ORIGINAL ARTICLE

SUBEPENDYMAL ZONE: IMMUNOHISTOCHEMICALLY DISTINCT COMPARTMENT IN THE ADULT MAMMALIAN FOREBRAIN

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Summary: The subependymal zone (SEZ) lining lateral walls of the lateral cerebral ventricles represents the site of active neurogenesis in the brain of adult mammals. Peroxidase immunohistochemistry performed in paraffin-embedded sections reveals that structural organization of the SEZ differs from other regions in the brain. The SEZ is devoid of synapses that are abundant in the adjacent striatal neuropil. Therefore immunostaining of synaptophysin detects sharp borders of the SEZ. Using immunophenotypization, we identified cell types constituting the SEZ in the intact rat forebrain. The presence of neural progenitor/stem cells was confirmed by finding of nestin-immunopositive cells. Detection of the astroglial marker GFAP confirmed that astrocytes represented major supporting elements responsible for creating a unique microenvironment of the SEZ. One type of the astroglia participated in covering surfaces of the blood vessels and boundaries of the SEZ. The second astroglial cell type formed branched elongated tubes that enwrapped other SEZ cell types with their cytoplasmic extensions. The interior of astrocytic channels was occupied with small densely aggregated NCAM-immunoreactive neuroblasts. Bipolar morphology indicated that these cells probably underwent migration. Immunodetection of other neuronal markers like β-III tubulin, MAP-2 and Pan neurofilaments identified positive cells in the neighbouring brain parenchyma but not in the SEZ. The rostral migratory stream (RMS) linked with the anterior SEZ had a similar structural arrangement. It contained a large amount of nestin$^+$ and vimentin$^+$ cells. The RMS consisted of GFAP$^+$ astrocytic tubes ensheathing NCAM$^+$ neuroblasts. On the contrary to the SEZ, the RMS neuroblasts expressed β-III tubulin. However, markers of postmitotic neurons MAP-2, Pan neurofilaments and synaptophysin were not expressed in the RMS. Our study describes a complex histological structure of the rat SEZ, identifies its individual cell types and demonstrates a usefulness of immunohistochemical detection of cell-specific markers in a study of microenvironment forming neurogenic zones in the mammalian brain.

Key words: *Subependymal zone; Rostral migratory stream; Immunohistochemistry; Phenotypization; Rat forebrain*

Introduction

Although for decades the brain had been considered as an organ incapable of regenerating the neuronal cells, nowadays it is generally accepted that neuronogenesis occurs within the adult mammalian central nervous system (CNS). A neural stem cell (NSC), a tissue-specific stem cell of the CNS, is responsible for generation of new neuronal and glial cells in situ. Its presence in the CNS has been confirmed by numerous studies based on incorporation of tritiated thymidine or bromodeoxyuridine (e.g. 1,29,37,38, 41), expression of nestin (which is considered to be a neural stem cell marker; 10) or cultivation of dissociated nervous tissue (6,35). The in vitro experiments gave the evidence that different regions of the adult CNS contained multipotent stem cells; so far NSCs have been isolated from the subependymal zone (SEZ; 3,6,9,30), rostral migratory stream (RMS; 12), olfactory bulb (12,21), ependymal lining (15), hippocampus (3,30,32), other parts of the cerebral hemispheres (3,30,35), retina (2), spinal cord (16) etc. Of these, the SEZ represents the largest neurogenic region that continuously produces new neuronal and glial cells; the neuroblasts emigrate from the SEZ and incorporate in the target structures, e.g. in the olfactory bulb (via a specific pathway of the RMS) or neocortex (1,8,11,23). Following an injury to the adjacent brain parenchyma or exposure to growth factors, SEZ NSCs become activated and production of new cells in the SEZ rapidly increases (7,11,29,30,41), which documents functional participation of this region in the maintenance of brain tissue homeostasis.

