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Full-length Review

Perineuronal nets — a specialized form of extracellular matrix in the adult nervous system

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Abstract

One century ago, Camillo Golgi described 'perineuronal nets' enwrapping the cell bodies and proximal dendrites of certain neurons in the adult mammalian central nervous system and suggested that they represent a supportive and protective scaffolding. Although other neuroanatomists validated the existence of these nets on selected neurons in the adult brain, there was a lack of agreement on their origins, composition and function. The application of modern molecular and ultrastructural methods has brought new insights and a renewed interest in these classic observations. Recent data suggest that perineuronal nets result from the visualization of extracellular matrix molecules that are confined to the space interposed between glial processes and the nerve cells that they outline. The material confined to these spaces can be visualized selectively by antibodies directed to glycoproteins (e.g., tenascin and restrictin/janusin), proteoglycans (e.g., chondroitin sulfates), markers for hyaluronan as well as by lectins recognizing *N*-acetylgalactosamine and by monoclonal antibodies directed to epitopes on unknown molecules (e.g., HNK-1, VC1.1 and Cat 301). This review examines the emerging clarification of classical observations of perineuronal nets and the functional implications suggested by their molecular composition. Also discussed are studies that further extend observations on the time of development and of the specificity in the occurrence of perineuronal nets. In the adult brain the molecules constituing the 'perineuronal nets of matrix' could serve as recognition molecules between certain neurons and their surrounding cells and participate in the selection and consolidation of their relationship.

Key words: Extracellular space; Lectin; Monoclonal antibody; Parvalbumin; Matrix molecule; Neuron–glia interaction; Golgi-net

1. What are 'perineuronal nets'?

There is general agreement that 'perineuronal nets' were first described in 1893 by Camillo Golgi. A consensus description of perineuronal nets is that they are "a finely reticular covering" [a] [64,65] (Fig. 1) of "finely granular aspect" [b] [133] (Fig. 2) adhering intimately to the surface of the cell body and proximal dendrites[a], excluding the axon initial segment, of certain neurons in the adult brain. Perineuronal nets appear as a honeycomb or mesh that delimits small polygonal alveolar compartments [64,65,104,133], which were interpreted as being holes through which the nerve terminals passed to impinge on the surface of the nerve cells [73,104]. Perineuronal nets were observed on short axon cells (interneurons) and, less frequently, on pyramidal cells in the cerebral cortex [44,45,133,134], in the hippocampus, the dentate nucleus of the cerebellum [64,65,104,162], the olivary nuclei [15] and anterior

[a] '...un sottile rivestimento,...,avente forma reticolare o di strato continuo, ...interessante non soltanto i corpi cellulari, ma anche i loro prolungamenti, ...' [64] (Page 450).

[b] '...de aspecto finamente granuloso con los más resolventes objectivos...' [133] (Page 202).

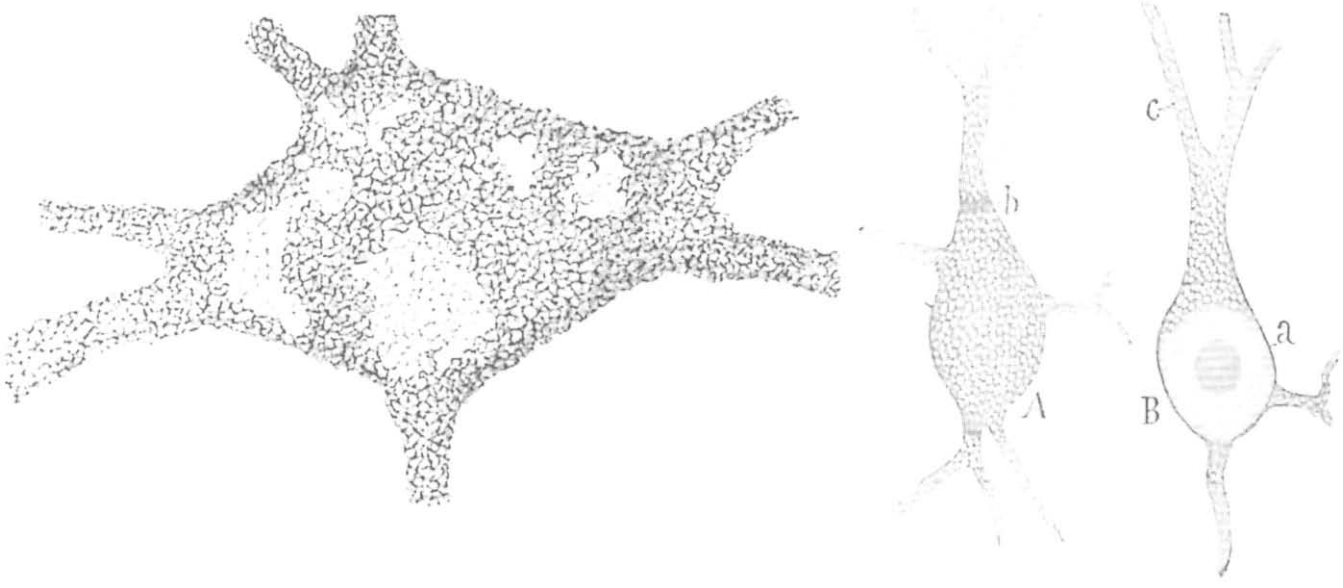


Fig. 1 (left). Nerve cell with reticular covering (nerve cell of the anterior horn of the cat spinal cord). Picture and legend from Camillo Golgi (1898).

Fig. 2 (right). Two cells of the cerebral cortex of the adult cat. Method of Ehrlich [methylene-blue]. Fixation with ammonium-molybdate. A: Focus on the surface of the cell. B: Focus on the middle of the cell. a: side view of the superficial membranous net [perineuronal net]; b: clot of stain at the attachment of a cell process c: net on a dendrite. Picture and legend from Ramón y Cajal (1898).

horn cells of the spinal cord [44,45,65] of many different animal species, including the human.

Since the turn of the century, only a handful of authors have again depicted the perineuronal nets [1,23,39,102,155]. Why are published descriptions of perineuronal nets so rare? Probably because the techniques used earlier were capricious, relying as they did on Golgi methods [64,65,73] and vital methylene-blue staining followed by ammonium molybdate fixation [15,133,134]. Furthermore, in low-power views of Golgi-stained specimens, neurons surrounded by a perineuronal net resemble those that are less well impregnated and are often ignored by the observers [23]. Although there was agreement as to their 'appearance' by classical anatomists, there was a lack of agreement as to their origins, composition and function.

