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Perineuronal Nets Provide a Polyanionic, Glia-Associated Form of Microenvironment Around Certain Neurons in Many Parts of the Rat Brain

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ABSTRACT The nature and function of previously described perineuronal nets are still obscure. In the present study their polyanionic components were demonstrated in the rat brain using colloidal iron hydroxide (CIH) staining. In subcortical regions, such as the red nucleus, cerebellar, and vestibular nuclei, most neurons were ensheathed by CIH-binding material. In the cerebral cortex perineuronal nets were seen around numerous nonpyramidal neurons. Biotinylated hyaluronectin revealed that hyaluronan occurs in perineuronal nets. Two plant lectins [*Wisteria floribunda* agglutinin (WFA) and *Vicia villosa* agglutinin (VVA)] with affinity for N-acetylgalactosamine visualized perineuronal nets similar to those rich in anionic components. Glutamic acid decarboxylase (GAD)-immunoreactive synaptic boutons were shown to occupy numerous meshes of perineuronal VVA-positive nets. Electron microscopically, VVA binding sites were scattered throughout perisynaptic profiles, but accumulated at membranes and in the extracellular space except not in synaptic clefts. To investigate the spatial relationship between glial cell processes and perineuronal nets, two astrocytic markers (S100-protein and glutamine synthetase) were visualized at the light and electron microscopic level. Two methods to detect microglia by the use of *Griffonia simplicifolia* agglutinin (GSA I-B_4) and the monoclonal antibody, OX-42, were also applied. Labelled structures forming perineuronal nets were observed with both astrocytic, but not with microglial, markers. It is concluded that perineuronal nets are composed of a specialized type of glia-associated extracellular matrix rich in polyanionic groups and N-acetylgalactosamine. The net-like appearance is due to perisynaptic arrangement of the astrocytic processes and these extracellular components. Similar to the ensheathment of nodes of Ranvier, perineuronal nets may provide a special ion buffering capacity required around various, perhaps highly active, types of neurons.

INTRODUCTION

Glial cell processes show structural relations to neuronal cell surfaces and thus form regional, neuron-specific, and subcellular distribution patterns. Substantial data indicate that especially astrocytes may adapt to morpho-functional features of the neuronal environment, which leads to an enormous variation in the number, volume fraction, and shape of cell processes (Fedoroff and Vernadakis, 1986; Reichenbach, 1989; Wolff, 1970). In particular, perisynaptic astrocytic processes may con-

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tribute to the transmitter regulation and ion homeostasis in the perisynaptic region (Kimelberg, 1988). Evidence for glial specialization was recently provided for nodes of Ranvier (ffrench-Constant et al., 1986), where the axolemma is partly invested by perinodal attachment of astrocytic processes (Black and Waxman, 1988; Waxman, 1986).

In the grey matter, another highly specialized array of glial processes appears to constitute perineuronal nets, which have been selectively stained with the Golgi impregnation (Brauer et al., 1982, 1984; Lafarga et al., 1984). Similar net-like structures enwrapping subpopulations of nonpyramidal neurons in the cerebral cortex and various types of neurons in subcortical regions were also detected by N-acetylgalactosamine (GalNAc)-binding lectins (Kosaka and Heizmann, 1989; Naegele and Katz, 1990) and by immunocytochemistry using antibodies against proteoglycans or epitopes related to them (Bertolotto et al., 1991; Fujita and Kudo, 1992; Fujita et al., 1989; Kosaka et al., 1989; Naegele and Barnstable, 1989; Naegele et al., 1988; Stephenson and Kushner, 1988; Zaremba et al., 1989, 1990). Although there are some indications that glial cells may be involved in the formation of the perineuronal proteoglycan sheath (Brückner et al., 1990; Delpech et al., 1989; Lüth et al., 1992; Schweizer et al., 1991; Yamamoto et al., 1988), most investigators, even after electron microscopic examination, interpreted these perineuronal staining patterns as being due to glycoconjugates of the extracellular matrix, which is mainly related to neuronal cell surfaces (Atoji et al., 1990; Bertolotto et al., 1991; Naegele et al., 1988; Zaremba et al., 1989; Yamamoto et al., 1988).

In the present paper cytochemical and structural evidence are given that such perineuronal nets are associated with glial cell processes, also forming net-like arrays around corresponding neurons. After confirming the presence of GalNAc-containing glycans and hyaluronan in perineuronal nets of cortical and subcortical brain regions, in the present investigation we could establish the polyanionic character of this specialized extracellular matrix and the existence of glial components at the light and electron microscopic levels. They were demonstrated by immunocytochemical detection of 2 glial markers, S100-protein and glutamine synthetase (GS), which are, unlike glial fibrillary acidic protein, ubiquitous cytoplasmic antigens (Boyes et al., 1986; Haan et al., 1982; Matus and Mughal, 1975; Norenberg and Martinez-Hernandez, 1979) and occur even in the most peripheral astrocytic processes, often being devoid of cell organelles.

MATERIALS AND METHODS

Animals

Adult male and female Wistar rats and Sprague Dawley rats were used for this study. The procedures of fixation and treatment of the tissue were performed according to necessities of the histochemical methods applied.

Preparation of Antisera and Reagents for Cytochemistry

Antiserum against glutamic acid decarboxylase (GAD) was raised in sheep (Oertel et al., 1981).

Monoclonal antibody against S100-protein was prepared by Haan et al. (1982), and G12B8 ascites fluid was kindly provided by these authors.

Antiserum against glutamine synthetase (GS) used in this study was prepared and described by Hallermayer and Hamprecht (1984). It was kindly provided by these authors.

Biotinylamidocaproyl-rabbit-anti sheep IgG was obtained after isolation of the IgG-fraction from anti-sheep serum (SIfIN) according to McKinney and Parkinson (1987) and subsequent biotinylation with Bio-cap-ONSu.

Biotinylamidocaproyl-*Vicia villosa* agglutinin (Bio-VVA) was prepared as follows: A solution of 1.35 ml of 0.05 M borate buffer, pH 8.5, containing 1.5 mg of VVA (Camon, Wiesbaden, L-1230) was mixed with 150 μl of dimethylformamide containing 150 μg of biotinylamidocaproyl-N-hydroxysuccimidester (Bio-cap-ONSu). After 1 h the reaction mixture was dialyzed against PBS 2 times at 4°C.

The hyaluronan-binding glycoprotein hyaluronectin was isolated from sheep brain as published by Delpech et al. (1991) and subsequently biotinylated (Bio-HN).

Biotinylamidocaproyl-horseradish peroxidase (Bio-HRP) was obtained by mixing of 8 mg HRP (Boehringer, Mannheim, grade I) in 0.72 ml of 0.1 M carbonate buffer, pH 8.5, with 1.4 mg Bio-cap-ONSu in 80 μl dimethylformamide for 1 h and following dialysis against several changes of PBS.

Streptavidin was affinity-chromatographically purified for a first set of experiments (detection of Bio-HN and GAD) as described by Bayer et al. (1986); otherwise, streptavidin purchased from Molecular Probes (Eugene) was used.

Histochemical Detection of Anionic Components

Colloidal iron hydroxide staining

Wistar rats were anesthetized with ether and decapitated. Brains were removed from the skull and fixed for 20 h in 5% paraformaldehyde containing 5% chromium potassium sulfate and 5% $HgCl_2$ at 4°C. Subsequently, they were rinsed in tap water for 4–5 h, dehydrated in increasing concentrations of ethanol, and after saturation in methylbenzoate, embedded in paraffin. Further steps were performed at room temperature. Then 10 μm sections mounted on slides were dewaxed and incubated for 10 minutes in a colloidal iron hydroxide solution (pH 1.15) prepared according to Graumann

and Clauss (1958). Slides were then rinsed twice in 5% acetic acid and thereafter reacted for 10 minutes in a solution containing 0.65% potassium ferrocyanide in 0.65% HCl (Berlin blue reaction). They were then rinsed twice in distilled water, dehydrated in increasing concentrations of ethanol, cleared in xylene, and coverslipped. In a series of sections, the following control procedure was performed prior to CIH staining: methylation with absolute methanol containing 0.8% HCl at 60°C for 4 h. Some of the sections were counterstained with lithium carmin.

Detection of hyaluronan with biotinylated hyaluronectin (Bio-HN)

Wistar rats were transcardially perfused under pentobarbital anesthesia with saline and 4% paraformaldehyde. The brains were postfixed in the same fixative overnight at 4°C. After cryoprotection in 30% sucrose, 30 μm thick sections were cut on a freezing microtome and treated with 0.6% H_2O_2 in TBS for 30 minutes and rinsed 3 times with TBS. The blocking of unspecific binding sites was performed with 3% BSA in TBS for 30 minutes immediately before the incubation with Bio-HN (10 μg/ml) for 2 h. Following 3 washes in TBS the sections were incubated with a preformed ABC-complex consisting of 5 μg of Bio-HRP and 10 μg of streptavidin/ml TBS, containing 2% BSA (TBS-BSA). After washing of sections, peroxidase was visualized using 3,3′-diaminobenzidine (DAB) as a chromogen.

Lectin-Binding Studies

Light microscopy

Wistar rats were perfused under pentobarbital anesthesia with 4% buffered paraformaldehyde. Using 30 μm frozen sections, the experiments were performed in the same manner as described for detection of hyaluronan. Biotinylated *Wisteria floribunda* agglutinin (Bio-WFA; Sigma, L-1766) and biotinylated *Vicia villosa* agglutinin (Bio-VVA), prepared as described above, were applied in the concentration range of 2–10 μg/ml. Controls were performed as published by Mulligan et al. (1992), with lectins preincubated with 0.2 M GalNAc for about 10 h before use in cytochemistry.

