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Immunocytochemical Localization of Substance P in the Spinal Trigeminal Nucleus of the Rat: A Light and Electron Microscopic Study

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ABSTRACT

The neuropeptide substance P is a transmitter candidate for certain primary afferent fibers which terminate in the substantia gelatinosa. In this study the light and electron microscopic localization of substance P in the substantia gelatinosa of the spinal trigeminal nucleus of the rat has been studied using immunocytochemical procedures.

Substance P immunoreactive fibers were observed mainly in lamina I and outer lamina II. Ultrastructural analysis revealed immunoreactivity in unmyelinated fibers and in axon terminals which contained agranular spherical vesicles and large dense-cored vesicles and which made predominantly simple asymmetric axodendritic synaptic contacts. Immunoreactive terminals only rarely formed the central terminal of synaptic glomeruli and in only one example was a stained terminal possibly postsynaptic to an unstained terminal. The majority of synapses were onto small dendrites in outer lamina II and in some cases these dendrites were themselves presynaptic to other dendrites. Immunoreactive terminals also synapsed with the soma and proximal dendrites of large neurons on the border of laminae I and II.

The results show that there are at least two distinct targets for substance P immunoreactive terminals in the substantia gelatinosa, namely the large lamina I neurons and lamina II probable interneurons. Some of the former may be projection neurons while some of the latter may correspond to the inhibitory islet cells described by Gobel and colleagues in the cat. In addition the results indicate that few substance P immunoreactive terminals receive axoaxonic synapses and emphasize instead the role of postsynaptic interactions. In particular the results suggest several sites at which substance P might interact postsynaptically with the neuropeptide enkephalin.

The substantia gelatinosa of the spinal cord is an important area for processing sensory information and in particular is thought to be involved in pain control mechanisms (see Cervero and Iggo, '80; Wall, '80). As early as 1953 Lembeck proposed that the peptide substance P might be a sensory transmitter and there is now a large amount of data in support of such a role (for review see Nicoll et al., '80). In particular a link between anatomy and physiology has been provided by immunohistochemical studies which have shown that substance P is contained in primary afferent fibers which terminate in the substantia gelatinosa (Hökfelt et al., '75) and which can be affected by the sensory neurotoxin capsaicin (Jessell et al., '78). A large number of preliminary ultrastructural immunocytochemical studies support the postulated transmitter role for substance P in the substantia gelatinosa of the spinal cord by showing that the peptide in this area is contained in synaptic terminals (Hökfelt et al., '77; Pickel et al., '77;

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Cuello et al., '77; Chan-Palay and Palay, '77; Pelletier et al., '77) and a detailed study by Barber et al. ('79) has described the distribution and synaptic relationships of substance P axons in the rat spinal cord.

Light microscopic immunocytochemical studies show that the distribution of substance P immunoreactive fibers in the substantia gelatinosa of the spinal trigeminal nucleus of the rat is similar to that seen in the spinal cord (Cuello et al., '78; Del Fiacco and Cuello, '80) and in the cat Gobel and colleagues have proposed that a close homology exists between the spinal cord and the spinal trigeminal nucleus on the basis of various anatomical criteria (Gobel et al., '77). Preliminary ultrastructural studies have described the appearance of substance P immunoreactive terminals in the substantia gelatinosa of the spinal trigeminal nucleus (Cuello et al., '80; Sumal et al., '80) and suggest also that the organization of substance P-containing fibers in the substantia gelatinosa of the spinal cord and the spinal trigeminal nucleus is similar. However, no detailed study on the synaptic relationships of substance P immunoreactive terminals in the spinal trigeminal nucleus has been published. In this paper, therefore, we describe at light and electron microscopic level the detailed immunocytochemical localization of substance P in the spinal trigeminal nucleus of the rat. The preembedding staining protocol introduced for transmitter immunocytochemical studies by Pickel and colleagues ('75) has been adopted and employed in a way which provides a direct correlation between light and electron microscopic results (Priestley and Cuello, '82) and a specific monoclonal antibody has been used (Cuello et al., '79). Preliminary reports of these results have appeared (Cuello et al., '80; Priestley, '81).

MATERIALS AND METHODS

Light microscopy

Routine light microscopic immunocytochemical procedures were used as described in previous publications (Cuello and Kanazawa, '78; Cuello et al., '80; Priestley et al., '82). Transverse sections (10–20 μm) from a rat perfused with 4% paraformaldehyde were cut on a cryostat and incubated with 1/100 monoclonal rat anti-substance P antibody (NCl/34, Seralab Ltd U.K.). The primary antibody was then localized using either the indirect fluorescence (Weller and Coons, '54) or the peroxidase antiperoxidase (PAP; Sternberger, '79) procedures. All antisera were diluted in phosphate-buffered saline (PBS) containing 0.1% Triton X-100.

Electron microscopy

A large number of different fixation and incubation conditions were explored and detailed observations relating to methodology are presented elsewhere (Cuello et al., '80; Priestley and Cuello, '82). The protocol given below is therefore generalized and particular information on fixative composition and on any other variables is included in the main text. Except where otherwise stated, electron micrographs in figures show material from an animal perfused with 4% paraformaldehyde 0.5% glutaraldehyde and processed without Triton.

Male Wistar rats (200–250 gm) were anaesthetized with equithesin and perfused through the abdominal aorta with 15 ml 0.1 M phosphate buffer (pH 7.4) followed by 100 ml fixative delivered over a period of 20–30 minutes. Fixative consisted of 4% paraformaldehyde (BDH) and 0.0–0.5% glutaraldehyde (EMScope) in phosphate buffer. The brain was then dissected out and kept in fixative for a further 2 $^1/_2$ hours after which small blocks were cut out from the brainstem and transferred to phosphate buffer containing 30% sucrose. When completely infiltrated (after 6–8 hours) the blocks were given a rapid freeze/thaw by immersion in liquid nitrogen followed by immersion in buffer. Transverse sections (40 μm) were then cut in 0.1 M phosphate buffer at 4°C on a Vibratome (Oxford Instruments) and incubated in PBS containing 0.0–0.2% Triton X-100 for 1 hour. Immunostaining was then carried out as follows: 30 minutes in 10% normal rabbit serum (Cappel), 15-minute wash, 12 hours at 4°C in 1/150 monoclonal anti-substance P, 1-hour wash, 1 hour in 1/10 IgG fraction rabbit antirat IgG (Miles), 1-hour wash, 1 $^1/_2$ hours in 1/50 rat PAP (Cappel), 1-hour wash. Unless otherwise indicated all incubations were carried out at room temperature and wash and antibody solutions were in PBS. For localization of peroxidase, sections were then preincubated for 10 minutes in 0.06% DAB in 0.05M Tris HCl (pH 7.6) followed by 15 minutes in the same solution containing 0.01% H_2O_2. Sections were then washed for 30 minutes in 0.1 M phosphate buffer, stained in 1% OsO_4 for 1 $^1/_2$ hours, dehydrated, and embedded in Durkupan (Fluka). Sections were either flat embedded directly in TAAB capsules or flat embedded on glass slides and covered with a plastic coverslip (Bel-ART, USA). After light microscopic examination such slide mounted sections were reembedded in EM capsules. Prior to thin sectioning semithin (2 μm) sections were taken and stained with Toluidine/Azur II. Groups of serial thin sections were mounted on formvar-coated single slot grids and some grids in a sequence were contrasted with lead citrate. Profiles were studied in the uncontrasted as well as in the serial contrasted sections. Phillips 201 and Jeol 100B electron microscopes were used and uncontrasted sections were examined using low-voltage (HT 40 kV) and small-aperture (object lens 20 μm) settings. For light microscopic examination of flat-embedded and semithin sections a Leitz Dialux 20 equipped with brightfield, phase, and interference contrast illumination was employed.

Colchicine

A group of animals received 100 μg colchicine (Sigma) in 25 μl saline injected stereotaxically into the lateral or the IVth ventricle. Animals were perfused 24 hours after injection and processed for light microscopy.

Immunostaining controls

The monoclonal antibody employed in this study has been characterized by radioimmunoassay and shown to cross react to identity with C terminal fragments of substance P and by 5% with the related nonmammalian peptide eledoisin. Full characteristics of this antibody have been reported elsewhere (Cuello et al., '79). Specific immunostaining is abolished by preadsorption of the antibody with 200 μg/ml synthetic substance P or by omitting the primary antibody in the staining sequence, but staining is unaffected by preincubation with other peptides found in the substantia gelatinosa such as CCK-8, leu enkephalin, met enkephalin, or somatostatin.

RESULTS

Light microscopy

Immunofluorescence or PAP processed cryostat sections showed an arc of staining in the substantia gelatinosa of subnucleus caudalis of the spinal trigeminal nucleus. Dif-

fuse staining was seen also in the adjoining nucleus reticularis lateralis. Caudally the arc of staining diminishes in size and bunches up in the dorsal quadrant to become continuous with the substantia gelatinosa of the cervical spinal cord while rostrally the arc of staining gives way to discrete clumps located within the spinal tract. For detailed light microscopic analysis and for the preparation of electron microscopic material sections were taken at a level of nucleus caudalis where the arc of substance P immunostaining was greatest (Fig. 1). On the outer border of the substantia gelatinosa fibers were seen running for some way in a transverse plane parallel to the arc of the gelatinosa. Such fibers were particularly prominant in colchicine-treated animals and were occasionally seen to be continuous with immunoreactive fibers in the spinal tract (Fig. 2). In colchicine-treated animals immunoreactive cell bodies were seen in the nucleus reticularis lateralis but not in the substantia gelatinosa.

In order to ascertain the distribution of immunostaining in terms of the classic cytoarchitectonic laminations described by Rexed ('52) in the cat, semithin (2 μm) plastic sections were examined and serial sections stained with Toluidine/Azur II. On the basis of the Toluidine/Azur II-stained sections the following laminations were identified in the rat (Fig. 3). Outermost lies lamina I, just inside the spinal tract and corresponding to the marginal zone described by Olszewski ('50). The lamina contains a number of quite large cells, often long portions of large dendrites, and also transversely cut isolated myelinated fibers. The lamina also contains bundles of myelinated fibers which run parallel to the axis of the brainstem and are referred to by Gobel and Purvis ('72) in the cat as the deep bundles. In contrast, lamina II is almost completely devoid of individual myelinated fibers, contains many small cells and a mass of small dendrites, and is equivalent to the substantia gelatinosa. Both laminae contain bundles of mye-

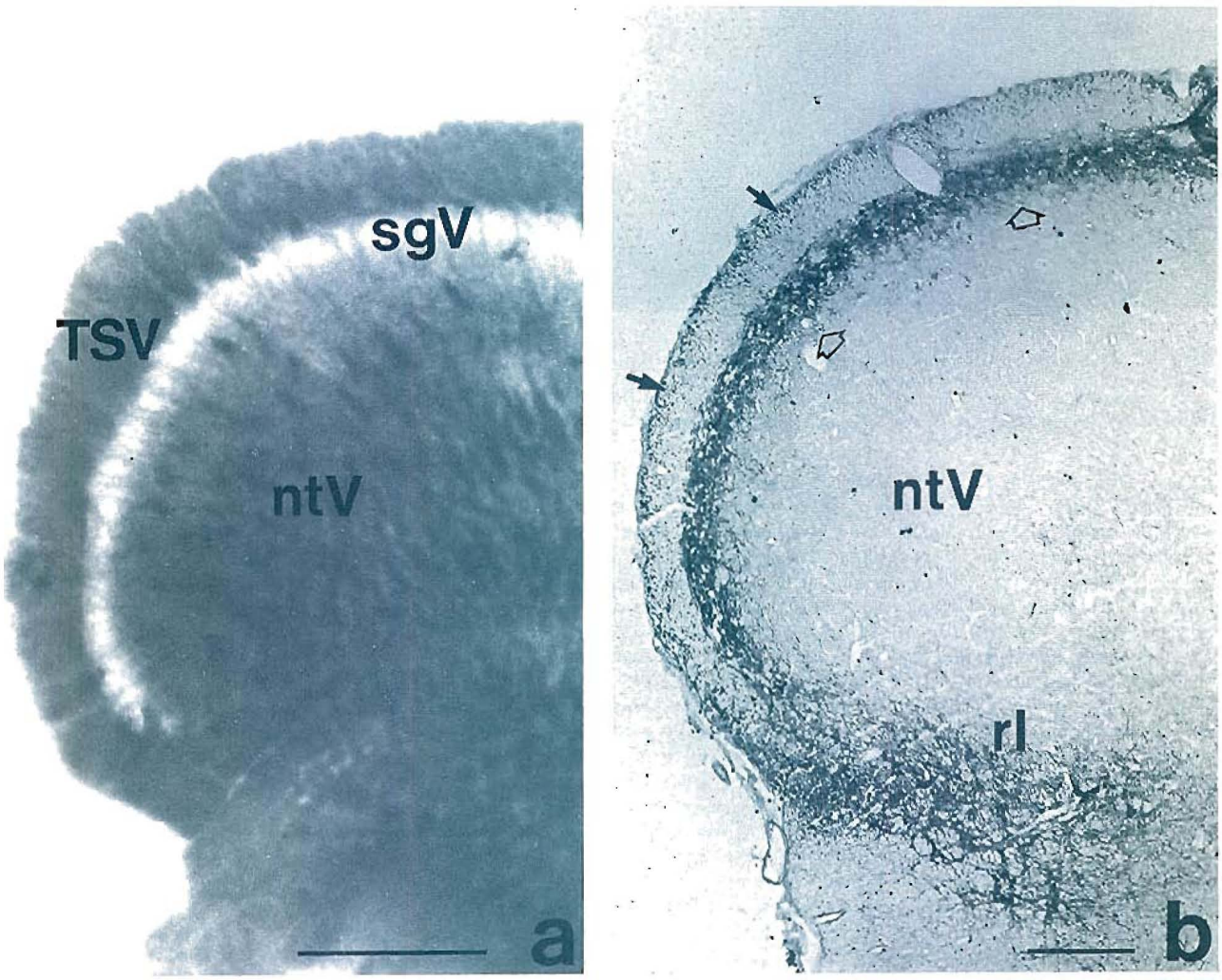

Fig. 1. Light micrographs showing the appearance of the spinal trigeminal nucleus pars caudalis in transverse sections at the level at which sections for EM processing were taken. a. Unfixed, unstained 200 μm tissue chopper section. The translucent band of the substantia gelatinosa (sgV) can be seen clearly. TSV, tractus spinalis nervi trigemini; ntV, nucleus tractus spinalis. b. Cryostat section (10 μm) processed for light microscopic substance P immunostaining as described in Methods. Colchicine-pretreated animal, PAP immunocytochemistry. The substance P immunostaining follows the arc of the substantia gelatinosa (hollow arrowheads) but extends also diffusely into the nucleus reticularis lateralis (rl). On the outer border of the spinal tract transversely cut immunostained axons can be seen (arrows). Scale bars = 200 μm.