In tissues, stem cells occupy specific microenvironments, the so-called niches, created by adjacent cells and extracellular structures that provide signals controlling the renewal of SCs and generation of their progeny. Due to

a large accumulation of NSCs along the SEZ, this zone structurally differs from the other brain regions. Whereas the white and gray matter of the brain contain nerve fibre tracts or interneuronal synapses making appropriate neuronal circuits, the SEZ is devoid of these structures. Permanent differentiated elements that likely play a key role in formation of the SEZ niche are represented by ependymal and astroglial cells. Doetsch et al. who analysed structure of the SEZ at the level of electron microscopy using serial sections identified two types of astroglial cells, the first one representing the element participating in delineation borders of this zone and the second type creating the tube-like channels ensheathing the immature SEZ cells (8). Similar astroglial tube-like structures were recognized in the RMS (34). These elongated channels delimitate pathway for neuroblasts emigrating from the SEZ to the olfactory bulb.

In the present study, we described a light microscopical architecture of the SEZ lining the lateral walls of lateral cerebral ventricles in paraffin-embedded sections of the intact rat forebrain. Using peroxidase immunohistochemistry, we identified precise boundaries of the SEZ and the cell types that coexist in the SEZ in a close relationship. Finally, we described expression of cell-specific markers in the RMS and compared its histological structure with the SEZ.

Materials and methods

Animals

Seven adult Wistar female rats (VELAZ, Prague, Czech Republic), approximately 3 months old and weighing 180–200 g, were used in this study. Experiments performed in this study were approved by the Ethical Committee supervising procedures on experimental animals at Charles University Medical Faculty in Hradec Králové.

Histology and Immunohistochemistry

Under deep anesthesia, rats were transcardially perfused with Carnoy's fixative. Brains were removed and immersed in the same fixative for at least three days, dehydrated in increasing concentrations of alcohols and embedded in paraffin. Serial seven-micrometre thick coronal sections were mounted on glass slides. Each 10th section was stained with haematoxylin and eosin, while other sections were processed for peroxidase immunohistochemistry.

Following deparaffinization, rehydration and microwave antigen retrieval, sections were incubated with the following primary antibodies for 45 min: anti-synaptophysin (SY38, 1:20, DAKO, Glostrup, Denmark), anti-GFAP (GA-5, 1:400, Sigma, Prague, Czech Republic), anti-β-III tubulin (TU-20, 1:200, Exbio, Prague, Czech Republic), anti-vimentin (V9, 1:40, Sigma), anti-MAP-2 (microtubule associated protein-2, HM-2, 1:500, Sigma), anti-PAN Neurofilament (clones DA2, FNP7 and RmdO20.11, 1:100, Zymed Laboratories Inc., San Francisco, CA), anti-NCAM (neural cell adhesion molecule, 5B8, 1:4), anti-nestin (Rat 401, 1:4). Antibodies against NCAM and nestin were obtained from the DSHB, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. After washing and incubation with the appropriate secondary antibody, streptavidin conjugated with horseradish peroxidase complex was applied for 45 min at room temperature. The intensity of faint immunoreactivity for synaptophysin was enhanced with the catalyzed signal amplification: after washing, the sections were incubated with 10 μL biotin tyramine and 0.03% hydrogen peroxide for 10 min and then again incubated with streptavidin coupled with peroxidase. After rinsing, visualization was achieved by incubating with 3,3'-diaminobenzidine tetrachloride (Sigma) as the peroxidase substrate and hydrogen peroxide. The sections were then counterstained with methyl green.

Results

The subependymal zone

The SEZ is easily identifiable in lateral walls of the lateral cerebral ventricles (LCVs) between the ependyma and the striatum (Figs. 1, 2). The thickness of the SEZ varies according to its location; in the anterior SEZ, cells are arranged in several layers whereas posterior SEZ consists of an irregular layer of SEZ cells. Cellular density is larger in the SEZ than in the surrounding neuropil. Tightly packed cells of the SEZ are easily recognizable from the adjacent ependymal cell monolayer with characteristic round nuclei and distinct cilia that separates the SEZ from the ventricular cavity. The cells in the SEZ differ in their sizes, which indicate the presence of distinct cell types. Rich vascularization provides a proper nutrition to cells generated in the SEZ. Structure of the SEZ differs from all the other CNS regions because it is devoid of nerve fibre tracts or neuronal circuits formed by neuronal cells in other parts of the brain parenchyma. For that reason the SEZ lacks interneuronal synapses and it does not reveal immunoreactivity for glycoprotein of synaptic vesicles, synaptophysin. Due to small sizes of synaptic vesicles, a routine immunohistochemical detection of synaptophysin provides just a faint punctuate signal distributed throughout the mature brain parenchyma. Amplification of the specific signal using biotinylated tyramine results in an enormous increase of the intensity that allows to demarcate a sharp boundary between the immunopositive neuropil and immunonegative SEZ (Fig. 2).

