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SYNAPTIC CONNECTIONS OF GABA-CONTAINING BOUTONS IN THE LATERAL CERVICAL NUCLEUS OF THE CAT: AN ULTRASTRUCTURAL STUDY EMPLOYING PRE- AND POST-EMBEDDING IMMUNOCYTOCHEMICAL METHODS

D. J. MAXWELL,* W. M. CHRISTIE* and P. SOMOGYI†

*Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, U.K.
†MRC Anatomical Neuropharmacology Unit, University Department of Pharmacology, Oxford OX1 3QT, U.K.

Abstract—The lateral cervical nucleus receives input from the spinocervical tract and projects to the thalamus and mesencephalon. The organization of this nucleus was examined using two immunocytochemical methods. Pre-embedding immunolabelling was performed using an antibody against glutamate decarboxylase, and post-embedding immunogold-reaction was performed with an antibody to glutaraldehyde-fixed GABA. Light microscopic analysis of material reacted for glutamate decarboxylase revealed that punctate structures were present throughout the nucleus and were associated with large cells in the dorsolateral region of the nucleus. Electron microscopy demonstrated that the punctate structures were synaptic boutons which formed symmetrical synaptic junctions with dendrites and somata of cells in the nucleus. The ultrastructural preservation of material prepared for the post-embedding immunogold technique was superior to that prepared for pre-embedding immunostaining. Positively labelled synaptic boutons exhibited high colloidal gold density and, like those prepared for the pre-embedding method, formed symmetrical synaptic junctions with dendrites and somata of neurons. Labelled boutons were densely packed with irregularly-shaped synaptic vesicles. They displayed characteristics which were distinct from those unlabelled boutons. Boutons, revealed by both immunolabelling methods, were not observed to form synaptic associations with other axon terminals and were presynaptic to dendrites and somata only.

Therefore, it is probable that such boutons are responsible for postsynaptic inhibition of cells in the nucleus. In view of this evidence, it is concluded that the lateral cervical nucleus is not simply a relay but is actively involved in processing sensory information.

The lateral cervical nucleus (LCN) in the cat forms a synaptic stage in the spino-cervico-thalamic pathway (see Ref. 3 for a review). The LCN is present on both sides of the spinal cord in the first two cervical segments and consists of an elongated column of gray matter in the dorsolateral funiculus, just lateral to the dorsal horn of the spinal gray matter. In the cat, it has been estimated that the nucleus contains approximately 5000 neurons.[1,10] However, Flink and Westman[16] recently concluded that this was probably an underestimate and the figure was closer to 8300. Neurons of the LCN may be classified arbitrarily into three groups on the basis of their projections. (1) Neurons which project to the thalamus and/or the mesencephalon.[10,16] These cells are situated throughout the nucleus and vary considerably in size; 94% of the cells of the LCN fall into this category. (2) Cells which project to the spinal cord[15,40] and tend to be located in the ventromedial part of the LCN. (3) Small cells (soma diameter about 15 μm) which also tend to be located in the ventromedial region and do not appear to project from the nucleus.[10,16] A proportion of these cells contain GABA and therefore may be regarded as putative local circuit neurons.[4] The principal projection to the LCN comes from the spinocervical tract (SCT),[3,7,9,12,41,42] however further afferent projections from the dorsal column nuclei, reticular formation, and spinal trigeminal nuclei may be present.[3,9,11,18] In addition to these sources, some axon terminals in the LCN probably originate also from recurrent collateral axons of ascending projection neurons[16,46] and from local axon arbors of small interneurons,[4,46] but this remains to be demonstrated conclusively. Several morphological classes of bouton have been described in the nucleus.[47] Some of these have been positively identified as terminals originating from the SCT.[41,48]

Anatomical and physiological studies indicate that the LCN is somatotopically organized.[11,12,42,45] Receptive fields of LCN neurons which project through the medial lemniscus have similar properties to SCT neurons; i.e. most of them are excited by gentle brushing of the hair and noxious stimulation.[5] The

Abbreviations: GAD, glutamate decarboxylase; LCN, lateral cervical nucleus; NDS, normal donkey serum; NGS, normal goat serum; PBS, phosphate-buffered saline; PEG, polyethylene glycol; SCT, spinocervical tract; TPBS, Tris–phosphate buffered saline.

majority of receptive fields of LCN neurons are similar in size to those of the SCT although a minority may be considerably larger.[8,11,24,26,27] LCN neurons also have inhibitory receptive fields and there is evidence for both pre- and postsynaptic inhibitory mechanisms.[14]

In view of the complex organization and physiology of the LCN, it seems unlikely that this nucleus is simply a relay in the spino-cervico-thalamic pathway. We embarked upon the present study with a view to improving our understanding of the inhibitory mechanisms that occur in the LCN by examining the morphology and synaptic organization of GABA-containing terminals in the nucleus. There were three principal aims of the study. (1) To examine the distribution of GABA-containing terminals in the LCN and, in particular, consider the relationship of such terminals with putative projection neurons in the dorsolateral region of the nucleus. (2) To examine the morphology of GABA-containing terminals and determine if such terminals have characteristic features which may differentiate them from the other terminals of the nucleus. (3) In view of the evidence for presynaptic inhibitory mechanisms in this nucleus, to establish if GABA-containing terminals form axoaxonic relationships with other terminals. To date there is no evidence for the existence of axoaxonic synapses in the LCN.[4,41,47]

In order to achieve these aims we have employed two different immunocytochemical techniques in conjunction with electron microscopy. The post-embedding immunogold technique[35] was used with an antibody raised against GABA conjugated to a carrier protein.[23] This technique has several advantages over the pre-embedding method (see below). Pre-embedding immunocytochemistry was accomplished using an antibody raised against glutamate decarboxylase (GAD),[32] the enzyme responsible for the synthesis of GABA. The pre-embedding method enabled us to examine the light microscopic distribution of labelled structures and also to compare the fine structure of labelled profiles with those revealed by the post-embedding technique. We also examined LCN tissue that was fixed and processed under standard conditions in order to compare it with tissue processed for immunostaining.

A short report previously described some aspects of GAD-immunoreactive terminals in the LCN.[2] However, during the course of our study a larger report was published by Broman and Westman[4] describing details of GABA-labelled terminals which were identified using pre-embedding immunocytochemistry. We felt that it was still appropriate to continue with our study since the post-embedding immunogold technique has two considerable advantages over the pre-embedding technique. Firstly, it may be performed on well fixed tissue with good ultrastructural preservation and, secondly, the gold particles do not obscure internal details of labelled structures. Therefore the post-embedding approach reveals greater details of labelled structures and enables them to be assessed more accurately.

EXPERIMENTAL PROCEDURES

Pre-embedding immunocytochemistry

Two adult cats were anaesthetized with sodium pentobarbitone (40 mg/kg i.p.). Animals were artificially ventilated during the early stages of transcardial perfusion. Initially, they were perfused with 500 ml of 0.9% saline with heparin (100 units/ml) and sodium nitrite (0.01%) at 37°C, and then with fixative. The fixative solution consisted of 4% paraformaldehyde, 0.1% glutaraldehyde, 150 ml/l saturated picric acid in 0.1 M phosphate buffer at pH 7.4.[37] One litre of this solution was given at 37°C and subsequently, 2 l at 4°C. Blocks of spinal segments C1 and C2 were kept for 8 h in the same fixative. Transverse sections (40 μm) were cut from blocks with a Vibratome and washed several times in 0.1 M phosphate-buffered saline (PBS). Sections were placed in PBS containing 10% normal donkey serum (NDS) for 30 min prior to incubation in a PBS solution containing the primary antiserum (diluted to 1:1000) and 1% NDS for 18 h. The primary antiserum (which was generously donated by Dr M. L. Tappaz) was raised in sheep using a GAD–anti-GAD complex isolated from rat brain.[32] Characteristics and specificity of this antiserum have been documented elsewhere.[32,43] Sections were next placed in donkey anti-sheep biotinylated whole antibody (1:100) (Amersham) for 45 min and finally in streptavidin–peroxidase complex (1:300) (Amersham) for 15 min. Both dilutions were made in PBS containing 1% NDS and sections were washed six times in PBS between each stage of processing. The presence of peroxidase was revealed using pyrocathechol/*p*-phenylenediamine as a chromogen[22] with cobalt intensification. Sections were then placed in a 1% solution of osmium tetroxide, dehydrated through a series of ethanol solutions and cleared in propylene oxide. They were subsequently flat-embedded in Araldite between cellulose acetate foils and, once polymerization was complete, they were examined with a light microscope, drawn and photographed prior to resectioning on an ultramicrotome. Series of thin sections were collected on single slot copper grids coated with Formvar. Contrasting was achieved using uranyl acetate and lead citrate according to standard procedures. Control sections were processed similarly except that the primary antiserum was omitted from the incubation medium.

