J. Anat. (1994) 185, pp. 267–278, with 6 figures Printed in Great Britain

The innervation of the splenic capsule in the guinea pig: an immunohistochemical and ultrastructural study

L.-G. ELFVIN, J. JOHANSSON, ANNA-STINA HÖIJER AND H. ALDSKOGIUS

Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden

(*Accepted 22 February 1994*)

ABSTRACT

The innervation of the capsule of the guinea pig spleen was studied by light microscopy using an indirect fluorescent-labelled antibody technique, as well as by electron microscopy. A dense network of nerve fibres immunoreactive to the general neuronal marker, protein gene product 9.5 was observed in tangential sections through the capsule corresponding to the subcapsular compartment. The PGP 9.5-immunoreactivity in the fibres appeared to a large extent to be colocalised with tyrosine hydroxylase and neuropeptide Y (NPY) immunoreactivities as well as with synaptophysin immunoreactivity. Only very occasional fibres with substance P or calcitonin-gene-related peptide immunoreactivity were observed in tangential sections of the capsular region. By electron microscopy unmyelinated nerve fibres in the capsule were found to contain a large number of small dense-cored as well as clear vesicles and large dense-cored vesicles in varicose parts of the axons. The axolemma of the varicose regions was often naked, devoid of Schwann cells, and sometimes appeared denser than the nonspecialised parts of the membrane. These naked regions were observed in single sections to be apposed to splenic cells with variable intervals of extracellular space and interposed basal lamina material. Another type of contact was characterised by a very close association with splenic cells with no basal lamina interposed between the plasma membranes of the axon and the splenic cell. An intimate ultrastructural relationship was often also seen between varicose vesicle-containing axons and neighbouring axons in the nerve fibre bundles. The results show that the splenic capsule and its immediate neighbouring regions are innervated by catecholaminergic, NPY-containing fibres, which appear to establish different types of relations with the splenic cells as well as with one another.

Key words: Spleen; immunohistochemistry; neuropeptides; protein gene product 9.5.

INTRODUCTION

The splenic capsule plays an important role in the regulation of volume and vascular control of the spleen. The innervation of the spleen is known to consist mainly of sympathetic unmyelinated post-ganglionic nerves (Utterback, 1944; Elfvin, 1958; Fillenz, 1970; Baron & Jänig, 1988; Saito, 1990) which in the rat and cat to a major extent originate in prevertebral ganglia (Meckler & Weaver, 1984; Felten et al. 1986; Bellinger et al. 1989; Chevendra & Weaver, 1991). In recent years the study of these nerves has intensified partly owing to the increasing interest in the possible connections and interactions between the autonomic nervous system and the immune system (Felten et al. 1987; Felten, 1991; Weihe et al. 1991). The sympathetic splenic nerve fibres have been shown to be mainly noradrenergic fibres, as demonstrated by immunohistochemical techniques (Felten et al. 1985). Several neuropeptides have been observed in nerves of the spleen such as neuropeptide Y (NPY), substance P (SP), and calcitonin gene-related peptide (CGRP) (Felten et al. 1985; Lundberg et al. 1985; Lorton et al. 1991; Weihe et al. 1991). The fibres containing the two last-mentioned peptides have been assumed to be of afferent sensory origin. Also nonnoradrenergic nerves of sympathetic origin containing vasoactive intestinal polypeptide (VIP) have been suggested to occur in the spleen (Lundberg et al. 1985). Close structural

Correspondence to Professor L.-G. Elfvin, Department of Neuroscience, Karolinska Institutet, S-171 77 Stockholm, Sweden.

relationships between tyrosine hydroxylase (TH)-immunoreactive nerve terminals (Felten & Olschowka, 1987) as well as between NPY-immunoreactive terminals (Romano et al. 1991) and some of the splenic cells have recently been described.

Although it is possible to localise the peptide-containing fibres with specific antibodies, the amount of antigen may limit their detection especially during situations when the peptide production or turnover may be altered. In addition, other nerve fibres may exist which are not visualised with the antibodies generally used. Additional neuronal markers are therefore of importance in revealing the total innervation of a particular splenic compartment. Jackson & Thompson (1981) described a neuronal cytoplasmic protein, protein gene product 9.5 (PGP 9.5), with a molecular weight of 27 kDa and a mobility of 9.5 cm in 1–dimension polyacrylamide gel electrophoresis. More recent work has disclosed that the protein is a ubiquitin carboxyl terminal hydrolase (Wilkinson et al. 1989). Studies on the peripheral innervation, using PGP 9.5 antibodies, have indicated that it may be a general cytoplasmic marker, possibly demonstrating all types of efferent and afferent nerve fibres (Gulbenkian et al. 1987; Lundberg et al. 1988; Dalsgaard et al. 1989; Rice et al. 1993). In the present study the distribution of PGP 9.5 immunoreactive nerve fibres was investigated in the splenic capsule of the guinea pig. For comparison, the distribution of immunoreactivity for synaptophysin, a membrane protein specific for small synaptic vesicles (Navone et al. 1986) and for tyrosine hydroxylase (TH), and NPY, SP, and CGRP-like immunoreactivities (LI) were studied. The ultrastructure of the capsule innervation was also analysed. Many nerve fibres were found in this part of the spleen of the guinea pig of which no detailed ultrastructural studies seem to have been made. In the extensive report by Saito (1990), dealing with the splenic innervation of the guinea pig, the nerves of the capsule were only mentioned in passing.

MATERIALS AND METHODS

The immunohistochemical experiments were performed on 5 male guinea pigs with a body weight of 200–300 g under anaesthesia with ketamine (Ketalar, Parke-Davis, Gwent, UK) and xylazine (Rompun Vet., Bayer Leverkusen, FRG). After perfusion transcardially with 100 ml Tyrode solution the animals were perfusion-fixed with 4% paraformaldehyde and 0.4% picric acid in a phosphate buffer (pH 7.4) as described by Zamboni & de Martino (1967). The spleen was removed and cut into 1 mm thick slices which were immersed in the same fixative for 90 min at +4 °C. The specimens were transferred to a phosphate-buffered 10% sucrose solution, stored overnight at 4 °C and cut on a cryostat at a section thickness of 14 μm. The sections were processed for indirect immunohistochemistry according to Coons (1958). The sections were incubated in a humid atmosphere at 4 °C for 12–15 h with antisera raised in rabbits towards PGP 9.5 (dilution 1:1000), (Ultraclone, Cambridge, UK), NPY, SP or CGRP (dilution 1:400) (Peninsula, Belmont, USA). For synaptophysin and TH the sections were incubated with mouse monoclonal antibodies (dilution 1:200) (Sigma, St Louis, USA and Seralab, Sussex, UK, respectively). After rinsing, the sections were incubated at 37 °C for 30 min with green fluorescent fluorescein isothiocyanate (FITC) or red fluorescent tetramethyl rhodamine isothiocyanate (TRITC)-conjugated swine antirabbit antibodies and goat antimouse antibodies respectively (dilution FITC 1:10, TRITC 1:20) (Dakopatts, Copenhagen, Denmark). All sera contained 0.3% Triton-X 100 and 0.5% BSA. To demonstrate possible colocalisation of messenger molecules, consecutive sections incubated with 2 different antibodies were analysed or, alternatively, double-staining with FITC and TRITC was made after incubation with the synaptophysin or TH antibody together with a neuropeptide antibody. The sections were examined in a Nikon epifluorescence microscope. Control sections were analysed after preabsorption with the corresponding antigen as appropriate or after incubation with the secondary antibody only.

