UNIVERSITA' DEGLI STUDI DI VERONA

SCUOLA DI DOTTORATO IN BIOMEDICINA TRASLAZIONALE

INDIRIZZO FARMACO-GENETICA

PhD THESIS

CHARACTERIZATION OF NEW STEM CELL NICHES WITH NEURONAL DIFFERENTIATION POTENTIAL

Coordinatore: Prof. CRISTIANO CHIAMULERA

Relatore: Dr. ILARIA DECIMO

Dottorando: Dr. EMANUELA BERSAN

XXII CICLO – Anno Accademico 2009-2010

SUMMARY

Adult neural stem cells (NSC), have been found in the main neurogenic regions of brain, i.e. hippocampus, sub ventricular zone (SVZ), olfactory bulb, and in some non-neurogenic regions, i.e. spinal cord. Other brain sites could host NSC niches and, in particular, considering the role of meninges in correct cortex development we were interested in exploring the region residing between arachnoide and the first layers of the cerebral cortex, called Leptomeninges.

Aim of this project is characterized the leptomeningeal compartment as potential niche for neural stem cells with *ex vivo* and *in vitro* approaches.

The leptomeningeal compartment has been characterized by immunohistochemistry at different rat ages, from embryo E20, postnatal day 0 (P0), P15 and adult. It appeared as a nestin (neuro-epithelial stem cells marker) positive layer with decreasing thickness from embryo up to adult. Nestin positive cells were distributed outside the basal lamina (marked by laminin), and as a distinct population from astrocytes (stained with GFAP) and oligodendrocytes (stained with NG2).

Once nestin positive cells were dissected and expanded *in vitro* from leptomeninges, they grew as an homogeneous nestin positive population with rare clusters of GFAP, NG2 and Laminin, while no Map2 (neuron marker) positive cells were found in culture. In differentiating condition, nestin positive cells mainly differentiate into MAP2 positive cells but also GFAP and O4 (marker for mature oligodendrocyte) positive cells were detected in culture.

As a first level of functional evaluation of differentiated cells, their ability to depolarize has been analyzed by calcium imaging assay after Fura-2 loading. *In vitro* differentiated neurones responded to fast applications of the depolarizing agent KCl suggesting the expression of voltage dependent calcium channels, similar to that of functional neurons.

As following step, the *in vivo* neuronal differentiation potential was assessed by infusion of expanded EGFP LeSC in rat hippocampus. Engrafted LeSC were monitored by immunofluorescence up two months and during this period LeSC were able to survive after injection. About half of EGFP cells engrafted in hippocampus, expressed neuronal markers (DCX, MAP2, NeuN, Neurofilament-160, GAD67) and shown differentiated neuronal morphology.

Because of the persistence of these cells up to adulthood, their proliferation capability in vitro, and their differentiation potential into neuronal cells in vitro and in vivo, we suggest to name them leptomeningeal stem/progenitor cells (LeSC) as a new population never described before. Since meninges cover whole brain, also Leptomeninges from rat spinal cord has been analyzed. Nestin positive cells were distributed as previously observed in the brain, outside the basal lamina, and as a distinct population from astrocytes and oligodendrocytes. Cells were dissected and kept in culture as neurosphere and resulted positive for nestin, MAP2, GFAP, O4, and Oct4. A new study In collaboration with professor M. Schwartz group (Weizmann Institute, Rehovot, Israel) is ongoing to understand the potential role of immune system in regulating leptomeninges and LeSC (as suggested by previous publications from Schwartz's group). Comparison of LeSC proliferation and nestin expression by immunohistochemistry in SCID vs wt mice, revealed a significant decrease of nestin positive LeSC in SCID mice. However total cell number and proliferating cells in leptomeninges were not changed. Further characterizations are ongoing to understand the phenotype of proliferating nestin negative cells in meninges.

The importance of Leptomeningeal stem cells reside in the easier reachable localization compared to the already known neural stem cell niches, and in their high neuronal differentiation potential. These characteristics will open novel studies in regenerative medicine.

INTRODUCTION

1. BRAIN DEVELOPMENT

The nervous system begins the development at a relatively late stage in embryogenesis. Prior to its formation three main cell layers have been generated. The endoderm, the innermost layer, gives rise to the gut, lungs, and liver; the mesoderm, the middle layer, gives rise to connective tissues, muscle, and the vascular system; the ectoderm, the outermost layer, gives rise to major central and peripheral nervous system.

Neural and glial cells derive from a sheet of ectodermal cells located along the dorsal midline of the embryo at the gastrula stage. As this ectodermal sheet acquire neural properties it forms the neural plate, a columnar epithelium.

Soon after the neural plate has formed it begins to fold into a tubular structure, called the neural tube, throughout a process called neurulation. The caudal region of neural tube gives rise to the spinal cord, the rostral region becomes the brain. During these early stages cells divided rapidly without a uniform extent along the neural tube (Fig.1).
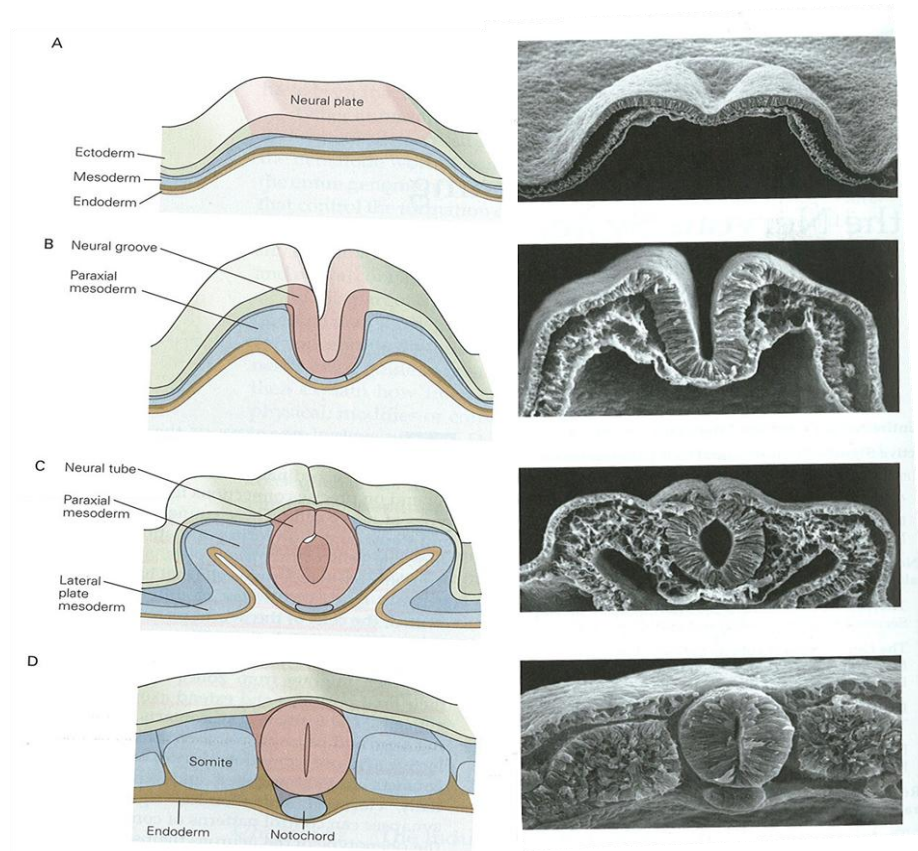
Fig.1: **Stages of neural plate fold**: **A** position of the neural plate in relation to non neural ectoderm, the mesoderm and the endoderm. **B** folding of the neural plate to form the neural groove. C dorsal closure of neural folds to form the neural tube. D maturation of the neural tube and its position relative to the notochord and somites. Modified from Kandel et al.2000

Individual regions of the neural epithelium expand at different rates and begin to form various specialized regions of the mature central nervous system. The proliferation of cells in the rostral part of neural tube initially forms three brain vesicles: the forebrain (prosencephalon), the midbrain (mesencephalon) and the hindbrain (rhombencephalon). Later in the development the forebrain gives rise to the telencepalon and diencephalon, and the hindbrain vesicle gives rise to the metencephalon and myelencephalon. These subdivisions together with the spinal cord, make up the six major regions of the mature central nervous system(Tab1). (Kandel et al., 2000)

Three-vesicle stage	Five-vesicle stage	Major mature derivatives	Related cavity
1. Forebrain (prosencephalon)	1a. Telencephalon (endbrain)	1. Cerebral cortex, basal ganglia, hippocampal formation, amygdala, olfactory bulb	Lateral ventricles
	1b. Diencephalon	2. Thalamus, hypothalamus, sub-thalamus, epithalamus, retina, optic nerves and tracts	Third ventricle
2. Midbrain (mesencephalon)	2. Mesencephalon (midbrain)	3. Midbrain	Cerebral aqueduct
3. Hindbrain (rhomb-encephalon)	3a. Metencephalon (afterbrain) 3b. Myelencephalon (medullary brain)	4. Pons and cerebellum 5. Medulla	Fourth ventricle Fourth ventricle
4. Caudal part of neural tube	4. Caudal part of neural tube	6. Spinal cord	Central canal

Tab1 Main subdivision of embryonic CNS and adult forms. Modified from Kandel et al., 2000.

2. ADULT NEUROGENESIS

Neurogenesis of mammals, rodents and non-human primates occurs primarily in 2 areas of the adult brain: the dentate gyrus (DG) of the hippocampus, and the SVZ. It is estimated that as many as 9000 new neuronal cells are generated per day in the rodent DG, contributing to about 3.3% per month or about 0.1% per day of the granule cell population. Indeed neurogenesis has been reported to occur in other areas of the adult brain – albeit at lower levels – like the CA1

area, striatum and 3rd ventricle in rodents, and neocortex in non-human primates. However, some of these data have been the source of debates and controversies, and remain to be further confirmed (Taupin 2006).

Adult neurogenesis and embryonic neurogenesis differ in at least two fundamental aspects. Adult neurogenesis proceeds in an environment that is not generally programmed to promote neurogenesis because its own development has long ceased. Neuronal development in the adult thus needs protection from the anti-neurogenic influences of the surrounding brain tissue and requires the maintenance of a permissive microenvironment. The second fundamental difference is that adult neurogenesis shows nothing of the orchestrated and massively parallel progression of developmental stages. In the adult hippocampus, neurons of all developmental stages can be found at any given time point. Thus, adult neurogenesis is an individualized process, not a population event. We use 'neurogenesis' as a term encompassing this entire multistep process, not the division of a neuronal progenitor cell alone.(Kempermann et al., 2004)

Persistent neurogenesis is limited to two areas of the adult brain: the olfactory bulb and the dentate gyrus of the hippocampus. Neurons in the olfactory bulb originated from precursor residing in the anterior part of subventricolar zone. These cells migrate along the rostral migratory stream toward the olfactory bulb where they differentiated into granule and periglomerular neurons (Encinas et al.,2008, Alvarez-Buylla et al., 2002).

Neurons in dentate gyrus derived from sub granular zone of dentate gyrus, here neuronal precursors migrate locally to the granule cell layer and differentiated into granule neurons.

Adult neurogenesis is modulated by a very wide range of intrinsic and extrinsic factors however it's important highlight that rules that govern neuronal specification during development may not be the same in the adult brain.

Intrinsic factors regulating adult neurogenesis belong to two main classes:

I. MOLECULES CONTROLLING CELLS PROLIFERATION. Among the cell cycle factors regulating cellular proliferation, Rb (retinoblastoma), necdin, and the E2F protein families are key players. During the G1 phase, Rb which predominates has an hypophosphorylated form, can bind to E2F, a positive regulator of the cell cycle thereby repressing its transcriptional activity and

preventing the cells from entering the S phase. In cycling cells, phosphorylated Rb accumulated during the late G1 phase leading to a release of E2F with a consequently entry in S phase.

Rb and E2F protein families are differentially expressed in proliferative and postmitotic cells of the adult brain. Thus, in the two neurogenic sites, the DG and the SVZ, Rb immunoreactivity is high in proliferating neuronal precursors and reduced during terminal differentiation (Okano et al., 1993, Abrous et al., 2005) which implies that the transient increase in the level of Rb is an important step in the initiation of terminal mitosis in neuronal progenitors.

II. MOLECULES CONTROLLING CELL FATE . Factors called bHLH derive from proneural and neurogenic genes and antagonistically control the switch from cell proliferation to neural differentiation. Cascades of neuronal bHLH induced genes promoting differentiation, whereas antineuronal bHLH induced genes repress differentiation keeping cells at a precursor stage.

Two classes of proneural genes can be distinguished: a. *the determination factors*, such as Mash1 (mammalian achaete-scute homolog), Math1 (mammalian atonal homolog), and Ngns (Neurogenins) including Ngn2, expressed early in mitotic neural precursor cells, and *b. the differentiation factors*, including NeuroD, NeuroD2, and Math2, expressed later in postmitotic cells. It was recently determined that these proneural genes are downstream effectors of Pax6, a transcription factor that promotes neurogenesis (Heins et al., 2002) These proneural genes are components of a cell-cell signaling mechanism whereby a cell that becomes committed to a neural fate inhibits its neighbor from doing likewise. (Abrous et al., 2005).

Extrinsic factors regulating neurogenesis belong to various classes of molecule:

I. HORMONES AND NEUROSTEROIDS. a. *Adrenal corticosteroids*. In vitro studies reported that corticosterone hampers cell proliferation and his mechanism could be a block of G1 phase of the cell cycle. Suppression of corticosterone secretion increases glial and neuronal births in the DG, whereas mitotic activity in the SVZ remains unchanged, suggesting a site-specific inhibitory influence of corticosteroids. However, it remains to be determined which of these mechanisms is involved in vivo (Pestell et al., 1999)

b. *Gonadal hormones*. The influence of female gonadal hormones has been investigated in the DG since estrogen replacement therapy seems to reduce the risk of age-related cognitive impairments. Although cell proliferation in the Granule Cell Layer (and not the hilus) is higher in

female than in male rats, the newly born cells do not survive, which explains the lack of sex differences in the number of BrdU-IR cells 2 wk after labeling Sex-dependent proliferative activity involves the stimulatory influence of estrogens, since the number of BrdU-labeled cells is highest during the proestrus phase of the estrus cycle, when circulating levels of estrogens are highest. In contrast, using calbindin as early and late neuronal markers, it has been shown that estradiol does not alter neuronal differentiation (Tanapat et al.,1999).

c. *Neurosteroids* Neurosteroids represent a subclass of steroids synthesized de novo in many regions of the brain, independently of peripheral sources. They are synthesized in the HF, by glial cells mainly, and influence hippocampal-mediated functions (Abrous et al.,2005).

II. NEUROTRANSMITTERS AND NEUROREGULATORS. a. *Glutamate* exerts a complex influence on hippocampal cell proliferation, increasing it through activation of AMPA receptors, and inhibiting it through activation of NMDA receptors. Finally, the recent discovery that GABA and glutamate are co-transmitted at the mossy fibers synapses adds further complexity to the respective role of each neurotransmitter in neurogenesis regulation.

b. *Serotonin* up-regulates cell proliferation in the adult *DG and SVZ. It was* proposed that early forming serotonin (5-HT) neurons act as humoral signals governing neuronal development and neurogenesis (Azmitia et al.,2001).

c. *Nitric oxide,* a free radical molecule, serves as a neurotransmitter in the brain. In adult mice SVZ, neuronal precursors have been found to be influenced by NO source and to express NO synthase at the sites of terminal differentiation. Moreover, administration of DETA/NONO-ate, a NO donor, to young adult rats significantly increases both cell proliferation and migration in the SVZ and the DG (Abrous et al., 2005).

III. TROPHIC FACTORS. Many trophic factors have been shown to have mitogenic actions in the adult brain neurogenic regions.

a. The proliferative effects of basic *fibroblast growth factor* (FGF-2) was showed in the SVZ where FGF-2 induce an increaseing number of newly born neurons (expressing DCX) that reach the OB where they express NeuN.

b. *Epidermal growth factor* (EGF) and *heparin binding EGF* (HB-EGF) stimulate cell birth in the SVZ

certainly via EGF receptors. However, these two factors probably have distinct actions: HB-EGF increases the number of BrdU-IR cells expressing DCX or NeuroD in the SVZ, whereas EGF reduces the number of newly born neurons reaching the OB (Kuhn,1997). In the DG, HB-EGF but not EGF increases cell proliferation by interacting most probably with EGF receptors expressed by the dividing cells.

c. *Transforming growth factor* (TGF)-α is required in SVZ to induce an increase in precursor proliferation (Craig, 1996). No data on the influence of TGF- α on hippocampal precursors are yet available.

d. *Growth-promoting peptide hormone* (IGF-I) is produced in the CNS by neurons and glial cells and exhibits neurotrophic properties in adulthood.

e. *Brain-derived neurotrophic factor* (BDNF) is a neurotrophic proteins which prevent neurons from dying during development. Moreover BDNF seems to influence survival and/or differentiation (Arsenijevic, 1998) of neuronal precursors and their progeny .

f. *Vascular endothelial growth factor* (VEGF) is a hypoxia-induced angiogenic protein that exhibits neurotrophic and neuroprotective properties. Given that neurogenesis occurs in close proximity to blood vessels and that clusters of dividing cells contain endothelial precursors, VEGF may constitute the link between neurogenesis and angiogenesis (Abrous et al.,, 2005).

IV. MORPHOGENIC FACTORS. Two classes of morphogens were mainly identify in neurogenesis regulation: Sonic hedgehog (Shh) and the bone morphogenic proteins (BMPs).

Shh is a signaling glycoprotein that acts to trigger various events during CNS development, including induction of oligodendrocyte precursors, proliferation of specific neuron progenitor populations, and modulation of growth cone movements (Marti et al., 2002). Differently BMPs belongs to the TGF-α superfamily, which includes TGF- α, activins. These glycoproteins play a crucial role in bone remodeling and in the regulation of dorsoventral patterning of the neural tube and cell fate during embryonic development (Gross et al.,1996).

V. REGULATION BY GLIAL CELLS. Astrocytes, which play an important role as sensors of changes in their extracellular microenvironment, could regulate neurogenesis by secreting local signals (Song et al., 2002). These signals, unknown so far, may be ionic fluxes, neurosteroids, cytokines, growth factors, and glutamate metabolites.

VI. REGULATION BY CELL DEATH. An equilibrium between neurogenesis and cell death probably ensures a homeostatic balance in the adult brain. This hypothesis, based on the observation that the neurogenic structures do not grow in size, implies that cell death provides a stimulus for increased neurogenesis, a hypothesis reinforced by several lines of argument: 1) during development, there is a balance between the birth and death of granule cells; 2) mechanical lesions stimulate neuronal precursor proliferation in the Granule Cell Layer and/or the SVZ (Johansson et al.,,1999;) proliferation in the SVZ is upregulated under inflammatory conditions (Calzà et al., 1998); 4) the apoptotic degeneration of corticothalamic neurons induces endogenous neural precursors to differentiate into mature neurons in regions of the cortex undergoing targeted neuronal death (Magavi et al., 2000).

The specificities of each neurogenic site may be related to differences in the intrinsic properties of the dividing cells and/or in their microenvironment, which corresponds to the summation of local neurogenic signals expressed or synthesized "locally" by healthy neighboring cells or by dying cells.

Factors that lead to a net increase or decrease in the number of new neurons may, in principle, affect any step in the differentiation cascade that converts neural stem cells into fully differentiated neurons, for example symmetric and asymmetric divisions of different types of precursors, their survival, or their differentiation. Both the cell populations that are targeted by neurogenic stimuli and the molecular mechanisms that mediate the action of these stimuli are only beginning to be understood.

2.1.FUNCTION: ENVIRONMENTAL STIMULI AND PATHOLOGICAL CONDITIONS

The most important function of adult NSCs is to generate neurons. NSCs in the SVZ generate neuroblasts that migrate a significant distance via the rostral migratory stream (RMS) to the olfactory bulb, where they generate interneurons essential for maintenance of the olfactory bulb. In contrast, neurons produced in the hippocampal SGZ integrate into the immediately adjacent granule cell layer, where they are important for learning and memory.

Adult NSCs are also involved in gliogenesis in the SVZ generating oligodendrocytes and in the SGZ generating astrocytes (Suh et al., 2007). Finally, although the brain is notoriously bad at

repairing itself, adult NSCs respond to neural injury with an attempt at repair, a finding that has led to therapeutic strategies aimed at recruiting and improving this endogenous ability (Zhao et al., 2008; Miller et al., 2009).

Evidence suggests that newly generated neuronal cells participate to processes like learning and memory, and depression. In learning and memory, hippocampal neurogenesis is involved in the formation of trace memories that depends on the hippocampal formation, but not in all types of hippocampal-dependent learning processes. Further support of the involvement of adult hippocampal neurogenesis in learning and memory come from studies where adult rats were subjected to brain irradiation. Hippocampal brain irradiation blocked the formation of new neurons in the DG, and in the following weeks after irradiation, the animals performed poorer than controls in various hippocampus-dependent tasks. Therefore, there are evidences of the involvement of adult neurogenesis in learning and memory, but its contribution in learning and memory remains to be elucidated.

Newly generated neuronal cell would be involved in processes like learning and memory, and depression. However, the contribution of newly generated neuronal cells to CNS functioning remains to be fully understood (Taupin 2006).

Neurogenesis in the adult DG and OB is modulated by various environmental stimuli, and in patho-physiological conditions. Environmental enrichment together with various form of physical exercises, like voluntary running, forced running and swimming, were reported to enhance hippocampal neurogenesis, an activity that was found to be dependent on the circadian rhythm – running activity significantly increases neurogenesis only in animals with wheel access during the middle of the dark period, when mice are normally active. Behaviors and social environments were also reported to modulate neurogenesis. Learning, novel environment and dietary restriction enhance neurogenesis in the adult hippocampus. Social isolation, alcohol consumption, stress and sleep deprivation decrease hippocampal neurogenesis.

The rate of neurogenesis in the DG and SVZ decreases with age while in pathological conditions, like neurological diseases, and traumatic brain injuries, neurogenesis increase and new neuronal cells have been reported to be generated at the sites of injury or degeneration. It is estimated that 0.2% of the degenerated nerve cells are replaced in the striatum after middle

cerebral artery occlusion, a model of focal ischaemia. Cell tracking studies revealed that new neuronal cells at the sites of degeneration originate from the SVZ. They migrate to the site of degeneration, partially through the RMS. The function and contribution of adult neurogenesis to the CNS pathophysiology remain to be understood (Taupin 2006).
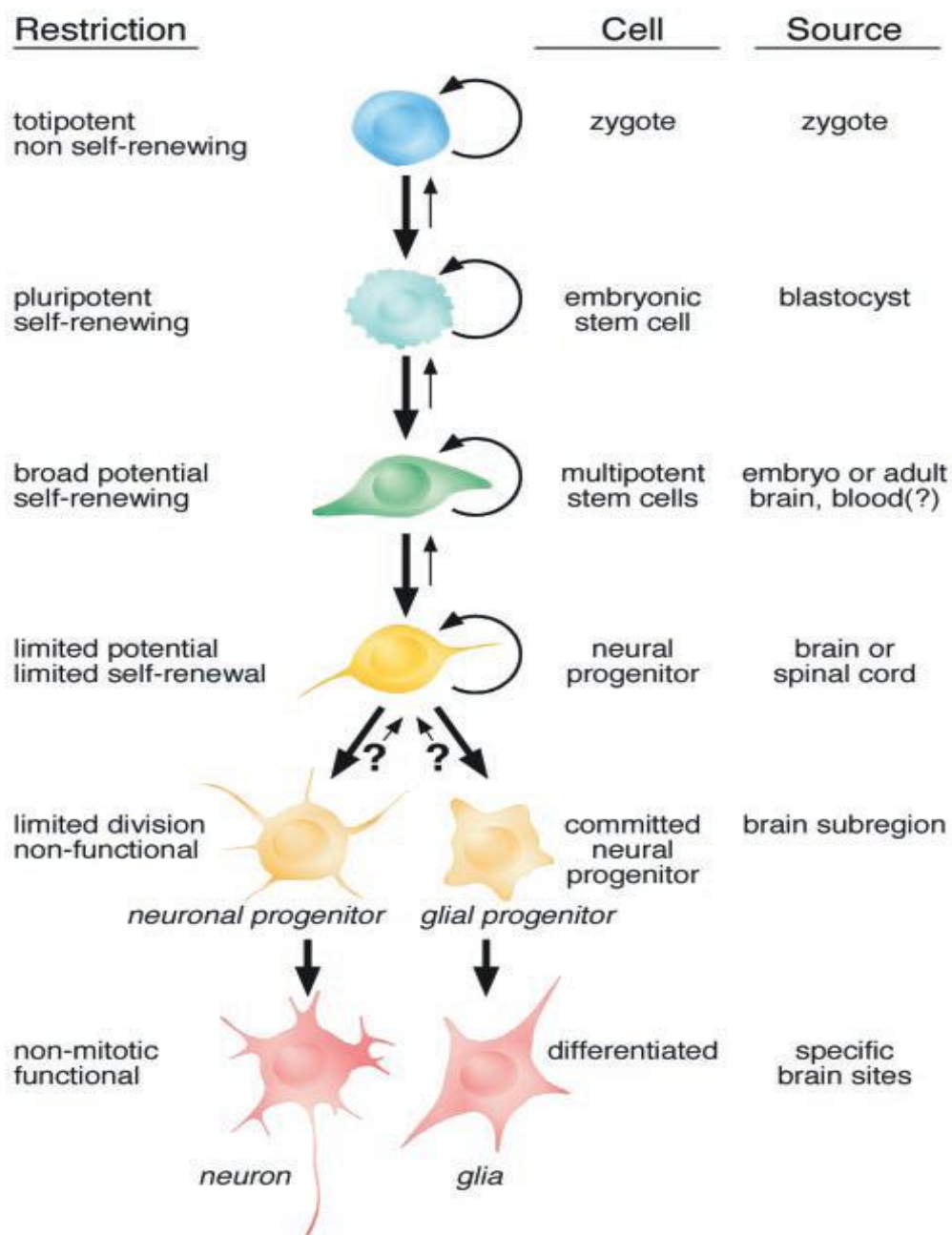

3. NEURAL STEM CELLS

Stem cells can be derived from early embryos after the formation of blastocyst or from fetal, postnatal, or adult sources. The working definition of a stem cell includes self renewal and the ability to differentiate into several cell types. There are also aspects of clonality and potency. Self-renewal is defined as the ability to generate daughter cell identical to their mother. The daughter cells can also produce progeny with more restricted potential. Thus a stem cell can divide to generate one daughter that is a stem cell and another daughter cell that can produce differentiated cells. This definition is easily applied to the totipotent embryonic stem cells that are derived from the blastocyst's cell mass (Thomson et al., 1998).