Detection of glial fibrillary acidic protein (GFAP), a marker of astroglial cells that represent the main glial cell type of the CNS tissues, revealed that the SEZ contained a larger accumulation of macroglia than was typical for other brain regions (Fig. 2). Therefore astroglia represented a major supporting element responsible for creating a unique microenvironment of the SEZ. $GFAP^+$ cells were adjacent to the ependymal lining where they extended many pro-

cesses; feltwork of processes formed a lamina underlying the ependyma. Only sporadic GFAP$^+$ cells were inserted in the ependymal cell monolayer. Some astrocytic processes ensheathed the blood vessels by forming the perivascular limiting membrane. Other GFAP$^+$ cells were localized at the interface with the striatal parenchyma. Immunohistochemistry visualized numerous cytoplasmic processes of astroglial cells due to the presence of bundles of intermediate filaments like GFAP, vimentin and nestin. In the SEZ, a dense meshwork of astrocytic processes formed tangentially oriented intercommunicating channels or glial tubes forming a characteristic complex system. Individual tubes ran nearly parallel to each other but they could branch and merge along the SEZ. Astrocytic meshwork forming the tubes enwrapped incompletely the aggregations of small basophilic cells.

Anti-NCAM (neural cell adhesion molecule) immunohistochemistry confirmed that these smallest cellular elements with a high nuclear/cytoplasmic ratio were neuroblasts. NCAM$^+$ cells exhibited two main processes emerging from the opposite poles giving the cell an elongated and bipolar shape. Such morphology with tangentially oriented leading and trailing processes is characteristic for migrating neuroblasts (Fig. 2). Neuroblasts in the SEZ tended to cluster in elongated groups arranged in the form of numerous longitudinal chains parallel to the wall of the LCVs. These chains formed an extensive network that extended from the caudal to the rostral lateral wall of the LCVs. The continuous splitting and converging chains formed a characteristic structure of the SEZ. Networks of chains of migrating neuroblasts were ensheathed by astrocytes. Occasionally small clusters were visible in contact with blood vessels or in the adjacent neuropil.

Immunodetection of markers specific for fully differentiated neuronal cells like MAP-2, Pan NF and β-III tubulin revealed no positive cells in the SEZ. On the contrary, cells expressing markers specific for adult neuronal cells were scattered throughout the mature brain parenchyma, e.g. in the adjacent striatum (see Fig. 2). When stained for nestin, a marker of immature neural cells, immunopositive neural stem/progenitor cells were recognized in the SEZ where they were juxtaposed by clusters of neuroblasts. In other

