HISTOCHEMICAL STUDY OF PERINEURONAL NETS IN THE RETROSPLENIAL CORTEX OF ADULT RATS

BY

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SUMMARY

The retrosplenic cortex of rats, similar to many cortical or subcortical regions, is provided with special subsets of neurons which exhibiting a fenestrated or reticular coat of condensed extracellular matrix on their soma, initial dendrites and axon's proximal segment. This coating, currently termed as "Perineuronal Nets", was detected at the surfaces of some neurons distributing throughout the cortical layers II-V. They presented direct interconnections with each other, and appeared in close association to the astroglial processes. In addition to their collagenous ligands, the perineuronal nets (PNs) were enriched with proteoglycans (PGs) and/or glycoproteins with terminal N-acetylgalactosamine (GPs). The PNs were differentially identified into three categories depending upon their organic nature or chemical composition. First, coats were exclusively formed of PGs (stained with iron colloid); second, coats which were formed of GPs (labeled with plant lectins binding to terminal N-acetylgalactosamine); and third, complex coats were formed of PGs networks intermingled with glycoprotein molecules (double stained with iron colloid and lectin). Since differential distribution of protein containing substances (GPs and/or PGs) in the extracellular matrix contributes to functional terms, we suggest that these biochemical or morphological differences in the microenvironment of some retrosplenial neurons might reflect certain functional aspects.

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INTRODUCTION

Recent studies of central nervous system in mammals, avians and other lower vertebrates have shown that certain subsets of neurons exhibited a reticular perineuronal coat of PGs reactive to cationic iron colloid and aldehyde fuchsin (Murakami et al., 1994, 1996). This coat is frequently intermingled with an additional extracellular chain of glycoprotein molecules (with terminal *N*-acetylgalactosamine) and collagenous ligands (Murakami et al., 1999). The extracellular GPs are labeled with lectin *Vicia villosa* agglutinin 'VVA', *Wisteria floribunda* agglutinin 'WFA' or lectin *Glycine max* agglutinin 'SBA' (Murakami et al., 1996) and that of the collagenous ligands are stainable with Gömöri ammonical silver (Murakami et al., 1999a). These investigations were systematically limited and insufficient to describe the histochemical characteristics of rat retrosplenial cortex, especially its coated neurons.

The present investigation supplements our previous studies and obtains more information on the chemical compounds and the different types of PNs. Furthermore, it demonstrates a spatial relationship between PNs and astrocytes. The granular and non-granular subfields of rat retrosplenial cortex were used for this study, which suggested to be involved in navigation and processing of episodic memory (Maguire, 2001). The obtained data would be contributed as baseline for future quantitative or experimental studies searching for the biological significance of pericoated neurons at this cortical region.

MATERIALS AND METHODS

Animals and tissue processing. Adult Wistar rats at four months of postnatal life (n= 8) were perfused transcardially under ether anesthesia with saline, followed by 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). The brains were thereafter isolated and 2-3 mm-thick tissue slices, which, traverse the retrosplenial cortex, were cut with a razor blade in saggital and coronal planes and immersed in the aforementioned fixative for 24 hours. After proper fixation, specimens were dehydrated, cleared and embedded in paraffin. Serial saggital or coronal sections (5-7 µm in thickness), that traversed the retrosplenial cortex were prepared and after deparaffinization by xylene they were treated as follows:

Colloidal iron histochemistry. Tissue sections were stained with a cationic iron colloid (CIC) at pH 1.5 (Murakami et al., 1986). The aim was to estimate the spatial distribution of polyanionic components or sulfated glycoconjugates (PGs). Counter staining with nuclear fast red was usually inserted prior to balsam embedding. Sections for control were not treated with iron colloid.

Lectin Cytochemistry. Tissue sections were incubated with lectin VVA (Kosaka and Heizmann, 1989), lectin WFA (Härtig et al., 1992) and lectin SBA (Lüth et al., 1992). The aim was to examine spatial distribution pattern of *N*-acetyl-galactosamine containing glycoconjugates. Peroxidase activity was demonstrated with diaminobenzidine (Nakagawa et al., 1986). Counter staining with Mayer's hematoxylin was inserted prior to balsam embedding. Controls for lectin-stained

sections consisted of adjacent sections treated with phosphate buffer containing no agglutinin.

Immunocytochemistry. For astroglial cell demonstration uses the intermediate filament protein glial fibrillary acidic protein (GFAP) after Bignami and Dahl (1974) was used. Briefly, the unspecific binding sites for immunoreagents were blocked with 5% normal goat serum in PBS. Subsequently, sections were processed with a rabbit antiserum to GFAP (1:500; RBI, Natick, MA, USA), followed by incubation with biotinylated goat anti-rabbit IgG (1:400; Vector Lab), then treated with HRP-conjugated streptavidin (Vector Lab), and finally the reaction was visualized by diaminobenzidine. Omitting the primary antibodies performed control experiments.