Stem cells have varying repertoires. A totipotent stem cell can be implanted in the uterus of a living animal and give rise to a full organism, including the entire central and peripheral nervous systems. A pluripotent stem cell is restricted in that it can give rise to every cell of the organism, including cells of the nervous system, except the trophoblasts of the placenta. Stem cells out of context are not able to give rise to the form and structure of the organism. The pluripotent cell is the same as an embryonic stem cell (ES cell) and is currently used to create transgenic animals; it is also the one being proposed for use in a wide variety of commercial and clinical applications (1). Most stem cells fall into the category of multipotent stem cells, a term that really does not provide much useful information, because the developmental potential of these cells has usually not been fully tested. Most often, stem cells are defined by the organ from which they are derived or by where they are observed in vivo.

The term "neural stem cell" is used to describe cells that (i) can generate neural tissue or are derived from the nervous system, (ii) have some capacity for self-renewal, and (iii) can give rise to cells other than themselves through asymmetric cell division. Whether stem cells from neural and other tissues are more defined by their tissue of origin or by their multipotentiality

is at present unclear. However, neural stem cells can also be derived from more primitive cells that have the capacity to generate neural stem cells and stem cells of other tissues (Fig2).

Potential Stem Cells with Neural Capability

Restriction		Cell	Source
totipotent non self-renewing		zygote	zygote
pluripotent self-renewing		embryonic stem cell	blastocyst
broad potential self-renewing		multipotent stem cells	embryo or adult brain, blood(?)
limited potential limited self-renewal		neural progenitor	brain or spinal cord
limited division non-functional	*neuronal progenitor* *glial progenitor*	committed neural progenitor	brain subregion
non-mitotic functional	*neuron* *glia*	differentiated	specific brain sites

Fig.2: **Classes of mammalian stem cells.** Mammal stem cellst can give rise to neurons, presented as a hierarchy beginning with the most primitive and multipotent stem cell and progressing
to the most restricted. The restrictions of fate at each step and examples of sites in the body where they can be obtained are also presented. As our understanding of the true potential and nature of stem cells is still unfolding, modifications will clearly be added. For example, the small arrows pointing up suggest the potential, although not well documented, dedifferentiation of the more restricted cell below. Image modified from Gage 2000.

The observation of stem cells in the adult nervous system has not been adequately integrated into our ideas of the function of the adult brain, which had long been thought to be entirely postmitotic (Gage 2000).

The identity of NSCs in the adult brain remains the source of debates and controversies. There are currently 3 theories with regard to the identity of the cell type at the origin of newly generated neuronal cells in the adult rodent brain.

I. The first theory contends that newly generated neuronal cells originate from a population of ependymal cells in the SVZ that express the intermediate filament protein nestin. In 1999, Johansson et al reported that isolated ciliated ependymal cells showed self-renewing, multipotent properties in vitro. An ependymal cell origin for newly generated neuronal cells has also been reported more recently in the 3rd ventricle.

II. The second theory identify them as astrocyte-like cells expressing glial fibrillary acidic protein (GFAP) in the SVZ and SGZ. Cell lineage analyses in vivo with markers of cell division, electron microscopy, and retroviral markers showed that newly generated neuronal cells in the adult SVZ and DG originate from a population of cells expressing GFAP and nestin that exhibit ultrastructural characteristics of astroglia. Imura et al (2003) used tissue culture techniques and transgenic mice expressing herpes simplex virus thymidine kinase (HSV-TK) from the mouse GFAP promoter to test the hypothesis that certain NSCs express GFAP. In this transgenic model, dividing GFAP-expressing cells are ablated selectively by treatment with the antiviral agent ganciclovir (GCV). GCV applied in vitro eliminated growth of multipotent neurospheres isolated from SVZ area of adult transgenic mice, indicating that the predominant multipotent NSCs isolated from adult SVZ express GFAP. The other strategy allowed fate mapping of progeny cells derived from GFAP-expressing cells by using the targeted expression of Cre recombinase (Cre) to excise a loxP-flanked stop signal and activate reporter gene expression from an independent ubiquitous promoter. Transgenically targeted ablation of dividing GFAP expressing cells in the adult mouse subependymal and subgranular zones stopped the generation of new neuronal cells in the OB and DG. These findings identify morphologically distinctive GFAP-expressing progenitor cells as the predominant sources of constitutive adult neurogenesis, further supporting a glial origin for newly generated neuronal cells in the adult brain.

III. The third theory identifies NSCs in the adult brain, as population of cells non-expressing GFAP. Two reports based on flow cytometry showed no expression or only partial expression of

immunohistologically detectable GFAP in partially purified populations of adult cells from the SVZ area that show neurosphere-forming potential in vitro (Taupin 2006).

There are several issues that need to be further investigated to confirm the identification and origin of NSCs in the adult brain. Particularly, the relationship between neurosphere-forming cells in vitro and NSCs in vivo need to be understood (Taupin 2006).

4. ADULT NEURAL STEM CELLS NICHES

Specialized microenvironments or niches support the lifelong self-renewal of stem cells and their production of differentiated cells Importantly, stem cells themselves extensively interact and participate in the niche. Furthermore, niches may in fact be dynamic structures that alter their location and characteristics over time concomitant with tissue remodeling.

Cell–cell interactions and diffusible signals are key elements allowing feedback control of stem cell activation and differentiation from progeny and/ or niche support cells. An emerging feature of several stem cell niches is the intimate association with endothelial cells, which regulate stem cell self-renewal and differentiation. Within a niche, stem cells are frequently anchored to a basal lamina or stromal cells that can provide a substrate for oriented cell division. The basal lamina is also an important regulator of the accessibility of growth factors and other signals, as associated extracellular matrix (ECM) molecules and glycoproteins can both concentrate and sequester factors in inactive or active forms. Moreover cell anchoring may orient cell division resulting in the segregation of key determinants into one or both daughter cells depending on the plane of division (Doetsch 2003).

Neurogenic niches, though distinct in many ways, are built so that NSCs can receive, integrate, and respond to signals from the outside world. In both cases, NSCs are (1) closely associated with the vasculature, (2) adjacent to a variety of neighboring cells, including their own neuronal progeny, resident mature astrocytes and microglia, blood vessel endothelial and smooth muscle cells, and (3) in close contact with basal lamina components. For example, SVZ precursors are closely associated with ''fractones,'' slender projections of the vascular basal lamina that are rich in extracellular matrix components like laminin and that might sequester and concentrate growth factors (Miller et al., 2009).

Adult neurogenic niches have an instructive role in directing neuronal production and stem cell maintenance and protect ongoing neurogenesis from possible external inhibitory influences. Although the components of adult neurogenic niches that mediate these processes are still being elucidated, it is clear that both neural and non-neural cell types are key players (Doetsch, 2003).

Neurogenesis occurs in close proximity to blood vessels and may be associated with angiogenesis. Moreover regions are enriched in ECM proteins and a prominent basal lamina has been described in the already known SVZ (Riquelme et al, 2008).

NICHE ASTROCYTES

Astrocytes have classically been considered support cells in the brain, with multiple roles including forming a capsule around the surface of the brain, buffering extracellular potassium ion concentrations, interacting with endothelial cells to form the blood–brain barrier and taking up neurotransmitters at the synaptic cleft.

Recent works revealed that astrocytes dynamically regulate many brain processes, including synaptogenesis and synaptic efficacy (Ullian et al., 2001;), support adult neurogenesis (Kornyei et al, 2005) and act as neural stem cells in the adult brain (Imura et al., 2003; Ahn et al, 2005).

Thus, astrocytes are emerging as key mediators of brain development, function and plasticity.

Within adult neurogenic niches, in addition to their role as stem cells, astrocytes are poised to be sensors and regulators of the environment. They have long processes that envelop and contact all cell types and structures in the niche including blood vessels and the basal lamina, allowing them to integrate diverse signals from many sources (Mercier et al., 2002).

Astrocytes also contribute to the neurogenic niche through contact-mediated cues and by secreting diffusible signals (Kornyei et al., 2005). It is still unknown whether the dual roles of astrocytes as stem cells and niche support cells are segregated into distinct astrocyte populations or whether individual astrocytes can have both roles.

EPENDYMAL CELLS: SUPPORT CELLS IN THE NICHE

Ependymal cells line the walls of the ventricles in the adult brain and are at the interface between the CSF and the brain tissue. They act both as a structural barrier and as a sensor of CSF components and osmotic pressure. Ependymal cells actively regulate the absorption of ions and transport of factors from the CSF into the parenchyma. Numerous growth factors that affect adult neurogenesis, including TGF-α; FGF-2 and amphiregulin, are made by the choroid

plexus, epithelial cells within the ventricles that produce the CSF. Ependymal cells may distribute and create gradients of factors produced by the choroid plexus through the directional beating of their cilia thereby, they have been proposed to guide the migration of SVZ neuroblast.

Ependymal cells do not appear to be endogenous neural stem cells as astrocytes (Johansson et al. 1999) however, they contribute to the niche in many ways, including as a source of secreted proneurogenic factors and of CSF components. In addition ependymal cells create a favourable environment for neurogenesis, together with astrocytes, through the secretion of noggin, an antagonist of bone morphogenic protein (BMP) signalling (Lim et al., 2000; Peretto et al.,2004). Within the adult SVZ, BMP signalling favours the production of astrocytes, but this pro-glial fate is reversed to a pro-neurogenic fate through the sequestration of BMP ligands by noggin (Lim 2000). In addition to noggin, ependymal cells also express CXCR4 (Stumm et al., 2002), a chemokine receptor for SDF1 that is important for migration and survival (Dziembowska et al.,2005), EphA7 (Holmberg et al., 2005), a member of the Eph family of tyrosine kinase receptors that guide many developmental processes, and pigment epithelium-derived factor (PEDF) (Ramirez-Castillejo et al.,2006). How these molecules and others yet to be identified interact merits further study.


VASCULATURE

Vasculature was revealed as an important component in adult neurogenic niches because common signals regulate both the development of the vasculature and the nervous system. For example vascular endothelial growth factor (VEGF), implicated in neurogenesis in the SVZ and SGZ, when infused into the lateral ventricle leads to an increase in SVZ proliferation and neurogenesis (Carmeliet 2003). Moreover brain microvascular endothelium elaborate BDNF and endothelium was observed to promote the survival and differentiation of neuronal precursors isolated from the rodent SVZ.

In addition, blood vessels are the way for the delivery of paracrine factors, such as hormones and cytokines, from distant sources. These 'long-distance' signals may act directly on neural stem cells and progenitors, endothelial cells or both to regulate angiogenesis and neurogenesis.

Furthermore, since neurogenesis is limited to restricted brain regions, angiogenic interface became necessary to guarantee trophic and metabolic support on niches.

The vascular recruitment closed to within the SGZ, for example, is likely caused by a simple increase in local metabolic demand, but the tight clustering of both cell types within each proliferative focus suggests that the CNS-vascular interface may also provide instructive signals. Endothelial cells are emerging as critical niche cells that regulate stem cell self-renewal and neurogenesis: Shen et al (2004) showed that co-culturing embryonic neural stem cells or adult SVZ cells with endothelial cells leads to enhanced stem cell self-renewal as well as increased neurogenesis from these expanded stem cells upon differentiation (Shen e al., 2004, Riquelme et al., 2008).

Palmer et al. (2000) studied neurogenesis within the SGZ with evidences that neurogenesis occurs in close proximity to blood vessels, with proliferative clusters containing neural progenitors, glial cells, newborn neurons and endothelial cells, suggesting that neurogenesis and angiogenesis are coordinated processes.
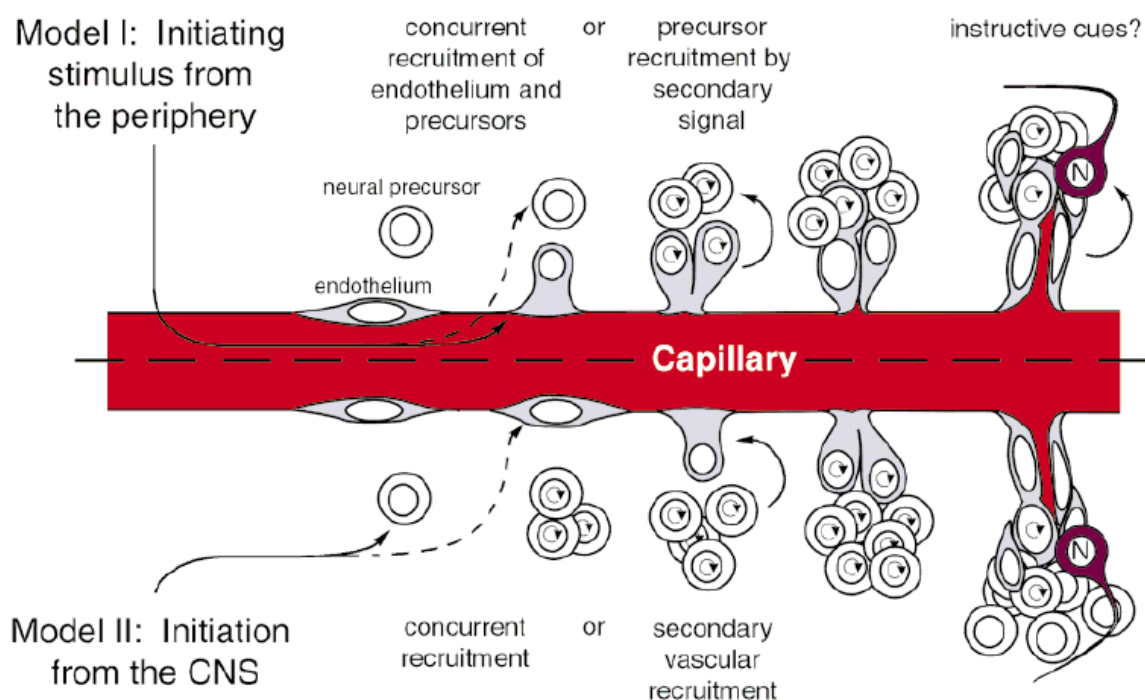
Fig.3: Schema of neuro- angiogenic recruitment mechanisms: neurogenic signals may originating from either somatic tissues (model I) or from either somatic tissues or the central nervous system (CNS; model II) modulating the production of new neurons Modified from Palmer et al., 2000

In the study they showed that the majority of precursors cells in SGZ proliferate in novel "neuro-angiogenic" foci where neuronal, glial, and endothelial precursors divide in tight clusters commonly found at a branch or terminus of fine capillaries, suggesting an active site of angiogenesis. It seems likely that neurogenic foci grew in response to discrete local signals.

Given the vascular involvement in neurogenesis, it is possible that signals originating from either somatic tissues (model I) or the central nervous system (CNS; model II) modulate the production of new neurons. In either model, signals may act to stimulate simultaneously neurogenesis and angiogenesis. Alternatively, an initiating stimulus might activate one cell type, which subsequently stimulates proliferation of the second cell type. Probably, these two hierarchies of signaling, extrinsic somatic cues vs. intrinsic CNS cues, act in concert to modulate neurogenesis. (Fig3) (Palmer et al. 2000).

Angiogenesis and neurogenesis are extensively interconnected and likely reciprocally influence each other in adult neurogenic regions.

BASAL LAMINA AND EXTRACELLULAR MATRIX

The extra cellular matrix (ECM) and associated molecules are integral components of stem cell niches creating a favorable microenvironment and architecture. They regulate signaling in the niche by providing, storing and compartmentalizing growth factors and cytokines indispensable for cell proliferation and differentiation, as well as acting as a substrate for anchoring cells. One common component of many stem cell niches is a basal lamina, overlaying connective tissue. Within the SVZ, a unique basal lamina, rich in laminin and collagen-1, extends as 'fractones' the extra vascular basal lamina with fractal organization (Fig4) (Mercier et al., 2002).

The branched configuration of fractones allows extensive interaction with all SVZ cells, especially with SVZ astrocytes and ependymal cells. This basal lamina, as well as other ECM components, is probably a key mediator of stem cells and their progeny. Fractones may represent sites at which growth factors and other signaling molecules interact with stem cells and progenitors to regulate their proliferation, activation and differentiation by modulating the availability of signaling molecules within the stem cell niche. The source of these factors is diverse: ependymal cells, CSF, endothelial cells and SVZ cells. It will be fascinating to see

whether fractones are dynamic, perhaps indicating that adult neural stem cell niches undergo constant remodeling. It is not known whether similar structures are present in the SGZ.
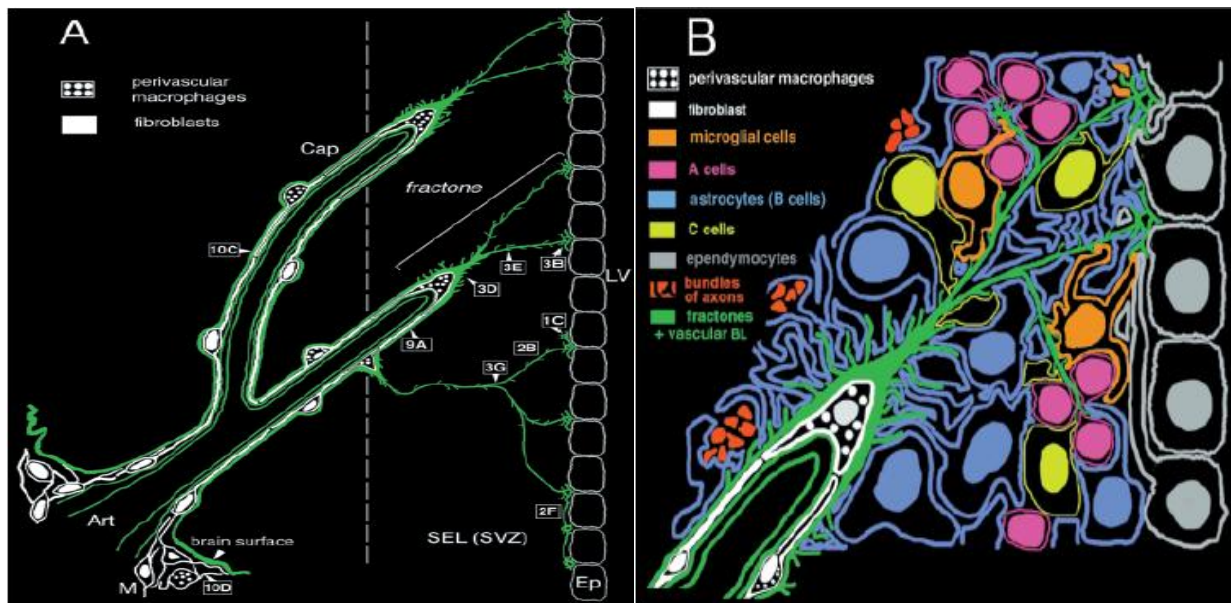
Fig.4: **Schematic representation of fractones.** Vascular, perivascular basal laminae (BL), and fractones are represented in green. **A:** Schema based upon the confocal distribution of laminin immunoreactivity but including ultrastructural details. A fractone is a continuous BL and consists of a base at the tip of a perivascular macrophage, stems crossing the SEL, and bulbs under the ependyma. The perivascular macrophage belongs to a fibroblast/macrophage network originating from the meninges. The boxed labels within the figure correspond to representative electron photomicrographs. Art: artery; Cap: capillary; Ep: ependyma; M: meninges; SEL: subependymal layer (also termed SVZ, subventricular zone); LV, lateral ventricle. **B**: Cytoarchitectonics of the sub ependimal layer and organization of cell types contacting fractones. "A" cells, "C" cells, astrocytes, microglial cells, and ependymocytes directly contact fractones. Macrophages and fibroblasts are reported in the model. Modified from Mercier et al.,2000.

Other ECM components known at present to be in adult neurogenic niches are chondroitin sulphate proteoglycans (CSPG), heparan sulphate proteoglycans (HSPG), tenascin-C, laminins and collagen-1, which together probably modulate accessibility of growth factors, cytokines and other signalling molecules. Integrins are receptors that provide structural links between the ECM and the cytoskeleton, allowing for oriented cell division. In addition, they cooperate with growth factor receptors to enhance signal transduction. Integrins coordinate spatial positioning within the niche with downstream cellular signaling and probably play a key role in maintaining adult neural stem cell niches, as they do in other stem cell niches (Fuchs et al.,2004).

CELL–CELL CONTACT AND DIFFUSIBLE SIGNALS IN THE NICHE

Intrinsic genetic programs as well as extracellular signals underlie stem cell fate choices. Whether a cell undergoes self-renewal or differentiation is the result of the spatial and temporal convergence of niche cues and the intrinsic state of the cell. Feedback signals from newly generated progeny can also regulate neural stem cells via either cell–cell contact or diffusible signals. When neuroblasts and transit-amplifying cells are depleted by an anti-mitotic treatment, SVZ astrocytes divide to rapidly regenerate the SVZ network of chains (Doetsch. 1999), perhaps reflecting loss of feedback inhibition from the neuroblasts onto their ancestors, such as loss of GABA signaling (Liu et al. 2005).

Another molecule involved in proliferation in adult neurogenic regions is Shh. During development, Shh acts as a morphogen, playing a crucial role in ventral patterning along the entire extent of the neuraxis, and as a mitogen, stimulating granule cell precursor proliferation in the cerebellum. In the adult SVZ, Shh regulates the proliferation of SVZ astrocytes (type B cells) and transit-amplifying type C cells (Ahn et al., 2005). Shh also affects proliferation in the SGZ (Lai 2003), although it is unknown which cells are Shh target.

Members of the Wnt family of soluble ligands play critical roles in various physiological processes during development and in the adult. Wnt signaling regulates stem cell self-renewal in several stem cell niches (Kleber et al.,2004). In adults neurogenic niches, Wnt signalling has thus far been shown to regulate neurogenesis in the SGZ. Increasing or decreasing Wnt activity in vivo leads to an increase or decrease of SGZ neurogenesis, respectively. Interestingly, astrocytes are the source of Wnts, highlighting their multiple roles in the niche.

4.1.SUB VENTRICULAR ZONE

In 1992, Reynolds and Weiss reported the first in vitro isolation and characterization of NPCs (Neural Progenitors Cells) from the adult brain. The investigators isolated, from the adult striatal area containing the sub ventricular zone (SVZ) of adult mice, a population of undifferentiated cells expressing nestin (an intermediate filament that has been characterized as a marker for neuroepithelial and CNS stem cell during development, and so considered as a marker for adult neural progenitor and stem cells), that differentiate into the main phenotypes of the nervous system, neuronal, astrocytic and oligodendrocytic. Isolated cells grew as neurospheres, in defined medium in the presence of epidermal growth factor (EGF) (Taupin 2006).

The SVZ consists of a thin layer of dividing cells that extends along the length of the lateral walls of the lateral ventricles and is largely separated from the cerebrospinal fluid (CSF) by a layer of multi-ciliated ependymal cells (Fig.5). Newly generated neuroblasts cross a network of chains which extends throughout the SVZ to join the rostral migratory stream (RMS) that leads to the olfactory bulb. There they differentiate into two kinds of inhibitory interneurons, and functionally integrate into the existing circuitry.
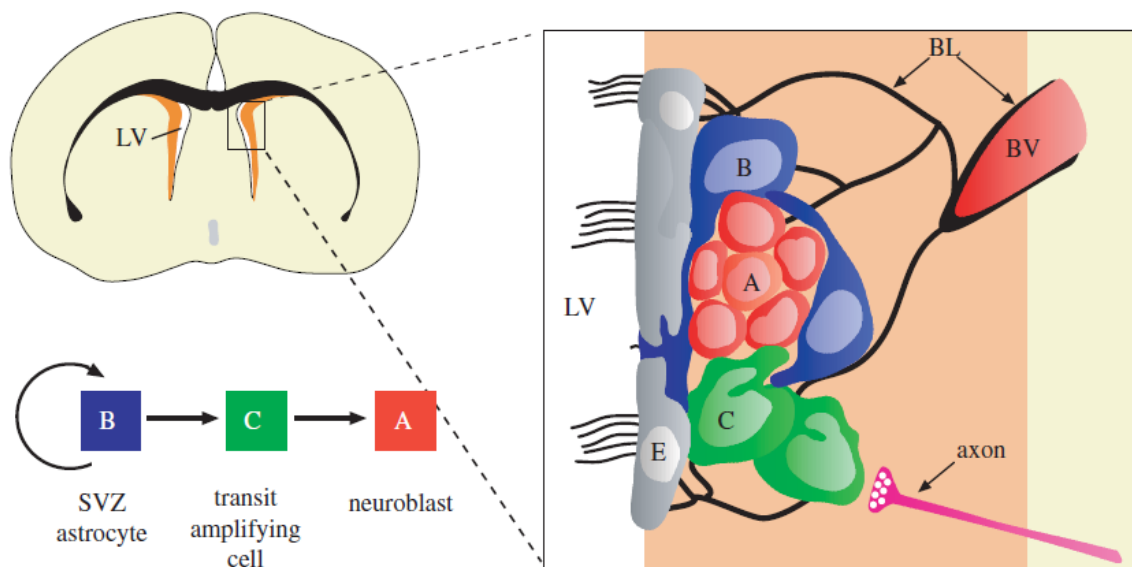
Fig.5: **Cell types and anatomy of the adult SVZ niche.** Schema of frontal section of the adult mouse brain showing the SVZ (orange) adjacent to the lateral ventricle (LV). SVZ astrocytes in this region (B, blue) are stem cells which generate migrating neuroblasts (A, red) destined for the olfactory bulb via a rapidly dividing transit-amplifying cell (C, green). Multi-ciliated ependymal cells (E, grey) line the walls of the lateral ventricle. A specialized basal lamina (BL, black) extends from perivascular cells and contacts all cell types. Modified from Riquelme et al., 2008.