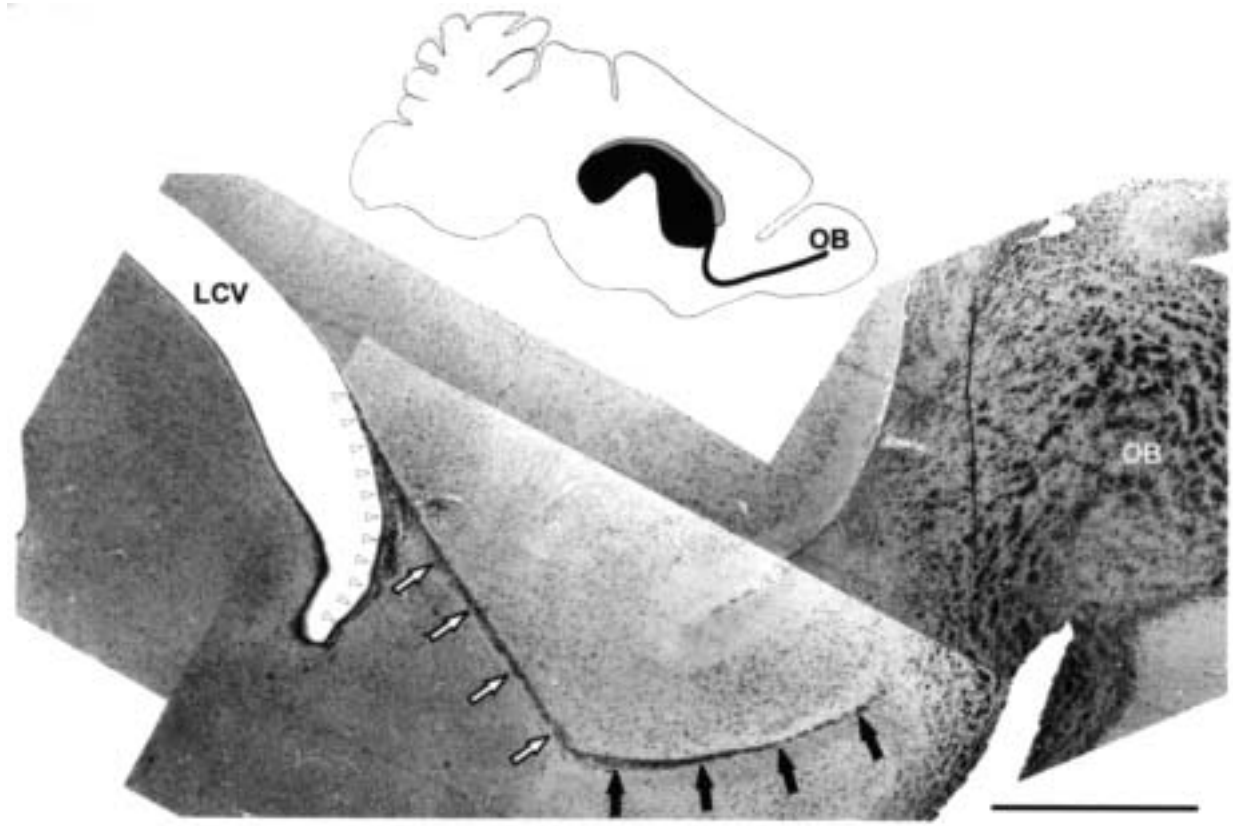

Fig. 1: A parasagittal section of the rat forebrain stained with haematoxylin and eosin reveals a variable thickness of the subependymal zone of lateral cerebral ventricles (LCV). In its anterior part, the SEZ reaches the largest thickness (arrowheads). At this site, the SEZ is associated with the rostral migratory stream (arrows) that serves to deliver immature cells to the olfactory bulb (OB). Vertical (white arrows) and horizontal limbs (black arrows) of the RMS are well visible. A schematic drawing of the corresponding parasagittal section through the rat brain depicts lateral cerebral ventricles lined by the SEZ and the RMS (black); the overlying corpus callosum is depicted in grey colour. Scale bar: 1 mm.