Dual Staining with Iron Colloid and WFA, VVA, SBA, GFAP or Bodian. Some sections were stained with WFA, VVA or SBA and then additionally with a cationic iron colloid. The aim was to allow differentiation of sulfated and unsulfated glycoconjugates. Double staining of GFAP and iron colloid staining would indicate a spatial relationship between perineuronal proteoglycans and astrocytes. Combination staining with iron colloid and Bodian procedure would promote a clear resolution of PNs (Murakami et al., 1997).

Photography. Photographic documentation was performed with an Olympus BX 50 transmission light microscope using Kodak technical pan TM film or Kodak TM (color) film and presented at their original magnifications.

RESULTS

General findings. The retrosplenial cortex in rats presented certain neuronal subsets exhibiting a fenestrated pericellular coat, which was recognized as network formations of condensed extracellular matrix (Figs. 1 and 2). This reticular structure usually ensheathed somata, dendritic arborizations and axon proximal segment of nerve cells (Fig. 2A,B,C). The subpopulation of coated neurons were distributed throughout the second to fifth (II-V) cortical layers; however, they could not be revealed at the molecular (I) layer. In general, the pericoated cells exhibited the morphological characteristics of multipolar neurons. The perineuronal coats varied in thickness and staining intensity from cell to cell, and presented direct interconnection between each other as revealed from reconstruction studies of serial sections (Fig. 1C).

Control experiments. In all experiments, control incubations resulted in a significant failure of the labeling.

Iron colloid histochemistry. Certain subsets of neurons in the retrosplenial cortex were ensheathed with bluish negatively charged pericellular coats of proteoglycans as indicated by iron colloid staining (Fig. 2A and Fig. 3A,B). The coated cells were distributed throughout the second to fifth cortical layers which, were relatively numerous at the fourth and second layers.

Apart from iron colloid staining, similar argyrophilic nets were also demonstrated around somata and cellular processes of certain neuronal subsets, as revealed with CIC/Bodian staining (Figs.1B and 2B). Notably, this combination procedure visualized the pericellular nets with clearer and sharper intensity than those stained with iron colloid alone.

The intracellular structures of retrosplenial neurons and their associated glial cells were reacted negatively to the iron colloid or its combination with Bodian staining.

Lectin cytochemistry. Reticular pericellular coats were revealed with three plant lectins from VVA, WFA and SBA ensheathed a special subpopulation of neurons (Fig. 1A and 2C). This sheath surrounded the surface of some neurons in layers II-V. Most labeled neurons were distributed in layer II and IV. Notably, labeling intensity of perineuronal coats differed considerably among neurons with respect to the lectin used. They exhibited the higher intensities in specimens treated with lectin VVA and WFA than SBA. Furthermore, staining intensity of neuropil was higher in sections stained with WFA than VVA or SBA.

Combination staining with CIC and VVA, WFA or SBA. The PNs were differentially labeled by these combination procedures. They solely stained with CIC (blue) or lectins (yellow) or were doubly labeled with CIC and lectin (light greenish to dark brown) (Fig. 1C). Accordingly, the perineuronal coats were identified into three categories: PGs coats (solely stained with iron colloid), GPs coats (solely stained with lectins) and coat complexes formed of PGs networks that intermingled with glycoprotein molecules (doubly stained with lectins and iron colloid). Notably, the intensity of coat complexes varied considerably among labeling lectins. It was the higher in sections stained with CIC/VVA or WFA/CIC than in those stained by SBA/CIC. Interestingly, the various colors of the complex coats properly reflected the relative quantities of unsulfated glycoconjugates (labeled with lectins) that intermingled with the sulfated proteoglycans nets (stained with CIC).

Immunocytochemistry. Immunostained sections with antibodies to the GFAP and additionally with CIC demonstrated large number of immunolabeled astroglial cells and associated glial fibers. They were distributed throughout the I-V cortical layers, which appeared in close approximation with blood capillaries and pericoated or non-coated neuronal cells. Notably, direct contact or interlacing between labeled astrocytes or associated glial fibers with perineuronal coats was clearly revealed (Fig. 3A,B).

DISCUSSION

The present investigation provided more information on the chemical components of PNs and demonstrated three neuronal circuits at the retrosplenial cortex of rats: PGs-, GPs-, and PGs/GPs-coated neurons.

The retrosplenial cortex in rats revealed special neurons that possessed reticular or net-like extracellular coatings on their surfaces that were enriched with PGs and/or GPs. The lattice-like or fenestrated structure of these coats has been

described to ensheath somata, dendretic arborizations and axon initial segments of nerve cells (Celio and Blümcke, 1994). The PNs 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 (Celio and Blümcke, 1994). They were visualized with various plant lectins, certain molecular markers, or other histochemical staining including iron colloid and aldehyde fuchsin (Brauer et al., 1982; Nakagawa et al., 1986; Kosaka and Heizmann, 1989; Atoji et al., 1992; Lüth et al., 1992; Brückner, et al., 1993; Brückner, et al., 1996a, b and Köppe et al., 1997). They were first described by Golgi as "Pericellular Nets" (Golgi, 1893), and after their re-investigation by Brauer et al., (1982), the currently accepted term "Perineuronal Nets" has came into use. The ultrastructure or fine organization of these perineuronal matrices was described elsewhere (Murakami et al., 1995, Ohtsuka et al., 2000).