In the SVZ, can be distinguished mainly three cells populations: the NSCs (called B cells), a relatively quiescent cell population that express markers of embryonic radial precursors, as astrocyte protein GFAP. B cells give rise to transit-amplifying cells (called C cells), a more rapidly dividing population that is GFAP negative but positive for nestin, EGF receptor and the transcription factor Dlx2. The third population is neuroblasts (called A cells) that express markers of newborn neurons such as doublecortin and PSA-NCAM (Miller et al., 2009, Doetsch, 1999).

An extensive blood vessel network spans the entire SVZ below the ependymal layer and it is closely associated with NSCs and their progeny. Many GFAP-positive NSCs are intercalated into the ependymal layer and have a short apical process with a single primary cilium contacting the ventricular wall and a second long basal process contacting a blood vessel (Riquelme et al., 2008).

After their generation and initial differentiation in the SVZ, neuroblasts migrate as neuronal chains, sliding along each other in the RMS, towards the olfactory bulb. This movement is highly directed toward the OB suggesting the presence of orienting cues in or around the RMS.

The GFAP+ population of astroglial cells adjacent to the ventricles is likely to be multifunctional: some functioning as neuronal precursors and others helping to orient and guide initial neuroblast migration away from the SVZ. Infact, radial processes of GFAP+ cells are highly polarized and oriented away from the SVZ towards the RMS (Ghashghaei et al., 2007).

A determinant key of this migration process seems to be a balance of chemorepulsive and chemoattractive signals from the SVZ environment. SLIT proteins, chemorepulsive signals for axonal growth and guidance, have been shown to repulse the newly generated cells from the SVZ. After exposure to SLIT, new neuroblasts in the SVZ might orient away from the SVZ and the surrounding striatal/septal areas, towards the olfactory bulb (Wu et al.,1999).

The lack of dispersion of migrating neuroblasts into surrounding tissues might be also due to astroglia, which encapsulate the migrating cells and form a cordon like a glial tube. The presence of these glial tubes might be use as a substrate for oriented migration. In addition, glial cells in the RMS secreted protein with migration-inducing activity (MIA), although in vitro RMS neuroblasts show diminished speed of migration in the presence of glial cells (Ghashghaei et al., 2007).

The maintenance and guidance of neuroblast migration through the mature forebrain requires a coordinated pattern of interactions between extracellular matrix (ECM) cues, cell adhesion molecules, and cell-surface integrin signaling receptors. An essential signal for the organization of neuroblast chain migration in the RMS is the polysialated form of neuronal cell adhesion molecule (PSA-NCAM) which control the strength of neuroblast adhesion allowing to form chains that 'slide along' each other within the glial tube. Integrins are another class of involved molecules: they convey the ECM-derived signals to the neuroblasts and show a differentiated expression during development in the RMS. Of the identified integrins in the RMS, the α1, β1 and β8 subunits are expressed mainly during early postnatal periods, whereas β3 and β6 integrin expression persists in the mature cortex. The substrates for intergrin signalling in the RMS during neuroblast migration remain to be fully elucidated. Another potential integrin ligand, tenascin-C, is expressed by the astroglial tubes and seems to fill the extracellular space between the migrating neurons and astrocytes in the RMS. Although tenascin-C is known to have anti-migratory effects and could potentially limit RMS neuroblasts from dispersing into the surrounding tissues (Riquelme et al. 2008).

Even though environmentally derived signals exert a potent influence on neuroblast migration, new neurons in the mature brain have an intrinsic capacity to migrate. The cell-intrinsic migratory mechanisms originate from the neuron's polarity and cytoskeletal machinery. In these neurons the microtubule cytoskeleton seems to be essential for the maintenance and growth of the leading processes, and the actin cytoskeleton is crucial for centrosomal reorientation. Recently, doublecortin (DCX), a microtubule- associated protein that predominantly localizes to the microtubule cage around the nucleus and to the leading processes, was shown to have a direct role in the intrinsic mechanisms modulating RMS neuroblast migration (Ghashghaei et al., 2007).

4.2.SUB GRANULAR ZONE OF HIPPOCAMPUS

The SGZ is located between the hilus and the granule cell layer of the dentate gyrus. Neurogenesis in the adult dentate gyrus originates from a precursor population that resides in the SGZ, a thin band of tissue between the granule cell layer and the hilus. Proliferative "hot spots" are identified close to the vasculature, and therefore, important regulation for neurogenesis has been supposed to come from blood vessels (Palmer et al., 2000). Newly generated granule neurons born in the SGZ migrate only at short distance to the granule cell layer, where they extend dendrites to the molecular layer and an axon along the mossy fibre path and integrate functionally into the circuitry of the dentate gyrus (Fig.6) (Riquelmeet al., 2008).
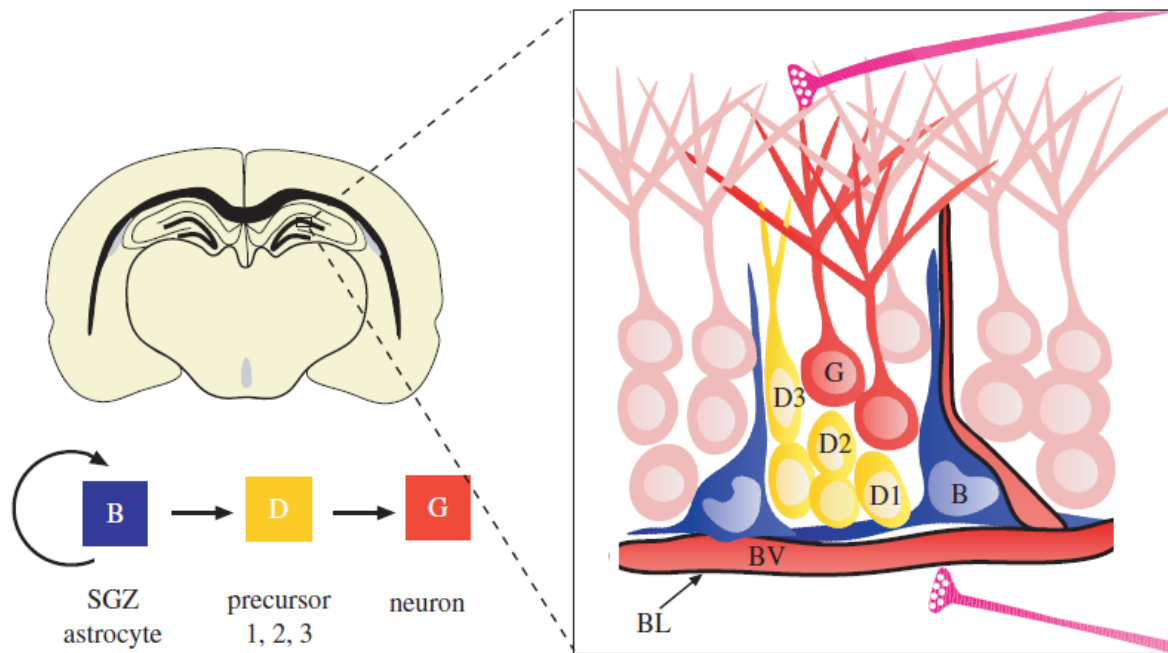

Fig.6: **Cell types and anatomy of the adult SGZ niche**. Schema of frontal section of the adult mouse brain showing the SGZ at the interface between the hilus (area below blood vessel) and the granule cell layer (light pink cells) of the dentate gyrus. SGZ astrocytes (B, blue) divide to generate intermediate precursors (type D cells; nomenclature according to Seri et al. 2004, yellow), which progressively generate more differentiated progeny (type D1/type D2/type D3), which mature into granule neurons (G, red). Neurogenesis occurs in pockets adjacent to blood vessels and although a specialized basal lamina has not yet been described in this region, the vascular basal lamina likely plays an important role in the niche. Afferent axons (pink) from the entorhinal cortex and axons from subcortical regions as well as from local inhibitory interneurons project to the SGZ. Modified from Riquelme et al., 2008

Adult hippocampal precursors were found to be multipotent, giving rise to neurons, astrocytes, and oligodendrocytes in vitro.

Hippocampus-derived astrocytes have been demonstrated to regulate neuronal differentiation from neural stem cells or progenitor cells in vitro (Song et al., 2002). Adult hippocampal neurogenesis originates from cells with morphological and functional characteristics of glial cells Fig7. Type 1 cells constitute the resident early precursor population that shares morphological and antigenic features with radial glia. The soma of type 1 cells is triangular-shaped and located in the SGZ. Type 1 cells usually extend a strong apical process into the molecular layer and may contact blood vessels through vascular end feet. Type 1 cells are abundant in the SGZ but rarely divide. They express astrocytic marker GFAP and intermediate filament nestin. Many type-1 cells express the radial glia marker BLBP and stem cell protein Sox2 (Kempermann et al., 2004).

Type 1 cells give rise to fast proliferating intermediate precursors (type 2 cells and type 3 cells). Most of the expansion of the pool of newly born cells occurs during the stage of the type 2 cell.
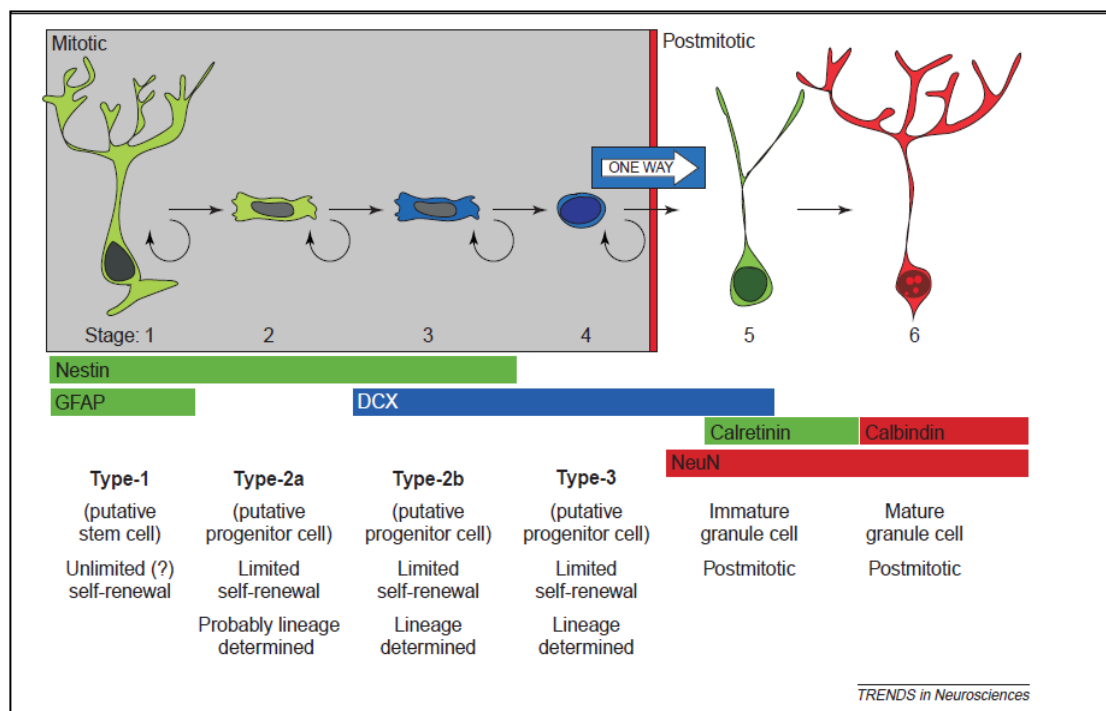
Fig.7: **Proposed sequence of cell types in adult hippocampal neurogenesis.** Six stages of neuronal development in the adult hippocampus can be readily identified on the basis of morphology, proliferative ability, and expression of markers such as nestin, glial fibrillary acidic protein (GFAP), doublecortin (DCX), calretinin, calbindin and NeuN. Modified from Kempermann et al. 2004

Type 2 cells are morphologically distinct from type 1 cells: their processes are short and horizontally oriented. The type 2 cells also show overlap in glial (BLBP, nestin) and neuronal marker (DCX and PSANCAM) expression. A recent study showed that type 2 Sox2-positive cells can self-renew and that a single Sox2- positive cell can give rise to a neuron and an astrocyte, providing the first in vivo evidence of stem cell properties of hippocampal neural progenitors (Suh et al., 2007). This study also suggested that the relationship between type 1 and 2 cells could be reciprocal.

The type 3 cell stage is one of transition from a slowly proliferating "neuroblast" to a postmitotic immature neuron. Under normal circumstances, type 3 cells show only little proliferative activity. These cells invariably express markers of the neuronal lineage (DCX, PSA-NCAM, NeuroD, Prox1) and lack glial markers. DCX expression shows almost complete overlap with PSA-NCAM and spans a developmental period that comprises the type 3 stage and the initial 2–3 weeks of postmitotic neuronal development. The highly variable morphology of type 3 cells reflects this developmental transition: type 3 cell processes have various lengths and complexities, and the orientation of their processes increasingly changes from horizontal to vertical. Radial migration into the granule cell layer to the final destination of the young neuron also occurs on the level of the type 3 cell. Exit from the cell cycle mostly occurs at the type 3 cell stage and coincides with the transient expression of the calcium-binding protein calretinin (Kempermann et al. 2004).
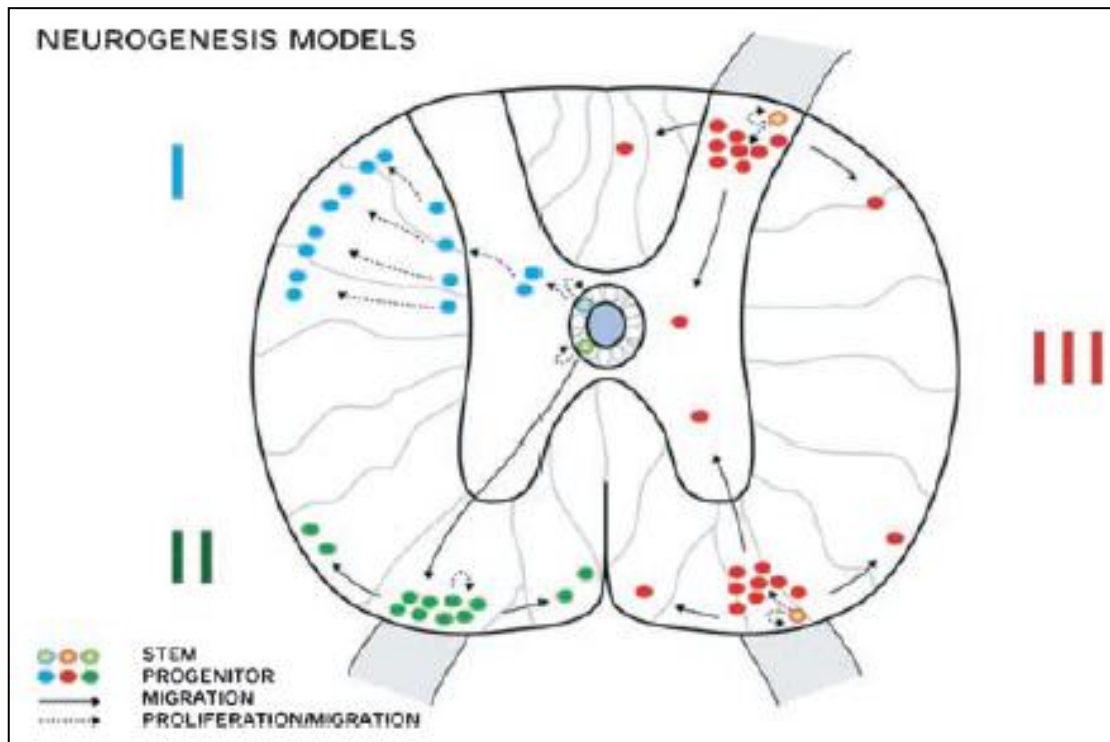
4.3.SPINAL CORD

Little is known about the in situ location, activity, regulation, and function of adult spinal cord SPCs (Stem/Progenitors Cells). Two models have been proposed regarding the location of these cells in the intact adult spinal cord: in the first, a slowly proliferating stem cell resides in the ependymal layer of the central canal (Johansson et al., 1999); in the second is suggest that stem cells and glial progenitors may exist in the parenchyma of the spinal cord (Obermair et al., 2008) and are independent of the proliferative ependyma.

In vitro expansion and characterization of SPCs, however, demonstrated that in vivo proliferating cells from the adult spinal cord have the ability to give rise to neurons in culture. Furthermore, cell transplantation studies have demonstrated that, although SPCs derived from spinal cord will differentiate into glial cells when re-implanted into the region of origin, they are able to give rise to neurons when grafted into the neurogenic hippocampus (Shihabuddin et al., 2000).These findings indicate that the neuronal differentiation in vitro is not a tissue culture artifact but reflect the multipotent differentiation potential of these cells. In addition, these results demonstrate that adult spinal cord SPCs are not intrinsically fate restricted but that environmental cues are able to instruct their fate choice (Obermair et al., 2008).

In non pathological conditions, the dividing spinal cord progenitor cells are glial-restricted progenitor cells and give rise to oligodendrocytes and astrocytes but not neurons. Horner and colleagues (Horner et al.,2000) found an active progenitor population that persists after postnatal glial cell formation. They provided evidence that proliferating cells do not commonly migrate from the central canal as previously described in the model for early postnatal gliogenesis. (Model I, Fig.8, I). They proposed two alternative models (Fig.8, II, III). In model II, a stem cell exists at the ependymal layer that divides asymmetrically. A daughter cell then migrates to the outer circumference of the spinal cord where it exists as a bipotent or glial progenitor and begins to divide more rapidly. This model separates rates the slowly dividing stem cell at the central canal from a proliferative progenitor that migrates first and then divides in the outer annuli of the spinal cord. The alternative model III, predicts that a glial progenitor and stem cell population may exist in the outer circumference of the spinal cord where cell

division is more common. This model functionally separates ependymal cell division from the proliferative zone of the outer annuli.

Fig.8: Models of stem cell proliferation and migration in the intact adult spinal cord. Three models illustrate how dividing stem cells may give rise to progenitors that migrate and proliferate. Model I corresponds to early postnatal gliogenesis. The current data suggest that in the adult, dividing cells are located primarily in the outer circumference of the spinal cord, and therefore Models II and III more likely reflect adult gliogenesis in the intact spinal cord. Modified from Horner et al., 2000.

Neural stem cells have also been isolated from the ependymal zone surrounding the central canal of the spinal cord. The spinal cord ependymal cell niche has been described for the first time from Hamilton and colleagues (Hamilton et al., 2009). They observed that central canal is lined with ciliated ependymal cells that express Vimentin. Ki67 proliferating cells are primarily found within the ependymal zone and are distributed in a dorsal to ventral gradient. Proliferating cells are found in doublets and are always closely opposed to PECAM-expressing blood vessels. There are Nestin-expressing and GFAP-expressing Vimentin cells at the dorsal pole that possess extensive basal projections. Within the sub-ependymal layer, there are GFAP positive astrocytes, NeuN positive neurons and Olig2 positive oligodendrocyte progenitors Fig.9.
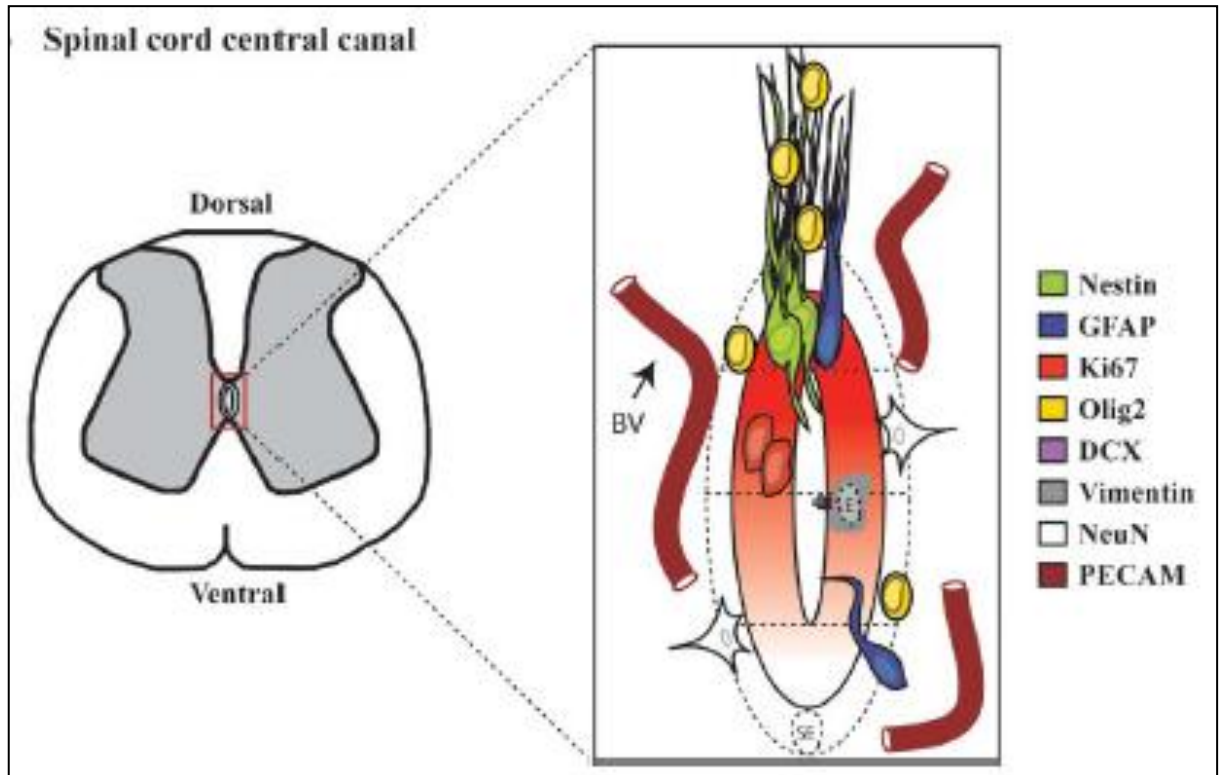
Fig.9: Key features of spinal cord ependymal cell niche. In the spinal cord, the central canal is lined with ciliated ependymal cells that likewise express Vimentin. Ki67 proliferating cells are primarily found within the ependymal rather than sub-ependymal zone and are distributed in a dorsal to ventral gradient (red gradient). Proliferating cells are found in doublets and are always closely apposed to PECAM-expressing blood vessels (however, this association is not clearly evident on coronal sections). There are Nestin-expressing and GFAP-expressing Vimentin cells at the dorsal pole that possess extensive basal projections. Within the sub-ependymal layer, there are GFAP astrocytes, NeuN neurons and Olig2 oligodendrocyte progenitors. Olig2 cells are often observed along the basal processes of dorsal ependymal cells. Modified from Hamilton 2009.

Hamilton and colleagues (2009) showed spinal cord ependymal cells have neural stem cell potential both in vitro and following tissue injury in vivo. Moreover they found that neurogenic astrocytic stem cell-derived spinal cord ependymal cells do not display multilineage potential or the ability to produce transit amplifying progenitors under normal conditions.

Regarding the spinal cord ependymal cell niche, ependymal cells are rapidly activated following spinal cord injury, proliferating and undergoing multi-lineage differentiation to contribute astrocytes and oligodendrocytes to the injured tissue. Thus, at least a sub-population of spinal cord ependymal cells has latent neural stem cell properties (Shihabuddin et al., 2000).

In pathological condition (Spinal Cord Injury, SCI) damage of neuronal and glial cells in and around the lesion site lead to disruption of neuronal circuitry and neurological dysfunction. It has been demonstrated that adult spinal cord stem cells are responsive to injury. The insult-induced progenitor cell response comprises division, migration, and maturation of SC progenitors cells (Okano 2007). After a contusive SCI in adult rodents, proliferation of cells expressing NG2 (oligodendrocyte progenitors cells marker) reaches a peak at 3 days, stays markedly increased in the epicenter of the lesion, and declines to baseline levels by 4 wk after injury. These newly formed oligodendrocytes likely contribute to remyelination.

The normally limited proliferation of ependymal cells increases after SCI, followed by migration of ependyma-derived progeny toward the site of injury. Moreover, ependymal cells differentiate and give rise to a substantial proportion of scar-forming astrocytes as well as to some oligodendrocytes after SCI (Meletis et al., 2008).

Since it is known that SC progenitors derived from the adult spinal cord produce neurons and oligodendrocytes when transplanted into neurogenic regions like the hippocampus (Shihabuddin et al.,, 2000), it is obvious that the environment plays an essential role in the fate decision made by progenitor cells. It was suggested that the properties of the local astrocyte populations play a major role in the creation of a neurogenic or gliogenic niche influencing differentiation of SC stem cells. Inflammation induced by SCI is another important player in SC progenitors cells induction by pro-inflammatory cytokines and peripheral immune cells. Infact pro-inflammatory cytokines upregulated after SCI (TNF-α, IL-6, IL-1α, and IL-1ß) can stimulate proliferation and survival of oligodendrocyte and neuronal precursors. Moreover the massive invasion of peripheral immune cells after SCI accompanied by the expression of proinflammatory may play a supportive role in neurogenesis as reported by Michal Schwartz group (Butovsky et al.,2005; Butovsky et al., 2006; Shechter et al., 2007).

Depending on whether microglial cells are stimulated by the T helper cell 1 (Th1) released cytokine interferon (IFN-α) or by IL-4 from Th2 cells, they differentially influence the fate choice of SC progenitors cells: microglia stimulated with IL-4 induced a predisposition toward oligodendrogenesis, whereas microglia stimulated with IFN-α preferentially induced neurogenesis. in co-cultured neural SC progenitors. Finally different growth factors have been used to stimulate proliferation of endogenous SC progenitor cells and to influence their differentiation:FGF-2, IGF-1 and BMP (Obermair et al., 2008).

4.4.RETINA

The neural retina is a complex tissue of the eye that mediate our vision by two structures: the rod and cone photoreceptors. The rods respond to single photons and are responsible for our night vision, while cones mediate high acuity color vision in the daylight. The photoreceptors respond to photons by changes in their membrane potential that are diffuse by synaptic connections through a circuit of neurons to the brain. Responses of the photoreceptors to changes in illumination lead to changes in their release of neurotransmitter at synapses with a group of retinal interneurons called bipolar cells which ,in turn relay the signals to ganglion cells, connecting by the optic nerve to visual centers in the brain.