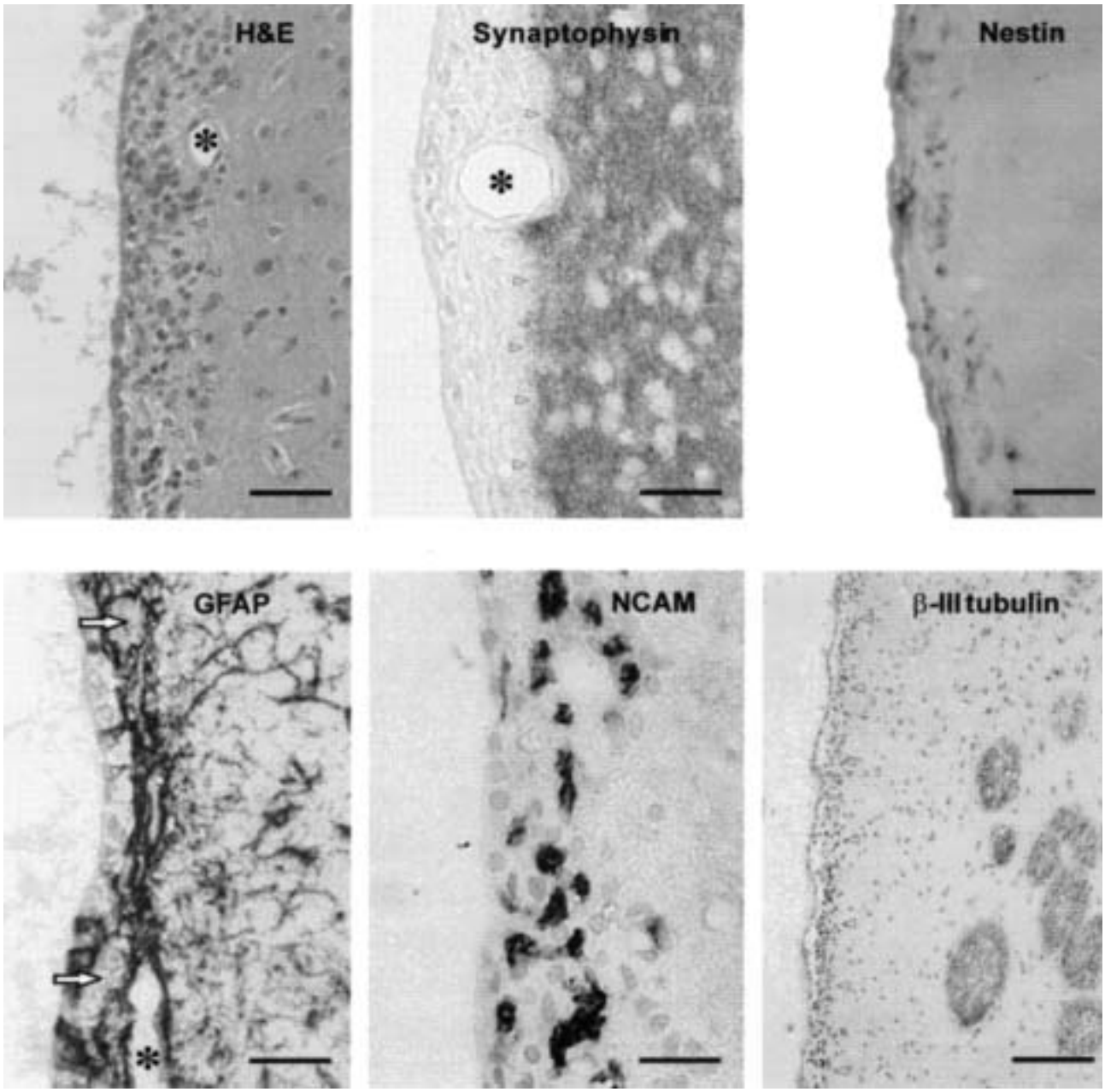

Fig. 2: Histological structure of the intact rat subependymal zone. Staining with haematoxylin and eosin shows a single layer of ciliated ependymal cells facing the cerebral ventricle and several layers of underlying basophilic SEZ cells. SEZ is characteristic by high cellular density due to a large accumulation of relatively small cells. Arrowheads indicate a boundary with the striatum; an asterisk marks a blood vessel inside the SEZ; scale bar: 25 μm. Synapses that normally occur in the brain neuropil are not found in the SEZ. Immunohistochemical detection and amplification of synaptophysin shows a sharp boundary between the SEZ and adjacent corpus striatum (arrowheads; scale bar: 25 μm). Anti-nestin staining reveals focal spots of neural stem and progenitor cells scattered throughout the SEZ; scale bar: 20 μm. $GFAP^+$ astroglial cells inside the SEZ are densely packed and form longitudinal channels that contain clusters of small $GFAP^-$ neuroblasts (arrows). Few $GFAP^+$ cells are found in the ependymal monolayer. In the striatum, protoplasmic astrocytes are not so densely packed and they are visible as individual cells; scale bar: 20 μm. Detection of NCAM visualizes small and densely clustered neuroblasts forming elongated chains; occasionally, short processes of these bipolar cells are visible; scale bar: 20 μm. A marker of fully differentiated neuronal cells, β-III tubulin, is not found in the SEZ; it is expressed in the striatal nervous tissue; scale bar: 50 μm.

brain areas, anti-nestin immunostaining detected thick processes of astroglial cells rich for intermediate filaments, e.g. those forming superficial limiting membrane, and tanycytes that were abundant especially in the lining of the third cerebral ventricle.