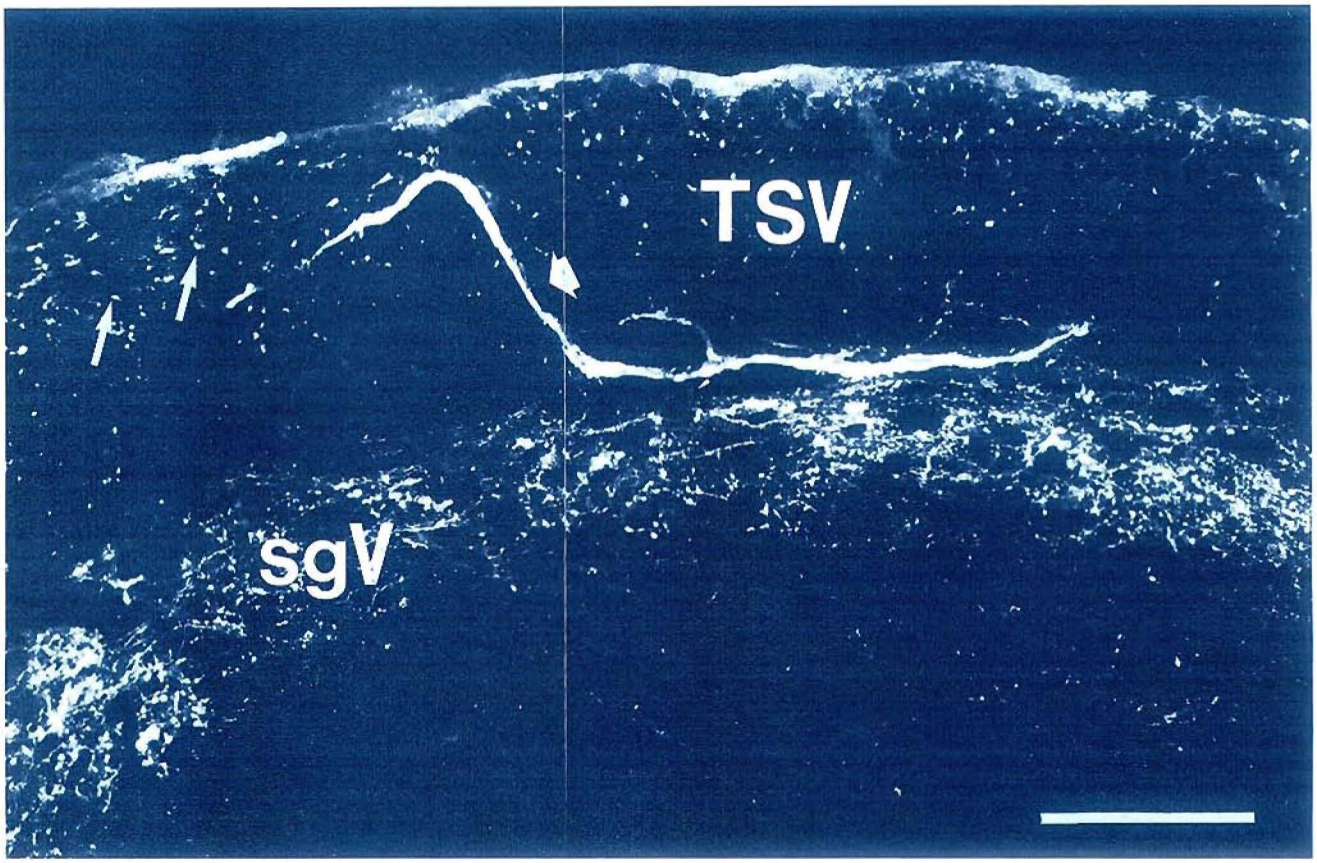

Fig. 2. Light micrograph showing the distribution of substance P immunofluorescence in a colchicine-treated animal processed for light microscopy as described in Methods. Heavy immunostaining is seen in the substantia gelatinosa (sgV) and in the cut ends of axons (arrows) in the spinal tract (TSV). A bundle of fibers (arrowhead) passes from the spinal tract and runs in an arc between the tract and the substantia gelatinosa. Scale bar = 100 μm.

linated fibers which course radially and enter the deeper laminae. Lamina III, deep to lamina II, is generally marked by the reappearance of large numbers of transversely cut myelinated fibers which occur both singly and in large groups. The division of the caudal medulla into laminae follows the homology between spinal trigeminal and spinal cord proposed by Gobel and colleagues ('77) in the cat and the substantia gelatinosa has been designated lamina II in line with the majority of recent studies on the spinal cord (see Cervero and Iggo, '80). The heaviest substance P immunostaining was seen in lamina I and the outer two-thirds of lamina II and on this basis lamina two is further subdivided into outer (II_o) heavily stained and inner (II_i) lightly stained areas (Fig. 3). Some immunostaining occurred also in deeper laminae. The thin sections used for electron microscopic analysis were immediately serial to the Toluidine/Azur II-stained semithin sections and so the position of any profile observed in the electron microscope can be identified in the light microscope on the stained semithin sections. Hence the position of immunostained structures described in the following pages will be presented with respect to the laminations as defined above.

Electron microscopy

The surface of the Vibratome sections show poor ultrastructural preservation and nonspecific background staining (see Priestley and Cuello, '82) and so thin sections for detailed analysis were always taken 5 μm or more into the section. Examination of uncontrasted sections revealed immunostaining scattered sparcely throughout laminae I and II and stained structures were easily visible against

Fig. 3. Distribution of substance P immunoreactivity in the various laminae of the substantia gelatinosa of the spinal trigeminal nucleus. Figures (a–c) show equivalent areas of a single tissue section processed for EM immunocytochemistry as described in Methods and photographed either directly in the EM embedding capsule (a) or in the semithin sections cut from the block prior to thin sectioning (b and c). 4% paraformaldehyde 0.5% glutaraldehyde fixative, no triton. a. Cut surface of the immunostained tissue section embedded in an EM capsule and illuminated by transmitted light. Approximate boundaries of laminae I and II are shown by dashed lines. Immunostaining is heaviest in the outer region of laminae II (II_o) while the inner region (II_i) shows relatively sparce staining. Ax indicates bundles of myelinated axons. b. Semithin (2 μm) section taken from the block shown in (a) and stained with Toluidine/Azur II to reveal the general cytoarchitecture. Using such a section and on the basis of the criteria described in the main text, the boundaries of laminae I and II as shown were assigned. The two myelinated fiber bundles shown in (a) marked Ax can be used as reference points to help relate features shown in (a), (b), and (c). c. Semithin section serial to (b) and observed with phase-contrast illumination. Under such conditions the immunostaining shows clearly and as in (a) is seen to be confined mainly to the outer region of lamina II. Thin sections for electron microscopic examination were taken serial to the semithin sections and small arrows in (b) and (c) show the exact position of various profiles observed in this particular tissue section and described in other figures of this paper. Numbers indicate the relevant figure number. TSV, tractus spinalis nervi trigemini. Scale bar = 50 μm.

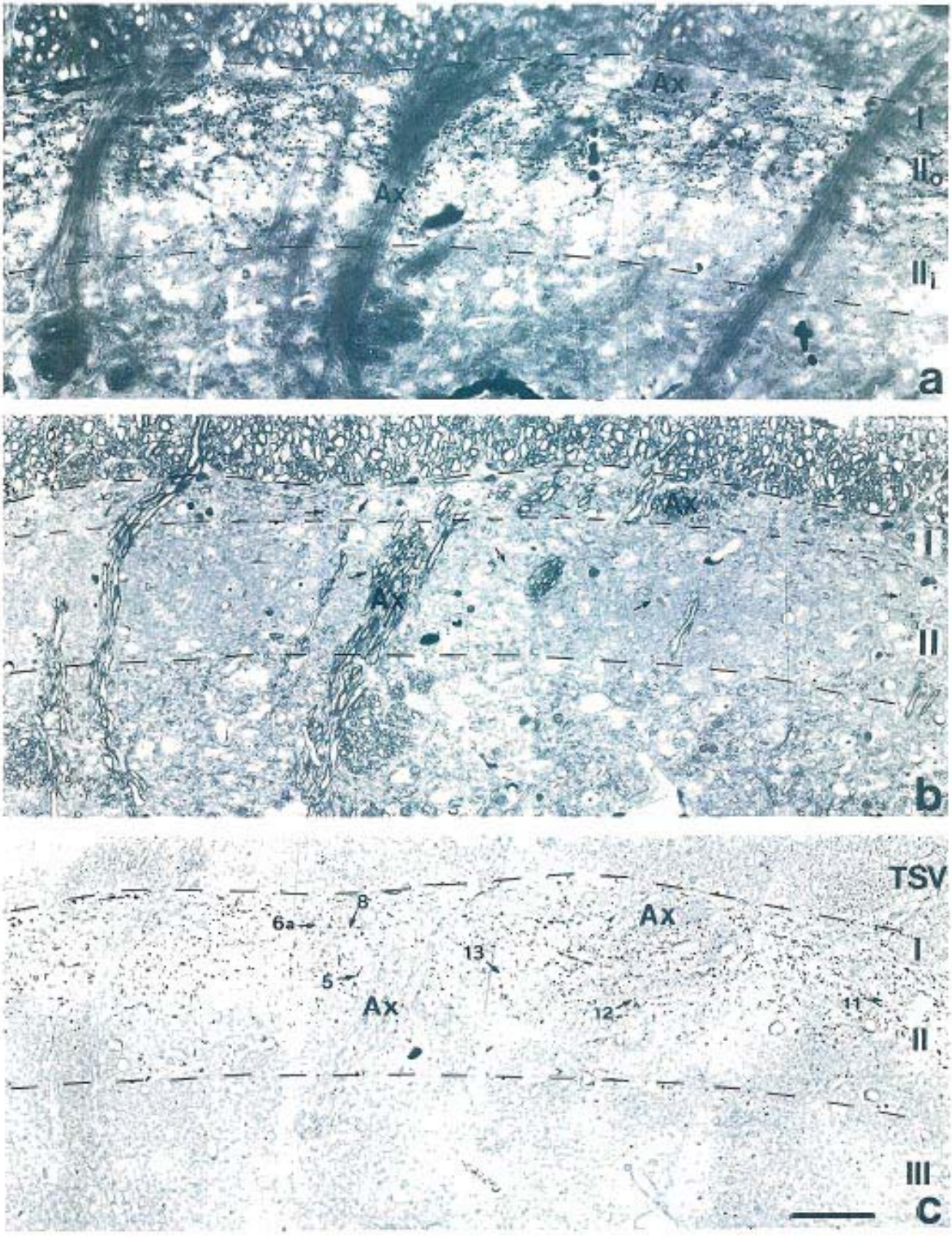

Figure 3

the uncontrasted background. In the lead citrate-contrasted serial sections the same profiles were less clearly visible against background but showed greater ultrastructural detail (Fig. 4). For this reason profiles were always examined in both contrasted and uncontrasted sections.

Axons. In lamina I immunoreactive axons cut in longitudinal section were observed. Such processes were unmyelinated, approximately 0.2 μm in diameter, and frequently several profiles were seen in a group. Immunostaining was never seen in myelinated axons. Immunoreactive unmyelinated axons cut in transverse section were also seen and occurred most frequently in lamina II where they sometimes formed bundles of six or seven axons which ran for some distance parallel to the axis of the brainstem (Fig. 5). Stained axons contained few small vesicles but frequently contained several large (100–120 nm) vesicles. Large vesicles were observed both with and without an obvious dense core and both with and without staining of the dense core (Fig. 5). Occasionally much larger heavily stained structures packed with vesicles were seen, probably representing axonal varicosities cut in transverse section.