In accordance with Celio and Blümcke, (1994), the expressed epitopes in the present investigation were deposited at the perineuronal spaces of certain neuronal populations including their related synaptic buttons and intercalated glial structures. These substances were not revealed in the synaptic clefts (Hagihara et al., 1999, Ohtsuka et al., 2000).

Ultrastructural investigations of PNs have demonstrated glycan components in close vicinity to astrocytic processes, suggesting that the extracellular matrix contribute differentially to the glianeuron interface (Derouiche et al., 1996). It is postulated that a single neuron may receive net-like contacts from several astrocytes and that a single astrocyte can contribute to a PNs on more than one neuron, confirming that the extracellular matrix molecules and astrocytic processes are topically associated to a high degree. In addition, different proportion of both components may specify the individual neuronal microenvironment (Derouiche et al., 1996).

Our previous ultrastructural and immunohistochemical study of PNs has revealed that each synaptic button fits into a mesh of the perineuronal network (Ohtsuka et al., 2000). It may be possible that perineuronal sheaths have a significant role in permanent stabilization of neuronal-synaptic interfaces and therefore assuring signal transmission at the axo-somatic synapses (Celio and Blümcke, 1994).

It is well known that lectin VVA and SBA bind selectively to terminal alpha and/or beta *N*-acetyl-galactosamine (Debray at al., 1981), while the lectin WFA binds selectively to terminal alpha *N*-acetyl-galactosamine (Härtig et al., 1992). In accordance with these studies, it was confirmed that the observed lectin binding epitope in the present investigation is a membrane bound glycoprotein with terminal *N*-acetyl-galactosamine.

The basic components of PNs in the retrosplenial cortex have been demonstrated to be somewhat variable. Notably, clear differences exist between individual PNs with respect to the quantity of PGs and/or GPs present in the microenvironment of the corresponding neurons. The differential concentration of

these epitopes, as was postulated by Brückner et al., 1996a and Köppe et al., (1997) appears to be a significant factor in the regulation of individual neuronal micromilieu and suggests a definite or certain function for each type.

Although the coated neurons comprise a distinct population in the retrosplenial cortex of rats, their anatomical or functional significance is quite uncertain. However, the histochemical and differential characterization of perineuronal coats, as presented in the present investigation, may provide a qualitative information that has a special significance when establishing correlation with experimental data.

Nevertheless, there are some putative roles for proteoglycans which include: neuronal maturation, stabilization of synapses, generation of polyanionic ion-buffering microenvironment, maintenance of extracellular spaces and concentration of growth or inhibitory factors (Matsui et al., 1999). Furthermore, the PNs possess a supportive or protective role, and serve as recognition molecules between certain neurons and their surrounding cells (Celio and Blümcke, 1994).

In conclusion, the central nervous system of vertebrates possesses certain subsets of PGs, GPs and PGs/GPs-ensheathed neurons, suggesting a significant role for each neuronal circuit. Future experimental studies should center on the molecular structure of perineuronal nets and knocking out of related genes, including their biochemical or physiological significance.

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LEGENDS

Figure 1.

Staining patterns and distribution of perineuronal coats at the retrosplenial cortex.

A. The glycoconjugates-coated neurons are distributed throughout the II-IV cortical layers. They are most frequent at layers IV and II than layers III and V. VVA-stained section and counter-stained by Mayer's hematoxyline. X: 33.

B. The proteoglycans-coated neurons are distributed throughout the II-IV cortical layers. They are most frequent at layers IV and II than layers III and V. CIC/Bodian-stained section. X: 50.

C. The perineuronal coats are differentially stained with CIC (blue color), lectin SBA (yellow color) or doubly labeled with CIC and SBA (green to dark-brown color). X: 200. Lower inset shows a sulfated proteoglycans coat (blue color) interconnected with a coat complex (light green color).
X: 330. Right inset shows a neuron-exhibiting coat complex .SBA/CIC-stained section and counter-stained by Mayer's hematoxylin. X: 330.

Figure 2.

Staining patterns and distribution of the expressed epitopes at the perineuronal coats.

A. Proteoglycans-coated neuron as revealed by CIC procedure and counter-stained by nuclear fast red. The CIC-binding sites surround the perikaryon, initial segment of the axon (arrowhead), and proximal parts of dendrites. X. 330.

B. Proteoglycans-coated neuron as expressed by CIC/Bodian procedure. Notice, this staining procedure appears with marked intensity than the CIC, which visualizes numerous denderitic arborizations with clear and sharp intensity. (Arrowhead = axon). X. 330.

C. Glycoproteins-coated neuron stained with VVA and counter-stained by Mayer's hematoxylin. (Arrowhead = axon). X. 330.

Figure 3

A, B. GFAP-immunoreactive glial cells and glial fibers are present in close association or interlacing with the perineuronal proteoglycans nets (arrowheads). Immunostained section with GFAP, doubled with CIC, and counter-stained by Mayer's hematoxylin. X: 200 (A), and X: 330 (B).

I
II
III
IV
V
1A

I
II
III
IV
V
1B

1C