The different types of retinal neurons are organized in three laminae: the nuclei of the rods and cones are in the outer nuclear layer (ONL), the nuclei of the bipolar cells and amacrine cells are in the inner nuclear layer (INL), and the innermost layer of the retina contains the ganglion cells and their axons (Fig.10).
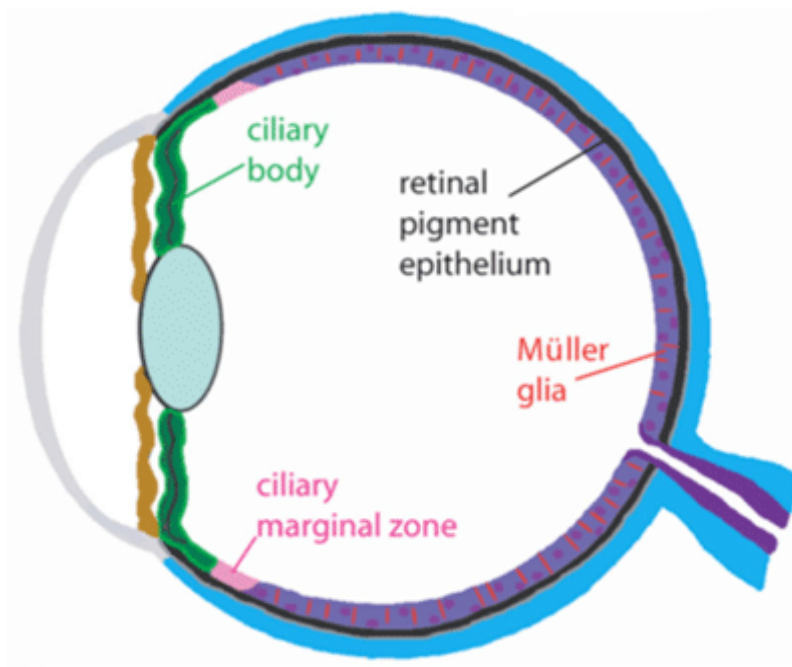
Fig.10: Laminar structure of mature retina: RPE retinal pigmented epithelium, ONL outer nuclear layer, INL inner nuclear layer, GCL Ganglion cellular layer.

In addition to the neurons of the retina, there are several types of non neuronal cells that are also critical for its function: Müller cells, a type of glia that perform functions similar to astrocytes in other regions of the CNS. Adjacent to the photoreceptor layer is a layer of pigmented cells, called the retinal pigmented epithelium (RPE), which are essential for the maintenance of the rods and cones.

The presumptive retina can be identified as early as neural plate: as the neural plate rolls into a tube, the lateral aspects of the anterior neural tube evaginate to form paired optic vesicles. These vesicles undergo a series of morphogenetic changes to form two-layered optic cups. The inner layer develops into the neural retina and the non pigmented layer of the ciliary epithelium, while the outer layer acquires pigmentation and develops into the RPE and the pigmented layer of the ciliary epithelium. In these steps different signaling pathways are required: FGF for the development of the neural retina (Vinothkumar et al.,2008); BMP and Wnt signaling promote ciliary epithelium development (Liu et al., 2007); Shh to promote both RPE and optic stalk development (Lamba et al., 2008).

Once the optic cup has formed, the cells within this structure are known as retinal progenitor cells. Similarly to neural progenitors found in the brain, retinal progenitors respond to mitogenic factors that stimulate proliferation of neural stem/progenitors in the CNS, notably EGF, FGF, and Shh. Moreover retinal progenitors express and require many of the genes like the pro-neural transcription factors bHLH, fundamental for the production of the different retinal neurons, as they are for neurons in other areas of the CNS.

A small peripheral zone at the junction between the cilliary epithelium and retina, is mitotically active and it is called ciliary marginal zone (CMZ). The CMZ is analogous to other neural stem cell
zone in the vertebrate CNS, it can persist throughout the lifetime of the animal and can generate all the types of neurons and glia normally present in the retina.

The cells in the zone proliferate and generate new neurons at the junction with the retina, which subsequently differentiate and are incorporated into the retinal circuitry.

The CMZ cells look like retinal progenitors in their pattern of gene expression and respond to at least some of the same mitogenic factors as the embryonic retinal progenitors (Moshiri et al., 2005). Infact Wnt signaling promotes ciliary epithelium development (Cho et al., 2006). The same signal may also be critical for the CMZ to form by maintaining the cells in a stem cell state.

Since Bmp4 also promotes ciliary epithelial formation (Zhao et al., 2002), Noggin might also play a role in maintaining this niche, as it does for the SVZ (Lim et al., 2000). Shh is an important mitogenic factor for retinal progenitors during embryonic stages and it is also expressed in the CMZ (Moshiri et al.,2004, Lamba et al., 2008).

5. NEURAL STEM CELLS AND IMMUNE SYSTEM

Immune cells and immune molecules have been recently shown to support neurogenesis in the adult brain. In particular, Hauben et al. (Hauben et al., 2000) observed an impaired neurogenesis in both SVZ and DG of T-cell-deficient mice [i.e. severe combined immune deficiency (SCID) and nude] compared to that in wild type mice. Moreover reconstitution of the T-cell pool in immune-deficient mice by injection of wild-type splenocytes showed a partially restore of proliferation and neuronal differentiation, reaching levels similar to those observed in wild-type mice (Sartori et al., 1997).

Experiments in models of CNS injury have shown that the neuroprotective effect of such CNS-specific T cells is mediated by modulation of the activity of the local microglia and macrophages at the site of injury (Butovsky et al., 2001). T-cell-based modulation of microglial activity also seems to have a role in the adult neurogenic niches (Ziv et a., 2008).

The proposed mechanism is that T cells residing within the choroid plexus and the meninges interact locally with antigen-presenting cells (such as dendritic cells and macrophages) and secrete cytokines or growth factors into the cerebrospinal fluid (CSF) (Schwartz et al., 2008). Both neurogenic niches of the adult brain (the SVZ and the dentate gyrus) are located 10–100 µm away from the ventricle walls, certainly within diffusion distance of the CSF. Such a 'remote access' mode of T-cell activity remains to be demonstrated experimentally, but the possibility that T-cell-derived cytokines could affect neurogenesis in vivo, regardless of T cell presence in the parenchyma, has already been demonstrated (Ziv et al., 2008).

Additionally Rolls et al. found that NPCs express Toll-like receptors (TLRs) usually associated with innate immunity in mammals. Additionally, Rolls et al. (2007) found that NPC activity is affected by TLR-2 and TLR-4 signaling in both adult neurogenic niches . TLR-2 and TLR-4 have opposing effects on NPCs; TLR-2 signaling positively regulates neuronal differentiation, whereas TLR-4 signaling negatively regulates progenitor-cell proliferation.

The finding that NPCs express TLRs, should not come as a surprise when considering the fact that these receptors were first characterized with regard to their roles during nervous system development in Drosophila (Anderson et al., 1985).

Similarly to neurogenesis in the classical neurogenic niches T cells, particularly T cells that recognize CNS antigens were found to support the formation of new spinal cord neurons. The generation of similar cells has been reported in the cerebral cortex as well (Dayer et al., 2005).

6. LEPTOMENINGES

6.1.ANATOMY

The organs of the central nervous system (brain and spinal cord) are covered by 3 tissue layers called meninges. The meninges include the dura mater and the leptomeninges (arachnoid and pia mater). Dura forms an outer endosteal layer in contact with the skull and spine bone and an inner layer strictly linked to the arachnoid mater. Leptomeninges have multiple functions and anatomical relationships. The outer parietal layer of arachnoid is impermeable to the cerebrospinal fluid (CSF) due to tight intercellular junctions; elsewhere leptomeningeal cells form desmosomes and gap junctions. Trabeculae of leptomeninges compartmentalize the subarachnoid space and join the pia to arachnoid mater (Fig. 11).
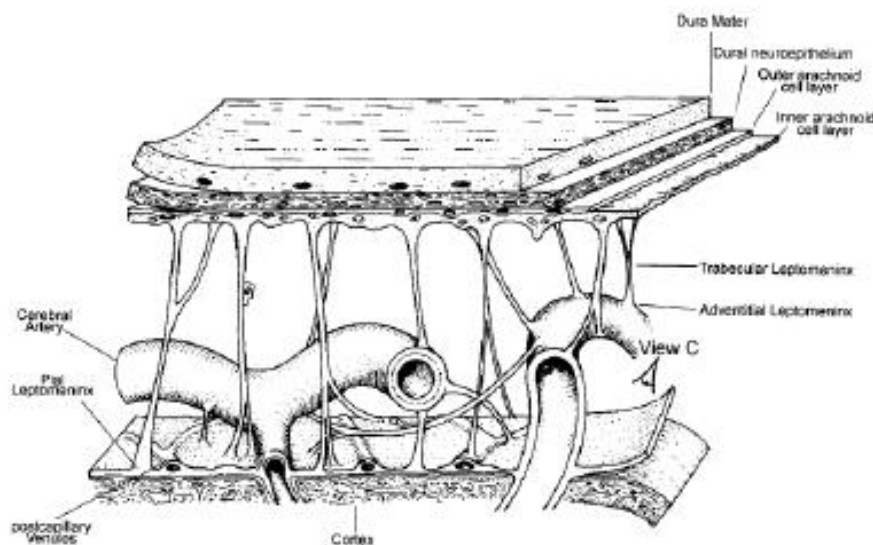
Fig.11: Schematic draw of meningeal layers in human brain. Modified from McMenamin et al. 2003.

The arachnoid consists of bundles of white fibrous and elastic tissue intimately joined. Its outer surface is covered with a layer of low cuboidal mesothelium. The inner surface and the trabeculae are covered by a cuboidal mesothelium which somewhere is flattened to a

pavement type (Vandenabeele et al., 1996). A few vessels of considerable size and a rich plexus of nerves derived from the motor root of trigeminal, facial and accessory nerves, are found in the arachnoid. Pia mater is reflected from the surface of the brain and spinal cord onto arteries and veins, thus separating the subarachnoid space from the brain and cord (Nicholas and Weller, 1988). The pia mater is a vascular membrane, consisting of a minute plexus of blood vessels, held together by an extremely fine areolar tissue and covered by a reflexion of the mesothelial cells from the arachnoid trabeculae. It is an incomplete membrane, absent at the foramen of Majendie and the two foramina of Luschka and perforated in a peculiar manner by all the blood vessels as soon as they enter or leave the nervous system. An anatomical basis for the separation of the subarachnoid from the perivascular (Virchow-Robin) spaces has been established in several mammalian species, including human. It has been shown that the pia mater on the surface of the brain and spinal cord is reflected onto the surface of blood vessels in the subarachnoid space, thus separating the perivascular and subpial spaces from the subarachnoid space (Pollock et al., 1997). In the perivascular spaces, the pia apparently enters as a mesothelial lining of the outer surface of the space; at variable distance from that, these cells become unrecognizable and are apparently absent, replaced by neuroglia elements. The inner walls of the perivascular spaces seem covered for a certain distance by the mesothelial cells, reflected with the vessels from the arachnoid covering these vascular channels as they pass through the subarachnoid spaces (Kida et al., 1993). Leptomeninges that accompanies arteries into the brain are involved in draining interstitial fluid that plays a role in inflammatory responses in the brain and appears to be blocked by amyloid-beta in Alzheimer's disease. Specialized leptomeningeal cells in the stroma of the choroid plexus form collagen whorls that become calcified with age. Leptomeningeal cells also form channels in the core and apical cap of arachnoid granulations to drain CSF into venous sinuses. In the spine, leptomeninges form highly perforated intermediate sheets of arachnoid and delicate ligaments that compartmentalize the subarachnoid space; dentate ligaments anchor subpial collagen to the dura mater and stabilize the spinal cord (O'Meara et al., 2007).

6.2.EMBRIOLOGY

In humans, the central cavity of the neural tube is formed during neurulation, which occurs during the fourth gestational week. The first milestone is occlusion of the spinal neurocele (the central canal in the neural tube) shortly after neurulation. This prevents free communication between the ventricular system and the amniotic cavity. The second milestone is development of the meninges, which separate the CNS from the rest of the body. The embryonic origin of the meninges varies across species. In birds (and probably in mammals), the spinal meninges are derived from the somitic mesoderm, the brain stem meninges from the cephalic mesoderm, and the telencephalic meninges from the neural crest. Differentiation of the meninges, which involves formation of the subarachnoid space, occurs early, before CSF begins to flow around the CNS (Catala, 1998). During ontogenesis, the meninges play a key role in regulating the growth of underlying nervous structures. They induce the formation of the superficial glial limiting layer and stimulate the growth of precursors located in the superficial blastemas of the cerebellum and hippocampus (McLone, 1980a). The choroid plexuses are complex specialized structures that produce most of the CSF. Their epithelium derives from the neural tube epithelium and their mesenchyma from the meninges (Keep and Jones, 1990). Development of pia-arachnoidal membranes in the mouse occurs in four stages: the first (prenatal days 10-13) follows closure of the neural tube and is a period of initial vascularization of the developing telencephalon; the second (prenatal days 14-16) is a period of delineation during which the limits of the subarachnoid space are defined; the third (prenatal day 17 to birth) is a period of formation of pia-arachnoidal blood vessels; and the fourth (birth to postnatal day 15) includes addition of smooth muscle cells to larger vessels, the appearance of macrophages in the subarachnoid space, and a general increase in extracellular collagenous and elastic fibers. The mesenchyme over the telencephalic surface in the 10-day fetus has a typically large extracellular space. By the 13th fetal day CSF begins to flow into and replace it. The mesenchymal extracellular compartment is reduced in the periphery, resulting in a compacted pia-arachnoidal tissue which limits the peripheral extent of the subarachnoid space. By the 15th postnatal day a subarachnoid space typical of the adult animal is established (McLone, 1980b).

6.3.LEPTOMENINGES IN CORTICOGENESIS

The cortical plate of the mouse cerebral cortex develops between embryonic day 12 (E12) and E18 with the migration of neuroblasts from the ventricular layer to the pial surface. The organizing framework for cortex histogenesis is provided by the spindle shaped radial glia cells that serve as substrate for the migrating neuroblasts . In the cortex, basement membranes are found in the pia and around blood vessels. Several reports have implicated the pial basement membrane as important player in brain development (Halfter et al., 2002): abnormal brain development has been observed after chemical ablation of the meningeal cells (Sievers et al., 1994) and after the targeted deletions of basement membrane constituents, such as perlecan (Arikawa-Hirasawa et al., 1999) or receptors for basement membrane proteins (Graus-Porta et al., 2001). Deletions of 10 of the current 14 laminin isoforms through inactivation of the laminin a1 chain resulted in the early lethal phenotype before neural tube formation (Smyth et al., 1999); however, mice that lack the laminin a5 chain have an obvious brain phenotype and develop exencephaly . In case of targeted deletion of the nidogen-binding site within the laminin a1 chain, b1III4 (Willem et al., 2002), the basement membranes in kidney and lung alveoli are disrupted. The mutant mice die at birth because of impaired lung and kidney development. Pial basement membrane of the b1III4-deficient mice disappers at early gestational stages and results in a disrupted neuronal migration. Defects of the ECM have been suggested to be the primary cause of type II (clobbeston) lissencephaly, where superficial neuroblasts migrate from the malformed cortical plate into the subarachnoid space through defects of the BM (Olson et al.,2002). In cobblestone lissencephaly the pial basal membrane is not necessarily smooth, but it frequently shows a broad smooth paving, like cobblestones. Here, leptomeninges contain astrocytes and are named gliovascular tissue. Strands of gliovascular tissue extend from the surface into the depth of the cortex, following the spaces between microgyri and polipoid protrusions. Thus, gliovascular tissue intersperses with islets of central nervous tissue, resulting in a dysplastic cortical pattern (Bornemann et al., 1997). Leptomeninges play an important role in the control of neuronal migration, because intact pial-basal membrane is absolutely required for proper cortical development; its disruption leads to the development of ectopic neuronal and glial cells in the subarachnoid space (Beggs et al., 2003).The cortical plate of the mouse cerebral cortex develops between embryonic day 12

(E12) and E18 with the migration of neuroblasts from the ventricular layer to the pial surface. The organizing structure for cortex histogenesis is provided by radial glia cells that serve as the substrate for the migrating neuroblasts (Rakic 1995). The localization of the neurons within the cortical plate is regulated by reelin an extracellular matrix glycoprotein secreted by the Cajal-Retzius cells.

AIMS

During recent years, embryonic and adult neural stem cells (NSCs) gained attention as major candidates for regenerative and cell replacement therapies in various neurodegenerative diseases. The clinical application of adult NSCs, despite their properties of self-renewal, neuro-glial differentiation potential and their possible use in autologous setting, is still of debate. Among technical concerns, of relevance is that NSCs have hardly accessible site of sampling, are difficult to expand in vitro as homogeneous stem cell population and show low rate of in vivo neuronal differentiation efficiency .

As an alternative to an homologous implant, human embryonic stem cells (hESCs) are strong candidates, however they are associated with a set of problems. First most of grafted cells died or partially differentiate; secondly undifferentiated grafted cells might continue to proliferate giving rise to tumors; finally hESC derived grafts could stimulate an immune reaction in the brain, and this might adversely affect surrounding brain circuitry (Li et al., 2008).

Based on previous observations, our hypothesis is that leptomeninges might host a neural stem/progenitor cells niche. Firstly, leptomeninges, which include arachnoid and pia mater, cover the entire CNS (brain and spinal cord) and are filled with CSF produced by choroid plexi. All the major arteries supplying the brain pass through leptomeninges and form branches while penetrating the cortex (Reina-De La Torre et al., 1998). Interestingly, every parenchymal vessels inside the CNS are surrounded by a perivascular space (Virchow-Robin space) formed by the extroflexions of leptomeninges filled with CSF (Jones, 1970; Rodriguez-Baeza et al., 1998). Thus, leptomeninges are widely spread inside the CNS parenchyma, including the choroid plexus.

Moreover leptomeninges are characterized by spatial-temporal interactions amongst environmental cells that ensure a correct cortex development (Halfter et al., 2002). They are present since the very early embryonic stages of cortical development and are known to produce the chemiotactic factors SDF-1 involved in neocortical development (Reiss et al., 2002, Borre et al.,l 2006). They are involved in multiple interactions among a large number of cell types such as pial basal membrane cells, radial glia, neural precursor cells, Cajal Retzius cells and extracellular matrices like laminin, and collagen IV, fibronectin that ensure the correct

cortical development. Abnormal function/structure of leptomeninges causes altered cortical histogenesis, as showed in Halfter et al (2002).

The peculiar spatial relationships of leptomeninges in CNS, their role in cortex development and our discovery of nestin-positive cells in leptomeninges, prompted us in determining whether leptomeninges could be a stem cell niche hosting stem/progenitor cells with *in vitro* and *in vivo* neuronal differentiation potential.

In order to demonstrate this hypothesis, the following experimental plan was followed (Fig.12):

- Describe the presence of different cellular markers in leptomeninges from brain and spinal cord slices at different rat ages to understand the features of leptomeninges as a stem/progenitors cells niche;

- Evaluate the morphology and functionality of differentiated Leptomeningeal cells previously expanded in culture to assess their in vitro neural differentiation potential.

- Evaluate the in vivo differentiation potential of leptomeningeal cells injected in living adult rat hippocampus.

- Explore the possible existence of immune-based regulation of the Leptomeningeal nestin positive cells (ongoing project).
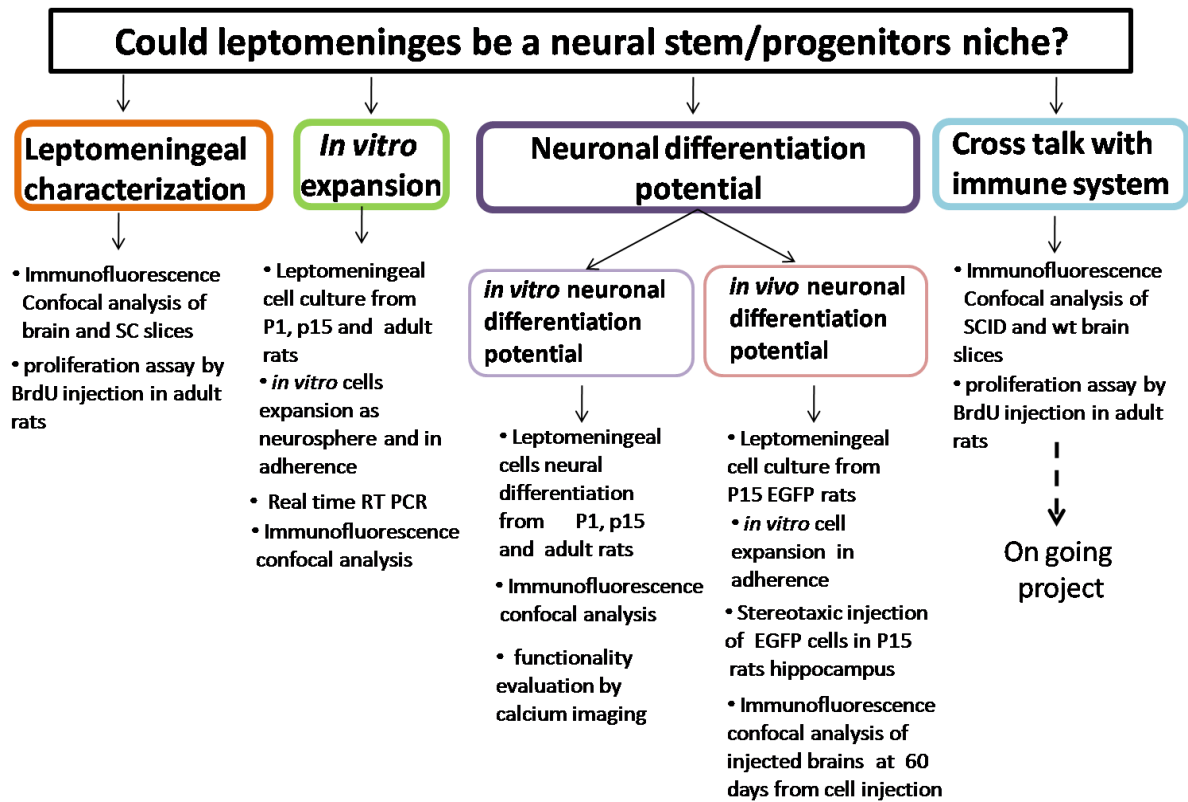
Could leptomeninges be a neural stem/progenitors niche?

Leptomeningeal characterization

- Immunofluorescence Confocal analysis of brain and SC slices
- proliferation assay by BrdU injection in adult rats

In vitro expansion

- Leptomeningeal cell culture from P1, p15 and adult rats
- *in vitro* cells expansion as neurosphere and in adherence
- Real time RT PCR
- Immunofluorescence confocal analysis

Neuronal differentiation potential

in vitro neuronal differentiation potential

- Leptomeningeal cells neural differentiation from P1, p15 and adult rats
- Immunofluorescence confocal analysis
- functionality evaluation by calcium imaging

in vivo neuronal differentiation potential

- Leptomeningeal cell culture from P15 EGFP rats
- *in vitro* cell expansion in adherence
- Stereotaxic injection of EGFP cells in P15 rats hippocampus
- Immunofluorescence confocal analysis of injected brains at 60 days from cell injection

Cross talk with immune system

- Immunofluorescence Confocal analysis of SCID and wt brain slices
- proliferation assay by BrdU injection in adult rats

On going project

Fig.12: Experimental plan.

MATERIALS AND METHODS

Brain perfusion and immunofluorescence

The animals were perfused with 4% paraformaldehyde in PBS. Brains were dissected, fixed in 4% paraformaldehyde solution, then left in 10% and subsequently 30% sucrose solution. 30 µm thick coronal brain sections were cut by freezing microtome and processed by immunofluorescence as described in Weimann 2003. Briefly, Brain slices were incubated for 2h in blocking solution (PBS/5%FCS/3%BSA/0.3% Triton X-100). Slices were then incubated for 12 hrs at 4°C in floating, with antibodies. The following primary antibodies were used: mouse monoclonal antibodies anti-nestin, anti-BrdU (all from Pharmingen/Becton Dickinson); anti-EGFP (rabbit, 1:2000, Invitrogen), MAP2 (mouse, 1:1000, Sigma), GFAP (mouse, 1:1000, BD Pharmigen), Laminin (rabbit, 1:1000 Sigma), Nestin (mouse, 1:1000, BD Pharmingen), NG2 (rabbit, 1:500, Chemicon), Neurofilament 160 (mouse, 1:100, Sigma), DCX (goat, 1:500, Chemicon), Synaptophhysin, GluR2, NR1, gently given by C.Sala.

Primary antibodies were detected with appropriated secondary antibodies for 4h at 4°C in blocking solution The following secondary antibodies were used: goat anti-mouse Ig/Alexa Fluor 488, IgM/FITC, IgG/PE, and chicken anti-rabbit/Alexa Fluor 488 (all from Molecular Probes), goat anti-mouse/Cy3, goat anti-rabbit/Cy3 (all from Amersham).

Cell cultures

Cells were harvested from the first cortical layers and overhead leptomeninges of Sprague-Dawley rats at embryo age 20days (E20) and post natal day 0 (P1), P15 and adult; or from enhanced green fluorescent protein (EGFP) transgenic rats (Leto 2006) at postnatal days P15, (n= 6 experiments, 10 animals each). Tissues were sampled with stereo-microscope from brain coronal sections. Mechanically/enzymatically- dissociated tissue extracts were cultured in neurosphere-inducing, adherent and differentiating conditions.

Media

Growing medium: Dulbecco modified Eagle medium (DMEM), with high glucose concentration, GLUTAMAX I, 18% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 µg/ml streptomycin (all from GibcoBRL/Life Technologies, Milan, Italy).

Differentiation medium: Differentiating medium: Neuron Chow (Neurobasal Medium, Gibco), with 2% B27 supplements (Gibco), 0,5mM glutamine, 1,25µM mM glutamate, 1% penicillin-streptomycin-fungizole) and 50 ng/ml brain-derived nerve growth factor (BDNF).

Colony Forming Unit (CFU) assay

Dissociated tissue extracts from E20, P0, P8, P15 and Adult were plated at five different cell concentrations. After 14 days of culture, every single cell-colony with more than 50 cells was considered as CFU. CFU assay was performed every following passage, by plating cells at four different concentrations. CFUs were stained with May-Grunwald Giemsa and then counted (mean ±SD of two different cultures).