Post-embedding immunocytochemistry

Spinal material was examined from cats which were fixed and prepared in a number of ways. The most successful method is outlined below and is similar to that described by Somogyi and Hodgson.[35]

Adult cats of either sex were anaesthetized with sodium pentobarbitone (40 mg/kg, i.p.). A cannula was inserted into the trachea and animals were artificially ventilated with the aid of a pump during the initial stages of perfusion. Transcardial perfusion was performed with the following solutions: (1) 500 ml 0.9% saline, with heparin (100 units/ml) and sodium nitrite (0.01%) at 37°C; (2) 1 l of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 37°C; and (3) 2 l of solution 2 at 4°C. Spinal segments C1 and C2 were identified, cut into blocks and placed in the fixative solution for 8 h at 4°C. The following day blocks of tissue were washed in phosphate buffer (pH 7.4) and 40-μm transverse sections were cut with a Vibratome. Sections were placed in 1% osmium tetroxide in phosphate buffer for 1 h, were dehydrated through a series of alcohol solutions, stained "*en bloc*" with uranyl acetate for 1 h (a 1% solution in 70% ethanol), and cleared in propylene oxide. The sections were flat-embedded in Durcupan between foils of cellulose acetate.

When polymerization was complete, sections were examined with a light microscope, the LCN was identified and

attached to blocks for sectioning on an ultramicrotome. Serial sections were collected on single slot gold grids coated with Formvar. Electron microscope sections were etched by floating grids on a solution containing 1% periodic acid which was followed by 1% sodium periodate. Grids remained in these solutions for 7 min each and were washed in distilled water between changes. The sections were next floated on drops of Tris (10 mM)–phosphate (10 mM) buffered saline (TPBS; pH 7.4) for two periods of 10 min and then transferred to TPBS containing 5% normal goat serum (NGS) for 30 min. They were transferred to TPBS containing 1% NGS for a period of 10 min prior to incubation in primary antiserum. Details of the anti-GABA (GABA-9) serum have been published in depth elsewhere.[23,35,38] Grids were floated on droplets of the primary antiserum for 2 h. Dilutions of GABA-9 in TPBS with 1% NGS ranged from 1:1000 to 1:10,000 and the optimal dilution (i.e. that which gave the best signal to background ratio) was found to be 1:6000. To test method specificity the antiserum was also used at the same dilution on some electrom microscope sections following its solid phase preadsorption to GABA coupled to polyacrylamide beads (see Ref. 38). Grids were subsequently washed in 0.05 M Tris–HCl buffer (pH 7.5) containing 0.05% polyethylene glycol (PEG) for four periods of 5 min and then placed in a similar solution containing goat anti-rabbit IgG coupled to 15 nm gold spheres (Janssen Pharmaceutica) for 1.5 h. Finally, the grids were washed several times in distilled water and stained with Reynolds' lead citrate.[34]

Standard procedure for electron microscopy

A further cat was prepared according to our standard electron microscope technique (e.g. see Ref. 30) to provide a comparison with tissue processed for immunocytochemistry. The animal was anaesthetized and fixed in the same manner as those prepared for immunostaining, however the fixative consisted of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 with 0.025 M calcium glycerophosphate. Initially 1 l of this was given at 37°C and subsequently 2 l at 4°C. Blocks were stored in fixative for 8 h and 40-μm transverse sections were cut on a Vibratome. The sections were then prepared for combined light and electron microscopy in an identical manner to that described in the pre-embedding immunocytochemistry method.

Electron microscopy and quantitative analysis

Material was examined from rostral (C1) and caudal (C2) regions of the nucleus.

Terminals examined in the post-embedding immunostaining study were followed through serial sections (up to 10) to determine consistency of staining (i.e. that the same terminal was consistently labelled or unlabelled through the series). Although a random sampling procedure was not adopted, thin sections of the nucleus were systematically scanned with the microscope and all GABA-positive terminals encountered were photographed at high and low magnifications. The sample of unlabelled boutons came from the same photographic fields as that of the labelled terminals. In some experiments, single sections were collected on grids in a series and alternate sections were treated with GABA-9 or serum that had been preadsorbed with conjugated GABA. Terminals were viewed through the series of sections for staining consistency. GAD-immunoreactive boutons were similarly viewed through series of sections.

Several parameters were measured in this study. Maximum diameters of boutons, diameters of dendrites in contact with them and lengths of synaptic appositions were measured directly from micrographs with the aid of a Reichert Videoplan measuring system. Vesicle densities and densities of gold grains were calculated as a function of unit area with the assistance of a Magiscan image analysis system (Joyce Lobel). The program (written by Dr R. J. Houchin) enabled a profile to be defined and the number of structures contained within it to be counted. Estimates of background labelling were obtained by dropping a card of unit area on micrographs from a standard height and measuring the numbers of gold grains contained within these randomly defined areas.

RESULTS

Light microscopy of the lateral cervical nucleus

Light microscopic analysis of thick (40 μm) sections established that GAD-immunoreactive structures are present throughout the nucleus (Figs 1A, B, 2A, B and 3A). The immunoreactive structures were punctate in form and corresponded to boutons (see below). Some boutons were observed to be associated with somata in the LCN, including somata of large cells which were 30 μm or greater in diameter (Figs 2C and 3B). However, the majority of labelled terminals were not associated with cell bodies but were found dispersed within the neuropil.

Immunoreactive neurons were not observed in the present material; reasons for this are discussed below. Staining was lacking in sections incubated in the absence of the primary antiserum.

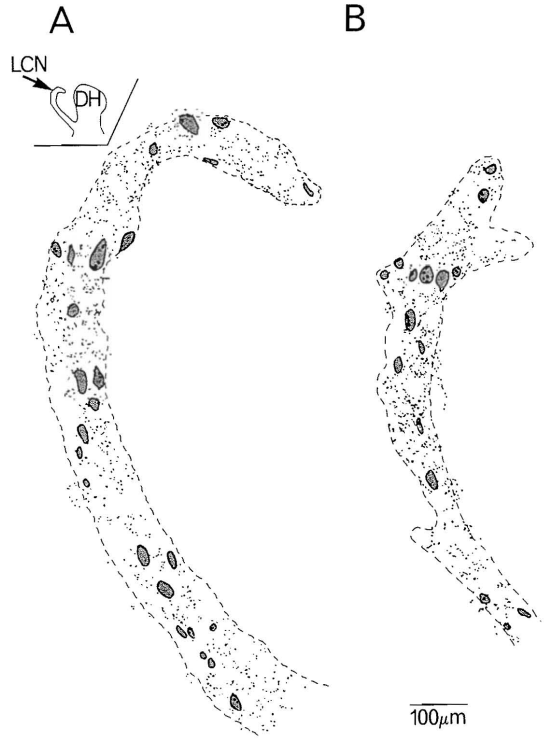

Fig. 1. Distribution of GAD immunoreactivity in rostral (C1) regions of the LCN. A and B. Camera lucida drawings of the LCN. The dashed line demarcates the nucleus and shaded structures represent LCN neurons. Numerous punctate structures are present throughout the nucleus. The inset illustrates the position of the LCN shown in A relative to the dorsal horn (DH). Note that the nucleus is continuous with the horn at this level.