2. What questions did the observation of perineuronal nets raise?

Not only have descriptions of the perineuronal nets been infrequent, but also few scientists have addressed the question of their exact derivation. Nets were assumed to originate from glia or neurons by different investigators. Some authors [15,111,112] thought nets were the terminal arborization of nerve processes. Others [73], made a clear distinction between what they thought were two types of cellular processes at the surface of nerve cells: the 'terminal boutons' of nerve fibres and the glially-derived perineuronal nets. They [73,111,112] described the terminal boutons as a "plexus in which each fibre maintains its independence" [c] and perineuronal nets as being "an interrupted wire-netting in which the fibres are fused together" [d].

The opinion that the nets were of glial origin [73] strongly influenced subsequent investigators [16,39,155] including more modern ones [23,25a,102,143].

3. What speculations accompanied the discovery of perineuronal nets?

Views on the composition and function of perineuronal nets differed significantly. Golgi (1893, 1898) interpreted them as a kind of corset or armour of "neurokeratin" [e]. At that time neurokeratin was defined as the delicate network which appears in myelin sheaths after boiling in absolute alcohol (e.g., Fig. 42 in ref. 151). Golgi put forward this interpretation despite the fact that he was able to visualize the perineuronal nets by much less harsh procedures. Some authors [104,133,134], contended that perineuronal nets occurred as the "result of the coagulation of a substance dissolved in the pericellular fluid" [f]. Another opinion [16] was that the extremely thin bars of the net could be elaborated processes of glial cells in which "the substance of the [glial] reticulum emancipates to a certain extent from that of the glial cell body and has peculiar staining properties" [g]. One author [104] argued astutely that the "interstitial substance obstructs the contact in some points, giving to the nervous action on the cell [body] a sharper local character" [h].

With the development of modern neuroanatomical methods that allow for the visualization of the nervous system at the ultrastructural level, as well as molecular methods that include the ability to visualize the tissue localization of different molecules, these classical observations have now been greatly extended and partly clarified.

4. Specific methods give hints about the chemical composition of perineuronal nets (Table 1)

Brauer's group (1984) revealed perineuronal nets of the deep cerebellar nuclei cytochemically by using the lectin, wheat germ-agglutinin, which recognizes *N*-acetyl-D-glucosamine. Steindler and Cooper (1986) applied the same lectin to stain perisomatic nets on Purkinje cells. A series of articles published by Samuel Spicer's group (reviewed in [146]) demonstrated the occurrence of strong binding sites for lectins recognizing *N*-acetylgalactosamine (GalNac) in perineuronal nets [123,124,125]. The distribution, appearance and the mesh-size of the net visualized by the lectins *Vicia villosa* (VVA B4) and *Soybean lectin* (SBA) (e.g., Fig. 3) are strongly reminiscent of the drawings of the

[c] '...ein Geflecht, in welchem jede Faser ihre Selbständigkeit behält.' [73] (Page 193).

[d] '...ein unterbrochenes Maschenwerk, dessen Fasern miteinander verschmolzen sind.' [73] (Page 193).

[e] 'Si direbbe trattarsi di una completa corazza o maglia che, a guisa di armatura riveste....' [64] (Page 2).

[f] '...ce réseau est dû à la coagulation d'une substance dissoute dans le liquide péricellulaire.' [134] (Page 157).

[g] 'Allerdings emanzipiert sich die Substanz des Reticulums bis zu einem gewissen Grade von derjenigen der Gliazellkörper und besitzt auch besondere färberischen Eigentümlichkeiten' [16] (Page 95). There are indeed some observations in support of a specialization of membrane-domain of glial cells. The activity of the enzyme 5′ nucleotidase is not homogeneously distributed on the membrane of glial cells [99] and ATP-ase activity was recognized only on astrocytic protrusions on vessels and neurons, but never on the membrane of the glial cell body [158]. Interestingly, the adhesion molecule on glia (AMOG) is a homologue of the β-subunit of the Na, K-ATPase [62]. Furthermore, the histochemical reaction for another supposed marker associated with the membrane of glial cells, butyrylcholinesterase, also reveals 'perineuronal nets' [109].

[h] '...la sostanza interstiziale ostacoli il contatto in altri punti dando così alle azioni nervose sulla cellula un carattere locale più definito'. [104] (Page 11).

Table 1
Markers labelling perineuronal nets

Marker	Immunogen	Recognized epitope	Recognized antigen(s)	Localization	Ultrastructural localization of staining	Development	Refs.
Lectins from: - Vicia Villosa (VVA Isolectin B_4) - Soybean lectin (SBA) - Dolichus biflorus, - Helix Pomatia - Wisteria floribunda		Terminal nonreducing *N*-acetylgalactosamine	Glycoprotein(s) of MW 20–100 kDa	Cortical interneurons hippocampal interneurons (PV), CA2 Pyramidal cells Basal forebrain, Substantia nigra, Betz-cells, superior olivary complex. (Neuromusc. junction).	Golgi-apparatus neuron; interstitial space Surface glial processes	3 weeks postnatal	8,9,25a,48,72,93,95, 105,118,121,123,143
Wheat germ agglutinin		*N*-acetylglucosamine		Deep cerebellar nuclei Purkinje cells, cerebellar molecular layer.	Surface of glial processes		24,147
Peanut agglutinin		gal β1,3 galNAc		Superior olivary complex	Interstitial space, Golgi apparatus neurons		8,9
Anti-tenascin (= cytotactin)	Chicken embryo fibroblast tenascin		Chick tenascin MW:220–250 kDa	Cortical and hippocampal interneurons	Interstital space around PV-neurons and diffuse in the extracellular matrix. Node of Ranvier in the PNS		33,38,69,139
Anti-restrictin (= janusin; = J1–160/180)	Chicken brain restrictin		Chick and rat restrictin (janusin); J1–160–180) MW: 170–180 KDa	Cortical and hippocampal interneurons, CA2-pyramidal cells.	Interstitial space around PV-neurons in the cerebral cortex. Node of Ranvier in the CNS		34,59,165a
McAB Cat-301	Homogenized, fixed adult cat spinal cord	Polypeptide	Chondroitin-sulfate proteoglycan (680 kDa).	Monkeys: cortical interneurons and pyramidal cells. thalamic relay neurons, magnocellular layers dLGN etc.. Species variability!	Interstitial spaces, endopl. reticulum of neurons, Golgi apparatus.	In the cat dLGN, start at 4 weeks, adult distribution after 24 months	71,74,75,78,80,85, 86,87,154,169,170
McAB Cat-304	Homogenized, fixed adult cat spinal cord	different epitope on same polypeptide					
McAB 473 (McAB 528: distinct epitope but same distribution; McAB 376: cortical interneurons partly overlapping with 473)	Extract of monkey brain conjugated to keyhole limpet haemocyanin.	Glycosaminoglycan (Chondroitinase ABC destroys immunoreactivity)	Portion of proteoglycan	Monkey: cortical interneurons (PV) and pyramidal cells, deep cereb. nuclei and Lugaro cells. rat: in addition NRTh, red nucleus, vest. nuclei, motoneurons.	Outer surface of neurons	4–5 postnatal week	57,98,116,164