Terminals. Many immunoreactive terminals were seen in lamina II and these exhibited various shapes and synaptic arrangements. Heavily stained profiles were frequently observed which were packed with small agranular vesicles (30–50 nm) and the occasional large dense-cored vesicle and which adjoined unstained terminals and dendrites but did not make any identifiable synapses (Fig. 6a). However, stained terminals were also frequently seen which made synaptic contacts and these were generally of asymmetric configuration although the degree of asymmetry varied (Fig. 4, 6b, 7, 8, 11). Occasionally prominent synaptic specializations were evident in the form of subjunctional dense bodies (Fig. 6b). Stained terminals normally made a single axodendritic synapse and frequently the terminals were of a simple dome shape (Fig. 6b). Immunoreactive terminals were observed close to blood vessels and glial cells (Fig. 6c,d) but membrane specializations or vesicle accumulations were not observed at contact points between stained terminals and nonneuronal profiles. Terminals always contained large numbers of agranular spherical vesicles with prominent membrane staining and usually also a few large dense core vesicles. Where large dense-cored vesicles were absent some could usually be located in neighboring serial sections and seemed generally to be most concentrated away from the area of synaptic specialization (Fig. 7). Immunoreactive terminals

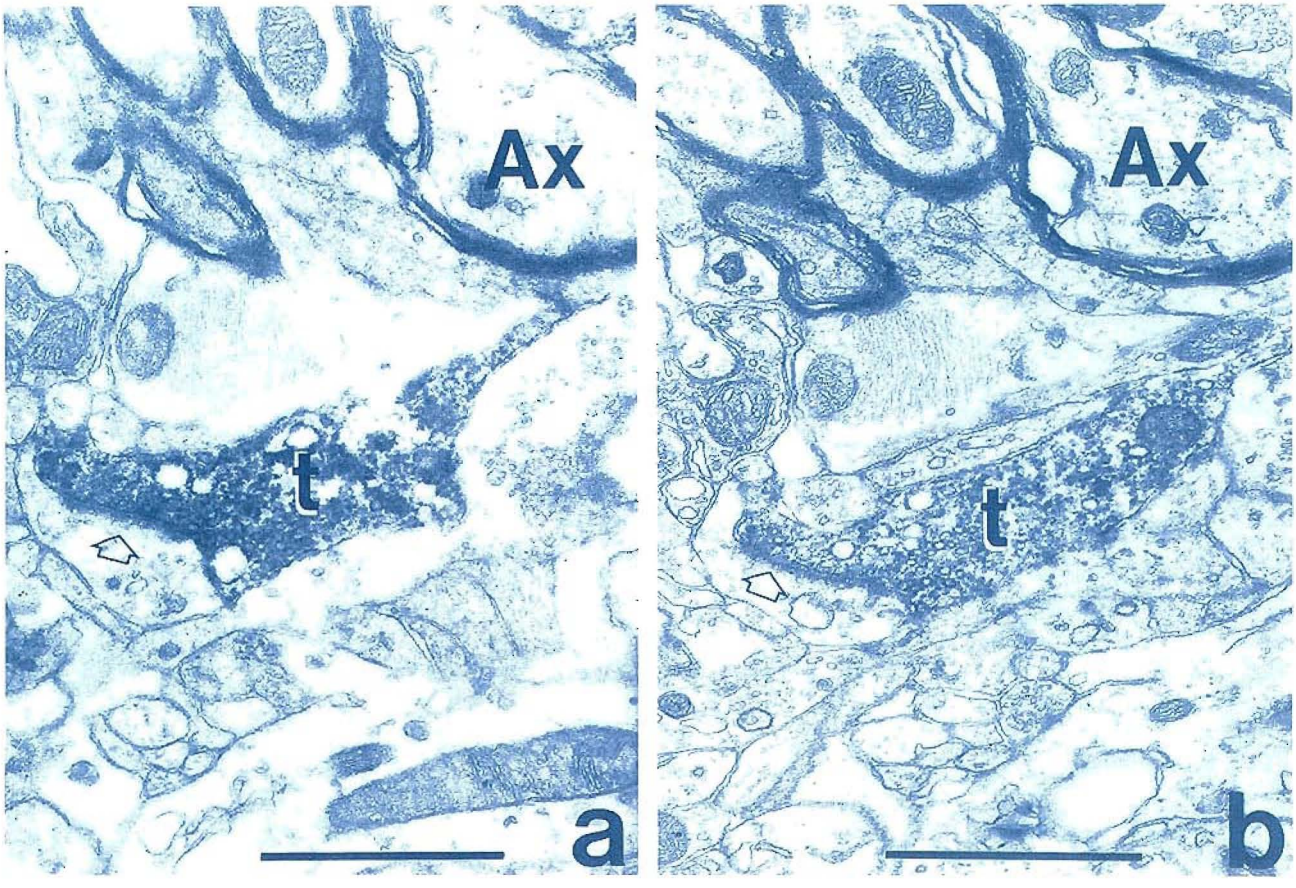

Fig. 4. Electron micrographs showing the appearance of an immunostained profile seen in uncontrasted (a) and contrasted (b) semiserial sections. Inner region of lamina II_o. a. Uncontrasted section. The immunostained terminal (t) can be seen clearly against the relatively indistinct background features. The terminal makes asymmetric synapse (clear arrowhead) with a probable dendritic spine. Ax indicates a myelinated axon. b. Contrasted section semiserial to (a). Lead citrate contrasting allows greater resolution of fine detail compared to (a). The immunostaining of the terminal (t) is less prominent but indicated by the characteristic granular reaction deposit in the axoplasm. Scale bar = 1 μm.

synapsed with both large and small dendrites and occasionally also with dendritic spines. Large dendrites in synaptic contact with a stained terminal were seen to receive synaptic input from unstained terminals and sometimes also from other stained terminals. Unstained terminals were either similar to the stained ones or contained pleomorphic vesicles and made symmetric synaptic contact (Fig. 8b). Terminals with prominent elliptical or flattened vesicles were never stained. It was generally not possible to characterize the structures postsynaptic to the substance P immunoreactive terminals further than to identify them as dendrites. However, certain more interesting synaptic arrangements were noted and these are described in greater detail below.

On the border of laminae I and II_o immunostained terminals were observed in synaptic contact with large dendrites cut in longitudinal section and running in a transverse plane parallel to the arc of the substantia gelatinosa. Stained terminals had agranular spherical vesicles and made asymmetric contact as described above and the large dendrites also received synapses from various unstained terminals (Fig. 8). In one case the plane of section cut through the cell of origin of the dendrite revealing a large oval cell body some 8–15 μm in diameter (Fig. 9). This cell also received an immunostained axosomatic synapse. The proximal dendrite of the cell was about 1.5 μm in diameter and could be followed for 40 μm in the plane of section. On the basis of its size, position, and dendritic orientation it is proposed that this cell and the other similar large dendrites belong to the class of large lamina I or marginal neurons described in the spinal cord of the gorilla originally by Waldeyer (1888). Immunoreactive terminals were also very occasionally seen on large dendrites situated deeper in lamina II and orientated radial to the lamina but the origin of these dendrites could not be determined.

A special search was made for immunoreactive terminals in contact with lamina II (substantia gelatinosa) neuron cell bodies and proximal dendrites. Small cells, ranging in size from 4 to 13 μm, were frequently seen arranged in groups and with many stained terminals situated amongst them. Stained terminals were in close contact with the cells and displayed prominent parallel membrane appositions and local accumulations of vesicles but synaptic specializations were only seen occasionally. When present,

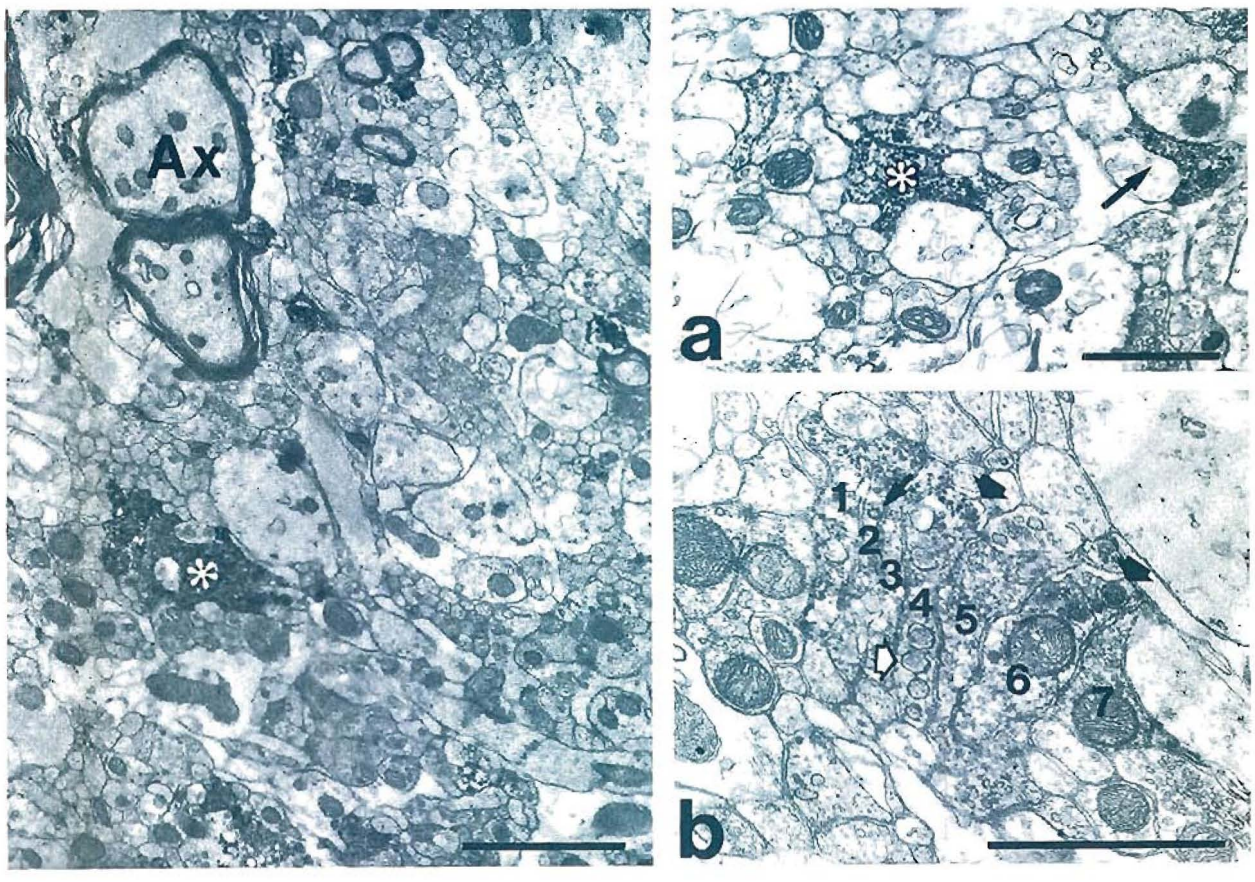

Fig. 5. A large bundle of immunoreactive unmyelinated fibers (asterisk) is shown in an uncontrasted section. The exact position of the bundle within lamina II is shown in Figure 3. Scale bar = 2 μm. Insets (a) and (b) show the bundle at high magnification. a. The bundle was traced in a long series of thin sections and seen to run parallel to the axis of the brain stem. In the section shown here one component of the bundle is now located separately from it (arrow). Scale bar = 1 μm. b. Examination of a semiserial contrasted section shows the bundle to consist of seven distinct axonal profiles (1–7). The axons contain large dense-cored vesicles and also occasional small agranular vesicles (arrow). The large vesicles are seen both with (black arrowhead) and without (hollow arrowhead) immunostaining of their dense cores. Scale bar = 1 μm.

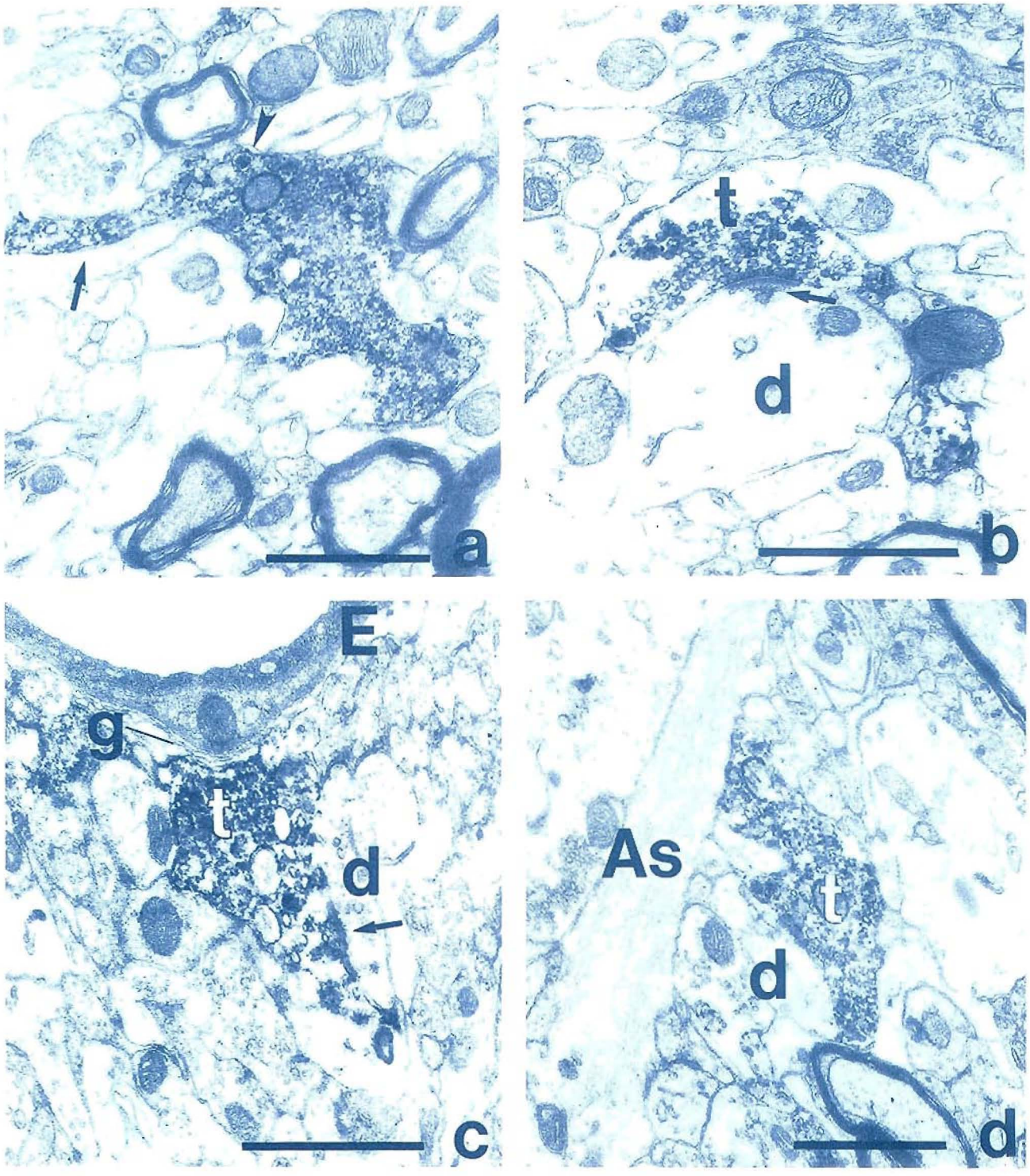

Fig. 6. Characteristics of substance P immunoreactive terminals. Scale bars = 1 μm. a. Immunoreactive vesicle-packed varicosity on the border of laminae I and II. The exact position of this structure is shown in Figure 3. Arrow shows probable intervaricose axon segment and arrowhead indicates an immunostained large dense-cored vesicle. b. A dome-shaped immunoreactive terminal (t) in synaptic contact with a small dendrite (d). A subsynaptic specialization can be seen (arrow). c. An immunostained terminal (t) is seen close to a blood vessel. However, the terminal is separated from the lumen of the vessel by an endothelial cell (E), the basal lamina, and by several glial lamellae (g). The terminal synapses (arrow) with a neighboring dendrite (d). d. A stained terminal (t) adjoins an astroglial process (As). No membrane specialization between the two profiles is evident and the terminal probably synapses with a dendrite (d).

