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ScienceDirect
Journal of Chemical Neuroanatomy 32 (2006) 143–158
Journal of CHEMICAL NEUROANATOMY
www.elsevier.com/locate/jchemneu

Organization of brain extracellular matrix in the Chilean fat-tailed mouse opossum *Thylamys elegans* (Waterhouse, 1839)

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Received 13 July 2006; received in revised form 8 August 2006; accepted 15 August 2006
Available online 25 September 2006

Abstract

We investigated the structural and molecular organization of the extracellular matrix in *Thylamys elegans*, a marsupial representative of the mammalian order Didelphimorphia. Perineuronal nets (PNs) associated with distinct types of neurons were visualized by detection of chondroitin sulfate proteoglycans and hyaluronan, and by labeling with *Wisteria floribunda* agglutinin (WFA), a marker for PNs in the mammalian brain. In the neocortex of *Thylamys*, these methods revealed PNs on pyramidal cells. In contrast, parvalbumin-immunoreactive interneurons in the neocortex and hippocampal formation (displaying robust, WFA-labeled PNs in placental mammals) were ensheathed only with a delicate rim of hyaluronan and proteoglycans not detectable with WFA. The absence of WFA staining was characteristic also of some subcortical regions which contained PNs intensely labeled for chondroitin sulfate proteoglycan and hyaluronan. However, corresponding to placental mammals, numerous subcortical nuclei showed clearly WFA-stained PNs. Similar as in placental mammals, cholinergic basal forebrain neurons and tyrosine hydroxylase-immunoreactive neurons of the substantia nigra and locus coeruleus were devoid of PNs. Together with our earlier study on *Monodelphis*, the present results reveal that South American opossums show either a particular ''marsupial'' or ''Didelphid'' type of extracellular matrix chemoarchitecture, supporting the view that these components may vary phylogenetically as integral parts of neuronal physiology at the systems and single cell level.

Keywords: Perineuronal nets; Chondroitin sulfate proteoglycans; Hyaluronan; Cerebral cortex; Brain evolution; Marsupials

1. Introduction

The extracellular matrix specifically contributes to functional characteristics of cells, tissues and organs in developmental and adult stages (for reviews see Comper, 1996). In the mammalian brain, the spatial and molecular organization of the extracellular matrix shows a great diversity that obviously mirrors the complexity of neuronal functions at the single cell and systemic level (Brückner et al., 1996; Carlson and Hockfield, 1996; Matthews et al., 2002). It can be assumed that brain evolution is accompanied by a corresponding evolution of the extracellular matrix.

Since perineuronal nets (PNs) have been shown to be a basic form of extracellular matrix organization in the central nervous system (Brauer et al., 1984; Hendry et al., 1988; Delpech et al., 1989; Brückner et al., 1993, 1994, 1996; Murakami et al., 1994; for reviews see Celio and Blümcke, 1994; Carlson and Hockfield, 1996; Celio et al., 1998; Yamaguchi, 2000), they appear as appropriate subjects for studies focused on evolutionary aspects. In the various vertebrate species investigated so far, PNs are composed of aggregating chondroitin sulfate proteoglycans, connected with hyaluronan, link proteins and tenascin glycoproteins (Yamaguchi, 2000; Brückner et al., 2003; Dityatev and Schachner, 2003; Carulli et al., 2006). These major components may form complexes in variable proportions resulting in distinct molecular properties, such as hydration and viscosity, or electrical load of the cellular microenvironment. Especially, the proteoglycans are assumed to have a great potential of molecular variation, by formation of splice variants of their core proteins and by variable glycosylation by the attached glycosaminoglycan chains (Matthews et al., 2002; Rauch, 2004). It is conceivable that

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doi:10.1016/j.jchemneu.2006.08.002

distinct characteristics of the extracellular matrix persist from ancient to recent animal species in association with neuronal systems conserved during brain evolution. Correspondingly, the extracellular matrix may undergo evolutionary alterations related to functional changes of anatomically defined neuronal systems. However, the great variability of extracellular matrix components also inherits the spontaneous formation of multiple new molecular combinations, which are endowed with analogous functional properties.

The present study was undertaken to investigate the association of PNs with defined types of neurons in the South American mouse opossum (*Thylamys elegans*), a marsupial representative of the phylogenetically ancient mammalian order Didelphimorphia (Steiner et al., 2005). Complementary to our previous study focussed on the cerebral cortex of the related species *Monodelphis domestica* (Brückner et al., 1998), we performed a detailed analysis of subcortical regions in the present investigation. Since, we used cytochemical methods established in extracellular matrix research, the results are comparable with data obtained from phylogenetically distant mammalian, including the human, brain. Our data suggest that there exist unique marsupial as well as common mammalian features of extracellular matrix organization.

2. Materials and methods

2.1. Animals and tissue processing

Four adult male mouse opossums (*T. elegans*) were captured in the wild and kept in a standard animal facility at the Universidad de Valparaiso (Chile). Permission to work on collected specimens was under authorization #3014 from Chilean Servicio Agricola y Ganadero (SAG). Animal care and experimental procedures complied with international regulations (NIH publications No. 80-23). For histochemical procedures, animals were euthanized by intraperitoneal lethal dose of ketamine hydrochloride (1 g/kg body weight) and xylazine hydrochloride (0.5 g/kg body weight). The brains were removed from the skull and placed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4 for 14 days, cryoprotected in 30% sucrose, frozen and cut into a series of 30 μm thick sections. All sections were collected and extensively rinsed in 0.1 M Tris-buffered saline, pH 7.4 (TBS).