Rostral migratory stream

The SEZ reaches its largest thickness in the site where the migrating neuroblasts enter the rostral migratory pathway (RMS). In parasagittal section of the rat brain (Fig. 1), it is well apparent that the RMS represents a thin strand of a tissue interconnecting the SEZ with the olfactory bulb (OB). Histological structure of the RMS mimics the SEZ because it represents a continuation of the SEZ. There were no ependymal cells due to absence of ventricular system filled with the cerebrospinal fluid. Immunoperoxidase detection of synaptophysin identified the precise boundaries of the RMS because it was devoid of any synapses on the contrary to the adjacent neuropil (Fig. 3). GFAP was expressed in astroglial cells that formed a characteristic tube-like structures ensheathing chains of small densely packed cells. These astroglial cells also expressed the intermediate filament vimentin. However, the anti-vimentin antibody did not allow to recognize these longitudinally oriented glial tubes because it provided an intense staining of all elements (i.e. mature glial cells and immature neural precursors) of the RMS. Immature cells migrating along the RMS could

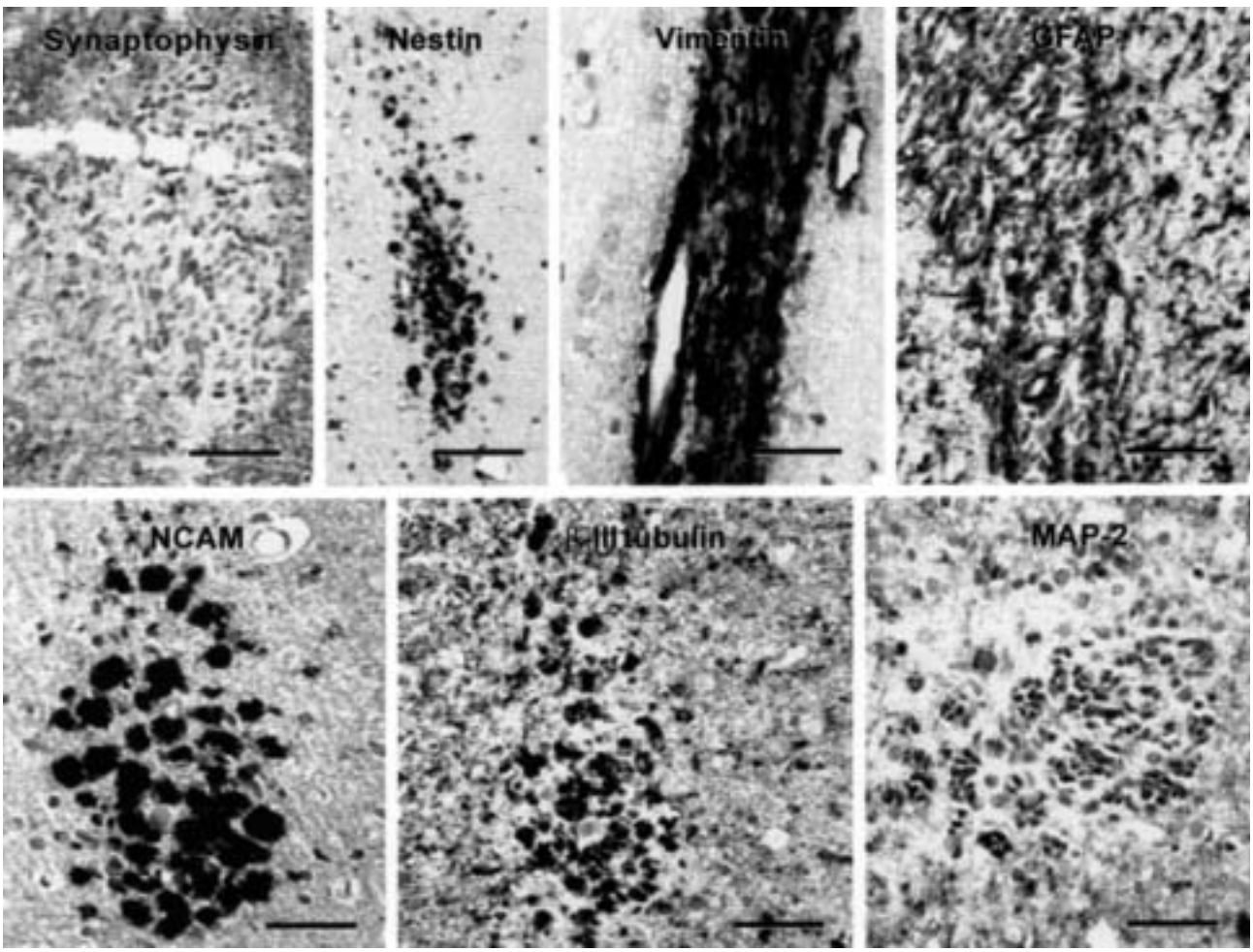

Fig. 3: Peroxidase immunohistochemistry defines antigenic profiles of cells in the rat rostral migratory stream. Synaptophysin is expressed only in the neuropil surrounding the RMS; scale bar: 20 μm. The RMS cells express large levels of intermediate filament proteins nestin (scale bar: 30 μm), vimentin (scale bar: 30 μm) and GFAP. GFAP expressed in RMS astrocytes permits to identify channel-like structures that contain immunonegative neuroblasts. $GFAP^+$ astrocytes outside the RMS do not form such channels; scale bar: 30 μm. The RMS neuroblasts express high levels of NCAM (scale bar: 20 μm) and moderate levels of β–III tubulin (scale bar: 30 μm). A marker of dendrites of postmitotic neurons, MAP-2, is expressed only in the surrounding fully mature parenchyma (scale bar: 30 μm). Sections were counterstained with haematoxylin to visualize nuclei of immunonegative cells.

be recognized with immunostaining of the nestin. Staining of markers specific for neuronal cells distinguished which cells belonged to a neuronal cell lineage. They expressed NCAM and β-III tubulin which identified migratory neuroblasts in the RMS. Detection of markers characteristic for postmitotic neurons did not reveal any immunoreactivity inside of the RMS; the only positive cells were located in the mature parenchyma outside the RMS.

Discussion

Immunohistochemical phenotypisation described in this study reveals a complex organisation of the subependymal zone (SEZ) of the intact rat forebrain. The major role of this specific CNS region is a production of new neural cell types, i.e. maintenance of neurogenesis. It is thought that this function is performed by primitive neural stem cells (NSCs) that reside inside the SEZ. Although their presence in the SEZ has been confirmed in numerous studies (see e.g. 6,8,18,29), the precise identity of the