McAB 3B3	Chondroitinase ABC treated chondroitin sulfate proteoglycan from Swarm rat chondrosarcoma.	Uronic acid residues adjacent to *N*-acetylgalactosamine-6-sulfate	chondroitin sulfate proteoglycans	Cortical interneurons (PV), pyramidal cells, deep cerebellar nuclei, NRTh, CA3-region.	Extracellular space		14,28,37,57,97
McAB 1B5		unsulfated, unsaturated disaccharide	chondroitin-sulfate proteoglycans	Cortical and hippocampal interneurons, NRTh, CA3 region, deep cerebellar nuclei, VII and XII motor nuclei, reticular formation, red nucleus, etc., diffuse in the brain.	Extracellular space		13,14,18,19,57
McAB 2B6		unsaturated 4-sulfate disaccharide	chondroitin-sulfate proteoglycans	Cortical interneurons, NRTh, deep cerebellar nuclei, etc. Immunoreactivity also scattered throughout the extracellular space of the CNS.			13,14,28
McAB 376	Monkey visual cortex in saline		600 kDa proteoglycan	Cortical and hippocampal interneurons, CA2 pyramidal cells, thalamic reticular nucleus, Golgi-cells cerebellar cortex (?), deep cerebellar nuclei, superior vestibular, lateral superior olivary, gigantocellular reticular, lateral reticular, plexiform layers of the retina.		Perineuronal staining appears only 3–4 week postnatal.	55,57,58
Anti-hyaluronectin (or hyaluronate binding protein followed by the antibody)	Human hyaluronectin	glycoprotein	365 kDa Versican and fragments of MW 45–50 kDa and 68 kd	Cortical interneurons, deep cerebellar nuclei, inferior olivary neurons, motoneurons. Finely dispersed in neuropil and around myelinated axons. Ranvier's node brain etc.	Extrasynaptic interstitial spaces	Third postnatal week	17,18,19,20,21,25,40, 41,60
McAB HNK-1 (= anti Leu-7)	Membrane extract of the human lymphoblastoid cell line HSB-2	Glucuronic acid 3-sulfate	Glycolipids, glycoproteins and proteoglycans	Cortical interneurons (PV), medial habenular nucleus, NRTh, horizontal and amacrine retinal cells, node of Ranvier in CNS, etc.			49,67,138

McAB 4F4	Cell suspension of embryonic rat forebrain	Glucuronate and sulfated hexoses of glycosaminoglycans (identical to HNK-1?)	Proteins ranging in molecular weight between 60 and 250 kDa	During prenatal development concentrated on postmigratory cells. In adults: Cortical and hippocampal interneurons, deep cerebellar nuclei, NRTh, Globus pallidus, inferior colliculus and various brain stem nuclei.	Nerve cell membrane and cytoplasm subjacent to glial cell processes. All Golgi-cisternae.	Appears 3 weeks postnatally in perineuronal nets	142,167
McAB VC1.1	unfixed cat Area 17	N-linked carbohydrates (identical to HNK-1?)	Integral membrane polypeptide (MW: 95/105; 145/170 kDa)+2 proteoglycans (680 and 650–700 kDa)	Cortical interneurons (PV), Nucl. Ret. thal., Perigeniculate nucleus, Deep cerebellar nuclei, Motoneurons, Horizontal+Amacrine cells	Extrasynaptic interstitial spaces		5,96,119,120,122,170
McAB VC5.1	fixed cat Area 17		Soluble polypeptides of 97 kDa and 150 kDa	Cortical interneurons/ Lugaro cells, Deep cereb. nucl., Motoneurons, optic nerve layer			5
McAB Leu-M1		3-fucosyl-*N*-acetyl-lactosamine (FAL)		Cortical neurons, Globus pallidus, Nucleus basalis of Meynert, striatum etc.			12
McAB Tor 23	Torpedo synaptosomes		175 kDa polypeptide	Cortical and hippocampal interneurons (PV), CA2 pyramidal neurons, NRTh, deep cerebellar nuclei, red nucleus			150
McAB 6A2	Drosophila Melanogaster head			Cortical and hippocampal interneurons, Betz-cells, deep cerebellar neurons, neurons in the nucleus of Clarke			56,77,113

Synoptic table summarizing the characteristics of the markers known to label perineuronal nets. An empty box indicates that the information is not as yet available. CNS, central nervous system; dLGN, dorsal lateral geniculate nucleus; NRTh, Nucleus reticularis thalami; PNS, peripheral nervous system; PV, subpopulation of nerve cells immunoreactive for the calcium-binding protein parvalbumin (see for reviews, refs. 3,31).

