UNIONE EUROPEA
Fondo sociale europeo

REGIONE AUTONOMA DELLA SARDEGNA

FSE 2007-2013
POR SARDEGNA
obiettivo competitività regionale e occupazione

Università degli Studi di Cagliari

DOTTORATO DI RICERCA

Scienze e Tecnologie per l'Innovazione

Ciclo XXX

Immunohistochemical markers of stem/progenitor cells in the developing human cerebellum

Settore scientifico disciplinare di afferenza

MED/08

Presentata da:	Dott.ssa Valeria Pibiri
Coordinatore Dottorato	Prof. Roberto Orrù
Tutor	Prof. Gavino Faa
Co-tutor	Prof. Rossano Ambu

Esame finale anno accademico 2016 – 2017
Tesi discussa nella sessione d'esame febbraio – marzo 2018

Summary

Introduction

The cerebellum is a part of the central nervous system (CNS) very well conserved within the vertebrates. Several evidences from the neuroanatomy, electrophysiology and gene expression studies, as well as from knockout phenotypes, demonstrate that the cerebellum is a highly organized structure, with a remarkably somatotopy in the latero-lateral and rostro-caudal axes, necessary for its functional role in integration of sensory perception and the coordination of voluntary movements. For these reasons, neurodevelopment represents a very important moment of embryogenesis, being involved in a precisely orchestrated sequence of molecular and cellular events. A premature interruption of any of these events during human brain development may lead an imbalance between excitatory and inhibitory circuits and it is responsible of motor, cognitive and behavioral deficits in childhood. Although in the past decades some disturbances of the cerebral white matter have been considered at the base of these deficits, it is now well known that an additional and clinically important component of neuronal/axonal disturbances can be due to abnormalities in the development of the cerebellum.

Over the past decades, the study of stem cells has focused on their possible role in the development and treatment of several neurological disease including Parkinson's disease, Alzheimer's disease, epilepsy, autism spectrum disorders.

A new branch of medicine called "regenerative medicine" aimed to exploit the potentials of these stem cells in order to replace damaged tissues in the adult organism and to produce new ones.

The great majority of studies regarding brain development have been focused on animal models.

This work is aimed to study the human cerebellum development and to focus on the identification of stem/progenitor cell markers that may play a key role during human cerebellar neurogenesis. These molecular markers may represent possible targets of potential regenerative therapy during the first weeks of postnatal life.

Establishment and organization of the cerebellar anlage

During embryogenesis, at the end of the 4th week of gestation, the most anterior portion of the neural tube is undergoing drastic changes during early development, generating the three primary brain vesicles: the forebrain (prosencephalon), the midbrain (mesencephalon) and the hindbrain (rhombencephalon). Caudal neural tube maintains a cylindrical shape while generating the spinal cord. As development proceeds, during the 5th week of gestation, the prosencephalon is further subdivided into the telencephalon and diencephalon and the rhombencephalon into the metencephalon and myelencephalon for a total of five secondary brain vesicles (Fig. 1).

Each vesicle gives rise to specific structures in the adult brain and spinal cord:

- the telencephalon gives rise to the cerebral cortex;
- the diencephalon gives rise to the thalamus, epythalamus and hypothalamus;
- the mesencephalon gives rise to the midbrain;
- the metencephalon gives rise to the pons and cerebellum;
- the myelencephalon gives rise to the medulla oblongata and the spinal cord.

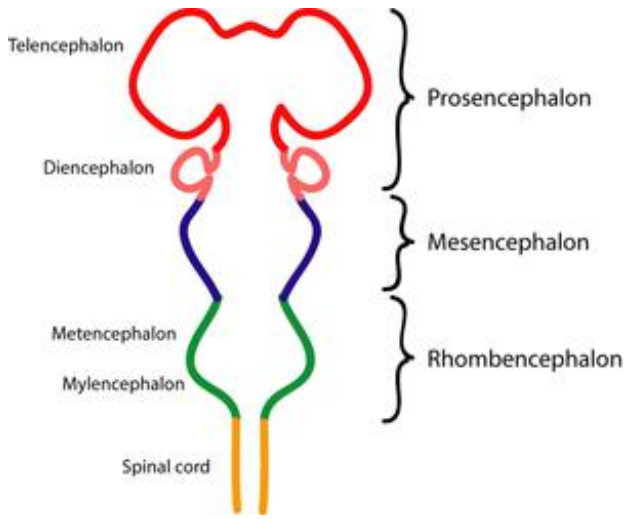

Figure 1. Human brain at the 5th week of gestation

The discovery that putative regulatory genes are expressed in regionally restricted patterns in the developing neural tube has provided new tools for defining histogenic domains and their boundaries at higher resolution. In the rhombencephalon, these regions are termed rhombomeres (r) that, from anterior to posterior, are known as r0 (the isthmus) and r1-r7, followed by the pseudorhombomeres r8-r11.

The mature cerebellar cortex

The cerebellum is located dorsally to the brainstem and inferiorly to the occipital lobes of the cerebral hemispheres; it has two major components: the cerebellar cortex and the cerebellar nuclei. In mammals, the cerebellar cortex consists of the vermis (or midline region) that is derived from the corpus cerebelli, as well as the flocculus, located laterally and derived from the auricles, and the hemispheres which are found only in mammals and birds [1,2]. The cerebellar cortex overlies the cerebellar nuclei. From medial to lateral, these are the fastigial, anterior interposed (or emboliform), posterior interposed (or globose) and lateral (or dentate) nuclei. The cerebellar nuclei, together with some vestibular nuclei, are the targets of the afferent projections from the cerebellar cortex [3,4].

The cerebellar lobes are pleated by an elaborately folded cortex; the lobes can be subdivided into ten rostro-caudal lobules (I-X). Lobes and lobules constitute the traditional way to describe cerebellar anatomy.

Superficially, the mature cerebellar cortex comprises a simple and uniform three-layered structure (Fig. 2). At the outside, beneath the pial surface, lies the *molecular layer*, which consists primarily of neurites, the dendritic trees of the Purkinje cells. In addition to Purkinje cell neurites, the molecular layer contains the cell bodies of some inhibitory interneurons, such as *basket cells* and *stellate cells*, that modulate Purkinje cell firing. The molecular layer is separated from the underlying granular layer by the *Purkinje cell layer*, a monolayer of large Purkinje cell somata. The *granular layer* consists of small and densely packed somata of granule cells, the most numerous neurons of the cerebellum.

The cerebellar cortex is organized around the Purkinje cells, discovered by the Czech anatomist Jan Evangelista Purkinje in 1837, and perhaps the first neurons to be recognized. Purkinje cells have elaborate and characteristics dendritic arbors in the molecular layer. The

dendrites consist of both smooth shafts and dendritic spines, each of them with distinct synaptic connections: climbing fibers and stellate interneurons terminate on the smooth dendritic shefts, whereas parallel fibers terminate on the dendritic spines [5].

The granule cells are the most numerous neurons of the cerebellum and outnumber Purkinje cells 1000-fold [6]. The somata of the granule cells are packed into the granular layer. Three to five short dendrites extends from the soma and terminate in synaptic glomeruli in the granular layer where they receive excitatory input from mossy terminal fibers and inhibitory input from Golgi cell axons. The granule cell axons extend into the molecular layer where they bifurcate and run mediolaterally as long parallel fibers that form glutamatergic synapses on the dendritic spines of Purkinje cells. In addition to granule cells, another class of excitatory interneurons has recently been recognized, the unipolar brush cells [7]. They receive excitatory input from mossy afferent fibers, which relay to multiple granule cells, thereby amplifying the effects of specific mossy fibers pathways. The cerebellar cortex contains several classes of inhibitory interneurons that include basket cells and stellate cells, confined to the molecular layer [8]. Basket cells are small multipolar interneurons located close to the Purkinje cell somata. Their dendrites extend through the molecular layer where they receive inputs from the parallel fibers. The basket cells axons envelop the Purkinje cell somata (the so-called “baskets”) and terminate on the Purkinje cell somata. Stellate cells are small, star-shaped interneurons that have their somata further away from the Purkinje cell layer. Like basket cells, stellate cells are also excitated by parallel fibers: they terminate on the Purkinje cell dendrites shafts. Two classes of inhibitory interneurons are located in the granular layer: *Golgi cells* and *Lugaro cells*. Golgi cell axons form GABAergic inhibitory synapses on granule cell dendrites. Lugaro cells have widespread connections that span many Purkinje cells and seem to play a prominent role in the integration of cerebellar activity.

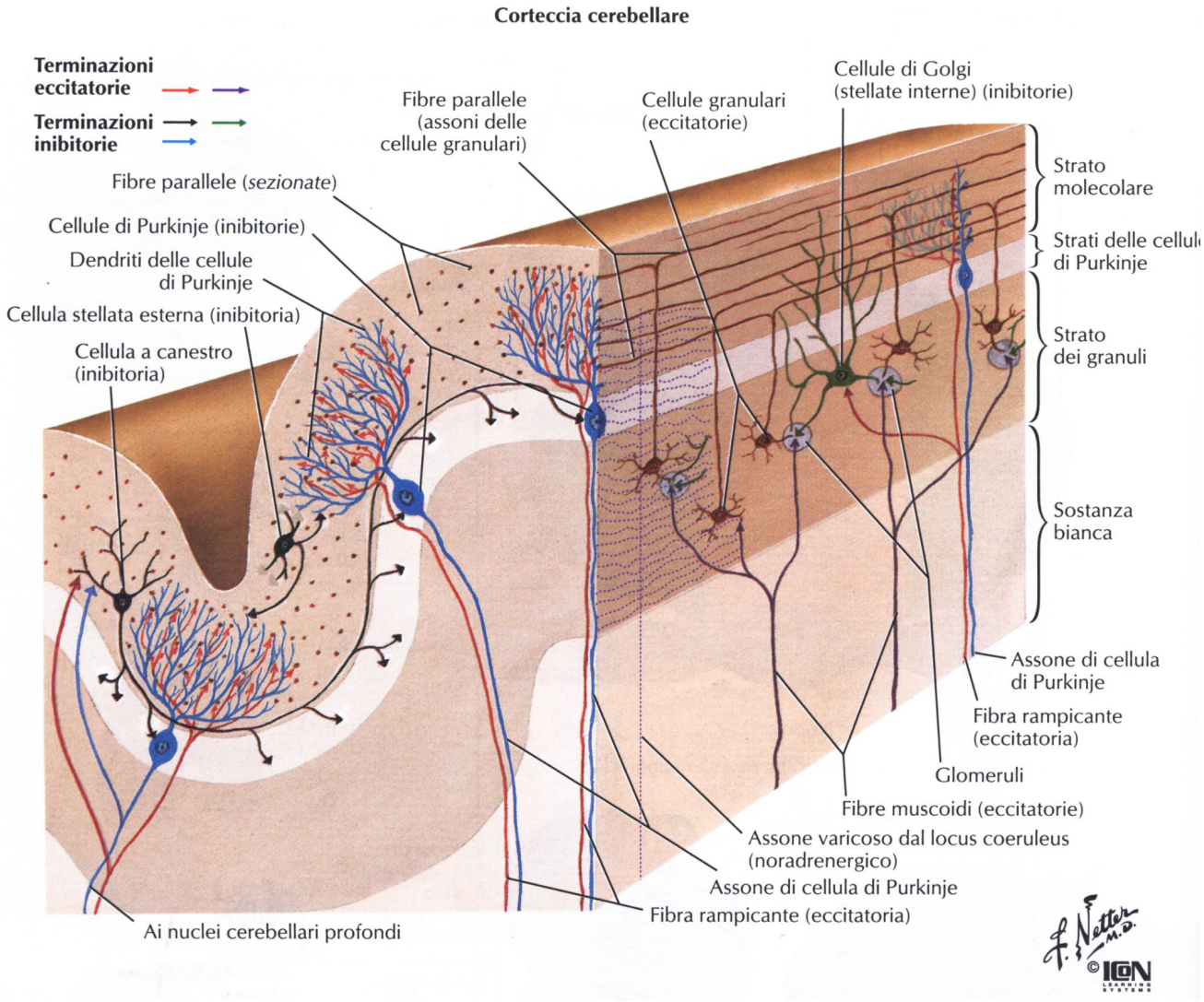

Figure 2. Anatomic cytoarchitecture of the three-layered cerebellar cortex

Products of the cerebellar anlage

The cerebellar anlage is comprised of two proliferative zones from which all neurons are generated: the ventricular zone (VZ) lining the dorsal aspect of the 4th ventricle, which generates inhibitory neurons, and the rhombic lip at the dorsal portion of rhombomere 1, which generates the excitatory neurons [9-11]. The distinction between the GABAergic lineage (e.g. Purkinje cells) and the glutamatergic lineage (e.g. granule cells) is conserved across vertebrates [12]. The VZ generates neuronal precursors fated to adopt GABAergic phenotypes, that is the inhibitory neurons of the cerebellum. The cerebellar VZ is delineated by the selective expression of the bHLH pancreas transcription factor *Ptf1a*. Indeed, deletion of *Ptf1a* in the mutant mouse, result in the loss of the entire cerebellar cortex [9]. The rhombic lip generates the glutamatergic neurons of the cerebellum, including the granule cells, most of unipolar brush cells and glutamatergic projection neurons of the cerebellar nuclei [13,14]. Granule cells are so numerous, more than 99% of the neurons in the cerebellum; this amount is due to a massive amplification of cells by the rhombic lip. This is achieved through the formation of a secondary germinal epithelium, the external granular layer (EGL). To this end, a cohort of rhombic lip-derived glutamatergic progenitors moves tangentially over the cerebellar surface to generate the EGL. These cells are fated to give rise to granule cells [15,16]. Rhombic lip progenitors express the proneural gene Atoh1/Math1 [17].

Stem/progenitor cells

During development, neural stem cells (NSCs) give rise to all the neurons of the mammalian Central Nervous System (CNS).

Usually, three criteria are applied to define a cell as a stem cell:

- ✧ *self-renewal*, ideally for an unlimited number of cell divisions;
- ✧ *unspecialized*: a stem cell is not has not any tissue-specific structures and, because of this, it is unable to perform specialized functions;
- ✧ *multipotency*, that is the ability to give rise to numerous types of differentiated cell in a process called differentiation.

The self-renewal of neural stem cells can occurs either by two different mitotic cell divisions: symmetrical and asymmetrical divisions (Fig. 3) [18]. The symmetric cell division generate two daughter cells identical to mother cell, which remain in the pool increasing the number of stem cells [19].

During the asymmetric division, can be generate one daughter cell that is identical to the mother cell and a second cell type that undergo to differentiation.

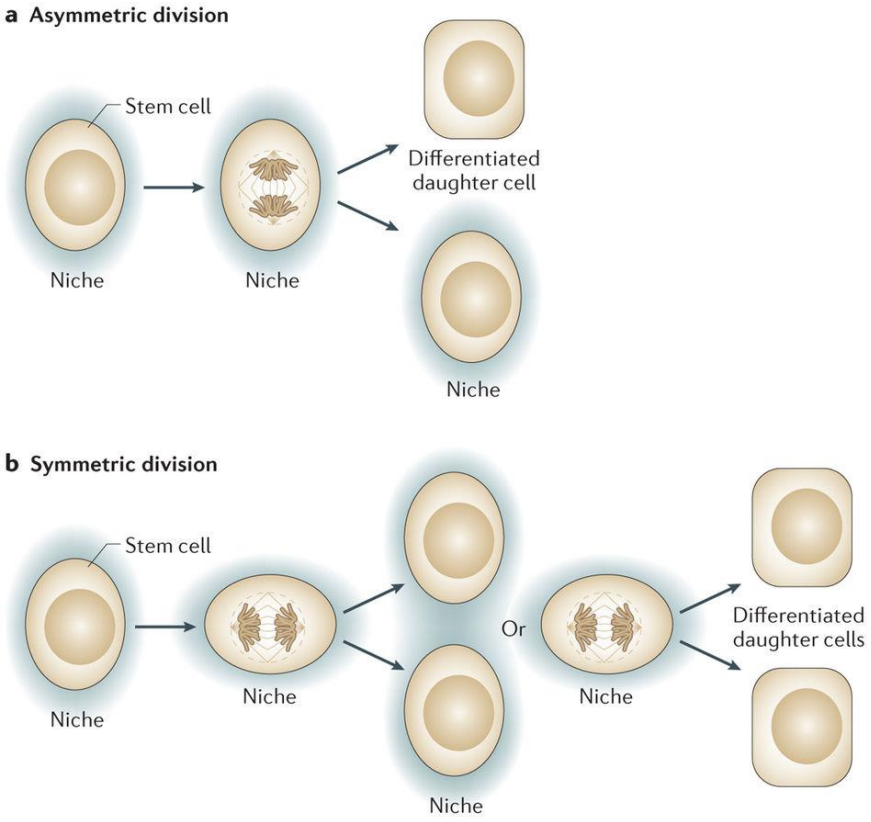

Figure 3. Symmetrical and asymmetrical divisions of stem cells

Stem cells have different potentials to differentiate into multiple cell types. According to their potential of differentiation, stem cells are classically distinguished into:

- *Totipotent stem cells*: stem cells can differentiate into embryonic and extraembryonic cell types, produced from the fusion of an egg and sperm cell;
- *Pluripotent stem cells*: they are characterized by a potential of differentiation to all cell types of the adult organism and to self-renewal. These cells appear at the blastocyst stage (4-14 days after fertilization) and they are capable of differentiating into embryonic tissues organized in three different germ layers (ectoderm, mesoderm and endoderm);

- *Multipotent stem cells*: they are stem cells able to differentiate into a number of cell types, but only those of a closely related family of cells;
- *Unipotent stem cells*: these cells are present in adult tissues. They maintain self-renewal property and they can differentiate only in one cell type of the tissue to which they belong.

Stem/progenitor cells niches in the developing human cerebellum

Cortical neurogenesis involve cell proliferation, migration and differentiation and leads to the three-layered structure, constituting by neuronal and glial cells [20]. During neurogenesis, neuronal and glial cells are generated from a common source, the proliferating neuroepithelial cells (NECs), which arise from the neural tube. A variety of signaling pathways are known to act during the process of neurogenesis [21]. These internal signals include Sonic hedgehog protein (Shh), Notch, Wnt/βcatenin signaling, Fibroblast Growth Factor (FGF) and Epidermal Growth Factor (EGF), and they are controlled by different genes that carry information for all the structures and functions of a cell [22-25]. In addition to this, evidence has emerges that epigenetic modifications such as DNA methylation and histone modifications are involved in the control of temporal and spatial gene expression during neurogenesis [26-28].

During the early stages of development, the neural tube is composed of a single layer of cells, the neuroepithelial cells, which form the neuroepithelium. The neuroepithelium looks layered ("pseudostratified"), because the nuclei of neuroepithelial cells migrate up and down the apical-basal axis during the cell-cycle [29]. The characteristic pseudostratification is mainly due to the interkinetic nuclear migration. Neuroepithelial cells show typical epithelial features and are highly polarized along their apical-basal axis.

Cerebellar cortical neurogenesis begins around the 7th week of gestation in the ventricular zone when NECs undergo to asymmetric division: through the property of self-renewal, a NEC can give rise to a daughter cell identical to the mother cell and capable of self-renewal; the other daughter cell becomes either an apical intermediate progenitor, a basal progenitor or a newborn neuron. After the onset of neurogenesis, NECs give rise to a distinct, but related, cell type, the apical radial glial cells (aRG), which exhibit residual neuroepithelial as well as astroglial properties [30,31]. Radial glial cells form radial glial fibers extending from from their apical and basal poles [32]. Like NECs, aRG undergo interkinetic nuclear migration in the ventricular zone. During neurogenesis progression, aRG cells switch from proliferation to differentiation. Apical intermediate progenitors, basal intermediate progenitors and basal radial glia can be generated either from NECs or aRG. Both types of basal progenitor cells are not attached to the ventricular surface and do not undergo interkinetic nuclear migration.

The accumulation of basal progenitor cells originate the subventricular zone, a germinal layer located above the ventricular zone [33,34]. Apical intermediate progenitor maintain contact only with the ventricular surface. Apical progenitor cells in the ventricular zone and basal progenitor cells in the subventricular zone are generally considered to represent the major source of cortical neurons [35]. For this reason, these two germinal zones represent a stem/progenitor cell niches in the developing human cerebellum [36]. During CNS development, radial glia exerts a dual function as a progenitor for neurons and glia as a structural scaffold to guide migration and homing of postmitotic cells. Radial glia follows a peculiar evolution during cerebellar development. Radial glial cells undergo a progressive morphological transformation into Bergmann glia (BG) that provide to guide the migration of different cell types and regulate the directional elongation of axons and dendrites [37]. Once that neurogenesis is complete, radial glial cells differentiate into glial lineage.

The EGL is composed of actively dividing cells representing by the granule cell precursors (GPCs) that arise from the rhombic lip and migrate anteriorly over the surface of the developing cerebellar anlage [38]. During cerebellar development, GPCs move inward from the EGL, along Bergman glial fibers, to form the internal granular layer (IGL) (Figure 4). The developing human cerebellar cortex consist of four layers: the outer external granular layer (EGL), the molecular layer, the Purkinje cell layer and the deepest internal granular layer (IGL). The EGL is a transient layer that disappear up to 1 year postnatally.

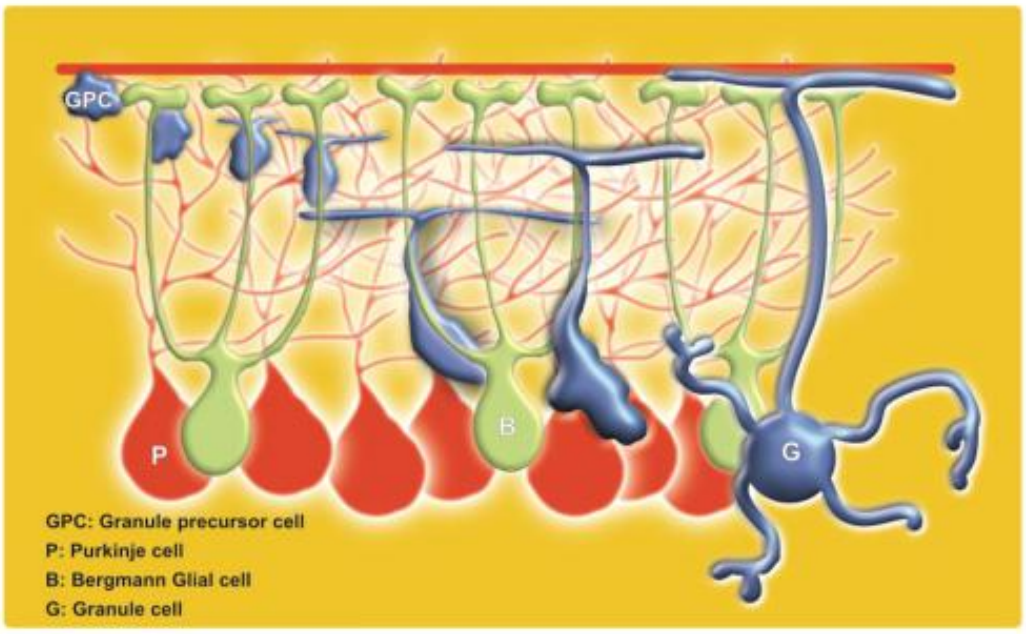

Figure 4. Schematic representation of migration of granule precursor cells along Bergmann glial fibers, from the external granular layer towards the internal granular layer

Cortical stem/progenitor cells markers

Several immunohistochemical markers have been used in previous experimental studies for the identification of stem/progenitor cells in the developing human cerebellum. This study is focus on the expression, by immunohistochemistry, of different proteins that play a key role in the mammalian CNS development: SOX2, Pax6, Pax2, WT1, Nestin, Vimentin and Calretinin.

SOX2

The transcription factor SOX2 [Sex determining region of Y chromosome (Sry)-related high mobility group box2] belongs to the SOX family of transcription factors characterized by the presence of a homologous sequence known as HMG (high mobility group) box, a DNA binding domain highly conserved among species [39-41]. SOX genes encode putative transcriptional regulators

implicated in cell fate during development and in different developmental processes control [42,43]. Experimental studies showed that SOX2 is expressed during the development of the mammalian central nervous system [44-46]. However, the expression of SOX2 persists also in adulthood, where SOX2 is found in the NSCs of the subventricular zone of the lateral ventricles and in the subgranular zone of the dentate gyrus of the hippocampus [47]. Experimental studies demonstrated that a constitutive expression of SOX2 maintains the properties of stem cells and inhibits neuronal development [48]. Therefore, SOX2 is highly expressed in proliferating neural progenitor cells, maintaining the self-renewal property [49,50], and it is down-regulated upon differentiation to post-mitotic neuronal and glial cells [51].

