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NOVOcan: a molecular link among selected glial cells

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Abstract

The nervous system is generated from cells lining the ventricular system. Our understanding of the fate potentials and lineage relationships of these cells is being re-evaluated, both because of recent demonstrations that radial glia can generate neurons and because of the identification of fate-determining genes. A variety of intrinsic and extrinsic molecules, including proteoglycans, regulate embryonic and postnatal brain development. Using probes modeled after species conserved domains of heparan sulfate proteoglycans, we cloned a novel gene called *novocan*, raised monoclonal antibodies against a segment of the predicted amino acid sequence of the expressed protein (NOVOcan) and used the antibodies to establish the cell and tissue localization of NOVOcan in postnatal rat brains by immunohistochemistry. NOVOcan was expressed in cells lining the ventricles, including a variety of radial glia during early postnatal development. Later, as radial glia disappeared and ependymal cells appeared, NOVOcan was detected in ependymal cells and in tanycytes, a specialized form of ependymal cell resembling radial glia. NOVOcan was absent in two known progeny of radial glia, mature astrocytes and neurons. Whereas NOVOcan was also absent in mature oligodendrocytes (OLGs), it was present in OLG precursors in developing white matter. These studies set the stage for determining the roles of NOVOcan in brain cell lineage patterns as well as in other aspects of development.
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1. Dedication

Whatever you can do,
or dream you can do,
begin it.
Boldness has genius,
power, and magic to it.
—Goethe

This manuscript is dedicated to Dr David Yphantis in whose laboratory I (SS) have sharpened and perfected the theoretical understanding and application of biophysical techniques as they apply to the study of proteins. Years later, when I switched to studying cells, it is this training that helped me to succeed where others have failed.

2. Introduction

Central nervous system (CNS) glial cells are morphologically, biochemically and functionally

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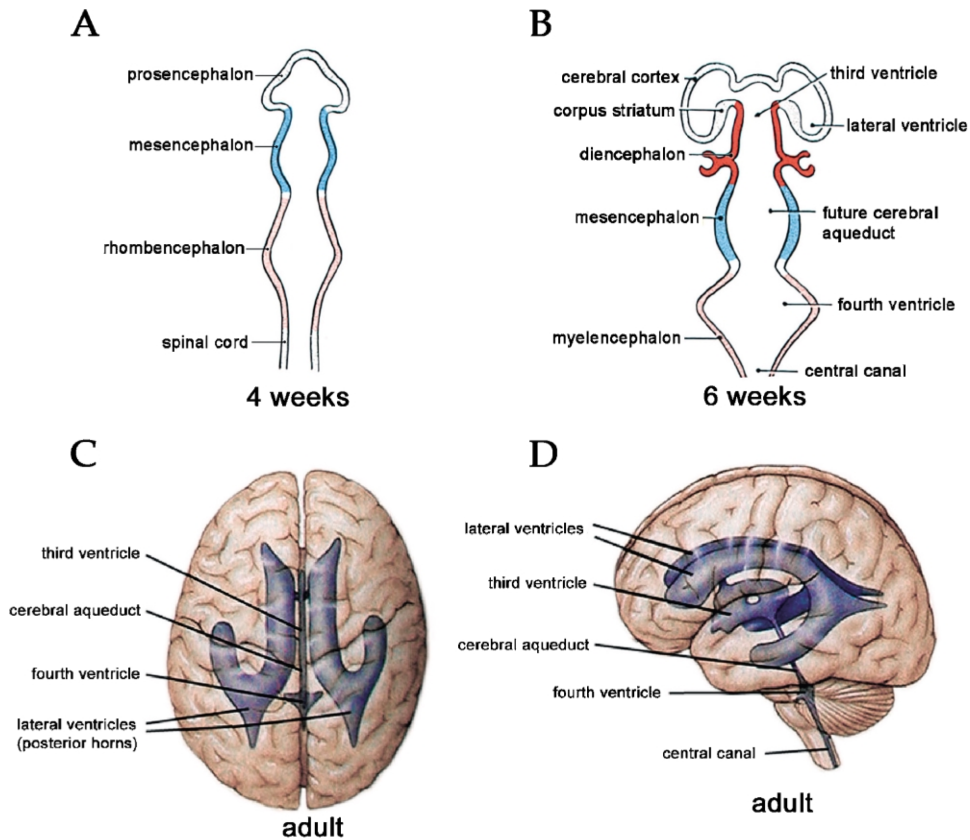

Fig. 1. Neural cells are born in the ventricular system or adjacent to it; the human ventricular system during embryogenesis (A and B) and in the adult (C and D). In (A) and (B) the regions of the brain derived from anterior posterior subregions of the VZ are indicated on the left and color coded. (A–C) dorsal views (anterior is up); (D) sagital view (anterior is to the left). (A) and (B) adapted from [1]; (C) and (D) adapted from Ref. [64].

heterogeneous. Originally relegated to roles such as structural scaffolds or merely 'glue' to sustain neurons, they have now come to occupy a central stage as stem cells that are capable of generating a variety of neural cells (reviewed in a series of articles in Ref. [17]), and also as active participants in neuronal function. The source of these and other neural cells is the thin layer of neuroectodermal cells that lines the ventricular system of the brain and spinal cord interior, Fig. 1 and Ref. [1]. During development these 'ventricular zone' (VZ) cells proliferate and give rise to the majority of the nervous system [2]. We consider four types of CNS glial cells: radial glia, oligodendrocyte (OLG) progenitors, ependymal cells and tanycytes linked here by their common expression of a novel gene, *novocan*.

2.1. The novocan gene, its expressed mRNAs and protein products

As part of an effort to characterize heparan sulfate proteoglycan constituents of the matrix that OLGs assemble during a regenerative process [3], we screened a cDNA library with oligonucleotide probes modeled after heparan sulfate proteoglycans important for the biology of other cell types. One of the isolated cDNAs (3500 nts) hybridized specifically to a probe designed for perlecan, a heparan sulfate proteoglycan present in basal lam-

ina and required for peripheral nerve myelination [4]. The sequence of the isolated cDNA proved to be novel, and was unrelated to perlecan except for the short segment corresponding to the probe. We called the gene *novocan* to stress the fact that it is new (*novo*) and *can*—a suffix for proteoglycans—because the sequence contains glycosaminoglycan-binding sites. We refer to the expressed protein as NOVOcan. Southern blot analysis of rat genomic DNA, utilizing a 3 kb *novocan* cDNA as a probe, indicated that *novocan* is a single copy gene. Northern blot analysis of mRNAs extracted from ovine OLGs and rat brains with fragments of *novocan* cDNA revealed at least three, possibly four, bands most likely representing alternatively spliced forms of the *novocan* mRNA. *Novocan* was expressed maximally in early postnatal murine brains. Among multiple rat tissues examined, brain showed the highest level of *novocan* expression, but small amounts of *novocan* mRNA were present in heart and muscle [5]. The predicted amino acid sequence of the open reading frame exposed domains that, apart from sites for *N*-glycosylation, glycosaminoglycan attachment and phosphorylation, are evolutionarily conserved from *Drosophila* to mammals. The conserved motifs, known as Broad-Complex, Tramtrack and Bric à brac (BTB), but also referred to as poxvirus and zinc fingers (POZ), are associated with a variety of processes, including pattern formation, eye and limb development and chromosomal translocation. They act by mediating homodimerization or heterophilic interactions that are gene-specific. We have generated a panel of monoclonal antibodies (mAbs) against a 28-amino acid synthetic peptide chosen from the predicted NOVOcan sequence (Section 3) and used them to investigate the cell and tissue localization of this protein in rat brains and cultured ovine OLGs. In rat brains, NOVOcan was detected in radial glial cells, oligodendrocyte progenitors (OLPs), ependymal cells and tanycytes. Whereas the expression of NOVOcan was transient and disappeared with development in radial glia and OLPs; it persisted in ependymal cells and tanycytes into adulthood.