For electron microscopy, 2 animals were perfused with a Tyrode rinse through the ascending aorta, followed by a 3% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4, 4°–6 °C). The spleen was removed, rinsed in buffer, cut into 1 mm cubes, osmicated in a 1% OsO_4 solution for 2 h, dehydrated in acetone and embedded in Vestopal W. Sections for electron microscopy were cut on an LKB Ultrotome, stained with lead citrate and uranyl acetate, and examined in a Philips 201 or 301 electron microscope.

RESULTS

Immunohistochemistry

Strongly fluorescent PGP 9.5-immunoreactive nerve fibres were seen abundantly in the splenic nerves accompanying the branches of the splenic artery in the region of the hilum as well as in the splenic

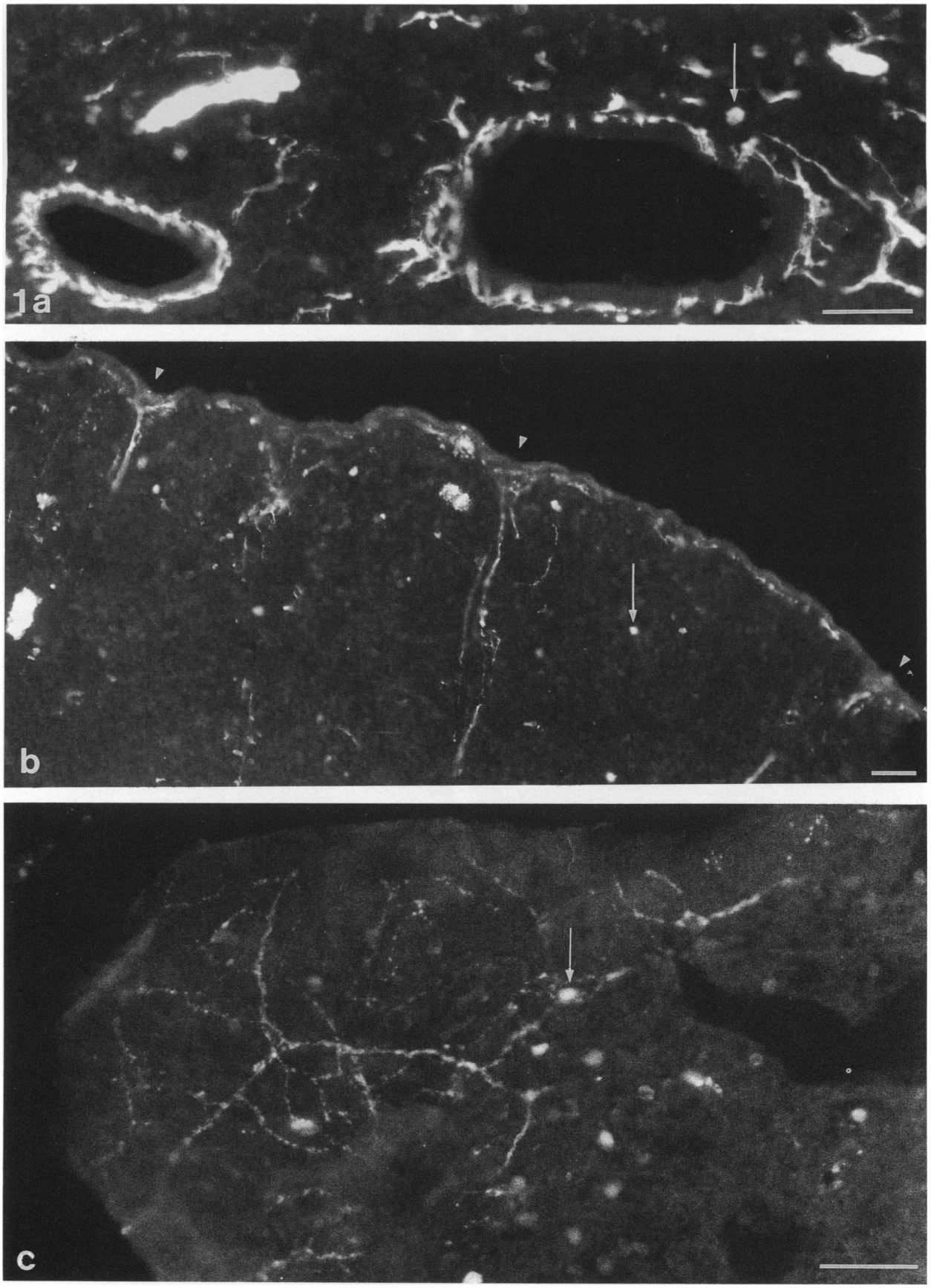

Fig. 1. Immunofluorescence micrographs of the spleen of the guinea pig after incubation with protein gene product (PGP) 9.5 antiserum. (*a*) Bundles of strongly fluorescent fibres as well as fluorescent fibres around blood vessels are seen in the splenic parenchyma close to the hilum. (*b*) Fluorescent fibres in the trabeculae are continuous with the capsular innervation (arrowheads). (*c*) Tangential section through the capsule showing a network of varicose fibres. Strongly fluorescent round cell bodies are seen in (*a–c*) (arrows). Bars, 50 μm.