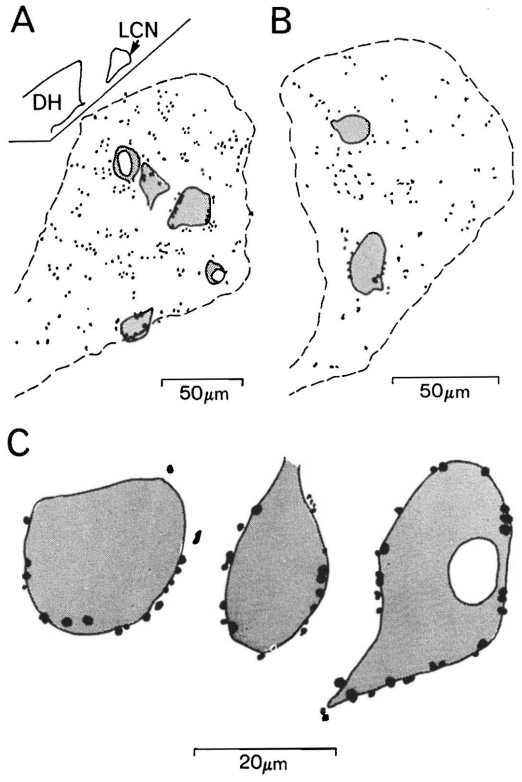

Fig. 2. Distribution of GAD immunoreactivity in caudal regions (C2) of the LCN. A and B. Camera lucida drawings of the LCN in transverse section. The dashed line demarcates the extent of the nucleus. Note the numerous punctate immunoreactive structures dispersed throughout the nucleus. Shaded structures are LCN neurons. Inset in A illustrates the position of the nucleus relative to the dorsal horn (DH) of the spinal grey matter. C. Associations between immunoreactive structures and three large LCN cells.

Ultrastructure of glutamate decarboxylase-labelled structures

GAD-immunoreactive structures were examined with the electron microscope and were found invariably to be synaptic boutons (diameter = 1.21 ± 0.36 S.D. μm; $n = 95$). The majority of boutons formed symmetrical (Gray[20] type 2) synapses with dendritic profiles which varied considerably in diameter (Fig. 4A and B). Usually each bouton formed a single synapse with one postsynaptic structure. On some occasions, several boutons were presynaptic to a single large dendritic profile (Fig. 4A). As predicted by the light microscopic analysis, some of them also were presynaptic to somata of LCN neurons (Fig. 4C). The labelled boutons appeared to contain irregularly-shaped vesicles which were densely packed but it was difficult to determine this with any degree of certainty because of the density of the reaction product. No examples of axoaxonic synapses were observed although labelled boutons were often closely apposed to unlabelled and labelled axon terminals. Also, no evidence of GAD-immunostaining was observed in dendrites or somata in the nucleus.

Ultrastructure of GABA-containing structures identified by post-embedding immunostaining

The ultrastructural preservation of material prepared by post-embedding staining was found to be far superior than that prepared for pre-embedding and was of a similar quality to the tissue processed by our standard procedure for electron microscopy (see below). Using dilutions of the antiserum at 1:6000 it was fairly obvious that some axon terminals were more densely labelled with gold grains than the background (e.g. see Fig. 6A). This became evident as serial sections were examined; some terminals were consistently heavily labelled whereas others were consistently unlabelled (i.e. displayed the same density of grains as the background) (see Fig. 8). In order to test this in a quantitative manner, 10 micrographs illustrating labelled boutons at relatively low magnifications ($\times$ 16,500–21,500) were chosen at random and the grain density of labelled profiles was assessed. A standard 1-μm square of card was dropped from a height of 60 cm onto the same 10 micrographs and the number of grains contained within this area was assessed to estimate the density of background labelling. Labelled profiles had an average density of 60.7 grains/μm^2 (range 29.9–113.7 grains/μm^2) and the average background was estimated to be 15.2 grains/μm^2 (range 5.4–30.7 grains/μm^2). Therefore at dilutions of 1:6000 boutons deemed to be positively labelled for GABA contain approximately four times the number of grains as the background.

In some experiments alternate serial sections were treated with anti-GABA or preadsorbed anti-GABA to test the specificity of the immunoreaction. Axon terminals in sections treated with the primary antiserum were consistently labelled with grains and could be followed through the series; however, the same terminals in adjacent sections treated with control serum were devoid of gold grains (Fig. 5). Therefore the antiserum is likely to recognize GABA fixed in the tissue.

In total 151 labelled boutons were examined with the electron microscope and photographed. These boutons had an average maximum diameter of 1.43 ± 0.37 (S.D.) μm and, characteristically, were densely packed with irregularly shaped vesicles (see

Fig. 3. A. A light-micrograph of the LCN (in transverse section at C2) illustrating punctate GAD-immunoreactive structures. The dotted line demarcates the extent of the nucleus which is located in the dorsolateral funiculus (DLF). A large neuron (*) is shown at greater magnification in B. Note the punctate immunoreactive structures which surround it.