cell type responsible for neurogenesis remains still a matter of debate. Johansson et al. believe that ependymal cells lining the SEZ act as multipotent NSCs (15) whereas Doetsch et al. consider the SEZ astrocyte as the NSCs (9). However, both mentioned cell types share features of differentiated cells like tufts of beating cilia or elongated or flattened cellular processes. Tissue-specific stem cells in other organs show primitive morphology that lacks any signs of cellular differentiation and therefore we can expect that there is a similar primitive NSC inside the SEZ. The findings described by Johansson et al. and Doetsch et al. can be interpreted as an evidence that ependymal and astroglial cells or their precursors (which appear in the SEZ as well) can dedifferentiate under certain conditions and thus give rise to multipotent NSCs. Morphologically NSCs are identified in situ according to the expression of protein nestin. This intermediate filament is strongly expressed in endogenous adult NSCs and it can be detected reliably in formaldehyde-fixed, paraffin-embedded sections following microwave antigen retrieval (28). Nestin$^+$ cells are found in focal clusters or as single cells scattered along the chains of migrating neuroblasts; these cells do not stain for neuronal or glial markers. It must be mentioned that nestin is also expressed in other cell types including astrocytes (reactive but also normal astrocytes), tanycytes, endothelial cells, precursors of neural cells and few other cell types found in non-neural tissues (e.g. 27,28, Fig. 2) and therefore a finding of nestin-immunoreactive cells has to be carefully interpreted. Recently, other markers expressed by NSCs have been identified, e.g. mCD133 (39), CD34 (33), mCD24(5); their combination could be useful for confirmation of the NSC phenotype. After activation, e.g. in response to lesion of adjacent brain structures (11,29,41) or after activation with proliferative growth factors (7,17,30), NSCs start to divide and represent the major proliferating cell type. NSCs and their progenitors can be detected according to the expression of proliferation markers like PCNA or BrdU (29); however, it is important to realize that other SEZ cell types including B2 astrocytes and neuroblasts also undergo cellular divisions (8,25). NSCs represent a minor SEZ population; it is estimated that they form just 0.3% of all SEZ cells (36). High rates of cell proliferation are still observed in the SEZ during ageing and the number of SCs does not appear to change throughout life (38). The SEZ NSCs are multipotent. Their differential potential was confirmed in vitro; NSCs isolated from the SEZ give rise to progenitors differentiating into neuronal and glial cells (18,19,40). In situ, gliogenesis is stimulated by bone morphogenic protein. This stimulation can be suppressed by noggin produced by ependymal cells which subsequently results in production of neuroblasts (20). These regulatory pathways require a close relationship of different cell types that is necessary to create a SEZ niche.

