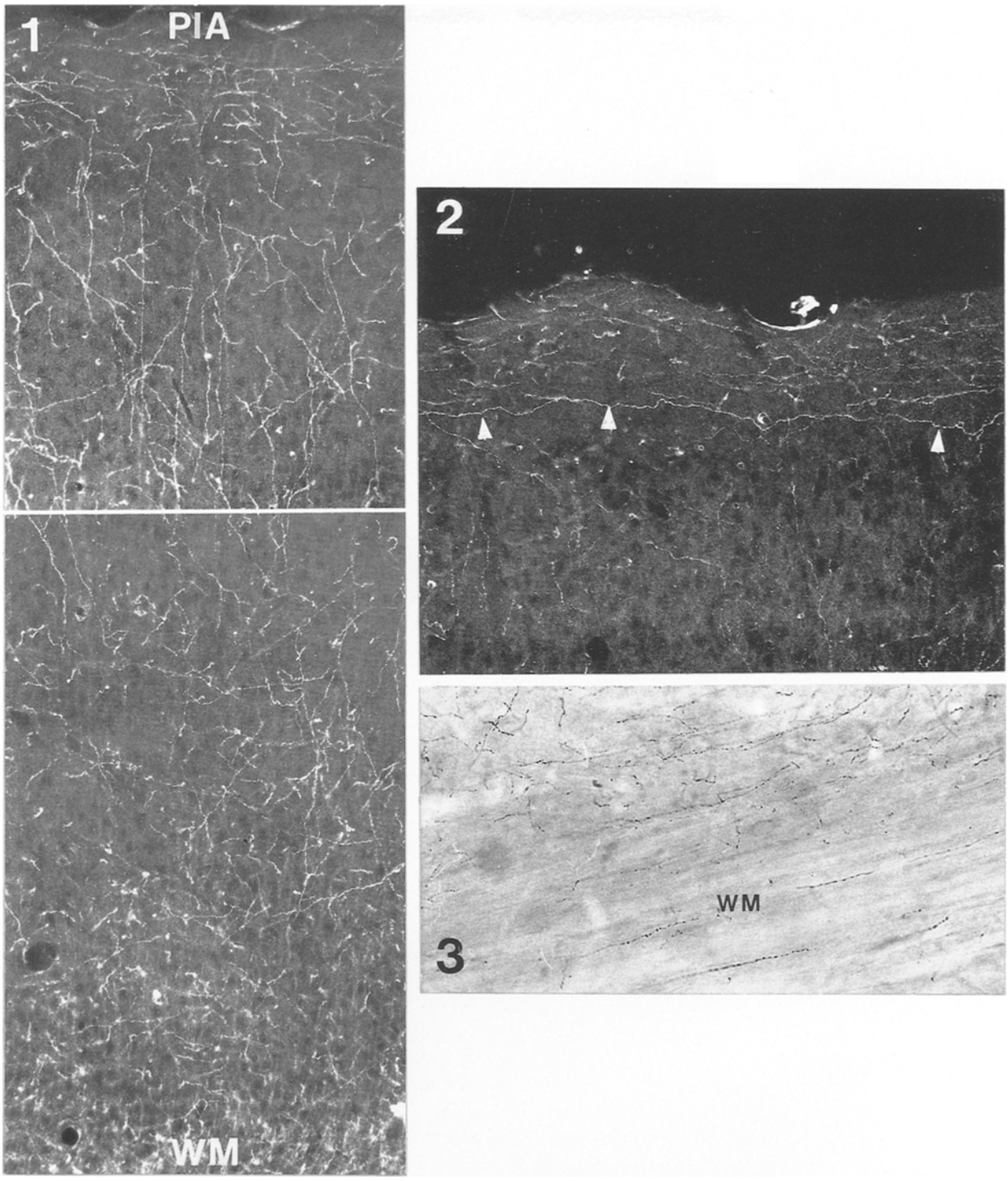


Fig. 1. Darkfield photomontage of a coronal strip through the rat visual cortex between the pia and the subcortical white matter (WM) illustrating the overall density and pattern of innervation of this area by the noradrenergic system. × 130.

