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PSA-NCAM in the developing and mature thalamus

Samanta Mazzetti [a], Barbara Ortino [b], Francesca Inverardi [b], Carolina Frassoni [b], Alida Amadeo [a,*]

[a] *Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy*
[b] *Unità di Epilettologia Clinica e Neurofisiologia Sperimentale, Fondazione I.R.C.C.S. Istituto Neurologico "C. Besta" di Milano, Italy*

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Abstract

The polysialylated form of the neural cell adhesion molecule (PSA-NCAM) is involved in several morphogenetic processes of the central nervous system. In the present study the expression of PSA-NCAM has been investigated in the rat thalamus during embryonic and postnatal development using light and electron microscopic immunocytochemical techniques. At all the examined ages, PSA-NCAM staining in the thalamus was mainly observed along neuronal plasmatic membranes and absent in astrocytes identified by labelling with cytoskeletal (vimentin and glial fibrillary acidic protein) and membrane (GABA transporter-3) markers. At embryonic day 14 the immunoreactivity was restricted to the dorsal thalamic mantle and to the region of reticular thalamic migration and subsequently it extended throughout the whole thalamic primordium. PSA-NCAM labelling remained intense and homogeneously distributed along perinatal period, but from P4 it began to decrease selectively, persisting throughout adulthood only in the reticular nucleus, ventral lateral geniculate nucleus and midline and intralaminar nuclei. The expression of this adhesion molecule differed in areas characterized by the presence of neurons containing distinct calcium binding proteins, as PSA-NCAM labelling was intense around calretinin-positive neurons, whereas it decreased in some calbindin-immunoreactive regions.

These findings show evidence of a selective neuronal expression of PSA-NCAM in developing thalamus, supporting its suggested role in cell migration and synaptogenesis as it occurs in the cerebral cortex. In adulthood PSA-NCAM could instead be a marker of thalamic nuclei that retain a potential for synaptic plasticity.

Keywords: Polysialic acid; Adhesion molecule; Neuronal migration; Axonal growth; Synaptic plasticity; Sensory systems

1. Introduction

Cell adhesion molecules are abundantly distributed in the central nervous system (CNS), where they are involved in important developmental processes [21,52]. Among them the neural cell adhesion molecule (NCAM) is concerned in neuronal migration and in neurite outgrowth [15,45,46,58]. The different effects of NCAM are often achieved by its post-translational modifications such as glycosilation, so that, after an early expression in neuroepithelial cells, NCAM is highly polysialylated later on, when cells begin to differentiate and migrate to their final destination [11,54]. The presence of the polysialic acid (PSA) confers to NCAM (PSA-NCAM) anti-adhesive properties that have been associated with neuronal migration, axonal growth and synaptogenesis [11,60,64]. In adulthood PSA-NCAM is expressed only in areas where structural modifications occur, such as in those undergoing synaptic and glial plasticity [11,15,62] and in those involved in some pathological conditions [13,20,41,53,67].

In accordance with the functions proposed for PSA-NCAM, its expression in adulthood has been reported in the hippocampus [57,58], subventricular zone and rostral migratory stream [31], olfactory bulb [51], piriform and enthorinal cortices [54] and hypothalamus [62], which are CNS regions retaining synaptic plasticity. So far, only scanty data exist on the expression pattern of PSA-NCAM in the thalamus, both in adulthood [10] and during development [1,46,55]. Aim of this study was therefore to investigate PSA-NCAM distribution in rat thalamus from the embryonic period to adulthood by immunohistochemistry at light and electron microscopic level. Moreover we analyzed the possible expression of PSA-NCAM in cells of glial lineage using antibodies against two cytoskeletal proteins, vimentin

* Corresponding author. Tel.: +39 02 50314886; fax: +39 02 50314881.
E-mail addresses: samanta.mazzetti@unimi.it (S. Mazzetti), alida.amadeo@unimi.it (A. Amadeo).

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(VIM) and glial fibrillary acidic protein (GFAP), and the astrocytic membrane protein GABA transporter-3 (GAT-3) at selected developmental ages. During the first postnatal days, VIM is gradually replaced by GFAP in mature astrocytes [24] that also express GAT-3 on their distal membranes [18,66]. Furthermore, the expression of PSA-NCAM in specific neuronal subpopulations, defined by their content in calcium binding proteins, such as calretinin (CR) and calbindin (CB), was here investigated. These two proteins are known as useful neuronal markers in the thalamus both in adulthood and during development [26,25,37,46].

2. Materials and methods

2.1. Animals

Sprague–Dawley rats (Charles River, Calco, LC) were used for immunoenzymatic and immunofluorescent assays focusing the analysis on the following developmental stages (E = days of gestation; P = days after birth): E14, E17, E18, P1, P2, P4, P5, P7, P12, P14, P21, P40 (adult, 250 g body weight). The University of Milan approved the care and handling of animals in accordance with the guidelines defined by the European Communities Council Directive of 24 November 1986 (directive 86/609/EEC). All efforts were made to minimize the number of animals used and their suffering. Animals were anaesthetized with chloral hydrate (4%; 1–2 ml/100 g body weight, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2; embryos were rapidly removed from deeply anesthetized pregnant rats and were perfused transcardially, with the same fixative, using a glass capillary [46]. Brains were then removed, postfixed in 4% paraformaldehyde at 4 °C for 12–48 h and cut with a Vibratome (Leica VT 1000S) in 50 μm-thick coronal sections.

Thalamic nuclei were identified according to atlases of the developing and mature rat brain [48,47] and following current criteria [29,49].