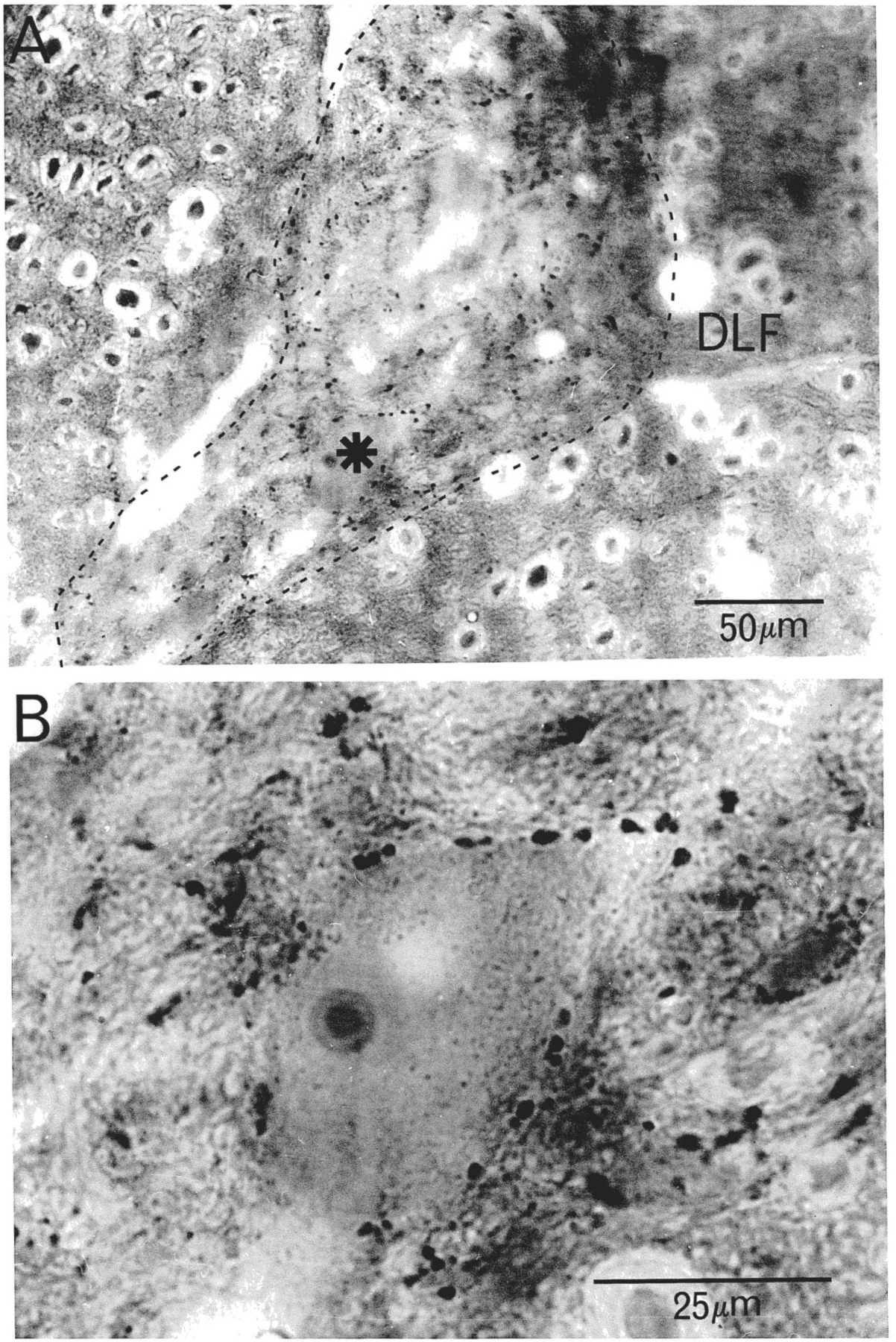

Fig. 3.

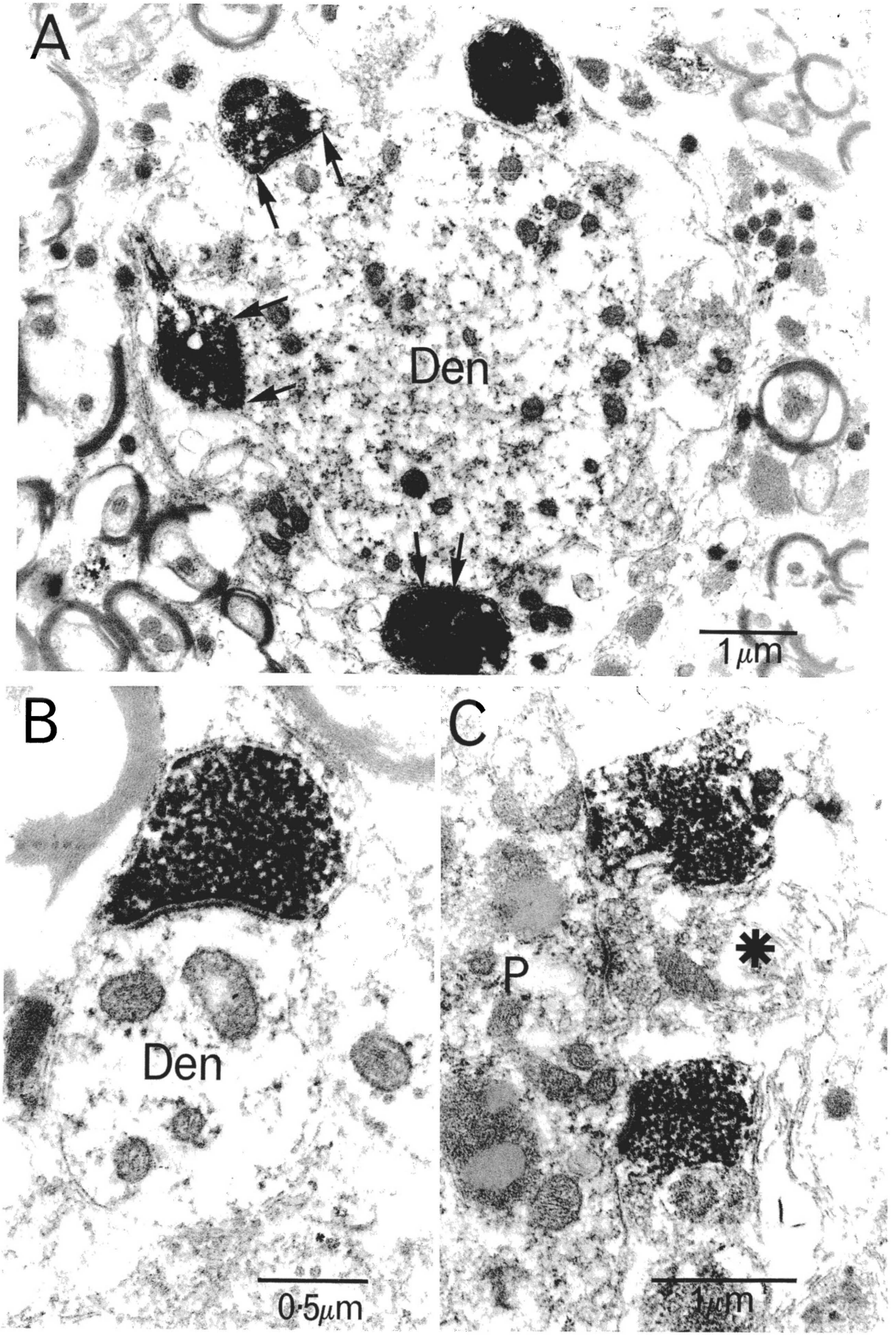

Fig. 4.