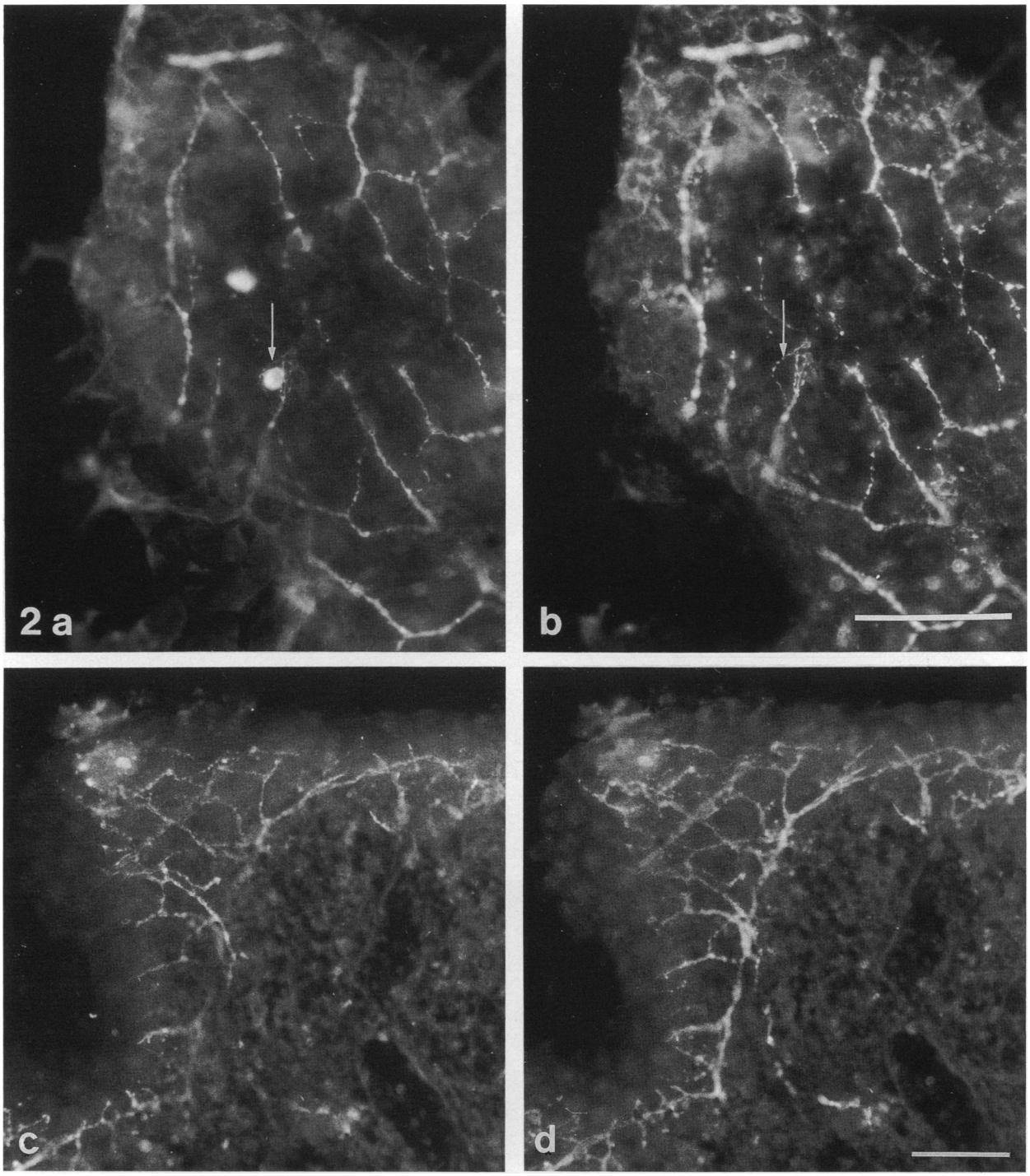

Fig. 2. Immunofluorescence micrographs of 2 tangential sections through the splenic capsule after incubation with antiserum to PGP 9.5 (*a*), tyrosine hydroxylase (TH) (*b*), neuropeptide Y (NPY) (*c*) and TH (*d*). There is a very close correspondence between the PGP 9.5 and TH-immunoreactive fibres as well as between the NPY and TH-immunoreactive fibres. Fluorescent cells are seen in (*a*) (arrow). There appear to be nerve fibre varicosities related to the cell both in (*a*) and (*b*). Bars, 50 μm.

parenchyma and capsule. In the hilum the fibres were arranged in several bundles around each vessel branch. The nerve bundles which followed the central artery in the white pulp split into smaller fascicles and ramified as individual nerve fibres in the periarteriolar lymphatic sheaths, in the red pulp, the trabeculae and the capsule. Groups of strongly fluorescent PGP 9.5-immunoreactive fibres were located in the adventitia of the central artery (Fig. 1*a*). Some round PGP 9.5-immunoreactive structures, about 10–12 μm in diameter, were observed in very close relation to some of the nerve fibres (Fig. 1). Haematoxylin-stained sections revealed that these structures were white blood cells or in some cases macrophage-like cells.