Intracellular Calcium Imaging

Intracellular calcium levels (Ratio 340/380) were evaluated by ratiometric imaging techniques. Cells were loaded with Fura2-AM for 30-45 minutes at 37°C. The loading solution contained 5µM Fura 2-AM in HEPES buffered solution(165mM NaCl, 5mM KCl, 2mM CaCl2, 10mM Glucose, 5mM Hepes; pH 7.4). Loading solution was removed and the culture was incubated another 10 minutes at 37°C in HEPES buffered solution to allow de-esterification of Fura2-AM. The coverslip were then placed onto a recording chamber, connected by a Tygon tube to a 1 ml syringe, used for substance fast application. All image processing and analysis was performed using OpenLab software (Improvision) with an inverted Zeiss microscope. Wavelength of 340 and 380nm were used to excite Fura2 and the emitted light was collected at 510nm.

Cells immunofluorescence

Cells were immunostained after previous plating on poli-lysine coated glass coverslips. Briefly, cells were fixed for 20 min with 4% paraformaldehyde and 4% sucrose in PBS buffer and subsequently rinsed in PBS. Cells were permeabilized for 30 min in PBS containing 0.2% bovine serum albumin and 0.2% Triton X-100 (PBS/BSA/TRITON). Neurons were incubated with primary antibodies in PBS/BSA/TRITON for 90 min at room temperature. After rinsing in PBS/BSA/TRITON, secondary antibodies were applied for 1 h. After final washing steps in PBS, preparations were mounted on anti-bleaching 1,4- diazabicyclo[2.2.2]octane (Sigma) in PBS containing 50% glycerol. Slides were observed by using a Zeiss LSM 510 confocal microscope equipped with argon (488 nm) and helium/neon (543 nm) excitation lasers. For 488 nm excitation, emission was selected with a 510–530 nm bandpass filter, whereas for 546 nm excitation emission was selected using a 560 nm longpass filter.

5-bromo-2-deoxyuridine (BrdU) cells labelling

Cells were incubated for 12h with BrdU (3 μM) before starting the differentiation protocol. After differentiation, cells were fixed on coverslips with 4% paraformaldehyde and rinsed with PBS. Cells were then treated for 15 minutes in 2N HCl/0.5% Triton X- 100 at room temperature, and the reaction were neutralized with 0.1N B4Na2 (pH 8.5).

5-bromo-2-deoxyuridine (BrdU) intra-peritoneal injection

SD rats at postnatal day P15, were injected IP with 50 mg/Kg body weight BrdU (three times every 8 hrs). Animals were perfused at 24 hrs from the first injection.

5-bromo-2-deoxyuridine (BrdU) positive cells count

Five wt C57Bl/6 and 5 SCID mice injected with BrdU (50mg/Kg body weight, 3 times, every 8hrs, IP) were donor of 30μm coronal hippocampal slices. Five sections were randomly selected for

each animal and processed in immunofluorescence for BrdU, MAP2 and DAPI markers. Five random field for each slice were acquired with a 40X objective in fluorescence microscopy. BrdU+/DAPI+, Nestin+/DAPI+ and BrdU+/Nestin+/DAPI+ cells were then count. Confocal analysis was performed with Zeiss 510 LSM.

Stereotaxical surgery in brain

All in vivo experiments were in accordance with the Italian Legislative Decree N.116/92. Male Sprague Dawley adult rats (Harlan, Italy) (n=12) were anesthetized and a guide cannula (26G, Plastics One) was stereotaxically (stereotaxic frame; Kopf Instruments) lowered to Hippocampus (AP -2.3mm, L 2.2 mm, V 3.4mm, incisor bar 3.0 below horizontal line; Paxinos and Watson). Either cell suspension (n=10) or vehicle (PBS, n=2), were loaded in a Hamilton syringe connected with a Tygon tube (ID 0.020mm, OD 0.060mm, Saint-Gobain PPL Corp) and with a needle (33G, Plastics one). The needle was inserted in the guide cannula. A volume of 1μl (3x103 EGFP+cells) was then infused with a syringe pump over 5 minutes and left in place for additional 5 minutes.

Transplanted cell counts

Frequency estimation of transplanted EGFP+/MAP2+ leptomeningeal cells were performed in hippocampal slices of 4 rats injected with EGFP+ leptomeningeal cells and one not-injected rat as negative control. Five of serial coronal sections (30 μm) of hippocampus were randomly selected and stained with antibodies against EGFP and MAP2. Five random fields for each slice were acquired with a 40X objective in confocal microscopy. EGFP-positive cells and MAP2/EGFP double-positive cells were then counted. The number of MAP2-positive injected cells were expressed as percentage of EGFP-positive cells examined.

Quantitative real-time RT(reverse transcription)-PCR analysis (qPCR)

Total RNA was purified with Trizol reagent (Invitrogen) and retrotranscribed to cDNA by reverse transcriptase AMV contained in the First Strand cDNA Synthesis Kit (Roche). qPCR reactions were carried out in 20 µl total volume containing 10 ng of cDNA (RNA equivalent), 1x Power SYBR Green I Master Mix or Taqman Universal PCR Master Mix (Applied Biosystems), 0.4 µM primers forward and reverse or 1/20 Taqman probe. After a starting denaturation for 10 minutes at 95°C, 40 PCR cycles (15s 95°C and 1 min 60°C) were carried out on ABI PRISM 7900HT SDS instrument (Applied Biosystems). The Taqman assays (Applied Biosystems) were as follows: Rn00566603_m1 for Gfap; Rn00565046_m1 for Mtap2; Rn00564394_m1 for Nes; Rn00578849_m1 for Cspg4; Rn00667869_m1 for Actb. Forward and reverse 5'-3' primer sequences and PCR product lengths were as follows:

Tdgf1, GCTGGTGAAGACCTCGACGT, CGGAAGGCACAAGCTGGA, 106 bp;

Smad4, GCACTACCACCTGGACTGGAA, TGTGAACCGGCCAGTAATGTC, 126 bp; P

ou5f1, GCCAAGCTGCTGAAACAGAAG, CTGGCTGAACACCTTTCCAAA, 96 bp;

Nanog, GGCCTGACTCAGAAGGGCTC, TGCCCCATACTGGAAGGTTTC, 106 bp;

Sox2, CGCCGAGTGGAAACTTTTGT, CGCGGCCGGTATTTATAATC, 111 bp;

Klhl1, GCTCATAGGCTTGTCCTGAGCT, GCTTGGCTTCACAGACATCG-3', 75 bp;

Eng GGACAGCCTCTCCTTCCAGC, TGCTCACCTGTACGAAGCCC 101 bp;

Col1a1 GCAGATTGAGAACATCCGCAG, CCAGTACTCTCCGCTCTTCCA 106 bp; 42

Cd44 CAACGCTATCTGTGCAGCCA, CAAGAGGAGCTGAGGCATTGA 101 bp;

Fgfr1 AAATTCAAATGCCCGTCGAG, GGCGTAACGAACCTTGTAGCC 111 bp;

Fgfr2 TTGGCAGCCAGAAATGTGC, CTTGACTGGAAGTCGCCCAT 126 bp; Fgfr3

CAAGGTGTACAGCGACGCAC, GTGGTGTTAGCTCCTGCAGTCTT 126 bp; Kit

GGCATCACCATCAAAAACGTG, GGGATAGCTTTGATGGCTGC 131 bp; Cd34

CACCAGCCATCTCAGAGACCA, CAGTGTGACGGTTGGGTAAGTC 113 bp.

The probe signal was normalized to an internal reference, and a cycle threshold (Ct) was taken significantly above the background fluorescence. The Ct value used for subsequent calculation was the average of three replicates. The relative expression level was calculated using

transcript level of Actb as endogenous reference. Data analysis was done according to the comparative method following the User Bulletin #2 (Applied Biosystems).

Unsupervised hierarchical clustering of qPCR data

Purified RNAs from undifferentiated and differentiated cells samples were analized as described above. Log2-transformed gene expression values were analyzed by Gene Cluster 3.0 software (De Hoon MJ)42. As unsupervised hierarchical clustering procedures, we used centered Pearson correlation as similarity measure and complete linkage. The resulting cluster tree was visualized by the 1.1.0 version of Java TreeView software.

Statistical analysis

Data were analyzed using GraphPad Prism4 software. Results were expressed as mean ± SD or SEM, when indicated. Differences between experimental conditions were analyzed using two-tail Student t-test. P value <0.05 was considered statistically significant.

RESULTS

1.Leptomeninges characterization

1.1 Brain Leptomeninges

In order to assess the expression of neural stem cells marker nestin in rat leptomeninges 30 μm brain slices were collected at different rat ages (embryo E20, postnatal day P1, P8, P15, and adult) and stained by immunofluorescence with specific anti-nestin antibody. Confocal microscopy analysis showed the presences of nestin positive cells in the peripheral layer of cortex (Fig.13). Sub Ventricular Zone (SVZ), a neural stem cell niche, was the positive control for nestin staining (Taupin, 2006).

Nestin positive cells in E20 and P1 brains slices showed a wide distribution, by increasing the rat age (P8, P15 and adult) the parenchyma nestin positivity decreased as expected while nestin positive cells residing in peripheral cortex persist up to adulthood.
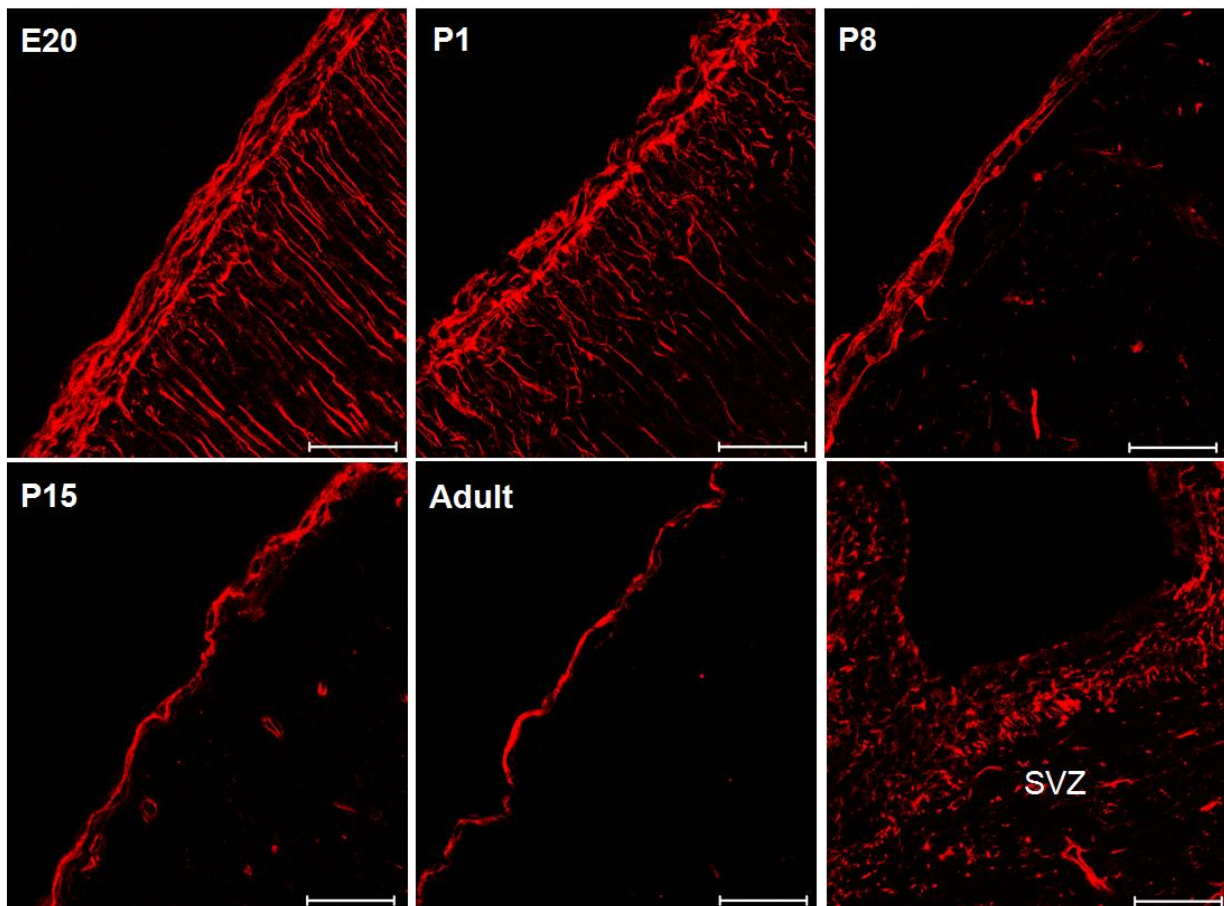
Fig.13: Distribution of nestinpositive cells in rat cortex at different ages.
Confocal images of coronal sections of parietal cerebral cortex stained by immunofluorescence with nestin (red) at different rat ages: embryonic day 20(E20), postnatal days P1 , P8, P15 and adults. As positive control staining for Nestin in rat P15 Sub Ventricular Zone (SVZ). Scale bar 50 μm.

To assess if nestin positive cells reside inside the cortex or in meningeal compartment, brain slices were stained for the pial basement membrane marker laminin (Halfter et al., 2002). Confocal microscopy clearly showed that nestin positive cells reside outside the pial basal lamina as a compact cell layer lining the cortex at different rat ages (Fig.14, first row). These data suggest that nestin positive cells resided in Leptomeningeal compartment.

To better describe the nestin positive cells population immunofluorescence confocal analysis was performed to understand if this population included astrocytes (marked by GFAP, an intermediate filament protein specific for astrocytes), NG2 (chondroitin sulphate proteoglycan) positive pericytes and oligodendrocytes precursor cells and neurons (MAP2, microtubule associated protein). GFAP, NG2 (Fig.14) and MAP2 (data not shown) positive cells had different pattern compared to that of nestin positive cells and no colocalization of this three markers was evident in the leptomeningeal layer.

Nestin positive cells appeared as a distinct population from astrocytes pericytes and oligodendrocytes precursor cells., Nestin positive layer did not include neurons and, as previously described in literature (Encinas et al., 2008).
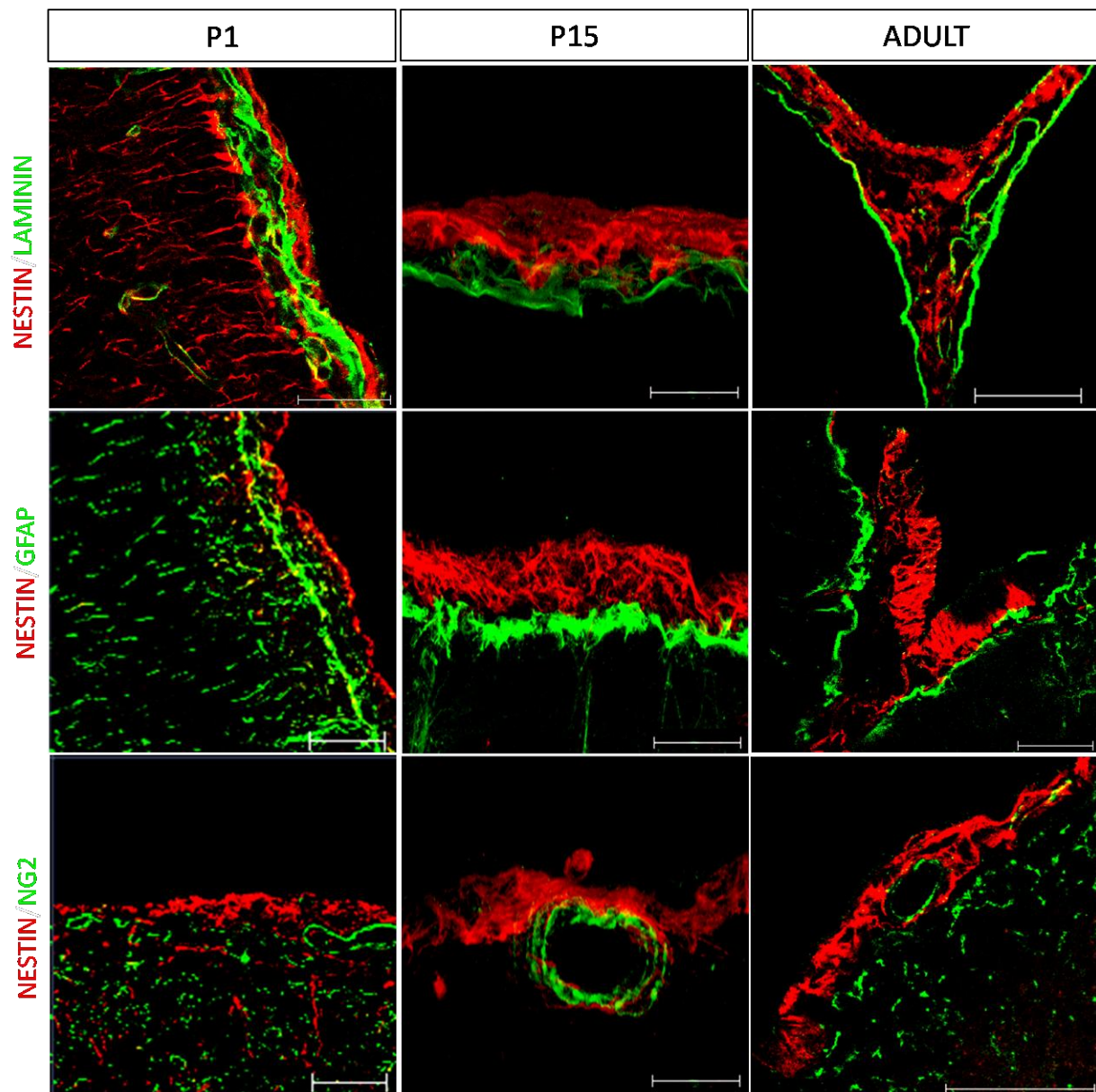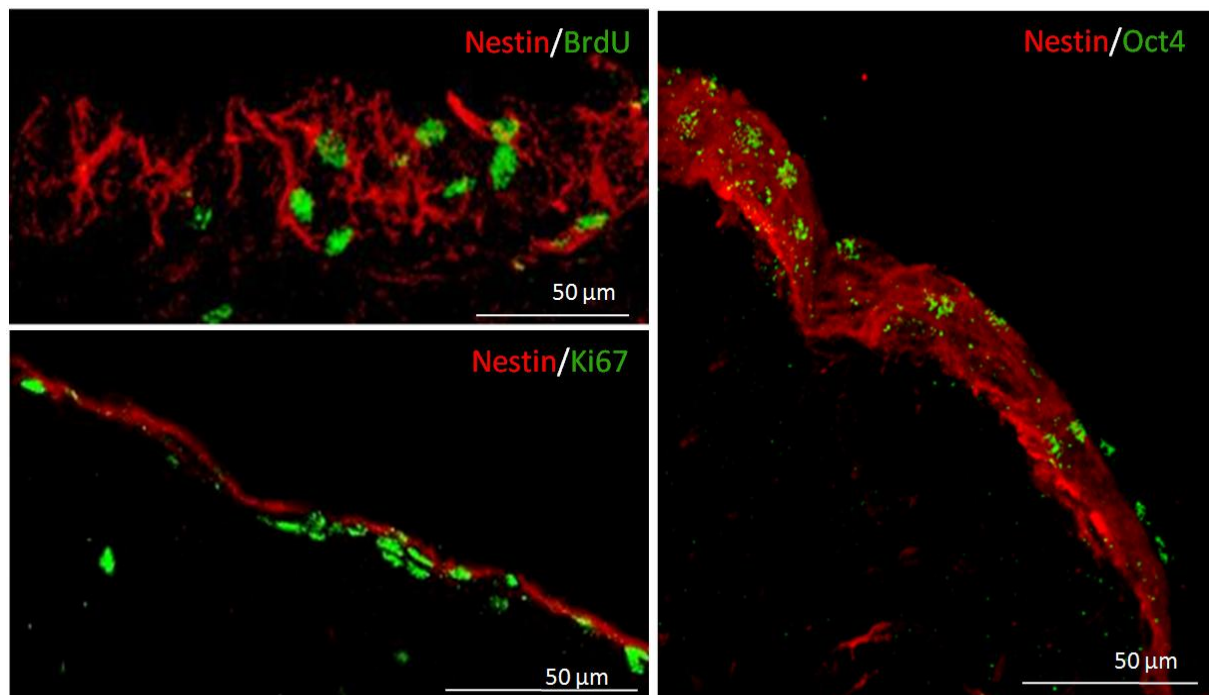
Fig.14: Description of nestin positive cells layer.
Confocal images of coronal sections of rat parietal cerebral cortex stained by immunofluorescence with nestin (red) in double staining with Laminin, GFAP and NG2 (green). Stainings were performed at different rat ages: P1 ,P15 and adults. Scale bar 50 µm.

1.2 Spinal cord Leptomeninges

As well reported in the literature, leptomeninges cover all central nervous system. For this reason, we analyzed leptomeninges in 30μm coronal sections of adult Spinal Cord (SC) for the presence of nestin positive cells. Immunolfuorescence confocal analysis of spinal cord leptomeninges, showed a compact layer of nestin positive cells lining all spinal cord, residing outside the pial basal lamina as a distinct population from astrocytes (GFAP) and pericytes (NG2), as previously described in brain LeSC (Fig.17, first row). Immunofluorescence confocal analysis showed that Nestin positive cells residing in CC were a different population from GFAP and NG2 positive cells, while there was no Laminin positive cells (Fig.16).

To asses if among the nestin positive cells population were proliferating cells, adult rats were injected with BrdU. Immunofluorescence confocal analysis of 30 μm SC slices revealed some nestin positive cells in co-localization with BrdU. Parallel staining for the proliferating marker Ki67 showed clusters of Nestin/Ki67 double positive cells along the spinal cord leptomeninges. Moreover, leptomeninges were also analyzed for the presence of Oct4, markers for stem cells self renewal, confocal microscopy showed the presence of rare Oct4+/Nestin+ cells. (Fig.15)
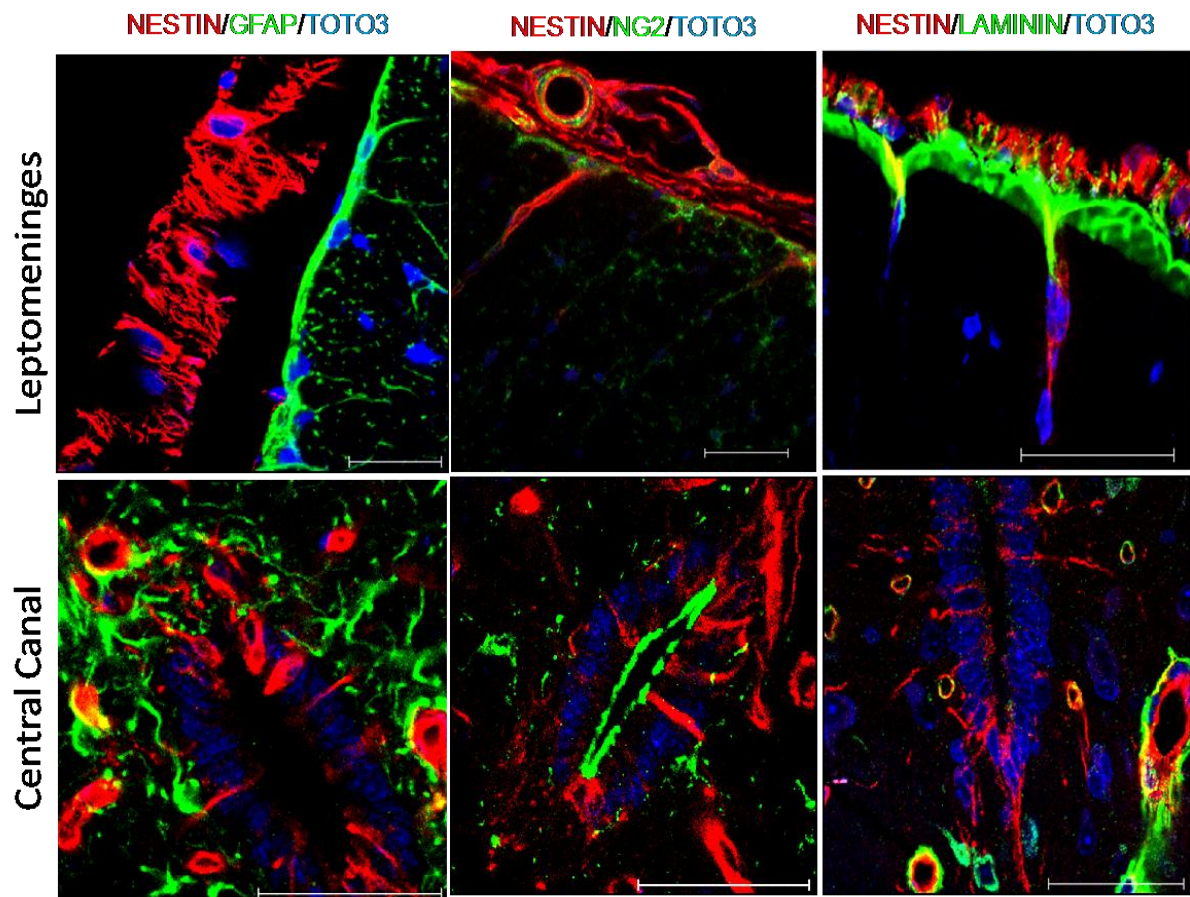

Fig.15: SC leptomeningeal nestin positive cells proliferation.
Confocal images of SC sections stained by immunofluorescence with nestin (red) in colocalization with proliferation markers BrdU, Ki67 and self renewal marker Oct4 (green). Scale bar 50 μm.