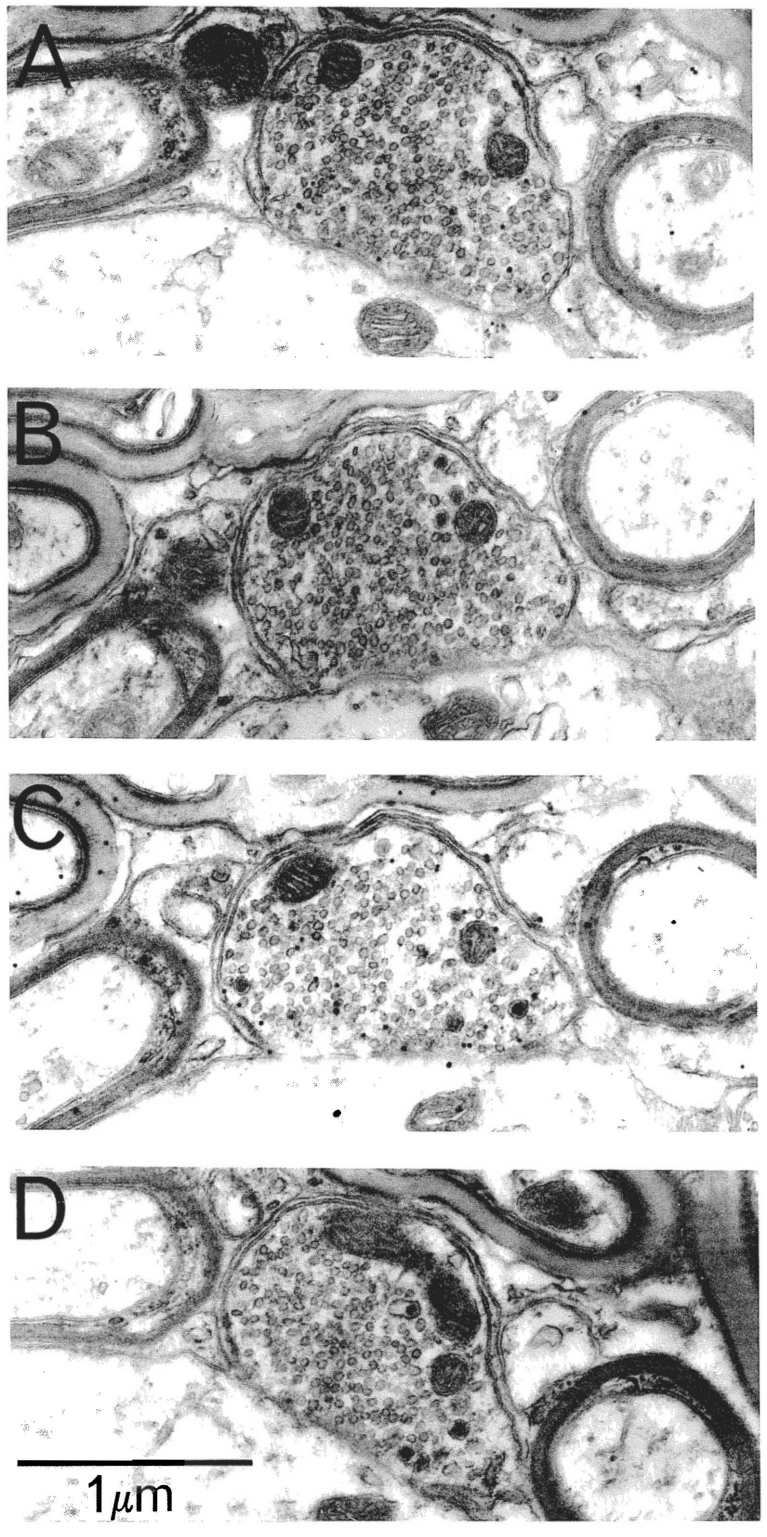

Fig. 5. Specificity of the immunoreaction used in the post-embedding immunogold technique. The same synaptic bouton is illustrated in A–D in a series of adjacent sections A and C were treated with antiserum to GABA whereas B and D were treated with the antiserum after adsorption to GABA coupled to polyacrylamide beads. Gold particles label the bouton in sections A and C but are absent in B and D, thus indicating that the antibodies recognize conjugated GABA.

Fig. 4. Electron micrographs of GAD-immunoreactive structures. A. A large dendrite (Den) is surrounded by immunoreactive boutons. Three of them form synaptic junctions with it (arrows). B. An immunoreactive terminal which is presynaptic to a small dendritic profile (Den). Note the symmetrical synaptic junction. C. Two immunoreactive boutons which form synapses with the perikaryon (P) of an LCN neuron. An unlabelled terminal (*) is also presynaptic to the cell.

Figs 5–9). The average vesicle density of labelled boutons was 172.2/μm^2 (range 82.7–287.1), whereas the average density of vesicles in unlabelled terminals was 62.6/μm^2 (range 40.5–89.5). Unlabelled boutons were similar, or slightly larger in size to labelled ones, but generally had the appearance of containing fewer vesicles (Figs 6A, 8 and 9). Vesicles in unlabelled boutons were often circular in profile although a sub-population of them contained irregular or flattened vesicles. Labelled boutons invariably formed symmetrical (type 2[20]) synaptic junctions with dendrites or somata (see Figs 5–9). The average length of the synaptic contacts was 0.77 ± 0.27 (S.D.)μm. Careful examination of serial sections revealed that labelled boutons formed synapses with only one post-synaptic structure. Some unlabelled boutons formed symmetrical junctions whereas others formed asymmetrical junctions.

A high proportion (75%) of labelled boutons examined were presynaptic to dendrites which varied considerably in size (Fig. 6); the average maximum diameter of dendritic profiles was 2.29 μm and the range was 0.43–6.07 μm. Often several labelled boutons were presynaptic to a large dendritic profile (Fig. 6A). Only 25% of labelled boutons were presynaptic to somata (Figs 7 and 8). These had the same morphological characteristics as terminals in contact with dendrites. The majority of cells contacted were large and situated in the dorsolateral region of the nucleus. As in the GAD study, axoaxonic synapses were not observed, although labelled profiles were periodically seen to be closely apposed to unlabelled boutons (see Fig. 9) and occasionally also to labelled axon terminals.

Occasionally a dendrite or a soma was observed which, on visual inspection, seemed to be associated with a greater density of gold particles when compared with the background. Such profiles were few in number and were never as densely labelled as boutons.

Comparison with standard electron-microscopic material

Material prepared according to our standard procedure for electron microscopy was identical in every respect to that prepared for the post-embedding study. Morphological classes of bouton were observed which corresponded to those described above; this included a class with similar characteristics to the GABA-labelled group. Although a careful search was made, unequivocal examples of axoaxonic synapses were not observed.

DISCUSSION

The post-embedding immunostaining method

The superior ultrastructural preservation of tissue prepared for the post-embedding immunostaining technique and the use of small gold grains as markers enabled details of labelled structures to be described and quantified with the same ease as material prepared for standard electron microscope studies.

A variety of dilutions of the GABA antibody was tested on LCN tissue to determine the dilution of serum that produced consistent and obvious labelling. Quantitative analysis demonstrated that profiles which were classified as labelled by visual inspection usually contained four times as many grains of gold as the background. In addition, individual labelled profiles were followed through series of sections and were invariably found to be densely labelled in each section. Therefore we are confident that labelled structures were identified correctly.

Characteristics of the GABA antibody have been described in a number of published accounts and evidence from several regions of the CNS indicates that it reacts with GABA conjugated to protein in glutaraldehyde-fixed tissue.[25,35,36,38] However, the specificity of the procedure in spinal material has not been documented. We observed that preadsorption of the primary antiserum with GABA coupled to polyacrylamide beads with glutaraldehyde abolished staining in profiles which could be demonstrated to be positively labelled with the original antiserum. The antiserum thus appears to react with aldehyde-fixed GABA in the LCN.

Comparison of glutamate decarboxylase- and GABA-containing terminals in the lateral cervical nucleus

Boutons in the LCN were labelled with the GAD and GABA antibodies. There was a small difference between the diameters of the two populations; the average diameter of GAD boutons was 1.21 μm (± 0.36 S.D.) and GABA labelled boutons was 1.43 μm (± 0.37 S.D.). Caution is required in the interpretation of this observation as variations in fixation methods could account for the difference. Both populations of boutons formed symmetrical synaptic junctions with dendrites and were organized in a similar fashion. For example, labelled boutons from both groups were seen to be clustered around large dendritic profiles and terminals were associated with large LCN cells. Boutons identified by both methods formed synaptic associations with single post-synaptic structures. In GABA-labelled boutons

Fig. 6. Synaptic associations between GABA-labelled boutons and dendrites. A. Two labelled boutons (*) are presynaptic to a large dendrite. Several unlabelled boutons are also in synaptic contact with the same dendrite. These contain vesicles which vary in shape and packing density and form symmetrical and asymmetrical junctions with the dendrite. Note the low background staining. B and C. Labelled boutons in contact with small dendritic profiles. Note the symmetrical synaptic junctions and the densely-packed irregular vesicles.