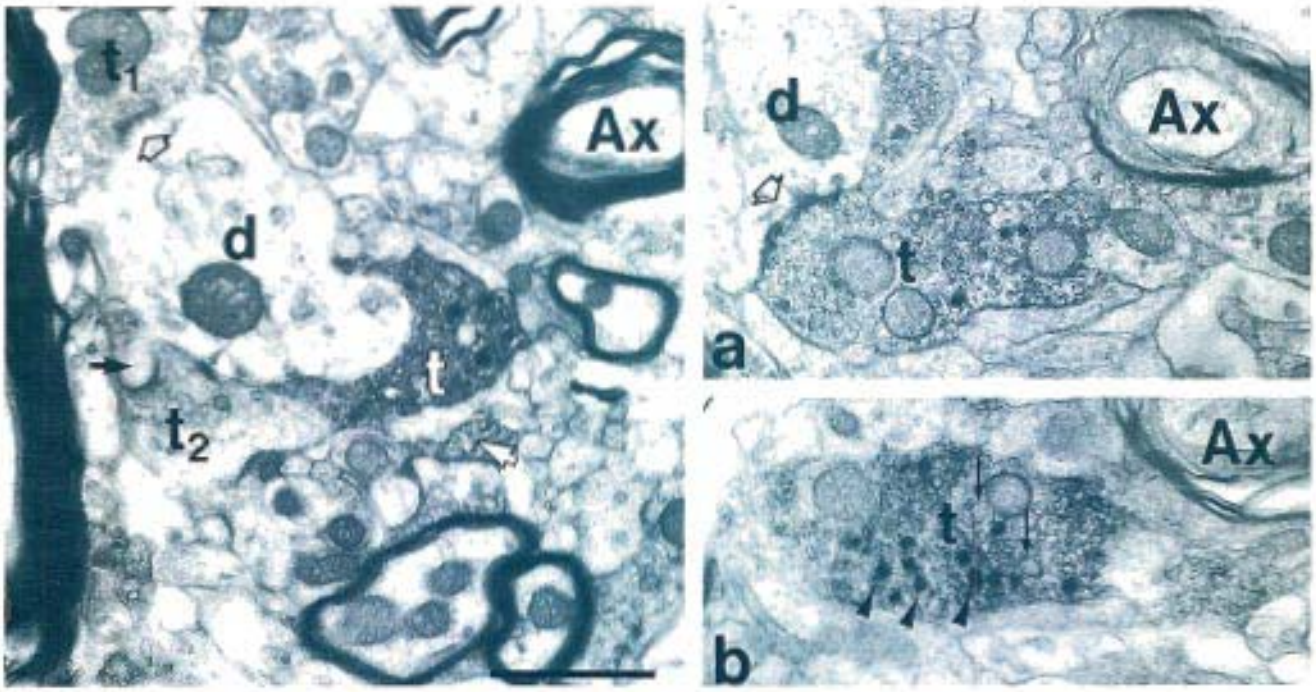

Fig. 7. A heavily immunostained terminal (t) partially envelops a dendrite (d) but makes no clear synapse. The dendrite receives synapses from two unstained terminals (t_1,t_2), one of which (t_2) contacts a dendritic spine (black arrow). White arrow indicates immunoreactive preterminal axon segment. Scale bar = 1 μm. Insets (a) and (b) show the same terminal identified in subsequent thin sections. a. The terminal makes a synapse with a dendrite (d) and is seen to contain mainly agranular unstained small vesicles. b. Away from the synaptic contact area the terminal displays very many large vesicles, some of which have prominent stained cores (black arrowheads). Other large vesicles are unstained (arrows). A myelinated axon which can be used as a landmark in each of the three plates is shown (Ax).

synaptic thickenings lasted for only a few serial sections. The same neurons received occasional synapses from unstained terminals. Contacts between immunoreactive terminals and slightly larger and more isolated neurons in lamina II were also observed (Fig. 10).

In lamina II membrane fragments and isolated vesicles were commonly seen in structures postsynaptic to axon terminals. Occasionally an immunostained terminal synapsed with a structure which was itself clearly presynaptic to a third profile, thus forming a serial synaptic arrangement (Fig. 11). The second profile had a localized accumulation of pleomorphic vesicles close to the presynaptic membrane (Fig. 11), and thus is probably a presynaptic dendrite. Less frequently another type of arrangement was seen. On the border of laminae II_o and II_i large sinusoid or scalloped immunoreactive terminals were observed which made synaptic contact with several dendrites (Fig. 12) in a similar way to that described for the central terminal of the characteristic substantia gelatinosa glomeruli (Réthelyi and Szentágothai, '69; Gobel, '74). In one example an unstained profile containing large round vesicles contacted the stained central terminal and the unstained profile appeared to be presynaptic to the stained terminal (Fig. 12).

In addition to the arrangements between vesicle-containing structures described in the previous paragraph, a third type of association was sometimes seen in lamina II. A stained profile and an unstained profile with rather similar vesicle populations were observed in close association and such that in certain sections the stained profile surrounded the unstained one (Fig. 13). No obvious synapses between the two structures occurred but both made synaptic contacts with neighboring dendrites.

DISCUSSION

Distribution of substance P immunoreactive fibers

The rostrocaudal distribution of substance P immunostaining in nucleus caudalis of the spinal trigeminal described in this paper agrees well with previous reports (Cuello and Kanazawa, '78; Ljungdahl et al., '78) as does the general localization of staining to the substantia gelatinosa and nucleus reticularis lateralis (Cuello et al., '78; Del Fiacco and Cuello, '80). The pattern of staining in the spinal trigeminal has not previously been described in terms of laminae but our results do agree with recent studies in the spinal cord showing that substance P fibers are most dense in lamina I and outer lamina II (Hunt et al., '81). The majority of substance P immunoreactive fibers in the substantia gelatinosa of the spinal trigeminal are probably of primary afferent origin, although a small proportion may also be derived from substance P-containing interneurons and from brainstem raphe neurons. Del Fiacco and Cuello ('80) obtained an almost complete disappearance of substance P immunostaining in the spinal trigeminal following electrolytic lesion of the Gasserian ganglion. The distribution of immunostaining observed in this study does correspond to the known pattern of termination of primary afferents and in addition fits in well

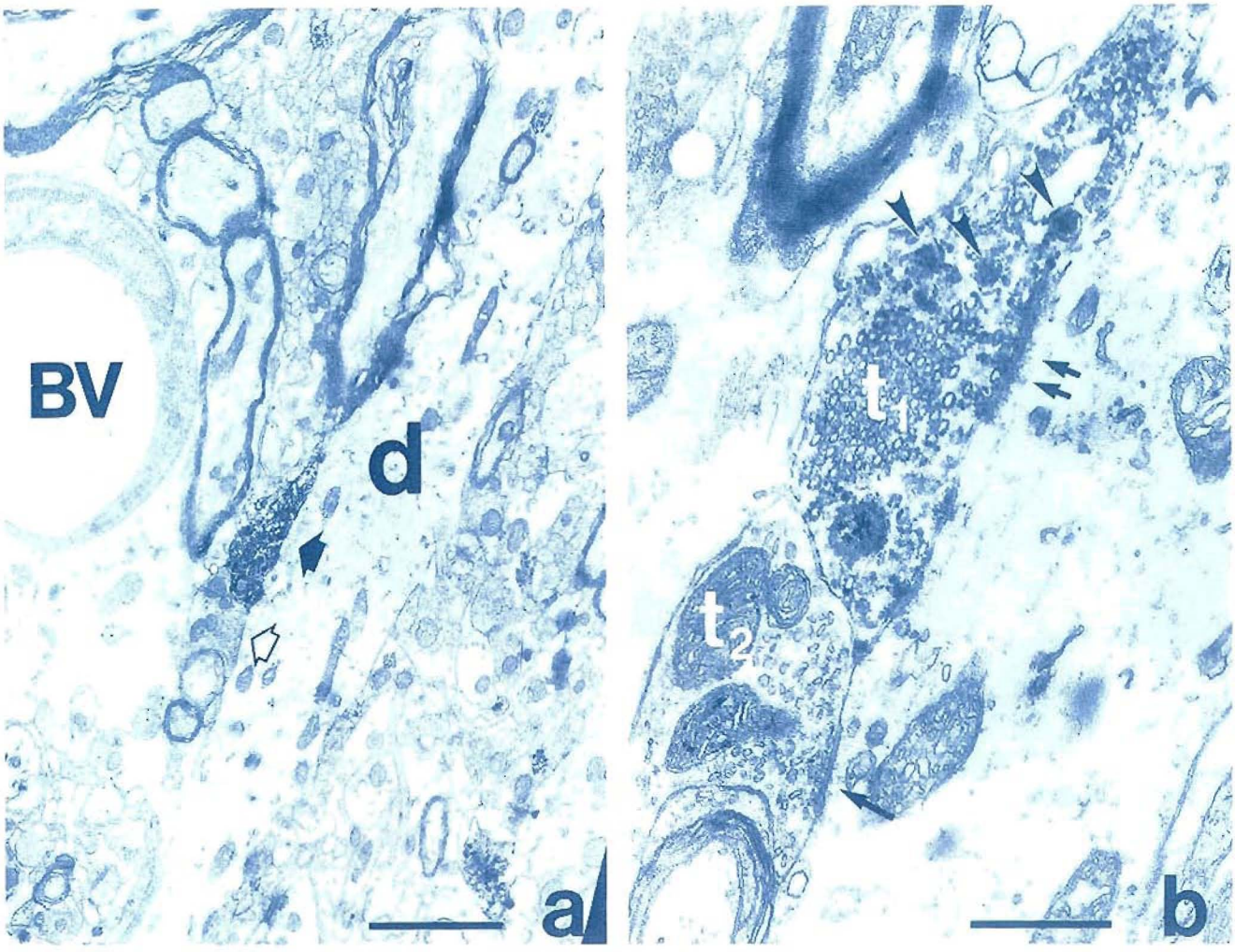

Fig. 8. Immunorective terminal in synaptic contact with a large lamina I dendrite. a. A large dendrite (d) on the border of lamina I and II makes contact with a stained (black arrow) and an unstained (hollow arrow) terminal. The exact position of the dendrite is shown in Figure 3. BV indicates blood vessel. Scale bar = 2 μm. b. Examination at high magnification of the terminals shown in (a) reveals that the stained terminal (t_1) contains small round agranular vesicles, a few stained large dense-cored vesicles (arrowheads), and makes asymmetric synaptic contact (two small arrows). In serial sections a prominent subsynaptic specialization was seen. The unstained terminal (t_2) contains pleomorphic vesicles and makes symmetric synaptic contact (single arrow). In serial sections no synaptic contact between t_1 and t_2 was seen. Scale bar = 0.5 μm.

with the generally accepted conclusion that substance P containing afferent fibers are of small diameter. Thus the localization of immunostaining in lamina I and outer lamina II matches the termination field of small-diameter primary afferents described in the spinal trigeminal and spinal cord of the cat (Light and Perl, '79; Arvidsson and Gobel, '81) and the rostrocaudal extent of staining matches that described for chemosensitive, presumably small-diameter, fibers in the rat (Jancsó and Király, '80). However, it is not clear whether the substance P-containing afferents are myelinated or unmyelinated. Studies with the sensory neurotoxin capsaicin suggest that in the spinal cord the majority of substance P immunoreactive fibers are unmyelinated (Nagy et al., '81) and this is in accordance with many anatomical studies which indicate that the major afferent input to lamina II is from C fibers (for review see Cervero and Iggo, '80). The ultrastructural results in this paper show substance P immunoreactivity in unmyelinated fibers and are in agreement with the results of several previous ultrastructural immunocytochemical studies in the spinal cord (Pickel et al., '77; Barber et al., '79). However, Gobel and colleagues have concluded that the major input to lamina II of the spinal trigeminal of the cat is from small myelinated Aδ fibers and that unmyelinated C fibers terminate mainly in lamina I (Gobel and Binck, '77; Gobel, '78b). Chan-Palay and Palay ('77) in the spinal cord and Sumal et al. ('80) in the spinal trigeminal have described substance P immunoreactivity in finely myelinated fibers. In our ultrastructural studies very few fibers were observed actually in the spinal tract and those seen in laminae I and II may represent preterminal or intervaricose portions of myelinated fibers which have lost their myelin sheath after leaving the spinal tract.