2.2. Radial glial cells

Radial glial cells have somata that line the ventricles and processes that are perpendicular to the lumen and form a dense network reaching the surface of the brain. The processes become longer and translocate as the thickness and surface area of the developing brain increases [6,7]. Due to their particular morphology, radial glial cells were traditionally thought to serve mainly as guides for neurons migrating out of the VZ, their birthplace [8]. Radial glia appear as early as embryonic day 9 (E9), and thus are placed in space and time for this function as neurons are generated shortly thereafter [9]. Radial glia are also positioned to give rise to neurons and have been known for some time to be clonally related to neurons; in general, these clones contain a single radial glia that guides the migration of its clonal relatives [10–12]. Recently, radial glia were shown to generate neurons in vitro if isolated during the period of neurogenesis and astrocytes if isolated later during gliogenesis [13]. This has been confirmed with real-time imaging of radial glia dividing to give rise to neurons [14]. Radial glia continue to divide throughout neurogenesis undergoing first symmetrical (vertical cleavage plane) and then asymmetrical (horizontal cleavage plane) cell division [15]. This pattern has also been observed in ‘neuroepithelial VZ cells, which have been accepted as the primary neurogenic cells of the CNS for decades. In fact, it has been proposed that radial glia, in addition to serving as substrates for neuronal migration, are the primary neurogenic cells in the brain [16].

Radial glial cells are molecularly heterogeneous in different regions and phases of the developing CNS [17], and this may be the reason for the pronounced regional and temporal differences in cell fate [18,19]. Radial glia have been shown to give rise to several classes of neurons, reviewed in [17,20], OLGs [19], astrocytes [7,21] and subsequently astrocyte-like adult neuronal stem cells [22,23], specialized adult radial glial cells such as Bergmann glia [24] and, finally, ependymal cells.

2.3. Oligodendrocyte progenitors

OLGs are morphologically and phenotypically heterogeneous cells found throughout the CNS. Their most celebrated function is that of investing axons with myelin that facilitates fast neuronal conduction. Failure in myelin assembly leads to crippling diseases such as multiple sclerosis. However, it was recognized long ago [25] that OLGs partake in other capacities such as acting as satellites for neuronal somata (perineuronal OLGs) or enveloping blood vessels (perivascular OLGs). The function of these non-myelinating OLGs is not known. While little progress has been made in understanding the OLG functional heterogeneity, there have been significant advances in establishing OLG lineage and their progression from a progenitor to a differentiated cell [26,27]. The identification of fate-determining genes has been instrumental in delineating lineage relationships [28]. It is postulated that OLPs arise from neuroepithelial cells in the ventral embryonic VZ as a result of local growth factor and transcription factor expression and then migrate into presumptive white matter tracts where they are exposed to positive and negative influences of axons and other cellular elements that reside therein. In turn, OLGs exert an influence on axonal maturation. As a result of this cross-talk, OLGs differentiate and ensheath axons with myelin. Another wave of OLPs originates from the subventricular zone (SVZ) in the brain [29]. These OLPs seem to have different developmental timing and lineages [30]. Thus, there is evidence indicating that there may be more than one population of OLPs, and that they may differ in the mechanism by which they become specified [26,27, and references therein].

2.4. Ependymal cells

Ependymal cells are similar to epithelial cells in that they form a single layer of regularly spaced squamous to cuboidal cells surrounding the ventricles in the adult brain [31]. Unlike epithelial cells, adult mammalian ependymal cells do not divide. Ependymal cells are morphologically and functionally heterogeneous cells, and include tanycytes and choroid plexus epithelial cells [32]. The latter cells from the lateral, third and fourth ventricles generate the cerebrospinal fluid (CSF). Ependymal cells have cilia and also microvilli in varying numbers [32]; coordinated beating of the cilia on the apical surface of ependymal cells helps to move the CSF through the ventricular system [33]. In addition to forming a CSF/brain barrier, ependymal cells actively regulate the flow of water, ions and small molecules between the brain parenchyma and the CSF [34]. Ependymal cells express the glial markers vimentin, glial fibrillary acidic protein (GFAP) and the secretory protein S-100, but there is molecular heterogeneity amongst developing and adult ependymal cells [35]. It has generally been assumed that ependymal cells are derived from VZ cells [2]. Given that approximately 90% of the VZ cells may actually be radial glia [16], ependymal cells may well be derived from radial glia either via differentiation or division.

2.5. Tanycytes

Tanycytes are a relatively unknown special class of ependymal cells that are ubiquitous in non-mammalian vertebrates, and are primarily found surrounding the third ventricle in adult mammalian brains. Tanycyte morphology is reminiscent of radial glia cells in that they also have long peripheral processes that connect the ependyma wall with the glia limitans, wrap around blood vessels or terminate at neurons or glia, and they express GFAP and vimentin [36–38]. The morphology of tanycytes, therefore, suggests a guiding function for migrating neurons, similar to radial glia. This function is observed particularly with neurons that express the gonadotropin releasing hormone (GnRH or LHRH), which have been shown to follow tanycyte processes quite closely [39]. Tanycytes in the mediobasal hypothalamus may also participate in the repair of lesioned monoaminergic neurons by promoting sprouting along their processes through an unknown mechanism [40]. Evidence for a lineage relationship between radial glia, tanycytes and common adult ependymal cells is found in the human lateral ventricle, where GFAP-positive radial glial cells are shown to develop into tanycytes with GFAP-positive basal processes that gradually lose their immunoreactiv-

ity as they, in turn, mature into common ependymal cells following their acquisition of cilia and loss of basal processes [41–43].

Several lines of evidence indicate that the four cell types mentioned above may originate from a multipotential radial glial cell, even though the details of proliferation and differentiation leading to these different fates have yet to be defined. Here we describe a putative keratan sulfate proteoglycan, NOVOcan, originally isolated from OLGs, that is also found in these other cell types. Significantly, this protein is regulated in a cell-specific fashion. Given that NOVOcan might constitute a family of proteins, the possibility must be entertained that the different molecular entities are not equally represented in each cell type. Nevertheless, the finding of a novel molecular link (NOVOcan) among these cell types with diverse phenotypes should open up new avenues to address issues concerning cellular interrelationships and the role of NOVOcan in these and other events.