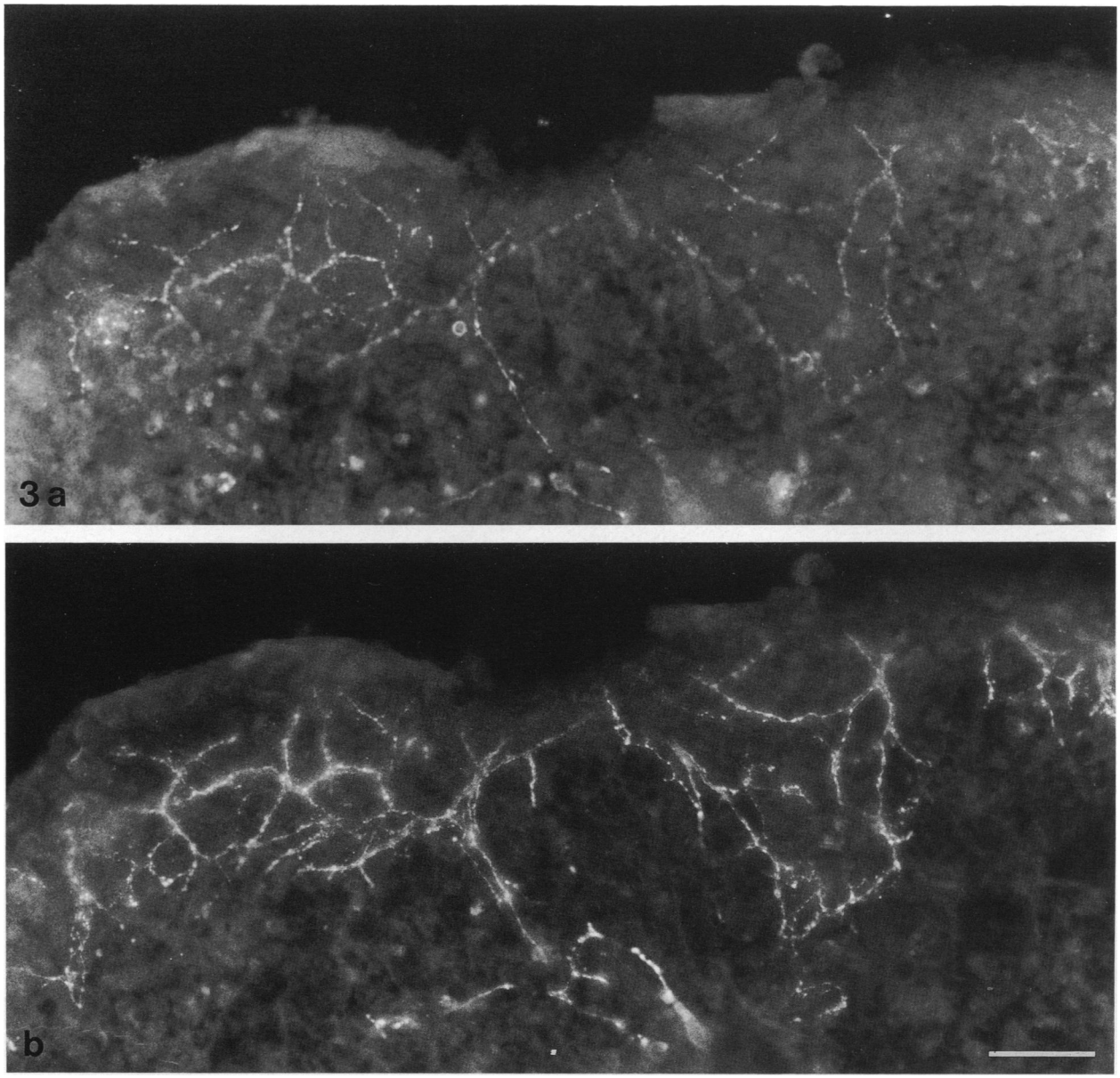

Fig. 3. Immunofluorescence micrographs of a tangential section of the capsule after incubation with antisera to synaptophysin (*a*) and NPY (*b*) showing close correspondence in immunoreactivity. The synaptophysin labelling is most pronounced in the axonal varicosities. Bar, 50 μm.

The PGP 9.5-positive fibres observed in the trabecular system, which fused with the approximately 10–30 μm thick capsule, appeared to be directly continuous with similar fibres of the capsule (Fig. 1*b*). In sections perpendicular to the capsule, the nerve fibres were either cross-sectioned or longitudinally oriented and it appeared that most fibres were present in the subcapsular region. In an attempt further to visualise the PGP 9.5-positive fibre network, tangential sections of the capsule including the subcapsular region were studied. The arrangement of the PGP 9.5 nerves was revealed to be that of a meshwork (Fig. 1*c*) partly consisting of very thin varicose elements that appeared to be single nerve fibres, partly of slightly thicker varicose or smooth elements, probably representing bundles of fibres. The network in the capsule was similar in appearance in all regions of the spleen.

The distribution in the capsule of TH-immunoreactivity (IR) was found to a very large extent to be similar to that of the PGP 9.5-IR as observed in double-stained sections where almost complete overlap was observed (Fig. 2*a*, *b*). Almost complete overlap of NPY and TH-IR was also seen in double-stained sections (Fig. 2*c*, *d*).

After application of synaptophysin antibody, fluorescent fibres were observed to be distributed in principle in the same fashion as the PGP 9.5- and

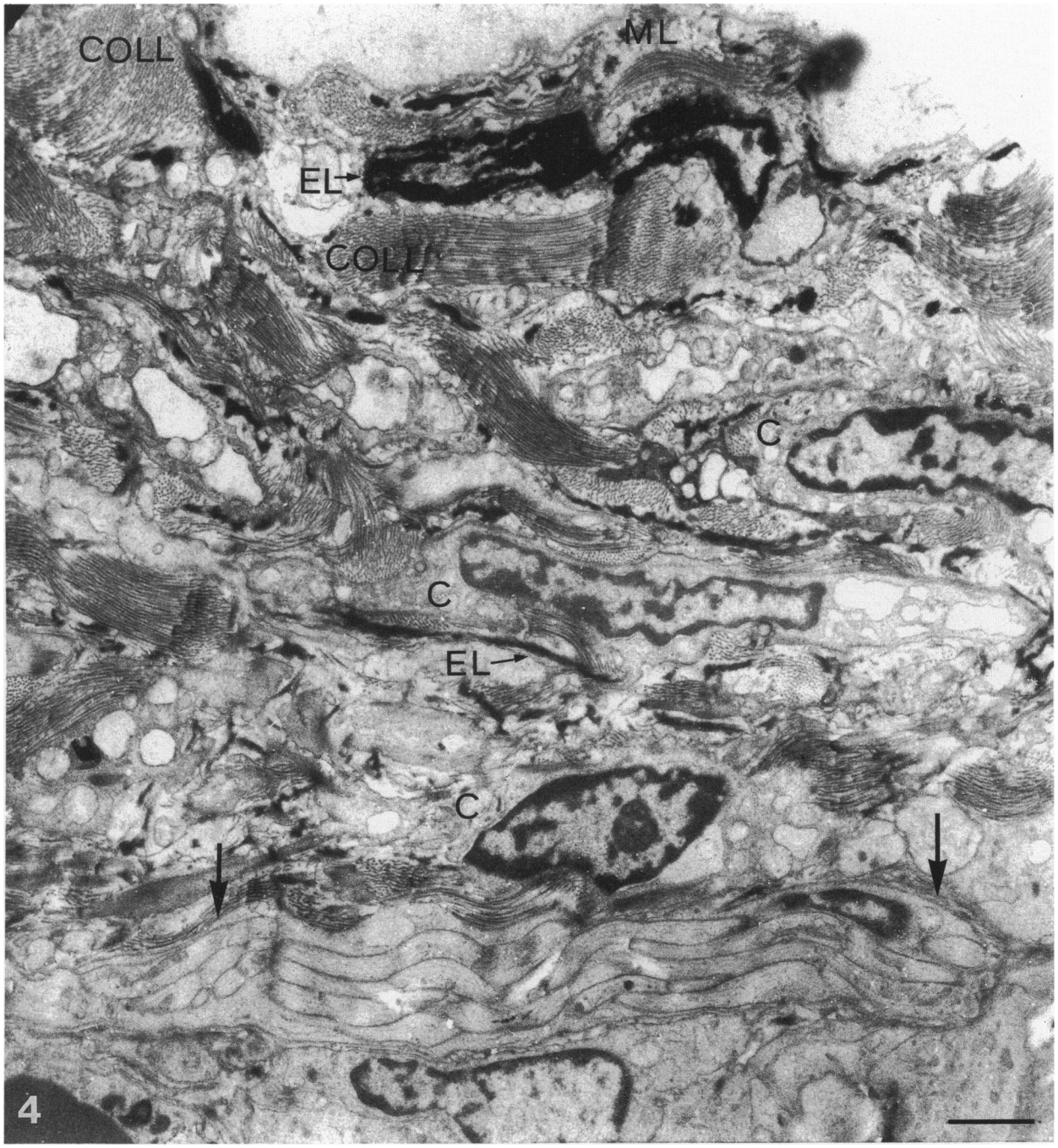

Fig. 4. Survey electron micrograph of the splenic capsule showing a longitudinally oriented nerve fibre bundle in the innermost part of the capsule (arrows). The capsule consists of several layers of cells (C), bundles of collagen fibrils (COLL), electron lucent elastin fibrils (EL) and, most superficially, mesothelial cells (ML). Bar, 1 μm.