Subcellular localization of immunostaining

In this study reaction deposit was observed over large dense core vesicles, the limiting membranes of small agranular vesicles, mitochondrial membranes, and also

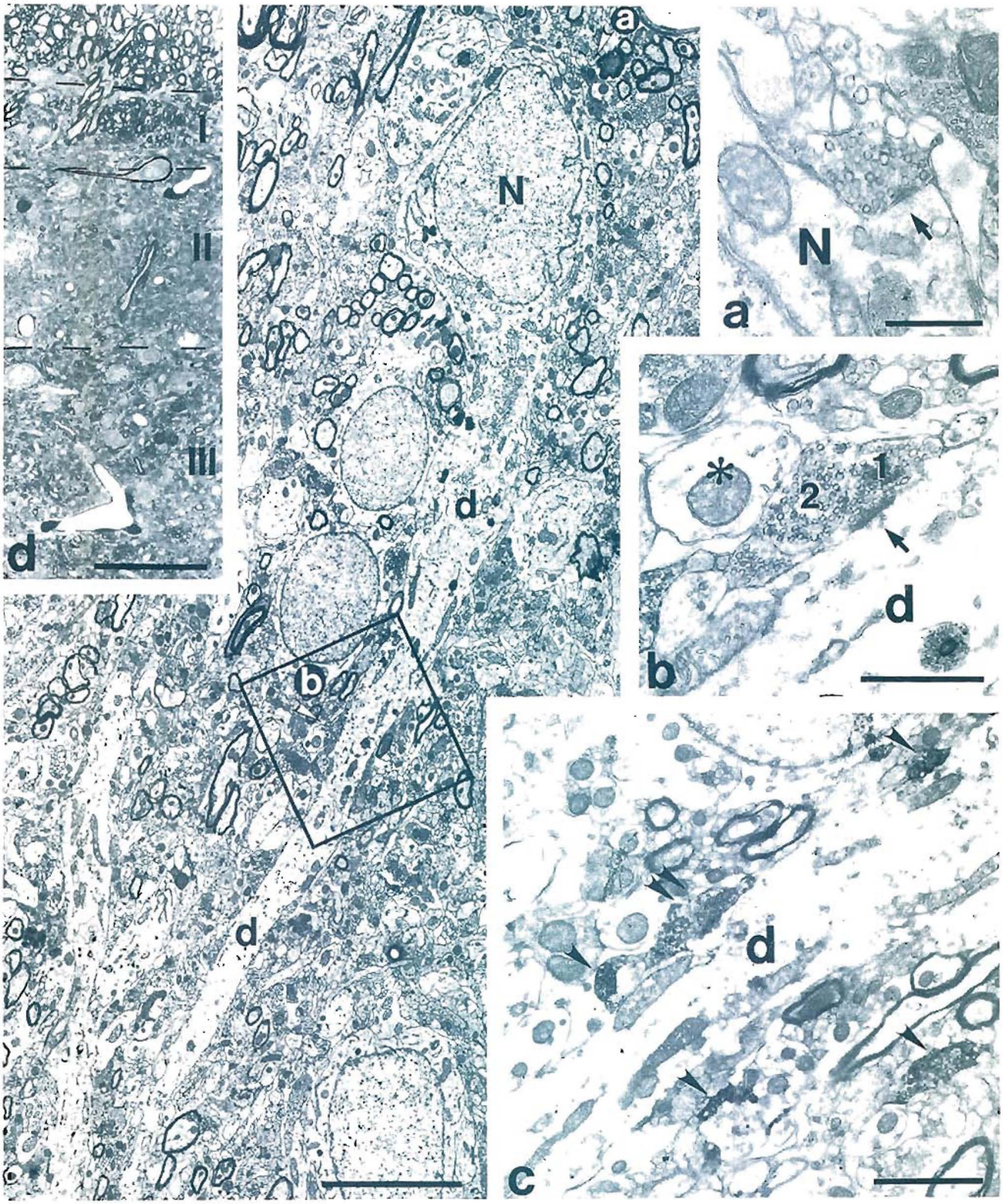

Fig. 9. Synaptic contacts on a lamina I neuron. A neuron on the border of lamina I and II is shown cut in a plane which reveals a large nucleus (N) and long proximal dendrite (d). The neuron receives two synapses from immunoreactive terminals, one (a) on the cell body and one (b) on the dendrite. These terminals are shown at high magnification in insets (a–c). The square indicates the area shown in inset (c). Scale bar = 1 μm. a. A lightly stained terminal makes synaptic contact (arrow) with the cell body (N). Scale bar = 0.5 μm. b. Two adjoining lightly immunostained terminals (1, 2) are shown. One of them (1) synapses (arrow) with the proximal dendrite (d) of the lamina I neuron. The other terminal (2) contacts a neighboring dendrite (asterisk) and in serial sections a clear synapse was seen. Scale bar = 1 μm. c. Uncontrasted section serial to (b). Various immunostained profiles are indicated (arrowheads) including the terminals (two arrowheads) labeled 1 and 2 in (b). Use of such uncontrasted serial sections allows lightly immunostained features to be easily detected. Scale bar = 2 μm. d. Light micrograph showing exact position of the neuron (outlined) on the border of lamina I and II. Scale bar = 50 μm.

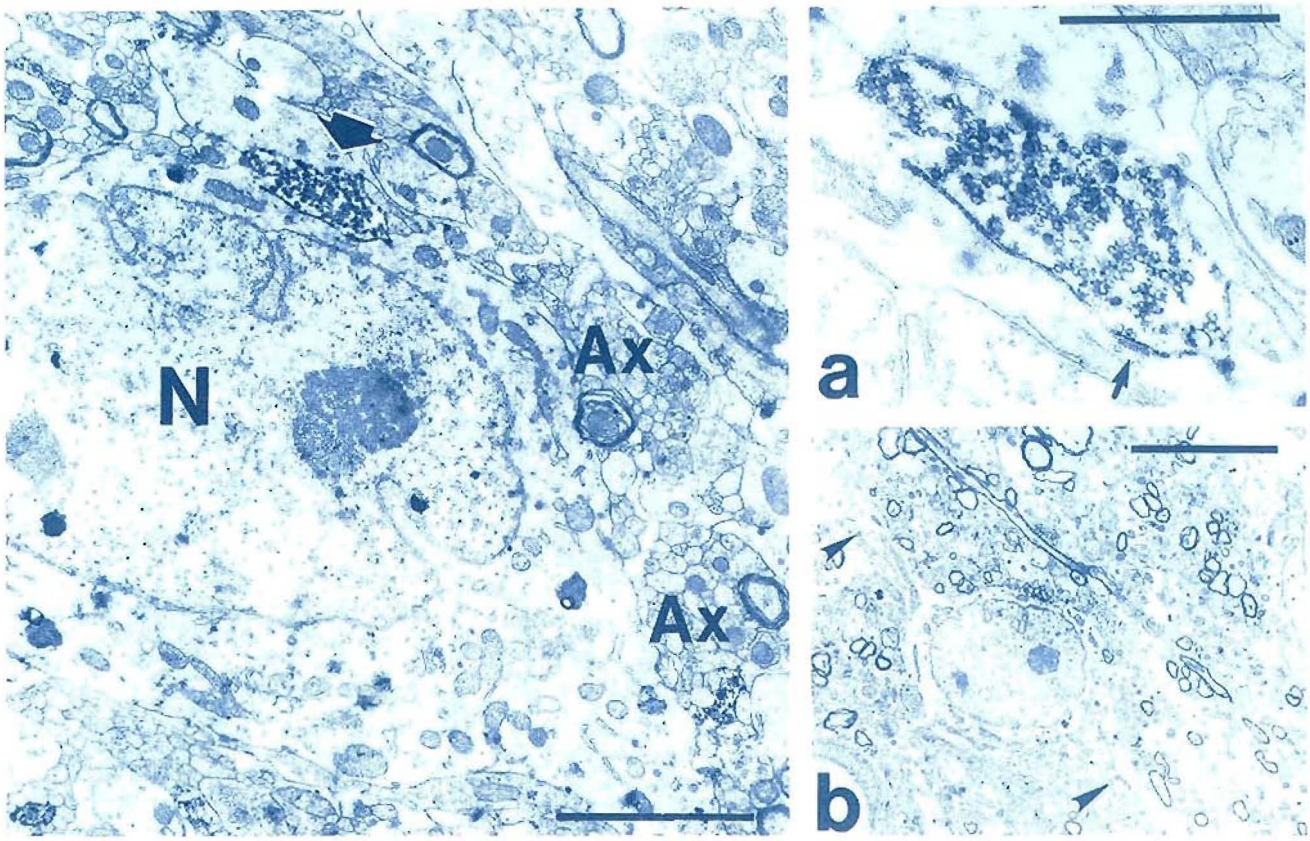

Fig. 10. An immunstained terminal (arrowhead) is shown in contact with a neuron (N) situated in lamina II_o. Ax, bundles of unmyelinated axons. Scale bar = 2 μm. Inset (a) shows the stained terminal at high magnification and a contact specialisation is shown (arrow). Scale bar = 1 μm. Inset (b) shows the same neuron in a semiserial section in which the plane of section has cut two proximal dendrites (arrowheads) at opposite poles of the cell. These dendrites were aligned parallel to the laminations of the substantia gelatinosa. Scale bar = 10 μm.

diffusely dispersed within the cytoplasm. Such a localization is in agreement with several previous preembedding staining studies on substance P in the spinal cord (Hökfelt et al., '77; Pickel et al., '77; Barber et al., '79). However, other reports have described immunostaining associated with agranular vesicles (Cuello et al., '77) or with both agranular and granular vesicles (Chan-Palay and Palay, '77). A postembedding staining study has described immunostaining associated exclusively with dense core vesicles (Pelletier et al., '77). The ultrastructural preservation in this study is comparable to that obtained in other recent immunocytochemical studies but not as good as that obtained in standard electron microscopic morphological work and therefore interpretation at the ultrastructural level must be carried out with caution. In addition, at the subcellular level interpretation of the distribution of immunostaining is complicated by various factors such as the degree of antigen preservation and antibody penetration and by the possible artifactual relocation of the antigen and the immunoreaction deposit during the preembedding staining procedure (see Priestley and Cuello, '82). Thus it is not possible to decide from our data or from previous reports whether substance P is contained solely in the dense core vesicles or also in the small agranular vesicles. However, it may be significant that in preterminal immunostained axons the dense-cored vesicles were present in large numbers whereas in terminals the small vesicles were the dominant population. Such a distribution is consistent with the hypothesis that substance P is synthesized and packaged into large vesicles in the cell body and then subsequently transported down the axon to the nerve terminal. In immunostained terminals dense core vesicles were observed both with and without stained cores. However, the problems of interpretation mentioned above apply also here and so it is not possible to decide whether unstained vesicles contain a neuroactive compound other than substance P or whether they simply contain substance P which has failed to be localized for various reasons.

Synaptic relationships of substance P-containing terminals

It has been proposed that substance P may be released from nonsynaptic sites to act in a diffuse or so-called "parasynaptic" fashion (Barber et al., '79). In this study little morphological evidence for a parasynaptic action for substance P was obtained and most of the substance P-containing varicosities in the substantia gelatinosa appear to make traditional synapses. However, unstained terminals containing large dense-cored vesicles were observed sometimes enveloped by similar stained terminals. The functional significance of such a relationship is not clear but

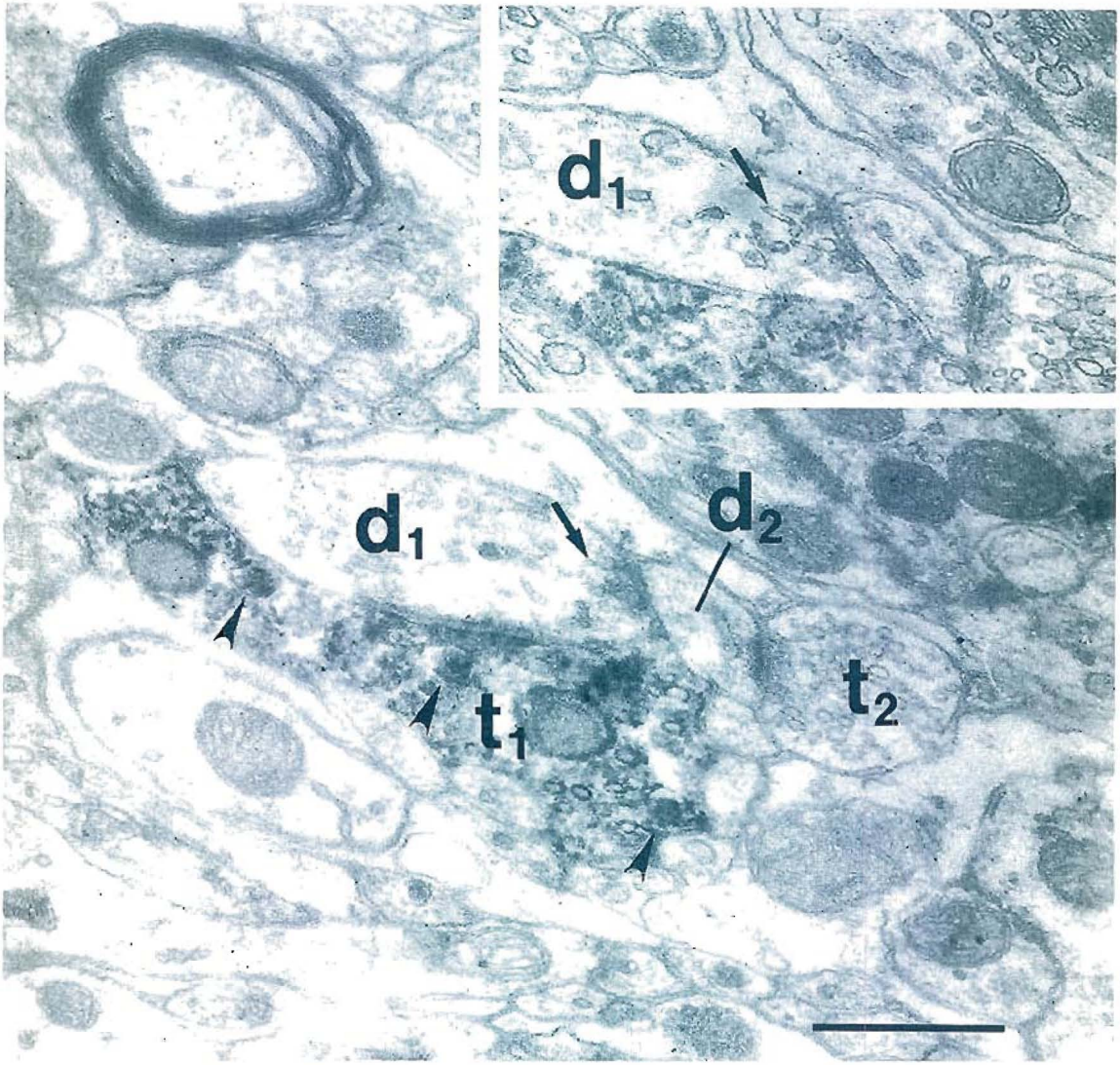

Fig. 11. An immunoreactive terminal is shown in synaptic contact with a presynaptic dendrite. The exact position of this structure is shown in Figure 3. A stained terminal (t_1) containing round vesicles and a few dense-cored vesicles (arrowheads) synapses with an unstained dendrite (d_1). The dendrite contains a cluster of pleomorphic vesicles (arrow) and synapses with a second dendrite (d_2). This dendrodendritic synapse is shown at the same magnification in the inset as it appears in a semiserial contrasted section. An unstained terminal (t_2) also synapses with the dendrite (d_2). Scale bar = 0.5 μm.

it might possibly represent the morphological substrate for a localized interaction between two peptide-containing terminals without the need for a specialized synaptic contact.