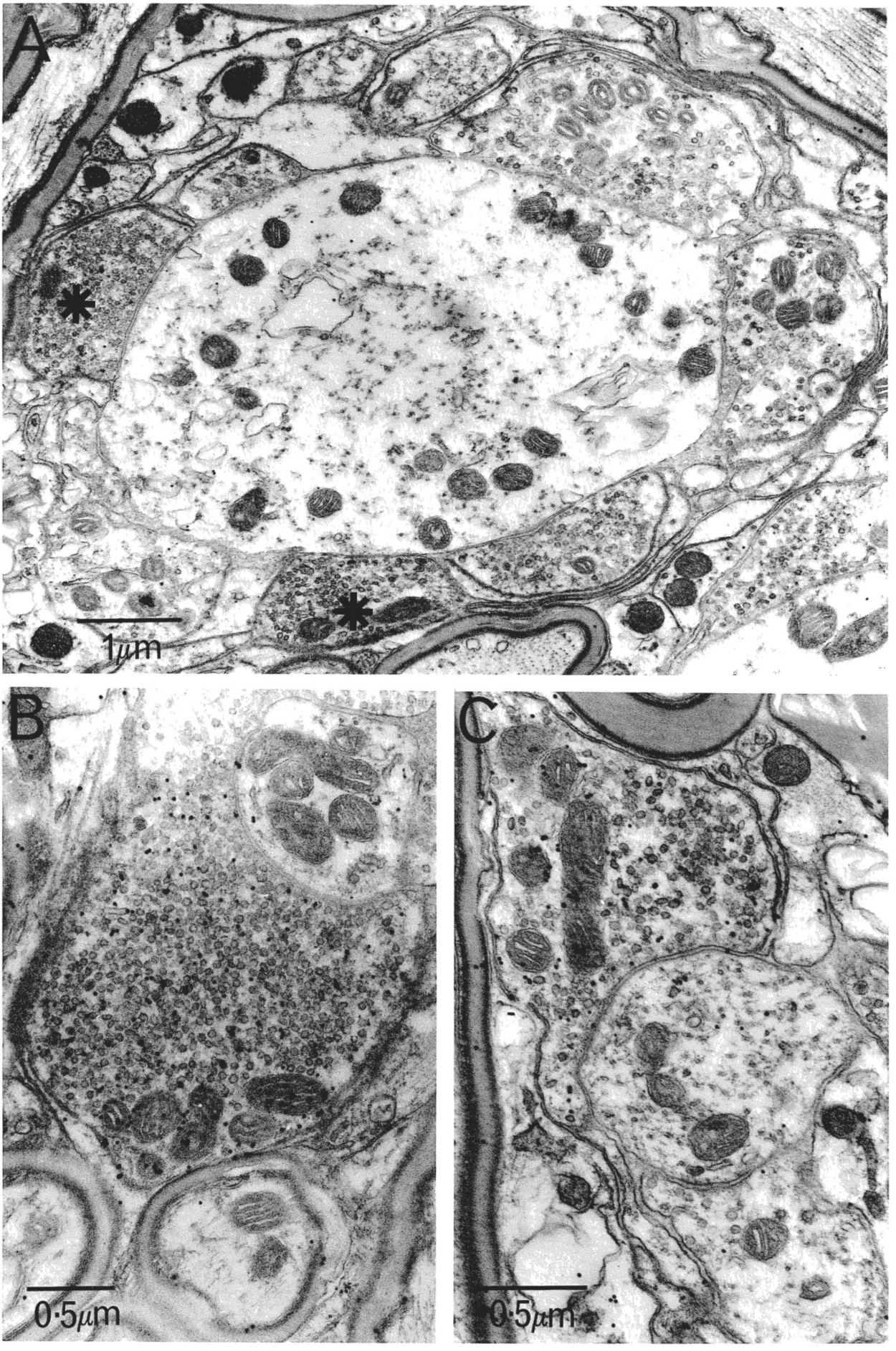

Fig. 6.

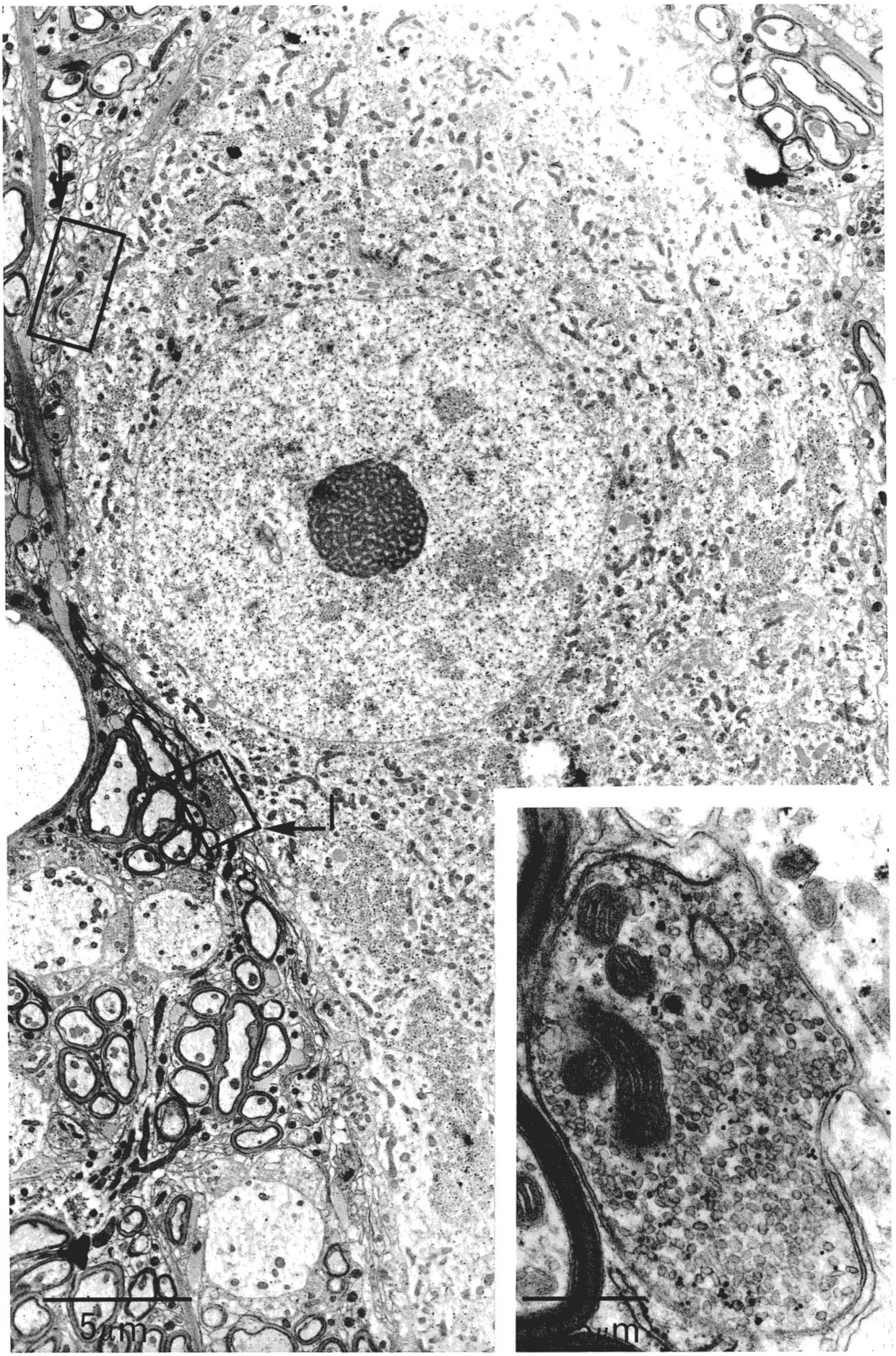

Fig. 7. A large LCN neuron. The area enclosed within the box designated I is shown at greater magnification in the inset. The other box is illustrated in Fig. 8. Inset: note the bouton which is heavily labelled with gold and forms a symmetrical synaptic junction with the perikaryon of the LCN neuron.