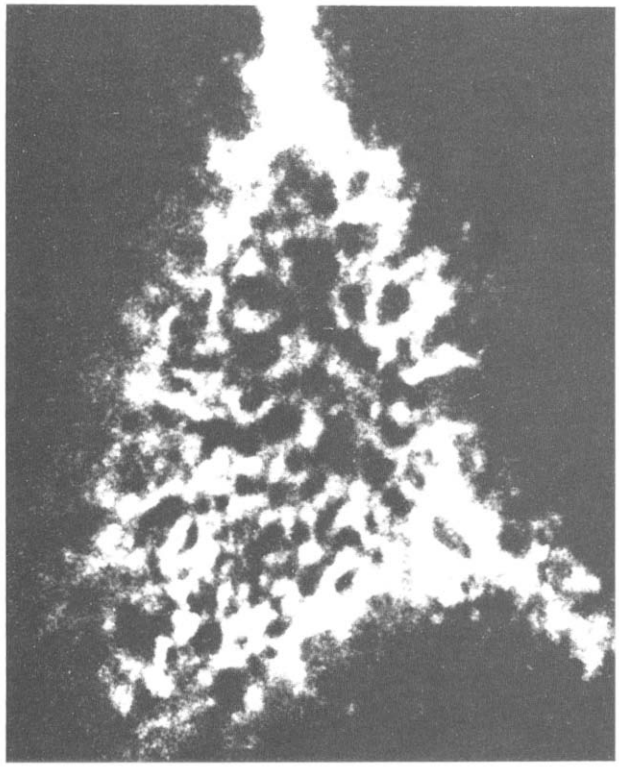


Fig. 3. Image of the "perineuronal net" enwrapping an interneuron of the rat somatosensory cortex acquired with a laser-scanning microscope (Bio-Rad). The net was labelled with biotinylated Soybean lectin (10 μg/ml 0.1 M Tris-buffered saline (TBS), pH 7.3 with 0.1% Triton X-100 and 0.1 mM $MgCl_2$, 0.1 mM Mn Cl_2 and 0.1 mM $CaCl_2$) followed by Avidin-FITC (in 0.1 M TBS but without detergent). Compare with Fig. 2. Work performed in collaboration with Dr. Peter Eggli, Anatomy Dept., University of Bern.

perineuronal nets made after demonstration by classical methods. The use of antibodies against proteoglycans on brain sections allowed the localization of different forms of chondroitin sulfate in a perineuronal situation [8–10,13,14,18,19,78]. The presence of hyaluronan in the same location was detected by various means, including the use of antibodies to hyalunorectin, a hyaluronan-binding protein [17,18,20, 21,25,40,41,60]. Recently, using highly specific antibodies we have detected the presence of the extracellular matrix-glycoproteins tenascin [33] and restrictin/janusin [34] on the circumference of a subset of neurons in the mammalian brain, corresponding to the location of perineuronal nets.

Independently, many different groups reported that a large number of monoclonal antibodies, raised against homogenates of brain areas of various species, stained the circumference of a subpopulation of neurons in a similar, fenestrated pattern. The immunogens used for the immunization of Balb/c mice for the production of the monoclonals which stain the perineuronal nets were very heterogeneous, including paraformaldehyde-fixed cat area 17, *Torpedo* synaptosomes and even whole *Drosophila* heads [5,56,77,150]. The molecules on which the epitopes recognized by these antibodies occur, are mostly proteoglycans [91]. Table 1 lists the lectins and antibodies which have been shown to reveal formations which most likely correspond to perineuronal nets.

Thus, perineuronal nets consist of an accumulation of at least three classes of substances: hyaluronan, glycoproteins and proteoglycans. Because of the established presence of many classical 'matrix molecules' in perineuronal nets [13,14,19,21,33,34,40,41,55] we have renamed them as 'perineuronal nets of extracellular matrix' (PNEM).

5. Glycoproteins, proteoglycans and hyaluronan of the 'perineuronal nets of extracellular matrix' are produced by glial and/or nerve cells [i]

Tenascin and hyaluronan are synthesized by glial cells [6,81,82]. Chondroitin-sulfate proteoglycans can be produced in vitro by both nerve and glial cells [47,81,82]. Restrictin/janusin mRNA is expressed by oligodendrocytes, astrocytes and a few nerve cells [59,165a]. The 'trans'-face of the Golgi-apparatus of neurons whose surface had been labelled using lectins recognizing *N*-acetylgalactosamine is also stained [123,152]. This suggests, but does not prove, that the glycoconjugate recognized by the lectins is synthesized inside the neuron and then transferred to its surface where it is exposed in the extracellular space. The antigen carrying the Cat-301 epitope may also be produced by the nerve cell, since the same antibody labels the endoplasmic reticulum of the neuron [78] and a perinuclear region [74], probably corresponding to the Golgi-apparatus.

Thus, although the three cellular components of the interstitial microenvironment around the soma of a nerve cell (postsynaptic neuron, glial cells and synaptic endings; see drawing of Fig. 4) may provide molecules to the perineuronal nets of extracellular matrix, circumstancial evidence has only been found for the first two.

[i] The site of the breakdown of components of perineuronal nets is unknown, but it can be assumed, that certain components are metabolized by lysosomes of nerve and glial cells. In fact, mucopolysaccharidoses, inborn deficiencies of specific lysosomal enzymes, are characterised by an accumulation of proteoglycans (e.g., chondroitin-sulfate) in lysosomes. Affected childrens suffer progressive mental and physical retardation [101,117.].

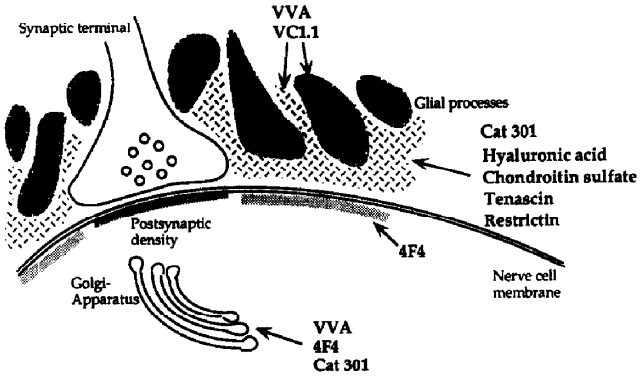


Fig. 4. Cartoon of the ultrastructural localization of the antigens recognized by the different markers at the interface between glial and nerve cells (see Table 1 for references). Markers can be classified in three different categories: (A.) those which stain mainly the surface of glial cells: VVA and VC1.1. (B.) those which label the extracellular matrix interposed between glial end-feet and neuronal surface: antibodies against Cat 301, hyaluronic acid-binding protein, chondroitin-sulfate proteoglycan, tenascin and restrictin, and (C.) those which label the cytoplasm of the nerve cell concerned: 4F4. This figure suggests that perineuronal nets are a site of focal expression of cell-matrix adhesion and extracellular matrix molecules destined to strengthen the ties between nerve and glial cells.

6. Most molecules of the 'perineuronal nets of extracellular matrix' are found extracellularly, interposed between glial processes and the surface of the neurons they outline [k]

The fact that a large number of the antigens recognized by the markers of Table 1 are located in the extracellular space, can be inferred from their solubility in isotonic buffer [antigen bound by Cat-301 [54,169], hyaluronectin [41] and chondroitin-sulfate [57,107, 127,137]].