3. Experimental

3.1. Antibodies and immunohistochemistry

A panel of anti-NOVOcan mAbs was generated of which two were used here, P_2C_6 and P_2G_7, at concentrations ranging from 1:200 to 1:400. Both mAbs were mouse IgM. The mAbs are directed against a 28 amino acid synthetic peptide modeled after the predicted sequence of NOVOcan. This segment was selected because of its high antigenicity, surface exposure and no-cross-reactivity with entries in databases. The mAbs were custom made at the Immunological Resource Center, University of Illinois at Champaign-Urbana. The specificity and titer of these mAbs were determined by ELISA. Rabbit polyclonal anti-GFAP was purchased from Sigma (St. Louis, MO) and used at a concentration of 1:1000. Rabbit polyclonal anti-vimentin was obtained from Sigma and used at 1:500. Secondary Abs were goat–anti-mouse IgM-Cy3-conjugated or goat–anti-rabbit IgG–FITC-conjugated (Pierce, Rockford, IL) at 1:200. Sections of 5–15 μm thicknesses were cut in the coronal plane from 3- to 60-day-old rat brains (purchased fast frozen from Pel Freeze, Rogers, AR) with a cryostat ($N>4$, all ages), placed on a slide and stored at −70 °C until further use. Prior to staining, sections were fixed in a 4% paraformaldehyde solution for 30 min and washed twice for 2 min each in Tris buffered saline (TBS; 150 mM NaCl+50 mM Tris–HCl pH 7.2). Non-specific binding of Ab was blocked with 10% normal goat serum (NGS) in TBS for 1 h at room temperature (slides were kept in a humidifying chamber throughout). After removing the blocking solution, the primary Ab in TBS containing 10% NGS was applied and left overnight at 4 °C. The slides were then washed 3×5 min each with TBS, and the secondary Ab conjugated to fluorescent probes was applied for 1 h at room temperature and washed with TBS 3×5 min. Nuclei were marked with a 1:50 solution of 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) in TBS for 30 s to 5 min. DAPI was removed with a 3 min wash with TBS. A single drop per slide of Prolong Antifade mounting medium (Molecular Probes, Eugene, OR) was added and slides were coverslipped. Images were acquired using either confocal (Olympus coupled to Fluoview) or epifluorescent microscopy (Leica coupled to Openlab). For control experiments the primary Abs were omitted.

3.2. Western analysis with anti-NOVOcan monoclonal antibodies

Frozen brain tissue was immersed directly in sample buffer and disrupted with an Ultra-Turrax T8 IKA® tissue disrupter (Works, Inc., Wilmington, NC), heated to 70 °C for 10 min, centrifuged to remove insoluble material, resolved on an SDS-PAGE gradient gel of 3.5–12%, and transferred to Immobilon™ PVDF membranes according to the vendor's instructions (Invitrogen, Carlsbad, CA). Membranes were exposed to one of the mAb (P_2C_6 or P_2G_7 at 1:200) overnight at 4 °C. Detection was done with a peroxidase conjugated anti-rabbit IgM (1:20 000) in conjunction with the SuperSignal® West Pico chemiluminescent substrate (Pierce, Rockford, IL). As controls, the membranes were stripped and reprobed using secondary Ab only.

3.3. Oligodendrocyte cultures

OLGs were isolated from 3- to 6-month-old lamb brains and cultured as previously described [44].

4. Results

We examined the expression of NOVOcan in rat brain extracts as a function of development (E17, postnatal (P)5 P20, P180) using Western blots. Three protein bands ($M_r \times 10^{-3}$ of 400, 240 and 200) were detected that had peak expression between P5 and P8 (Fig. 2) followed by a decline, such that by P180, the protein was barely visible (not illustrated). Because of this timing we examined the cellular expression of NOVOcan in the brains of postnatal rats at 3, 6, 9, 16 and 60 days after birth using immunohistochemistry. We also chose these times because major developmental changes occur during this period: transformation of radial glia, production of OLGs, disappearance of VZ cells and appearance of ependymal cells. To identify the cell types that synthesize NOVOcan, we performed double immunofluorescence on rat brain sections at different ages employing anti-NOVOcan mAbs in conjunction with recognized markers for OLGs, ependymal cells, radial glia and astrocytes. Morphology was utilized as a secondary factor in cell identification. We concentrated our analysis in three anterior posterior regions: the forebrain at mid striatal levels and at the level of the fimbria and the cerebellum. We found specific NOVOcan staining as evidenced by the lack of immunofluorescence in the absence of the primary antibody.

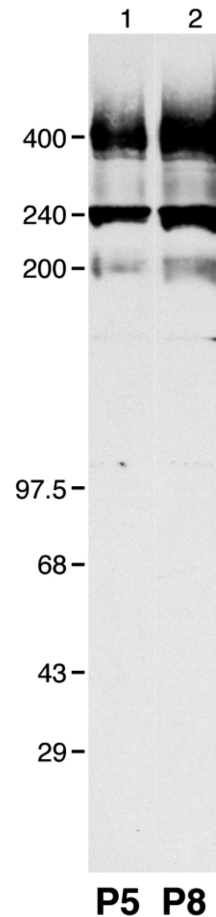

Fig. 2. Western blots. Staining of rat brain proteins with mAb P_2G_7. Lanes: 1. P5; 2. P8. By P20 there was a noticeable decline in the amount of protein detected. Notice that aside from the three bands, no other components were recognized by the mAb.

4.1. NOVOcan is expressed by postnatal radial glial cells

Although radial glial cells begin to retract their processes and transform into astrocytes by birth, many are still evident postnatally. In some species radial glia express the intermediate filament protein vimentin early during development but gradually replace it by switching to GFAP [45], which is the major intermediate filament and a widely accepted marker of astrocytes, further strengthening the notion of a lineal relationship between these cells [46] (Fig. 3B). In order to determine whether NOVOcan is expressed in late radial glia and also to examine its potential persistence in young astrocytes, we performed single and double immunofluorescence with NOVOcan and GFAP antibodies. We found NOVOcan to be present in radial glia-like processes in the brain areas examined (Fig. 3). In the walls of the anterior horn of the lateral ventricle radial glial cells are still present through day 7 [23,47]. Indeed, we observed positive