Meningeal nestin positive cells were compared to the already known stem cell population residing in Spinal Cord Central Canal (CC).
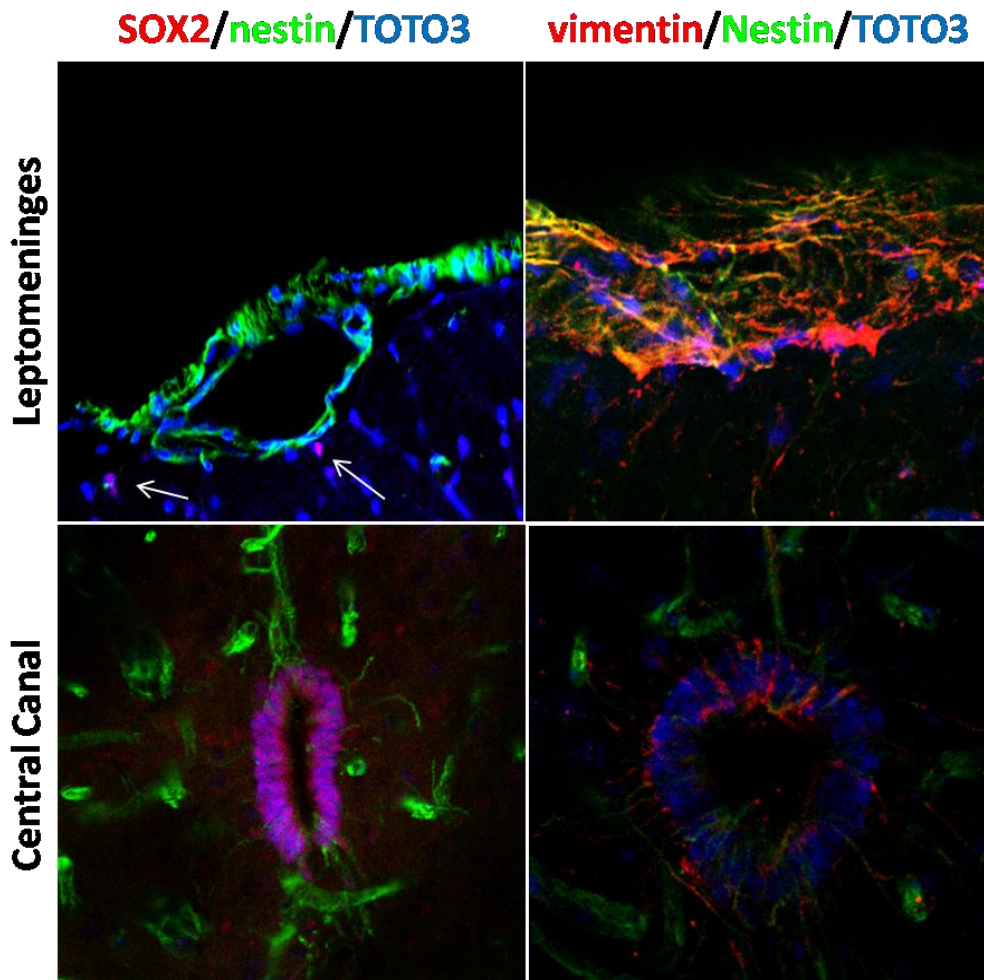

Fig.16: Description of nestin positive cells in spinal cord leptomeninges and comparison with Central Canal (CC).
Confocal images of coronal sections of adult spinal cord stained by immunofluorescence with nestin (red) in double staining with GFAP, NG2 and Laminin (green), the nuclear staining TOTO3 (Blue). In the first row stainings observed in Leptomeninges while in second row stainings observed in Central Canal (CC). Scale bar 50 μm.

Immunofluorescence confocal analysis of SC leptomeningeal nestin positive cells with CC stem cell markers SOX2, SOX9 and Vimentin (Hamilton et al., 2009), allowed a deeper description of nestin positive cells layer.

No SOX9+ and rare SOX2+/Nestin- cells were in Leptomeninges, while SOX9+ and SOX2+ cells were mainly astrocytes lining the parenchymal side of pial basal lamina or surrounding the central canal.

Vimentin positive cells were in both Leptomeninges and CC but remain to be investigate if LeSC co-express nestin and vimentin or if in leptomeninges reside two distinct populations (Fig.17a).

Fig.17a: Description of nestin positive cells in spinal cord leptomeninges and comparison with Central Canal (CC).
Confocal images of coronal sections of adult spinal cord stained by immunofluorescence with nestin (green) in double staining with SOX2 and Vimentine (red), the nuclear staining TOTO3 (Blue). In the first row stainings observed in Leptomeninges while in second row stainings observed in Central Canal (CC).

As following step the expression of specific receptors by nestin positive Leptomeninges was analyzed by immunofluorescence confocal microscopy. In particular the attention was focused on Toll Like Receptor 4 (TLR4) already known in literature to be important for regulation of proliferation and differentiation of neural stem cell (Rolls et al., 2007). TLR4+/ Nestin+ and TLR4+/ Nestin- cells were found in leptomeninges mainly concentrated around vessels and in surrounding nerves. The results on receptor expression in LeSC remain to be confirmed (Fig.17b).
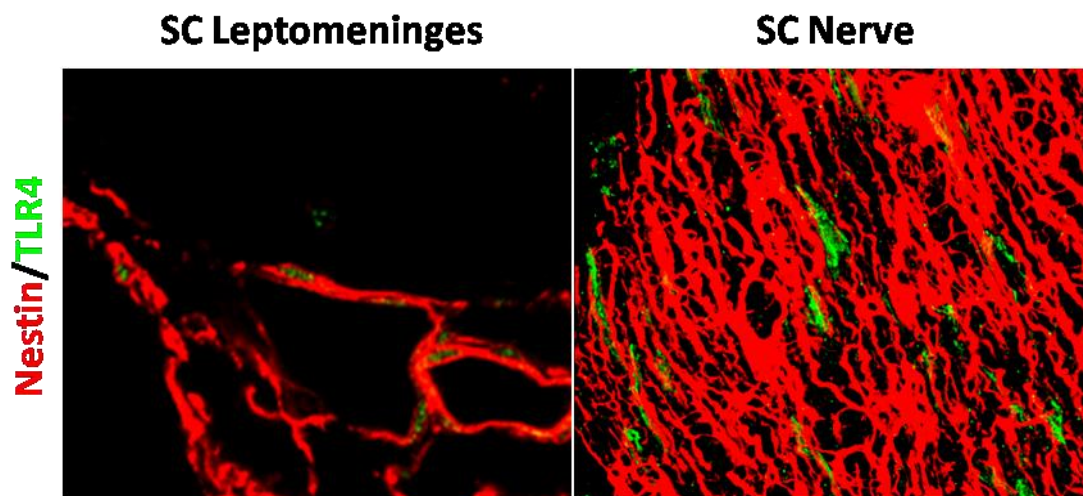
Fig.17b: TLR4 distribution in spinal cord leptomeninges and nerves.
Confocal images of coronal sections of adult spinal cord stained by immunofluorescence with nestin (red) in double staining with TLR4 (green).

2 Leptomeningeal nestin positive cells sampling and characterization

2.1 Tissue sampling

Samples from leptomeningeal sections of the parietal cortex were obtained from brains at the different post-natal stages P1, P15 and from adult rats (n=15 brains for each experiment). Figure 18 shows that dissection was limited to the outer layers of the cortex. Dissociated cells could be cultured in basal conditions for several passages when seeded at density >2.6 x10^{5}cells/cm^2 and split at 70-80% confluence, no more than 14 days after plating. Colony forming unit (CFU), an index of stem cell number in the tissue, was calculated after 14 days of culture in basal conditions. CFU number was high starting from P1 cells and decreased with rat age (Fig.18). Cell rate growth was higher for P1 cells than for P15 and adult rats.
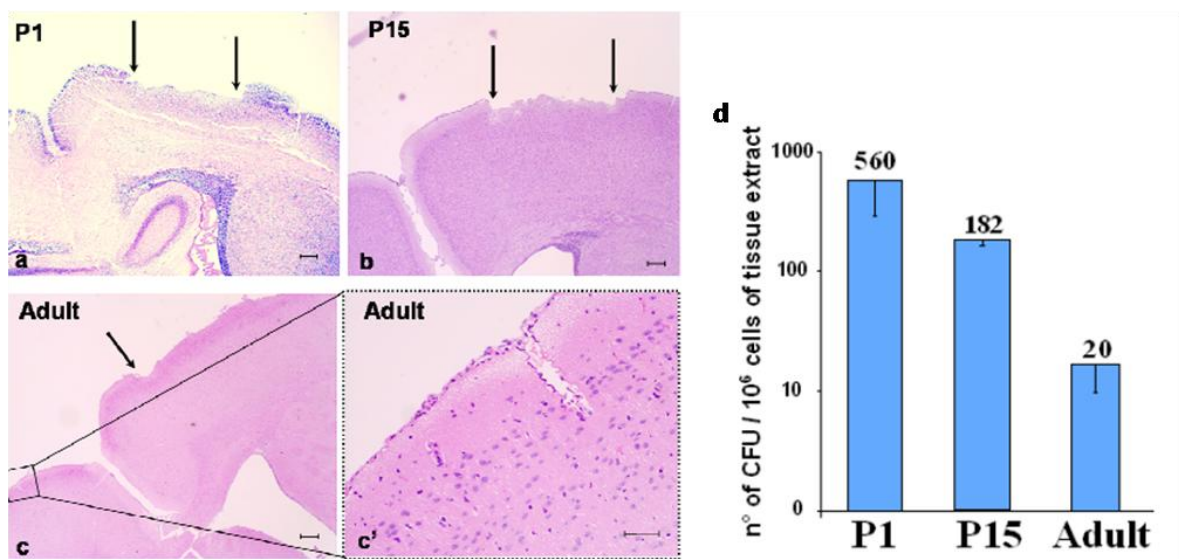
Fig.18: Tissue sampling.
a,b,c and c' Hematoxylin/eosin staining of a coronal section of P15 rat brain to show (arrows) the site and the extent of the biopsy. Scale bar 250 μm. **d** Colony Forming Unit (CFU) derived from nestin-positive cells at different ages. Bars represent the number (mean of 5 different dilution) of CFU/million of extracted cells ± SD, at different ages.

2.2 Cell culture in adherent condition

The whole tissue extract was plated in flasks with growing medium (see Materials and Methods). After 48h, non-adherent cells were removed and the medium completely changed. In 1-3 weeks, cell colonies could be recognized. Only a minority of cells from the whole tissue extract adhered and gave rise to colony forming units (CFU). Non- adherent cells underwent apoptosis or were removed by medium change.

Time course experiments were performed to assess whether a cell population of nestin+/GFAP-/NG2-cells (similar to what found in vivo in the leptomeninges) was present in the culture from the 5 hours after plating till the 6th day in colture (Fig.19).

As early as 5hrs after plating nestin+/GFAP-/NG2- cells represent the main population in culture (approximately the 90% of attached cells), only small percentage of Nestin /NG2 (5.1%) and Nestin/GFAP (4.4 %) double positive cells were present while rare GFAP and NG2 positive cells were detected.

From day 6 Nestin+/GFAP-/NG2- cell colonies were detectable and represent the 94% of whole population, nestin positive cells double positive for GFAP (1.5%) or NG2 (4,6%) were present in a small percentage, while no GFAP and NG2 positive cells were detected.
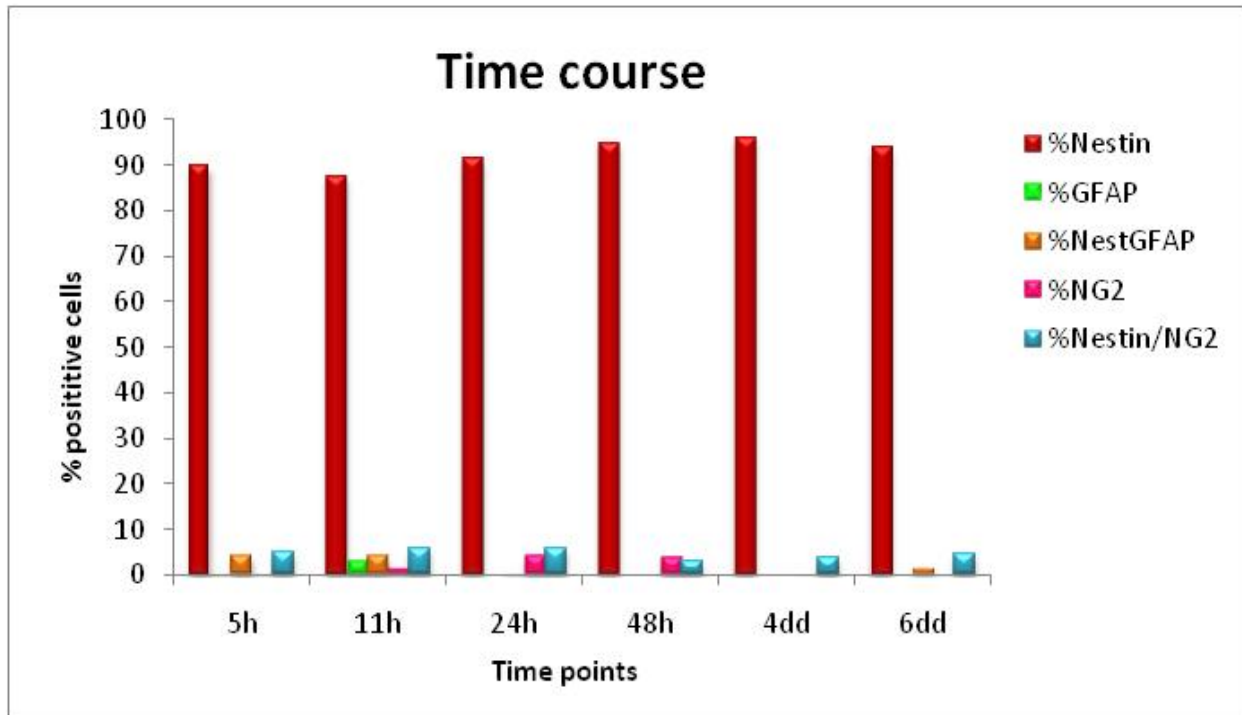
time point	%Nestin	%GFAP	%Nest/GFAP	%NG2	%Nest/NG2
5h	89,8	0,8	4,4	0,7	5,1
11h	87,6	2,9	4,4	1,5	5,8
24h	91,3	0,0	0,8	4,1	5,8
48h	94,9	0,0	0,0	4,0	3,0
4dd	96,1	0,0	0,0	0,3	3,8
6dd	93,8	0,0	1,5	0,2	4,6

Fig.19: Time course of the number of nestin positive cells from P15 rats.
Time course of nestin positive cells from P15 rats Leptomeninges to study the percentage of Nestin, GFAP, NG2, Nestin/NG2, Nestin/GFAP positive cells. In the tab were listed the percentage of positive cells for each markers in each considered time points.

All the adherent cells proliferating after several days derived from CFU and displayed a homogeneous immunophenotype. This cell population was expanded for several passages and was clonogenic, since CFU were present at each passage (Fig.20).
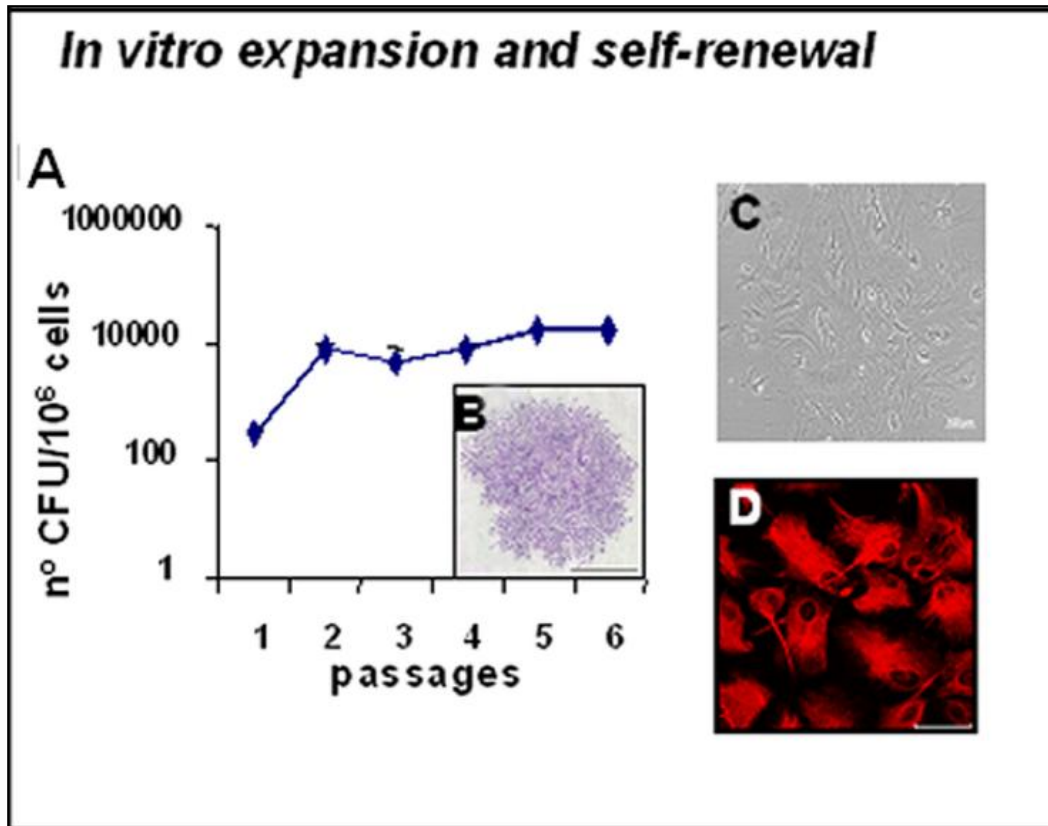
Fig.20: In vitro expansion of the tissue extract in adherent cells culture conditions
(A) CFU derived from leptomeningeal cells at different passages. Bars represent the CFU number/million of plated cells ± SD. (B) May-Grunwald Giemsa staining of a single colony of leptomeningeal adherent cells. (C) Transmitted light and (D) immunofluorescence, nestin (red), of adherent leptomeningeal cells.

Leptomeninges from P1,P15 and adult rats were cultured in adherent conditions and after several passages were analyzed using immunofluorescence as described above. Results revealed an homogeneous population of nestin positive cells in all considered rat ages. Majority of cells were nestin positive with a scarce presence of GFAP positive or NG2 positive or Laminin positive cells. MAP2 positive cells were not present in these culturing conditions. Colocalization in the same cells of nestin and any other markers was very rare. Figure 21 shows an example of expanded population obtained from P15 rats.
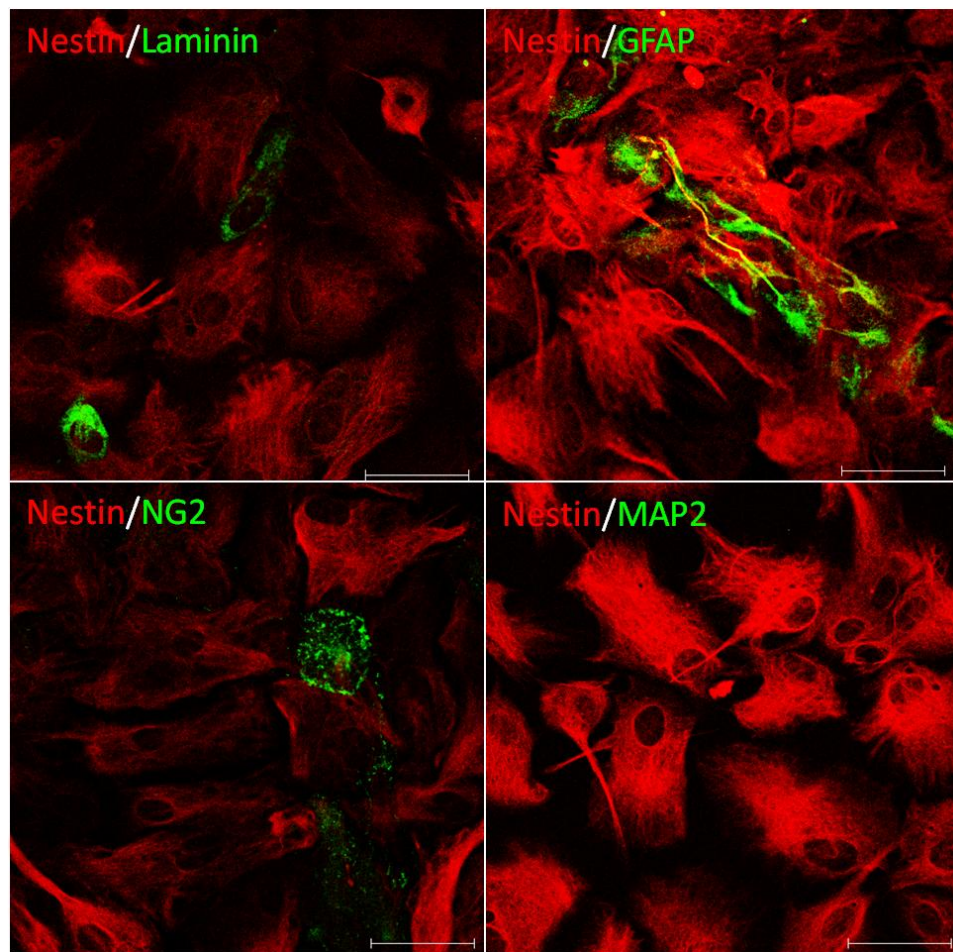

Fig.21: In vitro expansion of the tissue extract from P15 rats and expanded in adherent cells culture conditions.
Confocal images of cells kept in expansion in adherent condition. Nestin (red) was in co-localization with the neuronal marker MAP2, the astrocytes marker GFAP and the oligodendrocyte precursor cells marker NG2. Notably MAP2 and GFAP positive cells were co-expressed in the same neurosphere. Scale bar 50 μm.

2.3 Cell culture in neurosphere condition

To assess whether cells with neural differentiation potential were present in the tissue extracts from this region, we cultured these cells in the same conditions used to expand SVZ-NSCs. In NSC medium, cells from tissue extract generated floating neurospheres in 9-11 days . Neurospheres could be expanded in vitro up to several months (data not shown). Neurosphere, obtained from leptomeninges of rat P15, were cultured and expanded under conditions described in materials and method and subsequently were analysed by immunofluorescence.
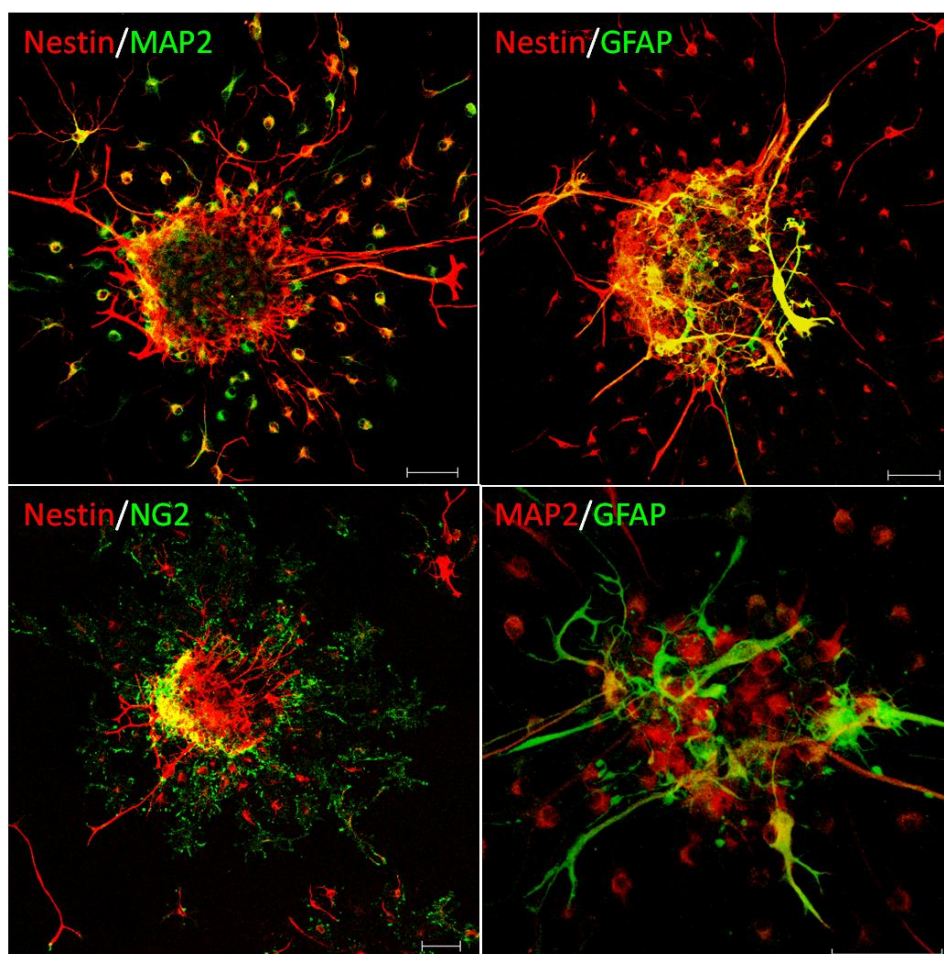

Fig.22: Neurosphere characterization.
Confocal images of neurospheres obtained from cells dissected from rat leptomeninges. Nestin (red) was in co-expression with the neuronal marker MAP2(green), the astrocytes marker GFAP (green) and the oligodendrocyte precursor cells marker NG2 (green). Notably MAP2 and GFAP positive cells were co-expressed in the same neurosphere. Scale bar 50 μm.

Neurosphere were positive for nestin in co-expression with the neuronal marker MAP2, the astrocytes marker GFAP and the oligodendrocyte precursor cell marker NG2. Moreover neurosphere showed contemporary presence of both MAP2 and GFAP positive cells (Fig.22).