Electron microscopy of *Vicia villosa* agglutinin (VVA)-binding

Sprague-Dawley rats were perfused under pentobarbital anesthesia with 5% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer. Then 40 μm thick vibratome sections were treated with VVA-HRP conjugate (Sigma, L-5641) used in a concentration of 2 μg/ml, which was visualized by DAB without nickel enhancement. The stained sections were dehydrated in series of graded ethanol, processed through propylene oxide, and embedded in epoxy resin.

Immunocytochemical Studies

Immunocytochemical detection of glutamic acid decarboxylase (GAD)

Animals were perfused under pentobarbital anesthesia with 4% paraformaldehyde and postfixed for 12–24 h at 4°C. In some experiments the fixative additionally contained 0.5% zinc salicylate, according to Mugnaini and Dahl (1983). Then 30 μm frozen sections were treated with 0.6% H_2O_2 in TBS for 30 minutes and rinsed 3 times with TBS. After blocking of unspecific binding sites with 5% normal rabbit serum (NRS), the sections were incubated with sheep-anti-GAD, diluted 1:4,000 in 0.5 M TBS containing 1% NRS. Following 3 washes with TBS, the incubation with Bio-rabbit-anti-sheep IgG (10 μg/ml TBS-BSA) was performed for 1 h. Washed sections were then reacted with a preformed ABC-complex and DAB was used as the chromogen, as described above. In control experiments the anti-GAD serum was omitted from the procedure.

Immunocytochemical detection of S100-protein

For S100-immunocytochemistry, Sprague Dawley rats were fixed by perfusion using 0.1 M Na-cacodylate, 0.1% $CaCl_2$ at pH 7.2 for rinsing the vascular system, and 4% paraformaldehyde, 0.2% glutaraldehyde in the same buffer for fixation. The overall fixation time was limited to 6 h. The ABC-method was used for preembedding staining of 50 μm thick vibratome sections. Monoclonal G12B8 antibody (Haan et al., 1982; dilution 1:1000) and rabbit antiserum (Dako; dilution 1:2,000), both of which detected S100a and S100b, were applied. Further detection was done as described by Rickmann et al. (1987).

For electron microscopy we resectioned the embedded vibratome section twice (semithin, ultrathin) to select that layer which was optimally penetrated by the immunological reagents.

Combination of S100-immunocytochemistry with colloidal iron hydroxide staining

In order to demonstrate the spatial relationship between S100-positive structures and polyanionic components, the paraffin sections of the same material used for CIH staining were treated for S100-immunocytochemistry. The sections were incubated in the rabbit S100 antiserum (Dako; dilution 1:600) for 16 h at 4°C. The primary antibodies were detected by biotinylated donkey-anti-rabbit IgG (Amersham; dilution 1:200), which

was visualized by the streptavidin/Bio-HRP complex, with DAB as the chromogen. After repeated rinsing in distilled water, the colloidal iron hydroxide staining procedure was subsequently performed. The sections were then rinsed in distilled water, dehydrated in alcohol, and coverslipped. This method resulted in double-stained sections in which S100-positive structures were brown and polyanionic components were blue.

Immunocytochemical detection of glutamine synthetase (GS)

Sprague-Dawley rats were transcardially perfused under ether anesthesia with 50 ml saline and a fixative containing 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After 2 h postfixation, the cerebellum was dissected and cut into 40 μm vibratome sections.

GS-immunoreactivity in sections containing the cerebellar nuclei was demonstrated according to Derouiche and Frotscher (1991). Briefly, after pretreatment with 1% $NaBH_4$ in order to prevent nonspecific, glutaraldehyde-induced immunostaining, and preincubation in 10% normal goat serum, the incubation followed with antiserum directed against GS (dilution 1:4,000) for 24 h. The immunoreactivity was visualized using the ABC-method and DAB as the chromogen. The DAB reaction product was silver-intensified, however, omitting the incubation in sulfuric acid for preservation of the ultrastructure. The silver grains were then stabilized by gold chloride. After dehydration and block-staining with 1% uranyl acetate, sections were flat embedded in epoxy resin, examined and documented in the light microscope, and mounted on resin blocks for ultramicrotomy.

Application of microglial markers

Microglia was stained by 2 methods in 30 μm frozen sections obtained from paraformaldehyde-fixed Wistar rats. Peroxidase-conjugated lectin from *Griffonia simplicifolia* (GSA I-B_4; Sigma, Munich) was used as described by Streit (1990), but mostly with nickel enhancement of the applied chromogen DAB. The immunocytochemical visualization of microglia was performed with the monoclonal antibody OX-42 (Camon, Wiesbaden) stained by a streptavidin-biotin method with DAB as the substrate.

RESULTS

Detection of Anionic Components by Colloidal Iron Hydroxide (CIH) Staining

The aim of this part of our study was to demonstrate the spatial arrangement of polyanionic components associated with perineuronal nets. Detailed mapping of all brain regions will be published elsewhere.

Perineuronal nets stained with the colloidal iron hydroxide method were found in varying numbers in many regions of the rat brain. These nets surrounded either small subpopulations of neurons, as in the cerebral cortex and basal forebrain, or major fractions of neurons, as in certain subcortical nuclei (e.g., red nucleus, motor nerve nuclei, cerebellar and vestibular nuclei, gigantocellular reticular nucleus).

In the cerebral cortex and in many subcortical nuclei, the dominant staining patterns were net-like arrays of CIH-positive structures surrounding cell bodies and proximal dendrites of certain neurons (Figs. 1–3 and 12). The characteristic staining pattern was based on CIH-positive meshes enclosing CIH-negative holes of varying sizes. Significant differences occurred in the average size of the mesh width; i.e., the size was relatively small in the cerebral cortex (0.5–1 μm) and large in the cerebellar and vestibular nuclei (1–3 μm). In some brain regions, such as the medial trapezoid nucleus, CIH-staining extended into the neuropil. Labelled nodes of Ranvier in both white and grey matter were clearly detected (Figs. 3, 12, and 13).

Consistently, the cytoplasm of neurons and glial cell bodies did not show any significant staining with the iron binding method.

Perineuronal nets were seen to be most frequent in layer IV and the upper layer V; they were rarely found in layer VI and were completely absent in layer I. A similar pattern was evident in all primary sensory areas. Differences between cortical layers did not correspond to differences in cell density, either of the total neuronal population or that of GABAergic interneurons in the cortical layers II-VI (compare Chronwall and Wolff, 1980; Wolff et al., 1984).

In most cortical areas the distribution of neurons surrounded by perineuronal nets showed a histotypic pattern. In the granular retrosplenial cortex, a row of neurons with a basket cell-like appearance was situated in the granular layer and showed a net-like ensheathment. In the hippocampus CIH-stained perineuronal nets could be correlated with nonpyramidal cells of the CA-regions and subiculum. Their position and number showed region-dependent variations. The surfaces of granular neurons of the dentate gyrus were not covered by CIH-positive nets.

In control experiments perineuronal nets were not revealed by the CIH-procedure, i.e., after methylation of anionic groups the stainability was abolished.

Detection of Hyaluronan by Biotinylated Hyaluronectin (Bio-HN)

The staining pattern obtained with Bio-HN was very similar to those found with the CIH-method, including the labelling of the nodes of Ranvier, except for a relatively intensive staining of the neuropil. The Bio-HN method clearly revealed the perineuronal nets surrounding several types of nonpyramidal neurons in comparable layers of the cerebral cortex and in subcortical regions, as mentioned in the previous section. A structural detail of a perineuronal net after reaction with Bio-HN