Astroglial cell represents another cellular element participating in formation of the SEZ niche. Astroglial cell endfeet cover the boundaries of the SEZ against the mature nervous tissue and ependyma, they are coupled with each other by gap junctions and are in an intimate contact with all the other SEZ cell types including neuroblasts, stem cells and blood vessels. They can detect alterations in neuronal and precursor number, translate signals from the vasculature and other cells and provide rapid propagation of signalling within the neurogenic niche. Moreover, astroglial cells can secrete factors supporting neurogenesis and therefore they are the key elements creating a neurogenic microenvironment in the SEZ (19). Astroglial cells in the SEZ contain glycogen particles and express markers of mature astroglial cells GFAP, vimentin and S100 protein (14,29). They form a lacunar system that incompletely enwrap small basophilic cells and compartmentalise the SEZ into longitudinally oriented tubes that run nearly parallel to each other. Detection of NCAM reveals spindle-shaped neuroblasts aggregated in elongated clusters arranged in network of interconnected chains. Bipolar morphology of NCAM$^+$ cells (leading and tail processes) is characteristic for migrating cells. The neuroblasts emigrate from the SEZ as they travel along the glial tubes and they divide giving rise to new neuroblasts. Although the above mentioned cell types are the most characteristic for the adult mammalian SEZ, other elements present here include small percentage of tanycytes within the ependymal layer, sporadic microglial cells and endothelial cells (8). The latter cell type participates in nutrition of other SEZ cells and also in formation of a unique neurogenic milieu (26). BDNF released by endothelial cells acts as a survival factor of newly generated neurons (24) and neurogenesis occurs in foci closely associated with blood vessels (31). A specialised basal lamina extends from blood vessels, contacts all SEZ cell types and terminates in small bulbs adjacent to the ependymal cells (26). This basal lamina and associated extracellular matrix plays an important role in cell tethering and ligand binding and forms an essential part of the SEZ stem cell niche.

Histological structure of the rostral migratory stream (RMS) closely resembles the forebrain SEZ. The reason is that the RMS also develops from the periventricular area. While the SEZ develops from the ventricular zone of the lateral cerebral ventricles, the RMS arises from the ventricular zone of the olfactory cerebral ventricle. At later developmental stages the olfactory ventricle obliterates and does not generate ependymal cells but its subventricular zone remains to be associated with a corresponding area of the LCVs. The RMS consists of two distinct cellular compartments corresponding to its two main cell types: longitudinally arranged $GFAP^+$ astroglial tubes and chains of fusiform $NCAM^+$ neuroblasts, which are contained within these fields (Fig. 3). As the neuroblasts travel inside of glial tubes they proliferate (22). Their migration and division are regulated by different beta1 integrins (13). The newly generated neural precursors start to express another neuronal marker, β-III tubulin. After reaching the olfactory bulb, the neuroblasts differentiate into GABAergic granule cells and dopaminergic periglomerular interneurons (4,22). A strong immunoreactivity for nestin in the RMS indicates the presence of immature cells including neural precursor and stem cells. Despite the ongoing differentiation of neuroblasts migrating throughout the RMS, several recent studies confirmed, that the RMS still contains a large amount of multipotent NSCs (12).

Immunohistochemical analysis of the RMS and SEZ permits to identify cell types constituting both areas and to study their intercellular relationship. Both areas represent a heterogeneous constellation of cells with differentiated and undifferentiated phenotypes. The mature cell compartment is represented by tangentially oriented intercommunicating glial channels forming a complex system enwrapping the immature neuroblasts and NSCs. Such structural arrangement is necessary for creation of specialized niches supporting neurogenesis and represents a histological feature unique to these CNS areas.

Acknowledgement

The authors thank to Mrs. Helena Rückerová and Mrs. Hana Hollerová for their skilful technical assistance. The work was supported by a project No. 304/03/1515 from Grant Agency of the Czech Republic.

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Submitted January 2004.
Accepted September 2004.

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