The characteristics of synapses made by substance P-immunostained terminals reported in this study are similar to those described in studies on the spinal cord (Hökfelt et al., '77; Pickel et al., '77; Cuello et al., '77; Pelletier et al., '77; Barber et al., '79) except that Barber et al. ('79) describe also symmetric axodendritic contacts. We have seen only asymmetric synapses between immunostained terminals and dendrites. This may be a genuine difference between our study and that of Barber and colleagues or may simply reflect different fixation and incubation conditions adopted in the two studies.

The majority of immunostained terminals observed in this study made single axodendritic contacts and very few large sinusoid or scalloped immunoreactive terminals at the center of synaptic glomeruli were seen. This is surprising since synaptic glomeruli are one of the characteristic features of the substantia gelatinosa of the spinal trigeminal and spinal cord (Réthelyi and Szentágothai, '69; Gobel, '74) and their central terminal is of primary afferent origin (Kerr, '70; Ralston and Ralston, '79) and contains a peculiar nonlysosomal acid phosphatase (Knyihár and Gerebtzoff, '73; Coimbra et al., '74) whose distribution and sensitivity to the sensory neurotoxin capsaicin parallels that of substance P (Jessell et al., '78). Recently Palermo and colleagues ('81) have reported that capsaicin selectively destroys glomerular terminals and they have proposed that this action is linked to the ability of capsaicin to deplete substance P from the substantia gelatinosa. Synaptic glomeruli are most easily identified in sagittal sections (Knyihár and Gerebtzoff, '73) and so the low

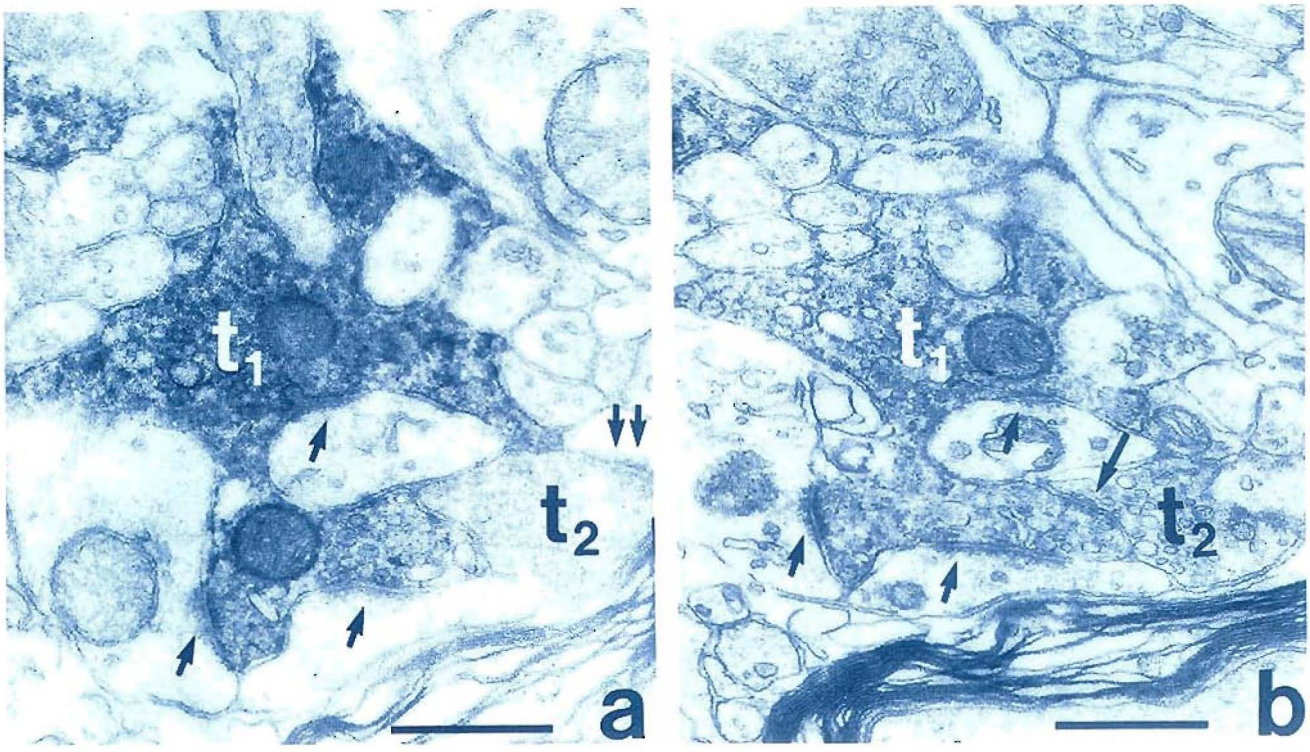

Fig. 12. A large immunoreactive scalloped terminal (t_1) is shown in semiserial uncontrasted (a) and contrasted (b) sections. The terminal forms the central profile of a synaptic glomerulus and its exact position in lamina II is shown in Figure 3. a. The stained terminal (t_1) makes synaptic contact (small arrows) with three different dendritic profiles. Adjoining the stained terminal is an unstained terminal (t_2) which synapses (two small arrows) with a dendrite. b. In the semiserial contrasted section the three synapses made by the stained terminal (t_1) are still evident (small arrows) and in addition a contact specialization between t_1 and t_2 can be seen (long arrow). t_2 appears to be presynaptic to t_1. Scale bar = 0.5 μm.

numbers observed in our study may partly be due to the use of transverse sections; however, this is unlikely to be the sole explanation. Our results are in accordance with several recent studies which indicate that the central terminal of many synaptic glomeruli is derived from relatively large-diameter primary afferents (Ralston and Ralston, '79) such as innocuous mechanoreceptor endings of the skin (Kerr, '75; Réthelyi et al., '79; Ribeiro da Silva and Coimbra, '82). In addition various other peptides are now known to be also affected by capsaicin (Nagy et al., '81; Priestley et al., '82) and Nagy and Hunt ('82) have recently reported that substance P and the nonlysosomal acid phosphatase are contained in different neuronal populations. The synaptic glomeruli form a heterogeneous population (Ribeiro da Silva and Coimbra, '82) and it seems likely that peptides other than substance P are contained in the primary afferent central terminals of many of them.

It is not possible to identify the origin of most of the postsynaptic structures described in this study but the substance P terminals on soma and proximal dendrites of large marginal neurons fits well with the reported significant primary afferent input to these cells (Narotzky and Kerr, '78). Previous light (LaMotte and de Lanerolle, '81) and electron microscopic (Barber et al., '79) immunohistochemical studies in the spinal cord have also described substance P terminals in association with marginal neurons. If substance P is a transmitter of primary afferents the evidence would point to it being the transmitter specifically of those afferents which convey noxious information (for review see Nicoll et al., '80) and lamina I is known to be a major site of neurons specifically excited by noxious stimuli (Christensen and Perl, '70). Although neurons in this area of the spinal trigeminal and spinal cord do not comprise a single morphological group (Gobel, '78a; Beal et al., '81) it is known from anatomical studies that a proportion of the larger cells project to the thalamus (Fukushima and Kerr, '79; Hockfield and Gobel, '78) and from electrophysiological studies that certain nociceptor-driven neurons in this area are projection neurons (see Willis, '80). It is not possible to tell from this study whether the large marginal zone neurons innervated by substance P terminals are indeed projection neurons but if they are this system might then represent a simple two-neuron substance P-mediated pathway for the transferance of nociceptive information to higher brain areas. We are presently exploring this possibility by extending to the ultrastructural level methodology which allows retrograde tract tracing procedures to be combined with immunocytochemistry (see Priestley et al., '81).

The identity of the various small dendrites seen in this study to receive synapses from immunostained terminals in lamina II is difficult to ascertain, but since axosomatic contacts were also seen it is likely that some of the dendrites are derived from neurons located within the lamina. The substantia gelatinosa of the spinal trigeminal nucleus contains few projection neurons (Hockfield and Gobel, '78) and so the lamina II neurons identified in this study are most likely to be interneurons. In the spinal trigeminal of

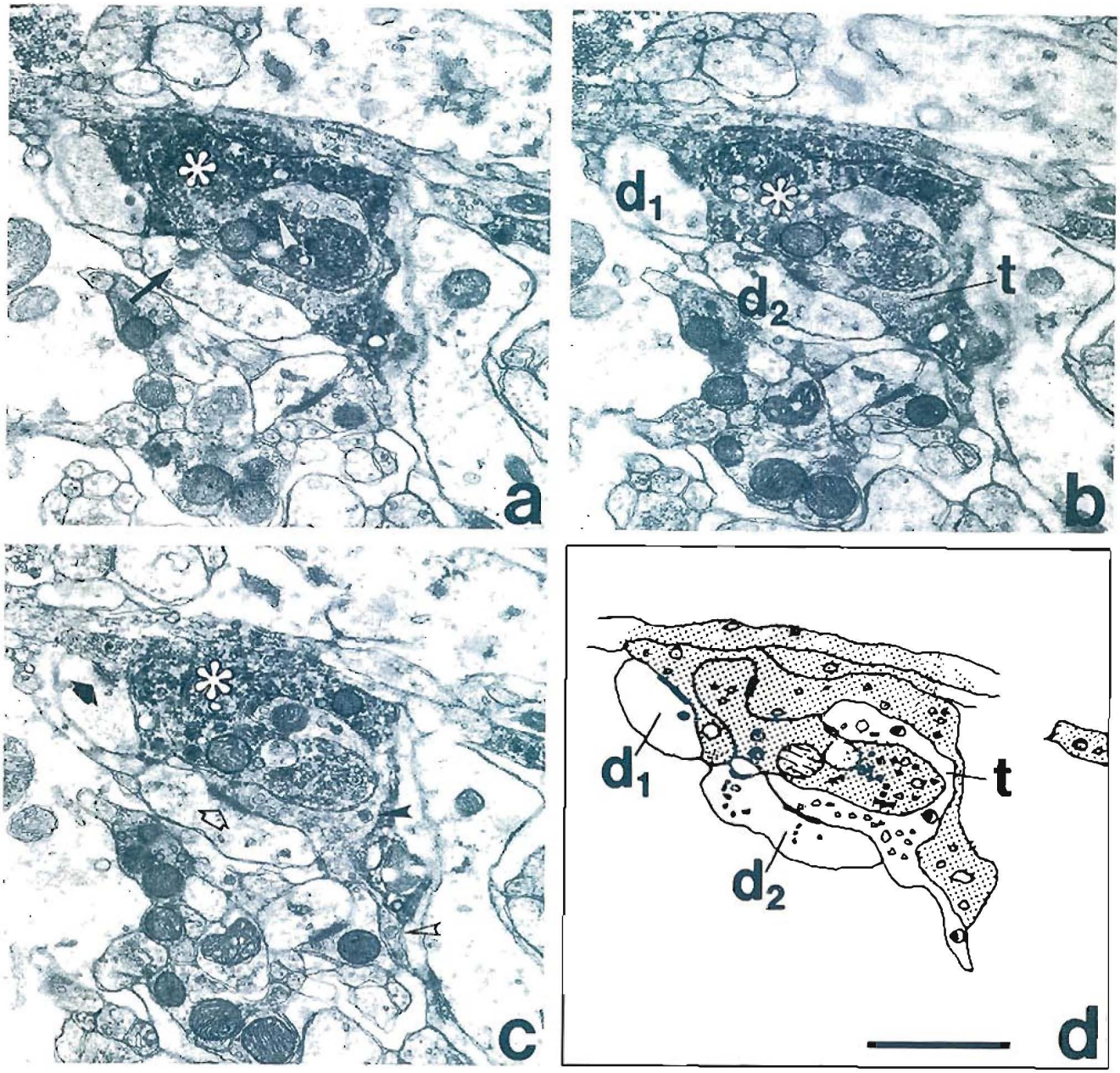

Fig. 13. A close association between immunostained and unstained vesicle-containing profiles is shown in three serial sections (a–c) and is represented diagramatically in (d). The exact position of this arrangement is shown in Figure 3. A number of immunostained profiles (asterisk) are seen to envelop an unstained terminal (t). In (a) the stained profiles surround three sides of the unstained terminal but in (c) the entry of the preterminal axon of the unstained terminal can be seen. The stained profile synapses with an adjoining dendrite d_1 (black arrow in c) and the unstained terminal synapses with another dendrite d_2 (hollow arrow in c). d_2 contains a cluster of vesicles at a membrane specialization (arrow in a) and is probably a presynaptic dendrite. The unstained terminal (t) contains large vesicles both with and without prominent dense cores (solid and hollow arrows in c). At points there are contact specializations between the stained and unstained profiles (white arrow in a). Scale bar = 1 μm.

the cat Gobel ('78b) has described four distinct types of interneuron in outer gelatinosa (our II_o). Immunostained terminals were seen synapsing with dendritic spines; however, all the types of interneuron identified by Gobel in lamina II have spines (Gobel, '78b). Recently Gobel and colleagues ('80), in an EM analysis of intracellularly HRP-filled substantia gelatinosa neurons in cat spinal cord, have identified a possible fine structural criterion for distinguishing between two major classes of neuron in lamina II_o. Aggregates of synaptic vesicles were seen in dendrites of islet cells but not in stalked cell dendrites and so it is possible that the presynaptic dendrites identified in this study as receiving synapses from immunostained terminals may belong to a cell analogous to the islet cell. Dendrites without synaptic vesicles seen in this study would presumably belong to another class of interneuron. Gobel

proposes that islet cells are inhibitory interneurons which receive input from primary afferent terminals and which in turn control the activity of other lamina II neurons and possibly also of neurons situated in deeper laminae (Gobel et al., '80). A substance P input to islet cells agrees with electrophysiological studies which indicate that such cells in lamina II_o are either nociceptive specific or wide dynamic range neurons (Bennett et al., '80). Iotophoretic studies have shown that substance P excites selectively neurons in laminae I–III of the cat spinal cord which are activated by noxious stimuli (Randić and Miletić, '77). However, it should be noted that the dendritic vesicles observed in this study in the rat are rather different to those described by Gobel et al. ('80) in the cat islet cells. This may merely reflect a species difference or may indicate that the substance P-containing terminals innervate a type of vesicle containing dendrite other than that of the islet cells. Gobel et al. ('80) describe a second type of presynaptic dendrite but the origin and function of this type is not known. Whatever the exact origin of the presynaptic dendrites described in this study, it is clear that a substance P input to such cells might provide a pathway for nociceptive information of higher integrative complexity than that via the lamina I neurons described in the previous paragraph.