NPY-positive fibres as demonstrated in double-stained sections (Fig. 3*a*, *b*). The synaptophysin-IR, however, was less intense and some of the PGP 9.5- and NPY-immunoreactive fibres were not visualised with the synaptophysin antibody. Although certain stretches of the synaptophysin fibres were immunoreactive, most of the IR was located in the varicosities which were particularly easy to observe in longitudinal sections of the wall of the central arteries and in tangential sections of the capsule (Fig. 3*a*). Some cell bodies were also stained after incubation with TH, NPY and synaptophysin antibodies.

As described in a previous paper (Elfvin et al. 1992) none or only occasional SP and CGRP immunoreactive fibres were observed in the capsule and subcapsular region. Colocalisation of SP and CGRP-IR with TH-IR was sought but could not clearly be demonstrated.

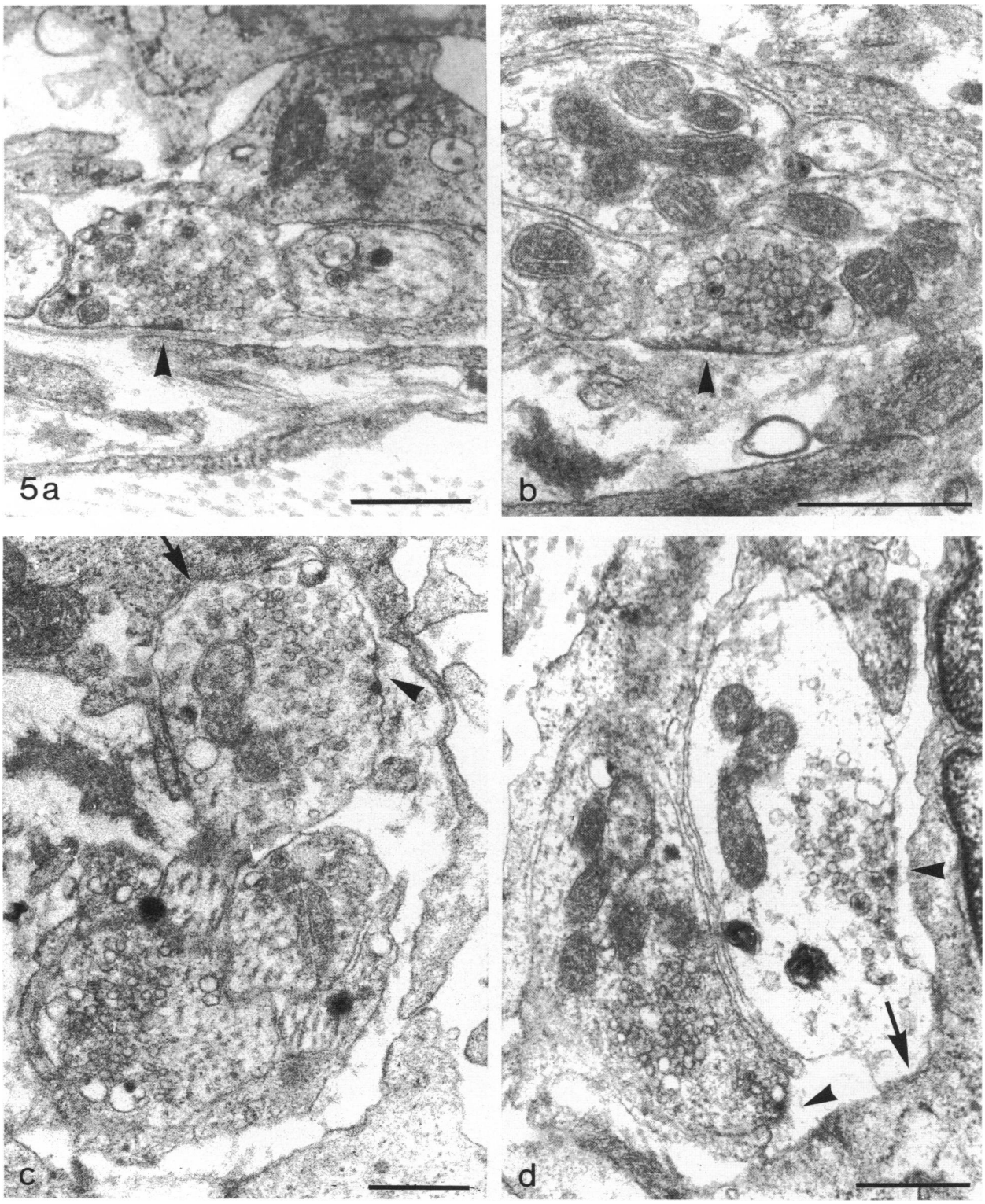

Fig. 5. Electron micrographs of axonal varicosities in fibre bundles in the subcapsular region of the spleen. In (*a*), the axonal membrane in one of the varicosities has an increased density along the surface close to a target cell with basal lamina interposed (arrowhead). A long specialised stretch of the axonal membrane is seen in (*b*) (arrowhead). In (*c*) and (*d*) accumulations of vesicles are located close to the membranes in axonal varicosities lacking a Schwann cell investment. Spot-like densities are observed in the membranes (arrowheads). Intimate contacts without basal lamina between the axonal membrane and the plasma membrane of adjacent cells are also observed (arrows). Bars, 0.5 μm.