At the ultrastructural level, the glycoconjugates localized by the lectins and the molecules recognized by the antibodies, are excluded from the synaptic cleft [l] and the flocculent reaction product is found in the extracellular space [m] and at the surface of the nerve cells and/or of the glial processes. This has been observed for the carbohydrates recognized by the lectins VVA and SBA [105,118], the epitopes recognized by the antibody Cat 301 [78] as well as for the matrix-molecules tenascin [33] and restrictin/janusin [34]. The antibody 4F4 seems to be the only known exception to this rule in that it labels the neuronal cytoplasm and the membrane directly subjacent to glial cell-processes [167]. Notwithstanding these efforts to locate components of the perineuronal matrix ultrastructurally by pre-embedding immunoperoxidase techniques, a definitive cartography must await high-resolution post-embedding electronmicroscopy with gold-labelled antibodies.

7. Is there a perineuronal net of anastomosing glial processes in register with the 'perineuronal nets of extracellular matrix'?

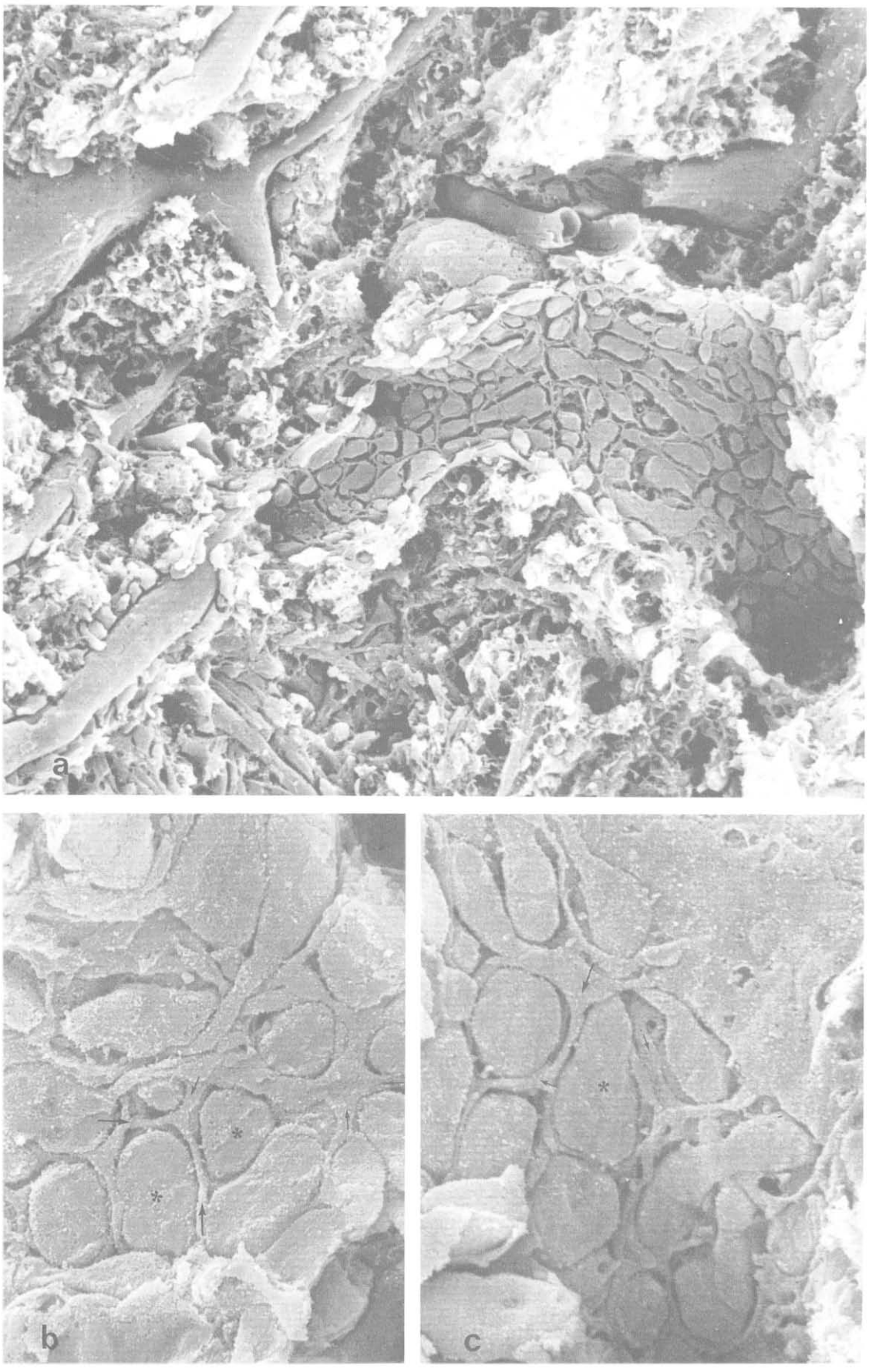
Does the lattice-like distribution of the material revealed by lectins and antibodies as the PNEM, coincide with the distribution of a meshwork of tiny, anastomosing processes of certain glial cells [16,23,25a, 39,102,155] or do a number of glial cell processes simply insert into the extracellular material surrounding these neurons? Two authors have drawn processes of Golgi-impregnated astrocytes [102] and microglial cells [23,24] coalescing with the perineuronal nets. They concluded that the perineuronal net is a reticulum of interlacing processes derived from these two glial cell types. However, studies of perineuronal nets by a combination of electronmicroscopy with the Golgi-impregnation method found that the reaction product was always located extracellularly and was never contained within glial processes [8]. Furthermore, perineuronal nets have never been described in preparations in which astrocytes and microglial cells have been selectively visualized [26,42,94,110,126,128]. Also the widespread use of antibodies against glial fibrillary acid protein and S-100 (for a review see [46]) to stain astrocytes, as well as the use of various markers to label microglial cells [27,153] and oligodendrocytes [53,148], have never led to the visualization of 'net-like' formations originating from glial cell processes. It is, however, true that these glial cell types are seen to protrude processes [n] which touch the surface of neighbouring neurons [39]. New electronmicroscopic tech-

[k] Various authors have reported or depicted, that some of these markers label the Golgi-apparatus but not the surface of subclasses of nerve cells (type 2 neurons [167]; Purkinje cells, Fig. 5A [77]). In these cases the epitope at the surface of the cell may be masked because the antigen was processed further before being exported.

[l] In this connection, the paradoxical situation in the peripheral nervous system should be mentioned. At the rat neuromuscular junction the GalNac residues, labelled by the VVA and SBA lectins, are concentrated in the basal lamina of the synaptic cleft and never occur in extrasynaptic localizations [144]. The expression is conserved phylogenetically and is co-localized with the enzyme acetylcholinesterase [144].