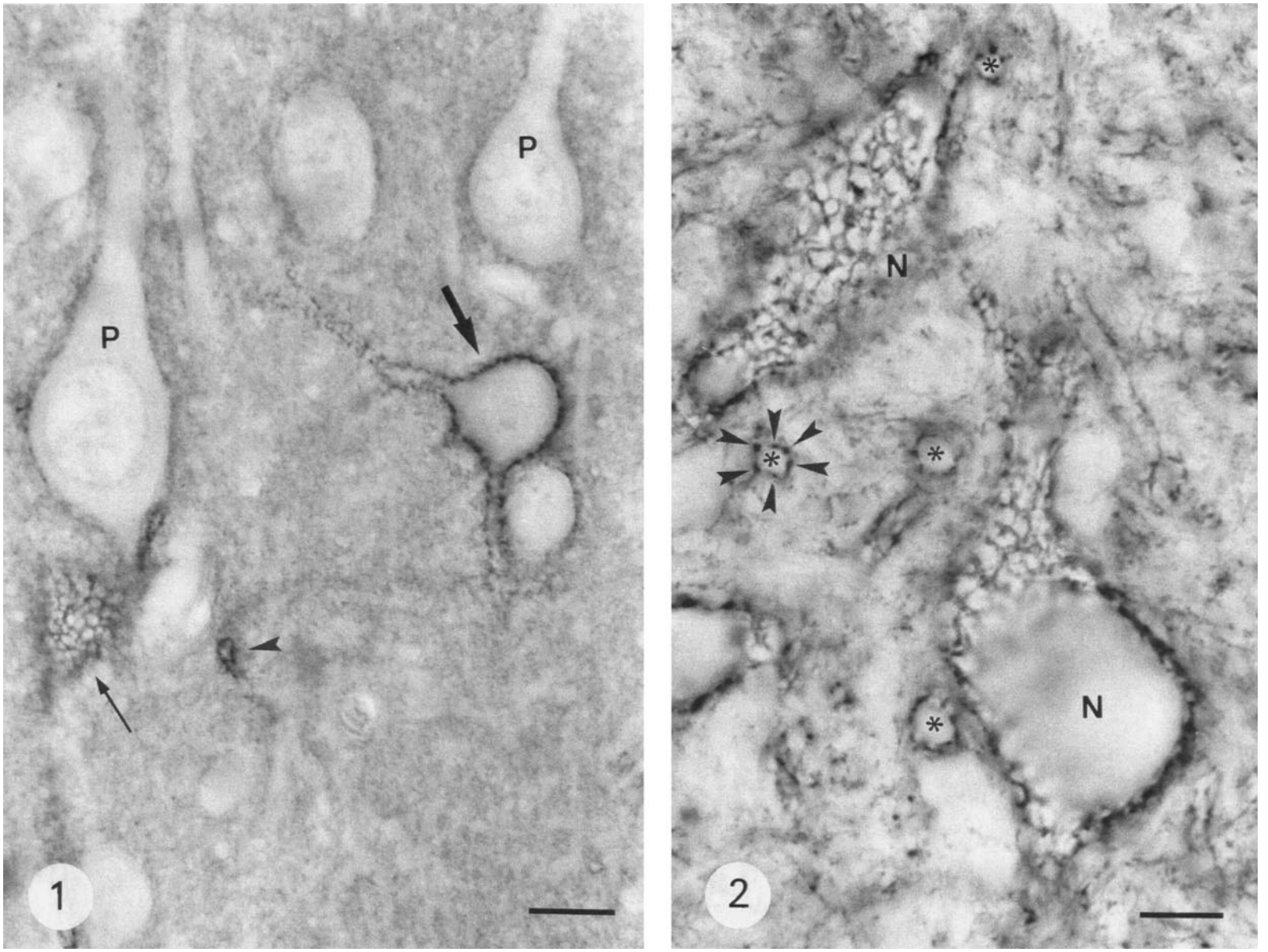


Fig. 1. Staining by the colloidal iron hydroxide (CIH) procedure of 2 nonpyramidal neurons in the upper level of layer V of the rat parietal cortex. Neuronal perikarya and the proximal dendrites are surrounded by an intensely stained net-like structure which is sectioned perpendicularly (large arrow) and in the tangential plane (small arrow). The staining intensity in the neuropil and at the surface of pyramidal cells (P) and other neurons is relatively low. The arrowhead indicates a CIH-positive dendritic profile in cross-section. Nomarski optics. Bar, 10 μm.

Fig. 2. CIH-staining of the rat medial cerebellar nucleus. Two large neurons are ensheathed by CIH-stained nets, which show the distinct pattern of meshes. Cross sections of large dendrites (asterisks) show a punctate staining (arrowheads). Glial cell bodies are not stained. N = neuron. Nomarski optics. Bar, 10 μm.

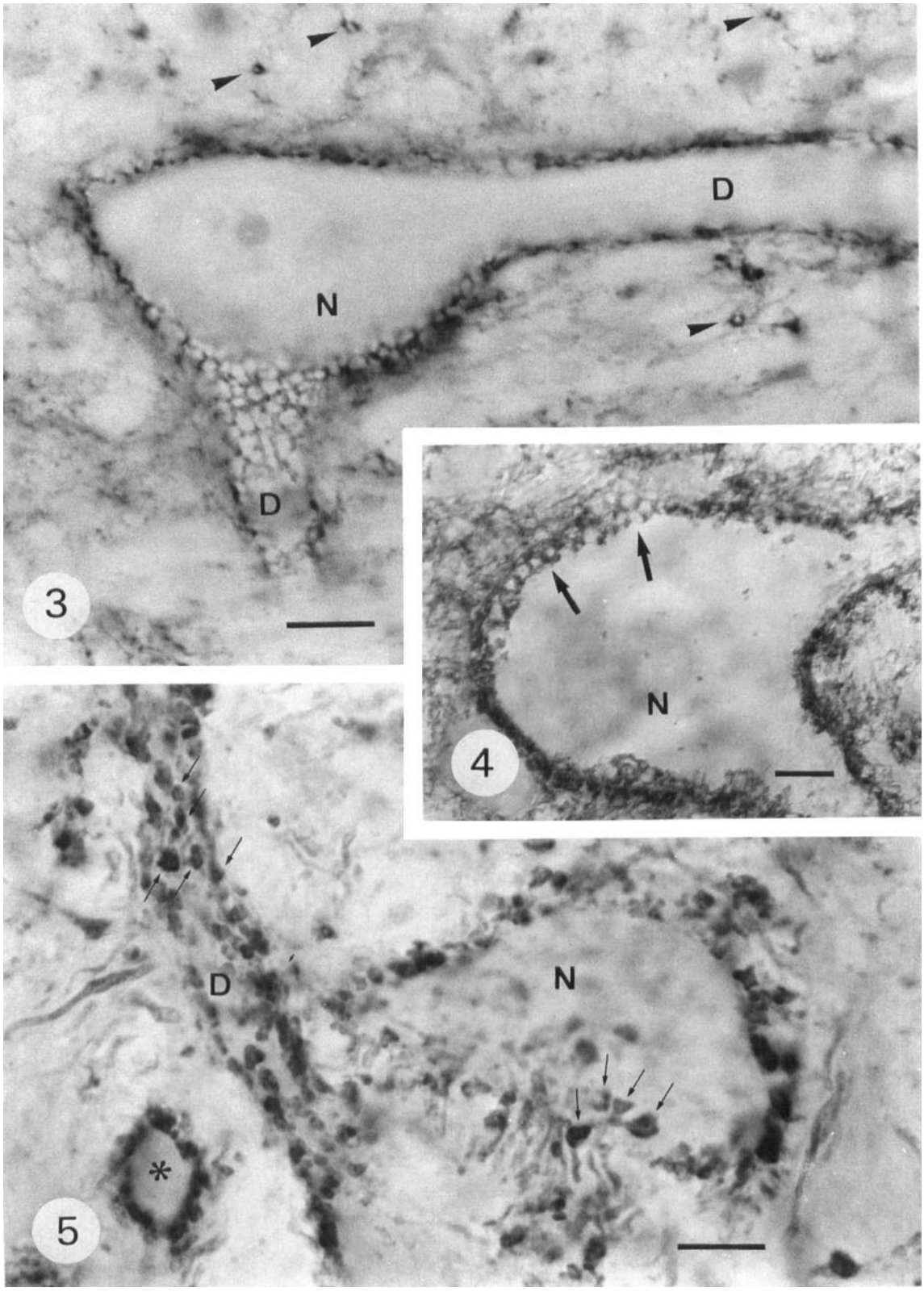


Fig. 3. CIH staining of the rat lateral vestibular nucleus. A large cell body of a Deiters neuron (N) with its proximal dendrites (D) is surrounded by an intensely stained net, partially seen in the tangential plane. Nodes of Ranvier exhibit strong CIH staining (arrowheads). Nomarski optics. Bar, 10 μm.

Fig. 4. Detection of hyaluronan with biotinylated hyaluronectin in the lateral vestibular nucleus. Structural details of the perineuronal net (arrows) resemble those of CIH staining shown in Figure 3. Nomarski optics. Bar, 10 μm.

Fig. 5. Detection of glutamic acid decarboxylase (GAD) immunoreactivity in the lateral vestibular nucleus. The Deiters neuronal cell body (N) and dendrites shown in the tangential plane (D) or in cross section (asterisk) are covered by GAD-positive synaptic boutons (arrows). The picture appears to be complementary to the net-like pattern shown in Figures 3 and 4. Bar, 10 μm.

is shown in Figure 4, which was taken from the lateral vestibular nucleus.

Lectin Binding

The 2 N-acetylgalactosamine binding lectins used in this study, *Wisteria floribunda* agglutinin (WFA) and *Vicia villosa* agglutinin (VVA), bound to components of perineuronal nets and, therefore, led to distribution patterns similar to those observed with the CIH-staining. This similarity was specifically checked and confirmed in the cerebral cortex, including the hippocampus, and in many subcortical structures, such as the red nucleus, oculomotor and motor trigeminal nucleus, and cerebellar and vestibular nuclei. Common structural details of the perineuronal nets, such as the shape and size distribution of meshes, were also found to be identical with these different methods. Particularly in the cerebral cortex, however, the lectins demonstrated the perineuronal nets with higher selectivity than CIH and Bio-HN staining due to the moderate or low lectin staining of the surrounding neuropil. Therefore, VVA was also applied at the electron microscopic level (see below).

Wisteria floribunda agglutinin (WFA)

Perineuronal nets were stained with varying intensity (Figs. 6, 7). In the cerebral cortex many nonpyramidal neurons were intensely stained, lying side by side with others which showed moderate lectin binding. The former were confined to layers II–VI. Pyramidal neurons, e.g., in layer V of the parietal motor cortex, frequently had very weakly stained nets ensheathing the perikarya.

WFA labelled perineuronal nets extended from neuronal perikarya via proximal to peripheral parts of first order branches of dendrites and very often also included the initial segment of the axon. Such nets were observed in the cerebral cortex and in various subcortical regions (Fig. 7a).

Vicia villosa agglutinin (VVA)

This lectin stained the perineuronal nets as already demonstrated by other authors in the rat brain (Kosaka and Heizmann, 1989; Nakagawa et al., 1986). Although both lectins are supposed to bind to GalNAc, the staining patterns were similar but not identical. In comparison to WFA, VVA was bound to a higher degree in the neuropil of some brain regions and led to a very high staining intensity at the nodes of Ranvier.