WT1

Wilms' tumor 1 (WT1) protein is a zinc finger transcription factor encoded by the human gene WT1 involved in the onset of Wilms' tumor, the most common primary renal tumor in childhood [52]. WT1 gene has a lenght of ~50 kb and consists of 10 exons (Call KM et al., 1990). and it shares a high degree of structural homology with the early growth response (EGR) transcription factor family [53,54]. The WT1 protein regulates the transcription of several genes and acts both as activator and as transcriptional co-activator or as repressor of gene expression [55]. Study on knock-out mice showed that WT1 is required for heart, spleen and adrenal gland development and for CNS development [56-59]. WT1 is involved in the development of several human organs during embryogenesis [59,60], including developing kidney [61,62]. Recent studies demonstrated that WT1 is an important marker involved in human CNS development, being expressed in radial glial cells during early phases of gestation [59].

Pax2

Pax2 belongs to the highly conserved DNA-binding paired box domain family which constitutes a group of developmental genes that encode several nuclear transcription factors. These genes play an important role in early mammalian embryogenesis, including kidney development [63]. Pax2 is a target of transcriptional suppression by WT1 during normal kidney development and it has been reported to be expressed during the formation of CNS in experimental animal studies, including eye development [64-67]. A number of researches showed that Pax2 was expressed in a subsets of post-mitotic neurons from different regions of CNS [68,69].

Pax6

Pax6 (paired box gene 6) belongs to the family of PAX gene class and encodes for a transcription factor containing a paired domain and a paired-type homeo-domain [70]. Pax6 plays a critical role in brain and eye development [71-73]. In the CNS, it is involved in neuronal specification, neuronal migration and axonal extension [74,75]. In the mouse cerebellum, Pax6-immunoreactive cells were found in migrating granule cell precursors from the EGL [76].

Nestin

Nestin is a cytoskeleton-associated class VI intermediate protein (IF) and it is a key regulator of various intracellular proteins involved in cell growth and differentiation [77]. Nestin regulates neural stem cell migration via controlling the cell contractility in murine neurogenesis studies [78]. During embryogenesis, Nestin expression is downregulated and gradually replaced by cell type-specific intermediate filaments such as Neurofilaments (NF) in neurons and Glial fibrillary acid protein (GFAP) in glial cells [79].

Vimentin

Vimentin is a III intermediate filament protein and acts as a crucial cytoskeletal component of mesenchymal cells being involved in cell migration and in epithelial-to-mesenchymal transition (EMT) [80]. During the reverse process, the mesenchymal to epithelial transition (MET), Vimentin is downregulated and consequently cell motility decreases and cell adopt epithelial features. Vimentin is overexpressed in various epithelial cancers, including prostate cancer, gastrointestinal tumors, CNS tumors, breast cancer, malignant melanoma and lung

cancer [81,82]. Vimentin play a key role during CNDS development, being expressed in radial glial cells during the early stages of gestation [83].

Calretinin

Calretinin (CR) is a calcium-binding protein (CaBPs) involved in the maintenance of intracellular calcium homeostasis. Similarly as other CaBP parvalbumin and calbindin, CR also belongs to the EF-family of CaBPs, characterized by six domains with different affinity to bind calcium ions [84]. Immunoreactivity to CR has been reported in different areas of the central nervous system of various animal species [85]. The study by Yew D.T. and coworkers [86] demonstrated that, in the developing human cerebellum, CR appeared approximately at the 21st week, more later than calbindin and parvalbumin, its expression increasing with developmental age. Since the 21st week, CR-immunostaining cells was found in Purkinje cells, basket cells and in neurons of deep nuclei [86]. Few data regarding the distribution of CR in the human cerebellum during postnatal development and adulthood have been reported [87], as well as the distribution of calretinin in granule cells in the adult human cerebellar cortex [88].

Aim of the study

The aim of this study was to identify the stem/progenitor cell markers, by immunohistochemistry, in order to highlight the cortical neurogenesis niches during the different gestational ages. To this end, the following stem/progenitor cell markers have been utilized: Sox2, Pax6, Pax2, WT1, Nestin, Vimentin and Calretinin. The expression of these markers have been compared with the expression of those markers of mature neurons and glial markers including Neurofilament (NF), neuron specific enolase (NSE), Synaptophysin (Syn), Glial fibrillary acid protein (GFAP) and S100β.

Evaluating both stem/progenitor and mature cell markers lead to the identification of multiple stages of differentiation of neuronal and glial progenitors during gestation, in order to better understand the development of human cerebellar cortex.

Materials and methods

The expression of different markers was investigated in human cerebellar specimens of 20 human fetuses aging from 11 to 38 weeks of gestation that we received from the Obstretric Division of the University of Cagliari. Regarding the cause of death, some of these fetuses underwent voluntary termination of pregnancy (VTOP); placental detachment was the cause of death in other cases and in another case therapeutic abortion followed the diagnosis of diaphragmatic hernia. All the fetuses examined had no brain congenital malformation.

Cerebellar samples have been histologically and immunohistochemically studied. Samples were fixed in 10% buffered formalin, routinely processed, and paraffin-embedded. Serial 3 µm-tick sections were obtained from each paraffin block; after dewaxing and rehydrating, one section was stained with hematoxylin-eosin, while the others were pre-treated for immunohistochemistry, with 10 minutes heat-induced epitope retrieval in buffer pH 9.00 (*EnVision™ FLEX Target Retrieval Solution High pH*; *Dako Denmark A/S, Glostrup, Denmark; Code K8004)*. Slides were then incubated for 20 minutes at room temperature with the antibodies reported in Table 1. Staining procedures were performed by Envision™ FLEX+ (*Dako; Code K8002*) Detection System and *AutostainerLink 48* instrument following dealer's instructions. Data were obtained by evaluation of positivity (+) and negativity (-) for the immunoreactivity of these markers in each cerebellar sample.

Antibody	Dilution	Source	Company	Code
WT1	1:100	Mouse monoclonal 6F-H2	Dako	M-3561
Vimentin	1:500	Mouse monoclonal 3B4	Dako	M-7020
Nestin	1:200	Mouse monoclonal 10C2	Dako	SC-23927
Pax2	1:400	Mouse monoclonal 3C7	Abnova	H00005076-M01
GFAP	1:100	Rabbit polyclonal	Novocastra	NCL-GFAP-GA5
NSE	1:200	Mouse monoclonal BBS/NC/VI-H14	Dako	M-0873
Synaptophysin	1:20	Mouse monoclonal SY38	Dako	M-0076
S100β	1:2000	Rabbit polyclonal	Dako	Z-0311
Neurofilament	1:50	Mouse monoclonal 2F11	Dako	M-0762
SOX2	1:50	Mouse monoclonal E-4	Santa Cruz	SC-365823
Pax6	1:50	Mouse monoclonal	Santa Cruz	SC-53108
Calretinin	1:1000	Mouse monoclonal	Dako	M-7245

Table 1. Antibodies used in this study

Results

Stem/progenitor cells markers

SOX2

From the 11th to the 15th week, immunostaining for SOX2 was particularly strong in both ventricular and subventricular zones, while in the intermediate and in the cortical zones ther immunostaining was weaker. At this gestational ages, especially at the 11th week, the ventricular and subventricular zones have a large thickness in comparison with the other zones of the developing human cerebellum (Figures 5). SOX2 nuclear immunoreactivity higtlights the nuclei of stem/progenitor cells in the ventricular and subventricular zones and in the newborn cells migrating from the ventricular and subventricular niches towards the cortical surface (Figure 6).

From the 18th to the 21th gestational week, SOX2 immunoreactivity showed a lesser intensity and a reduced distribution in the ventricular and subventricular niches and in the other cerebellar zones (Figures 7, 8).

Between 30th and 34th gestational week, the thickness of the ventricular and subventricular zones is visibly reduced in comparison with the previous weeks. Scattered SOX2-positive cells were also detected in migrating newborn neurons in the intermediate and in the cortical zones (Figures 9, 10).

No reactivity for SOX2 was detected in the cortical layers at the 38th week.

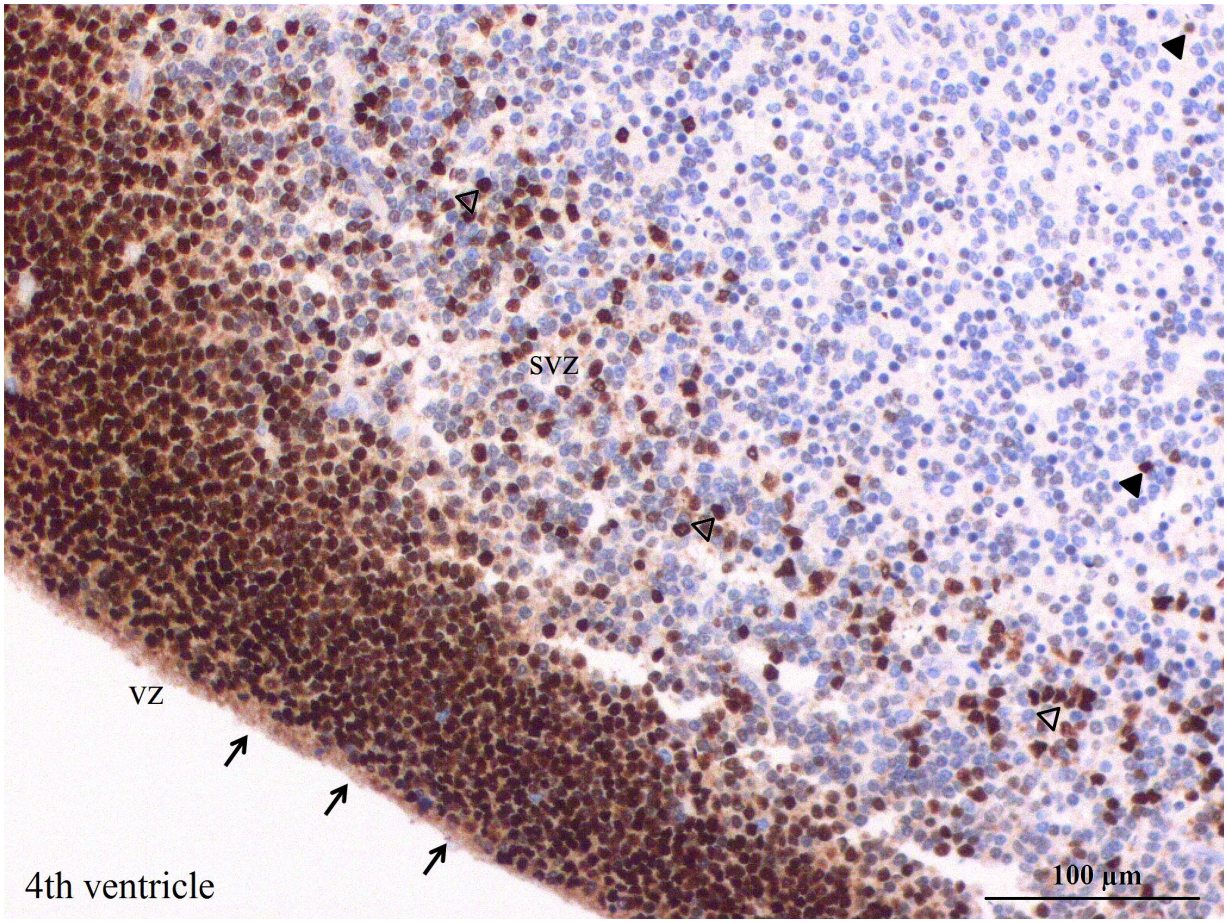

Figure 5. Gestational week 11. SOX2 nuclear reactivity in stem/progenitor cells in the VZ (arrows), in the SVZ (open arrowheads) and in migrating progenitor cells in the inner zone (arrowheads)

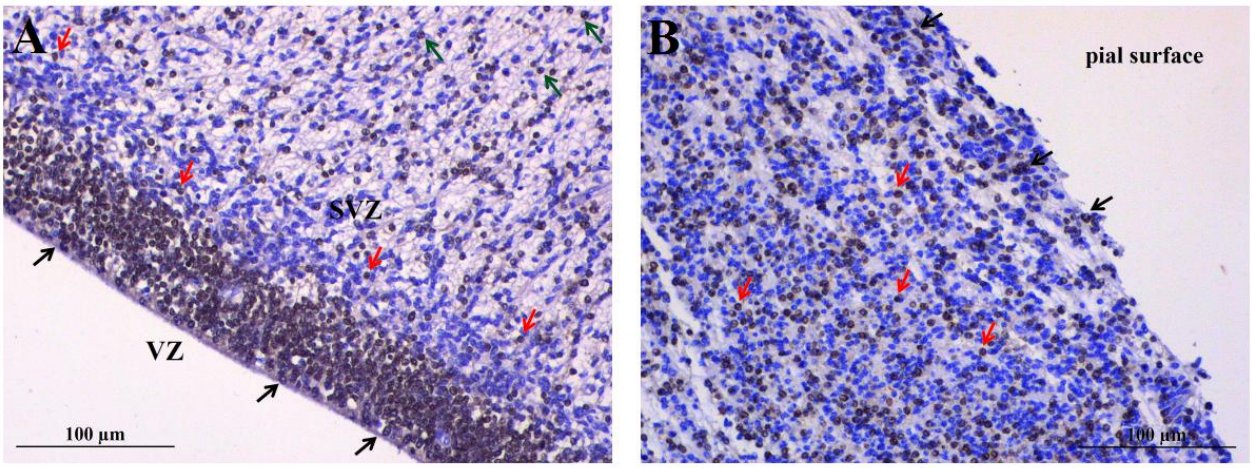

Figure 6. Gestational week 15. A: SOX2 nuclear immunoreactivity in the VZ (arrows), in the SVZ (red arrows) and in the inner zone (green arrows).B: SOX2 immunoreactivity in migrating progenitor cells in the inner zone (green arrrows) and in the cortical surface (arrows)

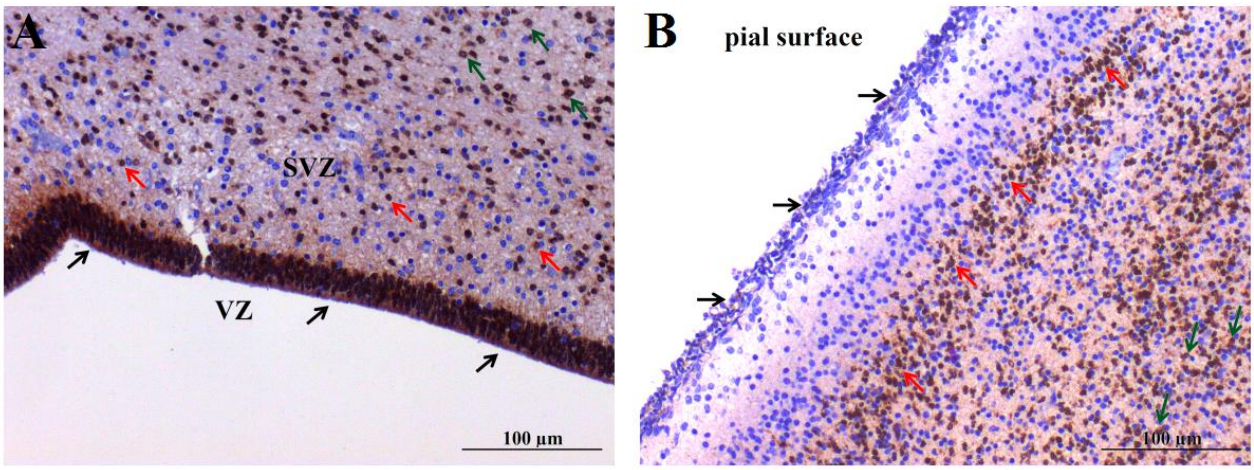

Figure 7. Gestational week 18. A: SOX2 nuclear immunoreactivity in the VZ (arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: SOX2 nuclear immunoreactivity in migrating newborn cells in the inner zone (green arrows), in the Purkinje cell layer (red arrows) and in the cortical surface (arrows)

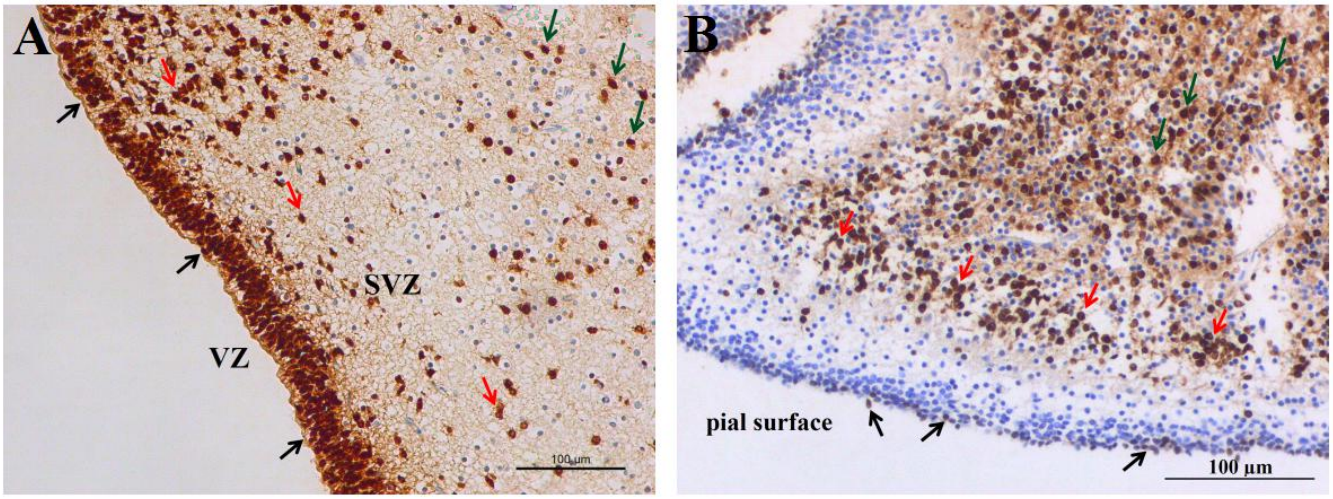

Figure 8. Gestational week 21. A: SOX2 immunoreactivity in stem/progenitor cells in the VZ (arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: SOX2 immunoreactivity in migrating progenitor cells in the cortical surface (arrows), in the Purkinje cell layer (red arrows) and in the internal granular layer (green arrows)

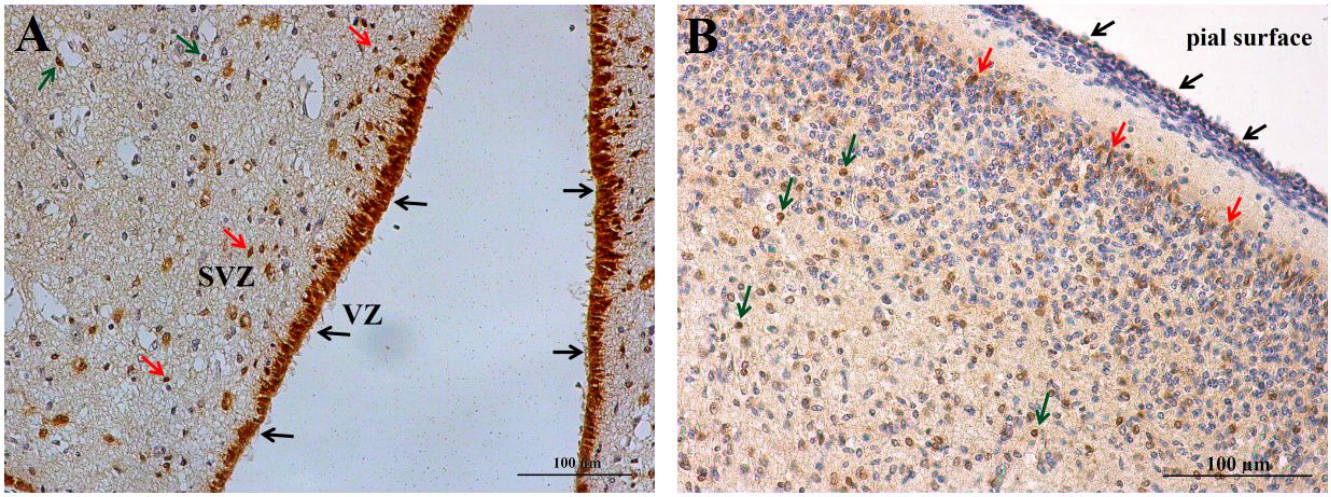

Figure 9. Gestational week 30. A: SOX2 immunoreactivity in stem/progenitor cells in the VZ (arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: SOX2 immunoreactivity in the cortical surface (arrows), in the Purkinje cell layer (red arrows) and in the internal granular layer (green layer)

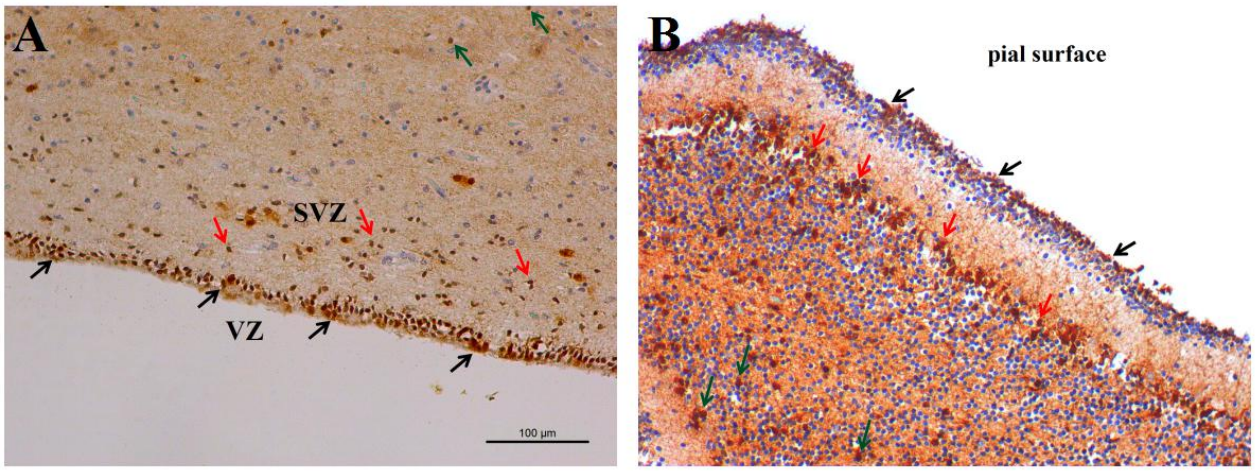

Figure 10. Gestational week 34. A: SOX2 reactivity in stem/progenitor cells in the VZ (arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: SOX2 reactivity in migrating progenitor cells in the cortical surface (arrows), in the Purkinje cell layer (red arrows) and in the internal granular layer (green arrows)

WT1

From the 15th to the 18th week of gestation, WT1 immunostaining was particularly strong in the cytoplasm of radial glia fibers extending from the ventricular zone towards the pial zone. Nuclei of progenitor cells in both ventricular and subventricular zones, as well as those of cortical neurons, did not show reactivity for WT1 (Figures 11, 12).

At the 21st week of gestation, WT1 cytoplasmic expression was detected in radial glia fibers extending from the ventricular and subventricular zones, evidencing a decrease in the number of radial glia fibers. Nuclei of both progenitor cells in the ventricular and subventricular zones and those of postmitotic cells showed no reactivity for WT1 (Figure 13).

At the 34th week of gestation, a mild WT1 cytoplasmic immunoreactivity was detected in the ventricular and subventricular zones, as well as in the cortical layers. A strong WT1 expression was observed in the epithelium of small blood vessels in the cortical layers (Figure 14).

At the 38th week of gestation, whereas a mild reactivity for WT1 was detected in cell extensions in the cortical surface, the immunostaining for this antibody was mainly observed in the epithelium of cortical small blood vessels (Figure 15).