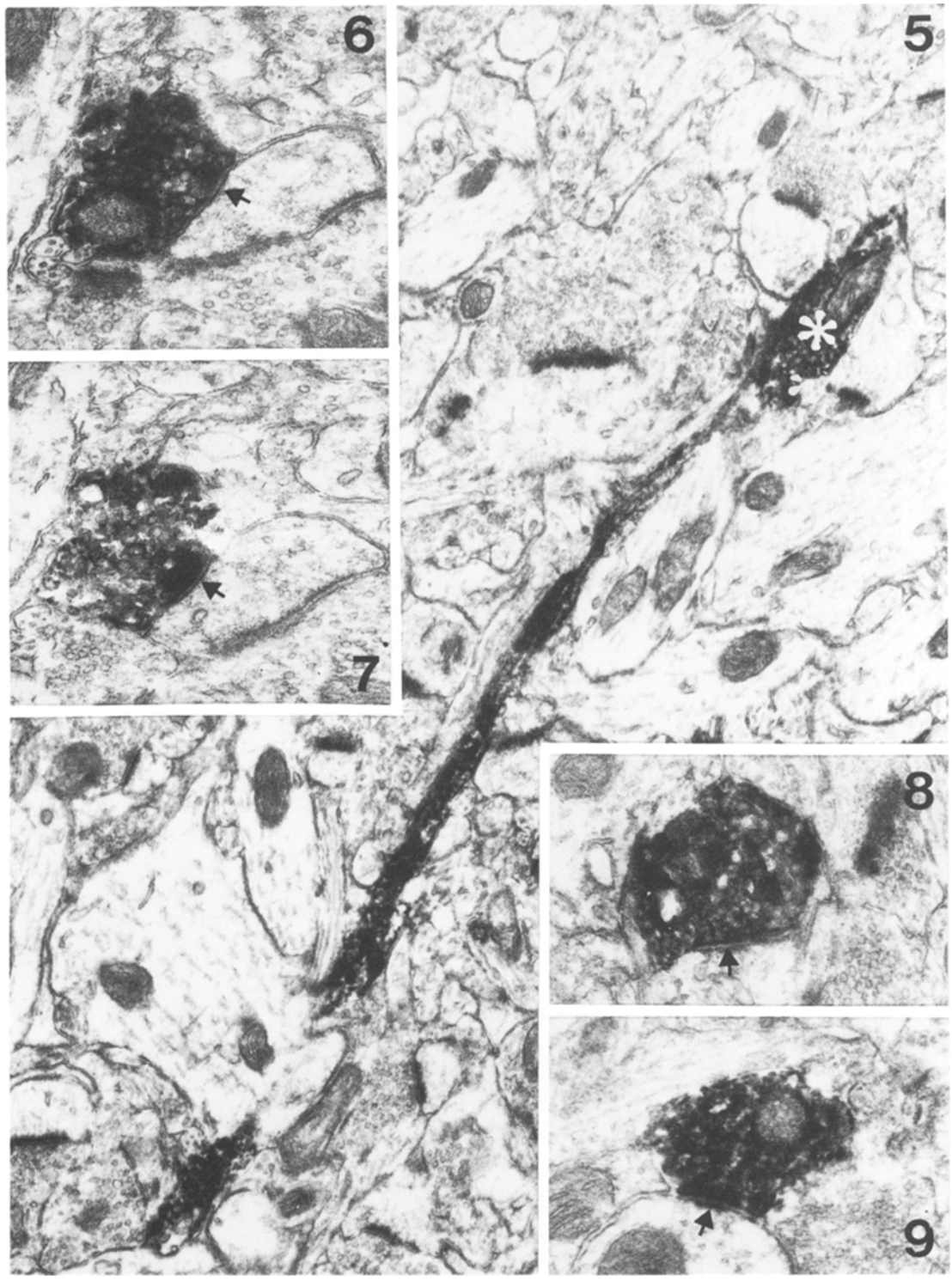
Fig. 2. This darkfield micrograph illustrates the distribution of labelled fibres in layer I. Tangentially oriented fibres (arrowheads) predominate in this layer, while a number of axonal segments seem to terminate in the outermost surface of the cortex. × 180.

Fig. 3. Tangentially oriented fibres in layer VI and in the subcortical white matter (WM). × 300.

an occasional mitochondrion and axially oriented microtubules, while varicosities usually contained one to three mitochondria surrounded by numerous synaptic vesicles.

The incidence of NA varicosities forming conventional synapses was examined in both single and serial ultrathin sections. Examination of immunoreactive varicosities in single ultrathin sections showed that

Fig. 4. Photomontage of a radial, noradrenaline-containing axon showing several oscillations between the upper portion of layer II (top) and deep layer IV (bottom) before leaving the plane of section. Coronal section; × 500.