Electron microscopic visualization of VVA binding sites

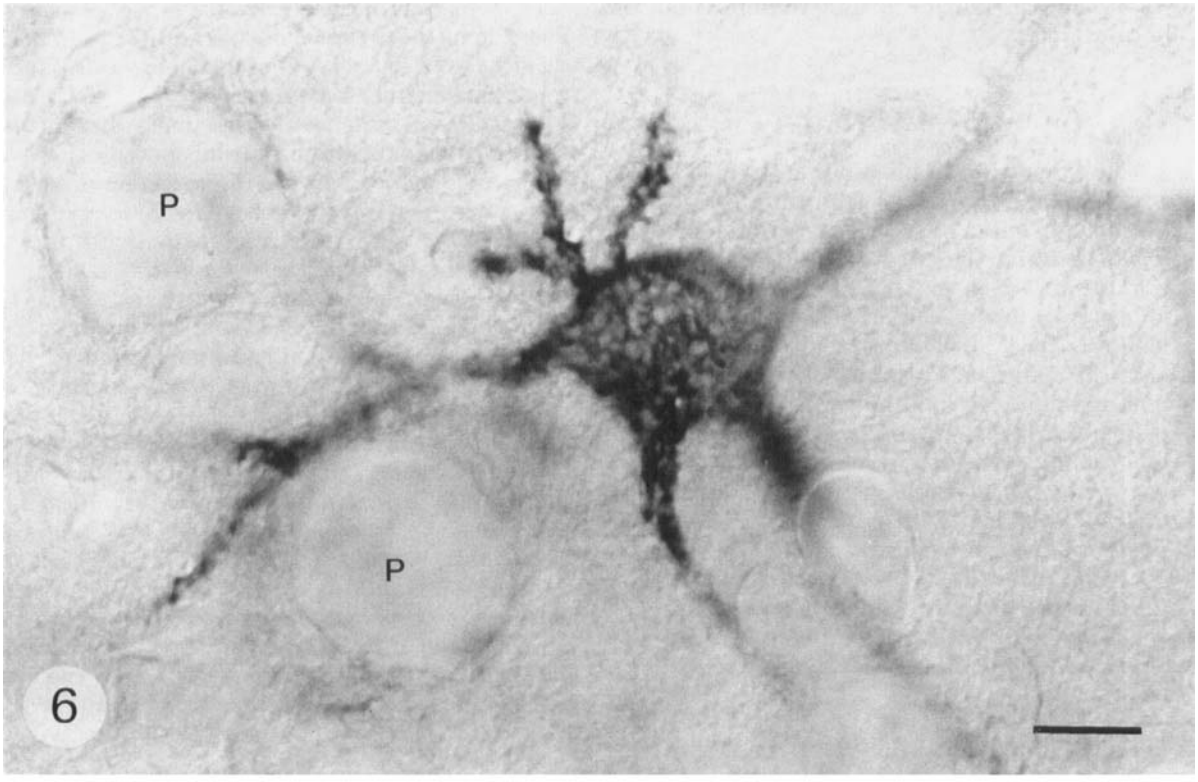
Net-like perineuronal structures identified by light microscopy on semithin sections of the cerebral cortex were studied with the electron microscope. The diaminobenzidine (DAB) precipitates indicated that VVA binding sites were distributed in translucent profiles in the immediate vicinity of neuronal surfaces, which could be either sections of dendrites or somata. In all cases the net-like arrangement of VVA-labelled profiles was obvious and depended on the interposition of VVA-negative axonal boutons and presynaptic elements of varying size (Fig. 8a,b). Parallel with light microscopic findings, there was a constant spatial relationship of VVA-positive profiles, which always surrounded synaptic contacts on somatic and dendritic surfaces. In general, each presynaptic bouton was surrounded by a VVA-reactive sheath, i.e., the presynaptic bouton occupied one mesh of the perineuronal net. DAB-precipitates were more or less diffusely distributed throughout labelled profiles, but accumulated along their outer border, extending into the extracellular space. Membrane-bound precipitates were found to be apposed to neighbouring presynaptic boutons, the surface of the postsynaptic neuron, and small, axonal, glial, or unidentified profiles (Fig. 8a,b). In constrast to this, the VVA-binding did not occur in the zones where presynaptic boutons were apposed to the postsynaptic neuron; the synaptic cleft was especially devoid of reaction product (Fig. 8a,b).

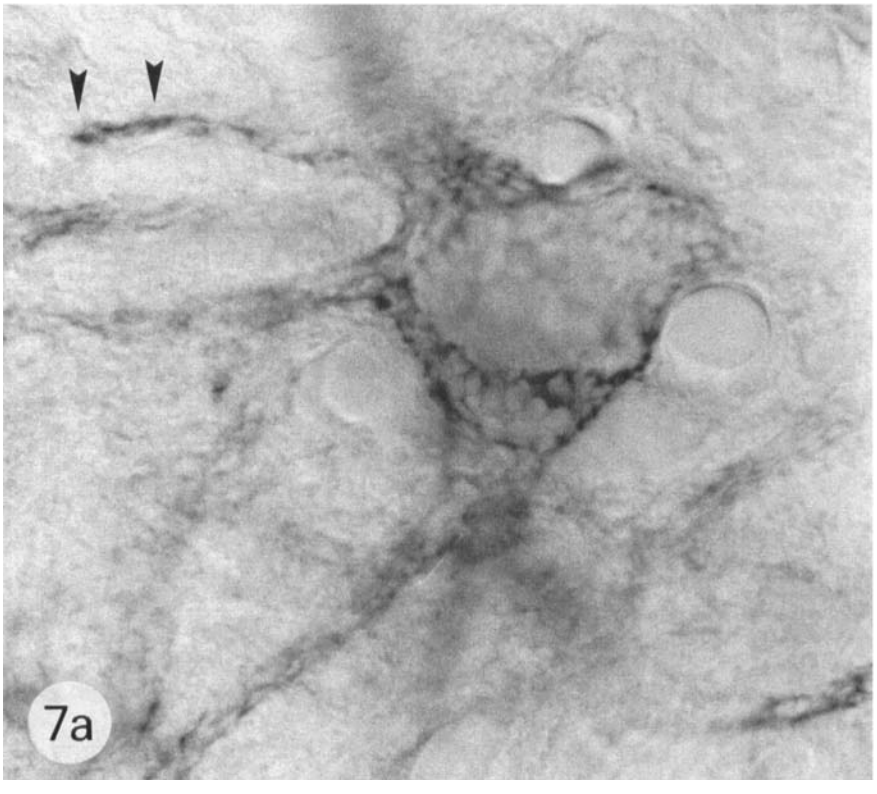
Detection of GABAergic Structures

It was a consistent observation in our study that the neuronal types characterized by the polyanionic nets received a significant GABAergic input. The distribution and size of the GAD-immunoreactive synaptic boutons fitted well into the hollows of the perineuronal nets. In light microscopic observations many of the meshes seemed to be occupied by GABAergic synaptic boutons. An example obtained from the lateral vestibular nucleus is shown in Figures 3 and 5. The neuronal cell bodies and the proximal dendrites were covered by tightly packed large GAD-immunoreactive axonal boutons. The same interlocking pattern was found in the cerebellar nuclei. In other brainstem regions with higher GAD-immunoreactivity in the neuropil, such as the striatum, the basal forebrain, the reticular thalamic nucleus, the superior colliculus, and the cerebellar cortex, the correlation with the CIH-staining requires further investigation on the afferent patterns and functional characteristics of the GABA containing terminals in these regions.

Immunocytochemical Detection of S100-Protein

This method allowed immunocytochemical identification of parent cells which contribute a glial component in perineuronal nets. In the lateral vestibular nucleus (Deiters), S100-immunoreactive cell processes formed net-like patterns similar to those of CIH-positive and lectin-binding perineuronal nets surrounding the large neurons. Otherwise, S100-antibodies appar-





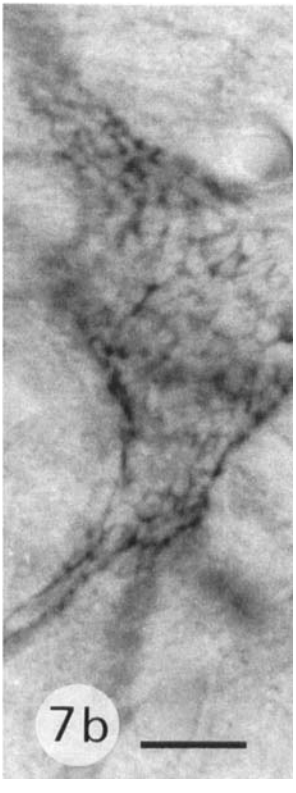


Fig. 6. Demonstration of a perineuronal net by *Wisteria floribunda* agglutinin (WFA) in layer V of the parietal cortex. A nonpyramidal cell is intensely labelled, even on intermediate sectors of dendrites. The surface of pyramidal cells (P) and the neuropil are almost unlabelled. Nomarski optics. Bar, 10 μm.

Fig. 7. WFA-binding in the medial cerebellar nucleus. A perineuronal net is shown at 2 levels of focus (**a**,**b**). The rat and the incubation conditions were the same as in Figure 6. Note the larger size of the meshes but the lower staining intensity in the cerebellar perineuronal net as compared to the neuron of the cerebral cortex. The arrowheads indicate a net-like pattern on what appeared to be the axon initial segment. Bar, 10 μm.