[m] The presence of an extracellular space in the brain has been the theme of passionate discussions. Classical electronmicroscopy of the nervous system does not visualize an extracellular space [130]. However, using rapid freezing, the presence of a consistent extracellular space was demonstrated [161]. It is not known if the extracellular space around different types of nerve cells varies in size, but it seems to be very large around neurons of the superior olivary nucleus, which are enwrapped in prominent 'perineuronal nets' [8,9,10.].

[n] Although the Fig. 8 of De Castro's article is of unsurpassed clarity and shows single astrocytic end-feet covering the circumference of a perikaryon and proximal dendrites of a motoneuron, the commentary on page 331 of his article is ambivalent. In fact he writes that a thin sheet of cytoplasm is stretched between the end feet ('prolongándose una sutil lámina de protoplasma glial entre ellos') [39].


a
b
c

niques reveal that these processes could interlace to form a net (Fig. 5) (ref. 156), which may coincide in location with the PNEM. Therefore, the only reasonable way to settle this question definitely is to inject a dye (e.g., Lucifer yellow) randomly into identified glial cells in brain slices and to look for perineuronal nets of glia processes. It will then be easy to recognize whether the surface of the neurons are covered by an anastomosing meshwork of glial cell processes or only by foot-like protrusions inserting in the PNEM.

8. Nerve and glial cells may carry receptors for these extracellular matrix molecules

The presence of an extracellular matrix rich in hyaluronan, glycoproteins and chondroitin sulfate calls for the presence of the corresponding receptors at the surface of the nerve and glial cells concerned. Functional CD44 [7], the receptor for hyaluronan [114,159], has been demonstrated on cultured astrocytes and F3/11 (contactin) the putative receptor for tenascin and restrictin/janusin [129,136,172] has been found in the brain. However, their direct demonstration in a perineuronal localization has not as yet been accomplished. Some of the epitopes recognized by the markers of Table 1 may belong to membrane-bound molecules of as yet unknown identity, which could function as receptors. The molecules recognized by the VC1.1 antibody and by the GalNac specific lectins, for example, can be extracted only after detergent treatment of a membrane fraction [5,121,122,170], suggesting that they are partly inserted in membranes.

9. The perineuronal nets of extracellular matrix are found preferentially around certain types of nerve cells

The descriptions of nets in the earlier literature were limited to a few examples in selected locations, e.g., spinal cord, deep cerebellar nuclei, hippocampus and cerebral cortex [15,44,45,64,65,73,104,133,162]. These regions contain large neurons, which may have been visualized more easily using impregnation methods. However, this cannot be the only explanation because some of the best modern descriptions of the perineuronal nets concern examples taken from these locations [23,24,78,102,121]. A glance at column 5 of Table 1 indeed confirms that certain cell types are consistently labelled by these different markers. This raises the question of whether PNEM are found only at the surface of a specific neuronal population.

In principle, all neurons are surrounded by a 'glycocalyx', a coat of carbohydrates arising from glycoproteins, glycolipids and proteoglycans. With the periodic-acid method of Schiff (PAS) which reveals glycoproteins, surface staining of many neurons in the central nervous system was observed [132], particularly in the motor nuclei of the medulla, on superior olivary neurons [8] and on motoneurons. With the colloidal iron-technique [25a,132], which reveals acidic carbohydrates, the surface of all neurons was outlined sharply °. Sometimes, however, particularly in the dentate nucleus and in the ventral cochlear nucleus (Figs. 6, 7, 46, 48 in ref. 132) the surface had a feltlike appearance, much resembling the perineuronal nets seen either with classical methods or using lectins and immunological markers.

Assuming that the nets represent the visualization of extracellular material in the meandering interstitial spaces around synaptic terminals, it can be imagined that postsynaptic cells receiving only a few synaptic contacts on their cell body may not show this lattice-like labelling at their surface. The surface of a nerve cell occupied by synaptic endings can vary between 1% for the small and 50% for the large neurons of the deep cerebellar nuclei [4,102,145]. Thus, large neurons of the deep cerebellar nuclei, which show well developed perineuronal nets [23,24,102], have indeed a more extensive surface area devoted to synaptic endings than small cells in the same nuclei, which lack nets [23,24,102].

The formation of the perineuronal nets of extracellular matrix does not seem to be correlated with the type of neurotransmitter used by the nerve endings impinging on the surface of the neuron in question

° It is interesting to note that Golgi had already revealed with his staining method the presence of a non riddled ('non bucherellato'), continuous, covering, around many categories of nerve cells [63,64,65.]. He contended that this situation was incompatible with the cellular theory of the nervous system because the transmission of information by contact was occluded by this homogeneous sheet! (ref. 64, page 450; ref. 65, pages 8 and 9).

←

Fig. 5. a). View at low magnification of a cerebellar nucleus macerated with NaOH. Nuclear neurons (N) reveal their round somata and thick proximal dendrites. A large soma was removed from its surrounding, exposing ellipsoidal axon terminals. Notice thin glial processes coursing among them. b) and c) High magnifications of axon terminals and glial processes attached to the soma of large nuclear neurons. Ellipsoidal terminals (asterisk) occasionally extending a thin tail. Attenuated glial processes intervene between them (arrows). The figures and the legend (slightly adapted) are reproduced from Takahashi-Iwanaga (1992).

[25a]. The deep cerebellar nuclei, which receive mainly Purkinje-cells terminals [36] employing the inhibitory neurotransmitter γ-amino butyric acid (GABA) have well developed PNEM [23,24,102]. But also the motoneurons, which mainly receive excitatory, glutamatergic synapses on their cell body, have prominent PNEM [40,41,65,78,79]. Thus, PNEM are found on those nerve cells with a considerable proportion of their perikaryal plasma membrane devoted to synaptic contacts, irrespective of the transmitter phenotype of the afferent terminals.

10. The distribution of the molecules of the perineuronal nets of extracellular matrix in the brain is very complex [p]

Some of the components of the PNEM (e.g., hyaluronan, chondroitin-4-sulfate, tenascin) infiltrate the interstitium of the whole central nervous system [84,107] [q] and are abundant in the perineuronal nets of extracellular matrix [13,19,33,84]. They may play the role of 'housekeeping' molecules. Other members of the class of substances occurring in PNEM (e.g., restrictin/janusin, *N*-acetylgalactosamine bearing molecules, chondroitin-6-sulfate, antigens recognized by antibodies VC1.1, Cat-301, etc.) 'exhibit exquisite cell type specificity' [80] and may lend to certain neurons molecular surface identity.