Figs 5–9. In Fig. 5 an intervaricose segment is seen in continuation with a varicosity (asterisk) which contains a mitochondrion and numerous synaptic vesicles. × 27 500.

Figs 6, 7. A labelled varicosity forming a symmetrical synapse (arrows) with a dendritic spine, as seen in two consecutive sections. The spine also receives a synaptic contact (asymmetrical) from an unlabelled axon terminal. × 41 200.

Figs 8, 9. Asymmetrical synapses (arrows) formed by labelled varicosities with a spine (Fig. 8) and a dendritic shaft (Fig. 9). × 41 200.

only 20% (40/200) formed synaptic contacts while the remaining lacked morphologically defined synaptic specializations. However, analysis of 800 NA varicosities sampled from all cortical layers and contained in 30 uninterrupted series of 10 to 15 serial sections showed that the great majority (701/800; 87.6%) formed synapses with postsynaptic targets. It should be mentioned that varicosities which were obscured by excessive amount of immunoreactive product or showed poor ultrastructural preservation were not included in the sample. An almost identical proportion (111/125; 89%) was obtained in a sample taken from 15 series of serial sections through the frontoparietal cortex. These extended counts confirm the proportion reported briefly in an earlier account (Papadopoulos *et al.*, 1987a). Synaptic specializations were typically present only in two to four consecutive sections in every series. The presence of immunoreactive product made it difficult at times to evaluate the types of the synaptic contacts. However, both symmetrical (Figs 6, 7) and asymmetrical (Figs 8, 9) varieties were observed. The postsynaptic targets were, with declining frequency, spines, dendritic shafts of various diameters or somata. Usually, dendritic spines were also recipients of asymmetrical synapses by unstained axon terminals. Neuronal perikarya receiving synapses were identified on the basis of established criteria (Parnavelas *et al.*, 1977) as pyramidal or non-pyramidal (Fig. 10), although identification was not always unequivocal.

Finally, NA-positive profiles were at times seen in close proximity to intracerebral blood vessels, particularly in layers II–IV. They were seen coursing near or surrounding capillaries and in some cases no astrocytic process could be seen between the labelled profile and the capillary basal lamina. This observation supports earlier findings of Swanson *et al.* (1977) in the paraventricular nucleus.

Discussion

Intracortical organization of NA fibres

Using a systematic series of lesions, Morrison and co-workers observed that the NA-innervation of the medial and dorsolateral rat neocortex is formed by two distinct groups of axons which arise in the locus coeruleus and ascend through the septal area or more rostrally through the ventral telencephalon. Upon reaching the neocortex, NA-axons turn caudally and then coursing tangentially, mainly within layer VI, innervate longitudinal slabs of cortex from the frontal to the occipital poles (Morrison *et al.*, 1979, 1981; Morrison & Magistretti, 1983). A similar intracortical trajectory of NA axons appears to exist also in gyrencephalic animals (Morrison *et al.*, 1982). Further details about the intracortical path of NA axons are lacking perhaps due to the relatively thin sections that have been used thus far. The use of thicker sections in the present study permitted tracing of individual fibres for quite long distances and the establishment of continuity between fibres running at various depths of field. For example, in some cases five or six radial axonal segments, each spanning the thickness of layers II–IV and present at different depths of field, were shown to be parts of the same axon. These axons were actually oscillating between layers II–IV as they proceeded in the mediolateral direction. An estimate of the mediolateral extent of these meandrous fibres may be obtained from the results of the lesion experiments of Morrison and colleagues (Morrison *et al.*, 1981). According to these authors, when a parasagittal incision was made in the dorsal cortex, a narrow cortical strip extending for approximately 1 mm lateral and 300–400 μm medial to the incision exhibited a decrease in the innervation density, especially in the superficial layers. Taking also into account the width of the lesion, one could infer that individual NA axons do not extend in the mediolateral direction for more than 2 mm. This is also supported by injections of multiple fluorescent retrograde tracers into different cortical regions of the rat (Loughlin *et al.*, 1982). In these experiments, when pairs of injections of different tracers were placed in cortical sites less than 2 mm apart in the parasagittal plane, a high percentage of cells in the locus coeruleus contained both tracers. It would seem to us that tangential fibres within layer VI are the stem axons which innervate longitudinal cortical strips, and radial branches of these fibres follow quite a geometric route to innervate coronal strips of cortex with limited (up to 2 mm) mediolateral extent. Individual NA axons are thus capable of influencing synchronously very large numbers of columns – if columnar organization exists in the rat neocortex – along the anteroposterior axis, and a relatively limited number in the coronal plane.