2.2. Anatomical mapping of brain regions and associated extracellular matrix

Every sixth section of one series of frontal and one series of sagittal sections were stained with a modified Nissl technique using 0.1% toluidine blue stem solution diluted 1:200 in 0.1 M acetic acid buffer, pH 4.6, overnight at room temperature (RT). Alternate sections of these series were used for extracellular matrix staining with biotinylated *Wisteria floribunda* agglutinin (Bio-WFA; Sigma, Deisenhofen) that was visualized with a standard streptavidin/peroxidase technique and nickel-enhanced diaminobenzidine as a chromogen. The treatment of the tissue and the staining followed the previously published protocol (Härtig et al., 1992, 1994).

Since no cytoarchitectonic brain atlas is available for *Thylamys*, brain regions were identified using the stereotaxic atlas of the brain of the opossum *Didelphis marsupialis* (Oswaldo-Cruz and Rocha-Miranda, 1968). Structural details of individual brain regions were identified with the help of studies describing the anatomy of the cholinergic basal forebrain nuclei (Semba, 2004), midbrain catecholaminergic regions (Hazlett et al., 1991), red nucleus (King et al., 1971), cerebellar nuclei (Martin et al., 1974), as well as cortical areas and hippocampus (Benevento and Ebner, 1971; Hamel, 1967, 1982; Rowe, 1990; Beck et al., 1996; Huffman et al., 1999; Frost et al., 2000) in American marsupials.

Most of structural abbreviations used in the present paper were adopted from the nomenclature applied for mice (Franklin and Paxinos, 1997) and rats (Paxinos and Watson, 1998).

2.3. Cytochemistry of extracellular matrix components

The reagents used for the fluorescence microscopic analysis of extracellular matrix components in the present study are specified in Table 1. Tissue sections showed no labeling when staining procedures were performed without the use of primary antibodies or WFA.

Antibodies proved to be non-reactive for *Thylamys* brain were mouse anti-tenascin-R (Mab 619 and Mab 596; M. Schachner, Hamburg) and rabbit anti-mouse aggrecan (AB1031; Chemicon, Temecula).

Lectin staining of N-acetylgalactosamine-containing components: Preceding the labeling with Bio-WFA, free-floating sections were treated for 1 h with a blocking solution consisting of 5% normal goat serum in 0.1 M Tris-buffered saline with 0.3% Triton X-100 (NGS-TBS-T). The sections were incubated with Bio-WFA (2.5 μg/ml NGS-TBS-T), rinsed with TBS and processed for 1 h with a second solution containing streptavidin conjugated to the red fluorescent Cy3 or green fluorescent Cy2 (Dianova, Hamburg; 20 μg/ml TBS containing 2% bovine serum albumin, BSA).

Immunoreaction for chondroitin sulfate proteoglycan components: Non-specific binding sites for subsequently applied immunoreagents were blocked with NGS-TBS-T for 1 h. Free-floating sections were then incubated overnight at room temperature with solutions containing the primary anti-chondroitin sulfate proteoglycan (CSPG) antibodies specified in Table 1. The sections were then rinsed with TBS and processed for 1 h with a solution containing Cy3-goat anti-rabbit IgG or Cy3-goat anti-mouse IgG (Dianova, Hamburg; 20 μg/ml TBS containing 2% BSA) as secondary antibodies.

Detection of hyaluronan: The presence and distribution of hyaluronan was detected by using biotinylated hyaluronic acid-binding protein (BHABP, 1 μg/ml TBS containing 2% BSA) overnight at room temperature. The labeling was then visualized by Cy3-streptavidin as described for WFA-staining.

To test the specificity of the BHABP binding, free-floating sections were pretreated with hyaluronidase from *Streptomyces hyalurolyticus* (50 U/ml 0.1 M PBS, pH 5.0; Sigma H1136; Köppe et al., 1997) for 4 and 16 h at 37 °C. Hyaluronidase treatment of the tissue sections for 4 h resulted in decreased staining intensity. Binding of BHABP was at the background level after 16 h of enzymatic treatment.

Table 1
Cytochemical detection of extracellular matrix components

Detected components	Marker	Dilution	Source	References
N-acetylgalactosamine	Bio-WFA[a]	2.5 μg/ml	Sigma (Deisenhofen)	Härtig et al. (1992, 1994)
Chondroitin sulfate proteoglycan core protein	Rabbit anti-CSPG[b]	1:800	Quartett (Berlin)	Bertolotto et al. (1986)
Hyaluronan	BHABP[c]	10 μg/ml	Seikagaku America	

[a] Biotinylated *Wisteria floribunda* agglutinin, reduced form.
[b] Antigen from chondroitinase ABC-digested bovine nasal cartilage proteoglycan.
[c] Biotinylated hyaluronic acid binding protein, isolated from bovine nasal cartilage proteoglycan.