A number of biochemical and physiological studies indicate that opiates and opiate peptides may act presynaptically on primary afferent terminals (LaMotte et al., '76; Macdonald and Nelson, '78) and may inhibit the release of substance P from such terminals (Jessell and Iversen, '77; Mudge et al., '79). Axoaxonic synapses have long been regarded as the morphological substrate for presynaptic inhibition (Gray, '62) and so substance P- and enkephalin-containing terminals might be expected to participate in such synapses. In this study only one example was seen of an immunostained terminal possibly postsynaptic to an unstained terminal. In the spinal cord Barber et al. ('79) describe few substance P-containing terminals participating in axoaxonic contacts, and in addition, when the immunostained terminal was the postsynaptic component of the synapse the presynaptic terminal was also lightly immunostained. Thus the substance P immunocytochemical studies indicate that if enkephalin neurons modulate substance P actions via direct axoaxonic contacts this is a relatively unusual arrangement. Recent enkephalin immunocytochemical studies (Hunt et al., '80; Aronin et al., '81; Priestley, '81) and preliminary EM studies detecting simultaneously enkephalin and substance P antigenic sites (Cuello et al., '82) have reached the same conclusion. In fact this result is not unexpected because quantitative ultrastructural studies on the substantia gelatinosa of the spinal cord reveal that axoaxonic contacts are rare. Thus in the rat Zhu et al. ('81) have shown that they represent less than 0.1% of identified synaptic contacts while in the cat and monkey they represent only 0.4% (Duncan and Morales, '78) and 0.8% (Ralston, '79).

The nature of the round vesicle-containing profile observed in the study to be possibly presynaptic to a substance P-containing terminal is unclear. It is possible that this structure represents a presynaptic enkephalin-containing terminal since ultrastructural studies have shown that a few enkephalin immunoreactive terminals participate in axoaxonic synapses (Hunt et al., '80; Aronin et al., '81). However, this arrangement is probably not the axoaxonic synapse classically described in the literature for the substantia gelatinosa since the presynaptic component of the synapse is normally described as containing vesicles which are discoid (Ribeiro da Silva and Coimbra, '82) or flattened (Gobel, '74; Duncan and Morales, '78; Ralston, '79; Zhu et al., '81). Alternatively, the profile observed in this study may be a presynaptic dendrite since Gobel ('76) has shown that such dendrites participate in dendroaxonic synapses. However, it is not possible to decide between these alternatives from the results of this or of previous immunocytochemical studies and so a final resolution of the anatomical basis of any presynaptic enkephalin action must await the results of further investigation.

Postsynaptic interactions between substance P and enkephalin

The results of this study suggest several possible postsynaptic targets for substance P-containing terminals and these include the large lamina I possible projection neurons and two classes of lamina II interneurons, one of which possesses presynaptic dendrites (Fig. 14). Many biochemical and physiological studies indicate that enkephalins may play a role in the substantia gelatinosa in modulating the transmission of nociceptive information (for reviews see Zieglgänsberger, '80; Jessell, '81) and one of the most frequently cited mechanisms for such an action is that originally proposed by Jessell and Iversen ('77) whereby enkephalin acts presynaptically to inhibit the action of substance P on lamina IV–V projection neurons. However, as discussed in the previous section, the anatomical results show that substance P and enkephalin immunoreactive terminals participate in relatively few axoaxonic contacts and make primarily axodendritic synapses. Most electrophysiological studies on the action of enkephalin in the substantia gelatinosa cannot distinguish between pre- and postsynaptic mechanisms but recently Zieglgänsberger and Tulloch ('79) have shown, using intracellular recordings, that enkephalin effects on neurons in deeper laminae (IV–VI) of the dorsal horn are primarily postsynaptic. In fact the structures identified in this study as postsynaptic to substance P-containing terminals have also featured in recent immunocytochemical studies on the localization of enkephalin and this suggests some possible postsynaptic sites for the interaction of the two peptides.

Two different types of interaction are indicated by comparing the results of substance P and enkephalin immunocytochemical studies. The first possibility is that substance P- and enkephalin-containing terminals synapse with common second-order neurons, and a likely target is the large lamina I neurons. Thus LaMotte and de Lanerolle ('81) in light microscopic studies on the human spinal cord have described both substance P- and enkephalin-containing terminals in close apposition to the soma and dendrites of certain lamina I (marginal zone) neurons. Our ultrastructural studies and those of Barber et al. ('79) have shown substance P immunoreactive terminals in synaptic contact with large lamina I neurons in the rat and Aronin et al. ('81) have shown that enkephalin terminals in the monkey synapse with lamina I neuronal soma and dendrites. Another possible target is the lamina II probable interneurons since Aronin et al. ('81) and Basbaum and Glazer ('81) have shown that enkephalin terminals contact dendrites which themselves receive synapses from terminals considered to be primary afferent terminals. The

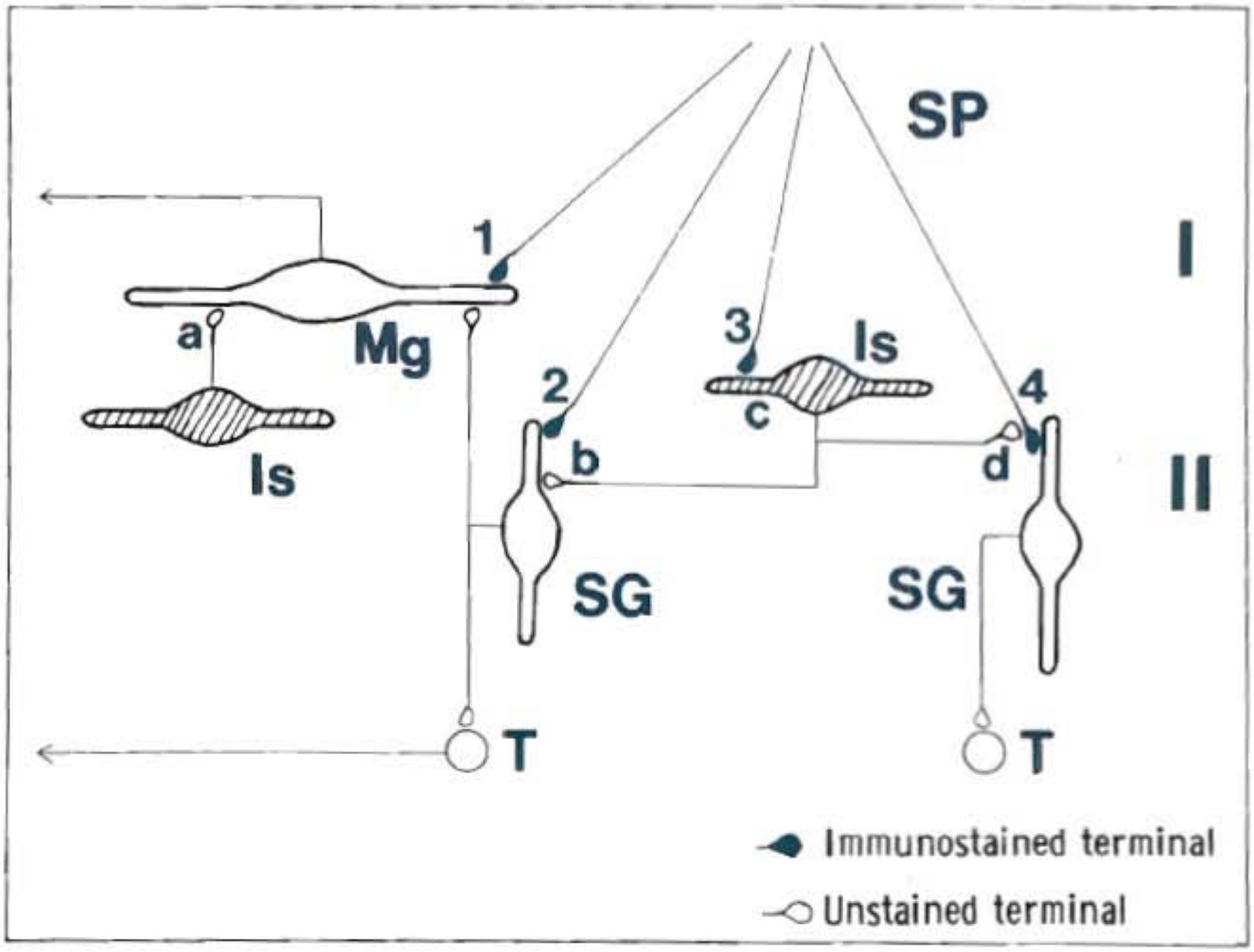

Fig. 14. Diagram showing the relationship of substance P immunoreactive terminals to various neuronal elements of the substantia gelatinosa. Fine lines indicate connections which are probable but which have not been demonstrated in this particular study. Four sites of termination for substance P (SP) fibers are indicated (1–4): 1. Synapses on large marginal zone neurons (Mg). A proportion of these neurons are likely to be projection neurons. 2. Synapses on substantia gelatinosa probable interneurons (SG). 3. Synapses on a second class of substantia gelatinosa interneurons which are characterized by the presence of presynaptic dendrites and which may be analogous to the islet cells (Is) described by Gobel and colleagues in the cat. 4. Synapses in which substance P terminals are postsynaptic to other unstained terminals. The diagram shows also four possible ways (a–d) in which enkephalin-containing neurons (striped) may interact with substance P. a. Synapses by enkephalin immunoreactive terminals onto the marginal zone neurons which receive a substance P input. b. Synapses by enkephalin immunoreactive terminals onto the substantia gelatinos interneurons which receive a substance P input. c. Synapses by substance P immunoreactive terminals onto enkephalin containing islet (Is) cells. d. Axoaxonic synapses between enkephalin and substance P immunoreactive terminals. T indicates transmission cells in deeper laminae. I, II indicate laminae I and II, respectively.

possible targets for substance P and enkephalin are summarized in Figure 14.

The other basic type of interaction indicated by comparison of our data with the enkephalin immunocytochemical results is that substance P-containing terminals synapse with enkephalin-containing neurons. In the recent detailed study on the monkey spinal cord by Aronin et al. ('81) enkephalin immunoreactivity was observed in dendrites which received synapses from terminals with some of the characteristics of those seen to be stained for substance P in our study and in addition some of the enkephalin immunoreactive dendrites contained accumulations of vesicles and made dendrodendritic synapses. As discussed earlier, it is possible that these vesicle-containing dendrites are derived from the islet cells described by Gobel et al. ('80), and two research groups have recently proposed that islet cells contain enkephalin (Hunt et al., '81; Bennett et al., '82). Immunocytochemical studies have generally produced comparable results in different species but Glazer and Basbaum ('81) have described prominent enkephalin immunoreactive neurons in lamina I (marginal zone) of the cat in addition to those in lamina II observed in rat (Hunt et al., '81), cat (Glazer and Basbaum, '81), and monkey (Aronin et al., '81). Hence the situation is likely to be far more complex than that outlined in Figure 14 and may vary from species to species.

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LITERATURE CITED

Aronin, N., M. DiFiglia, A.S. Liotta, and J.B. Martin (1981) Ultrastructural localization and biochemical features of immunoreactive leu-enkephalin in monkey dorsal horn. J. Neurosci. *1*:561–577.

Arvidsson, J., and S. Gobel (1981) An HRP study of the central projections of primary trigeminal neurons which innervate tooth pulps in the cat. Brain Res. *210*:1–16.

Barber, R.P., J.E. Vaughn., J. Randall Slemmon, P.M. Salvaterra, E. Roberts, and S.E. Leeman (1979) The origin, distribution and synaptic relationships of substance P axons in rat spinal cord. J. Comp. Neurol. *184*:331–352.

Basbaum, A.I., and E.J. Glazer (1981) Anatomical studies of serotonin/enkephalin interactions in the control of spinal nociceptors. In A.G. Brown and M. Réthelyi (eds): Spinal Cord Sensation. Edinburgh: Scottish Academic Press, pp. 241–242.

Beal, J.A., J.E. Penny, and H.R. Bicknell (1981) Structural diversity of marginal (lamina I) neurons in the adult monkey (Macaca mulatta) lumbosacral spinal cord: A golgi study. J. Comp. Neurol. *202*:237–254.

Bennett, G.J., M. Abdelmoumene., H. Hayashi, and R. Dubner (1980) Physiology and morphology of substantia gelatinosa neurons intracellularly stained with horseradish peroxidase. J. Comp. Neurol. *194*:809–827.

Bennett, G.J., M.A. Ruda, S. Gobel, and R. Dubner (1982) Enkephalin immunoreactive stalked cells and lamina IIb islet cells in cat substantia gelatinosa. Brain Res. *240*:162–166.