In the present as well as in the previous studies, the origin and the fate of the long tangential fibres of layer I could not be traced. Morrison and co-workers (Morrison *et al.*, 1981) have reported that coronal incisions which only penetrated the upper cortical layers had no apparent effect on the density of innervation of adjacent cortical areas. This suggests that the long tangential fibres of layer I, unlike the fibres of layer VI, contribute very little to the NA innervation of the remaining cortical layers. It is possible, then, that axons in layer I comprise a separate system of noradrenergic afferents from those which emanate from the axons coursing in layer VI. Developmental studies have shown that during early development, the cortical anlage is innervated by two independent bundles of NA axons running in the marginal (primordial layer I) and subplate zones, respectively (Levitt & Moore, 1979; Schlumpf *et al.*,

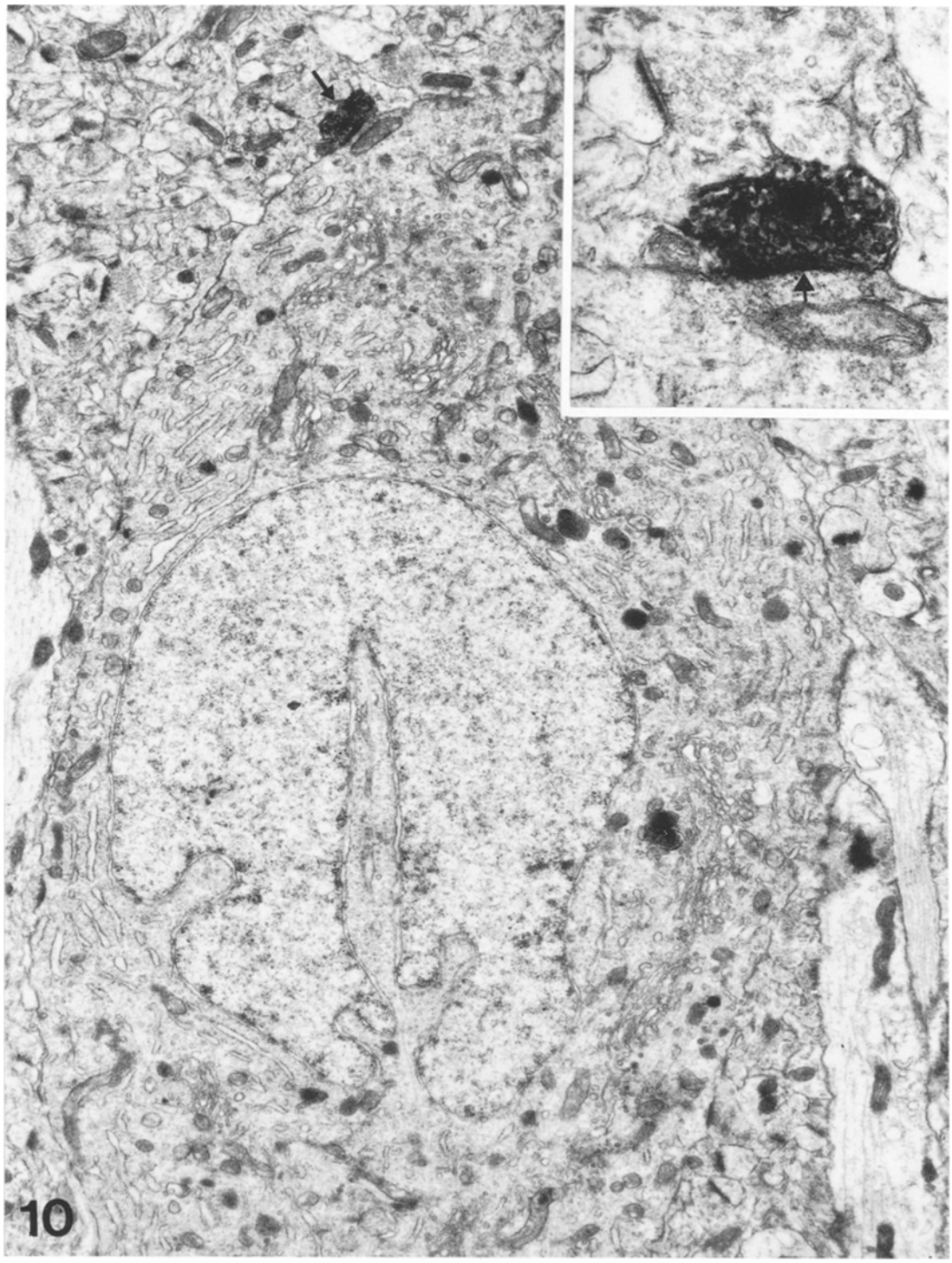


Fig. 10. A labelled varicosity (arrow) contacting the soma of a cortical non-pyramidal neuron. Inset shows this asymmetrical synaptic contact at higher magnification in a consecutive section. × 12 500; inset, × 41 200.

1980; Verney *et al.*, 1984). Neurons of these zones, considered to be both phylogenetically and ontogenetically old cell types (König *et al.*, 1977; Marin-Padilla, 1978), are the first to be contacted by NA fibres in the rat neocortex (Kristt, 1979). Retzius–Cajal cells, the early cells of layer I, remain in the adult cortex in the form of typical non-pyramidal neurons (Parnavelas & Edmunds, 1983). Perhaps a relationship between the long tangential fibres of layer I and the descendants of embryonic layer I cell types continues into adulthood.

Ultrastructure of NA terminals

The incidence of cortical NA varicosities exhibiting synaptic specializations has been a source of con-

troversy. This, which also holds in other parts of the brain (Koda & Bloom, 1977; Swanson *et al.*, 1978; Groves, 1980; Groves & Wilson, 1980; Olschowka *et al.*, 1981), has been attributed to the differential sensitivity and the inherent drawbacks of the methods used (see Beaudet & Descarries, 1984; Parnavelas *et al.*, 1985). Serial section analysis utilized here has shown that virtually all NA varicosities examined formed conventional synapses. Serial section analysis has been applied previously in studies which used potassium permanganate fixation (Maeda *et al.*, 1975; Itakura *et al.*, 1981), or radioautography after topical application of [^{3}H]NA (Descarries & Lapierre, 1973; Descarries *et al.