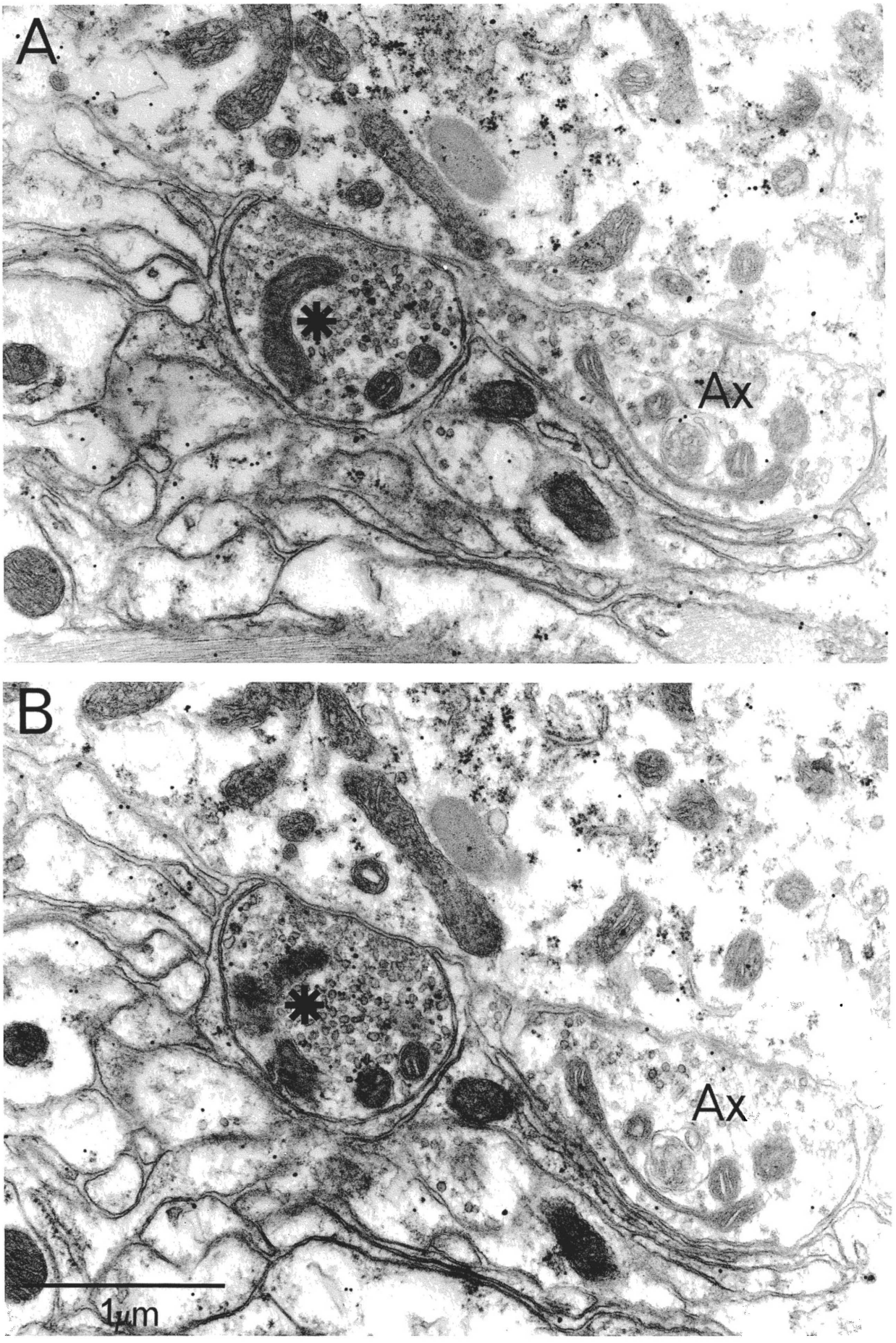

Fig. 8. Serial sections through a labelled bouton (*) and an unlabelled bouton (Ax) which are both presynaptic to the LCN neuron illustrated in Fig. 7. Note the differences in vesicle packing density and shape, the symmetrical junction formed by the labelled bouton and the asymmetrical junction formed by Ax in A.

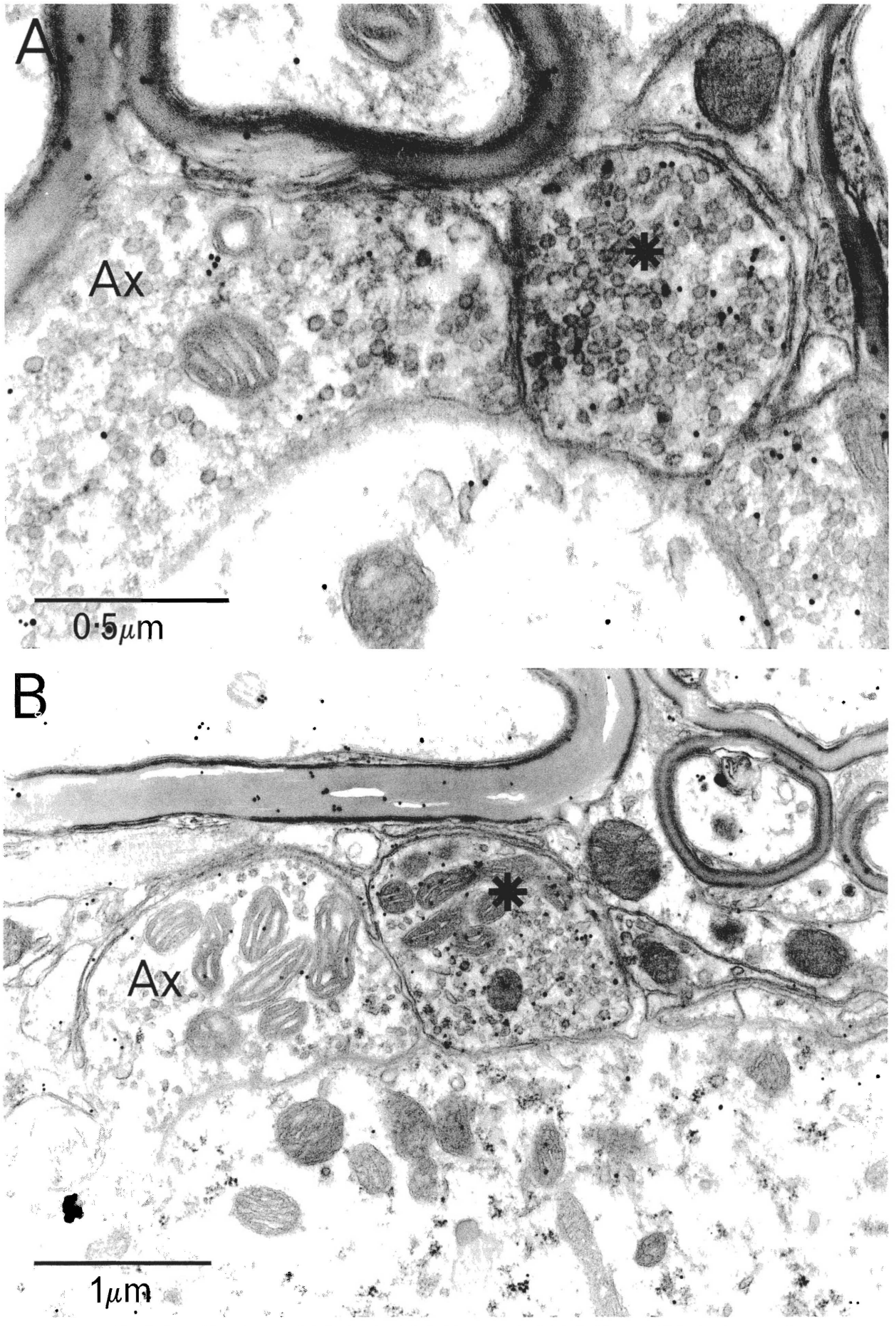

Fig. 9. Appositions between labelled and unlabelled boutons. A. A labelled bouton (*) is closely apposed to an unlabelled bouton containing circular vesicles. B. A labelled bouton (*) is closely apposed to an unlabelled bouton containing flattened vesicles. In both examples criteria for the identification of axoaxonic synapses are not fully satisfied.

it was obvious that vesicles were densely packed and irregular in shape; this seemed to be the case also with GAD although the density of reaction product obscured much of the detail. Broman and Westman[4] have described a population of GABA-containing boutons in the LCN which were identified by pre-embedding immunoreaction; this population had features in common with our GAD- and GABA-containing boutons. In studies by Somogyi *et al.*[38] of the cerebellar cortex, neocortex and hippocampus, staining properties of the GABA and GAD antisera were assessed. The authors concluded that both antibodies labelled identical populations of neurons and their terminals. The presence of GAD and GABA immunoreactivity has also been demonstrated in the same neurons of the hippocampus.[39] It seems probable, therefore, that the same population of boutons is labelled by the GAD and GABA antibody methods in the LCN.