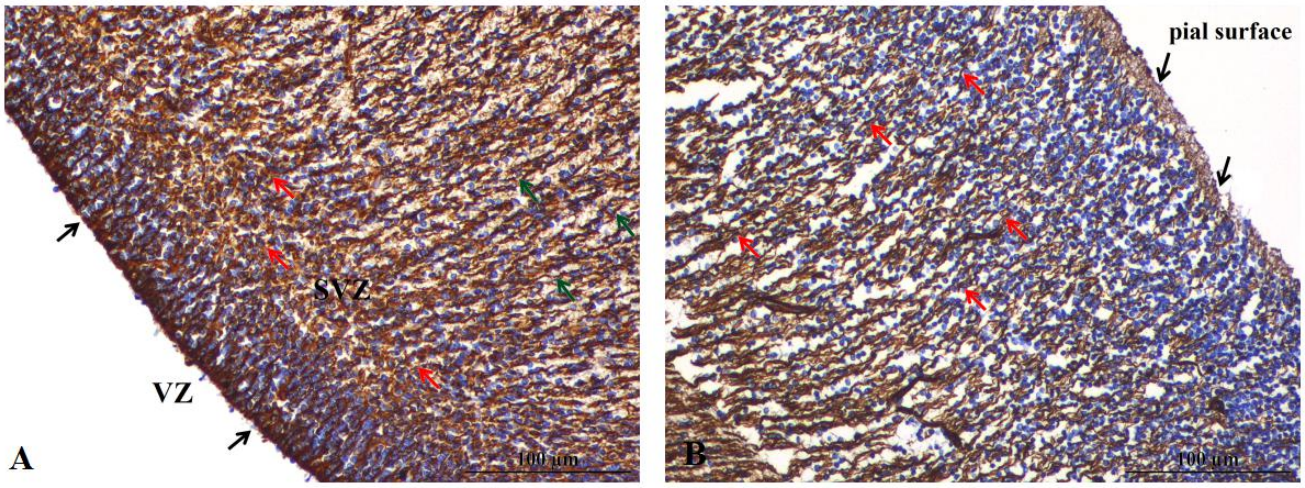

Figure 11. Gestational week 15. A: WT1 expression in the cytoplasm of progenitor cells extensions in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: WT1 expression in the cytoplasm of progenitor cells extensions migrating towards the cortical layers (red arrows) and the pial surface (black arrows)

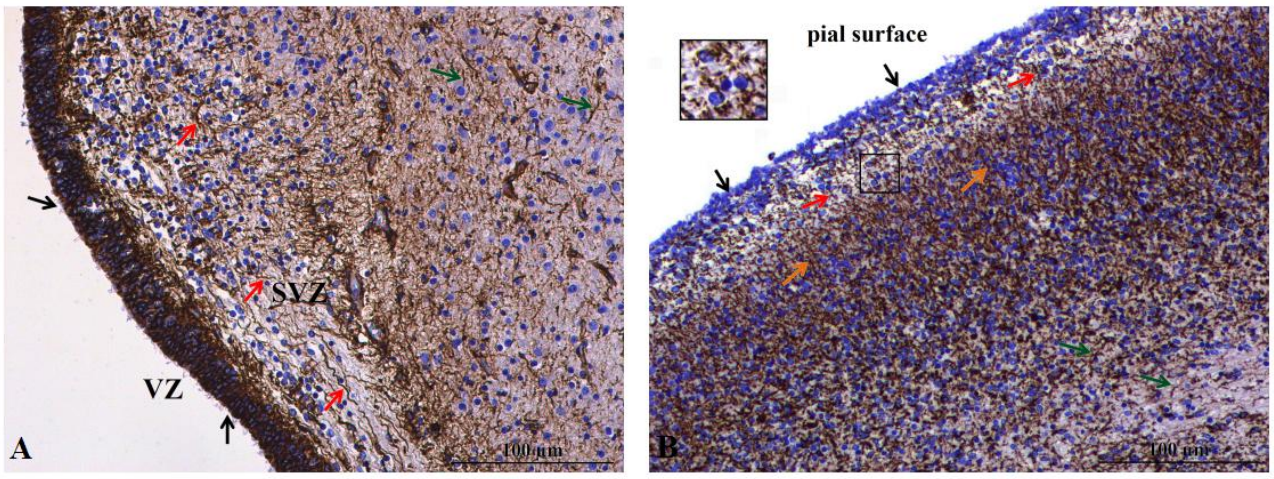

Figure 12. Gestational week 18. A: WT1 expression in the cytoplasm of progenitor cells extensions in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: WT1 expression in the cytoplasm of progenitor cells extensions migrating towards the pial surface (black arrows), the molecular layer (red arrows), the Purkinje cell layer (orange arrows) and in the inner zone (green arrows)

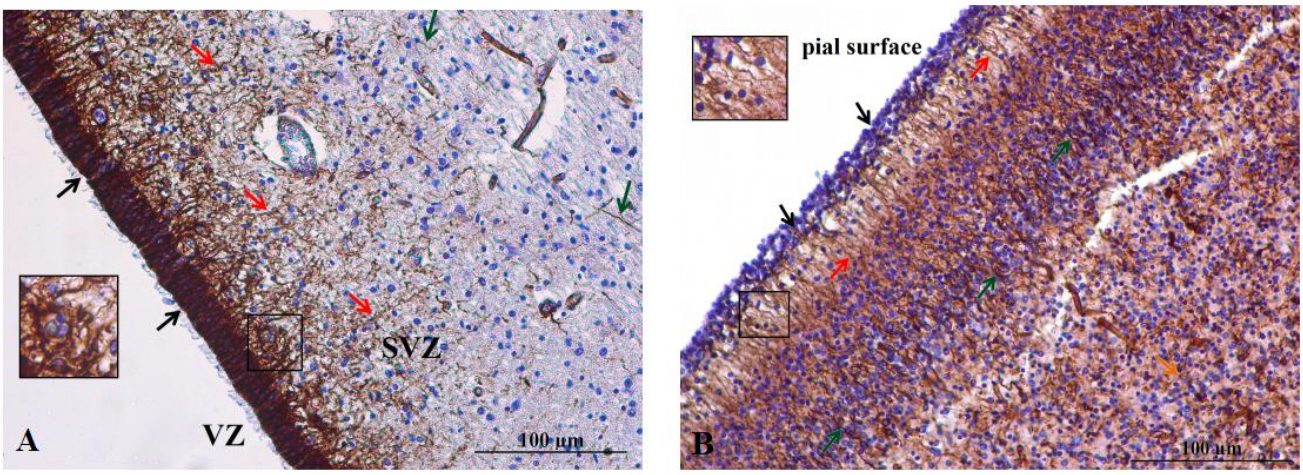

Figure 13. Gestational week 21. A: WT1 expression in the cytoplasm of progenitor cells extensions in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: WT1 expression in the cytoplasm of progenitor cells extensions migrating towards the pial surface (black arrows), the molecular layer (red arrows), the Purkinje cell layer (green arrows) and in the inner zone (orange arrows)

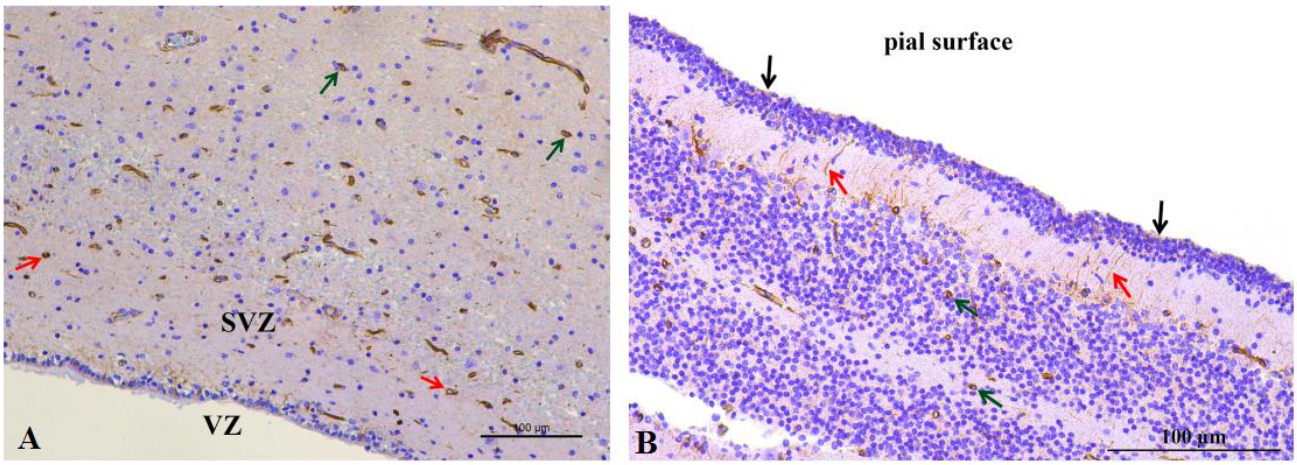

Figure 14. Gestational week 34. A: WT1 expression in the epithelium around small blood vessels in the SVZ (red arrows) and in the inner zone (green arrows). B: WT1 expression in the cytoplasm of progenitor cells extensions migrating towards the pial surface (black arrows), the molecular layer (red arrows) and in the epithelium around small blood vessels (green arrows)

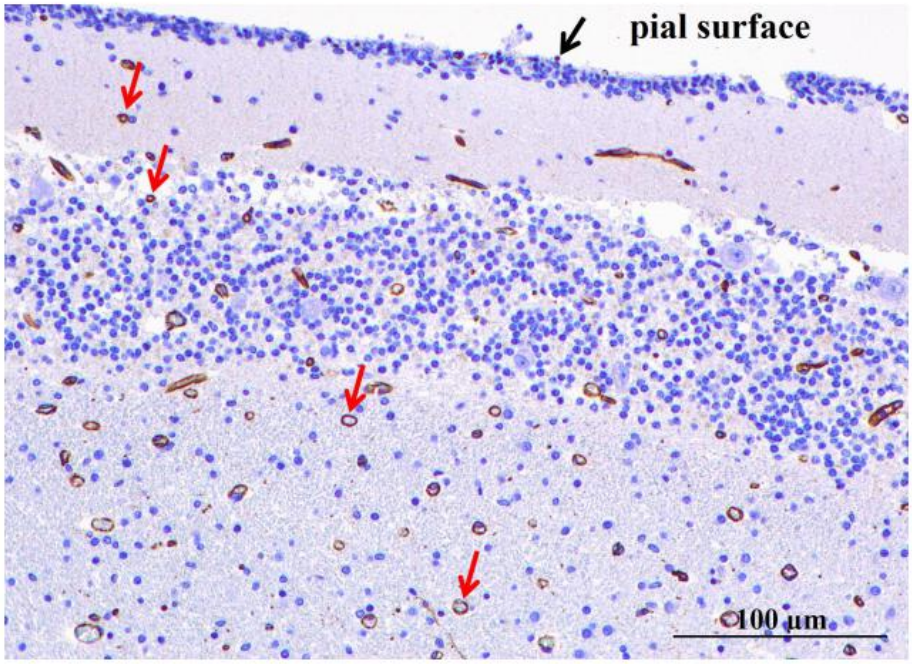

Figure 15. Gestational week 38. WT1 expression in the epithelium around small blood vessels in the cortical layers (red arrows) and a mild reactivity for WT1 in cell extensions in the cortical surface (black arrow)

Vimentin

From the 15th to the 18th week of gestation, Vimentin immunoreactivity was detected in the ventricular and subventricular zones, being restricted to cytoplasmic projection of radial glia cells extending from the ventricular zone towards the cortical zone. No immunostaining for Vimentin was detected in nuclei of radial glia cells (Figures 16, 17).

At the 21st week of gestation, Vimentin immunoreactivity evidenced a decrease in radial glia projections extending from the ventricular and subventricular zones (Figure 18).

At the 34th week of gestation, a mild Vimentin immunopositivity was found in the ventricular zone, as well as in the epithelium of small blood vessels in the subventricular zone. At the same gestational age, Vimentin-positive radial glia projections were observed in the cortical layers (Figure 19).

At the end of gestation, no reactivity for Vimentin was found in cortical radial glia projections, being restricted only in the cortical epithelium of small blood vessels (Figure 20).

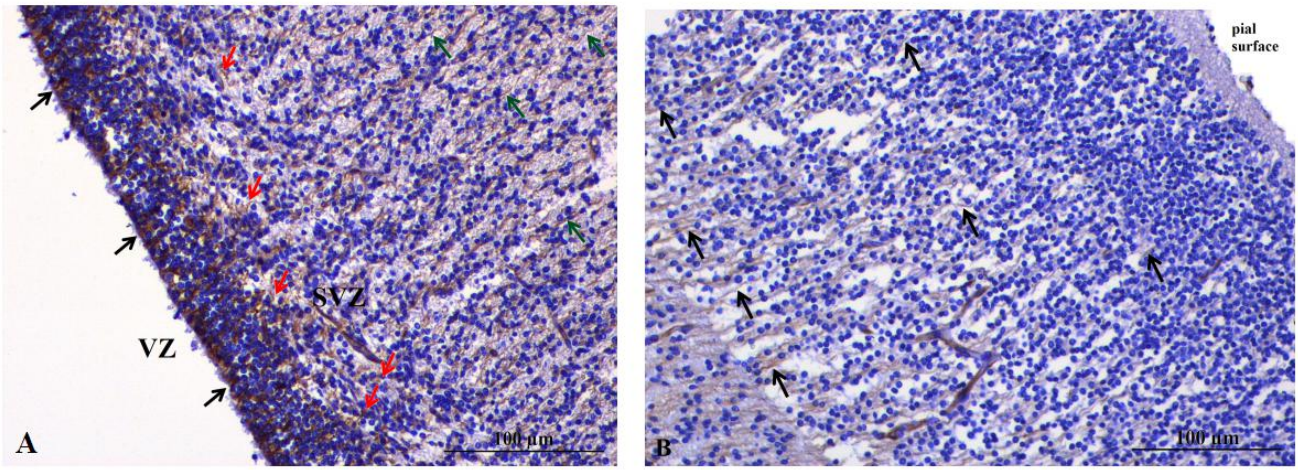

Figure 16. Gestational week 15. A: Vimentin expression in the cytoplasm of progenitor cell projections in the VZ (black arrows), in the SVZ (red arrows) and in the inner zones (green arrows). B: Vimentin immunoreactivity in the cytoplasm of progenitor cell projections reaching the cerebellar cortex (black arrows)

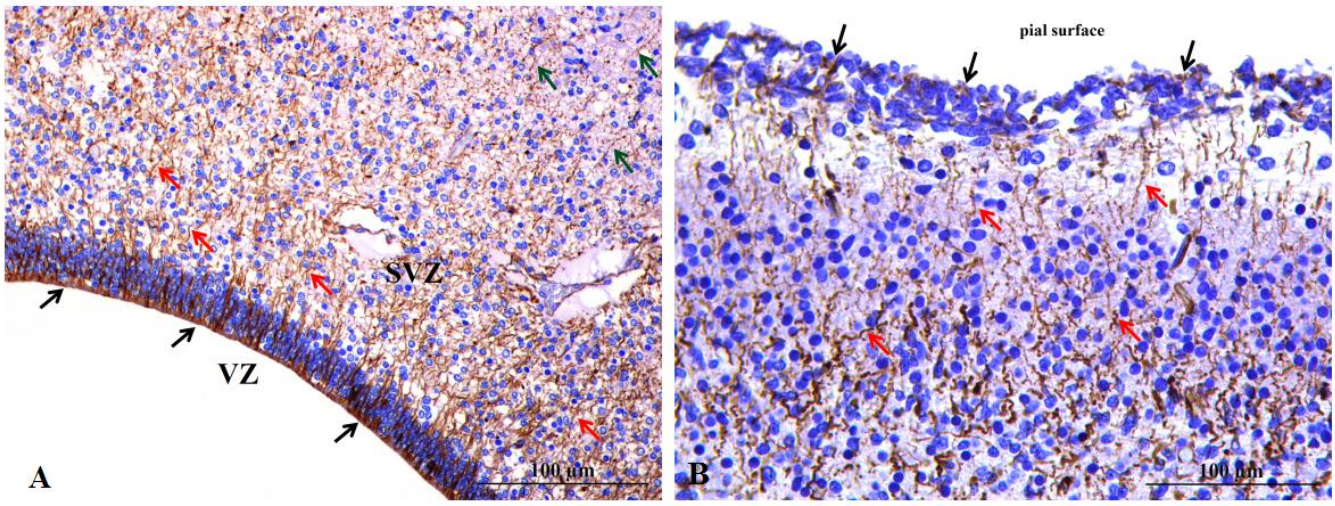

Figure 17. Gestational week 18. A: Vimentin immunoreactivity in the cytoplasm of progenitor cell projections in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: Vimentin immunoreactivity in progenitor cell projections in the pial surface (red arrows) and migrating progenitor cell projections in the cortical layer (black arrows)

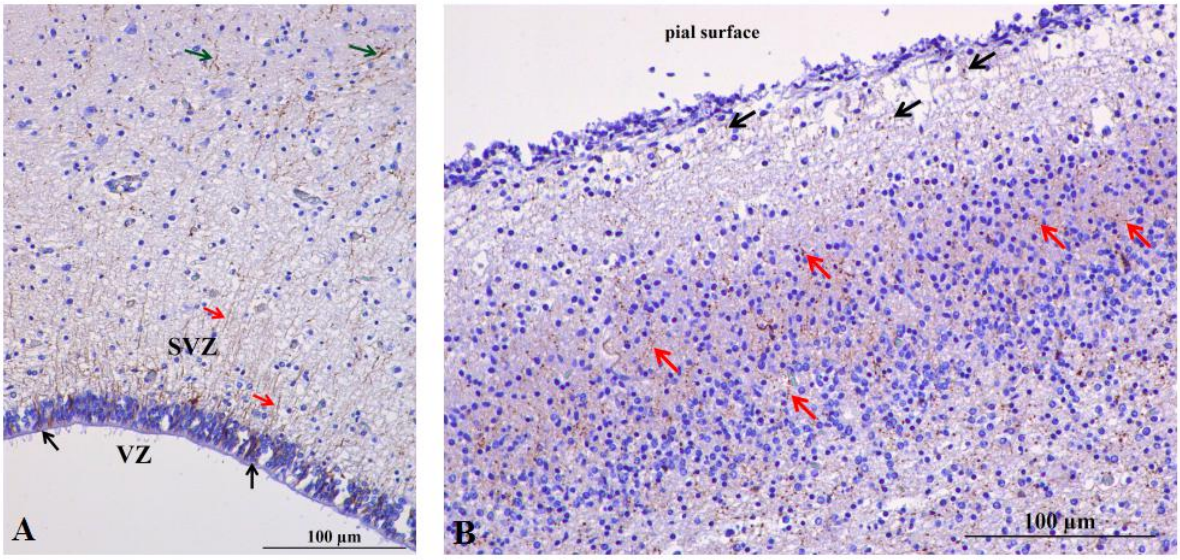

Figure 18. Gestational week 21. A: Vimentin expression in the cytoplasm of progenitor cell projections in the VZ (black arrows), in the SVZ (red arrows) and in the inner zones (green arrows). B: Vimentin immunoreactivity in the cytoplasm of progenitor cell projections reaching the cerebellar cortex (black arrows)

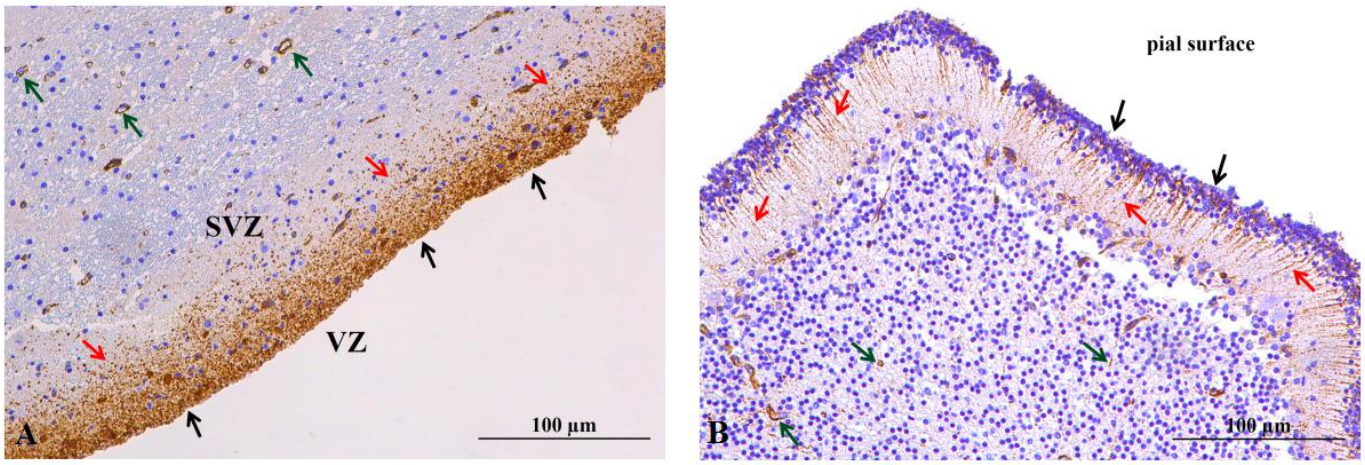

Figure 19. Gestational week 34. A: Vimentin immunoreactivity in the citoplasm of progenior cell projection in theVZ (black arrows) and in the SVZ (red arrows) and also in the epithelium around small vessels in the inner zone (green arrows). B: Vimentin immunoreactivity in progenior cell projection in the pial surface (black arrows) and in the molecular layer (red arrows) and in the epithelium around small vessels in the inner zone (green arrows)

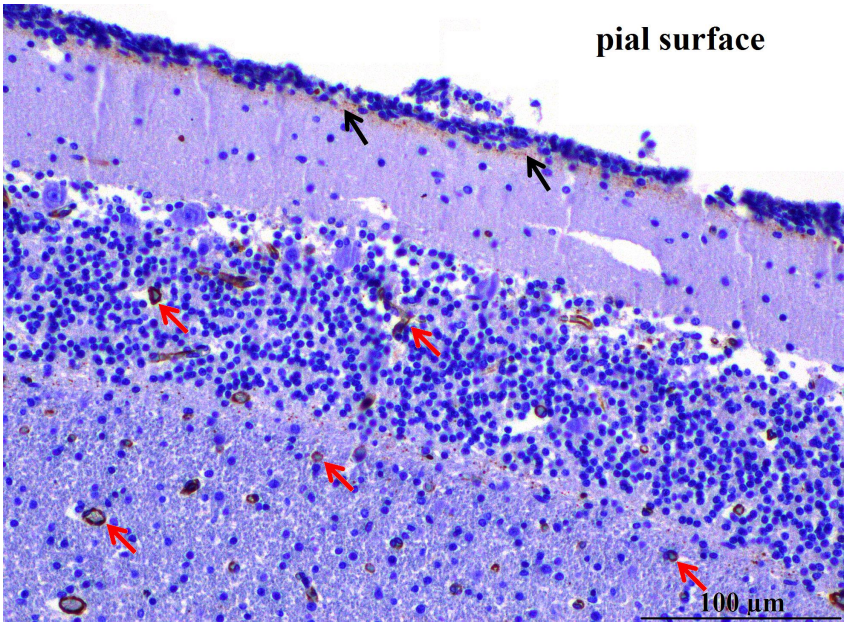

Figure 20 Gestational week 38. Vimentin immunoreactivity in scattered progenitor cell projections in the pial surface (black arrow) and in the epithelium around small vessels in the different cortical layers (red arrows)

Nestin

From the 15th to the 21st week of gestation, a strong Nestin cytoplasmic immunoreactivity was detected in the ventricular and subventricular zones, as well as in the cortical layers. At higher magnification, immunostaining for Nestin was detected in the projection of radial glia cells extending from the ventricular zone towards the cortical zone (Figures 21, 22, 23). At 21st week of gestation, we found a lesser the number of cortical radial glia fibers positive for Nestin (Figure 23).

At the 34th week of gestation, no reactivity for Nestin was found in radial glia projection from the ventricular zone, while the epithelium around blood vessels and the progenitor cell extensions in the molecular layer showed a mild immunoreactivity for this antibody (Figure 24).

At the 38th week of gestation, immunoreactivity for Nestin was only restricted to the epithelium of small cortical blood vessels (Figure 25).