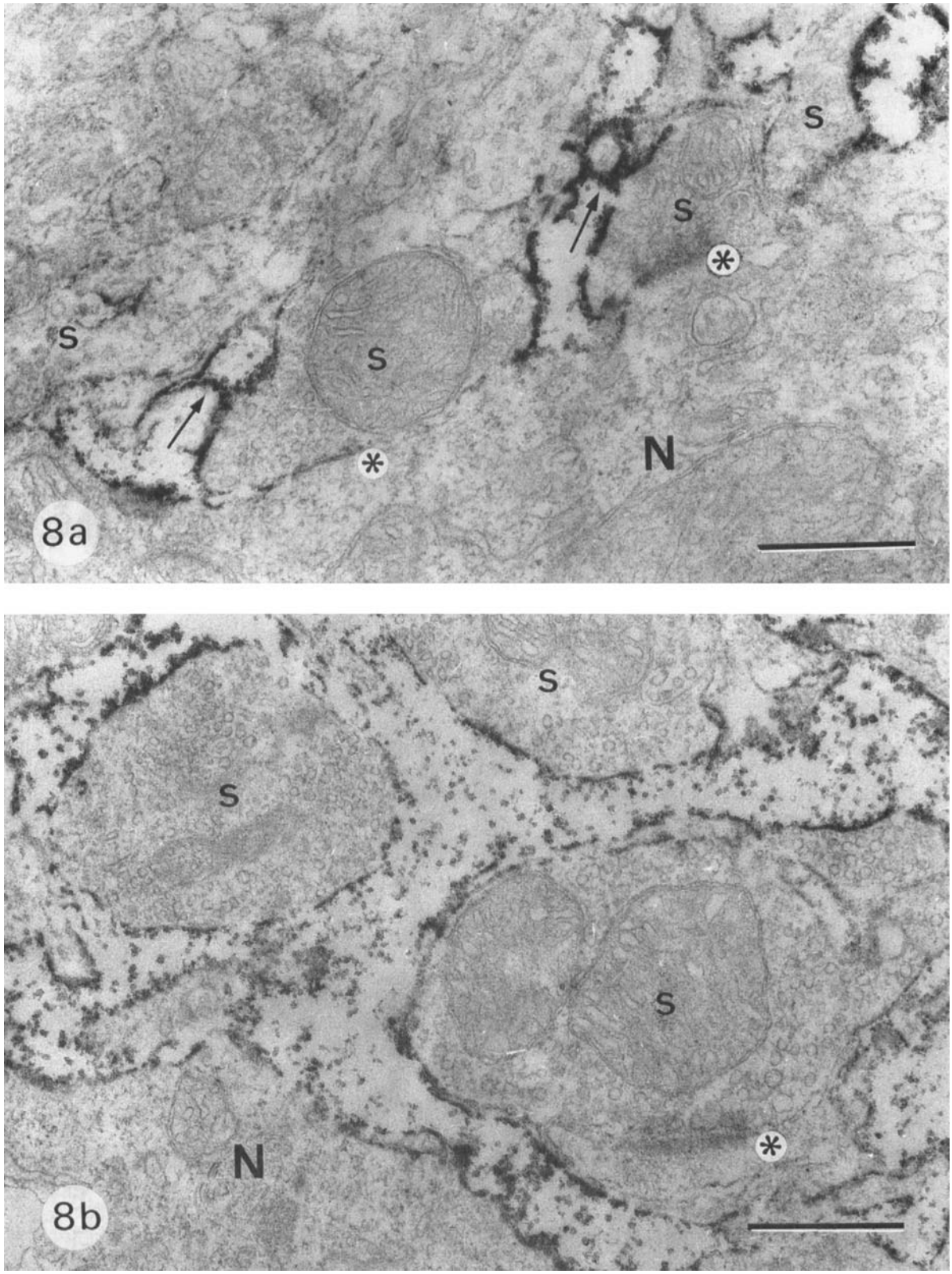


Fig. 8. Electron microscopic detection of *Vicia villosa* agglutinin (VVA) binding in the visual cortex. The distribution of diaminobenzidine precipitates in the microenvironment of neurons (N) is shown in the cross-sectional (**a**) and tangential planes (**b**). Presynaptic boutons (S) are ensheathed by lectin-binding profiles in which membrane-associated (arrows) or extracellularly located precipitates predominate. The extracellular space near synaptic contact zones (asterisks) is free of label. Bar, 0.5 μm.

ently labelled all astrocytes (Fig. 9a). Our observations agreed with the generally accepted notion that S100 is predominantly localized in astrocytes (Moore, 1988), while cells with ultrastructural criteria of microglial cells or oligodendrocytes never contained any detectable amounts of S100. In addition to astrocytes, S100 was seen in cell bodies and proximal dendrites of Deiters neurons. However, their distinct morphological characteristics allowed a definite identification of astrocytic elements.

Since S100 occurred in all parts of astrocytic processes, even in the finest lamellae, the tissue was traversed by irregularly shaped immunopositive glial profiles. Around Deiters neurons, they condensed to form a meshwork with pores of about 1 μm size (Fig. 9a).

In the electron microscope, the perikarya of Deiters neurons were almost completely covered by large immunonegative presynaptic elements (Fig. 9b). These axosomatic synapses were ensheathed by S100-positive cell processes, which showed concave outlines, gave rise to fine lamellae, contained S100-protein, and could reach the surface of Deiters neurons (Fig. 9c). According to these immunocytochemical and structural criteria, perineuronal nets included glial components, which consisted of distal portions of astrocytic processes and ensheathed large, densely packed, axosomatic synapses.

Combination of S100-Immunocytochemistry With the Colloidal Iron Hydroxide (CIH) Staining

The detection of S100-protein was also performed in the sublimate-fixed, paraffin-embedded tissue with the intention to show the spatial relation between the polyanionic components and S100-immunoreactive astrocytic structures. As in paraformaldehyde-fixed brains, there was a significant labelling of numerous glial cells, which exhibited the structural criteria of astrocytes in the white and grey matter.

In sections first stained with S100-immunocytochemistry using the ABC-technique, the blue staining of CIH-labelled structures could be clearly distinguished from the brown DAB reaction product (Fig. 13). By CIH-binding, perineuronal nets and nodes of Ranvier were identified in various brain regions. The CIH-stained perineuronal nets were surrounded and penetrated by S100-positive cell processes, which apparently formed an outer net often in continuity with the CIH-positive material (Fig. 13). This pattern suggested that CIH-positive components of perineuronal nets were somehow interposed between the neuronal surface and S100-positive cell processes of astrocytes.

Immunocytochemical Detection of Glutamine Synthetase (GS)

Light microscopy

In the cerebellar nuclei investigated here, GS-immunoreactivity appeared much stronger than in the neighbouring white matter. Many small cells were GS-immunopositive. These cells were frequently seen in satellite position to neurons (Fig. 11). Stained cells in the neuropil did not show the classical morphology of either microglial or protoplasmic astroglial cells. The small size of their roundish perikarya suggested that there was only a narrow rim of cytoplasm surrounding the nucleus. These features resembled glioblastic cells or oligodendrocytes. GS-positive fine cell processes could not be followed over long distances. This was probably due to their irregular shape and branching, which also gave rise to numerous silver grains evenly scattered throughout the neuropil. However, GS-positive branches contacting each other were observed where these were apposed to neuronal surfaces and collectively outlined more or less completely some perikarya and proximal dendrites (Fig. 11).

Electron microscopy

Apart from the very surface of the section, where silver grains were randomly distributed, grains were confined to the interior of cellular profiles, often without the concomitance of visible DAB reaction product. As observed in hippocampal astrocytes (Derouiche and Frotscher, 1991), silver crystals were preferentially localized in the tapering ends and finger-like profiles of glial processes (Fig. 10a,b). Such processes were diffusely distributed in the neuropil and contained few if any organelles. These GS-positive cell processes contacted axon terminals and capillaries, and therefore probably belonged to astrocytes. On neuronal somata or larger dendritic profiles, contacts of GS-positive processes were either occasional or numerous, as within perineuronal nets. Typically, the meshes were filled in by large boutons, which were rich in mitochondria and predominantly formed symmetric synaptic contacts (Fig. 10b). The nucleus of immunoreactive cells was always unstained, displaying a rather homogeneous chromatin distribution. Its shape was usually round, ovoid or kidney-shaped but nuclei were never elongated, as would be typical of microglial cells. Intermediate filaments could occasionally be found in the perinuclear cytoplasm, indicating that GS-positive cells may belong to the class of astrocyte-like macroglial cells.

Detection of Microglial Structures

Lectin and immunocytochemical methods commonly used for detection of microglial cells were applied to test the possibility that this glial cell type participates in the formation of perineuronal nets. This proposal was derived from the morphological characteristics of Golgi-impregnated cells being continuous with perineuronal nets (Brauer et al., 1982, 1984). Although these markers (GSA I-B_4, OX 42) visualized cells with the typical morphology of microglial cells, we did not see a staining resembling the typical pattern of perineuronal nets.