Comparison of GABA-labelled terminals with unlabelled terminals

Boutons containing GABA appeared to form a distinct and homogeneous population within the LCN, regardless of their location in the nucleus; they were densely packed with irregular vesicles ($172.2/\mu m^2$), formed symmetrical synaptic junctions and were generally rather small (less than $2\,\mu m$ in diameter). Boutons displaying these characteristics were invariably observed to be labelled. In the study by Broman and Westman[4] of GABA labelled terminals in the LCN, they estimated that the average vesicle density was $82.5/\mu m^2$ in labelled boutons and $61.1/\mu m^2$ in unlabelled boutons. This latter figure corresponds well with our estimate of $62.6/\mu m^2$ for unlabelled boutons but our figure for GABA-containing boutons is considerably larger. This probably reflects a difference in the methods employed; the post-embedding method used in the present study is free from the encumbrance of dense reaction product which obscures much of the intracellular detail of labelled structures. Unlabelled boutons contained circular or irregular vesicles and formed symmetrical or asymmetrical synaptic junctions with dendrites or somata but, in general, the most obvious difference between them and boutons containing GABA was the density of vesicle packing.

Clearly, the unlabelled boutons form a heterogeneous population and probably originate from a variety of sources. Some of them are similar to the terminations of SCT cells described by Svensson *et al.*,[41] others may originate from the dorsal column nuclei, spinal trigeminal nucleus, reticular formation[9] or (in the ventromedial region) collateral axons of neurons projecting to the thalamus.[16] A proportion of the GABA terminals could arise from small cells in the ventromedial region of the nucleus which are known to contain GABA,[4] however, it is by no means certain if this is the only source of GABA in the LCN. At present little is known about the neurotransmitter content of the LCN; there is evidence for substance P-containing fibres in the medial portion of the nucleus,[2,17] therefore a small proportion of unlabelled boutons may contain this substance.

Postsynaptic targets of GABA-containing boutons

Terminals labelled with GABA and GAD were presynaptic to single dendrites or somata in the LCN and therefore probably are responsible for postsynaptic inhibition in the nucleus. It is probable that a proportion of the neurons contacted are thalamic projection neurons. Inhibitory receptive fields for such neurons have been described[8,11,19,26] but the nature of the inhibition and where it occurs in the pathway has not been investigated in any detail (but see the recent report by Brown *et al.*,[8] discussed below). Fedina *et al.*[14] reported that inhibitory postsynaptic potentials could be elicited in LCN neurons by stimulating appropriate receptive fields which are generally outside excitatory fields and are not of the "surround" type encountered in the dorsal column nuclei.[19] The presence of inhibitory postsynaptic potentials in LCN neurons therefore is consistent with our observations of synaptic associations between GABA-containing boutons and neurons in the nucleus.

The study by Fedina *et al.*[14] also produced evidence for presynaptic inhibition in the LCN. They observed that stimulation of contralateral cutaneous nerves and skin areas resulted in depolarization of SCT terminals and that the monosynaptic excitatory postsynaptic potentials from the SCT to the LCN could be reduced without depressing the resting membrane potential of LCN cells. No evidence for axoaxonic synapses involving GABA-containing terminals or indeed any other category of terminal was found in this study. Previous electron microscope studies of the LCN by Westman and colleagues[4,41,47] (including one of SCT terminals) also failed to demonstrate the presence of such synaptic arrangements. It is generally accepted that axoaxonic synapses are the morphological correlates of presynaptic inhibition;[21] therefore we would have expected to find such arrangements in the LCN. It is difficult to reconcile these apparently contradictory observations; the reduction of excitatory postsynaptic potentials observed by Fedina *et al.*[14] could be accounted for by a postsynaptic mechanism such as shunting inhibition, but the absence of axoaxonic contacts indicates that the changes in excitability of SCT terminals were not mediated by a chemical neurotransmitter but occurred as a consequence of some other process such as an accumulation of potassium ions in the extracellular space or even the generation of electrical fields within the nucleus (for a discussion see Ref. 13). This is an area which requires clarification.

The absence of axoaxonic synapses has also been noted for boutons which are presynaptic to identified SCT neurons;[28,29] therefore this may be a feature of

the organization of the spino-cervico-thalamic pathway.

Recurrent inhibition has been demonstrated in LCN neurons[19] following stimulation of the contralateral medial lemniscus and is thought to be mediated through collateral axons of projection cells which terminate in the nucleus. Flink and Westman[16] have suggested, on the basis of anatomical studies, that these collateral axons may terminate in the ventromedial part of the nucleus, possibly on GABA-containing putative local circuit neurons.[4] Therefore some GABA terminals in the LCN could participate in such a circuit. Peto[33] has shown that electrical stimulation of the facial regions of cortical area 3a causes profound inhibition in LCN neurons which project via the medial lemniscus, but the organization of this, and other inhibitory circuits which may utilize GABA as a transmitter in the nucleus, is at present obscure.

GABA-containing neurons in the lateral cervical nucleus

In the present study, we were unable to demonstrate the existence of GABA-immunoreactive neurons in the LCN. However, the study of Broman and Westman[4] indicated that GABA-immunoreactive neurons were present in the ventromedial part of the nucleus. This apparent discrepancy between our study and that of Broman and Westman[4] is best explained by differences in the methods employed. For example, it is probable that omission of pretreatment with colchicine resulted in the absence of labelled neurons in the pre-embedding study. A similar finding was reported by Blomqvist *et al.*,[2] who also used antiserum raised against GAD. Although the occasional neuron in the post-embedding study appeared to be positively labelled for GABA, the labelling was always equivocal; the density of gold particles associated with such structures was never as great as that associated with boutons. Thus our study neither refutes or confirms the claim that GABA-containing neurons are present in the LCN.

Function of the lateral cervical nucleus

The lateral cervical nucleus exists in a number of species including cats, dogs and several species of monkey (see Ref. 3 for a review) but its existence in man is still a matter of some controversy. The function of the LCN and, indeed, the spino-cervico-thalamic pathway, remains uncertain. It is one of the most rapidly conducting pathways in the CNS[5] and thus could be a rapid and general arousal system. The existence of very large receptive fields for some LCN neurons[8,11,24,26,27] lends support to this hypothesis. Information from receptors responding to light tactile and noxious stimuli is conveyed through the pathway[8,26] and so it probably contributes to the awareness of the external environment and the perception of pain. The LCN is somatotopically organized,[11,42,45] hence spatial representation of this information appears to be important. It is probable that this pathway subserves several functions.

Recently a series of experiments investigated sensory processing mechanisms in the LCN[8] and attempted to determine if information conveyed through the spino-cervico-thalamic pathway was modified by the nucleus. A similar series was performed on the SCT[3] which revealed new details of the organization of receptive fields. In particular, two types of inhibitory process could be demonstrated to depress excitation in the SCT. The first type of inhibition could be elicited by stimulating receptive fields which were adjacent to the excitatory fields of the SCT. This "out of field" inhibition had a short time course which appeared to correspond to a postsynaptic mechanism. The second type of inhibition came from within the excitatory field itself and had a long time course which appeared to correspond to presynaptic inhibition. Both "in field" and "out of field" inhibition could also be detected in the LCN but it was concluded that the LCN was simply relaying this pattern of organization which had already been laid down by the input circuitry of the SCT. LCN and SCT receptive fields had similar properties; the only difference was that some LCN fields were considerably larger than those of the SCT thus suggesting a degree of convergence onto LCN neurons. In the cat it is estimated that 3000 SCT axons enter the LCN[44] and that approximately 8000 LCN neurons project to the thalamus,[16] therefore divergence of SCT input would also be expected. The concept of a relay nucleus has largely been discredited (e.g. see review by Brown and Gordon[6]) and yet, at present, there is scant physiological evidence to suggest that the LCN performs any other function. Nevertheless, the present study clearly demonstrates that the LCN receives dense innervation from GABAergic neurons. The large numbers of GABA-containing terminals on LCN neurons indicates that information is not simply relayed through the nucleus but may be modified by postsynaptic inhibitory mechanisms, but the circuitry responsible for this inhibition and its effects upon the physiology of the LCN remain to be determined.

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