International Journal of Developmental Neuroscience 69 (2018) 97–105

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Disappearance of cerebrovascular laminin immunoreactivity as related to the maturation of astroglia in rat brain

Mihály Kálmán[a,*], Erzsébet Oszwald[a], Károly Pócsai[a,1], Zsolt Bagyura[a,2], István Adorján[a,b]

[a] Department of Anatomy, Histology, Embryology, Semmelweis University, Budapest, Hungary
[b] Department of Physiology, Anatomy, Genetics, Univ. of Oxford, UK

ARTICLE INFO

Keywords:
brain development
GFAP
glio-vascular
glutamine synthetase
laminin
S100

ABSTRACT

The present paper provides novel findings on the temporo-spatial correlation of perivascular laminin immunoreactivity with the early postnatal astrocyte development. The cerebrovascular laminin immunoreactivity gradually disappears during development. The fusion of the glial and vascular basal laminae during development makes the laminin epitopes inaccessible for antibody molecules (Krum et al., 1991, Exp Neurol 111:151). The fusion is supposed to correlate with the maturation of the glio-vascular connections. Glial development was followed by immunostaining for GFAP (glial fibrillary acidic protein), S100 protein, glutamine synthetase as glial markers and for nestin to visualize the immature glial structures. Our investigation focused on the period from postnatal day (P)2 to P16, on the dorso-parietal pallium. In the wall of the telencephalon the laminin immunoreactivity disappeared between P5 and P10; in subcortical structures it persisted to P12 or even to P16. Its disappearance overlapped the period when GFAP-immunopositive astrocytes were taking the place of radial glia. Despite the parallel time courses, however, the spatial patterns of the two processes were just the opposite: disappearance of the laminin immunoreactivity progressed from the middle zone whereas the appearance of GFAP from the pial surface and the corpus callosum. Rather, the regression of the vascular laminin immunoreactivity followed the progression of the immunoreactivities of glutamine synthetase and S100 protein. Therefore, the regression really correlates with a 'maturation' of astrocytes which, however, affects other astrocyte functions rather than cytoskeleton.

1. Introduction

Laminin is a ubiquitous major component of basal laminae, however, the vessels of adult brains are not visualized by immunostaining for laminin (Jucker et al., 1992; Krum et al., 1991). It is supposed that the fusion of the glial and vascular basal laminae makes the laminin epitopes inaccessible for the antibody molecules (Krum et al., 1991). This fusion occurs during development and it is considered as a 'maturation' of the glio-vascular connections (Bär, 1980; Caley and Maxwell, 1970; Marin-Padilla, 1985). Recent papers (Franciosi et al., 2007; Gama Sosa et al., 2014) also mention this correlation.

An important mark of the astrocyte maturation is the appearance of GFAP, the major cytoskeletal element of astrocytes (Bignami et al., 1980; Pixley and de Vellis, 1984), which occurs gradually during the first two postnatal weeks. It approximately coincides with the period of the disappearance of laminin immunoreactivity (until P11, Krum et al., 1991).

Since not all the astrocytes can be detected by immunohistochemical reaction against GFAP, it was also necessary to use other astroglial markers, such as glutamine synthetase and S100 protein (Ghandour et al., 1981; Ludwin et al., 1976; Norenberg, 1979). To visualize the immature glia we used immunostaining for nestin (Hockfield and McKay, 1985; Zerlin et al., 1995; Kálmán and Ajtai, 2001).

The investigation period lasted from P2 to P16, i.e. it finished after that the radial glia disappeared (Kálmán and Ajtai, 2001; Pixley and de Vellis, 1984; Stichel et al., 1991) and the vascular laminin immunoreactivity became undetectable (Krum et al., 1991). The investigation was focused on the dorso-parietal pallium, so the data refer to this area unless specified otherwise.

* Corresponding author at: Department of Anatomy, Histology, Embryology, Semmelweis University, H-1094, Tuzolto 58, Budapest, Hungary.
E-mail addresses: kalmanprof@gmail.com (M. Kálmán), oszwald.erzsebet@med.semmelweis-univ.hu (E. Oszwald), pocsai.karoly@gmail.com (K. Pócsai), bagyura@zortal.hu (Z. Bagyura), adorist@freemail.hu (I. Adorján).
[1] Present address: 2nd Dept. of Internal Medicine, Semmelweis University, Budapest, Hungary
[2] Present address: Heart, Vascular Center, Semmelweis Univ., Budapest, Hungary

https://doi.org/10.1016/j.ijdevneu.2018.07.003
Received 27 February 2018; Received in revised form 24 May 2018; Accepted 9 July 2018

Our description includes the whole telencephalic wall not only the cortex. Since the cortical layer system alters during the period studied, the term 'layer' was avoided and 'zone' was used instead, which did not relate to any conventional layer either in the mature cortex (e.g. external granular, etc.) or in the developing one (e.g. the cortical plate).

2. Materials, Methods

2.1. Animals

Postnatal rats were used at the age of P2, P4, P5, P6, P7, P8, P10, P12, P14, and P16, from 3 litters, 4-4 pups of either sex at each age. Experiments were performed according to the Committee on the Care, Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary (22.1/3491/003/2008) in accordance with the guidelines of European Union Directive (EU Directive 2010/63/EU). Animals were deeply anesthetized with ketamine and xylazine (80 and 20 mg/kg, respectively, intramuscularly) and perfused through the aorta with 0.9% sodium chloride followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), After perfusion brains were post-fixed in the same fixative for 4 days at 4 °C. Then the brains were embedded into agarose, serial coronal sections of 50 µm were cut with a vibration microtome (Leica VT 1000S) and rinsed in phosphate buffered saline (PBS, Sigma) overnight.