2.2. Antibodies

For the immunocytochemical detection of PSA-NCAM we used the mouse monoclonal antibody Mab 12E3 (IgM, 1:1200; kindly provided, through Dr. Alfonso Fairen, by Dr. Seki, Department of Anatomy, Juntendo University School of Medicine, Tokyo, Japan), which specifically recognizes the highly polysialylated 140 and 180 kDa isoforms of NCAM [54]. For double-labelling experiments, Mab 12E3 was associated with a mouse monoclonal antibody anti-VIM (IgG, 1:200, Dako, Glostrup, DK) or with one of the following rabbit polyclonal antibodies: anti-CB D-28k (1:5000, SWant, Bellinzona, CH), anti-CR (1:2000, SWant), anti-GAT-3 (1:250, Chemicon, Temecula, CA, USA) and anti-GFAP (1:50, Incstar, Stillwater, Minnesota, USA).

2.3. Immunocytochemistry

Some sections representative of the rostrocaudal extent of the thalamus were sequentially pretreated with 0.05 M NH_4Cl in phosphate buffer saline (PBS) for aldehydes quenching and with 1% H_2O_2 in PBS to inactivate endogenous peroxidases (20 min each). After treatment in 10% normal horse serum (NHS) and 0.2% Triton X-100 as permeabilizing agent, sections were incubated, overnight at room temperature, in the primary antibody MAb 12E3 solution containing 1% NHS in PBS. The antigen localization was revealed using the biotinylated horse anti-mouse IgM (1:200, Vector), the avidin-peroxidase-biotinylated complex (75 min each) and diaminobenzidine tetrahydrochloride as a chromogen. After the immunoreaction the sections were mounted, dehydrated, coverslipped and examined under the light microscope. In control sections, in which the primary antibody was omitted, no immunolabelling was observed.

A semi-quantitative analysis of PSA-NCAM immunoreactivity has been obtained by matching the evaluation of direct microscopic observation performed by two different observers. Serial thalamic sections from two animals for each of selected representative stages of postnatal development (P4, P7, P14, adult) were examined and the relative intensity of PSA-NCAM staining in the neuropil of thalamic nuclei was evaluated (Table 1).

Table 1
Semi-quantitative evaluation[a] of the intensity of PSA-NCAM staining in the neuropil of thalamic nuclei during postnatal rat development

Thalamic nuclei	P4	P7	P14	Adult
Association				
Anterodorsal nucleus	+	+	−	−
Anteroventral nucleus	+++	+++	+	−
Anteromedial nucleus	+++	+++	++	−
Mediodorsal nucleus	++	++	++	−
Laterodorsal nucleus	+++	+++	+++	++
Lateroposterior nucleus	+++	+++	+++	+
Relay				
Ventrobasal nucleus	+	−	−	−
Dorsal lateral geniculate nucleus	+++	+	+	+
Medial geniculate nucleus	+++	+	+	−
Ventromedial nucleus	++	+	−	−
Ventrolateral/ventroanterior nucleus	+++	+	−	−
Posterior complex	+++	+	−	−
Midline nuclei	+++	+++	+++	+++
Intralaminar nuclei	+++	+++	+++	++
Reticular nucleus	+++	+++	++	++
Ventral lateral geniculate nucleus[b]	+++	+++	+++	+++

[a] Intensity of PSA-NCAM immunoreactivity has been evaluated as follows: (−) undetectable; (+) faint; (++) moderate; (+++) intense.

[b] The ventral lateral geniculate nucleus is considered a "relay" nucleus according to Groeneweger and Witter [29], but it is here grouped with the reticular nucleus, because they share the same embryological origins, diffuse projections to the thalamus and lack of projections to the cerebral cortex [42,61].

2.4. Immunoelectron microscopy

The immunocytochemical localization of PSA-NCAM was also performed on selected sections obtained from E17, P5 and adult brains. Vibratome sections were permeabilized, instead of Triton X-100, alternatively with: (a) 0.2% PhotoFlo (Kodak, Rochester, NY, USA); (b) ethanol in PB (10%, 25%, 10%, 10 min each). After the immunoreaction the sections were postfixed with 1% osmium tetroxide, dehydrated and flat-embedded in Epon-Spurr. Ultrathin sections of different thalamic regions were cut by a Reichert UltraCut E ultramicrotome, counterstained with lead citrate and examined with a Jeol T8 electron microscope.

2.5. Confocal analysis

For double labelling experiments the thalamic sections were permeabilized using 0.2% Triton X-100 diluted in 1% bovine albumin serum (BSA) in PBS for 30 min and then incubated overnight in a mixture of the antibody Mab 12E3 and each one of the antibodies against glial and neuronal markers. The MAb 12E3 antibody was usually revealed using a biotinylated horse anti-mouse IgM and sequentially streptavidin conjugated with Rhodamine RedX (1:200, Jackson Immunoresearch, West Grove, PA, USA). A goat anti-rabbit conjugated with FITC (Sigma) was used for antibodies against CB, CR, GAT-3 and GFAP. For VIM/PSA-NCAM double immunofluorescence, the sections were incubated in anti-VIM antibody followed by Cy2-conjugated horse anti-mouse IgG (1:600, Jackson Immunoresearch) and subsequently, after several rinses, in anti-PSA-NCAM antibody followed by biotinylated goat anti-mouse IgM and Cy3-conjugated streptavidin (1:600, Jackson Immunoresearch). All the preparations were analyzed using a TCS NT confocal laser scanning microscope equipped with a 75 mW Kripton-Argon mixed gas laser (Leica Lasertechnik, Heidelberg, Germany). Fluorochromes were excited at 488 (FITC and Cy2) and 568 nm (Rhodamine RedX and Cy3), imaged separately and merged with Leica Power Scan software.