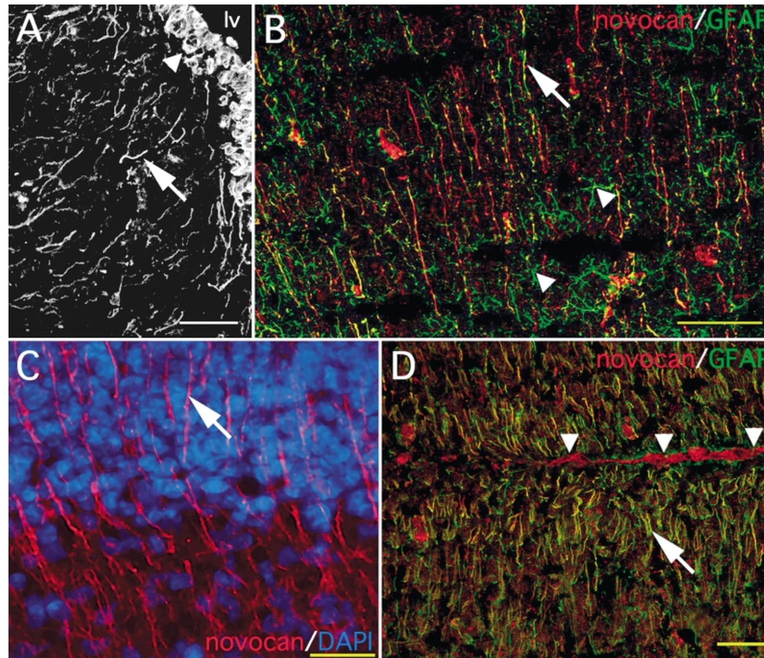

Fig. 3. NOVOcan is found in radial glial cells in different subregions of the CNS. (A) NOVOcan expression in radial glia cell bodies in the SVZ (arrowhead) and lining the lateral ventricle (lv) in the forebrain. NOVOcan was also detected in radial glia processes (arrow) emanating into adjacent nuclei such as the striatum in a 3-day postnatal rat. (B) NOVOcan immunofluorescence (red) in the cerebral cortex of a 9-day postnatal rat was often found in radial glia processes labeled with an anti-GFAP (green) antibody (arrow). GFAP marks astrocytes (arrowheads). (C) NOVOcan (red) is observed in specialized radial glia of the cerebellum, Bergmann glia, (arrow) that are interspersed amongst the dense granule cells (blue) migrating ventrally from the cerebellar surface; 16-day postnatal rat. (D) NOVOcan is co-expressed with GFAP in almost all Bergmann glia of 16-day postnatal rats. NOVOcan is also found in cells in the fissures between folia of the cerebellum (arrowheads). Scale bars: $A=50$ μm, $B=100$ μm, $C=10$ μm, $D=25$ μm.

NOVOcan staining in cell bodies lining the ventricles and in the processes emanating from them into the adjacent striatum and septum (Fig. 3A). This staining continued to be seen up to day 16 (Fig. 3A–C). NOVOcan detection in these processes was frequently discontinuous, in agreement with previous immunohistochemistry findings for markers of radial glia in this area [47]. The discontinuous appearance of NOVOcan could also be due to a combination of factors such as the thickness of sections and fragmentation of radial glia processes as they transform into astrocytes. With postnatal development radial glia processes become shorter and less dense. Co-labeling with GFAP confirmed that many (Fig. 3B and C), but not all (Fig. 3B), NOVOcan$^+$ radial glia also contained this intermediate filament protein at the level of the striatum and in the cerebral cortex (Fig. 3B). NOVOcan$^+$ radial glial cells were also found emanating from the intermediate bridge (posterior to the anterior horn) of the lateral ventricle at the level of the fimbria (not shown). In the cerebellum, we found NOVOcan$^+$ Bergmann glia, the specialized radial glia of the cerebellum [9,24]. Fig. 3C shows NOVOcan$^+$ Bergmann glia processes dispersed among the dense DAPI labeled external granule layer. Fig. 3D confirms that similar to the forebrain regions examined there was good overlap (yellow) between NOVOcan (red) and GFAP (green) immunostaining of Bergmann glia in the cerebellar cortex.

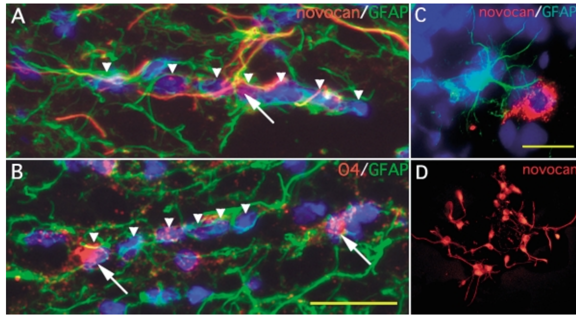

Fig. 4. NOVOcan is expressed in OLG precursors (OLPs) in the brain and in vitro. (A) and (B) are adjacent sections from a 9-day-old rat processed for NOVOcan (red) and GFAP (green) shown in the top panel, and for O_4 (red) and GFAP (green) shown in the bottom panel. Double immunohistochemistry for NOVOcan and O_4 could not be performed as both antibodies were raised in mouse cells. Note that the DAPI counterstain (blue) shows rows of horizontally aligned cells in the fimbria, a white matter tract. Cells in these rows expressed both NOVOcan (A) and O_4 (B) but did not co-express GFAP. (C) Two cells in white matter from a 9-day-old rat: one only expresses GFAP, one only NOVOcan. (D) NOVOcan was also found in cultured OLGs. Illustrated here is a 15-day-old culture; notice that cell somata and processes are immunostained. Scale bars: *A* and *B*=25 μm, *C*=10 μm.

4.2. NOVOcan is not expressed by astrocytes or neurons

We found that GFAP$^+$ cells with the morphology of adult astrocytes were not recognized by anti-NOVOcan mAbs, both in gray and white matter, in all sub-regions and ages examined. For example, we were able to detect typical GFAP$^+$ astrocytes in the cerebral cortex (gray matter) 9 days after birth, but these cells were not labeled by the anti-NOVOcan mAb (arrowheads in Fig. 3B). In the adult, GFAP$^+$ astrocytes were found in the striatum and septum, but they did not exhibit NOVOcan immunofluorescence (green cells in Fig. 5D). Similarly, GFAP$^+$ astrocytes in white matter did not express NOVOcan (Fig. 4A and C). Given that astrocytes are heterogeneous cells, the possibility that some subtypes in CNS regions not investigated might carry NOVOcan cannot be excluded.

We also sought to determine if NOVOcan was synthesized by neurons, which are amongst the first cells to be born in the brain. One could argue that the NOVOcan expression seen in the cerebral cortex postnatally corresponds to neuronal dendrites, but we do not favor this interpretation because many of these processes co-expressed GFAP, which is not found in neurons (Fig. 3B). Mature neurons are easy to identify morphologically by their typical dendrites and axons [48]. We never saw NOVOcan$^+$ cells with the morphology of neurons in the locations and time points exam-