2.2. Fluorescent immunohistochemistry

Floating sections were pretreated with 20% normal goat serum diluted in PBS, for 90 min at room temperature, to block the non-specific binding of antibodies. This and the following steps were followed by an intense rinse in PBS (30 min at room temperature). The sections were incubated with primary antibodies (Table 1) for 40 h at 4 °C. The primary antibodies were diluted as shown in Table 1 in PBS containing 0.5% Triton X-100, 0.01% sodium azide. Fluorescent secondary antibodies (Table 2) were applied at room temperature for 3 h. The sections were finally rinsed in PBS (1 h at room temperature), mounted onto microscope slides, cover-slipped in a mixture of glycerol and distilled water (1:1) and sealed with lacquer. Control sections were treated identically, but the primary antibodies were omitted. No structure-bound fluorescent labeling was observed in these specimens.

2.3. Fluorescent microscopy and digital imaging

High-resolution microphotographs were taken with a Radiance-2100 (BioRad, Hercules, CA) confocal laser scanning microscope whereas low-power ones with a DP50 digital camera mounted on an Olympus BX-51 microscope (both from Olympus Optical Co. Ltd, Tokyo, Japan). Digital images were processed using Photoshop 9.2 software (Adobe Systems, Mountain View, CA) with some adjustments for brightness and contrast. The confocal photomicrographs of double-labeled specimens were presented in color figures, the others in black and white figures.

2.4. Pre-embedding electron microscopic immunohistochemistry

In this case 0.5% glutaraldehyde was added to the perfusion solution for a better fixation. The immunohistochemical reaction against laminin was performed on vibratome sections according to the avidin-biotinylated peroxidase (ABC) method. Endogenous peroxidase was inactivated with 3% H_2O_2 in PBS (5 min at room temperature) followed by an intense rinse in PBS (30 min at room temperature). Incubations with 20% normal goat serum and anti-laminin (see in Table 1) were carried out as above except for that Triton X-100 detergent was reduced to 0.1% to decrease tissue destruction. The procedure continued by applying biotinylated anti-rabbit serum (Vector Laboratories, CA, USA, 1:100) followed by the avidin-biotinylated peroxidase complex (Vector Laboratories, CA, USA). Both incubations lasted for 90 min at room temperature and were followed by intense rinses in PBS (30 min, at room temperature). To visualize the immunohistochemical reaction product 0.05% 3,3' diaminobenzidine-tetrahydrochloride (DAB), 0.01% H_2O_2 in Tris-HCl buffer (0.05 M, pH 7.4, at room temperature) were used. The peroxidase-reaction was stopped at visual color control by replacing the solution with PBS.

2.5. Electron microscopic investigation

Following the immunohistochemical reactions tissue areas were selected under light microscope and cut from the vibratome sections. They were immersed for 30 min into a 1% osmium tetroxide solution then rinsed in phosphate buffer and dehydrated through a graded series up to absolute ethanol. Following immersion in propylene oxyde (10 min) the tissue samples were embedded into epoxy resin (Durcupan, Fluka). Semithin sections were cut with a Reichert Ultracut S ultramicrotome, areas were selected under light microscope and the samples were adequately trimmed. The ultrathin sections were prepared with the same ultramicrotome and mounted on grids. The photomicrographs were taken by a JEOL 100B elecron microscope equipped with a Sys Morada digital camera.

3. Results

3.1. The postnatal regression of the vascular laminin immunoreactivity

At P2 laminin immunostaining delineated the vascular pattern in the whole thickness of the brain wall (Fig. 1a). During the following days up to P10, however, the vascular laminin immunoreactivity withdrew from the vessels gradually but almost completely. In the telencephalic wall this process started in the middle zone of its thickness at P5 (Fig. 1b) and extended toward the superficial and deeper parts. By P7 this zone widened (Fig. 1c), by P10 the laminin immunoreactivity was confined to the entering (subpial) segments of vessels and to the deepest part of the cortex (Fig. 1d, e). Corresponding

Table 1
The primary antibodies applied in the study.

Against	Host	Company	Code Nr.	Dilu-tion	final con-centration (µg/mL)
GFAP	Mouse*	Novocastra, Newcastle, United Kingdom	ga5	1:100	100
GFAP	Rabbit**	DAKO, Galstrup, Denmark	Z0334	1:500	5.6
Glutamine synthetase	Mouse*	Transduction Laboratories, Erembodegem, Belgium	610518	1:100	2.5
Glutamine synthetase	Rabbit**	Novus Biologicals Littleton, Co, USA	NB110-41404	1:500	***
Laminin 1	Rabbit**	Sigma, San Louis, MO, USA	l 9393	1:100	5
Nestin	Mouse*	Millipore, Temecula, CA, USA	MAB-353	1:1000	1
S100-protein	Rabbit**	Sigma, San Louis, MO, USA	s-2644	1:100	81

*- monoclonal, **- polyclonal, ***- the original concentration is not given by the firm,