Comparison of gene expression between SVZ- and leptomeninges-derived neurospheres,. Quantitative gene expression assay was performed to analyze self-renewal regulators (Pou5f1/Oct4) (Nichols et al., 1998), Nanog (Loh et al., 2006), Sox2 (Avilion et al., 2003), genes related to the undifferentiated state maintenance (Tdgf1/Cripto-1, Smad4 and Nestin) (Ramalho-Santos et al., 2002), and genes related to neural differentiation (Klhl1, Gfap, Mtap2) (Jiang et al., 2007). In Figure 23 gene expression of SVZ- and leptomeninges-derived neurospheres are shown. Data were normalized for beta-actin expression level and were shown as ratio between the two population. A tight correlation between the gene expression pattern of SVZ-NSC-derived neurosphere and the leptomeninges-derived neurospheres was observed. Statistically significant differences($p<0.05$) were observed for differentiation genes Gfap and Mtap2 only.
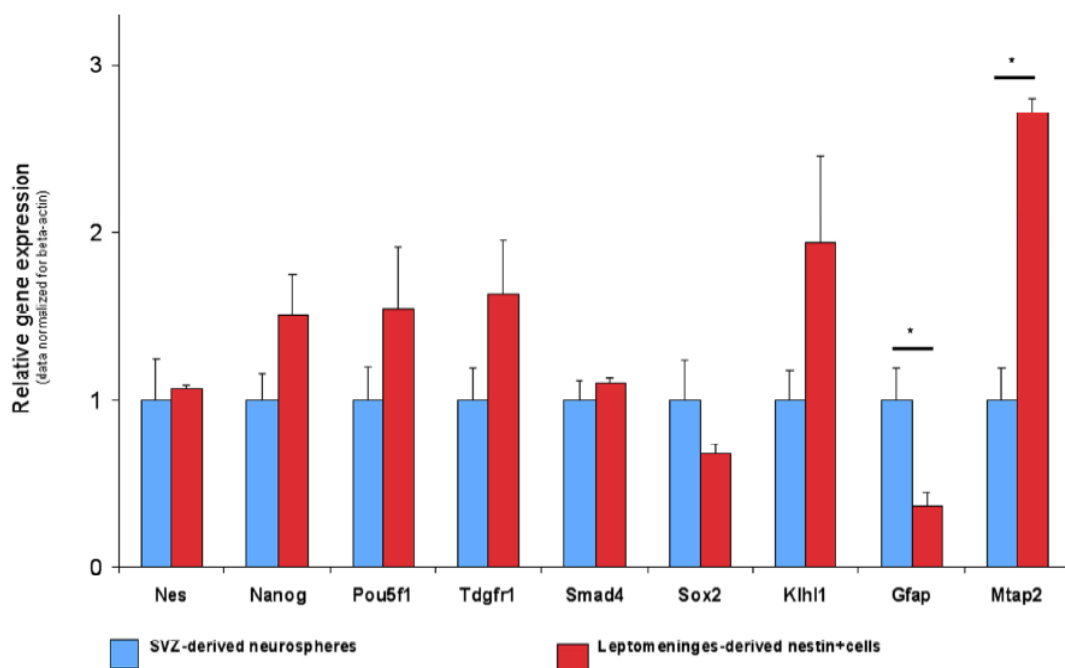
Fig.23: Neurospheres relative gene expression analysis
For each sample, expression levels of different genes were normalized to levels of beta- actin mRNA. The bars show fold change ± SD in transcription of normalized mRNA expression levels mesured for letomeninges-derived neurosphere compared to SVZ-NSC derived neurosphere

3 Neural differentiation potential

3.1 *In vitro* neural differentiation potential

To determine whether the in vitro-expanded cells had neuro-glial differentiation potential, leptomeninges-derived nestin-positive cells from P1, P15 and adult rats, were cultured with differentiating medium for at least one month, and then stained with antibodies against markers of neurons (MAP2), astrocytes (GFAP), oligodendrocytes (O4). For the efficient differentiation, an appropriate cells density of $10^5/cm^2$ was necessary (Fig. 24-25).

Under differentiating conditions, cells differentiated also into GFAP and rarely in O4 (specific marker for mature oligodendrocyte) positive cells (Fig.25).To determine whether the neurons found in differentiated cultures originated from replicating cells or from residual adult cells from tissue extract, cells were incubated with BrdU for 9 hours before the induction of differentiation. Immunofluorescence analysis for BrdU and MAP2, highlighted positive cells for both markers giving confirmation that neurones observed in culture derived from nestin positive cells after exposure of differentiating agents (Fig. 24 A',B',C').

To quantify the neuronal differentiation potential of cells expanded from leptomeninges of P15 rats, MAP2 positive cells were counted after exposure to differentiating conditions for one month. Quantitative analysis showed MAP2-positive neurons differentiated in cultures with high efficiency (30-50% of cells, n=6 experiments).

As a first level of functional evaluation, differentiated cells from P1, P15 and adult rats were studied by calcium imaging analysis after Fura-2 loading. Cells depolarization with 55mM KCl caused increase of the intracellular calcium concentration indicated by the shift of the 340/380 ratio.
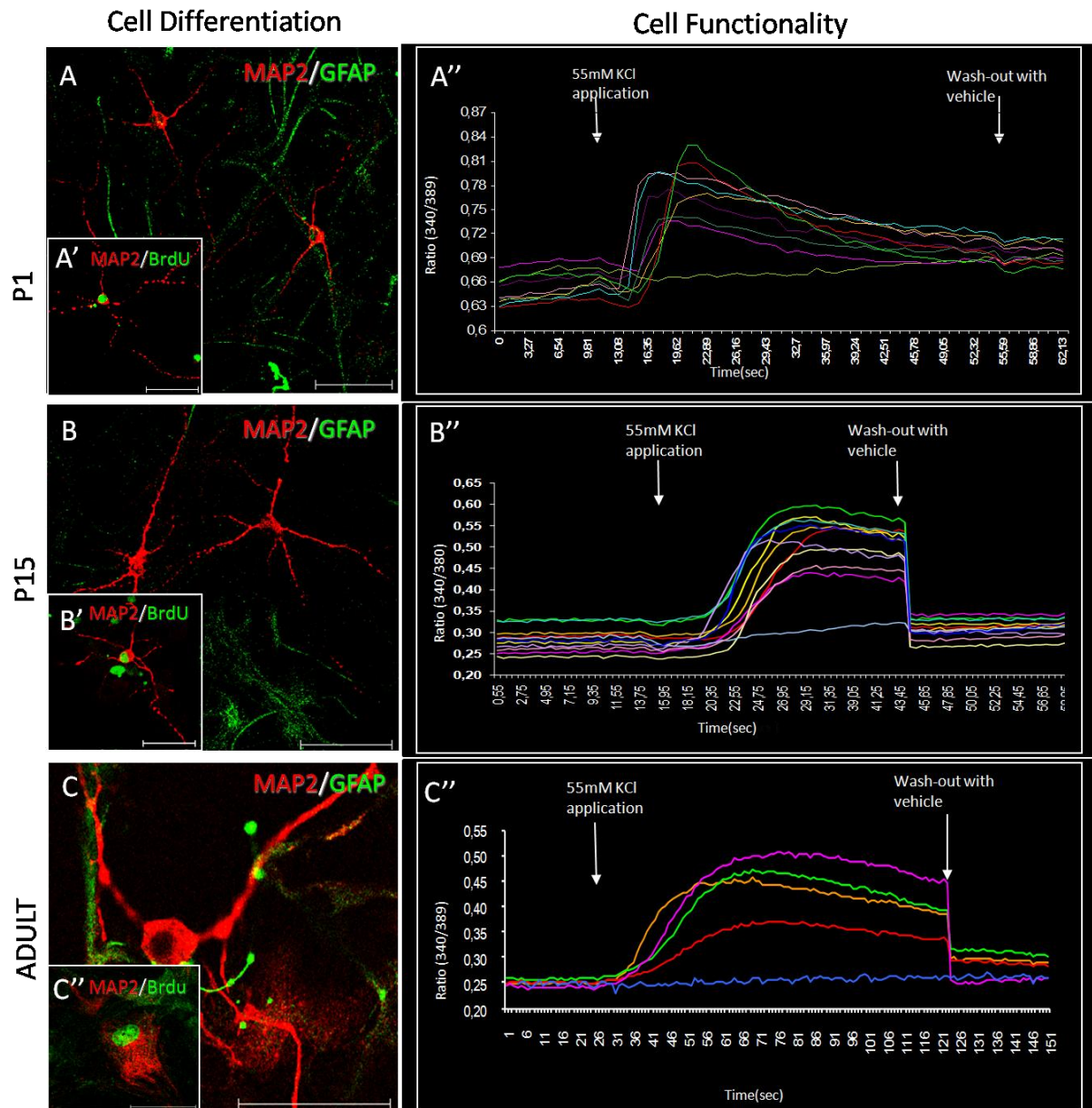
Fig.24: In vitro differentiation and functional assay of Leptomeningeal nestin positive cells from P1, P15 and adult rats. **A,B,C** Cultured Nestin positive cells dissected from P0, P15, adult rats were differentiated in MAP2 and GFAP positive cells. **A',B',C'** Double staining MAP2/BrdU (C') prove the origin of MAP2 positive cells from differentiated Nestin positive cell. **A'', B'', C''** Functional evaluation of differentiated cells shown ability to respond at 55mM KCl application. In graphs are represented examples of responding cells. Scale bar 50μm.

Removal of the KCl solution and wash out with HEPES buffered solution, abolished the potassium induced calcium increase, indicating that the increase in intracellular calcium was dependent on external calcium sources (Fig. 24 A'',B'',C'').

As positive control, comparable data were obtained in the same experimental conditions with primary neuronal culture. These data suggest that *in vitro* differentiated neurones respond to the depolarizing agent KCl and express functional voltage dependent calcium channels, similar to that of functional neurons.
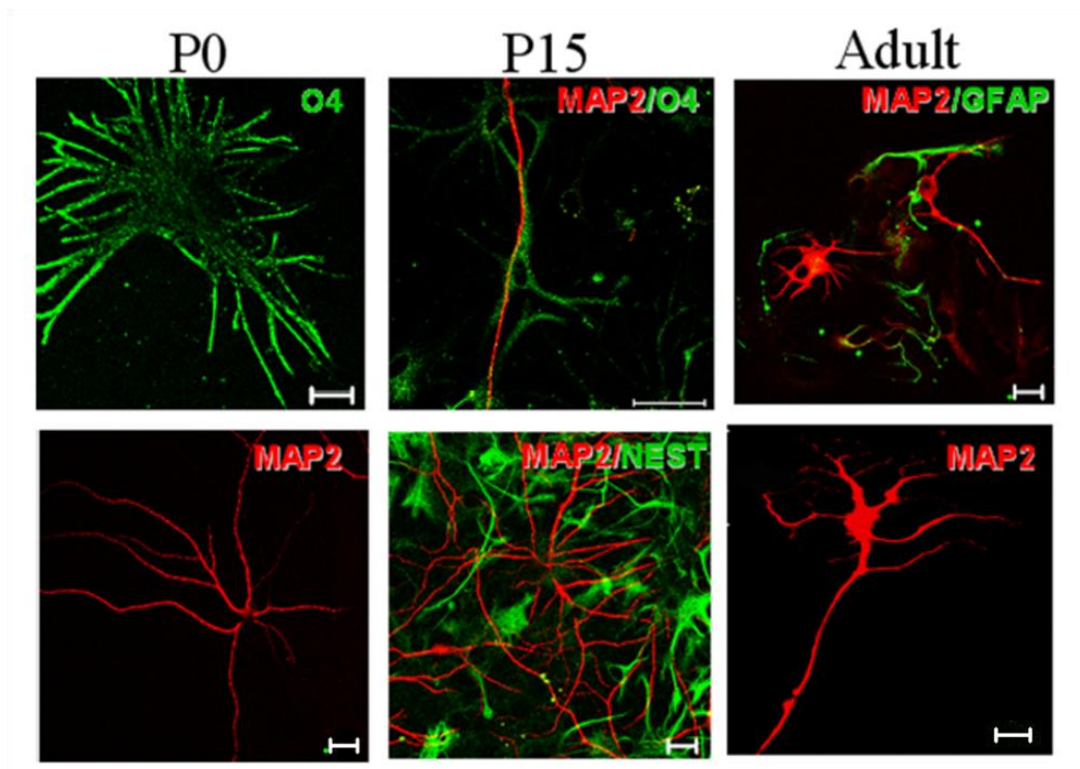
Fig.25:Neuro-glial differentiation potential of the Leptomeningeal-derived expanded population. Expanded cells after differentiation from P0 , P15 and adult rats, stained with antibodies against MAP2 (red). GFAP- or O4-positive cells (green) from P0, P15 and adult rats are shown. 20μm scale bar.

In addition, differentiated cells from P15 rats were more extensively analized for other specific neuronal markers. Cells expressed the presynaptic protein synaptophysin, the GluR2 subunit of the ionotropic AMPA-glutamate receptor, and the glutamate decarboxylase (GAD67), marker for GABAergic neurons (Fig26).
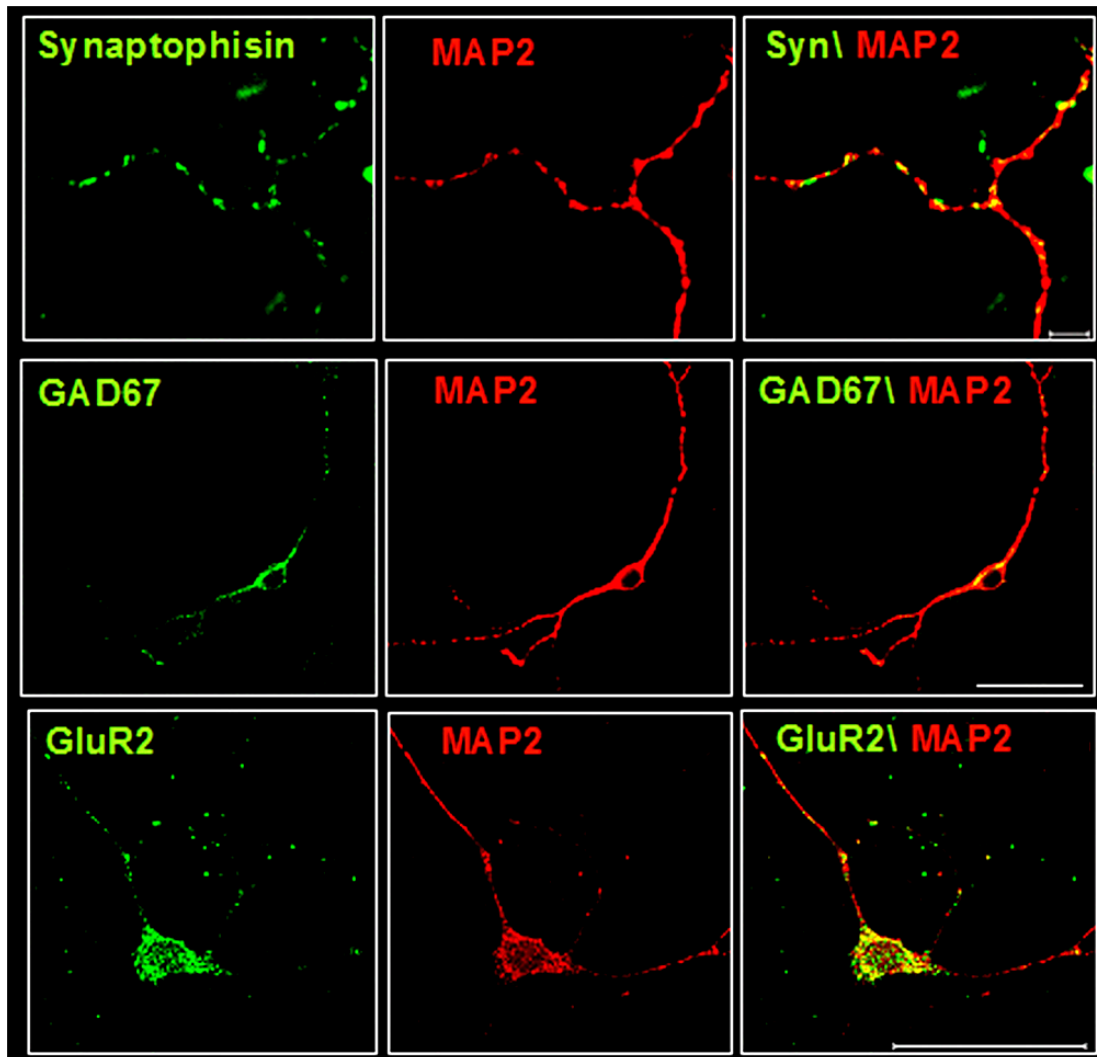
Fig.26: In vitro expansion of the tissue extract from P15 rats and expandied in adherent cells culture conditions.
Confocal images of differentiated cells. Immunostaining showed that MAP2-positive cells (red) expressed components of the synaptic apparatus, including the presynaptic marker synaptophysin (green), glutamate decarboxylase (GAD67) (green) and the glutamate ionotropic receptor subunit GluR2 (green). Scale bars 50 μm.

3.2 *In vivo* neural differentiation potential

On the bases of in vitro differentiation results, expanded leptomeningeal nestin positive cells were tested for their in vivo differentiation potential. Cells dissected from P15 EGFP transgenic rats (1µl of cells suspension corresponding to $3x10^3$cells/µl) were stereotaxically infused in two months rats hippocampus (n=10). As negative controls, animals (n=2) were injected with vehicle (PBS). Infused animals were perfused and analyzed at 4 and 8 weeks from the stereotaxic surgery.

Immunofluorescence analysis for anti-EGFP antibody on 30µm brain slices revealed that EGFP cells survived up to two months on hippocampus, while negative control infused with vehicle did not show any EGFP positive cell.
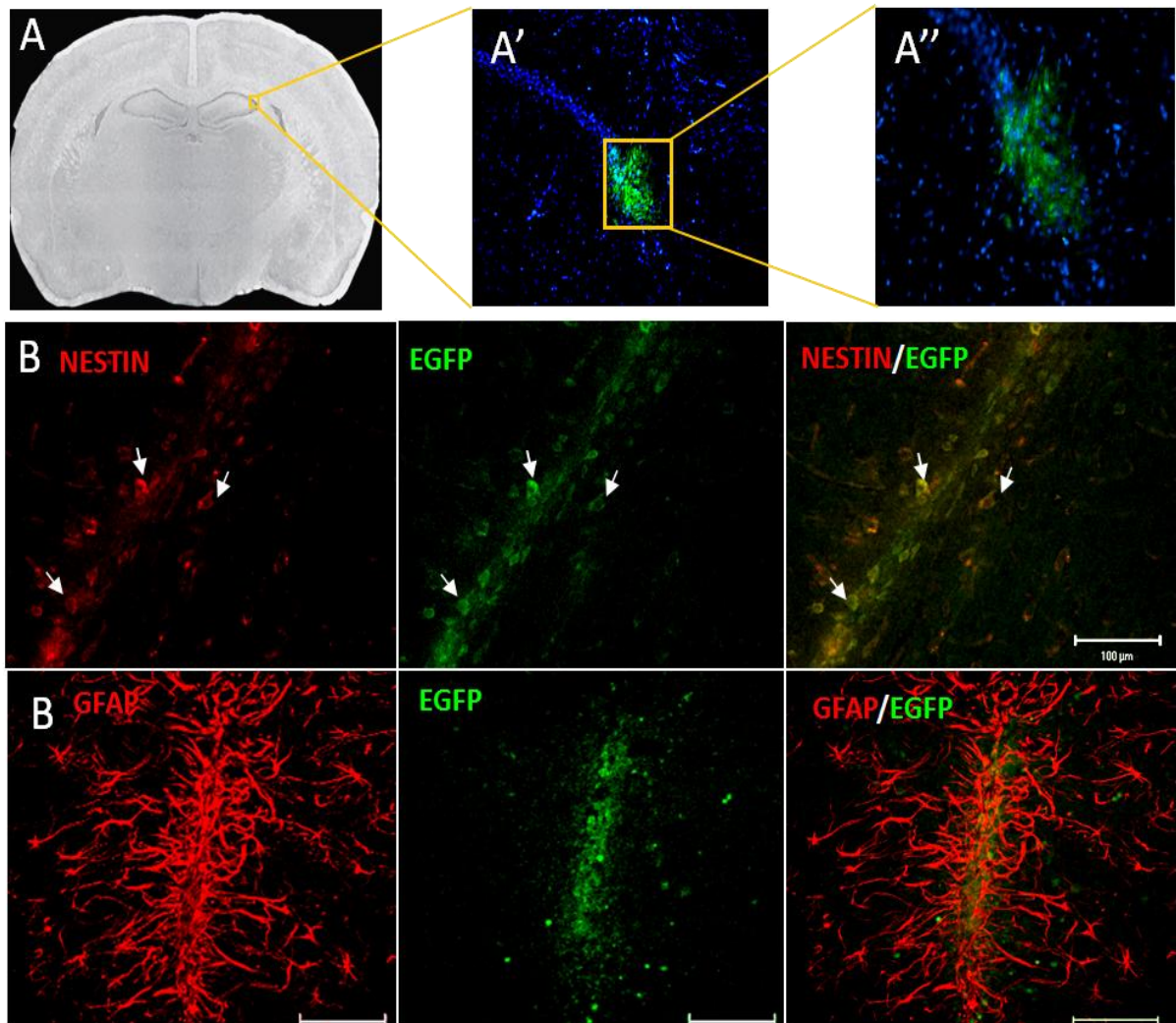
Fig.27: EGFP cells injection track localization and description.
A Injection point in hippocampus, **A'** and **A''** :two different magnifications of the injection point in a brain slice, in blue the nuclear marker DAPI and in green the EGFP antibody .**B, C** Confocal images of injection track: in B double staining with nestin (red) and EGFP(green); in C double staining of GFAP(red) and EGFP (green). Scale bar 50 µm.

Injection track was in CA2-CA3 hippocampal regions (Fig.27 A, A', A''). Cells along the injection track were surrounded by abundant GFAP expressing astrocytes, but rare EGFP cells co-localized with GFAP. Most of EGFP cells closed to injection track were positive for Nestin (Fig.27 B,C) and rare co-localizations were observed between EGFP cells and the oligodendrocyte precursor cells marker NG2. Moreover EGFP cells did not differentiate as neurons (MAP2 positive cells) or neuroblasts (DCX positive cells) around the injection track (data not showed).
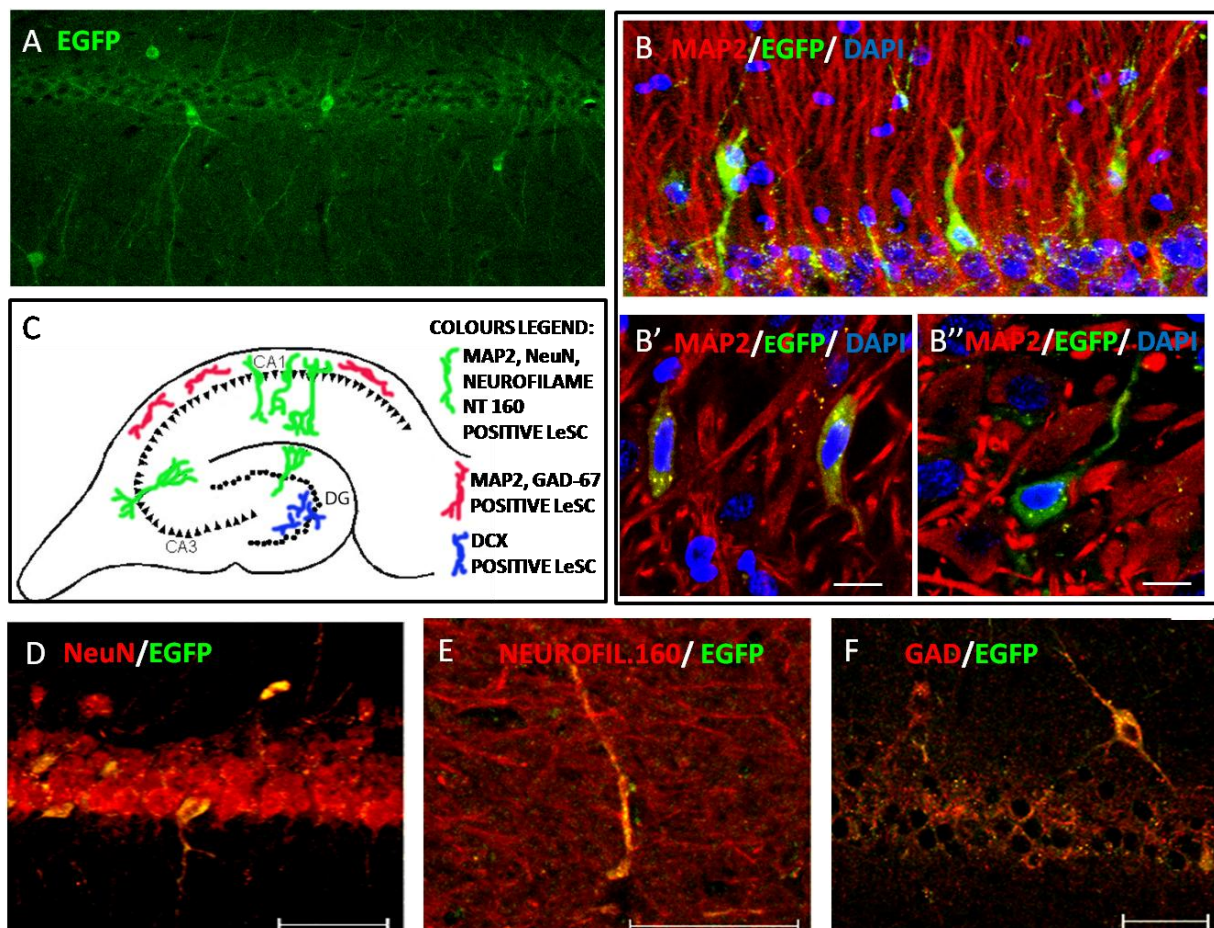
Fig.28: Distribution and differentiation of EGFP cells in hippocampal CA regions.
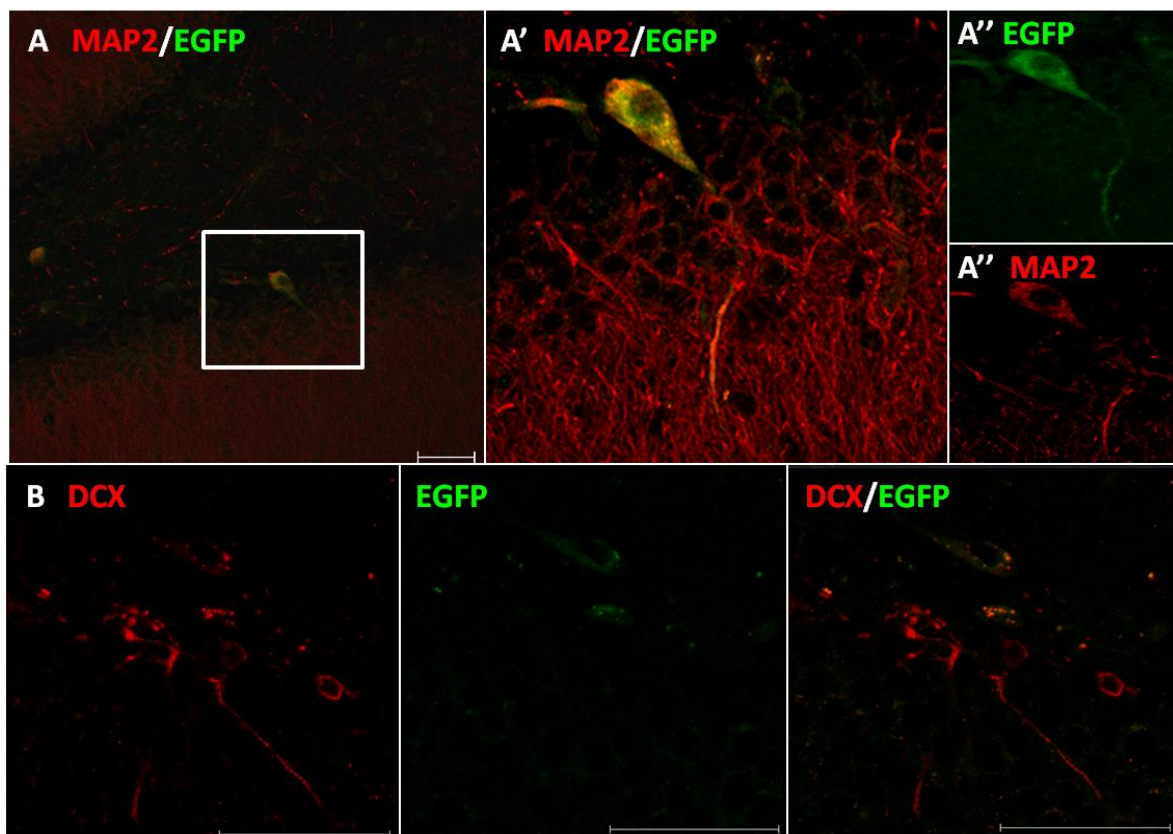**A** EGFP cells (Green) distribution in hippocampus. **B, B'** Confocal images of EGFP cells positive for MAP2 (red) and **B''** an example of EGFP positive cell negative for MAP2. **C** schematic distribution of EGFP cells in hippocampus. **D, E,F** examples of EGFP cells positive in co-localization for neuronal markers NeuN, Neurofilament-160 and GAD 67. Scale bar 50 µm.