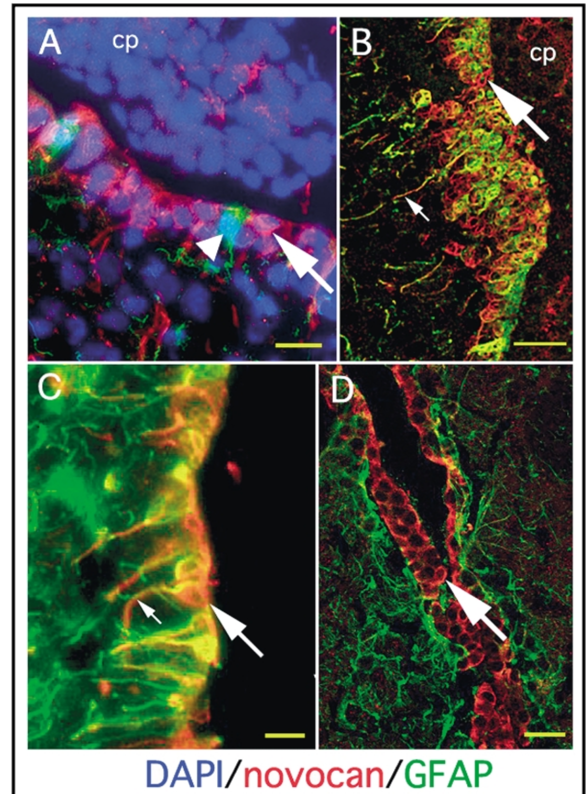

Fig. 5. NOVOcan is expressed in ependymal cells. All panels are from the lateral ventricle (lv) of 6-, 9-, 16- and 60-day-old rats, respectively (A–D). DAPI counterstain (blue), NOVOcan immunofluorescence (red), GFAP immunofluorescence (green). (A) NOVOcan-positive cells (red immunofluorescence, ex. arrow) line the lateral ventricles. (B–D) NOVOcan is present in ependymal cells (arrows) at 9–60 days. Note that NOVOcan is also weakly expressed by choroid plexus (cp) cells. NOVOcan is expressed in radial glia cell-like processes (small arrows) from 9 to 16 days, but is restricted to ependymal cells at 60 days (D). Scale bars: *A* and *C*=10 μm, *B* and *D*=25 μm.

ined. It is possible, however, that very young neurons without adult morphology express NOVOcan transiently or that other fixation methodologies will reveal neuronal NOVOcan immunoreactivity.

4.3. NOVOcan is present in OLPs but not in mature cells

We have previously shown that when OLGs are severed from their connection to myelin, purified and placed in culture on their own, they will survive and, if plated on a selected substratum—GRASP [49]—they will re-initiate their myelinogenic program [50]. *Novocan* is one of the many genes activated by this adhesion event, which we presume mimics an OLG neuron contact. Indeed, 24 h after adhesion to a substratum, cultured OLGs contain NOVOcan (not illustrated). The intensity of staining is maintained after the cells have been in vitro for 15 days, at which time they have extended numerous processes, which are also $NOVOcan^+$ (Fig. 4D).

Having determined that neither neurons nor astrocytes carry NOVOcan, we focused on white matter tracts, specifically the fimbria, because a detailed description of the development of OLGs in this tract is available [51,52]. When a 9-day-old rat fimbria was exposed to mAb P_2C_6 (NOVOcan) and anti-GFAP (radial glia and/or astrocytes), the pattern illustrated in Fig. 4A and B was obtained. We saw scattered DAPI-stained nuclei as well as nuclei organized in unicellular rows (arrowheads in Fig. 4A and B)—a feature characteristic of mature white matter tracts. $GFAP^+$ immunoreactivity revealed a few astrocytes (not shown). Striking elements were the $GFAP^+$ processes organized in an almost parallel pattern occupying the full width of the fimbria. These processes arose, most likely, from radial glial cells and a few of them co-expressed NOVOcan (Fig. 4A). This arrangement corresponds closely to what one would expect at this time of development [51]. To confirm that these cells are OLGs, we used the mAb O_4 [53], a recognized and specific marker for OLGs at early stages of differentiation. Because O_4 and P_2C_6 are both mAbs, the staining had to be done on sequential sections rather than on the same section. It is clear that the two Abs recognize the same type of cell (Fig. 4A and B). We examined another white matter tract, corpus callosum, at 9 days, employing a different anti-NOVOcan mAb, P_2G_7, together with anti-GFAP. The specific localization of NOVOcan in OLGs and not in astrocytes was evident (Fig. 4C). Additionally, we performed double immunofluorescence with anti-NOVOcan and markers of mature OLGs (e.g. myelin basic protein) in tissues older than 9 days and never found co-localization. These observations indicated that mature OLGs no longer have NOVOcan or that the level is below detection by the method utilized.

4.4. Adult ependymal cells express NOVOcan

In the walls of the lateral ventricles of rodents, radial glia are gradually replaced by ependymal cells during the first two postnatal weeks. It has recently been suggested that the two cell types may be lineally related [23,47]. We tested for NOVOcan immunostaining and found it to be positive in cells immediately lining the lateral ventricles at all ages probed (large arrows in Fig. 5A–D). Some of these cells had processes (Fig. 5A–C; small arrows) spreading into the striatum or septum, attesting to their radial glia nature. At early time points, NOVOcan was detected in cells found several cell diameters from the lateral ventricle (Fig. 5B), suggesting that it is synthesized by cells in the SVZ—an area of major neurogenesis in the adult. The identification of the $NOVOcan^+$ cells in the SVZ is not straightforward, but the fact that some of them expressed GFAP (Fig. 5B) may be taken to indicate that they are radial glia transforming into astrocytes [23,47]. GFAP/NOVOcan double immunofluorescence also revealed some cells adjacent to the lateral ventricle (arrowhead in Fig. 5A, large arrow in Fig. 5B), which did not co-localize NOVOcan and GFAP. This may be due to the heterogeneity of cells in the nascent VZ/SVZ; the two $GFAP^+$/$NOVOcan^-$ cells seen in Fig. 5A may correspond to the specialized astrocytes contacting the ventricles [23,54,55]. At postnatal days 16 and 60, NOVOcan immunoreactivity became restricted to

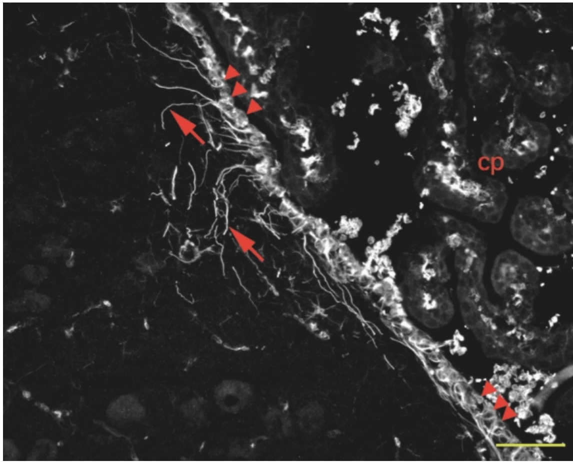

Fig. 6. NOVOcan expression in tanycytes of the fourth ventricle at 16-day postnatal. Tanycytes exhibit NOVOcan staining in cell bodies (arrowheads) as well as in their processes (arrows). NOVOcan immunoreactivity is also seen in some cells of choroid plexus (cp) of the fourth ventricle. Scale bar = 50 μm.

the innermost layer, which corresponds to the actual ependymal cells (Fig. 5C and D). Thus, NOVOcan was not seen in adult SVZ astrocytes, in agreement with our finding that astrocytes do not have NOVOcan (see above). In contrast, ependymal cells were positive for NOVOcan at day 60 in all regions investigated. Note that in Fig. 5D the left ependymal layer has been cut obliquely making the single cell layer appear multicellular. To confirm that the NOVOcan^{+} in the ventricles were ependymal cells, we double immunostained with P_2C_6 and vimentin—an intermediate filament found in ependymal cells—there was clear co-localization (not shown).