Cervero, F., and A. Iggo (1980) The substantia gelatinosa of the spinal cord. A critical review. Brain *103*:717–772.

Chan-Palay, V., and S.L. Palay (1977) Ultrastructural identification of substance P cells and their processes in rat sensory ganglia and their terminals in the spinal cord by immunocytochemistry. Proc. Natl. Acad. Sci. USA *74*:4050–4054.

Christensen, B.N., and E.R. Perl (1970) Spinal neurons specifically excited by noxious or thermal stimuli: Marginal zone of the dorsal horn. J. Neurophysiol. *33*:293–307.

Coimbra, A., B.P. Sodré-Borges, and M.M. Magalheas (1974) The substantia gelatinosa Rolandi of the rat. Fine structure, cytochemistry (acid phosphatase) and changes after dorsal root section. J. Neurocytol. *3*:199–217.

Cuello, A.C., T.M. Jessell, I. Kanazawa, and L.L. Iversen (1977) Substance P: Localization in synaptic vesicles in rat central nervous system. J. Neurochem. *29*:747–751.

Cuello, A.C., and I. Kanazawa (1978) The distribution of substance P immunoreactive fibers in the rat central nervous system. J. Comp. Neurol. *178*:129–156.

Cuello, A.C., M. Del Fiacco, and G. Paxinos (1978) The central and peripheral ends of the substance P containing sensory neurons in the rat trigeminal system. Brain Res. *152*:499–509.

Cuello, A.C., G. Galfre, and C. Milstein (1979) Detection of substance P in the central nervous system by a monoclonal antibody. Proc. Natl. Acad. Sci. USA *76*:3532–3536.

Cuello, A.C., C. Milstein, and J.V. Priestley (1980) Use of monoclonal antibodies in immunocytochemistry with special reference to the central nervous system. Brain Res. Bull. *5*:575–587.

Cuello, A.C., J.V. Priestley, and C. Milstein (1982) Immunocytochemistry with internally labelled monoclonal antibodies. Proc. Natl. Acad. Sci. USA *79*:665–669.

Del Fiacco, M., and A.C. Cuello (1980) Substance P and enkephalin-containing neurones in the rat trigeminal system. Neuroscience *5*:803–815.

Duncan, D., and R. Morales (1978) Relative numbers of several types of synaptic connections in the substantia gelatinosa of the cat spinal cord. J. Comp. Neurol. *182*:601–610.

Fukushima, T., and F.W.L. Kerr (1979) Organization of trigeminothalamic tracts and other thalamic afferent systems of the brainstem in the rat: Presence of gelatinosa neurons with thalamic connections. J. Comp. Neurol. *183*:169–184.

Glazer, E.J., and A.I. Basbaum (1981) Immunocytochemical localization of leucine-enkephalin in the spinal cord of the cat; Enkephalin-containing marginal neurons and pain modulation. J. Comp. Neurol. *196*:377–389.

Gobel, S., and M.B. Purvis (1972) Anatomical studies of the organization of the spinal V nucleus: The deep bundles and the spinal V tract. Brain Res. *48*:27–44.

Gobel, S. (1974) Synaptic organization of the substantia gelatinosa glomeruli in the trigeminal nucleus of the adult cat. J. Neurocytol. *3*:219–243.

Gobel, S. (1976) Dendroaxonic synapses in the substantia gelatinosa glomeruli of the spinal trigeminal nucleus of the cat. J. Comp. Neurol. *167*:165–176.

Gobel, S., and J.M. Binck (1977) Degenerative changes in primary trigeminal axons and in neurons in nucleus caudalis following tooth pulp extirpations in the cat. Brain Res. *132*:347–354.

Gobel, S., W.M. Falls, and S. Hockfield (1977) The division of the dorsal and ventral horns of the mammalian caudal medulla into eight layers using anatomical criteria. In D.J. Anderson and B.M. Matthews (eds): Pain in the Trigeminal Region. Amsterdam: Elsevier/North Holland Biomedical Press, pp. 443–453.

Gobel, S. (1978a) Golgi studies of the neurons in layer I of the dorsal horn of the medulla (trigeminal nucleus caudalis). J. Comp. Neurol. *180*:375–394.

Gobel, S. (1978b) Golgi studies of the neurons in layer II of the dorsal horn of the medulla (trigeminal nucleus caudalis). J. Comp. Neurol. *180*:395–414.

Gobel, S., W.M. Falls., G.J. Bennett., M. Abdelmoumene., H. Hayashi, and E. Humphrey (1980) An EM analysis of the synaptic connections of horseradish peroxidase-filled stalked cells and islet cells in the substantia gelatinosa of adult cat spinal cord. J. Comp. Neurol. *19*:781–807.

Gray, E.G. (1962) A morphological basis for presynaptic inhibition? Nature *193*:82–83.

Hockfield, S., and S. Gobel (1978) Neurons in and near nucleus caudalis with long ascending projection axons demonstrated by retrograde labeling with horseradish peroxidase. Brain Res. *139*:333–339.

Hökfelt, T., J-O. Kellerth., G. Nilsson, and B. Pernow (1975) Experimental immunohistochemical studies on the localization and distribution of substance P in cat primary sensory neurons. Brain Res. *100*:235–252.

Hökfelt, T., O. Johansson., J.O. Kellerth., Å. Ljungdahl., G. Nilsson., A. Nygårds, and B. Pernow (1977) Immunohistochemical distribution of Substance P. In U.S. von Euler and B. Pernow (eds): Substance P. New York: Raven Press, pp. 117–145.

Hunt, S.P., J.S. Kelly, and P.C. Emson (1980) The electron microscopic localisation of methionine-enkephalin within the superficial layers (I and II) of the spinal cord. Neuroscience *5*:1871–1890.

Hunt, S.P., J.S. Kelly., P.C. Emson, J.R. Kimmel., R.J. Miller. and J.-Y. Wu (1981) An immunohistochemical study of neuronal populations containing neuropeptides or γ-aminobutyrate within the superficial layers of the rat dorsal horn. Neuroscience *6*:1883–1898.

Jancsó, G., and E. Király (1980) Distribution of chemosensitive primary sensory afferents in the central nervous system of the rat. J. Comp. Neurol. *190*:781–792.

Jessell, T., and L.L. Iversen (1977) Opiate analgesics inhibit substance P release from rat trigeminal nucleus. Nature *268*:549–551.

Jessell, T.M., L.L. Iversen, and A.C. Cuello (1978) Capsaicin-induced depletion of substance P from primary sensory neurones. Brain Res. *152*:183–188.

Jessell, T.M. (1981) The role of substance P in sensory transmission and pain perception. In J.B. Martin, S. Reichlin, and K.L. Bick (eds): Neurosecretion and Brain Peptides, Advances in Biochemical Psychopharmacology Vol. 28. New York: Raven Press, pp. 189–198.

Kerr, F.W.L. (1970) The organization of primary afferents in the subnucleus caudalis of the trigeminal. A light and electron microscopic study of degeneration. Brain Res. *23*:147–165.

Kerr, F.W.L. (1975) Neuroanatomical substrates of nociception in the spinal cord. Pain *1*:325–356.

Knyihár, E., and M.A. Gerebtzoff (1973) Extra-lysosomal localization of acid phosphatase in the spinal cord of the rat. Exp. Brain Res. *18*:383–395.

LaMotte, C., C.B. Pert, and S.H. Snyder (1976) Opiate receptor binding in primate spinal cord: Distribution and changes after dorsal root section. Brain Res. *112*:407–412.

LaMotte, C.C., and N.C. de Lanerolle (1981) Human spinal neurons: Innervation by both substance P and enkephalin. Neuroscience *6*:713–723.

Lembeck, F. (1953) Zur Frages der zentralen Übertragung afferenter Impulse. III Mitteilung, Das Vorkommen und die Bedentung der Substanz

P in den dorsalen Wurzeln des Ruckenmarks. Naunyn-Schmiedebergs. Arch. Pharmakol. Exp. Pathol. *219*:197–213.

Light, A.R., and E.R. Perl (1979) Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibres. J. Comp. Neurol. *186*:133–150.

Ljungdahl, Å., T. Hökfelt, and G. Nilsson (1978) Distribution of substance P-like immunoreactivity in the central nervous system of the rat. I. Cell bodies and nerve terminals. Neuroscience *3*:861–943.

Macdonald, R.L., and P.G. Nelson (1978) Specific opiate induced depression of transmitter release from dorsal root ganglion cells in culture. Science *199*:1449–1451.

Mudge, A.W., S.E. Leeman, and G.D. Fischbach (1979) Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. Proc. Natl. Acad. Sci. USA *76*:526–530.

Nagy, J.I., S.P. Hunt., L.L. Iversen, and P.C. Emson (1981) Biochemical and anatomical observations on the degeneration of peptide-containing primary afferent neurons after neonatal capsaicin. Neuroscience *6*:1923–1934.

Nagy, J.T., and S.P. Hunt (1982) Fluoride-resistant acid phosphatase-containing neurons in dorsal root ganglia are separate from those containing substance P or somatostatin. Neuroscience *7*:89–97.

Narotzky, R.A., and F.W.L. Kerr (1978) Marginal neurons of the spinal cord: Types, afferent synaptology and functional considerations. Brain Res. *139*:1–20.

Nicoll, R.A., C. Schenker, and S.E. Leeman (1980) Substance P as a transmitter candidate. Annu. Rev. Neurosci. *3*:227–268.

Olszewski, J. (1950) On the anatomical and functional organization of the trigeminal nucleus. J. Comp. Neurol. *92*:401–413.

Palermo, N.N., H.K. Brown, and D.L. Smith (1981) Selective neurotoxic action of capsaicin on glomerular C-type terminals in rat substantia gelatinosa. Brain Res. *208*:506–510.

Pelletier, G., R. Leclerc, and A. Dupont (1977) Electron microscope immunohistochemical localization of substance P in the central nervous system of the rat. J. Histochem. Cytochem. *25*:1373–1380.

Pickel, V.M., T.H. Joh, and D.J. Reis (1975) Ultrastructural localization of tyrosine hydroxylase in noradrenergic neurons of brain. Proc. Natl. Acad. Sci. USA *72*:659–663.

Pickel, V.M., D.J. Reis, and S.E. Leeman (1977) Ultrastructural localisation of substance P in neurons of rat spinal cord. Brain Res. *122*:534–540.

Priestley, J.V. (1981) Ultrastructural localisation of substance P and enkephalin in the substantia gelatinosa of the spinal trigeminal nucleus. Br. J. Pharmacol. *74*:893P (Abstract).

Priestley, J.V., P. Somogyi, and A.C. Cuello (1981) Neurotransmitter specific projection neurons revealed by combining PAP immunohistochemistry with retrograde transport of HRP. Brain Res. *220*:231–240.

Priestley, J.V., and A.C. Cuello (1982) Electron microscopic immunocytochemistry: CNS transmitters and transmitter markers. In A.C. Cuello (ed): IBRO Handbook Methods in The Neurosciences. Immunohistochemistry. New York: John Wiley, in press.

Priestley, J.V., S. Bramwell, L.L. Butcher, and A.C. Cuello (1982) Effect of capsaicin on neuropeptides in areas of termination of primary sensory neurones. Neurochem. Int. *4*:57–65.

Ralston, H.J., III (1979) The fine structure of laminae I, II, and III of the macaque spinal cord. J. Comp. Neurol. *184*:619–642.

Ralston, H.J., III, and D.D. Ralston (1979) The distribution of dorsal root axons in laminae I, II and III of the macaque spinal cord: A quantitative electron microscope study. J. Comp. Neurol. *184*:643–684.

Randić, M., and V. Miletić (1977) Effect of substance P in cat dorsal horn neurones activated by noxious stimuli. Brain Res. *128*:164–169.

Réthelyi, M., and M. Szentágothai (1969) The large synaptic complexes of the substantia gelatinosa. Exp. Brain Res. *7*:258–274.

Réthelyi, M., A.R. Light, and E.R. Perl (1979) Ultrastructure of synaptic terminations of functionally identified myelinated afferent fibers. Soc. Neurosci. Abstracts *5*:728 (Abstract).

Rexed, B. (1952) The cytoarchitectonic organization of the spinal cord in the cat. J. Comp. Neurol. *96*:415–496.

Ribeiro da Silva, A., and A. Coimbra (1982) Two types of synaptic glomeruli and their distribution in laminae I–III of the rat spinal cord. J. Comp. Neurol. (in press).

Sternberger, L.A. (1979) Immunocytochemistry. Second Edition. New York: John Wiley.

Sumal, K.K., V.M. Pickel., R.J. Miller, and D.J. Reis (1980) Immunocytochemical and degenerative study of enkephalin and substance P in the spinal trigeminal nucleus. Soc. Neurosci. Abstracts *6*:353 (Abstract).

Waldeyer, W. (1888) Das Gorilla-Ruckenmark. Abhandlungen der Koniglichen Akademie der Wissenchaften. pp. 1–147.

Wall, P.D. (1980) The role of substantia gelatinosa as a gate control. In J.J. Bonica (ed): Pain. Association for Research in Nervous and Mental Disease Vol. 58. New York: Raven Press, pp. 205–231.

Weller, T.H., and A.H. Coons (1954) Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro. Proc. Soc. Exp. Biol. Med. *86*:789–794.

Willis, W.D. (1980) Neurophysiology of nociception and pain in the spinal cord. In J.J. Bonica (ed): Pain. Association for Research in Nervous and Mental Disease. Vol. 58. New York: Raven Press, pp. 77–92.

Zhu, C.G., C. Sandri, and K. Akert (1981) Morphological identification of axo-axonic and dendro-dendritic synapses in the rat substantia gelatinosa. Brain Res. *230*:25–40.

Zieglgänsberger, W., and J.F. Tulloch (1979) The effects of methionine and leucine-enkephalin on spinal neurones of the cat. Brain Res. *167*:53–64.

Zieglgänsberger, W. (1980) An enkephalinergic gating system involved in nociception. In E. Costa and M. Trabucchi (eds): Neural peptides and neuronal communication. New York: Raven Press, pp. 425–434.