*, 1977; Beaudet & Descarries, 1978). The former studies reported that 7% (subsequently modified to 25%) of NA varicosities in the rat neocortex and 10–20% in the kitten visual cortex possessed synaptic membrane specializations. An even smaller proportion (< 5%) was reported in the radioautographic studies in the rat frontoparietal cortex. More recently, Beaudet & Descarries (1984) have reported that stereological analysis has shown that the incidence of NA terminals engaged in synaptic contacts is 18%. However, apart from the criticisms concerning the effectiveness of these experimental approaches in the study of the fine features of monoaminergic terminals (see Parnavelas *et al.*, 1985), the number of consecutive sections examined in the above studies was inadequate. In the present study, a given varicosity was usually present in most, if not all, of the 10–15 consecutive sections of each series, and signs of synaptic specialization were recognized only in two to four serial sections. However, Descarries and his colleagues examined series of no more than three consecutive sections and, curiously, only 18% of the varicosities were found in more than one section. Thus, it is reasonable to assume that the difference between the results presented here and those of other workers is at least partly due to the different number of consecutive sections examined. It has also been suggested that such differences may be due to the peculiarities of the various cortical regions examined (Parnavelas *et al.*, 1985). However, examination of visual and frontoparietal (the area examined by Descarries and co-workers) cortices resulted in similar findings. The small proportion (about 10%) of the varicosities which was not found to exhibit synaptic specializations may represent either varicosities that were not cut in their entirety or sites from where release of transmitter does not occur. The latter possibility has been already proposed to apply for other types of axon terminals (Buijs, 1982).

Until now it was believed that NA axons synapse only upon dendritic profiles, although examples of NA varicosities adjacent to neuronal somata have been observed previously (Itakura *et al.*, 1981). The demonstration here of conventional synapses on somata of both pyramidal and non-pyramidal neurons modifies this view. The nature of the postsynaptic effects caused by NA terminals synapsing on different parts (proximal versus distal) of target neurons and their influence on information processing is elusive. In conclusion, the NA system, usually referred to as diffuse and non-specific, appears to exhibit constraints in its distribution (see also Caviness & Korde, 1981: Caviness, 1982 for work in reeler mouse) and acts at restricted sites similar to other transmitter-specific systems in the cortex.

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