4.5. Tanycytes and choroid plexus cells contain NOVOcan

Tanycytes are specialized ependymal cells with a morphology reminiscent of radial glia; they may have multiple functions, but as of yet these are unclear [36,37]. Choroid plexus cells are special-

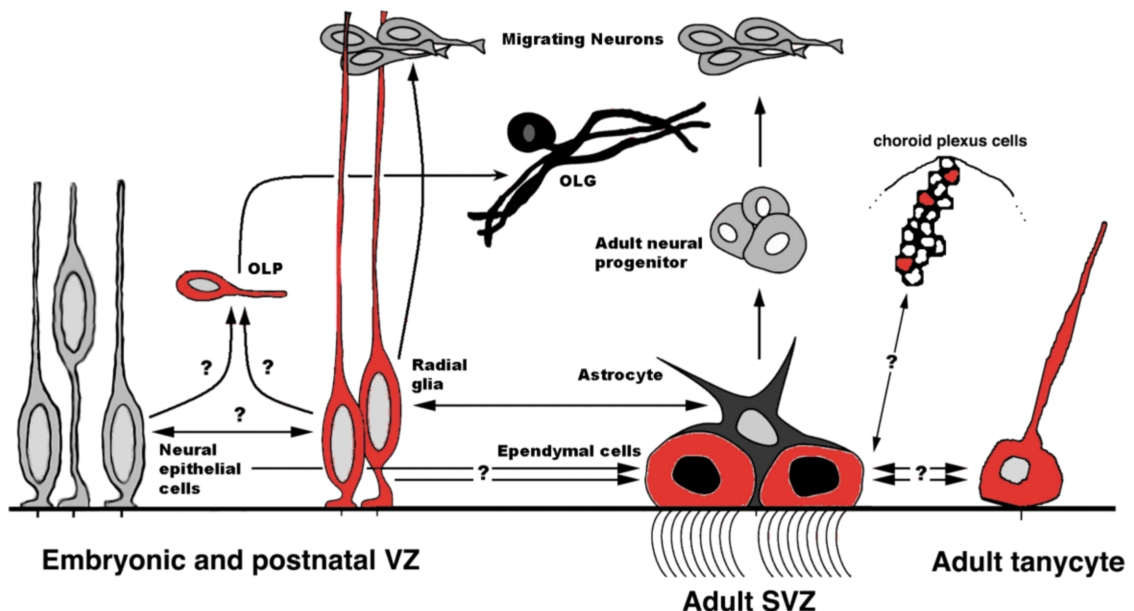

Fig. 7. Schematic (adapted from Tramontin et al. [23]) illustrating cells that exhibit NOVOcan immunoreactivity (red) in postnatal development and adult neural cells with known lineage relationships (arrows) and potential lineage relationships (arrows with question marks). The origin of OLG precursors, ependymal cells, tanycytes and choroid plexus cells is unclear. They may be derived from VZ cells, radial glia or one another. Choroid plexus cells expressed NOVOcan weakly, and we do not know which embryonic cells contain it.

ized ependymal cells that hang in the ventricular system and make CSF [56]. Not surprisingly, tanycytes and their processes of the third and fourth ventricles were immunolabelled by the NOVOcan mAbs (Fig. 6). In contrast to the strong labelling of tanycytes, choroid plexus cells were weakly and unevenly stained with anti-NOVOcan (Figs. 5 and 6).

5. Discussion

We describe here a novel protein, NOVOcan, and show it to be manifested in specific subsets of cells in the late developing rat brain. NOVOcan was recognized with specific mAbs in radial glia, OLPs, ependymal cells, tanycytes and, possibly, choroid plexus cells. The time course and locations of NOVOcan expression in radial glia and ependymal cells in the lateral ventricle suggest that these cells may be lineally related [57]. Fig. 7 is a cartoon (adapted from Tramontin et al. [23]) depicting cells that exhibited NOVOcan immunostaining (red) during postnatal development and in adulthood. This cartoon embodies the current notion [23] that postnatal radial glia in the walls of the lateral ventricles give rise to neurons and the neurogenic astrocytes of the SVZ and, possibly, even ependymal cells. Lineage analysis with retroviral vectors combined with NOVOcan immunohistochemistry may be a useful approach to confirm the hypothesis that radial glial cells and various ependymal cells are clonally related. As depicted in Fig. 7, the traditional view that ependymal cells in the lateral ventricles are daughters of VZ cells, i.e., neuroepithelial precursors lacking processes that reach the pial surface, is still viable [2,38]. Ependymal cells are heterogeneous, but the presence of NOVOcan in all ependymal cells, in the areas examined, suggests that it is a feature of all subtypes and we propose that NOVOcan may be used as a pan-ependymal marker.

The presence of NOVOcan in radial glia is especially interesting because radial glia are neurogenic [13,14,58,59]. Unlike the suggested radial glia to ependymal cell progression, which is characterized by the continued presence of NOVOcan, two other major cell types, which derive from radial glia, neurons and astrocytes, do not synthesize NOVOcan. Since we never detected NOVOcan in adult neurons, it seems likely that during asymmetric divisions of radial glia, *novocan* mRNA is only inherited by the radial glial cell and not by the neuronal daughter cell. The possibility remains, however, that newborn neurons may make NOVOcan transiently. Hence, co-expression studies of NOVOcan with class III β-tubulin, which is prominent in young neurons, should resolve this issue. We have demonstrated that NOVOcan is present in radial glia towards the end of development, but it is essential to determine how early it is expressed in these cells. More neuronal cells are produced early in development than any other cell type. Accordingly, radial glia produce a larger ratio of neurons to glia if isolated from early embryonic brains than if isolated from late embryonic brains [13]. Thus, if the absence of NOVOcan from young neurons is confirmed, it may indicate that NOVOcan is only found in radial glia during their gliogenic phase. Alternatively, asymmetric mRNA distribution may be at play. Experiments to find out the embryonic cellular expression of NOVOcan will help to resolve this issue and may also shed light on the role, if any, of NOVOcan in neurogenesis.

Radial glia also give rise to astrocytes in the cerebral cortex [21,60] and possibly to the astrocyte-like stem cells in the SVZ [23]. Not unlike adult neurons, astrocytes are NOVOcan negative. In other words, this may be another example of an asymmetric mRNA distribution during radial glia to astrocyte transition. However, it remains to be proven whether NOVOcan is not transcribed or translated in adult neurons or astrocytes or whether it is merely below the level of detection. It would be of interest to establish whether injuries that cause reactive astrocytosis would result in gene transcription and/or activation of NOVOcan translation. Such a scenario can be inferred from the report that adult astrocytes when exposed in vitro to embryonic cell conditioned medium [61], or in vivo to injuries [62,63], can exhibit radial glia-like morphologies and molecules.