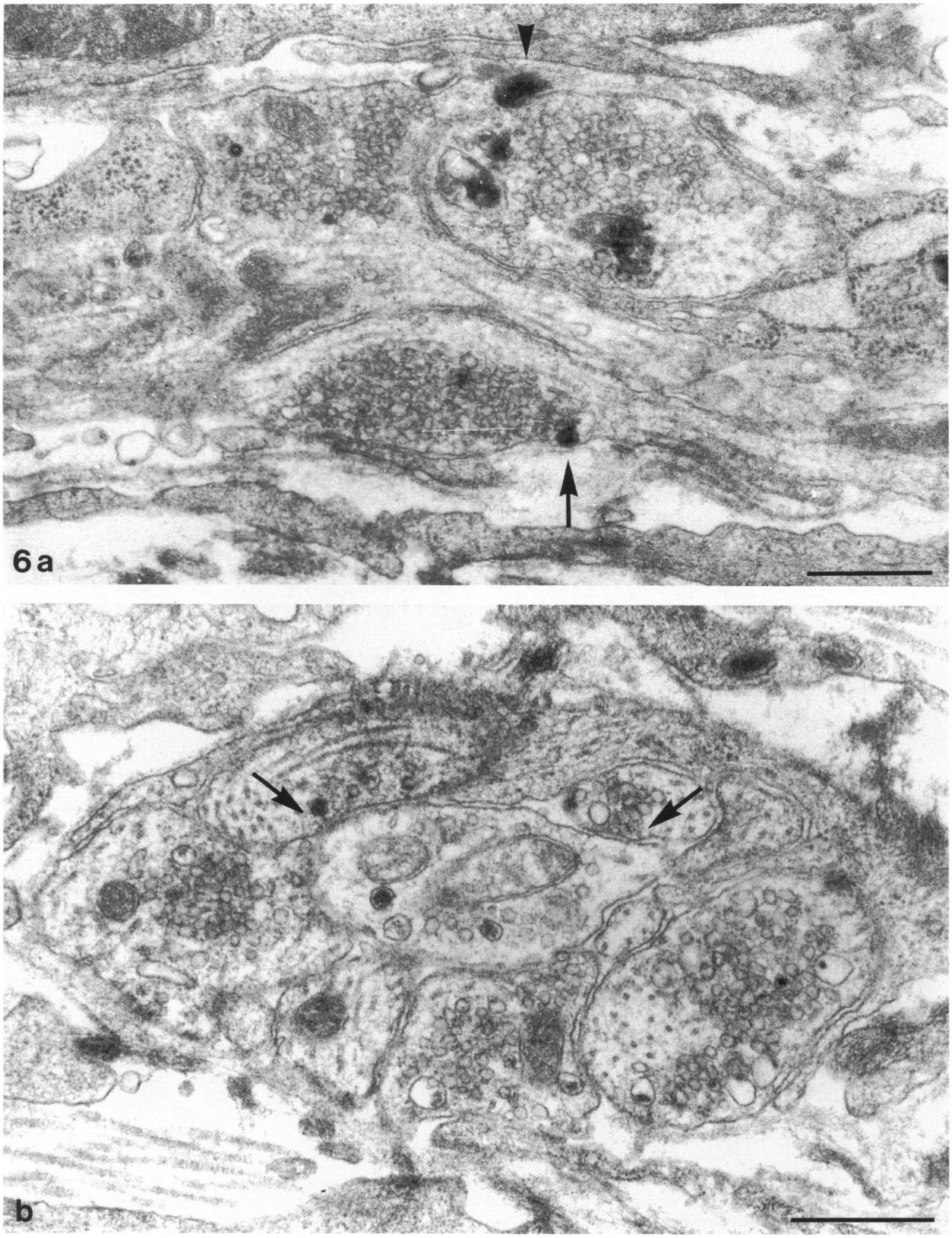

Fig. 6. Electron micrographs of varicosities in a nerve bundle in the splenic capsule, some of which are directly facing the extracellular compartment or/and other nerve fibres. In (*a*), the large dense cored vesicle is very close to the axonal membrane (arrow). A larger dense body is also seen, closely related to the axolemma (arrowhead). This body may be extracellular and possibly part of an elastin fibril. In (*b*), vesicle accumulations are seen in varicosities which are closely apposed to one another (arrows). Bars, 0.5 μm.

In control sections no nerve fibres were stained, but some fluorescent cells were seen.

Electron microscopy

The capsule of the guinea pig spleen consists mainly of connective tissue with dense networks of collagenous and elastic fibres as described by Saito (1990) (Fig. 4). Smooth myofibroblast-like elements were also observed. The surface of the capsule was covered by a layer of mesothelial cells. The nerve fibres were observed to lie in the innermost part of the capsule, the subcapsular region, facing the splenic parenchyma (Fig. 4). All nerve fibres which have so far been observed were unmyelinated. The electron microscopic analysis of the splenic nerves was made on transverse, oblique and tangential sections of the capsule where nerve fibres were easily identified (Figs 4–6). Bundles of varying numbers of nerve fibres, both nonvaricose and with widenings (varicosities) were observed. The varicosities contained small vesicles 30–50 nm in diameter, some clear-centred and some dense-cored, and large dense-cored vesicles about 100 nm in diameter. The dense cores, both in the small and large vesicles showed frequent variations in size and in location within the vesicles. The lengths of the nerve varicosities varied but usually lay within the range 1–1.5 μm.

Some areas of the plasma membrane of the nerve fibres were found, usually in connection with a swelling of the fibre and accumulation of vesicles in the axoplasm, which appeared slightly denser than the rest of the axolemma. This type of membrane appearance was mainly present in a fibre varicosity—occurring singly or within a fibre bundle—without complete Schwann cell covering, facing directly onto the extracellular space. The specialisation consisted of dense material accumulated on the axoplasmic surface of the membrane in close association with the vesicles (Fig. 5*a*, *b*). The distance between the varicosity and other cellular splenic elements varied and was in some instances about 25–50 nm (Fig. 5*a*). Basal lamina material was always present between the axolemma and plasma membrane of the other cells. In some varicosities, the dense axolemma material was located in a spot-like fashion in close association with vesicles (Fig. 5*c*). At these sites also basal lamina material was found between the widened axon and the splenic cells.

In addition to the above-described junctional specialisations some closer contacts between axons and splenic cells were seen. In these instances the varicose portion of an axon was directly apposed, with an interval of 10 nm or less, to a splenic parenchyma cell without any interposed basal lamina. There was no apparent vesicle accumulation at the axonal membrane at these contacts. An indication of increased density of the cytoplasm close to the adjacent membrane surfaces was, however, sometimes seen (Fig. 5*c*, *d*).

In some widened portions of the axons large dense-cored vesicles and dense bodies with a diameter often larger than 100 nm, i.e. exceeding the approximate diameter of the large dense-cored vesicles, were observed. Sometimes the membrane of these structures was very close to, or appeared to be fused with the axolemma (Fig. 6*a*).

Often varicosities without complete Schwann cell wrapping were observed directly facing onto other nerve fibres in the nerve bundles (Fig. 6*b*). An accumulation of vesicles was often observed in the varicose axons at the surface close to the neighbouring axon (Fig. 6*b*). In the same section other parts of the axolemma were sometimes also seen to be directly exposed to the extracellular space.

DISCUSSION

The present study gives evidence for the existence of a large number of nerve fibres in the splenic capsule of the guinea pig. In a comprehensive electron microscope analysis of the guinea pig spleen, Saito (1990) observed very few nerve fibres in the capsule and subcapsular region. The reason for the difference between the results of that study and the present one is probably partly due to the fact that we have studied tangentially sectioned material. Such material is more favourable for analysing the nerve network in this narrow compartment of the spleen.

In a previous report by Weihe et al. (1991) the PGP 9.5-IR fibres in the spleen of various mammals, including the guinea pig, were described as being particularly numerous in the red pulp but were also found in the white pulp. The PGP 9.5-positive fibres were observed to outnumber all other studied nerve fibre immunoreactivities, including TH and NPY-IR, which mostly coincided (Weihe et al. 1991). The capsular innervation was not described. In the present study it appears justified to assume that the PGP 9.5-IR-fibres in the capsule to a very large extent correspond to sympathetic, NPY-containing aminergic fibres. This assumption is based on the observation that TH and NPY-IR are present in a large number of nerve fibres having the same distribution and appearance as the fibres with the PGP 9.5-IR. Analysis of double-stained tangential sections of the

capsule shows an almost complete overlap of the PGP 9.5 and TH-IR. A similar overlap of TH and NPY-IR was also observed. Colocalisation of peptides and classical transmitters observed at the light microscope level does not necessarily mean costorage in the same neurons (see Klein & Thureson-Klein, 1990). The possibility therefore cannot be excluded that some of the TH-IR and NPY-IR is located in different fibres. However, Fried et al. (1986) have demonstrated that NPY, enkephalin and noradrenaline coexist in sympathetic neurons innervating the bovine spleen. There might, however, also be other neuromodulatory substances in fibres of these bundles which we have not detected with our technique. Such fibres may not be immunoreactive for TH or NPY.