Detailed mapping of the staining of PNEM in the nervous system with different markers has been carried out by various authors (see Table 1). Kosaka first had the intuition of comparing the distribution of neurons, labelled by the lectins recognizing GalNac, with that of those stained by some of these monoclonal antibodies [95–98]. He found very good correspondence between the distribution of neurons labelled with VVA and those labelled with VC1.1, HNK.1 and 473, at least in the rat cerebral cortex [95–98]. From the literature it is evident that other partially matching distributions can be postulated.

Notwithstanding the superficial similarity in the regional staining pattern, at the cellular level the distribution is microheterogeneous. The markers stain the circumference of partly overlapping populations of neurons in different combinations and permutations. For example, VC1.1 and VVA both stain the perimeter of interneurons, but the first marks only 90% of the cells labelled by the second [95–98]. VC1.1 and VC5.1 have similar staining patterns in the cerebral cortex but not in the retina or in the cerebellum [5]. VC1.1 and 3B3 are coexpressed in the same interneurons but in varying amounts: e.g., some neurons express VC1.1 weakly and 3B3 strongly and some the reverse [97]. Nerve cells positive for the antibodies HNK-1/VC1.1 form a subpopulation of the VVA/3B3 neurons [98] and these epitopes are coexpressed at the surface of a subset of parvalbumin-immunoreactive neurons [95–98].

Despite this extremely complex pattern of distributions, several molecular markers which label PNEM in the cerebral cortex by recognizing different epitopes, appear to converge onto a particular subpopulation of non-pyramidal, inhibitory, GABA-ergic neurons [30] expressing the calcium-binding protein parvalbumin [95–98,105,120]. Do these neurons have a peculiar chemical environment [r] or is their preferential labelling a pure coincidence? Their frequent staining could imply that the 'cell coat' and the extracellular matrix surrounding parvalbumin-positive neurons contain more powerful immunogens than those surrounding, e.g., pyramidal cells. In fact, only two out of the many monoclonal antibodies of Table 1, Cat-301 and 304, label the surface of a subpopulation of pyramidal cells in addition to that of interneurons [108]. However, the recurrence of this peculiar distribution of labelling and the exclusion of other staining patterns could also be due to the use for immunization of inbred mice who share a similar complement of immunoglobulin genes [149].

Notwithstanding the suggestive association of perineuronal nets of extracellular matrix with chemically defined populations of nerve cells, it is not clear as yet if these surface markers subdivide neurons into coherent groupings. The collective of neurons sharing the same markers may 'form a unit in which neurons are related by common functional or connectional attributes' [75]. However, at the moment only Cat-301 seems to conform to this idea in that it marks a functional pathway in the mammalian visual system [74,75].

[p] Most lectins used to visualize 'perineuronal nets' [25a,143], as well as antibodies against hyaluronectin [40] and against HNK-1 [52] also bind to nodes of Ranvier in the peripheral nervous system. The node of Ranvier has a specialized astrocytic process associated with it [22] and at this same location cell adhesion molecules as well as cytotactin [139] (= tenascin) and restrictin [59] have been found.

[q] These results rehabilitate many ancient controversial publications [15] on the presence of a 'reticulum' and 'Grundsubstanz' [76] old, discredited terms for extracellular matrix (see also [19,128]).

[r] Why this preferential association of 'perineuronal nets' with parvalbumin-immunoreactive neurons? One answer may be that these cells have functional peculiarities which make it necessary for them to be covered with a 'perineuronal nets of matrix'. Parvalbumin neurons are notoriously fast-firing cells [29,30,31,88,89.]. In order for them to be able to fire with high frequency, the concentrations of K^+ and neurotransmitters in their surrounding has to be kept under tight control. Glial end-feet inserted in the perineuronal net could assume the task of keeping the surface of parvalbumin-neuron free of interfering ions and neurotransmitters [11,25a,161a]. Additional attempts to explain the presence of 'perineuronal nets' around parvalbumin-neurons are to be found in another article [32].

11. The perineuronal nets of extracellular matrix appear only after birth (Table I, 7th column)

Appearing only during postnatal development [125,142,143,164], most antigens occurring in PNEM are probably not involved in developmental aspects like nerve cell migration, recognition, synaptogenesis or process elongation. The late postnatal development of PNEM (3–4 weeks postpartum for most, two years for Cat-301 in the cat dorsal lateral geniculate nucleus (dLGN, see Table 1) implies that the consolidation of the relationship between neuronal surface and its surrounding is a protracted phenomenon which may have consequences for the onset of functional activity of nerve cells. The time course of development of the Cat-301 epitope in the dLGN [79,80] and of lectin binding-sites in the cat visual cortex [143] matches the time course for the development of mature physiological properties by the neurons they outline and marks the termination of the period of experience-dependent plasticity. Thus, the appearance of some of the molecules collectively named in this article the 'perineuronal nets of extracellular matrix' seems to mark the definitive wiring of the brain. In this context it would be interesting to determine if experimental disruption of the PNEM can restore experience-dependent brain plasticity.

12. There is a critical period for the development of some molecules composing the perineuronal nets of extracellular matrix

In lid-sutured or dark-reared cats [154] as well as in newborn animals with nerve-crush or spinal cord lesion [85,87], Cat-301 labelling does not develop on neurons in the dorsal lateral geniculate nucleus or on motoneurons in the spinal cord, respectively. Staining with an antibody recognizing another epitope on the same chondroitin-sulfate molecule (Cat-304), is similarly affected in the dLGN and in the visual cortex [71]. In adult animals, the same interventions have no visible consequence on staining with these antibodies, suggesting that once established, the expression of the molecules of the PNEM is fairly robust.

In view of the plausible extracellular localization of the antigen recognized by antibody Cat-301 [78] one may imagine certain mechanisms which could lead to the lack of its expression in deprived animals. Deprivation may silence certain pathways which contact Cat-301 positive nerve cells. The absence (or inactivity during a critical period) of synapses on the cell bodies of these neurons may fail to induce the neuron to produce molecules which are contributed to the extracellular space. Perhaps excitatory aminoacids trigger the expression of the Cat-301 antigen on motoneurons [86] but they cannot be the only factor, since Cat-301 antigen is present also on deep-cerebellar neurons [170], which receive mainly an inhibitory GABA-ergic input from Purkinje cells. Unfortunately only one marker, antibody Cat-301, has been used in these studies [85,86,87], so that it is not known whether deprivations of this type affect this antigen specifically or have a general disruptive effect on the expression of other constituents or even of the whole perineuronal net of extracellular matrix. Undoubtedly these are important experiments to be extended.