The finding of NOVOcan in cells of the OLG lineage in the fimbria—a white matter tract—at the time when cells in adjacent sections expressed O_4—a marker for OLGs at the initial stages of

differentiation—demonstrates that NOVOcan marks immature OLGs (i.e. OLPs) but not the mature cell. This was confirmed by the failure of co-staining with myelin basic protein. It is interesting that, in vitro, when adult OLGs regenerate and re-enact their myelinogenic program [50], they also re-express NOVOcan. Indeed, we could not detect NOVOcan in ovine brain slices taken from the same tissue that was used for OLG isolation (not shown), confirming that its transcription/translation was induced in vitro upon regeneration. The birthplace of OLGs in the developing nervous system has recently been defined and is in general confined to ventral subdivisions from which cells migrate to white matter tracts [27,28]. However, it may well be that there are separate lineages of OLGs [26]. It will be important to examine NOVOcan immunoreactivity in other CNS areas such as the spinal cord where the genesis of OLGs has been well described. Collectively these data indicate that NOVOcan is present in OLPs and OLGs in their early stages of differentiation, but as the program of myelination gets underway they downregulate NOVOcan. We speculate that NOVOcan—a putative proteoglycan—may well play a role in the initial OLG–neuron cross-talk.

What might the functional role of NOVOcan be? At this stage of our investigations we know very little about the function of NOVOcan and can merely speculate based on sequence analysis and domain comparison with well characterized proteins. NOVOcan is a glycoprotein, *N*- and, possibly, *O*-glycosylated; preliminary data also suggest that it might be a proteoglycan. Initial immunoprecipitation experiments indicate that the 400 kD protein (Fig. 2) may interact with other proteoglycans as part of a matrix. It is conceivable that the function of NOVOcan differs in each of the cell types in which it is found. The subcellular localization of NOVOcan may eventually lead to clues as to its function. With light and confocal microscopy, NOVOcan appears to be localized in bands around the nucleus, and/or in punctate aggregates that look like Golgi or endoplasmic reticulum, which suggests that NOVOcan may be a secreted protein. We further speculate that one of its functions is to bind metals; the row of 11 histidines predicted in the amino acid sequence of NOVOcan, of which 9 are strictly conserved across species, is ideally suited for such a function. Thus, in ependymal cells NOVOcan may clear the CSF of toxic metals.

In summary, we have identified a gene, *novocan*, and show here that its expressed protein, NOVOcan, is found in a number of cell types that might all be lineally related to radial glial cells. Unraveling the function of NOVOcan may provide clues to an understanding of the interrelationships among these cells.

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References

[1] M.B. Carpenter, J. Sutin, Human Neuroanatomy, Williams & Wilkins, Baltimore, 1983.

[2] C. Kintner, Neurogenesis in embryos and in adult neural stem cells, J. Neurosci. 22 (2002) 639–643.

[3] S. Szuchet, K. Watanabe, Y. Yamaguchi, Differentiation/regeneration of oligodendrocytes entails the assembly of a cell-associated matrix, Int. J. Dev. Neurosci. 18 (2000) 705–720.

[4] R.P. Bunge, M.B. Bunge, C.F. Eldridge, Linkage between axonal ensheathment and basal lamina production by Schwann cells, Annu. Rev. Neurosci. 9 (1986) 305–328.

[5] S. Szuchet, M.S. Domowicz, D. Arvanitis, W. Macklin, The NOVOcans: a family of developmentally regulated proteoglycans, Mol. Biol. Cell 11 (2000) 225.

[6] J.P. Misson, M.A. Edwards, M. Yamamoto, V.S. Caviness Jr., Mitotic cycling of radial glial cells of the fetal murine cerebral wall: a combined autoradiographic and immunohistochemical study, Brain Res. 466 (1988) 183–190.

[7] J.P. Misson, C.P. Austin, T. Takahashi, C.L. Cepko, V.S. Caviness Jr., The alignment of migrating neural cells in relation to the murine neopallial radial glial fiber system, Cereb. Cortex 1 (1991) 221–229.

[8] P. Rakic, Mode of cell migration to the superficial layers of fetal monkey neocortex, J. Comp. Neurol. 145 (1972) 61–83.

[9] J.P. Misson, M.A. Edwards, M. Yamamoto, V.S. Caviness Jr., Identification of radial glial cells within the developing murine central nervous system: studies based upon a new immunohistochemical marker, Brain Res. Dev. Brain Res. 44 (1988) 95–108.

[10] G.E. Gray, J.R. Sanes, Lineage of radial glia in the chicken optic tectum, Development 114 (1992) 271–283.

[11] A.L. Halliday, C.L. Cepko, Generation and migration of cells in the developing striatum, Neuron 9 (1992) 15–26.

[12] F.G. Szele, C.L. Cepko, The dispersion of clonally related cells in the developing chick telencephalon, Dev. Biol. 195 (1998) 100–113.

[13] P. Malatesta, E. Hartfuss, M. Gotz, Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage, Development 127 (2000) 5253–5263.

[14] S.C. Noctor, A.C. Flint, T.A. Weissman, R.S. Dammerman, A.R. Kriegstein, Neurons derived from radial glial cells establish radial units in neocortex, Nature 409 (2001) 714–720.

[15] Y. Kamei, N. Inagaki, M. Nishizawa, O. Tsutsumi, Y. Taketani, M. Inagaki, Visualization of mitotic radial glial lineage cells in the developing rat brain by Cdc2 kinase-phosphorylated vimentin, Glia 23 (1998) 191–199.

[16] S.C. Noctor, A.C. Flint, T.A. Weissman, W.S. Wong, B.K. Clinton, A.R. Kriegstein, Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia, J. Neurosci. 22 (2002) 3161–3173.

[17] A.R. Kriegstein, M. Gotz, Radial glia diversity: a matter of cell fate, Glia 43 (2003) 37–43.

[18] E. Hartfuss, R. Galli, N. Heins, M. Gotz, Characterization of CNS precursor subtypes and radial glia, Dev. Biol. 229 (2001) 15–30.

[19] P. Malatesta, M.A. Hack, E. Hartfuss, et al., Neuronal or glial progeny: regional differences in radial glia fate, Neuron 37 (2003) 751–764.

[20] G. Fishell, A.R. Kriegstein, Neurons from radial glia: the consequences of asymmetric inheritance, Curr. Opin. Neurobiol. 13 (2003) 34–41.

[21] R.S. Cameron, P. Rakic, Glial cell lineage in the cerebral cortex: a review and synthesis, Glia 4 (1991) 124–137.

[22] F. Doetsch, I. Caille, D.A. Lim, J.M. Garcia-Verdugo, A. Alvarez-Buylla, Subventricular zone astrocytes are neural stem cells in the adult mammalian brain, Cell 97 (1999) 703–716.

[23] A.D. Tramontin, J.M. Garcia-Verdugo, D.A. Lim, A. Alvarez-Buylla, Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment, Cereb. Cortex 13 (2003) 580–587.

[24] S. Yuasa, Bergmann glial development in the mouse cerebellum as revealed by tenascin expression, Anat. Embryol. (Berl) 194 (1996) 223–234.

[25] P.D. del Rio Hortega, Tercera apportacion al conocimiento morfologico e interpretacion funcional de la oligodendroglia, Mem. R. Soc. Esp. Hist. Nat. 14 (1928) 5–122.

[26] N. Spassky, C. Olivier, E. Perez-Villegas, et al., Single or multiple oligodendroglial lineages: a controversy, Glia 29 (2000) 143–148.

[27] R.H. Miller, Regulation of oligodendrocyte development in the vertebrate CNS, Prog. Neurobiol. 67 (2002) 451–467.