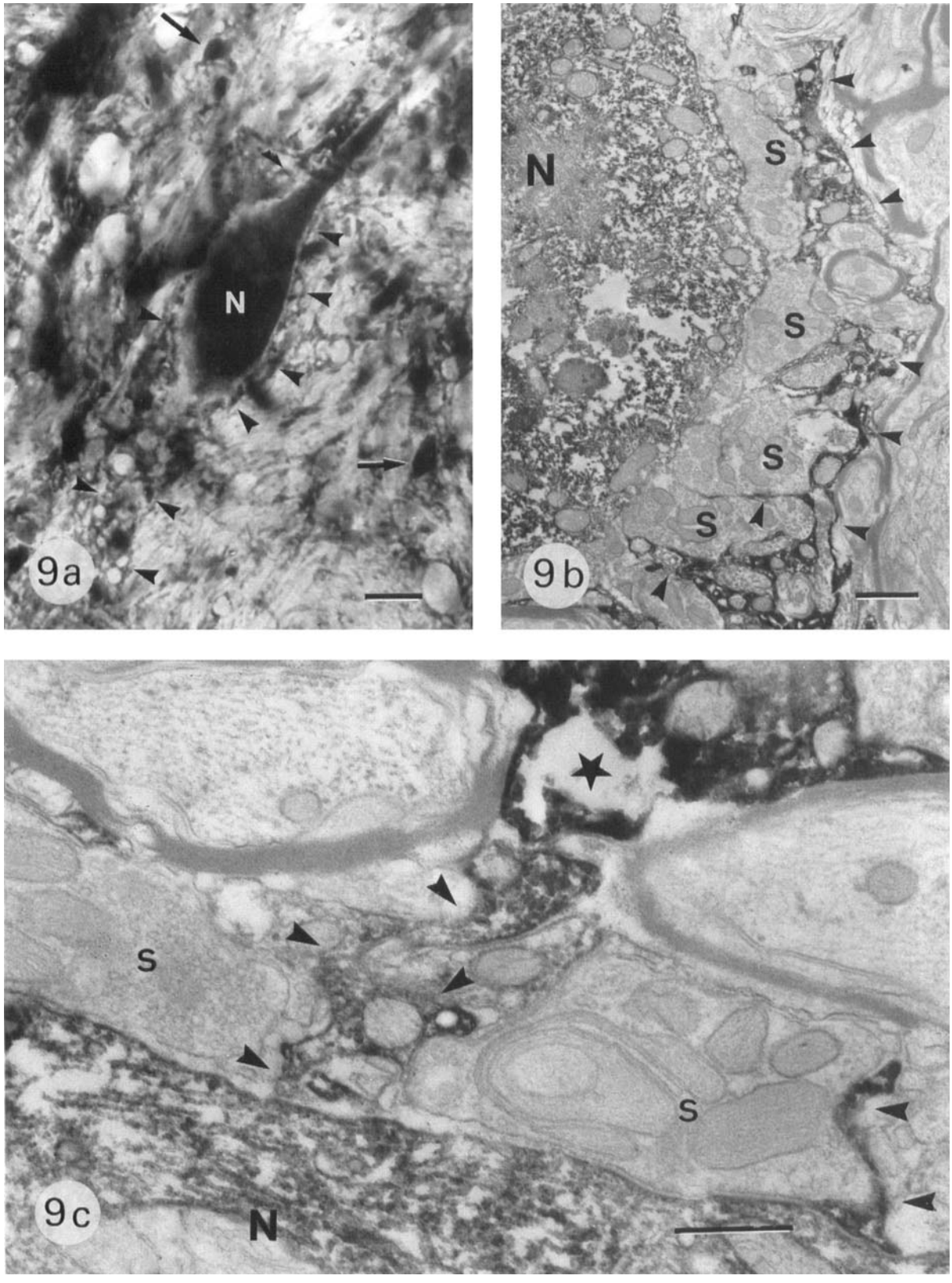


Fig. 9. Immunocytochemical detection of S100-protein in the lateral vestibular nucleus. a: Light micrograph showing the distribution of S100-positive structures. The immunoreactive Deiters neuron (N) is surrounded by a net of astrocytic processes (arrowheads). In the lower left the neuron's process has been cut off so that the covering astroglial sheath can be viewed tangentially. Arrows point to immunoreactive astrocytic cell bodies. Bar, 10 μm. b: Low-power electron micrograph of a Deiters neuron (N) which is S100-positive and is covered by numerous immunonegative presynaptic boutons (S). This assembly is ensheathed by a net of strongly S100-positive astroglial processes and lamellae (arrowheads), which partly penetrate spaces between boutons. Bar, 1 μm. c: The high power electron micrograph demonstrates astroglial lamellae (arrowheads), which originate from a larger process (star), interdigitate with the covering of synaptic boutons (S), and touch the surface of the Deiters neuron (N). Bar, 0.5 μm.

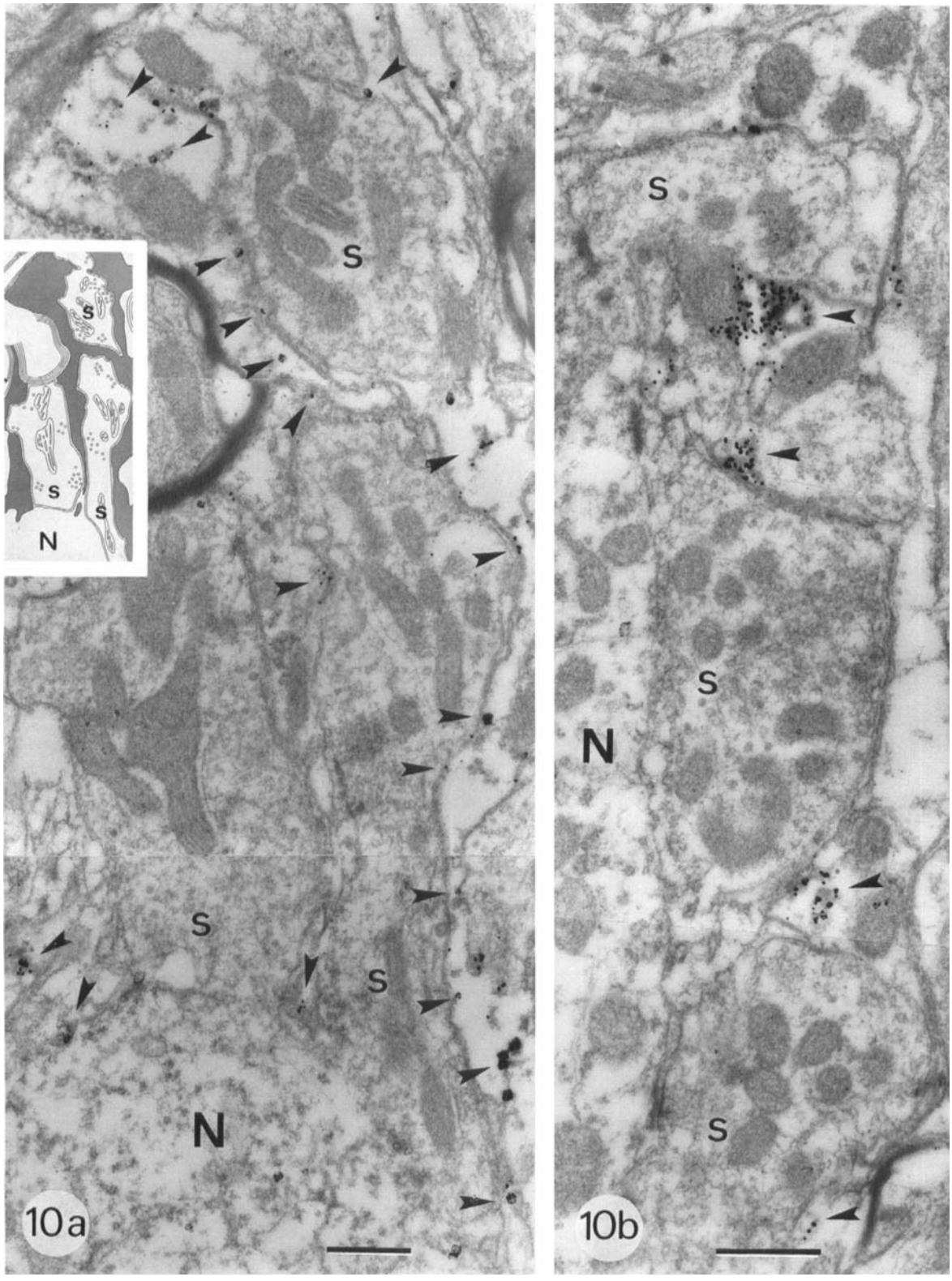


Fig. 10. Electron microscopic detection of glutamine synthetase (GS) in the medial cerebellar nucleus. A neuron (N) is contacted by synaptic boutons (S), which are ensheathed by GS-positive glial profiles. Arrowheads indicate grains labelling GS immunoprecipitates. **a:** Tangential view. The inset schematically illustrates the net-like appearance of the perineuronal glial sheath (shaded areas) shown in the electron micrograph. The pattern corresponds to perineuronal nets observed in light microscope (see Fig. 12). Bar, 0.5 μm. **b:** Cross-sectional plane. Three synaptic boutons (S) are partially ensheathed by labelled glial processes. Bar, 0.5 μm.

DISCUSSION

Perineuronal nets were stained with the basic dyes described, and their possible functions have been discussed already by Ramon y Cajal (1906). By this time perineuronal nets of Golgi (1891) had been controversially interpreted (for details see Brauer et al., 1982, 1984; Szentagothai, 1975). Some of the original ideas have to be reconsidered on the basis of the present findings.