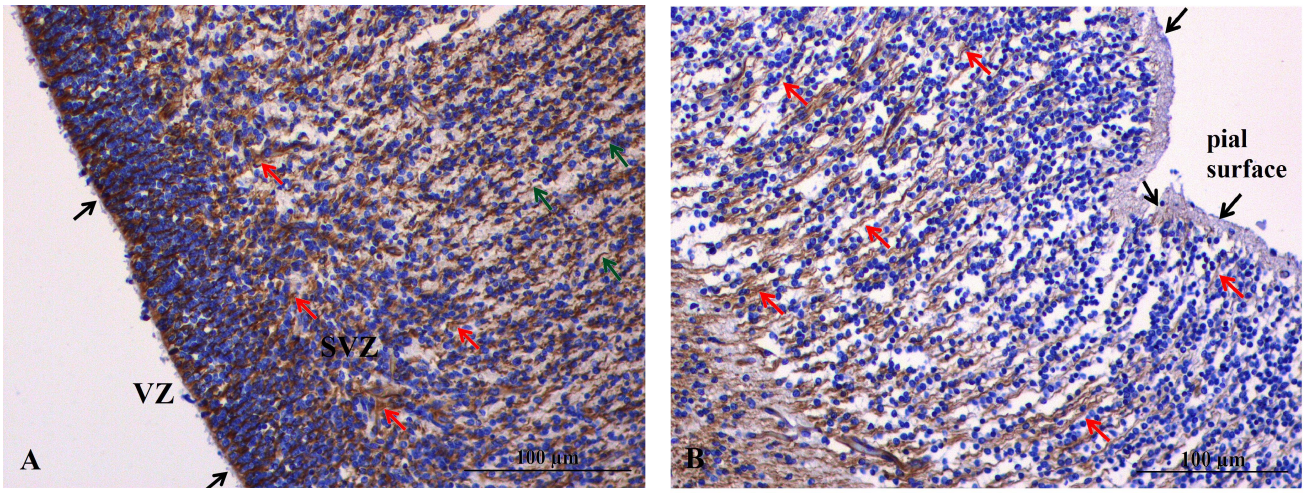

Figure 21. Gestational week 15. A: Nestin cytoplasmic expression in progenitor cells projections in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: Nestin cytoplasmatic expression in progenitor cell projections in the pial surface (black arrows) and in the cortical layers (red arrows)

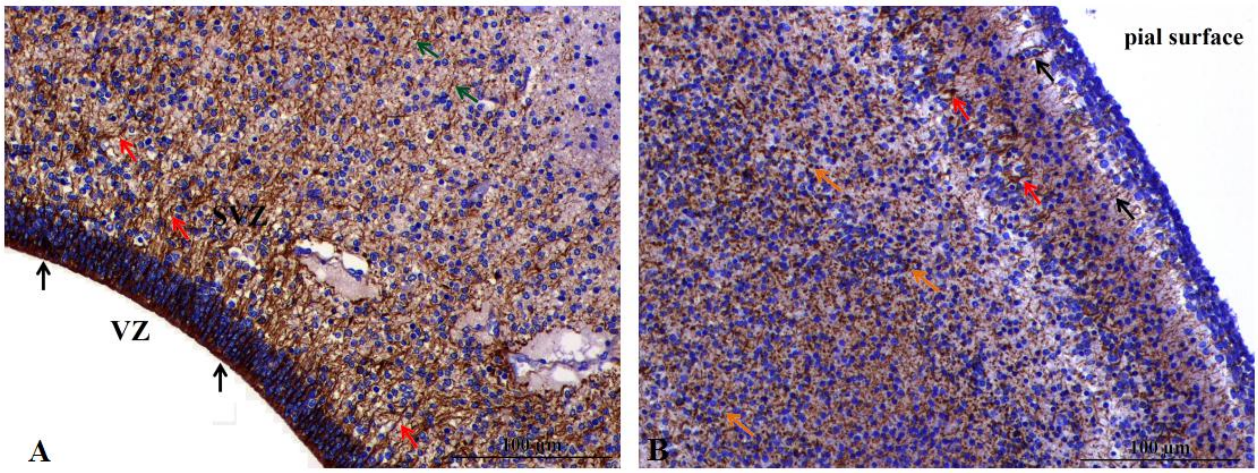

Figure 22. Gestational week 18. A: Nestin cytoplasmic expression in progenitor cells projections in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: Nestin cytoplasmatic expression in progenitor cell projections in the molecular layer (black arrows), in the Purkinje cell layer (red arrows) and in the inner zone (green arrows)

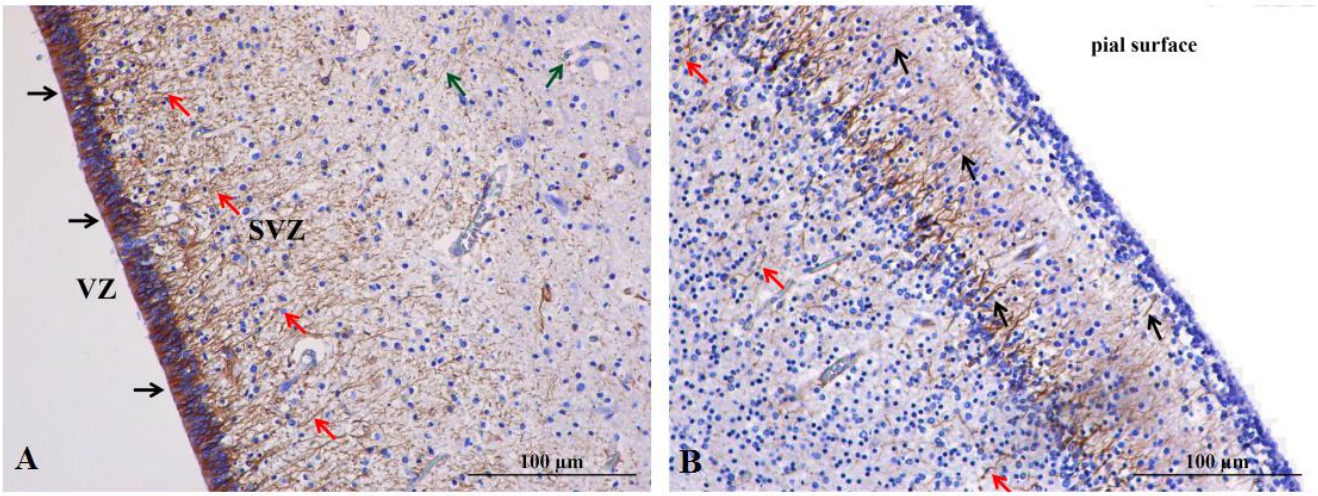

Figure 23. Gestational week 21. A: Nestin immunoreactivity in progenitor cell extensions in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: Nestin immunoreactivity in progenitor cell extensions in the molecular layer (black arrows) and in the inner zone (red arrows)

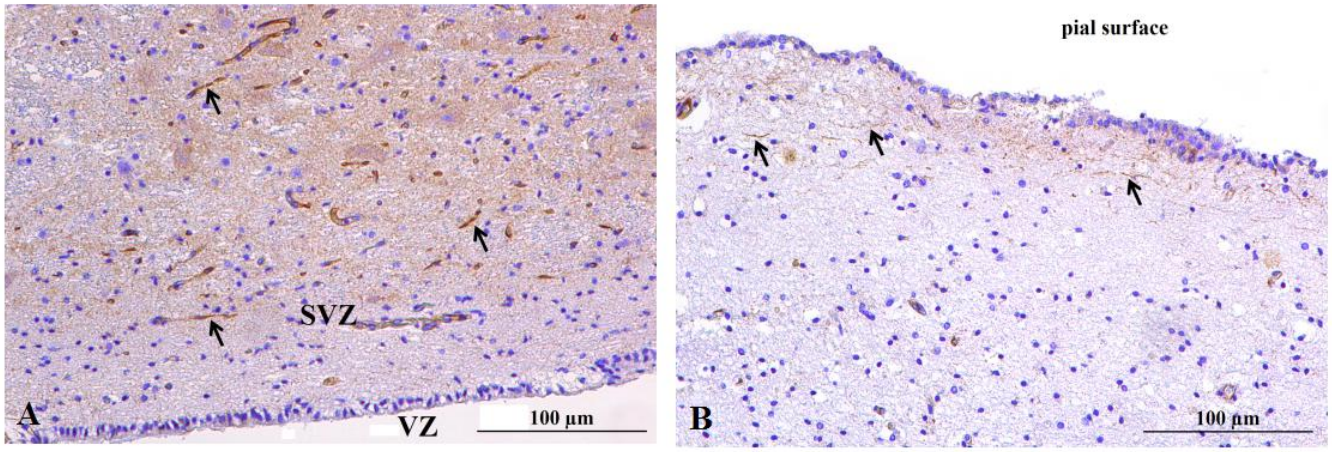

Figure 24. Gestational week 34. A: Immunoreactivity for Nestin in the epithelium around small vessels in the SVZ (black arrows). B: Mild immunoreactivity for Nestin in progenitor cell extensions in the molecular layer (black arrows)

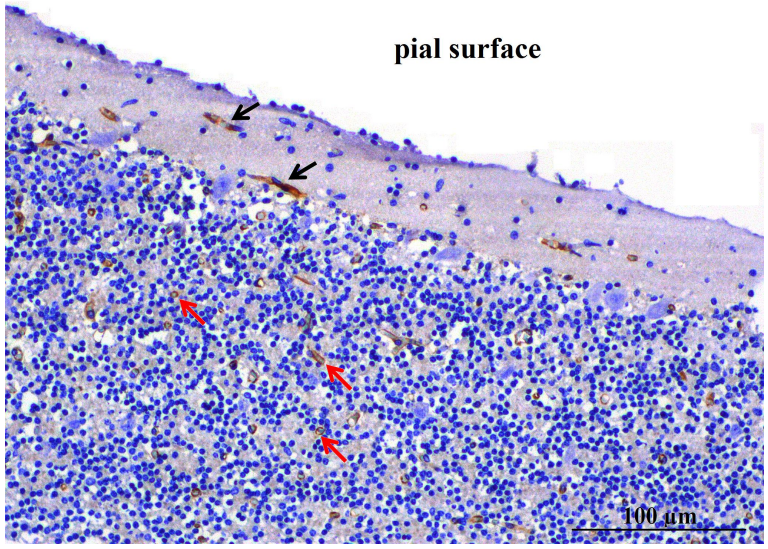

Figure 25. Gestational week 38. Immunoreactivity for Nestin in the epithelium around small vessels in the molecular layer (black arrows) and in the inner zones (red arrows)

Pax2

From the 15th to the 18th week of gestation, Pax2 immunoreactivity progressively increased from the ventricular and subventricular zones towards the pial zone. At higher magnification, Pax2 highlights the nuclei of progenitor cells in the ventricular and subventricular zones, as well as the nuclei of cortical progenitor cells (Figures 26, 27).

By the 21st week of gestation, nuclear immunoreactivity for Pax2 decreased both in the ventricular and subventricular zones and in the cortical layers (Figure 28).

At the 34th week of gestation, Pax2 showed a mild reactivity in scattered cells in the ventricular and subventricular zones and in the cortical layers (Figure 29).

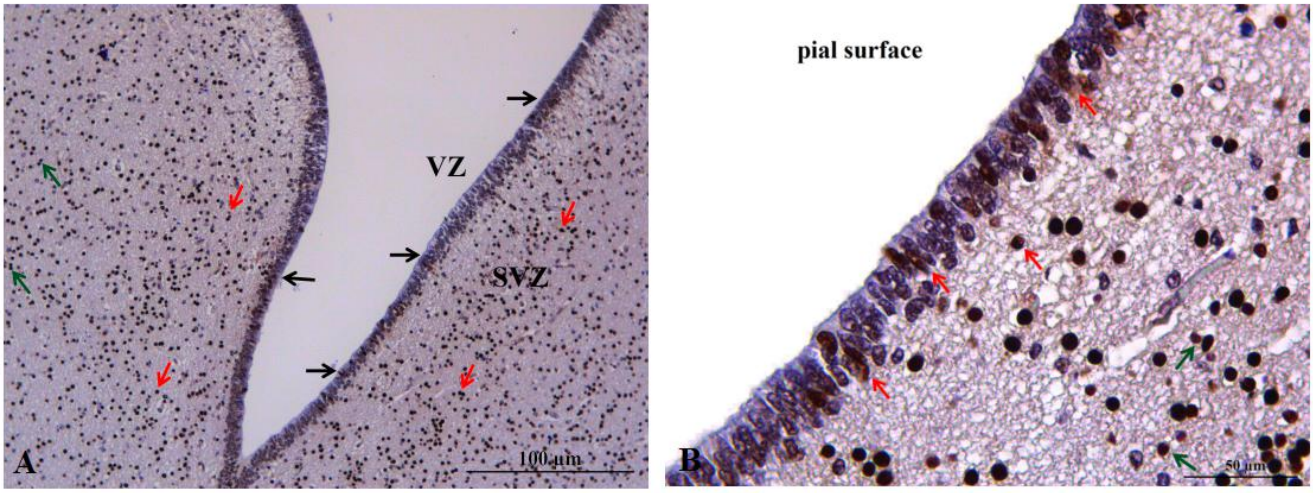

Figure 26. Gestational week 15. A: Pax2 nuclear expression in stem/progenitor cells in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: Pax2 nuclear expression in stem/progenitor cells in the pial surface (red arrows) and in the inner zone (green arrows)

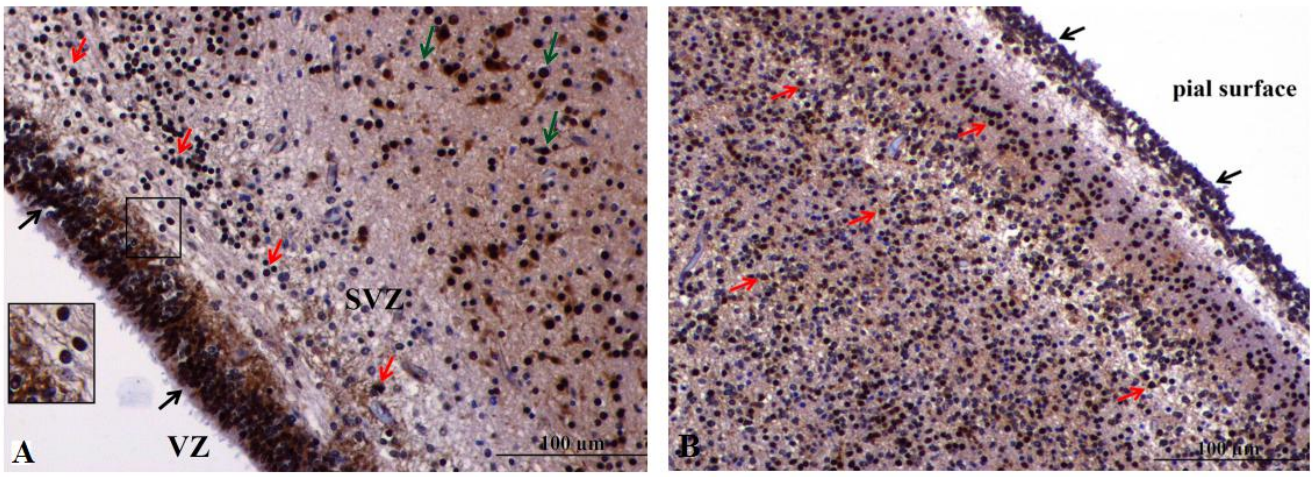

Figure 27. Gestational week 18. A: Pax2 nuclear expression in stem/progenitor cells in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). Insert: 50 µm. B: Pax2 nuclear expression in stem/progenitor cells in the pial surface (black arrows) and in the inner zone (red arrows)

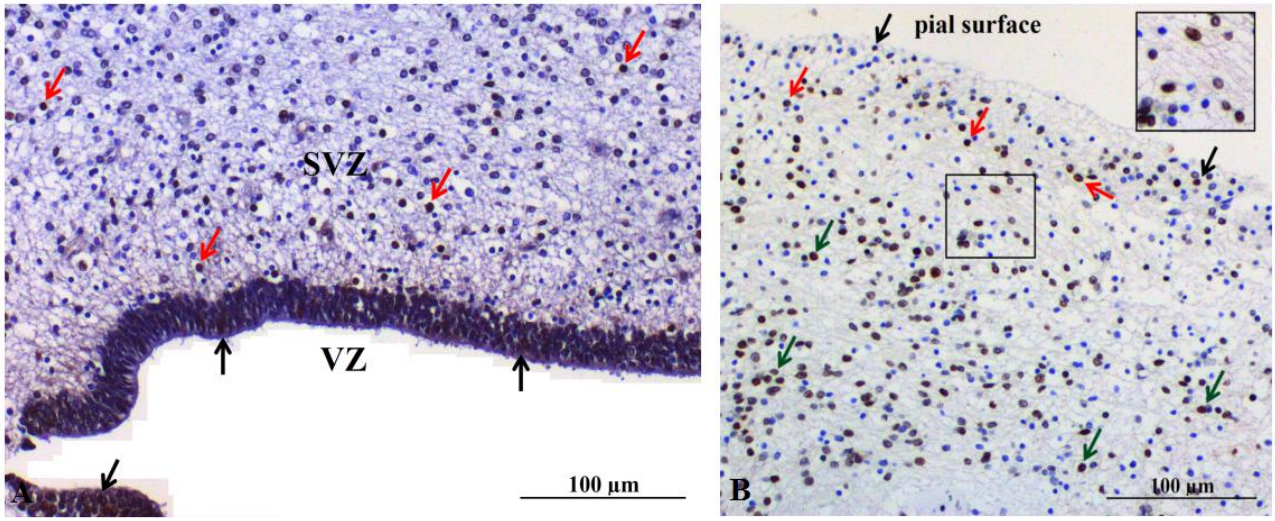

Figure 28. Gestational week 21. A: Pax2 nuclear expression in stem/progenitor cells in the VZ (black arrows), in the SVZ (red arrows). B: Pax2 nuclear expression in migrating progenitor cells in the cortical surface (black arrows), in the molecular layer (red arrows) and in the inner zones (green arrows)

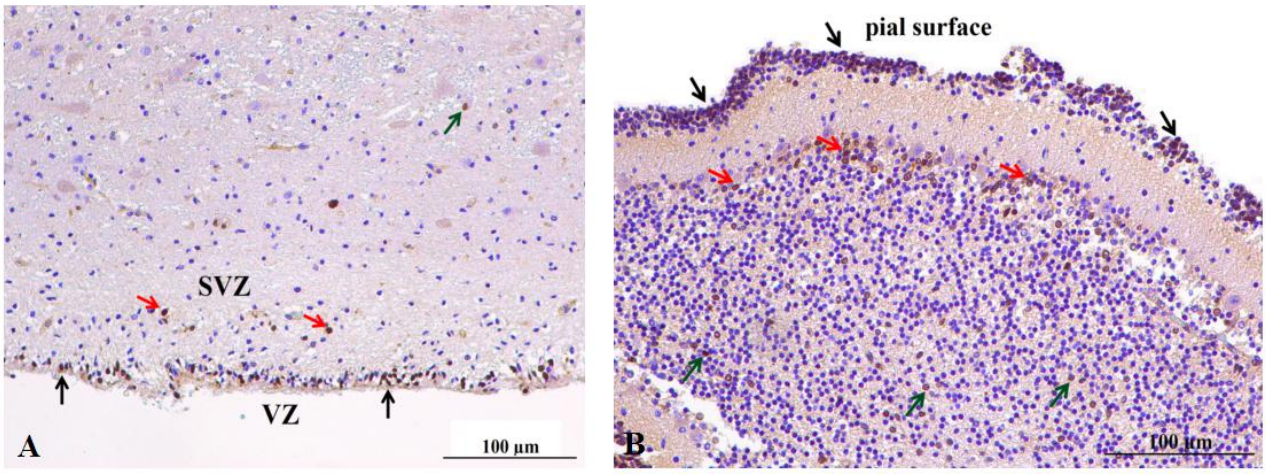

Figure 29. Gestational week 34. A: Pax2 nuclear expression in stem/progenitor cells in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrow). B: Pax2 nuclear expression in migrating progenitor cells in the cortical surface (black arrows), in the Purkinje cell layer (red arrows) and in the inner zone (green arrows)

Pax6

While at the 11st week of gestation we did not found immunoreactivity for Pax6, from the 20th to the 34th week of gestation, nuclear PAX6 immunostaining highlighted granule cell precursors in the cerebellar external granular layer (EGL). At higher magnification, Pax6 highlights the nuclei of granule cell precursors. Scattered PAX6-positive cells were also detected in the inner zone, suggesting the migration of these progenitor cells from the external granular layer towards the internal granule layer (Figures 30, 31).

At the 38th week of gestation, we observed an increase in the number of Pax6-immunoreactive cells in the cortical layers (Figure 32).

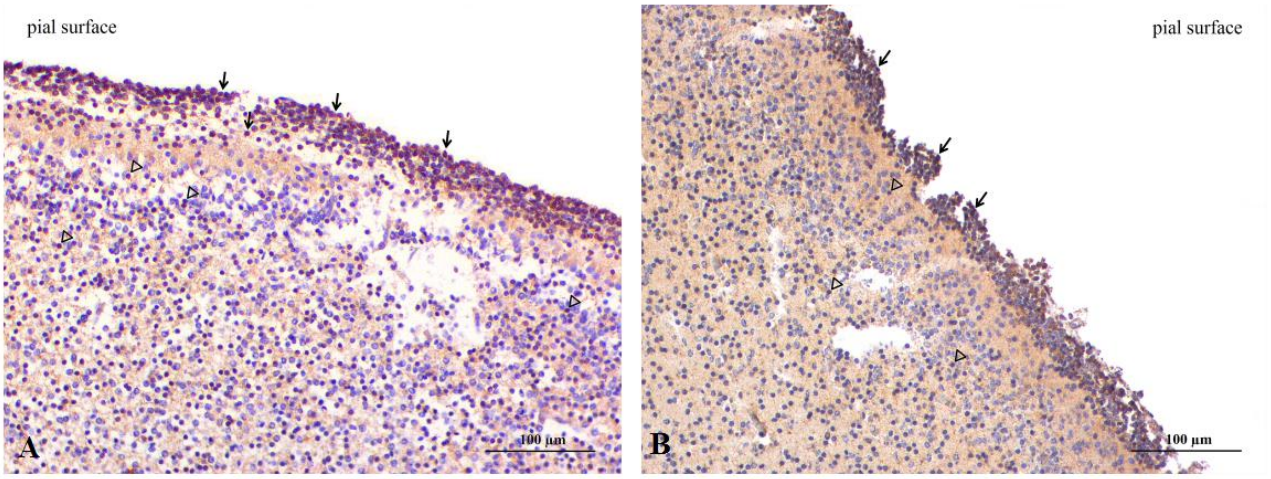

Figure 30. A: Gestational week 20. Pax6 immunoreactivity in the nuclei of granule cell precursors (GCPs; black arrows) in the external granular layer (EGL) migrating towards the internal granular layer (IGL; open arrowheads). B: Gestational week 24. Pax6 immunoreactivity in the nuclei of GCPs (black arrows) in the EGL migrating towards the IGL (open arrowheads)

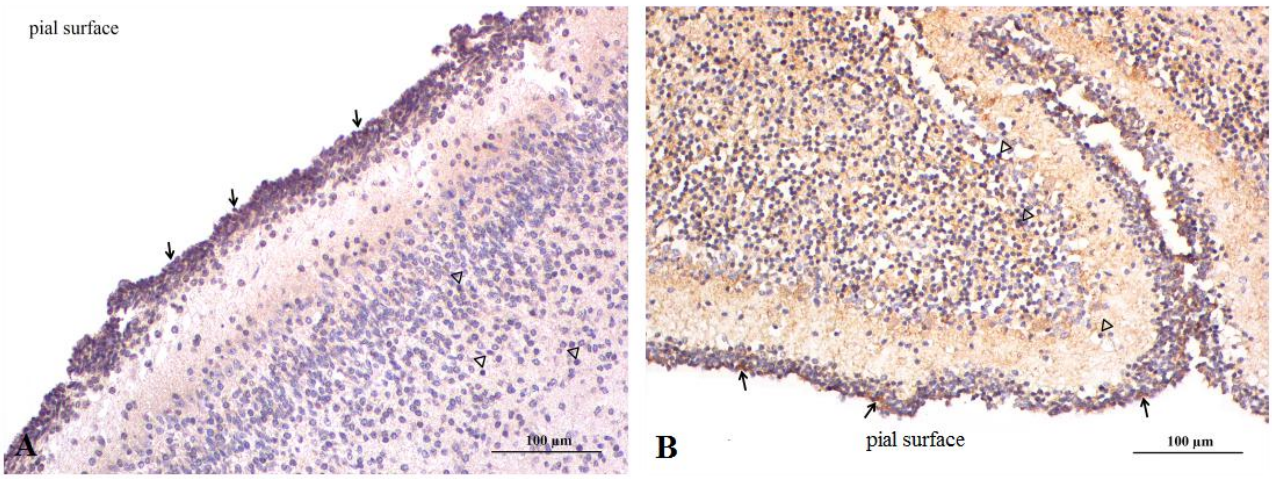

Figure 31. A: Gestational week 30. Pax6 immunoreactivity in the nuclei of GPCs in the EGL (black arrows) migrating cells towards the IGL (open arrowheads). B: Gestational week 34. Pax6 immunoreactivity in the nuclei of GPCs in the EGL (black arrows) migrating towards the IGL (open arrowheads)

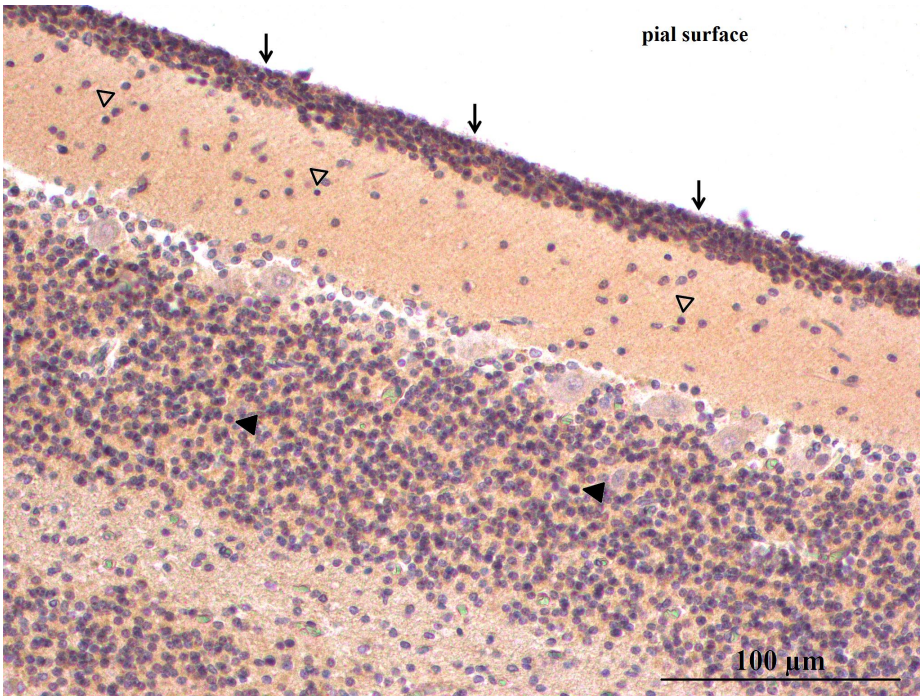

Figure 32. Gestational week 38. Pax6 immunoreactivity in the nuclei of GPCs in the EGL (black arrows), in migrating GPCs in the molecular layer (open arrowheads) and in migrating GPCs in the IGL (arrowheads)

Calretinin

Whereas at 11 weeks of gestation, Calretinin (CR) immunoreactivity was not detected in cerebellar samples, from the 18th to the 38th week CR was expressed in migrating Purkinje cell from the ventricular neuroepithelium towards the cortex, and in different interneuronal populations that migrate from the germinal neuroepithelium towards the cerebellar cortex. Interneurons such as Golgi cells and UBCs appeared to be stain by CR from the 18th week, in contrast to Lugaro cells that appeared at the 20th week (Figures 33-38).