The findings in the present study of a high density of NPY-immunoreactive nerve fibres in the splenic capsule of the guinea pig differ from observations made on the cat. In the cat splenic capsule many fibres are TH-positive but only few weakly fluorescent NPY-immunoreactive fibres are detected (Lundberg et al. 1985). Differences in neuropeptide content in sympathetic neurons are often found when different species are compared (see Elfvin et al. 1993). For the splenic capsule innervation it is probable that the difference in neuropeptide content may reflect a difference in function of the capsule in the guinea pig versus the cat. In the cat, experiments showed that the local administration of noradrenaline causes a more pronounced volume reduction of the spleen as compared with NPY, whereas NPY is comparatively more effective in increasing splenic perfusion pressure (Lundberg et al. 1985). The reduction in volume of the cat spleen accompanied by an increase in blood flow from the splenic vein is due to capsule contraction. In the guinea pig the contraction may not be as pronounced as in the cat, an assumption which is supported by the fact that only a few well developed smooth muscle cells are seen in the capsule as shown in this study and also reported by Saito (1990). The high TH and NPY-IR in the capsular nerves of the guinea pig spleen may thus reflect other functions.

No or very occasional SP- or CGRP-immunoreactive fibres were observed in sections cut at various angles through the capsule of the guinea pig spleen (Elfvin et al. 1992). This would indicate that the SP and CGRP-containing fibres barely contribute to the PGP 9.5-immunoreactive fibres in this compartment. Also in the splenic capsule of the cat there are few SP fibres (Lundberg et al. 1985). The presence of labelling of cells in the control material indicates an, at least partly nonspecific fluorescence in these cells.

The present ultrastructural findings indicate that the nerves may form several different types of contacts with splenic cells in the capsule and immediately subcapsular compartment. With respect to the content of small clear and dense-cored vesicles as well as large dense-cored vesicles, the present observations are in agreement with the findings of Saito (1990) in the nerve fibres of the capsule. The observed presynaptic specialisations of the axolemma have, however, not been described earlier in nerves of the spleen in the guinea pig, although closely apposed nonspecialised membranes separated by a space containing only basal lamina material were disclosed in the parenchyma between nerve varicosities and splenic cells (Saito, 1990). Contacts between terminal axons and smooth muscle cells with an intercellular gap of 20 nm was described in the cat spleen by Fillenz (1970) and with a gap of 20–40 nm in the human spleen by Heusermann & Stutte (1977). In these reports nothing was said about possible interposed basal lamina material. In other tissues, presynaptic membrane specialisations have been identified at nerve-smooth muscle cell junctions of arterioles such as in the submucosa of the guinea pig ileum (Luff & MacLachlan, 1988) and of arterioles in the rabbit kidney (Luff et al. 1992). Basal lamina material always appeared to be present at such junctions and at places the distance between axon and target cell was described as being about 50 nm. The membrane specialisations observed here are very similar to those described in these earlier studies.

It may be suggested that the membrane specialisations of different size observed in the nerve varicosities of the spleen, i.e. the spot-like and the elongated type, reflect different ultrastructural appearances of vesicular and plasma membrane dynamics. The differences may in part be ascribed to different degrees of packing of vesicles in the process of fusing with the axolemma, emptying their content and possibly reflecting the existence of high versus low output areas. The structural variation of this type of specialisation may be found at vascular walls, and myofibroblast cells and may be partly related to the volume and vascular regulation of the spleen.

Another type of contact, in which the axolemma and the plasma membrane of the target cell are more closely packed with no interposed basal lamina material, is also found. Felten & Olschowka (1987) have described, at the ultrastructural level, close contacts between the plasma membranes of TH-immunoreactive nerve fibres and lymphocytes in the rat spleen. The plasma membranes were estimated to be approximately 6 nm apart and exhibited no specialisations, and the contacts were interpreted to

indicate possibilities for synaptic interactions between the sympathetic nerves and the immune system. Also, in a recent electron microscopical study of the rat spleen, nerve fibres containing NPY-IR were shown to establish similar close contacts with splenic cells, presumably lymphocytes (Romano et al. 1991). The function of the close contacts observed in the present study and which are similar to those described by Felten & Olschowka (1987) and Romano et al. (1991) is not clear. It is possible that these contacts may be important in neuroimmunological processes, not only involving lymphocytes but also other immunologically active cells in the spleen. These contacts may be rather transient in contrast to the junctions with specialised morphology.

The organs innervated by the autonomic nervous system are generally believed to contain postjunctional receptors widely distributed over their cell membranes. The transmitters are usually thought to diffuse various distances through the extracellular space to interact with the receptors, although an alternative view has recently been proposed. According to this view transmission occurs at organised neuroeffector contacts, at which points there are restricted pools of specialised junctional receptors (Hirst et al. 1992). This suggestion was partly based on the finding made on serial ultrathin sections analysed in the electron microscope. All axon varicosities which were studied were always at some point as close as about 50 nm to the effector cell. The finding in the present investigation of various types of close relationship between nerve fibres and target cells seems to be compatible with this latter alternative. Ultrastructural analyses of serial sections are presently being undertaken in order to clarify further the structural relationship between the axonal terminals and the target cells in the guinea pig spleen.

The frequent direct relationship between varicose vesicle-containing segments of neighbouring axons is an interesting finding. The contacts are not clear-cut synaptic structures as defined in the central nervous system or autonomic ganglia, but modulatory effects onto one neuron of substances released from an adjacent neuron must clearly be facilitated.

In summary, the innervation of the splenic capsular compartment has in the present investigation been revealed to show interesting complexities. Additional work using serial sectioned material and electron microscope immunohistochemical techniques is needed to further clarify structurally the intricate synaptology of the innervation of the cellular elements and the identity of the latter, in the capsule and its immediate vicinity.

ACKNOWLEDGEMENTS

Excellent technical assistance was given by Kristina Holmberg and skilled secretarial assistance by Marianne Rapp. This work was supported by grants from the Swedish Medical Research Council, project numbers 5189 and 5420, as well as from funds of Karolinska Institutet, Stiftelsen Lars Hiertas Minne and the Swedish Society of Medicine.

REFERENCES

Baron R, Jänig W (1988) Sympathetic and afferent neurons projecting in the splenic nerve of the cat. *Neuroscience Letters* **94**, 109–113.

Bellinger DL, Felten SY, Lorton D, Felten DL (1989) Origin of noradrenergic innervation of the spleen in rats. *Brain, Behaviour and Immunology* **3**, 291–331.