Cytochemical Aspects

A common cytochemical principle of the perineuronal nets is the high concentration of polyanionic, GalNAc-containing components. Staining patterns similar to those observed in the present study have been demonstrated with the periodic acid-Schiff reaction and colloidal iron binding (Rambourg et al., 1966), with the immunocytochemical detection of hyaluronectin (Delpech et al., 1982) and monoclonal antibodies recognizing glycosaminoglycan-related epitopes (Bertolotto et al., 1990, 1991; Fujita et al., 1989; Fujita and Kudo, 1992; Kosaka et al., 1989; Morino-Wannier et al., 1992; Naegele et al., 1988; Stephenson and Kushner, 1988; Zaremba et al., 1989, 1990). The perineuronal material was sensitive to chondroitinase ABC, which confirmed its proteoglycan character (Fujita et al., 1989). Chondroitin sulphate was identified as a major component of these high molecular-weight glycoconjugates, which despite their common properties reveal a remarkable degree of structural heterogeneity (Bertolotto et al., 1990, 1991; Fujita and Kudo, 1992; Zaremba et al., 1989, 1990).

Lectins such as *Vicia villosa* agglutinin (VVA) and soybean agglutinin (SBA) (Kosaka and Heizmann, 1989; Lüth et al., 1992; Naegele and Katz, 1990; Nakagawa et al., 1986) as well as *Wisteria floribunda* agglutinin (WFA), which was recently introduced as a marker for perineuronal nets (Härtig et al., 1992), probably all recognize N-acetylgalactosamine in the repeating unit of chondroitin sulphate. However, other components of the macromolecular proteoglycan complex may additionally be detected by lectins. The core proteins of chondroitin sulphate proteoglycans are characterized by poly(N-acetyllactosaminyl) oligosaccharides (Gowda et al., 1989) and may therefore be further candidates for visualization of perineuronal nets.

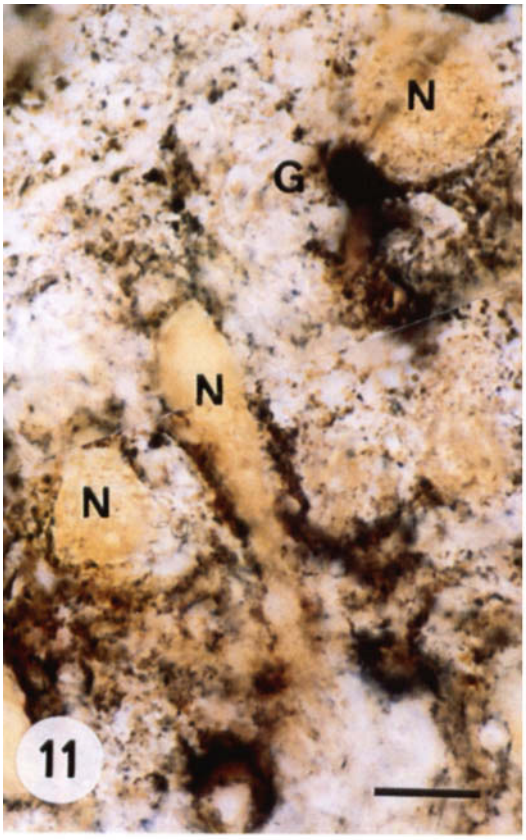
As shown in previous studies (Delpech et al., 1989; Girard et al., 1986, 1992) and confirmed here, hyaluronan is an anionic component of the perineuronal nets. The hyaluronan-binding glycoprotein hyaluronectin is not restricted to perineuronal nets, but it was immunocytochemically detected in association with these structural complexes (Brückner et al., 1990; Delpech et al., 1982, 1989). From other studies it has been concluded that hyaluronan can form aggregates with sulfated proteoglycans in glial cultures (Norling et al., 1978) and in neuronal tissue in situ (Margolis and Margolis, 1989). Thus there may be a special relationship of glial cells to perineuronal nets. Essentially the same cytochemical properties as in perineuronal nets can be found at nodes of Ranvier which are contacted by perinodal astrocytic processes (Black and Waxman, 1988; Waxman, 1986). In the nodal region, the polyanionic extracellular milieu contains hyaluronan and hyaluronectin (Delpech et al., 1982; Girard et al., 1986, 1992) and the astrocyte-derived molecule tenascin, formerly named J1-glycoprotein or cytotactin (ffrench-Constant et al., 1986; Rieger et al., 1986).

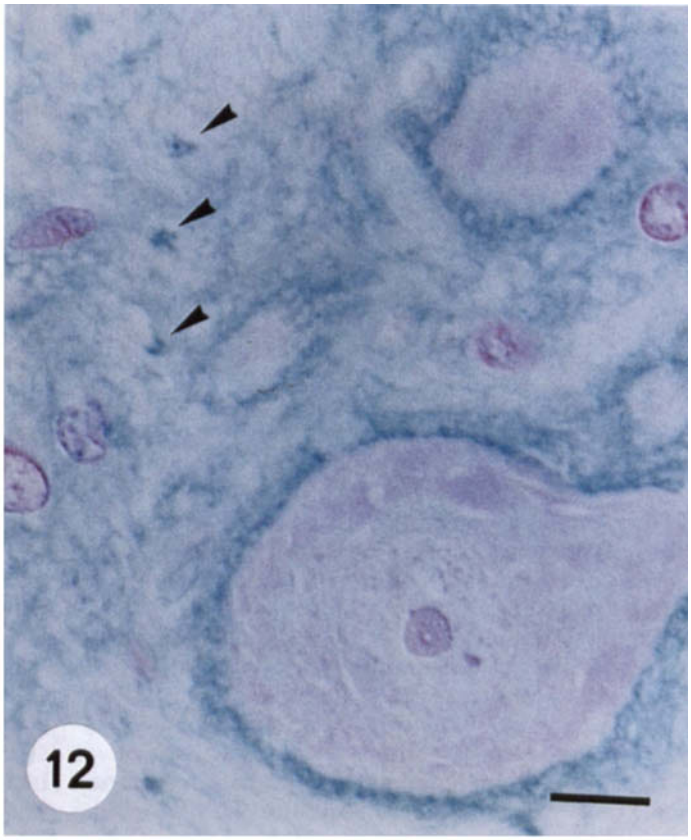
Structural Aspects

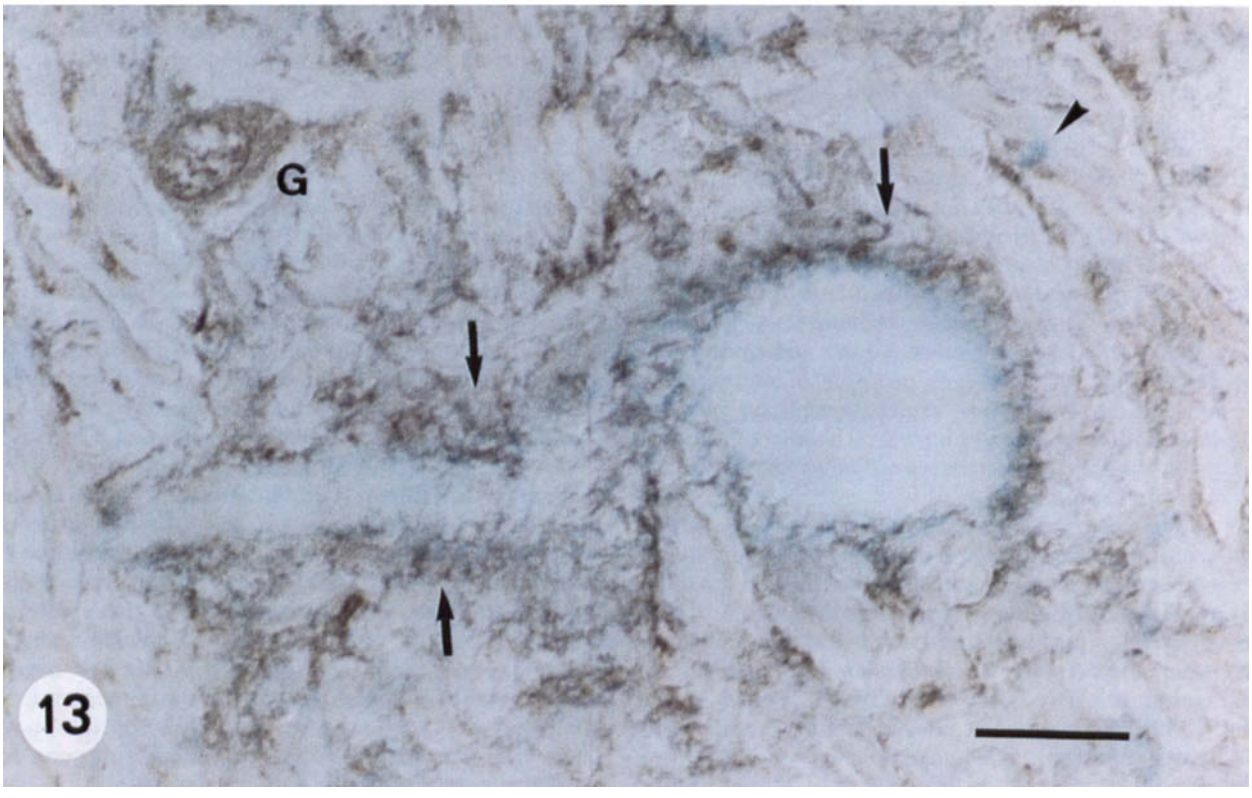
Light microscopical analysis of Golgi-impregnated material (Brauer et al., 1982, 1984; Lafarga et al., 1984) indicated that perineuronal nets identified by the presence of polyanionic glycans show some structural relations to perineuronal nets which can be visualized by glial markers and may even depend on the presence of certain glial cell processes. For the first time it is here demonstrated directly that a close spatial relation exists between glial components and polyanionic components.