[28] R.H. Woodruff, N. Tekki-Kessaris, C.D. Stiles, D.H. Rowitch, W.D. Richardson, Oligodendrocyte development in the spinal cord and telencephalon: common themes and new perspectives, Int. J. Dev. Neurosci. 19 (2001) 379–385.

[29] S.W. Levison, J.E. Goldman, Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain, Neuron 10 (1993) 201–212.

[30] S.O. Suzuki, J.E. Goldman, Multiple cell populations in the early postnatal subventricular zone take distinct migratory pathways: a dynamic study of glial and neuronal progenitor migration, J. Neurosci. 23 (2003) 4240–4250.

[31] J.E. Bruni, M.R. Del Bigio, R.E. Clattenburg, Ependyma: normal and pathological. A review of the literature, Brain Res. 356 (1985) 1–19.

[32] A. Mitro, M. Palkovits, Morphology of the rat brain ventricles, ependyma, and periventricular structures, Bibl. Anat. (1981) 1–110.

[33] W.H. Oldendorf, Cerebrospinal fluid formation and circulation, Prog. Nucl. Med. 1 (1972) 336–358.

[34] M.R. Del Bigio, The ependyma: a protective barrier between brain and cerebrospinal fluid, Glia 14 (1995) 1–13.

[35] H.B. Sarnat, Histochemistry and immunocytochemistry of the developing ependyma and choroid plexus, Microsc. Res. Tech. 41 (1998) 14–28.

[36] W. Wittkowski, Tanycytes and pituicytes: morphological and functional aspects of neuroglial interaction, Microsc. Res. Tech. 41 (1998) 29–42.

[37] J. Flament-Durand, J.P. Brion, Tanycytes: morphology and functions: a review, Int. Rev. Cytol. 96 (1985) 121–155.

[38] J.E. Bruni, Ependymal development, proliferation, and functions: a review, Microsc. Res. Tech. 41 (1998) 2–13.

[39] G.P. Kozlowski, P.W. Coates, Ependymoneuronal specializations between LHRH fibers and cells of the cerebroventricular system, Cell Tissue Res. 242 (1985) 301–311.

[40] N. Chauvet, M. Prieto, G. Alonso, Tanycytes present in the adult rat mediobasal hypothalamus support the regeneration of monoaminergic axons, Exp. Neurol. 151 (1998) 1–13.

[41] S.J. Gould, S. Howard, An immunohistochemical study of the germinal layer in the late gestation human fetal brain, Neuropathol. Appl. Neurobiol. 13 (1987) 421–437.

[42] S.J. Gould, S. Howard, L. Papadaki, The development of ependyma in the human fetal brain: an immunohis-

tological and electron microscopic study, Brain Res. Dev. Brain Res. 55 (1990) 255–267.
[43] U. Roessmann, M.E. Velasco, S.D. Sindely, P. Gambetti, Glial fibrillary acidic protein (GFAP) in ependymal cells during development. An immunocytochemical study, Brain Res. 200 (1980) 13–21.
[44] S. Szuchet, K. Stefansson, R.L. Wollmann, G. Dawson, B.G.W. Arnason, Maintenance of isolated oligodendrocytes in long-term culture, Brain Res. 200 (1980) 151–164.
[45] D.E. Schmechel, P. Rakic, A Golgi study of radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes, Anat. Embryol. (Berl) 156 (1979) 115–152.
[46] A. Bignami, D. Dahl, Astrocyte-specific protein and radial glia in the cerebral cortex of newborn rat, Nature 252 (1974) 55–56.
[47] J.A. Alves, P. Barone, S. Engelender, M.M. Froes, J.R. Menezes, Initial stages of radial glia astrocytic transformation in the early postnatal anterior subventricular zone, J. Neurobiol. 52 (2002) 251–265.
[48] S. Ramon y Cajal, Histologie du systeme nerveux de l'homme & des vertebres (Consejo Superior de Investigaciones Cientâificas, Instituto Ramâon y Cajal, Madrid, 1953).
[49] E.C. Schirmer, J. Farooqui, P.E. Polak, S. Szuchet, GRASP: a novel heparin-binding serum glycoprotein that mediates oligodendrocyte–substratum adhesion, J. Neurosci. Res. 39 (1994) 457–473.
[50] S.H. Yim, S. Szuchet, P.E. Polak, Cultured oligodendrocytes. A role for cell–substratum interaction in phenotypic expression, J. Biol. Chem. 261 (1986) 11808–11815.
[51] M. Suzuki, G. Raisman, Multifocal pattern of postnatal development of the macroglial framework of the rat fimbria, Glia 12 (1994) 294–308.
[52] M. Suzuki, G. Raisman, The glial framework of central white matter tracts: segmented rows of contiguous interfascicular oligodendrocytes and solitary astrocytes give rise to a continuous meshwork of transverse and longitudinal processes in the adult rat fimbria, Glia 6 (1992) 222–235.
[53] I. Sommer, M. Schachner, Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system, Dev. Biol. 83 (1981) 311–327.
[54] F. Doetsch, L. Petreanu, I. Caille, J.M. Garcia-Verdugo, A. Alvarez-Buylla, EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells, Neuron 36 (2002) 1021–1034.
[55] F. Doetsch, J.M. Garcia-Verdugo, A. Alvarez-Buylla, Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain, J. Neurosci. 17 (1997) 5046–5061.
[56] T.H. Milhorat, Structure and function of the choroid plexus and other sites of cerebrospinal fluid formation, Int. Rev. Cytol. 47 (1976) 225–288.
[57] F. Szele, S. Szuchet, Cells lining the ventricular system: evolving concepts underlying developmental events in the embryo and adult, Adv. Mol. Cell Biol. 31 (2003) 127–147.
[58] T. Miyata, A. Kawaguchi, H. Okano, M. Ogawa, Asymmetric inheritance of radial glial fibers by cortical neurons, Neuron 31 (2001) 727–741.
[59] N. Tamamaki, K. Nakamura, K. Okamoto, T. Kaneko, Radial glia is a progenitor of neocortical neurons in the developing cerebral cortex, Neurosci. Res. 41 (2001) 51–60.
[60] J.P. Mission, T. Takahashi, V.S. Caviness Jr., Ontogeny of radial and other astroglial cells in murine cerebral cortex, Glia 4 (1991) 138–148.
[61] K.E. Hunter, M.E. Hatten, Radial glial cell transformation to astrocytes is bidirectional: regulation by a diffusible factor in embryonic forebrain, Proc. Natl. Acad. Sci. USA 92 (1995) 2061–2065.
[62] F.G. Szele, M.F. Chesselet, Cortical lesions induce an increase in cell number and PSA-NCAM expression in the subventricular zone of adult rats, J. Comp. Neurol. 368 (1996) 439–454.
[63] B.R. Leavitt, C.S. Hernit-Grant, J.D. Macklis, Mature astrocytes transform into transitional radial glia within adult mouse neocortex that supports directed migration of transplanted immature neurons, Exp. Neurol. 157 (1999) 43–57.
[64] J.P.J. Pinel, Biopsychology, Allyn and Bacon, Boston, 2000.