13. The perineuronal nets of extracellular matrix in brain diseases

Perineuronal nets of extracellular matrix labelled by the lectin VVA or by the monoclonal antibody 6A2 are constantly found in adulthood and are preserved in old age. They do not seem to disappear in neurodegenerative diseases of the Alzheimer or Pick type [113] (and our own unpublished observation, but see [93]). It therefore seems that perineuronal nets of matrix are long-lived. The fate of PNEM in other neurologic or psychiatric diseases has not as yet been studied [s]. Given the variety of molecules composing the perineuronal matrix and their probable double source from glial and nerve cells, one may predict defects of PNEM in brain diseases affecting both neurons and/or glial cells. On the other hand, disruption of the expression of molecules specific for the PNEM during development and in adulthood may disturb the function of subclasses of neurons and cause subtle neurologic or psychiatric disorders.

14. What could be the role of the molecules composing the perineuronal nets of extracellular matrix?

14.1. Stabilization of synapses?

A direct function of these extrasynaptic molecules in synapse stabilization, as proposed by some authors [14,79,80,169], is conceivable but difficult to reconcile with experiments in which the neutralization of one of them with a lectin [92] had no effect on synaptic transmission. Furthermore, a family of synapse-associated cell-surface proteins, the neurexins, which may be in a better position to stabilize synapses, has recently been discovered [160].

14.2. Concentration of growth factors around certain neurons?

The cores of the glycosaminoglycan side chains of cell-surface and matrix proteoglycans, bind proteases

[s] We have preliminary results indicating a decrease of VVA-binding sites in perineuronal nets in HIV-encephalopathy [35].

and protease-inhibitors and act as low-affinity receptors which concentrate neurotrophic factors [51,140] such as bFGF [61,135,168] and TGFβ [163]. The binding of these factors to a heparan-sulfate proteoglycan is even necessary for their biological activity [135,163]. At the moment, nothing is known about any particular requirements in proteolytic or growth factor activity of those neurons surrounded by a PNEM or of the synaptic terminals and glial processes impinging upon them. It is worth noting that CA2 pyramidal cells, which are surrounded by a PNEM rich in various molecules (see Table 1), are immunoreactive for basic fibroblast growth factor [66,166] and hippocampus-derived neurotrophic factor (NT3) [50], molecules which promote neuronal survival and neurite extension [68]. It is unknown if these growth factors are secreted by CA2 neurons and remain trapped in the meshwork of PNEM to perform a local role.

14.3. Linkage of the extracellular matrix with the cytoskeleton?

In his detailed study, Bethe depicted intracellular neurofibrils which were inserted directly into the nodal points of the meshes of the perineuronal net [15]. The existence of a preferential relationship between elements of the neuronal cytoskeleton and the extracellular matrix has not been studied in the brain so far. In view of Bethe's observations, however, this aspect of the subject should be re-examined using modern markers, since transmembranous links between intermediate filaments and extracellular matrix molecules are an established fact for a range of other cell types [2]. This argument would be strengthened if the hyaluronate receptor CD44 [7,159] and/or cell adhesion molecules [26a] of the immunoglobulin- [49,113a], cadherin- [106,157] or integrin-type [103] could be detected in register to perineuronal nets of extracellular matrix. N-CAM in fact, is known to interact with extracellular matrix molecules [165] and the cytoskeleton [131], and CD44 and cadherins to interact with intracellular cytoskeletal elements [100,106].

14.4. Non-permissive substrate which blocks the formation of new synaptic contacts on the cell body of neurons?

Tenascin, as well as restrictin/janusin, which are found in the PNEM [33,34], promote adhesion of astrocytes but are repellent substrates for neurons and their processes [50a,115]. Chondroitin sulfate proteoglycans also inhibit the adhesion between nerve cells [70]. Could PNEM represent a barrier which hinders the formation of new synaptic contacts on the surface of the nerve cells which they surround? This view would be in conformity with the observation that PNEM form late in postnatal life and that their appearance coincides in time with the end of the period of experience-dependent plasticity [79,80,143]. However, although a repulsive effect of single components of PNEM on axon growth has been observed in vitro, it is not as yet proven that the highly complex meshwork of aggregated molecules in the PNEM subserves a similar role in vivo.

14.5. Help in targeting glial relationships with neurons?

The extrasynaptic localization and the biochemical nature of the components of the PNEM suggest a function for these formations in neuron-glia recognition [1]. Perineuronal nets of extracellular matrix could represent the accumulation of specialized matrix molecules to which certain nerve and glial cell membrane adhere through specific membrane receptors. An antibody directed against the HNK-1 epitope, present on tenascin and a variety of other molecules occurring in PNEM, indeed modulate neuron-to-astrocyte adhesion in vitro [90]. With the rapidly growing knowledge on the molecular components of the PNEM, it may be possible to reconstitute them in vitro and to test this hypothesis under favourable experimental conditions.

15. Conclusions

Perineuronal nets, described at the turn of this century as visualized with unspecific stains, have been rediscovered recently through the use of lectins and antibodies. They probably represent a particular form of extracellular matrix expressed around certain neurons of the central nervous system. This review attempts to summarize information scattered in the literature and to reconcile different views on this subject. It emphasizes the very preliminary nature of most of the ideas about the ultrastructural localization, cellular contribution, biochemical composition and functional role of the PNEM. In addition it points out that the regulation of the synthesis and breakdown of the molecules composing these 'matrices', as well as the timing of their development and the signals necessary for their maintainance, remain largely unknown.

The role of the extracellular glycoproteins, proteoglycans and hyaluronan which form the PNEM could consist in the attraction and retention of glial processes at the surface of certain nerve cells. The glial processes immersed in this specialized extracellular matrix could themselves carry out important 'nursing' functions for the neuron [161a], in neutralizing neurotransmitter by readsorption [43,83,141,161a] and in buffering ions [11,25a,161a]. The consistent presence of the molecules forming the perineuronal nets of extracellular matrix in restricted sites and their concerted action, could thus play a key role in the interaction between certain neurons and a subpopulation of glial cells.

Given the resurgent interest in the extracellular matrix of the brain and particularly in the special form called here the perineuronal nets of extracellular matrix (PNEM), this review aims to stimulate more investigators to invest in the elucidation of the functional roles of these, long neglected, components of the nervous system.

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