By the 30th week, CR was also expressed in the cytoplasm of granule cells in the internal granular layer (Figure 36-38).

A different immunohistochemical localization of CR was found in the Purkinje cells during development of human cerebellum. At the 30th week, CR was expressed in Purkinje cells arranged in an evident monolayer; in these cells, the expression was mainly detected at the cell membrane level (Figure 36). At the 34th week, the intensity of CR immunoreactivity increased in Purkinje cells of the monolayer. At this gestational age, CR immunostaining was found at the cell surface and also in Purkinje cell dendrites (Figure 37). At the 38th week, some Purkinje cells maintained CR expression at the whole cell surface, in the vast majority of Purkinje cells CR expression was punctuate at the cell surface. Other Purkinje cells were almost completely negative (Figure 38).

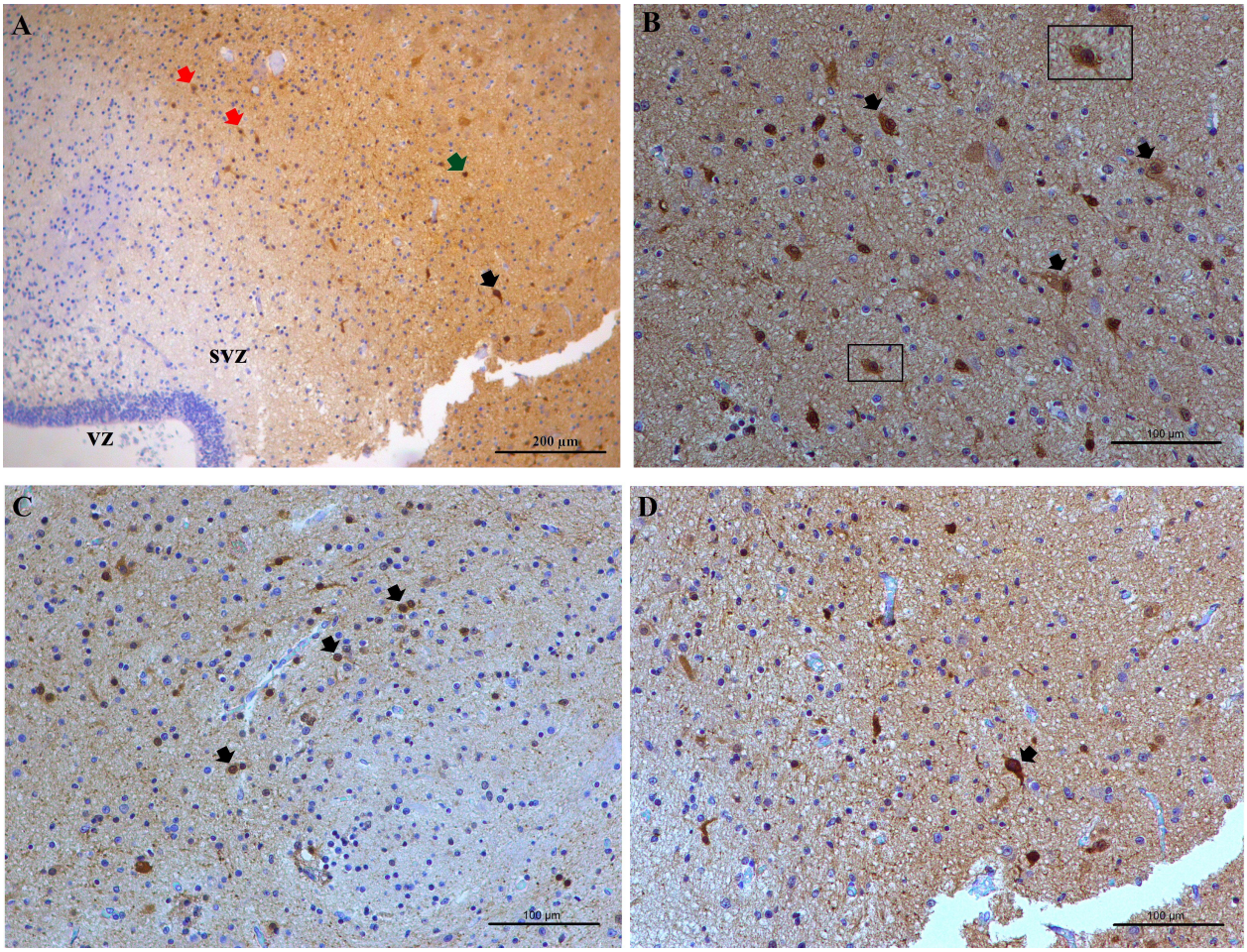

Figure 33. Gestational week 18. A: CR expression in migrating Purkinje cells (red arrows), Golgi cell (green arrow) and unipolar brush cell (black arrow) from the SVZ. B: CR expression in migrating Purkinje cells (black arrows). Insert: 50 µm. C: CR expression in migrating round cells concerning to cerebellar Golgi cells (black arrows). D: CR expression in migrating unipolar brush cell (black arrow)

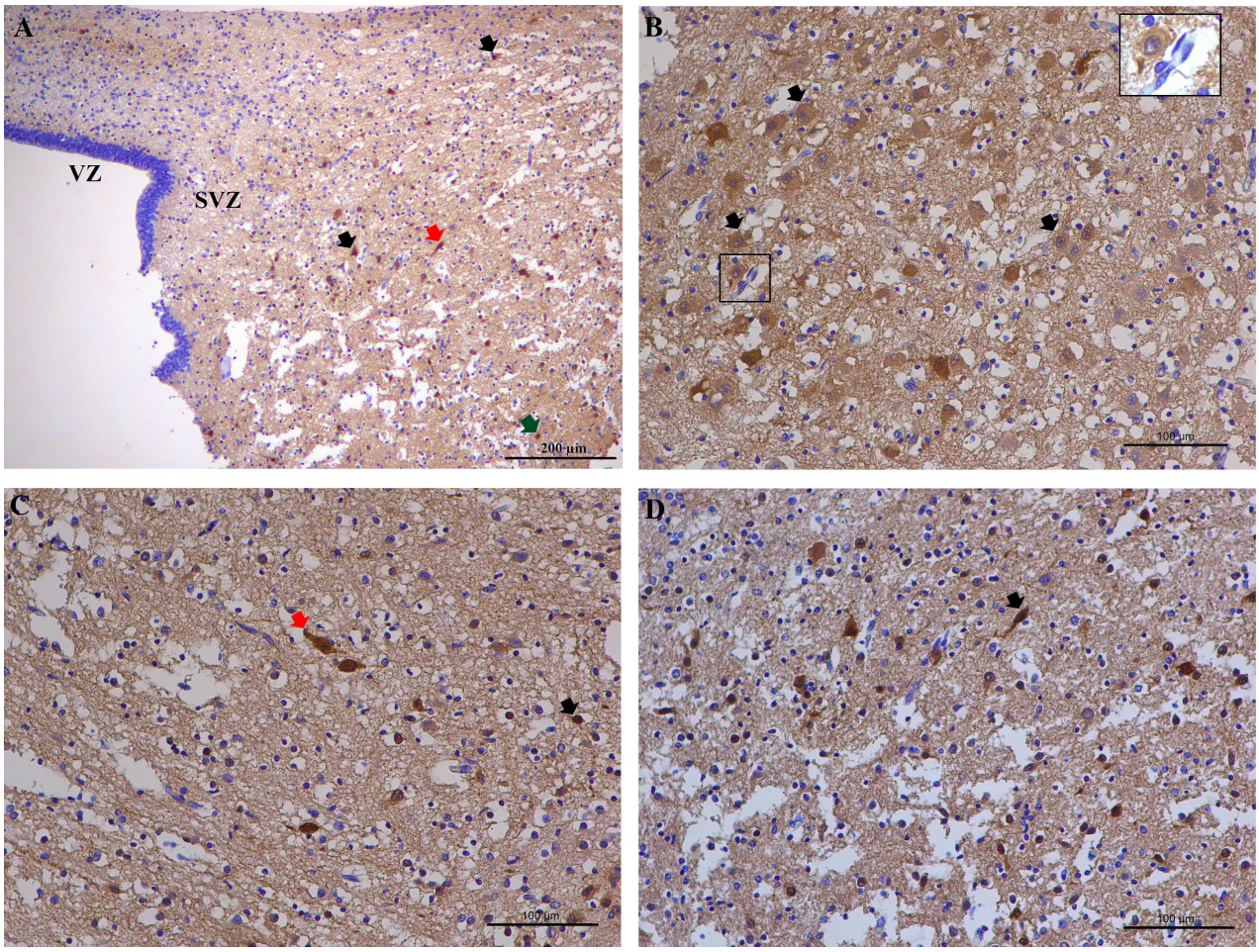

Figure 34. Gestational week 20. A: Immunoreactivity for CR in migrating Purkinje cells (black arrows), Golgi cell (green arrow) and unipolar brush cell (red arrow) from the SVZ. B: Immunoreactivity for CR in migrating Purkinje cells (black arrows). Insert: 50 µm. C: CR immunoreactivity in migrating Golgi cell (black arrow) and unipolar brush cell (red arrow). D: Immunoreactivity for CR in migrating Lugaro cell (black arrow)

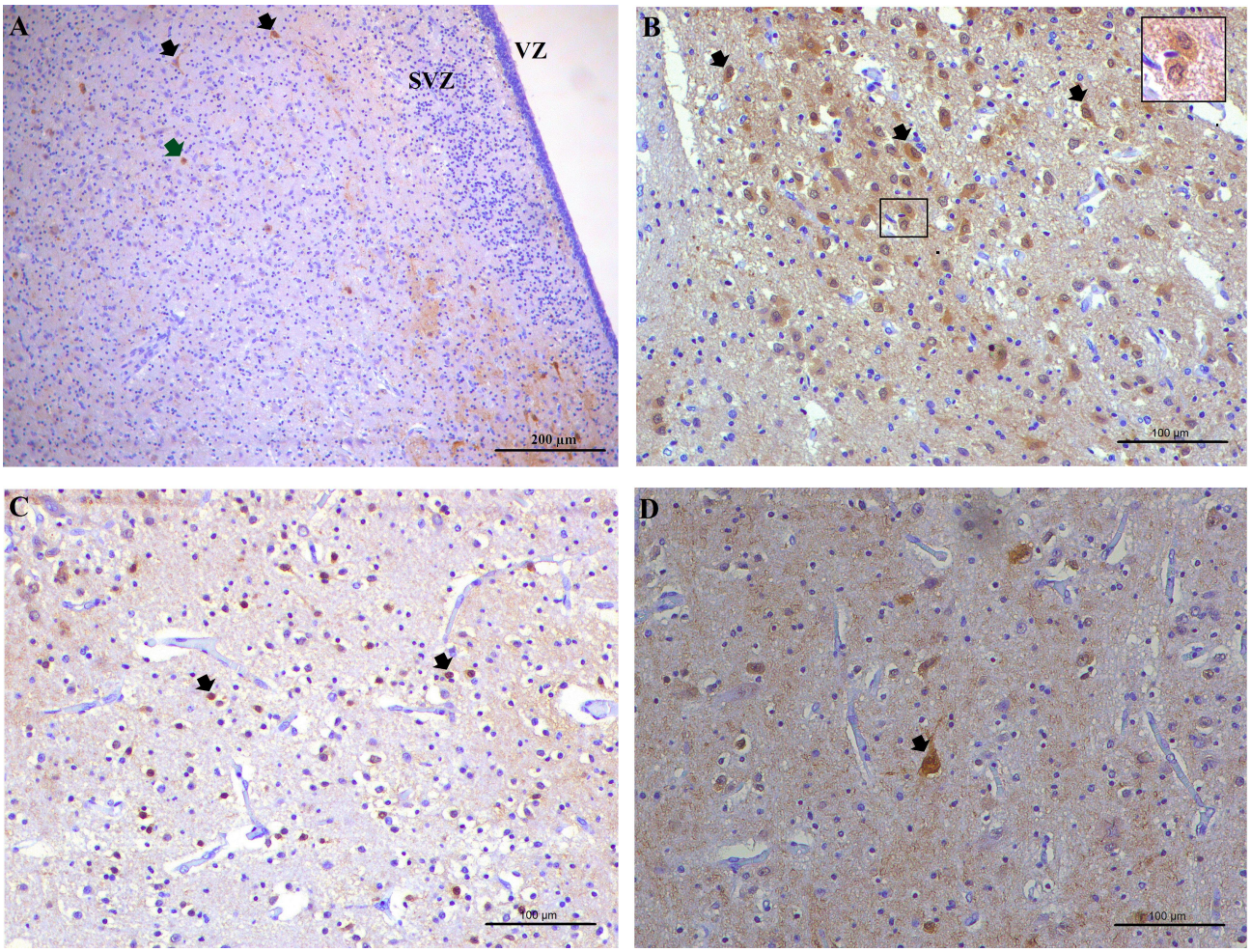

Figure 35. Gestational week 24. A: CR expression in migrating Purkinje cells (black arrows) and Golgi cell (green arrow) from the SVZ. B: CR expression in migrating Purkinje cells (black arrows). Insert: 50 µm. C: CR expression in migrating Golgi cells (black arrows). D: CR expression in migrating unipolar brush cell (black arrow)

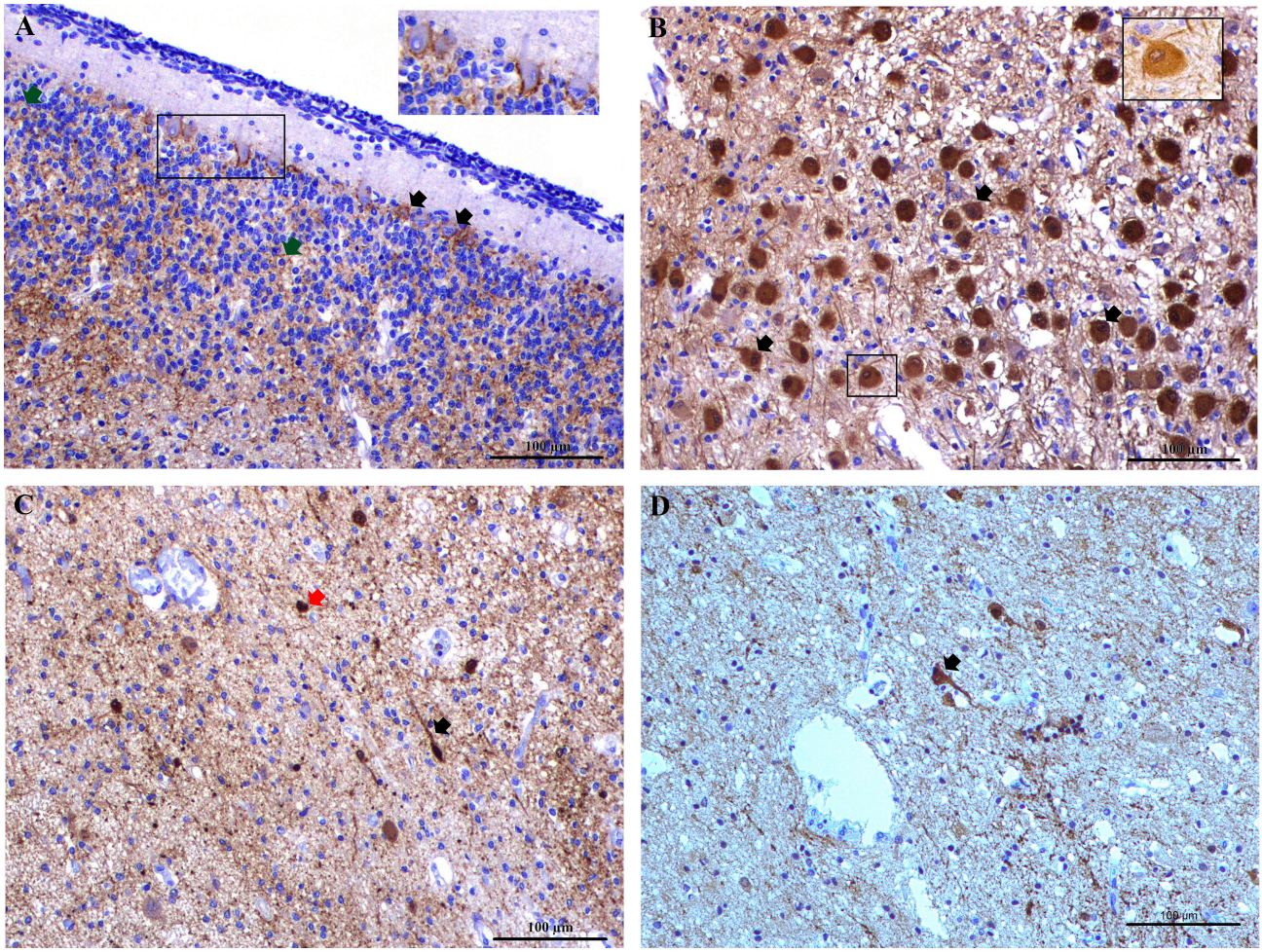

Figure 36. Gestational week 30. A: Immunoreactivity for CR in the cell membrane of Purkinje cells (black arrows). Insert: 50 µm. CR immunoreactivity in granule cells (green arrows). Insert: 50 µm. B: Immunoreactivity for CR in migrating Purkinje cells (black arrows). C: CR immunoreactivity in migrating Lugaro cell (black arrow) and in Golgi cell (red arrow). D: Immunoreactivity of CR in migrating unipolar brush cell (black arrow)

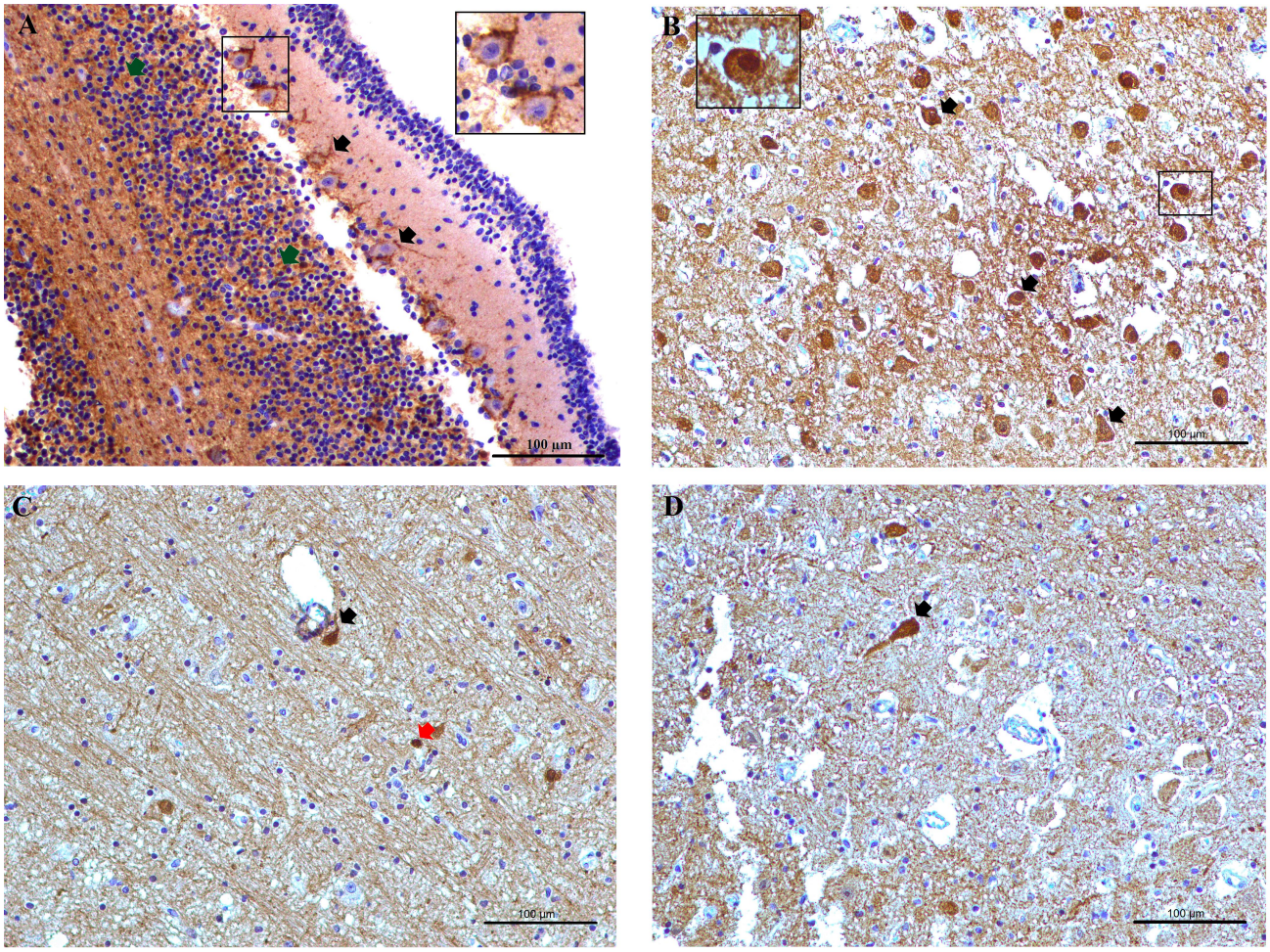

Figure 37. Gestational week 34. A: Immunoreactivity for CR in the cell surface of Purkinje cells (black arrows). CR immunoreactivity in granule cells (green arrows). Insert: 50 µm. B: CR immunoreactivity in migrating Purkinje cells (black arrows). Insert: 50 µm. C: Immunoreactivity for CR in migrating unipolar brush cell (black arrow) and in Golgi cell (red arrow). D: Immunoreactivity of CR in migrating Lugaro cell (black arrow)

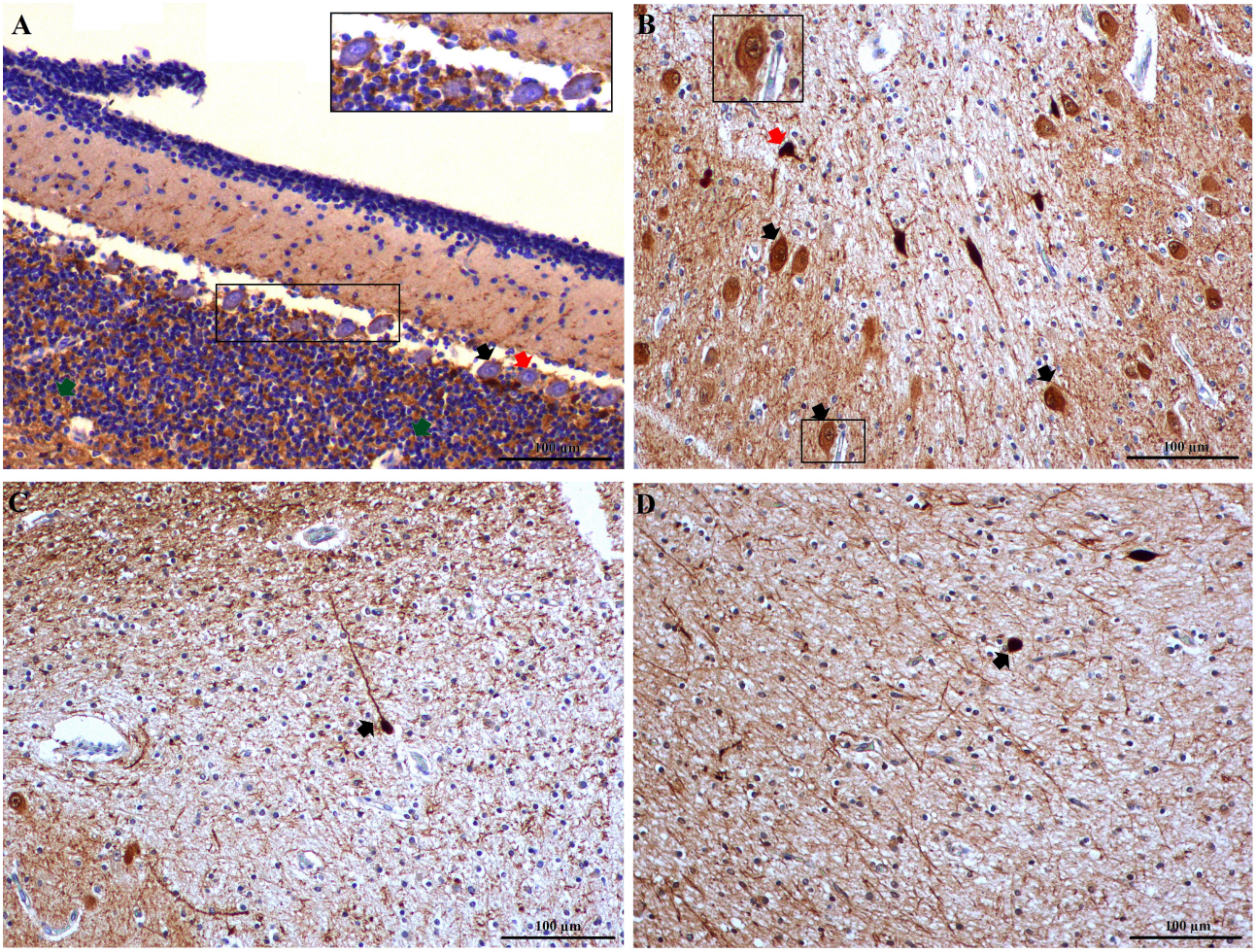

Figure 38. Gestational week 38. A: CR expression in the cell membrane of Purkinje cells (black arrow). Negative expression for CR in some Purkinje cells (red arrow). CR expression in granule cells (green arrows). Insert: 50 µm. B: CR expression in migrating Purkinje cells (black arrows) and unipolar brush cell (red arrow). Insert: 50 µm. C: CR expression in migrating Lugaro cell (black arrow). D: Expression of CR in migrating Golgi cell (black arrow)

Mature neuron cell markers

Neurofilament (NF)

From the 15th to the 34th week, a strong immunoreactivity for NF-L was detected in cytoplasmatic projections of intermediate progenitor cells (iPCs) in the SVZ. No reactivity was found in the VZ (Figures 39-42).