Chevendra V, Weaver LC (1991) Distribution of splenic, mesenteric and renal neurons in sympathetic ganglia in rats. *Journal of the Autonomic Nervous System* **33**, 47–54.

Coons AH (1958) Fluorescent antibody methods. In *General Cytochemical Method* (ed. J. F. Danielli), pp. 399–422. New York: Academic Press.

Dalsgaard C-J, Rydh M, Haegerstrand A (1989) Cutaneous innervation in man visualized with protein gene product 9.5 (PGP 9.5) antibodies. *Histochemistry* **92**, 385–390.

Elfvin L-G (1958) The ultrastructure of unmyelinated fibers in the splenic nerve of the cat. *Journal of Ultrastructure Research* **1**, 428–454.

Elfvin L-G, Aldskogius H, Johansson J (1992) Splenic primary afferents of the guinea pig demonstrated with anterogradely transported wheat germ agglutinin horseradish peroxidase conjugated. *Cell and Tissue Research* **269**, 229–234.

Elfvin L-G, Lindh B, Hökfelt T (1993) The chemical neuroanatomy of sympathetic ganglia. *Annual Review of Neuroscience* **16**, 471–507.

Felten DL (1991) Neurotransmitter signalling of cells of the immune system: important progress, major gaps. *Brain, Behaviour and Immunology* **5**, 2–8.

Felten DL, Felten SY, Carlson SL, Olschowka JA, Livnat S (1985) Noradrenergic and peptidergic innervation of lymphoid tissue. *Journal of Immunology* **135**, 755s–765s.

Felten DL, Bellinger DL, Ackerman KD, Felten SY (1986) Denervation of splenic sympathetic fibers in the young adult rat. *Society for Neuroscience* **12**, 1064. (Abstract.)

Felten DL, Felten SY, Bellinger, DL, Carlson SL, Ackerman KD, Madden KS et al. (1987) Noradrenergic sympathetic neural interactions with the immune system: structure and function. *Immunological Reviews* **100**, 226–259.

Felten SY, Olschowka JA (1987) Noradrenergic sympathetic innervation of the spleen: II. Tyrosine hydroxylase (TH)-positive nerve terminals form synaptic like contacts on lymphocytes in the splenic white pulp. *Journal of Neuroscience Research* **18**, 37–48.

Fillenz M (1970) The innervation of the cat spleen. *Proceedings of the Royal Society of London* B **174**, 459–468.

Fried G, Terenius L, Brodin E, Efendic S, Dockray G, Fahrenkrug J et al. (1986) Neuropeptide Y, enkephalin and noradrenaline coexist in sympathetic neurons innervating the bovine spleen. *Cell and Tissue Research* **243**, 495–508.

Gulbenkian S, Wharton J, Polak JM (1987) The visualization of cardiovascular innervation in the guinea pig using an antiserum to protein gene product 9.5 (PGP 9.5). *Journal of the Autonomic Nervous System* **18**, 235–247.

Hirst GDS, Bramich NJ, Edwards FR, Klemm M (1992) Transmission at autonomic neuroeffector junctions. *Trends in Neuroscience* **15**, 40–46.

HEUSERMANN U, STUTTE HJ (1977) Electron microscopic studies of the innervation of the human spleen. *Cell and Tissue Research* **184**, 225–236.

JACKSON GD, THOMPSON RJ (1981) The demonstration of new human brain-specific proteins by high resolution two-dimensional polyacrylamide gel electrophoresis. *Journal of the Neurological Sciences* **49**, 429–38.

KLEIN RL, THURESON-KLEIN ÅK 1990 Neuropeptide co-storage and exocytosis by neuronal larger dense-cored vesicles: How good is the evidence? *Current Aspects of the Neurosciences*, vol. 2 (ed. N. N. Osborne), pp. 219–258. Basingstoke: Macmillan.

LORTON D, BELLINGER DL, FELTEN SY, FELTEN DL (1991) Substance P innervation of spleen in rats: nerve fibers associate with lymphocytes and macrophages in specific compartments of the spleen. *Brain, Behaviour and Immunology* **5**, 29–40.

LUFF SE, MCLACHLAN EM (1988) The form of sympathetic postganglionic axons at clustered neuromuscular junctions near branch points of arterioles in the submucosa of the guinea pig ileum. *Journal of Neurocytology* **17**, 451–463.

LUFF SE, HENGSTBERGER SG, MCLACHLAN EM, ANDERSON WP (1992) Distribution of sympathetic neuroeffector junctions in the juxtaglomerular region of the rabbit kidney. *Journal of the Autonomic Nervous System* **40**, 239–254.

LUNDBERG JM, ÄNGGÅRD A, PERNOW J AND HÖKFELT T (1985) Neuropeptide Y-, substance P- and VIP-immunoreactive nerves in cat spleen in relation to autonomic vascular and volume control. *Cell and Tissue Research* **239**, 9–18.

LUNDBERG J-M, ALM P, WHARTON J, POLAK JM (1988) Protein gene product 9.5 (PGP 9.5): a new neuronal marker visualizing the whole uterine innervation and pregnancy-induced and developmental changes in the guinea pig. *Histochemistry* **90**, 9–17.

MECKLER RL, WEAVER LC (1984) Comparison of the distributions of renal and splenic neurons in sympathetic ganglia. *Journal of the Autonomic Nervous System* **11**, 189–200.

NAVONE F, JAHN R, DI GIOIA G, STUKENBRUK H, GREENGARD P, DE CAMILLI P (1986) Protein p 38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *Journal of Cell Biology* **103**, 2511–2527.

RICE FL, KINNMAN E, ALDSKOGIUS H, JOHANSSON O, ARVIDSSON J (1993) The innervation of the mystacial pad of the rat as revealed by PGP 9.5 immunofluorescence. *Journal of Comparative Neurology* **357**, 366–385.

ROMANO AT, FELTEN SY, FELTEN DL, OLSCHOWKA JA (1991) Neuropeptide-Y innervation of the rat spleen: another potential immunomodulatory neuropeptide. *Brain, Behaviour and Immunology* **5**, 116–131.

SAITO H (1990) Innervation of the guinea pig spleen studied by electron microscopy. *American Journal of Anatomy* **189**, 213–235.

UTTERBACK RA (1944) The innervation of the spleen. *Journal of Comparative Neurology* **81**, 55–68.

WEIHE E, NOHR D, MICHEL S, MÜLLER S, ZENTEL H-J, FINK T et al. (1991) Molecular anatomy of the neuro-immune connection. *International Journal of Neuroscience* **59**, 1–23.

WILKINSON KD, KEUNMYOUNG L, DESHPANDE S, DUERKSEN-HUGHES P, BOSS JM, POHL J (1989) The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science* **246**, 670–673.

ZAMBONI L, MARTINO C DE (1967) Buffered picric acid formaldehyde: a new rapid fixative for electron microscopy. *Journal of Cell Biology* **35**, 148A.