Golgi-impregnated glial cell processes obviously continuing into perineuronal nets showed a microglia-like pattern of ramification (Brauer et al., 1982, 1984) in some forebrain regions. Other light microscopic studies in the cerebellar nuclei suggested that perineuronal nets may originate from protoplasmic astrocytes (Lafarga et al., 1984). In contrast, immunocytochemistry of glial fibrillary acidic protein (GFAP), which is commonly used as a marker for astrocytic intermediate filaments, did not reveal astrocytic structures resembling perineuronal nets (Boyes et al., 1986; Hajós and Kálmán, 1989; Kálmán and Hajós, 1989). Therefore, in the present study GS and S100-protein have been used as cytosolic markers for astroglial cells at the light and electron microscopic levels. Both antigens labelled glial cell bodies and cellular processes, including those involved in the formation of perineuronal nets. These observations clearly suggested a close spatial association between the polyanionic perineuronal nets and glial processes.

The immunocytochemical localization of GS in the rat brain established this enzyme as an astrocytic marker. Many studies have confirmed this finding in vivo (e.g., Cammer and Tansey, 1989; Derouiche and Frotscher, 1991; Norenberg and Martinez-Hernandez, 1979) and in vitro (Hallermayer and Hamprecht, 1984). In some observations (D'Amelio et al., 1990) based on neurochemical and light (but not electron) microscopic evidence, it is suggested that GS may also be expressed by oligodendrocytes. This possibility was not systematically investigated in the present study. However, we could not observe GS-immunoreactivity in the interfascicular type of oligodendrocytes. In our material, GS-immunoreactivity was present in cellular perikarya with astrocytic ultrastructure and in pericapillary end feet, pointing to an astrocytic localization of GS. No other cell types, including microglia, were found to contain GS-immunoreactivity.







Figs. 11–13.

Thus, although the morphological heterogeneity of glial cells still hinders an unequivocal assignment of net-forming glial cells, we conclude from our cytochemical studies that perineuronal nets are apparently associated with astrocytic-like processes, rather than those of oligodendrocytes or microglial cells. Uncertainties in the structural identification of glial cells involved in the formation of perineuronal nets may arise from astrocytic cell types and glioblastic precursor cells, which are even present in the adult central nervous system (see Raff, 1990) and show shapes intermediate to classical glial cell types.

Site of Synthesis of the Polyanionic Compounds

The net-like pattern of the perineuronal staining was correlated with the neuronal surface in most previous studies, and a glial cell association was not suggested by any of these authors, even after electron microscopic investigation (Bertolotto et al., 1991; Naegele et al., 1988). In a few of the studies it was, however, mentioned that glycosaminoglycan-detecting immunoreactivity occurred where the neuronal membrane was in close apposition to glial processes (e.g., Yamamoto et al., 1988). Glial cell bodies showing unequivocal continuity with perineuronal nets were not labelled with any of the methods used. In the present study it was shown that VVA-binding, not only in the extracellular matrix, but also in some structures, occurred which appeared as perineuronal glial cell processes. A cytoplasmic staining was also obtained using wheat germ agglutinin in the cerebellar nuclei (Steindler and Cooper, 1986). The site of synthesis of defined molecules contributing to perineuronal nets has to be unequivocally identified, because the localization of extracellular molecular components may be different from their origin. For example, surface adhesion molecules and glycosaminoglycans associated with them (Werz and Schachner, 1988) may be formed by neurons and/or glial cells. On the other hand, the glia-derived molecule tenascin, which also binds to proteoglycans, has been shown to be adsorbed to certain neuronal surfaces in a topographically restricted pattern (Bartsch et al., 1992). Some proteoglycans were found in association with protoplasmic astrocytes in the cerebellum (Levine and Card, 1987) and with subpopulation of cerebellar glial cells in culture (Faissner, 1989). It was shown that primary astrocytes in culture can produce and release hyaluronan, heparan sulphate, chondroitin sulphate, and dermatan sulphate (Johnson-Green et al., 1991). Type-2 astrocytes and their progenitors selectively produced chondroitin 4-sulphate (Gallo and Bertolotto, 1990). According to these data glial cells could contribute to the formation of the polyanionic perineuronal nets by synthesis of at least some of the components of their macromolecular proteoglycan complexes. But finally, the detection of precursors and enzymes involved in these processes may elucidate the question of glial vs. neuronal origin of these polyanionic compounds.

Functional Aspects

Considering the possible functions of the polyanionic perineuronal nets associated with glia, one may wonder whether the neurons concerned or the presynaptic boutons making synaptic contacts on them may use specific neurotransmitters. In the rat cerebral cortex, the perineuronal microenvironment stained by hyaluronectin antibodies was found to surround about 10% of all neurons. All of these neurons belonged to the group of nonpyramidal cells possibly, including inhibitory interneurons (Delpech et al., 1982). Lectin binding studies (Lüth et al., 1992; Nakagawa et al., 1986) and immunocytochemical studies using proteoglycan-recognizing antibodies (Arimatsu et al., 1987; Naegele et al., 1988) revealed the correlation of this pattern of perineuronal staining with a subpopulation of GABAergic interneurons (Naegele and Barnstable, 1989; Naegele and Katz, 1990), which could be further characterized by the intracellular coexistence of GABA with the Ca^{++}-binding protein parvalbumin in the rat cortex (Celio, 1986).

It can be concluded from our study in subcortical regions, brainstem, i.e., mesencephalon, rhombencephalon, and cerebellum, that a correlation with GABAergic neurons was not consistently found. The neurons ensheathed by the polyanionic nets may rather belong to various transmitter systems, such as GABA in the medial septum/diagonal band complex and thalamic reticular nucleus, aspartate in the cerebellar nuclei (Kumoi et al., 1988), and acetylcholine in the motor nerve nuclei (McGeer et al., 1986). This leads to the question of whether the polyanionic character of the glia-associated perineuronal nets corresponds with some properties of presynaptic elements which contact these neurons. A common finding at the various neuronal cell populations considered was that all receive a significant GABAergic input from axosomatic and axodendritic synaptic boutons (Mugnaini and Oertel, 1985). However, it has to be noted that many other neuronal populations, which also receive a massive GABAergic input, are not surrounded by polyanionic nets on soma and stem dendrites. Well-known examples of the latter group are pyramidal cells which are densely covered by GABAergic axosomatic boutons (Houser et al., 1984) but do not show perineuronal nets.

Fig. 11. Immunocytochemical detection of glutamine synthetase in the medial cerebellar nucleus. Immunoreactive structures are glial cell bodies (G) and their processes partly ensheathing neurons (N) in a net-like pattern. Compare with Figure 10a,b for electron microscopy. Bar, 10 μm.

Fig. 12. Perineuronal nets and nodes of Ranvier (arrowheads) stained with the colloidal iron hydroxide method (blue) in the lateral vestibular nucleus. Counterstaining with lithium carmin (red). Bar, 10 μm.

Fig. 13. Demonstration of S100-immunoreactivity (brown) combined with the colloidal iron hydroxide staining (blue) in the lateral vestibular nucleus. S100-immunoreactivity labels a glial cell body (G), showing astrocyte morphology and numerous cell processes, some of which surround the cell body of a Deiters neuron and its proximal dendrite in a net-like pattern (arrows). The CIH-stained perineuronal net is closely apposed by S100-immunoreactive structures. A node of Ranvier with blue labelling is indicated by an arrowhead. Bar, 10 μm.

Therefore, simple correlations between the occurrence of perineuronal nets and defined transmitter systems can be ruled out. A functional correlation may exist which depends on the pattern of neuronal activity in which inhibitory inputs are involved.

According to findings obtained by Kettenmann and coworkers, astrocytes possess high affinity GABA receptors which are coupled to Cl^- channels (Kettenmann et al., 1988; MacVicar et al., 1989). Glia-associated Cl^- channels may be clustered in the cell membrane, suggesting a possible accumulation in perisynaptic glial cell processes. This spatial heterogeneity of GABA-mediated glial ion buffering may be supported by the occurrence of polyanionic proteoglycans associated with perisynaptic glial cell processes, which could highly increase the ion buffering capacity and water movement within the perisynaptic region (Delpech et al., 1989; Jenkins and Bachelard, 1988). The polyanionic perineuronal nets associated with glia could thus facilitate the presynaptic inhibitory input in several cell types ensheathed by perineuronal nets.

Strong indications for correlations between perineuronal nets and functional activity of neurons were obtained in visual deprivation experiments during brain development (Guimarães et al., 1990; Sur et al., 1988) or after disruption of motor neuron activity (Kalb and Hockfield, 1990). These investigations have shown that the expression of cell surface proteoglycans is markedly reduced on neurons in layers II/III and V/VI of the cortical area 17 and on Y cells in the cat lateral geniculate nucleus following dark rearing (Guimarães et al., 1990; Sur et al., 1988). As suggested by Hockfield et al. (1989) and Zaremba et al. (1989), the stabilization of synaptic contacts may be one of the functions of perineuronal proteoglycans. It remains to be elucidated why the perineuronal nets consisting of proteoglycans are only found on distinct subpopulations of neurons and how glial cells are functionally involved.

CONCLUSIONS

It may be concluded from the present findings that perineuronal nets formed by polyanionic proteoglycans are spatially associated with glial processes which were cytochemically identified as astrocytic. This specialized kind of neuron-glia-interaction is associated with various types of neurons. A common property of the neurons enwrapped by the described glial nets may be their demand of high perineuronal ion buffering capacity. The known structural variability and heterogeneity of glial cells leave questions open, especially with regard to the subtype of macroglial cells involved in the formation of perineuronal nets.

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