At the same gestational ages, NF-L immunoreactivity was also detected in the cytoplasm of neuron projections reaching the cerebellar cortex (Figures 39-42).

At the 34th and the 38th week, a strong nuclear and cytoplasmatic expression was found in the Purkinje cells in the Purkinje cell layer, highlighting also the Purkinje cell dendrites in the molecular layer (Figures 43, 44).

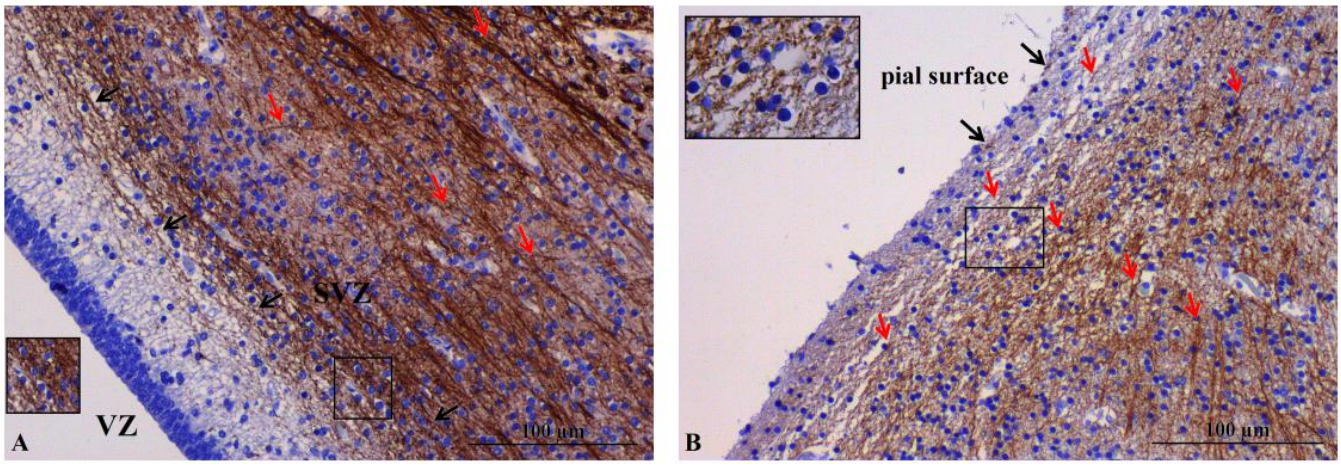

Figure 39. Gestational week 15. A: Immunoreactivity for NF-L in the cytoplasm of progenitor cells projections in the SVZ (black arrows) and in the inner zones (red arrows). Insert: 50 µm. B: Immunoreactivity for NF-L in the cytoplasm of progenitor cells projections in the pial surface (black arrows) and in the inner zones (red arrows). Insert: 50 µm

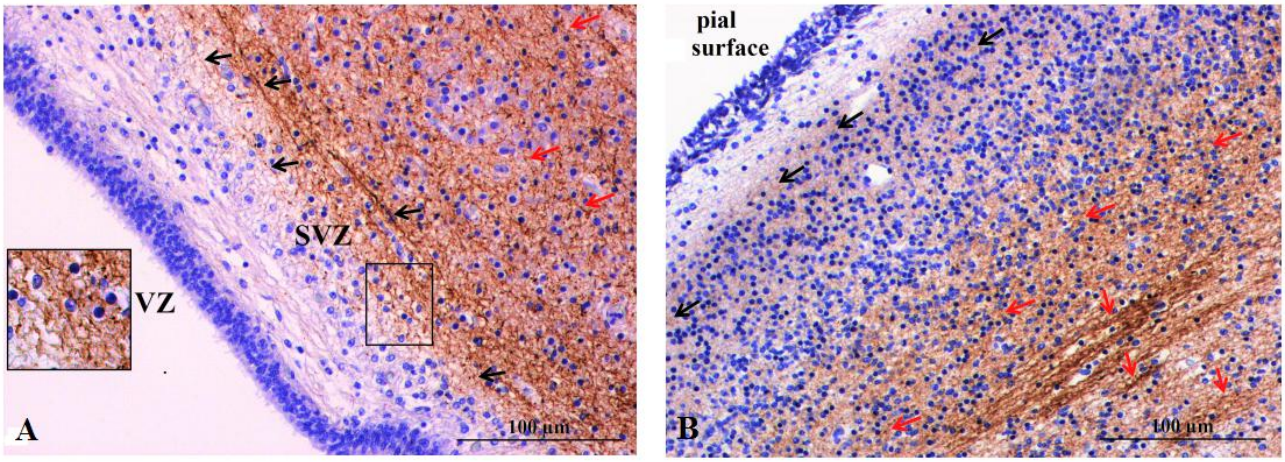

Figure 40. Gestational week 18. A: Immunoreactivity for NF-L n the cytoplasm of progenitor cells projections in the SVZ (black arrows) and in the inner zones (red arrows). Insert: 50 µm. B: Immunoreactivity for NF-L in the cytoplasm of progenitor cells projections in the Purkinje cell layer (black arrows) and in the inner zones (red arrows)

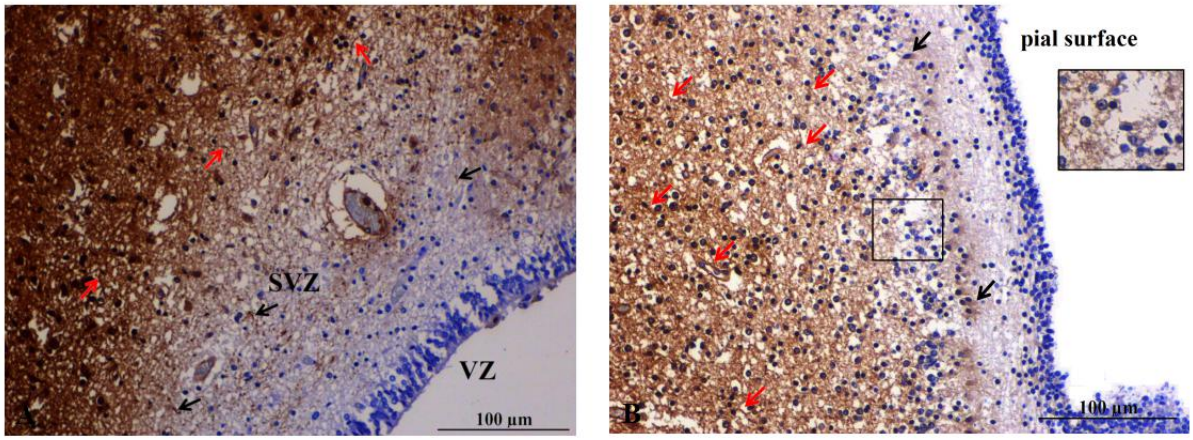

Figure 41. Gestational week 21. A: Immunoreactivity for NF-L in the cytoplasm of progenitor cells projections in the SVZ (black arrows) and in the inner zones (red arrows). B: Immunoreactivity for NF-L in the cytoplasm of progenitor cells projections in the Purkinje cell layer (black arrows) and in the inner zones (red arrows). Insert: 50 µm

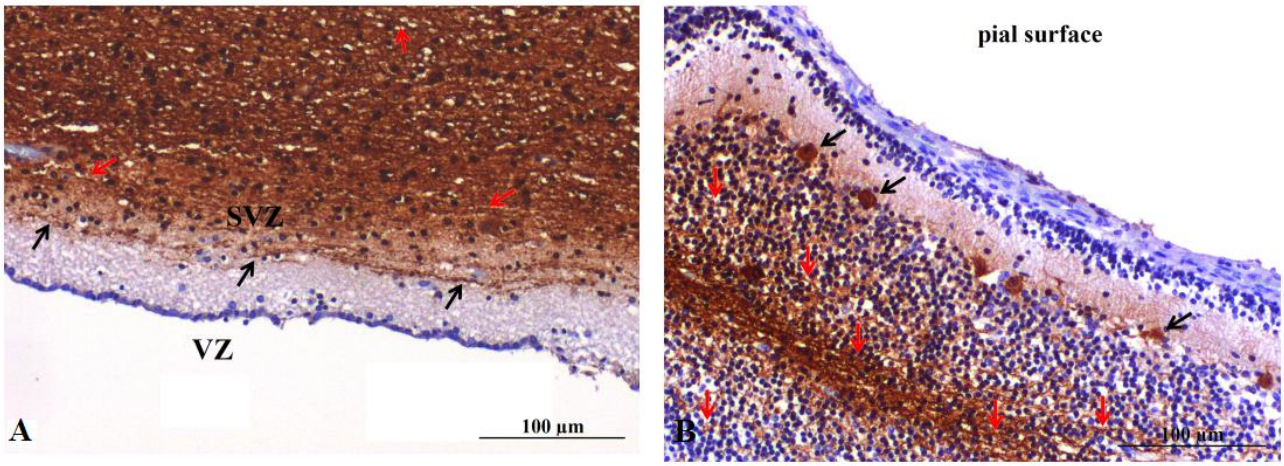

Figure 42. Gestational week 34. A: Immunoreactivity for NF-L in the cytoplasm of progenitor cells projections in the SVZ (black arrows) and in the inner zones (red arrows). B: Immunoreactivity for NF-L in the cytoplasm of progenitor cells projections in the Purkinje cell layer (arrows) and in the inner zones (red arrows). Insert: 50 µm

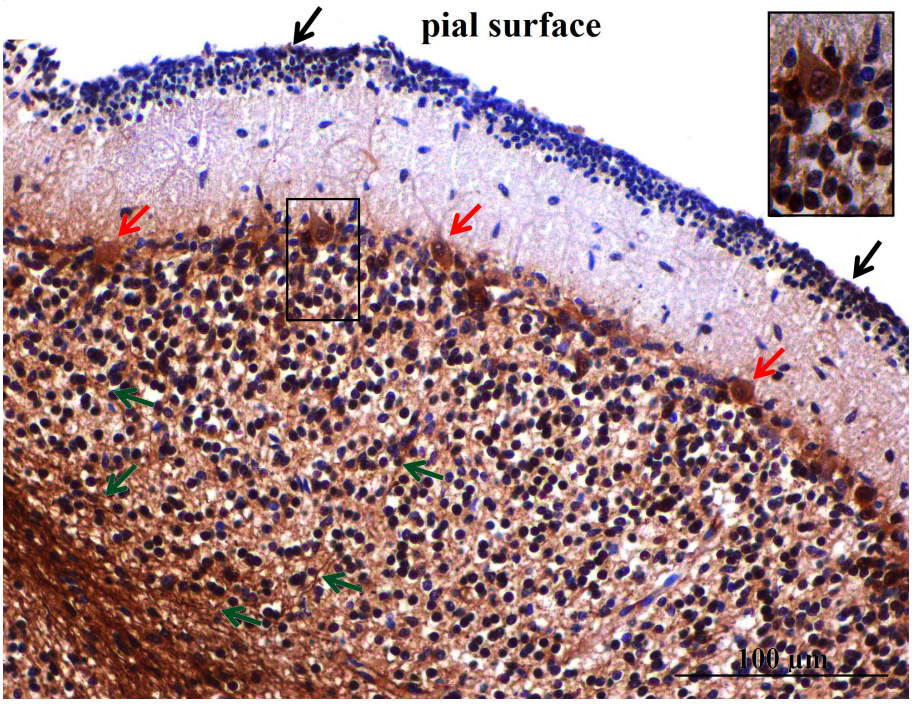

Figure 43. Gestational week 38. Immunoreactivity for NF-L in the cytoplasm of progenitor cells projections in the pial surface (arrows), in the molecular layer (red arrow), in the Purkinje cell layer (green arrows) and in the inner zones (orange arrows). Insert: 50 µm

NSE (neuron specific enolase)

From the 15th to the 18th week, a strong nuclear and cytoplasmatic immunoreactivity for NSE was found in progenitor cells in the VZ, SVZ and in the intermediate zone (Figures 44, 45).

At the 21st and the 34th week, a mild expression for NSE was detected in the VZ (Figures 46, 47).

From the 15th to the 38th week, a strong immunoreactivity for NSE was also observed in progenitor cells in the cortical zones.

By the 34th week, to the 38th week, NSE expression was found, at the cytoplasmatic but not nuclear level, in Purkinje cells in the Purkinje cell layer of the cerebellar cortex (Figure 48).

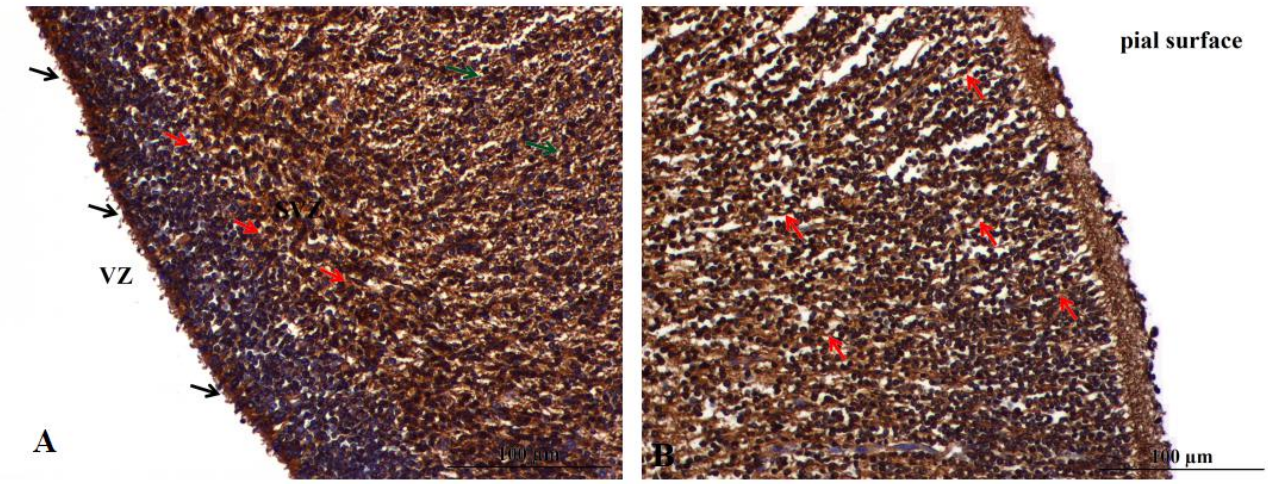

Figure 44. Gestational week 15. A: Immunoreactivity for NSE in the cytoplasm of progenitor cells projections in the VZ (black arrows), in the SVZ (red arrows) and in the inner zones (green arrows). Insert: 50 µm. B: Immunoreactivity for NSE in the cytoplasm of progenitor cells projections in the cortical intermediate zones (red arrows)

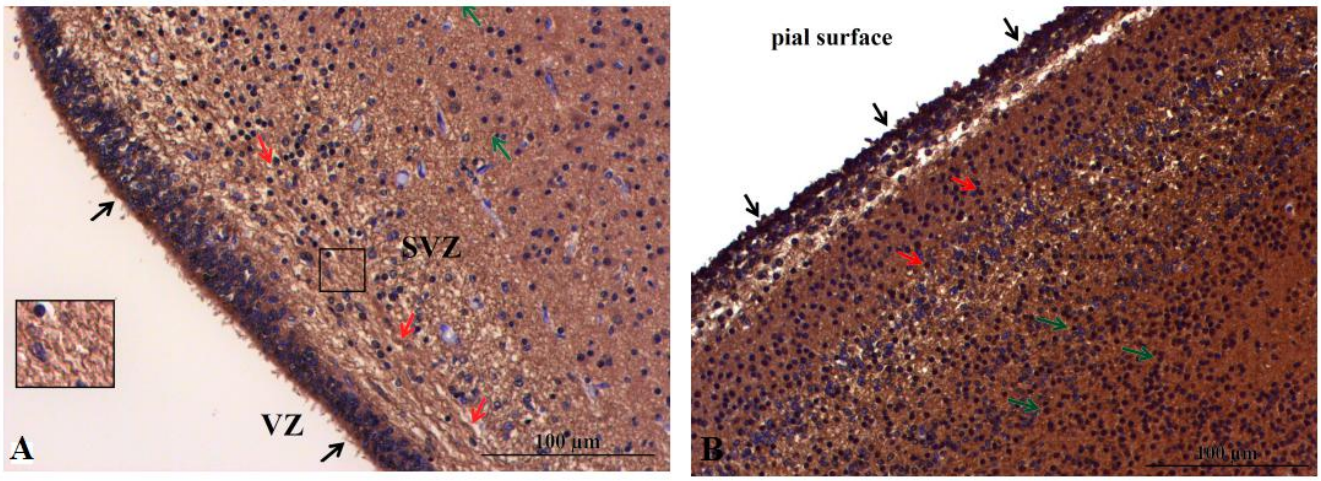

Figure 45. Gestational week 18. A: Immunoreactivity for NSE in the cytoplasm of progenitor cells projections in the VZ (black arrows), in the SVZ (red arrows) and in the inner zones (green arrows). Insert: 50 µm. **B:** Immunoreactivity for NSE in the nuclei and cytoplasm of progenitor cells in the pial surface (black arrows), in the molecular layer (red arrows), and in the inner zones (green arrows)

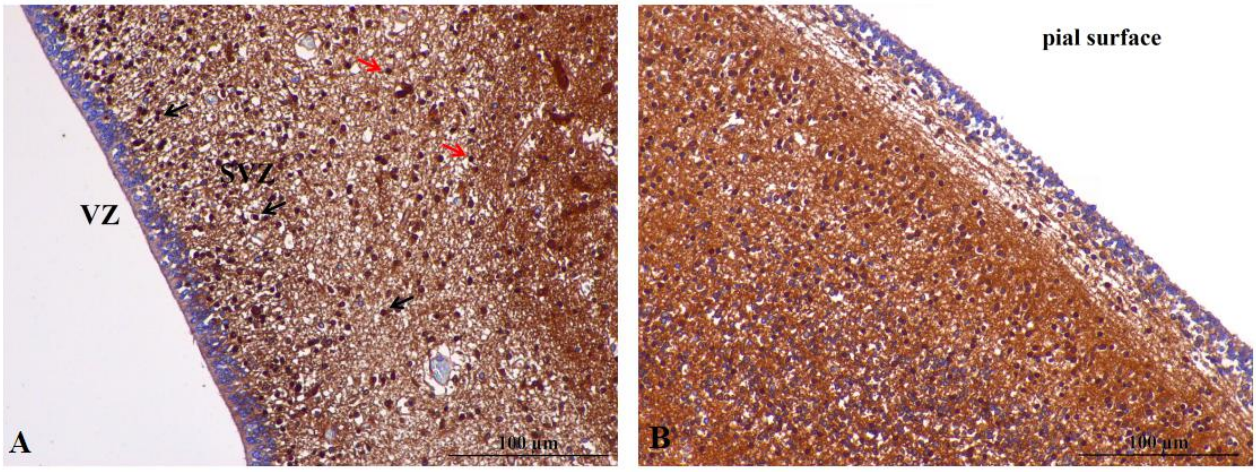

Figure 46. Gestational week 21. A: Immunoreactivity for NSE in the cytoplasm of progenitor cells projections in the SVZ (black arrows) and in the inner zones (red arrows). B: Immunoreactivity for NSE in cytoplasmatic and nuclear progenitor cells in the cerebellar cortical zones

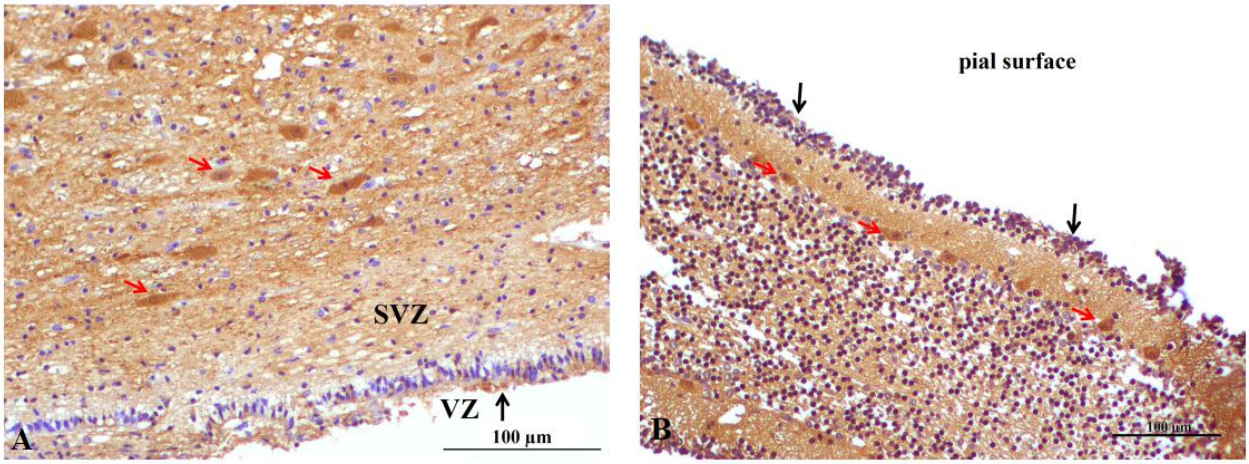

Figure 47. Gestational week 34. A: Immunoreactivity for NSE in the nuclei and cytoplasm of progenitor cells in the VZ (black arrows) and in the SVZ (red arrows). B: Immunoreactivity for NSE in the nuclei and cytoplasm of progenitor cells in the pial surface (black arrows) and in the Purkinje cells (red arrows). Insert: 50 µm

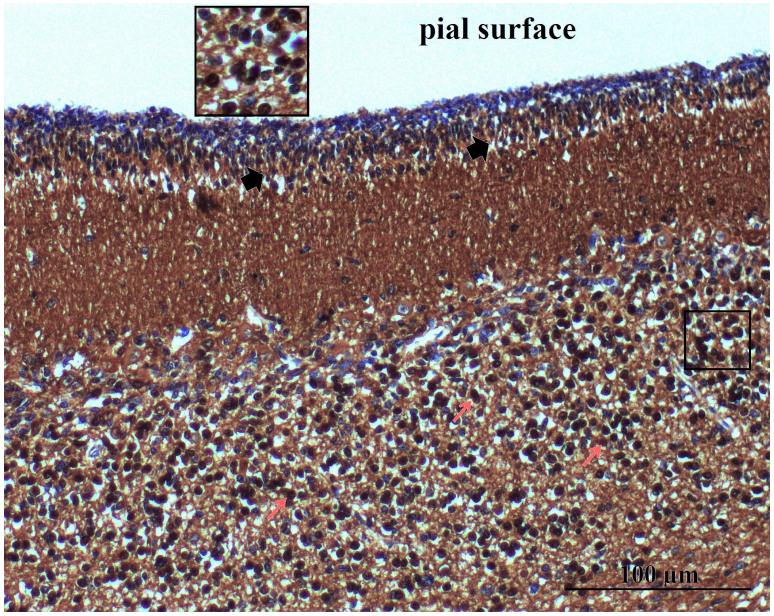

Figure 48. Gestational age 38. Immunoreactivity for NSE in the nuclei and cytoplasm of progenitor cells in the molecular layer (black arrows) and in the inner zones (red arrows). Insert: 50 µm

Mature glial markers

S100β

At the 15th week of gestation, immunoreactivity for S100β was detected in the nuclei and cytoplasm of scattered astrocyte-like cells localized in the inner zone and in the cortical layers. No significant staining was observed in the ventricular zone (Figure 49).

From the 18th to the 34th week of gestation, immunoreactivity for S100β was also found in the ventricular and subventricular zones (Figures 50, 51), although at the 34th week this staining was weaker than in the previous developmental stages (Figure 52).

After the 34th until the 38th week of gestation, nuclear and cytoplasmatic immunoreactivity for S100β was particularly strong in the cortical layers, being localized in the cytoplasm of glial projections in the molecular layer and in glial projections around the Purkinje cells in the Purkinje cell layer, and also in nuclei of progenitor glial cells in the inner zone (Figure 53).

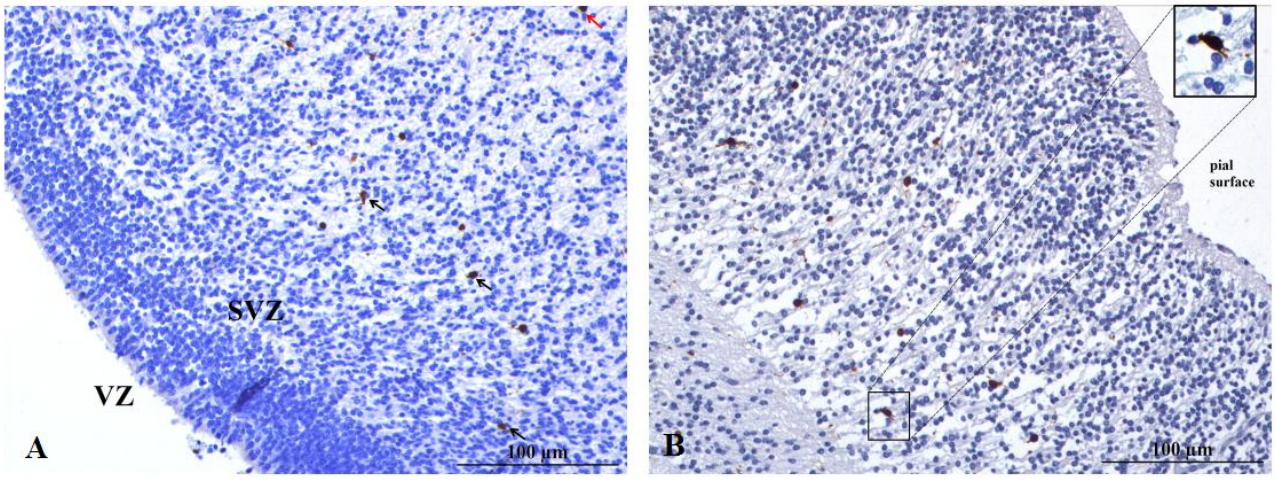

Figure 49. Gestational age 15. A: Immunoreactivity for S100β in scattered progenitor cells in the SVZ (black arrows) and in the inner zone (red arrow). B: Immunoreactivity for S100β in progenitor cells in the cortical zones. Insert: 50 µm

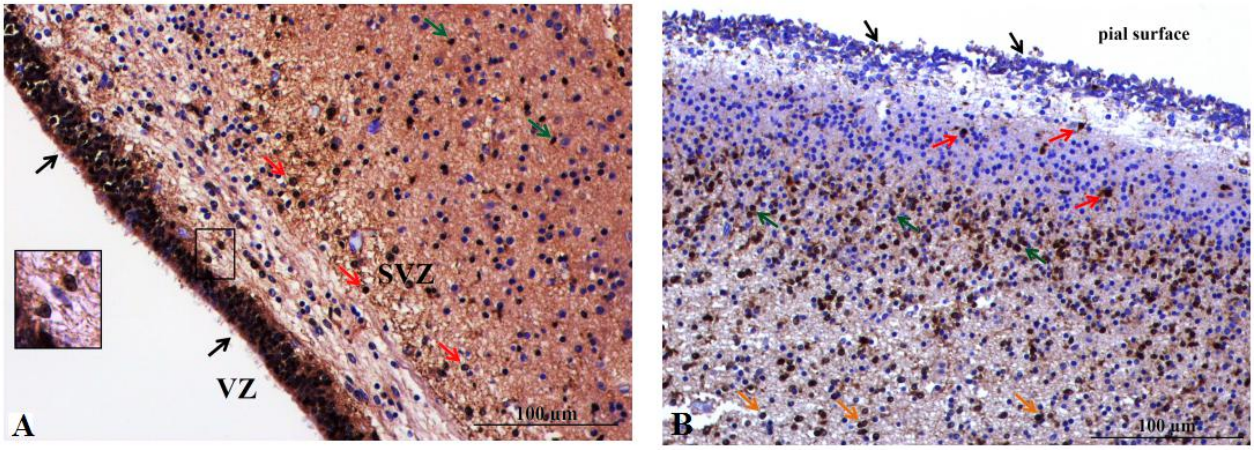

Figure 50. Gestational week 18. A: Immunoreactivity for S100β in progenitor cells in the VZ (arrows), in the SVZ (red arrows) and in the inner zones (green arrows). Insert: 50 µm. B: Immunoreactivity for S100β in progenitor cells in the pial surface (black arrows), in the molecular layer (red arrows), in the Purkinje cell layer (green arrows) and in the inner zones (orange arrows)

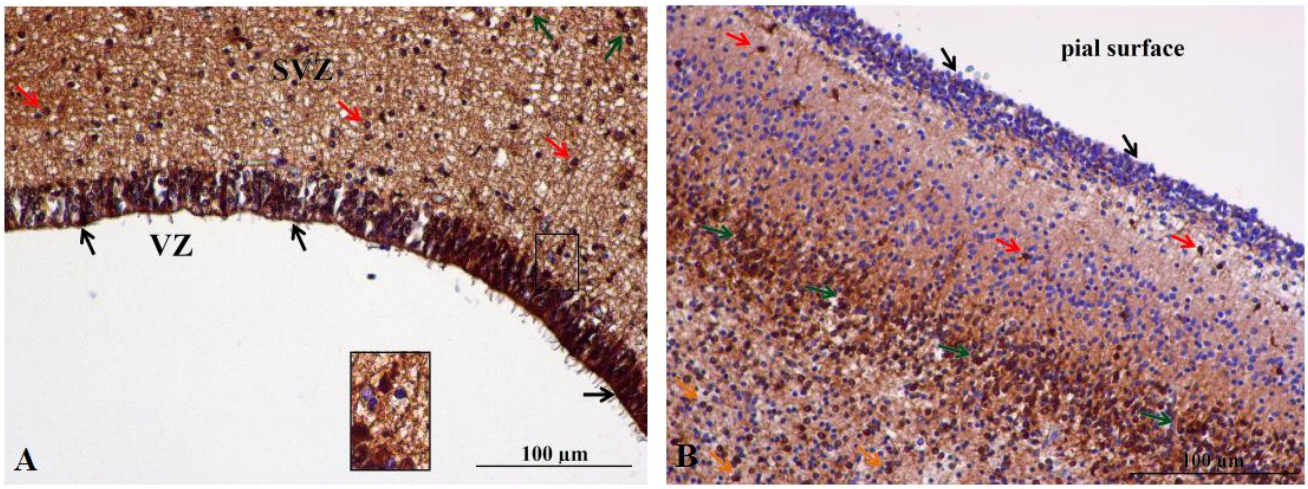

Figure 51. Gestational week 21. A: Immunoreactivity for S100β in progenitor cells in the VZ (arrows), in the SVZ (red arrows) and in the inner zones (green arrows). Insert: 50 µm. B: Immunoreactivity for S100β in progenitor cells in the pial surface (black arrows), in the molecular layer (red arrows), in the Purkinje cell layer (green arrows) and in the inner zones (orange arrows)

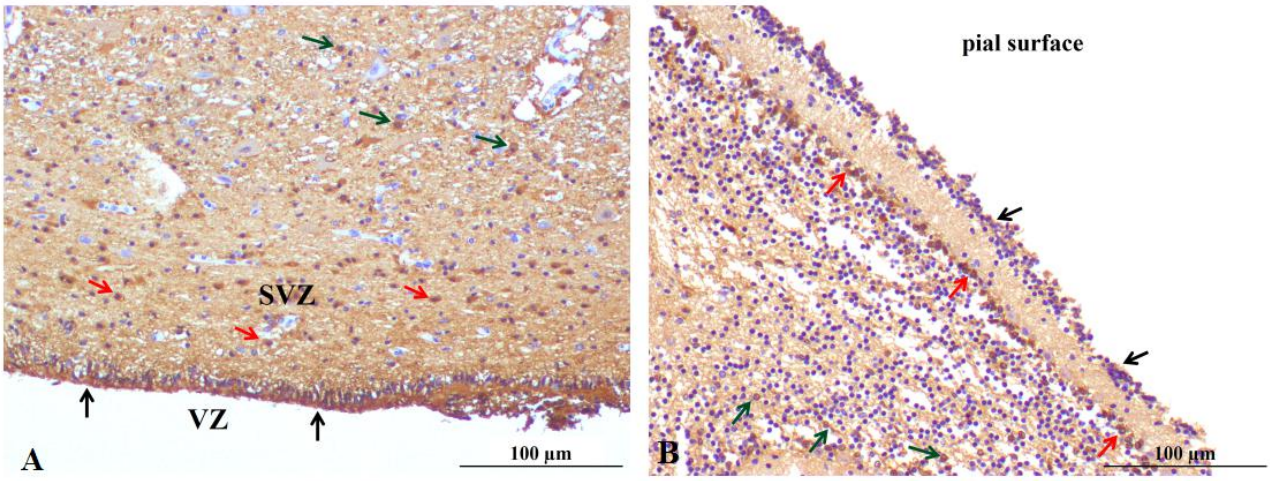

Figure 52. Gestational week 34. A: Immunoreactivity for S100β in progenitor cells in the VZ (arrows), in the SVZ (red arrows) and in the inner zones (green arrows). B: Immunoreactivity for S100β in progenitor cells in the pial surface (black arrows), in the Purkinje cell layer (red arrows) and in the inner zones (orange arrows)

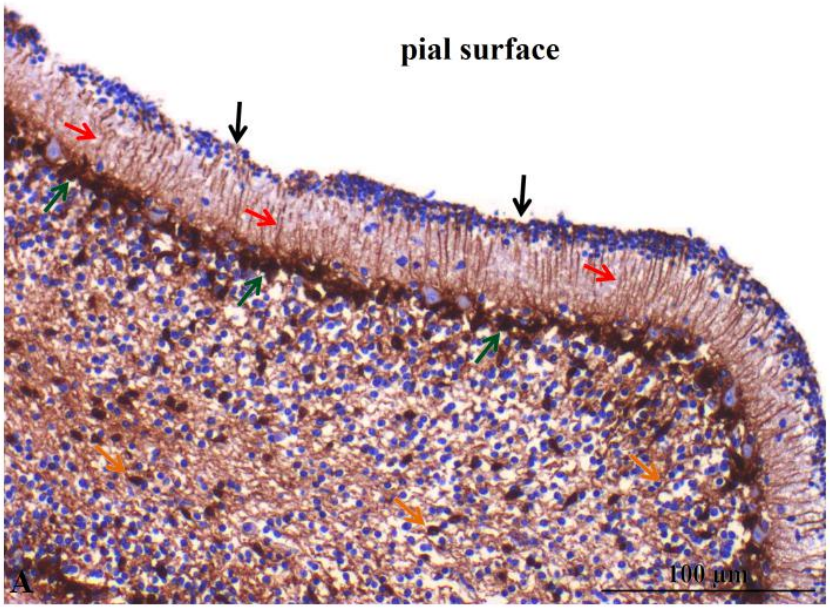

Figure 53. Gestational week 38. Immunoreactivity for S100β in progenitor cells in the pial surface (black arrows), in the molecular layer (red arrows), in the Purkinje cell layer (green arrows) and in the inner zones (orange arrows)

GFAP

At the 15th week of gestation, immunoreactivity for GFAP was detected in the cytoplasmatic radial glial projections in the ventricular zone and in the subventricular zone, reaching the cortical layers (Figure 54).

From the 18th to the 34th week of gestation, that immunostaining for GFAP increased both in the ventricular and in the subventricular zone (Figure 55, 57). Moreover, a strong nuclear and cytoplasmatic expression of GFAP was detected in glial cells of the intermediate zone in absence of reacivity of mature neurons. At the same gestational ages, immunoreactivity for GFAP was also detected in the cortical layers.

At the 38th week of gestation, a strong nuclear and cytoplasmatic expression of GFAP was found in mature glial cells, being well characterized in the molecular layer, around the soma of the Purkinje cells and in the internal granular layer (Figure 58).

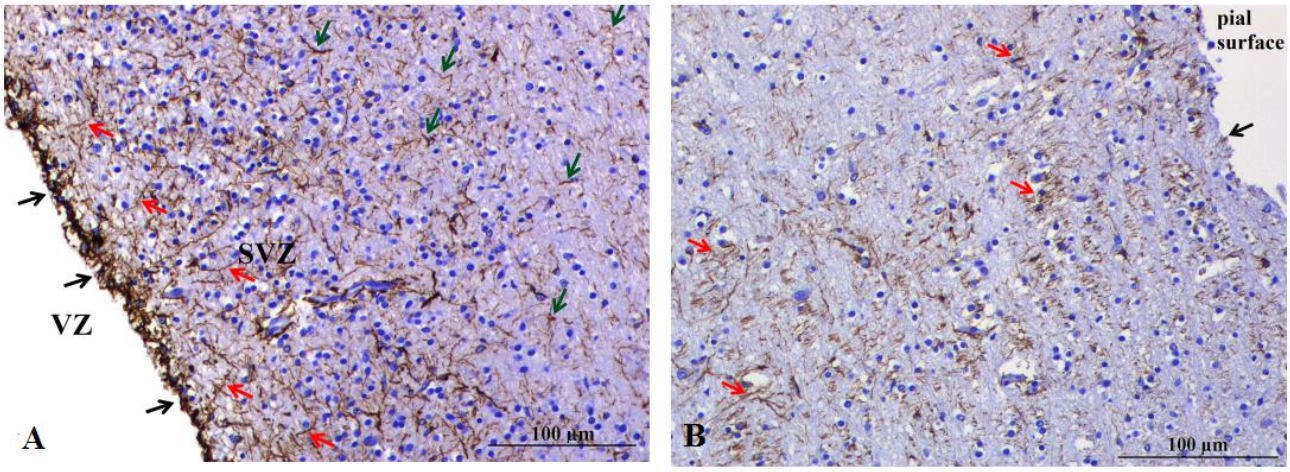

Figure 54. Gestational week 15. A: Immunoreactivity for GFAP in radial glial projections in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: Immunoreactivity for GFAP in radial glial projections in the pial surface (black arrow) and in the inner zones (red arrows)

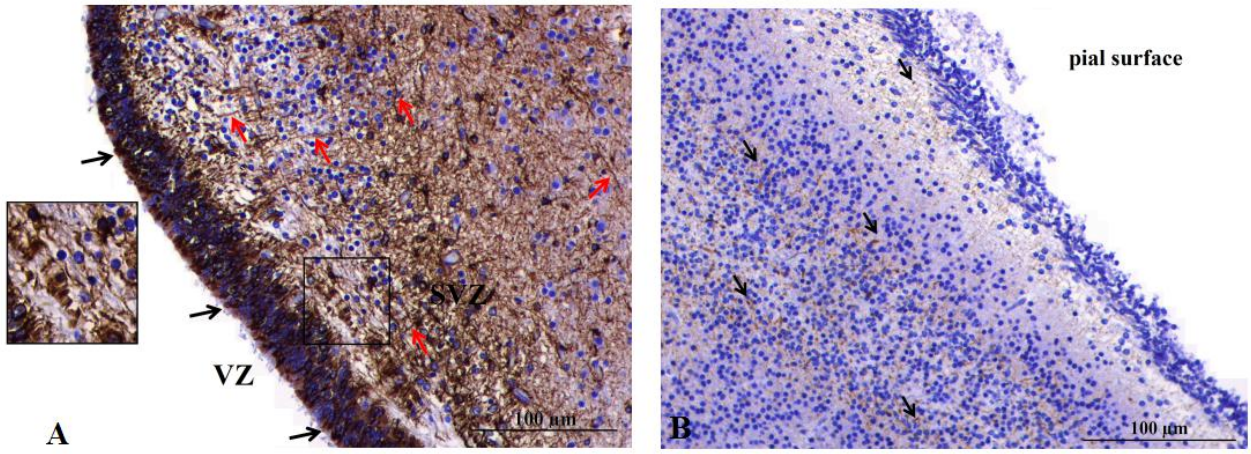

Figure 55. Gestational week 18. A: Immunoreactivity for GFAP in radial glial projections in the VZ (black arrows) and in the SVZ (red arrows). Insert: 50 µm. B: Immunoreactivity for GFAP in radial glial projections in the cortical zones (black arrows)

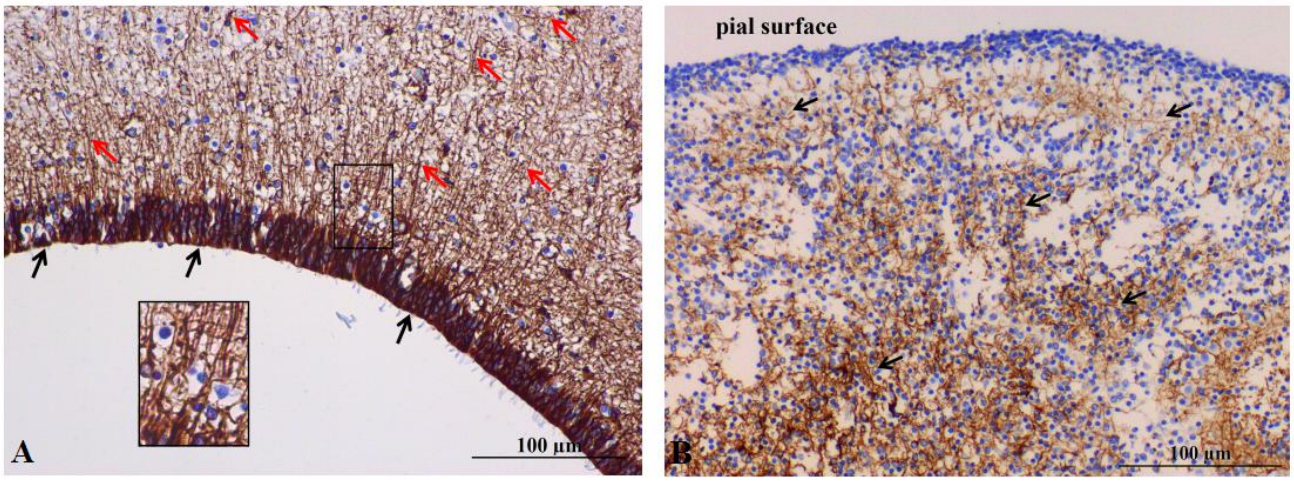

Figure 56. Gestational week 21. A: Immunoreactivity for GFAP in radial glial projections in the VZ (black arrows) and in the SVZ (red arrows). Insert: 50 µm. B: Immunoreactivity for GFAP in radial glial projections in the cortical zones (black arrows)

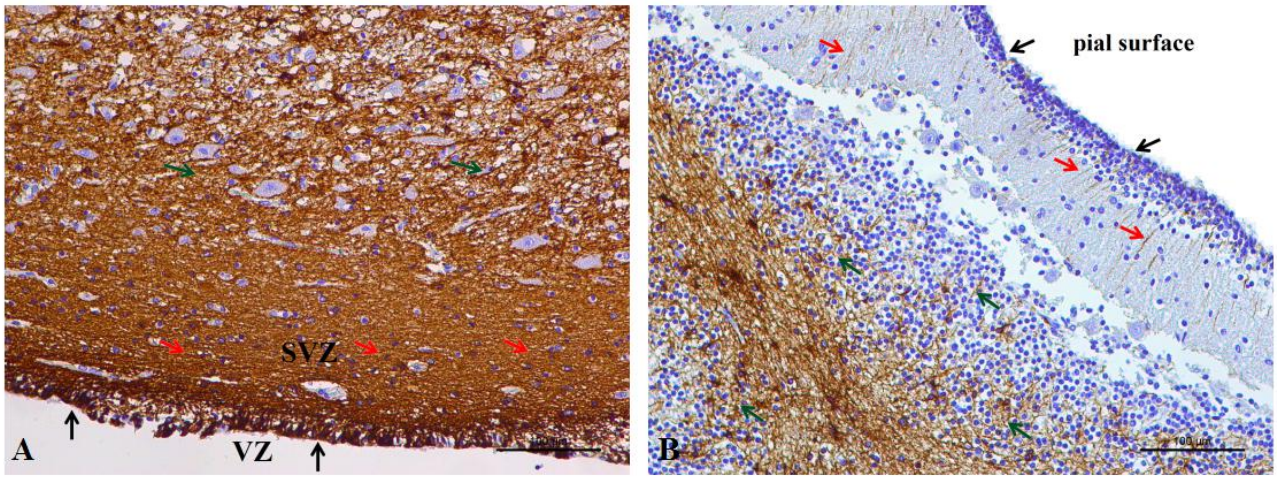

Figure 57. Gestational week 34. A: Immunoreactivity for GFAP in radial glial projections in the VZ (black arrows), in the SVZ (red arrows) and in the inner zone (green arrows). B: Immunoreactivity for GFAP in radial glial projections in the pial surface (black arrows), in the molecular layer (red arrows) and in the inner zone (green arrows)

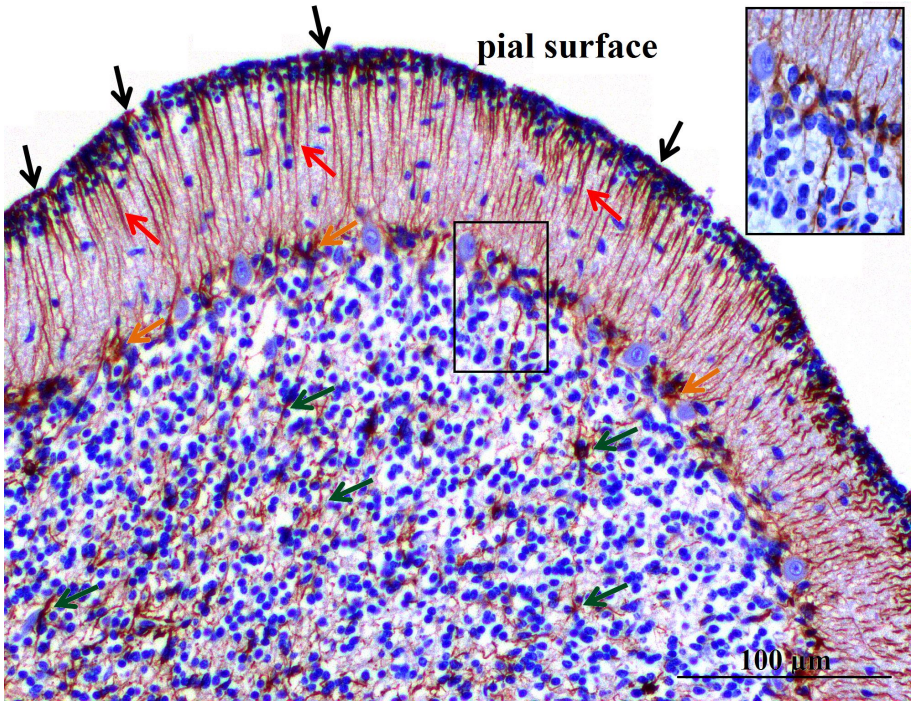

Figure 58. Gestational week 38. Immunoreactivity for GFAP in glial projections in the pial surface (black arrows), in the molecular layer (red arrows), in the Purkinkje cell layer (orange arrows) and in the inner zone (green arrows)

Discussion

The development of the human cerebellum, as well as for other organs, represents a very tricky period of embryogenesis, being characterized by critical molecular and cellular events including proliferation, migration and differentiation of multiple cell types. Previous studies, carried out on experimental models, evidenced that immunohistochemistry may allow the identification of different neural and glial precursor cells in the different cerebellar zones during development. This study confirms other previous researches focused on the developing cerebellum based on immunohistochemistry as a useful tool for the identification of stem/progenitor cells. Hence, immunoreactivity for several molecular markers allow the identification of different stages of differentiation of the neuronal and glial lineages.

As it has already been described, human cortical neurogenesis begins in the ventricular and subventricular zones when NECs undergo to asymmetric divisions giving rise to progenitor cells of the developing cerebellar cortex, radial glial cells and intermediate progenitor cells, which are generally considered to represent the major source of cortical neurons.

Stem cell self-renewal and progenitor cell differentiation is regulated by specialized microenviroment - or "niche"- in which these cells reside. Indeed, the ventricular and subventricular zones, surrounding the 4th ventricle, are considered the "stem/progenitor cell niches" of the developing cerebellar cells. Such niches are composed of soluble factors as well as membrane bound molecules and extracellular matrix (ECM). In addition, to promote normal growth and tissue homeostasis, progenitor cell niches are particularly rich in blood vessels. Blood vessels are formed by vascular endothelial cells and mural cells and they are generally required to provide cells and tissues with oxygen and nutrients [89].

The most relevant finding of this study is the ability of the different immunohistochemical markers to highlight the different zones of the developing human cerebellum, focusing on the

stem/progenitor cell niches. Indeed, immunohistochemistry allow to identify the presence of stem/progenitor cells inside the ventricular and subventricular zones during human cerebellar development. Nuclear expression of SOX2 and Pax6 allow to the identification of stem/progenitor niches during cerebellar corticogenesis.

This study shows a predominant expression of SOX2 in the nuclei of stem/progenitor cells in the ventricular and subventricular zones and, because this marker has been detected by the 11th week of gestation, we can affirm that this transcription factor represent one of the earlier stem/progenitor cell marker in the developing human cerebellum. These data confirm previous experimental studies carried out on mice in which SOX2 was expressed during the development of the CNS. In our data, SOX2 expression was found in progenitor intermediate cells located in the ventricular and subventricular zones as well as in newborn neurons migrating from the neuroepithelium towards the pial surface. The strongest expression for SOX2 has been found between the 11th and the 18th week of gestation.

Nuclear expression of PAX6 was observed from 20 to 38 weeks of gestation, being restricted to the external granular layer (EGL). Our study demonstrates that, in the development of human cerebellum, precursor cells of the EGL are PAX6+ and give rise to the granular cells of the human cerebellum. We also observed immunoreactivity for PAX6 both in granule cell precursors in the EGL and in migrating granule cell precursors in the internal granular layer (IGL) from the 20th gestational week to term. These data confirm experimental studies in the developing mouse cerebellum that demonstrated PAX6 expression in granule cell progenitors in the EGL and in those cells migrating from the EGL towards the IGL [90].

Another interesting finding emerging from our study is the expression of several markers of radial glial cells: Vimentin, Nestin and WT1. The cytoplasmatic expression of these three markers highlights the function of radial glial cells that, thanks to their parallel long fibers, represent the most important guide for the radial migration of newborn neurons from the

ventricular and subventricular zone towards the pial surface. In this study, the expression of these markers have been detected from the 15th to the 21st week of gestation; after that, the expression decreased until the 34th gestational week and disappeared at the 38th week. In fact, once the neuronal migration is complete the radial glial fibers in the cortical layers disappeare and they differentiate in glial lineages giving rise to astrocytes, oligodendrocytes or ependymal cells.

Nestin has been reported to be expressed in neural stem cells. Nestin is expressed in many dividing cell types during development of the CNS and other anatomical structures. In this study, Nestin immunoreactivity was mainly localized in radial glial cells in the ventricular and subventricular zones and in radial glial projections in the intermediate zone and in the pial surface. At the 34th week of gestation, a mild reactivity for Nestin was observed in the cortical layers and it disappears at term. These findings revealed Nestin as a possible marker involved in neural proliferation and migration during the development of human cerebellum. Moreover, this study indicates Vimentin as a useful marker for radial glial cells in the developing human cerebellum. WT1 highlights also radial glial fibers in early stages of CNS development. WT1 is a transcription factor highly expressed in several organs during human and mouse embryogenesis. In this study, the strong expression of WT1 in radial glial fibers in the first half of gestation indicates a possible role for this transcription factor in cell migration and differentiation during neurogenesis. These data confirm other data from a previous study of our group carried out on human fetuses in which WT1 was found in radial glial fibers in the developing human cerebral cortex [91]. On these bases, WT1 may be considered a useful marker of radial glial cells during human brain development.

Another interesting finding of this study is the expression of the transcription factor Pax2. Pax2 nuclear immunoreactivity was observed in all the developmental phases of human cerebellum. This antibody highlights the progenitor cells in the ventricular and subventricular

zone, the migrating newborn neurons of the inner zone and those reaching the cortical surface. The expression of Pax2 decreased during the advanced stages of development and in particular after the 34th week, in which a mild immunoreactivity was detected in scattered cells of the cerebellar cortical layers.

Regarding the expression of markers in mature neurons, the absence of immunoreactivity of NF, GFAP and NSE before the 15th week reflects the lack of maturation of glial and neuronal cells in the early phases of human cerebellar development.

NSE is a glycolitic protein known to be expressed in central and peripheral neurons and also in neuroendocrine cells [92]. This protein is generally considered a marker involved in neural differentiation [93]. In this study NSE is expressed in progenitor cells inside the ventricular and subventricular neuroepithelium from the 15th until the 21st week of gestation, as well as in progenitor cells of the cortical layers. At the 34th week, the immunoreactivity for NSE in the germinal neuroepithelium decreased, in contrast to the evident reactivity in the cortical layers from the 34th to the 38th week of gestation.

Neurofilament is an intermediate filament that represents a major component of the neuronal cytoskeleton being particularly abundant in axons. Neurofilaments are important for the radial growth of axons during development and the transimission of electrical firing [94]. In this study, the expression of NF in the developing human cerebellum has been detected from the 15th week both in the SVZ and in the cerebellar cortex, whereas the VZ showed no reactivity for NF. By the 34th week of gestation, the immunoreactivity for NF was particularly strong in the Purkinje cell layer of the cerebellar cortex, highlighting the dendrites of Purkinje cells.

S100β is a protein of the S100 protein family that represents a glial-specific marker primarily expressed in astrocytes [95]. Several studies have been demonstrated the expression of S100β in human fetal hippocampus, enthorial cortex and occipital cortex [96]. In this study,

at the 15th week S100β is expressed in scattered cells in the SVZ and in the cerebellar cortex; its immunostain increased in the followed ages, when it become evident also in the VZ. At the 38th week, S100β is expressed in the nuclei of glial cells of the inner zone and, at cytoplasmic level, in the glial projections in the molecular layer.

Glial fibrillary acid protein (GFAP) is an intermediate protein expressed in glial cells including astrocytes and ependymal cells [97]. A strong immunoreactivity for GFAP was detected from the 18th week, being localized in the cytoplasm of immature glial projections in the VZ and in the SVZ and in scattered glial projections in the cerebellar cortex. From the 21st to term, the number of radial glial projection GFAP-positive increased, as well as those in the cerebellar cortex, showing that the gliogenesis mainly occurs in the second half of gestation. Hence, when neuronal migration is completed, radial glial progenitors can differentiate into glial lineages.

Conclusions

In conclusion, this study show the relevant role of the immunohistochemistry as a useful tool for the detection of multiple stage of differentiation of neuronal and glial progenitor cells during the human cerebellar development. Future studies are needed in order to evaluate mRNA expression of these markers and to evaluate other markers of stem/progenitor cells in other gestational ages, in order to better clarify their role in cell proliferation, migration and differentiation of the human cerebellum during development, post-natal and adult life.

Bibliography

1. Larsell O. The development and subdivisions of the cerebellum of birds. 1948. J Comp Neuro. 89: 123-89

2. Marzban H, Chung SH, Pezhouh MK, Feirabend H, Watanabe M, Voogd J, Hawkes R. Antigenic compartmentation of the cerebellar cortex in the chicken (Gallus domesticus). 2010. J Comp Neurol. 518(12):2221-39.

3. Voogd J, Ruigrok TJ. The organization of the cortico-nuclear and olivo-cerebellar climbing fiber projections to the rat cerebellar vermis: the congruence of projection zones and the zebrin pattern. 2004. J Neurocytol. 33(1):5-21.

4. Sugihara I, Shinoda Y. Molecular, topographic, and functional organization of the cerebellar nuclei: analysis by three-dimensional mapping of the olivonuclear projection and aldolase C labeling. 2007. J Neurosci. 27(36):9696-710.

5. Ito M. The cerebellum and Neural Control. Raven Press. New York.

6. Lent R, Azevedo FA, Andrade-Moraes CH, Pinto AV. How many neurons do you have? Some dogmas of quantitative neuroscience under revision. 2012. Eur J Neurosci. 35(1):1-9.

7. Mugnaini E, Sekerková G, Martina M. The unipolar brush cell: a remarkable neuron finally receiving deserved attention. 2011. Brain Res Rev. 66(1-2):220-45

8. Sotelo C. Molecular layer interneurons of the cerebellum: developmental and morphological aspects. 2015. 14(5): 534-56

9. Hoshino M, Nakamura S, Mori K, Kawauchi T, Terao M, Nishimura Y, et al. Ptf1a, a bHLH, transcriptional gene, defines GABAergic neuronal fates in cerebellum. Neuron. 47(2): 201-213

10. Machold R & Fishell G. Math1 is expressed in temporally discrete pools of cerebellar rhombic-lip neural progenitors. Neuron. 48(1):17-24

11. Pascual M, Abasolo I, Mingorance-Le Meur A, Martinez A, Del Rio JA, Wright CV et al. Cerebellar GABAergic progenitors adopt an external granule cell-like phenotype in the absence of Ptf1a transcription factor expression. 2007. Proceedings of National Academy of Science of the United States of America. 104(12):5193-5198

12. Butler AB and Hodos W. Comparative Vertebrate Neuroanatomy: evolution and adaptation. 1996. 2nd ed. Wiley-Liss.

13. Englund C, Kowalczyk T, Daza RAM, Dagan A, Lau C, Rose MF et al. Unipolar brush cells of the cerebellum are produced in the rhombic lip and migrate through developing white matter. 2006. The Journal of Neuroscience. 26(36): 9184-9195

14. Chung SH, Marzban H, Hawkes R. Compartmentation of the cerebellar nuclei of the mouse. 2009a. Neuroscience. 161 (1): 123-138

15. Hallonet ME, Teillet MA & Le Douarin NM. A new approach to the development of the cerebellum provided by the quail-chick marker system. 1990. Development. 108(1) 19-31

16. Alvarez Otero R, Sotelo C, Alvarado-Mallart RM. Chick/quail chimeras with partial cerebellar grafts: an analysis of the origin and migration of cerebellar cells. 1993. Neurology. 333(4): 597-615

17. Wang VY, Rose MF, Zoghbi HY. Math1 expression redifines the rhombic lip derivates and reveals novel lineages within the brainstem and cerebellum. 2005. Neuron. 48(1): 31-43

18. Merckle FT and Alvarez-Buylla A. Neural stem cells in mammalian development. 2006. Current Opinion in Cell Biology. 18: 1-6

19. Rakic P. A small step for the cell, a giant leap for mankind: an hypothesis of neocortical expansion during evolution. 1995. Trends of Neurosci. 18(9): 383-8

20. Marzban H, Del Bigio MR, Alizadeh J, Ghavami S, Zachariah RM, Rastegar M. Cellular commitment in the developing cerebellum. 2014. Front Cell Neurosci. 8: 450

21. Paridaen JT and Huttner WB. Neurogenesis during development of vertebrate central nervous system. 2014. Embro Rep. 15(4): 351-64

22. Han YG. Sonic Hedgehog signaling: a conserved mechanism for the expansion of outer radial glia and intermediate progenitor cells and for the growth and folding of the neocortex. 2016. Neurogenesis. 3(1): e

23. Bengoa-Vergniory N and Kipta NM. Canonical and non-canonical Wnt signaling in neural stem/progenitor cells. 2015. Cell Mol Life Sci. 72(21): 4157-72

24. Inestrosa NC and Varela-Nallar L. Wnt signaling in neuronal differentiation and development. 2015. 359(1): 215-23

25. Woodbury ME and Ikezu T. Fibroblast growth factor-2 in neurogenesis and neurodegeneration. 2014. J Neuroimmune Pharmacol. 9(2): 92- 101

26. Roidl D and Hacker C. Histone methylation during neural development. 2014. Cell Tissue Res. 356(3): 539-52.

27. Contestabile A, Sintoni S. Hystone acetylation in neurodevelopment. 2013. Curr Pharma Des. 19(28): 5043-50.

28. Olynik BM, Rastegar M. The genetic and epigenetic joutney of embryonic stem cells into mature neural cells. 2012. Front Genet. 18;3:81.

29. Gotz M and Huttner WB. The cell biology of neurogenesis. 2015. Nat Rev Mol Cell Biol. 6(10): 777-88

30. Kriegstein AR and Gotz M. Radial glia diversity: a matter of cell fate.

31. Campbell K ang Gotz M. Radial glia: a multi-purpose cells for vertebrate brain development. 2002. Trends Neurosci. 25(5): 235-8

32. Malatesta P, Apolloni I, Calzolari F. Radial glia and neural stem cells. 2008. Cell, Tissue Res. 331(1): 165-78

33. Zecevic N, Chen Y, Filipovic R. Contributions of cortical subventricular zone to the development of the human cerebral cortex. 2005. J Comp Neurol. 491(2): 109-22

34. Hansen DV, Lui JH, Parker PR, Kriegstein AR. Neurogenic radial glia in the outer subventricular zone of human neocortex. 2010. Nature. 464(7288): 554-561

35. Florio M and Huttner WB. Neural progenitors, neurogenesis and the evolution of the neocortex. 2014. Development. 141(11):2 182-94

36. Pollen AA, Nowakowski TJ, Chen J, Retallack H, Sandoval-Espinosa C, Nicholas CR, Shuga J, Liu SJ, Oldham MC, Diaz A, Lim DA, Leyrat AA, West JA, Kriegstein AR. Molecular identity of human outer radial glia during cortical development. 2015. Cell. 163(1): 55-67

37. Buffo A, Rossi F. Origine, lineage and function of cerebellar glia. 2013. Prog Neurobiol. 109: 42-63

38. Altman J and Bayer SA. Development of the cerebellar system in relation to its evolution, structure and functions. 1997. New York. CRC Press

39. Laudet V, Stehelin D, Clevers H. Ancestry and diversity of HMG box superfamily. 1993. Nucleic Acids Res. 21(10): 2493-501

40. Koopman P. Sry and Sox9: mammalian testis-determining genes. 1999. Cell Mol Life Sci. 55(6-7): 839-56

41. Weiss MA. Floppy Sox: mutual induced fit in hmg (high-mobility group) box in DNA recognition. 2001. Mol Endocrinol. 15(3): 353-62

42. Arsic N, Rajic T, Stanojcic S, Goodfellow PN, Stevanovic M. Characterization and mapping of the human Sox14 gene. 1998. Cytogenenet Cell Genet. 83(1-2): 139-46

43. Sarkar A, Hochedlinger K. The Sox family of transcription factors: versatile regulators of stem and progenitor cell fate. 2013. Cell Stem Cell. 12(1): 15-30

44. Bani-Yaghoub M, Tremblay RG, Lei JX, Zhang D, Zurakowski B, Sandhu JK, Smith B, Ribecco-Lutkiewicz M, Kennedy J, Walker PR, Sikorska M. Role of Sox2 in the development of the mouse neocortex. 2006. Dev Biol. 295(1): 52-66

45. Sasai Y. Roles of Sox factors in neural determination: conserved signaling in evolution? 2001. Int J Dev Biol. 45(1):3 21-6

46. Feng R, Wen J. Overview of the roles of Sox2 in stem cell and development. 2015. Biol Chem. 396(8): 883-91

47. Alcock J, Lowe J, Engand T, Bath P, Sottile V. Expression of Sox1, Sox2 and Sox9 is maintained in adult human cerebellar cortex. 2009. Neurosci Lett. 450(2): 114-6

48. Thiel G. How Sox2 maintains neural stem cell identity. 2013. Biochem J. 450: 1-2

49. Graham V, Khudyakov J, Ellis P, Pevny L. Sox2 functions to maintain neural progenitor identity. 2003. Neuron. 39(5): 749-65

50. Fong H, Hohenstein KA, Donovan PJ. Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. 2008. Stem Cells. 26(8): 1931-8

51. Zhang S, Cui W. Sox2, a key factor in the regulation of pluripotency and neural differentiation. 2014. World J Stem Cells. 6(3): 305-11

52. Hastie ND. The genetics of Wilm's tumor, a case of disrupted regulation. 1994. Annu Rev Genet. 28: 523-58

53. Rauscher FJ, Morris JF, Tournay OE, Cook DM, Curran T. Binding of the Wilm's tumor locus zinc finger protein tothe EGR-1 consensus sequence. 1990. Science. 250(4985): 1259-62

54. Hamilton TB, Barilla KC, Romaniuk PJ. High affinity binding site for the Wilm's tumor suppressor protein WT1. 1995. Nucleic Acids Res. 23(2):2 77-84

55. Lee SB, Haber DA. Wilm's tumor and the WT1 gene. 2001. Exp Cell Res. 264(1): 74-99

56. Moore AW, McInnes L, Kreidberg J, Hastie ND, Schedl A. 1999. Development. 126(9): 1845-57
57. Herzer U, Crocoll A, Barton D, Howells N, Englert C. The Wilm's tumor suppressor gene WT1 is required for the development of the spleen. 1999. Curr Biol. 9(15): 837-40
58. Wagner KD, Wagner N, Bondke A, Nafz B, Flemming B, Theres H, Scholz H. The Wilm's tumor suppressor Wt1 is expressed in the coronary vasculature after myocardial infarction. 2002. FASEBJ. 16(9): 1117-9.
59. Ambu R, Vinci L, Gerosa C, Fanni D, Obinu E, Faa A, Fanos V. WT1 expression in the human fetus development. 2015. Eur J Histochem. 59(2): 2499
60. Parenti R, Salvatorelli L, Musumeci G, Parenti C, Giorlandino A, Motta F, Magro G. Wilm's tumor (WT1) protein expression in human developing tissues. 2015. Acta Histochem. 117(4-5): 386-96
61. Fanni D, Fanos V, Monga G, Gerosa C, Locci A, Nemolato S et al. Expression of WT1 during normal human kidney development. 2011. J Mater Fetal Neornatal Med. 2: 44-7
62. Sanna A, Fanos V, Gerosa C, Vinci L, Puddu M, Loddo C, Faa G. Immunohistochemical markers of stem/progenitor cells in the developing human kidney. 2015. Acta Histochem. 117(4-5): 437-43
63. Wehr R, Gruss P. Pax and vertebrate development. 1996. Int J Dev Biol. 40(1): 369-77
64. Ryan G, Steel-Perkins V, Morris JF, Rauscher FJ, Dressler GR. Expression of Pax2 by WT1 during normal kidney development. 1995. Development. 121(3): 867-75
65. Alvarez-Bolado G, Dressler G, Urbanek P, Busslinguer M, Gruss P. Pax2/5 and Pax6 subdivide the early neural tube into three domains. 1999. Mech Dev. 82(1-2): 29-39
66. Matsunaga E, Araki I, Nakamura H. Pax6 defines the di-mesencephalic boundary by repressing En1 and Pax2. 2000. Development. 127(11): 2357:65
67. Schwarz M, Cecconi F, Bernier G, Andrejewski N, Kammandel B, Wagner M, Gruss P. Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6. 2000. Development. 127(20): 4325:34.
68. Burrill JD, Moran L, Goulding MD, Saueressig H. Pax2 is expessed in multiple spinal cord interneurons, including a population of EN1+ interneurons that require Pax6 for their development. 1997. Development. 124(22): 4493:503
69. Matise MP, Joyner AL. Expression pattern of developmental genes in normal and Engrailed-1 mutant mouse spinal cord reveal early diversity in developing interneurons. 1997. J Neurosci. 17(20): 7805-16

70. Callaerts P, Halder G, Gehring WJ. Pax6 in development and evolution. 1997. Annu Rev Neurosci. 20: 483-532
71. Georgala PA, Carr CB, Price DJ. The role of Pax6 in forebrain development. 2011. Dev Neurobiol. 71(8): 690:708
72. Hever AM, Williamson KA, van Heyningen V. Developmental malformations of the eye: the role of Pax6, SOX2 and OTX2. 2006. Clin Genet. 69(9): 459-70
73. Hill RE, Favor J, Hogan BL, Ton CC, Saunders GF, Hanson IM, Prosser J, Jordan T, Hastie ND, van Heyningen V. Mouse small eye results from mutations in a paired-like homebox-containing gene. 1991. Nature. 354: 522-525
74. Matsumoto Y, Osumi N. Role of Pax6 in the development of the central nervosu system. 2008. Brain Nerve. 60(4): 365-74
75. Mastick GS, Andrews GL. Pax6 regulates the identity of embryonic diencephalic neurons. 2001. Mol Cell Neurosci. 17(1): 190-207
76. Chung SH, Kim CT, Jung YH, Lee NS, Jeong YG. Early cerebellar granule cell migration in the mouse embryonic development. 2010. Anat Cell Biol. 43(1): 86-95
77. Liu J, Ji X, Li Z, Zheng H, Zheng W, Jia J, Shen H, Zhang Q, An J. Nestin overexpression promotes the embryonic development of heart and brain through the regulation of cell proliferation. 2015. Brain Res. 1610: 1-11
78. Yan S, Li P, Wang Y, Yu W, Qin A, Liu M, Xiang AP, Zhaang W, Li W. Nestin regulates neural stem cell migration via controlling the cell contractility. 2016. Int J Biochem Cell Biol. 78: 349-360
79. Halliday GM, Cullen KM, Kril JJ, Harding AJ, Harasty J. Glial fibrillary acid protein (GFAP) immunohistochenistry in humna cortex: a quantitative study using different antisera. 1996. Neurosci Lett. 209(1): 29-32
80. Mendez MG, Kojima SI, Goldman RB. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. 2010. FASEBJ. 24(6): 1838-1851
81. Satelli A, Li S. Vimentin in cancer and its potential as a molecular target for cancer therapy. 2011. Cell Mol Life Sci. 68(18): 3033-46
82. Kidd ME, Shumaker DK, Ridge KM. The role of vimentin intermediate filaments in the progression of lung cancer. 2014. Am J Respir Cell Mol Biol. 50(1): 1-6
83. Stagaard M, Mollgard K. The developing neuroepithelium in human embryonic and fetal brain studied with vimentin-immunocytochemistry. 1989. Anat Embriol (Berl). 180(1):17-28.

84. Rogers JH. Calretinin: a gene for a novel calcium-binding protein expressed principally in neurons. 1987. J Cell Biol. 105: 1343
85. Resibois A. Rogers JH. Calretinin in rat brain: an immunohistochemical study. 1992. Neuroscience. 46: 101–134
86. Yew DT, Luo CB, Heizmann CW, Chan WY. Differential expression of calretinin, calbindinD28K and parvalbumin in the developing human cerebellum. 1997. Dev Brain Res. 103: 37–45.
87. Víg J, Takács J, Ábrahám H, Kovács GG, Hámori J. Calretinin-immunoreactive unipolar brush cells in the developing human cerebellum. 2005. Int J Dev Neurosci. 23: 723–729
88. Braak E, Braak H. The new monodendritic neuronal type within the adult human cerebellar granule cell layer show calretinin-immunoreactivity. 1993. Neurosci Lett. 154(1-2): 199-202
89. Wurmser AE, Palmer TD, Gage FH. Neuroscience. Cellular interactions in the stem cell niche. 2004. Science. 304(5675): 1253-5
90. Yamasaki T, Kawaji K,Ono K, Bito H, Hirano T, Osumi N, Kengaku M. Pax6 regulates granule cell polarization during parallel fiber formation in the developing cerebellum. 2001. Developmental. 128(16):3 133-44
91. Ambu R, Vinci L, Gerosa C, Fanni D, Obinu E, Faa A, Fanos V. WT1 expression in the human fetus development. 2015. Eur J Histochem. 59(2): 2499
92. Marangos PJ, Schmechel DE, Parma AM, Goodwin FK. Developmental profile of neuron-specific (NSE) and non-neuronal (NNE) enolase. 1980. Brain Res. 190(1): 185-93
93. Mareschi K, Novara M, Rustichelli D, Ferrero I, Guido D, Carbone E et al. Neural differentiation of human mesenchymal stem cells: evidence for expression of neural markers and K+ channel types. 2006. Exp Hematol. 34(11): 563-72
94. Yuan A, Rao MV, Veeranna RA. Neurofilaments at a glance. 2012. J Cell Sci. 125: 3257-3263
95. Zimmer BD, Cornwall EH, Landar A, Song W. The S100 protein family: history, function and expression. 1995. Brain Res Bull. 37(4): 417-29
96. Adami C, Sorci G, Blasi E, Agneletti AL, Bistoni F, Donato R. S100B expression and effects on microglia. 2001. Glia. 33(2): 131-42
97. Roessmann U, Velasco ME, Sindely SD, Gambetti P. Glial fibrillary acid protein (GFAP) in ependymal cells during development. 1980. Brain Res. 200(1): 13-21

” Valeria Pibiri gratefully acknowledges Sardinia Regional Government for the financial support of her PhD scholarship (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007-2013 - Axis IV Human Resources, Objective l.3, Line of Activity l.3.1.)”.