EGFP positive cells found in hippocampus were in the internal and external CA1, CA2, and CA3; in dentate gyrus throughout fascia dentate and in the hilus. EGFP cells appeared as well integrated in hippocampus architecture (Fig.28 A,B). Most of cells integrated in hippocampus principally in CA regions were double positive for neuronal markers MAP2, NeuN and Neurofilament 160 (Fig.28 B',D,E) although undifferentiated EGFP cells were present (Fig.28 B'').

In dentate gyrus EGFP cells mainly differentiated into MAP2 positive neurons and neuroblasts (positive for DCX marker) (Fig.28) while in external CA1 and CA2 EGFP LeSC mainly differentiated into GABA-ergic neurons (marker by GAD-67, (Fig.29 F). Our assay did not reveal any EGFP cells differentiated into GFAP positive astrocytes or NG2 positive oligodendrocytes in hippocampus.

No differences were observed in the two considered time point.

Fig.29: Distribution and differentiation of EGFP cells in hippocampal dentate gyrus.
A Confocal images of EGFP cells positive for MAP2 (red). **A',A' '**Biggest magnifications of squared cell.
B EGFP cells differentiated neuroblast positive for DCX. Scale bar 50 μm.

Five rats (four rats injected with EGFP LeSC and a one single non-injected rat), were analyzed for the frequency estimation of EGFP/MAP2 double positive cells in hippocampus. LeSC-derived neurons in hippocampus were 49.8% (236 MAP2/EGFP double positive LeSC out of 463 EGFP positive LeSC) suggesting a high neuronal differentiating potential of LeSC, once injected in adult rat hippocampus.

3.3 Homing

Expanded leptomeningeal nestin-positive EGFP cells were stereotaxically injected into the ventricle of adult rats (n=3). Homing of intra-ventricular injected cells were analyzed at 4 weeks after transplantation.

EGFP cells were along the injection track and spread in the neighbouring tissue: corpus callosum, cortex and also meninges.
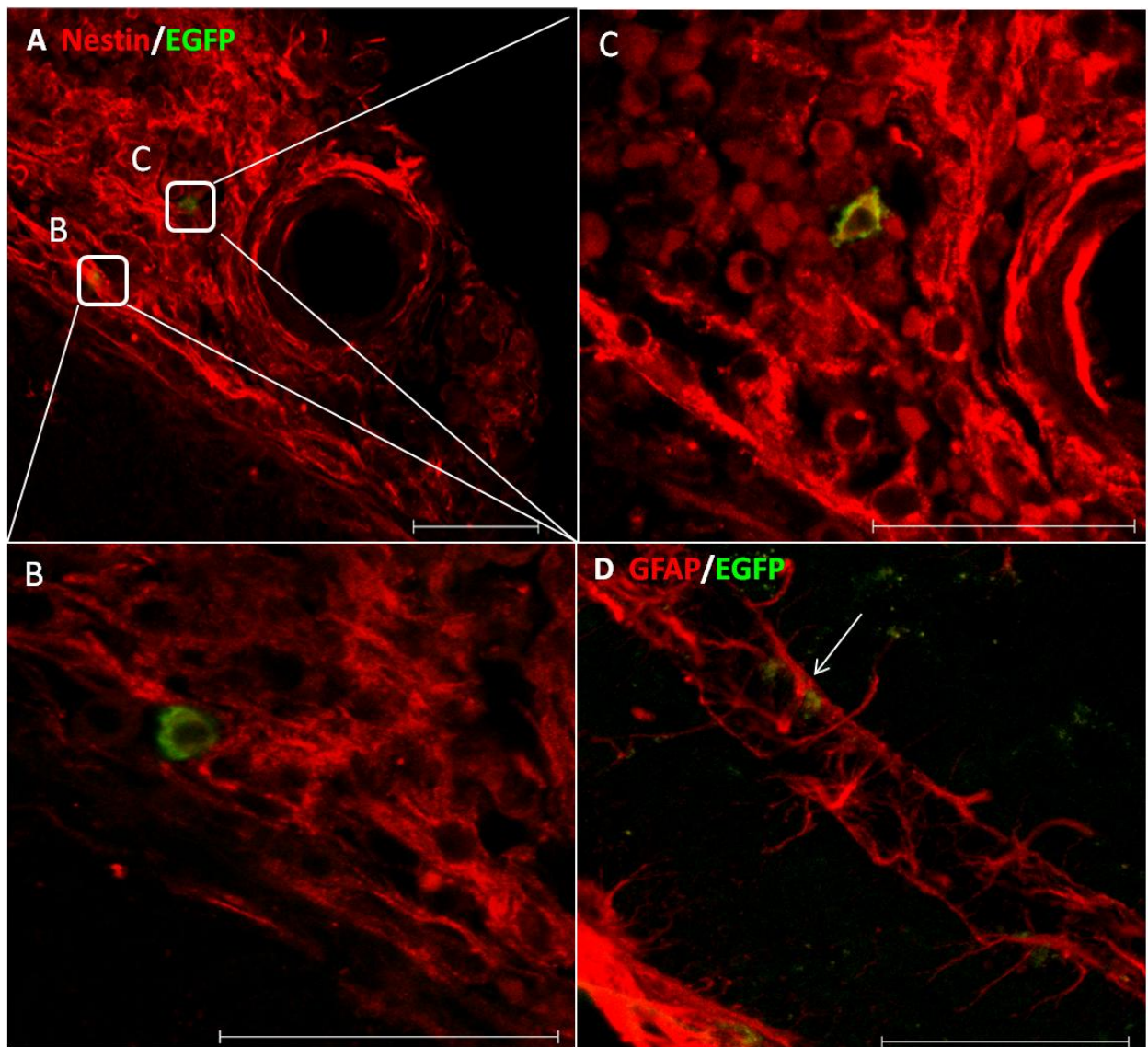
Fig.30: Distribution and differentiation of EGFP cells in hippocampal dentate gyrus.
A,B,C EGFP cells in Leptomeninges, 3D reconstruction. **D** Confocal images of EGFP cells along parenchymal blood vessels. Scale bar 50 μm.

EGFP+ cells were found also found along parenchymal blood vessels, in particular, cells appeared to be located in the perivascular space, between astrocyte extroflexions, delimiting the blood-brain-barrier, and the lumen of vessels (Fig.30 D). EGFP positive cells found in leptomeninges appeared well integrate with resident nestin-positive cells and some of them was also positive for nestin (Fig.30 A,B,C).

4. Crosstalk with the immune system

In order to assess the influence of immune system on Leptomeningeal niche, a study in collaboration with professor Schwartz started to compare leptomeningeal niche in wt and SCID mice was performed.

In the lab of professor Schwartz (Weizmann Institute, Rehovot, Israel), wt and SCID mice injected with BrdU (50 mg/Kg body weight, 3times every 8hrs, IP) were sacrificed and perfused at 24hrs from the first BrdU injection. 30µm brain slices were cut at freezing microtome and analyzed by immunofluorescence assay.

To evaluate the expression of the stem cell marker Nestin, five slices from each wt and SCID mice were stained for BrdU, Nestin and DAPI. Five fields were randomly chosen for each slices and the number of DAPI, BrdU+/DAPI+, Nestin+/DAPI+, Nestin+/BrdU+/DAPI+ cells were count in each fields at the fluorescence microscope.

Wt vs SCID mice had no statistically differences in both total cell number (DAPI: 248±42 vs 243±28) and total BrdU positive cell number (BrdU+/DAPI+: 64±11.3 vs 50.5±14.4). In contrast, the number of Nestin positive cells was statistically different between two strains (Nestin+: 206±49.3 vs 50.5±14.4, p=0.000172, Ttest analysis). This data reflected the slower number of Nestin+/BrdU+ double positive cells which showed a significant difference in wt vs SCID (Nestin+/BrdU+: 54±12 vs 14±5, p= 0.000147, Ttest analysis) (Fig.31A).

These results highlighted that differences between SCID and wt mice in the number of leptomeningeal nestin positive cells.

In parallel, Nestin+/BrdU+ cells of choroid plexus meninges were also compared in SCID vs wild type animals. Data showed a trend similar to that previously observed in Leptomeningeal cells (Fig.31 B): the total number of nestin positive cells were different in wt vs SCID mice (Nestin:23.33±5.66 vs 1.65±12.36) while total cell number and proliferating cells number were similar in both mice strains. However, these data had to be considered just preliminary due to a limited amount of choroid plexus that reduced the number of replicates and fields analyzed for each animals.
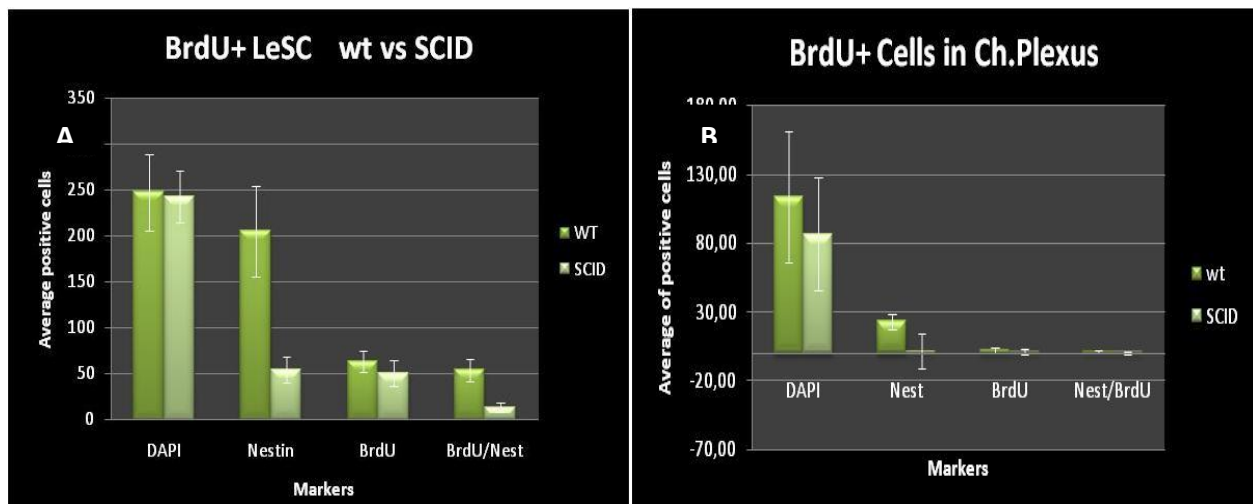
Fig.31: A Count of BrdU/Nestin positive cells in Leptomeninges expressed as average of positive cells count for each marker (DAPI, Nestin, BrdU, Nestin/BrdU double positive cells). Sideways average, standard deviation and ttest analysis are listed for each markers. **B Count of BrdU/Nestin positive cells in Choroid Plexus** expressed as average of positive cells count for each marker (DAPI, Nestin, BrdU, Nestin/BrdU double positive cells). Sideways preliminary average, standard deviation and ttest analysis are listed for each markers.

Leptomeningeal nestin positive cells were defined as an homogeneous layer of nestin positive cells ,residing outside the basal lamina (Laminin positive cells) as a distinct population from pericytes (NG2 positive cells) and astrocytes (GFAP positive cells). Based on this definition, markers for astrocytes and pericytes were first analyzed followed by DCX and βIII Tubulin.

Results showed that GFAP positive cells remained in parenchyma below basal lamina while few NG2 positive BrdU negative cells were in leptomeninges in both wt and SCID mice. SCID and wt mice did not showed differences in DCX and βIII Tubulin analysis, these two markers were not detect any positive cells in mice stains leptomeninges although the positive control (SVZ and DG) showed a strong signal (data not show).

The presence of immune system cells in leptomeninges were investigated by the distribution of immunological markers MHCII, CD8, CD4, CD3.

Three different wt mice (2 slices each) showed the presence of MHCIII, CD8, CD4, CD3 positive cells in Leptomeninges. These cells did not co-localized with BrdU or nestin and were mainly closed to vessels (data not show).

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DISCUSSION

The peculiar spatial relationships of leptomeninges in CNS, their role in cortex development and our discovery of nestin-positive cells prompted us to investigate leptomeninges as stem cell niche hosting stem/progenitor cells with *in vitro* and *in vivo* neuronal differentiation potential.

In this work, we analyzed the leptomeningeal compartment of the rat brain to assess whether a stem cell population with neuronal differentiation potential is present in this structure.

We found that (i) nestin-positive cells are present in the leptomeningeal compartment at the embryonic stages and persist up to adulthood, (ii) leptomeningeal nestin-positive cells can be extracted and cultured as neurospheres with features similar to the NSC-derived neurospheres, (iii) leptomeningeal nestin-positive cells can be cultured as adherent cells and expanded in vitro as homogeneous population of nestin-positive cells that highly express many of the stemness-related genes, (iv) expanded nestin-positive cells can be induced to differentiate in vitro with high efficiency to generate excitable neurons and (v) expanded cells can differentiate into neurons when injected into brains of living rats.

Leptomeningeal characterization

As far as literature reported, the presence of stem/progenitor cell in leptomeninges was never described. Indeed, this region is strictly associated with pia mater cells that secrete important chemotactic factors, such as SDF-1 (Borrell et al., 2006), as well as several extracellular matrix components endowed of trophic functions (Erickson et al., 2000). In addition, it may be in contact/communication with other known neural stem cell niches such as the ventricular zone mediated by the cerebrospinal fluid. Thanks to all these interactions, leptomeninges are a potentially favorable niche, a discrete areas of the brain where complex microenvironments, ensure a balance between proliferation and self-renewal of stem/precursor cells (Scadden 2006).

Previous studies described the distribution of stem cell markers inside the brain (Meletis et al., 2008; Okano et al., 2007), but there was no evidence that leptomeninges could be a stem cell niche.

In this work we analyzed the leptomeningeal compartment of the rat brain and spinal cord to assess if in this compartment reside stem cell population with neuronal differentiation potential. Nestin-positive cells were seen outside the pial basal lamina, as a different population from glial cells or pericytes. Nestin-positive cells persisted in brain and spinal cord leptomeninges from embryo up to adulthood.

These data suggest that leptomeninges might represent a progenitors/stem cells niche and prompted us to investigate in vitro properties of nestin positive leptomeningeal cells.

In vitro expansion

Term "neural stem cell" is used to describe cells that can generate neural tissue or are derived from the nervous system, have some capacity for self-renewal, and can give rise to cells other than themselves through asymmetric cell division (Gage 2000). Self renewing and clonality of SVZ precursor cells was described by their ability to behave *in vitro* as multipotent neurospheres (Taupin 2006).

Leptomeningeal nestin positive cells were extracted from all rat ages. To avoid any contamination with other cell types, we carried out a precise sampling of the very superficial portions of the cortex in the rats at all developmental stages.We can reasonably exclude that the stem cell population we have characterized could arise from any known site of neurogenesis because of the morphological examination of the stripped brains showing that SVZ, dentate gyrus and olphactory bulb remained intact.

The extracted cells were expanded as both neurospheres, as previously reported for SVZ-NSC (Taupin 2006), and in adherent cultures. Real-time PCR showed that leptomeninges-derived neurospheres and SVZ-NSCs had comparable gene expression and multipotent differentiation potential .

In addition, the number of cells that could be obtained from the biopsies of P15 rats was large enough to conduct extensive characterization of the leptomeningeal cells.

The nestin-positive cell population grown *in vitro* originated from the nestin-positive cells present in the stripped tissue (40.3% by facs analysis, data not showed) and not from a process of transformation occurring *in vitro*. This was shown by the presence of the nestin signal in cells as early as the cells become adherent to the flask following extraction.

Previous works showed that SVZ-NSC and DG-NSC derive from glial precursor cells (Riquelme et al., 2008) and that NG2/Nestin double positive cells extracted from the whole brain cells are capable of neural differentiation *in vitro (*Dore-Duffy et al.,2006). The newly identified population of nestin-positive cells extracted from the leptomeninges were neither NG2- nor GFAP- double positive cells and grew as an homogeneous population of nestin positive cells once kept in adherent conditions as showed by time course experiment.

Neuronal differentiation potential

Under neural differentiating conditions, expanded nestin-positive cells from P1, P15 and adults showed neural differentiation potential. Interestingly, they could differentiate into MAP2 positive neurons with distinctive morphology, including dendritic spines, GFAP-positive astrocytes and O4-positive oligodendrocytes.

Considering the high efficiency of neuronal differentiation (30-50% of cells), further studies characterized the neuronal differentiation potential of leptomeningeal-derived expanded cells from P15 rat. In one month, up to half of the cell population differentiated into MAP2-positive cells showing many features of terminally differentiated neurons, including dendritic spines, pre-synaptic proteins, receptors for neurotransmitters, enzymes involved in neurotransmitter synthesis and depolarization-induced changes of [Ca2+]. These data suggest that the nestin-positive stem/progenitor cells extracted from leptomeninges grown *in vitro*, have the potential to generate cells with several of the properties that identify mature and functional neurons (Rowe et al., 2005).

Data from literature showed how SVZ and DG NPC are conventionally cultured as neurosphere to obtain differentiated cells, which contain a mixture of stem cells, committed progenitors,

and differentiated cells (Suslov et al., 2002) while adherent culture is mainly choose to obtain an homogeneous population of proliferating stem cells with minimal differentiation (Pollard et al., 2006).

Leptomeningeal nestin positive cells grew as both neurosphere and in adherence as SVZ and DG NPC. Despite known NPC these cells can be both expanded as an homogeneous stem/progenitor cell population and differentiate with high neuronal potential in adherence conditions.

To determine more completely the fate potential of nestin positive cells characterized *in vitro*, literature suggest to test in parallel the *in vivo* differentiation potential in order to evaluate presence of possible artifact (Gage 2000). For these reasons brain leptomeningeal nestin positive cells, expanded from EGFP transgenic rats, were injected in living rats to assess the *in vivo* differentiation potential (Leto et al., 2006). Expanded cells were injected in hippocampus and their fate was followed up to 60 days. Following intra-brain transplant, the EGFP-positive cells persisted up to 60 days into the hippocampus where approximately half of the transplanted cells differentiated into MAP2-positive neurons with morphological aspect of differentiated neurons. We cannot exclude that the EGFP+ engrafted cells may fuse with resident neurons. Fusion of stem cells with resident neurons has been shown to occur in some experimental setting when *in vivo* differentiation is rare (Kozorovitskiy et al., 2003; Wurmser et al., 2004). This does not appear to be the case for leptomeningeal cells since up to 50% the engrafted cells express the neuronal MAP2 antigen and none of these cells appeared to be multinucleated.

Moreover is well demonstrated that after NPC transplantation into the hippocampus, transplanted cells that integrated in the granular cell layer differentiated into cells characteristic of this region, whereas engraftment into other hippocampal regions resulted in the differentiation of cells with

astroglial and oligodendroglial phenotypes (Shihabuddin et al. 2000). In contrast, leptomeningeal nestin positive cells differentiate into MAP2 positive cells with mature neuronal phenotype all over the hippocampal area suggesting that nestin-positive stem/progenitor cells extracted from leptomeninges may have capability of neural differentiation also *in vivo*.

Long-term maintenance of stem cells requires their migration and engraftment within supportive stem cell niches. Stem cell homing, trafficking and interstitial migration are processes by which stem cells recognize and interact with micro vascular endothelial cells or with extra vascular tissue-specific structures (Ghashghaei et al.,2007; Shen et al.,2008). In our *in vivo* experiments, EGFP+ cells, after intra-ventricular injection, were integrated inside leptomeninges and retained nestin-positivity. Moreover, EGFP+ cells followed the blood vessel distribution inside the brain parenchyma. At this site, injected EGFP+ leptomeningeal nestin-positive cells seem to reside in the perivascular spaces of the brain vessels. These data may indicate that leptomenigeal nestine-positive cells could migrate inside the brain and in choroid plexus through perivascular spaces.

Interestingly, preliminary data are consistent with the presence of GFP+/newly generated neurons in cerebral cortex (data not shown). As described for other NSCs derived from non-neurogenic regions, transplantation into SVZ, RMS or SGZ can generate neurons specific to that region (Temple, 2001).

Cross talk with immune system

The function of a healthy brain is largely affected by the immune system. However, no traces of T cells are to be found in the parenchyma of the CNS under normal physiological conditions. The meninges and choroid plexus, however, are heavily populated by antigen-presenting cells (APCs) and by substantial numbers of T cells (McMenamin et al., 2003; Ziv et al., 2006). It is therefore plausible that neuroimmune interactions affecting cognitive function take place in the meninges and ventricular areas rather than in the parenchyma (Kipnis et al., 2008).

The first indication that the immune system plays an important role in CNS came from the observation that the lack of T cells was found to primarily affect the proliferative capacity of NPCs at their niches: an impaired neurogenesis in both SVZ and DG of T-cell-deficient mice [i.e. severe combined immune deficiency (SCID) and nude] compared to that in wild type mice. Moreover reconstitution of the T-cell pool in immune-deficient mice by injection of wild-type splenocytes showed a partially restore of proliferation and neuronal differentiation, reaching levels similar to those observed in wild-type mice (Hauben et al., 2000). Additionally Rolls et al.

found that NPCs express Toll-like receptors (TLRs) and that NPC activity is affected by TLR-2 and TLR-4 signaling in both adult neurogenic niches (Rolls et al., 2007). TLR-2 and TLR-4 have opposing effects on NPCs; TLR-2 signaling positively regulates neuronal differentiation, whereas TLR-4 signaling negatively regulates progenitor-cell proliferation.

Leptomeninges were investigated for their regulation from the residing immune system. After the confirmation that our investigation system was able to detect CD8 and CD4 residing cells, the distribution in leptomeniges of neuronal stem cell marker nestin was investigate in wt and SCID mice. As previously described in literature for SVZ and DG, nestin expression was down regulated also in leptomeninges, but surprisingly the total number of residing cells was not affected.

A first investigation about the nature of nestin negative cells residing in leptomeninges of SCID animals let us to exclude the presence of glial cells or DCX positive neuroblasts (data not showed) but a deeper characterization will be fundamental to understand how the immune system might modify leptomeningeal population.

Final considerations

During recent years, embryonic and adult neural stem cells (NSCs) gained attention as major candidates for regenerative and cell replacement therapies in various neurodegenerative diseases. In this setting, stem cell-based therapies raised important ethical, technical and immunological concerns (Li et al., 2008). The clinical application of adult NSCs, despite their properties of self-renewal, neuro-glial differentiation potential and their possible use in autologous setting, is still of debate. It can be highlighted that NSCs have hardly accessible site of sampling, are difficult to expand in vitro as homogeneous stem cell population and show low rate of in vivo neuronal differentiation efficiency (Rosser et al., 2007).

The novel stem/progenitor cells described in this work is located in a more easily accessible brain site that might be reached without impair cortex structure and functioning. The superficial location of these cells and the high *in vivo* neuronal differentiation potential suggest the possible application of leptomeningeal stem/progenitor cells for autologous sampling

opening novel studies and encouraging perspectives in brain/spinal repair following injury or neuronal degeneration.

Because of the persistence of these cells during the brain development , their proliferation capability *in vitro* and their differentiation potential into neuronal cells *in vitro* and *in vivo*, we have suggested to name them Leptomeningeal Stem/progenitor Cells (LeSC), as a new entity (Bifari et al., 2009).

Further studies are needed to establish the *in vivo* role of leptomeningeal nestin-positive cells in embryonic, post-natal and adult CNS. Since leptomeninges cover the entire CNS surface and follow vessels into the brain parenchyma (O'Meara et al., 2007), and also have a roles in cortex development (Halfter et al., 2002), it is possible that in adult CNS, leptomeniges host regenerative stem cells over a large proportion of its volume.

Further characterization of LeSC in normal and pathological conditions may help to better understand the cortical neurogenesis, as well as their potential usefulness in the treatment of neurological diseases when used as source of NSC-like population.

The easier reachable localization compared to the already known neural stem cell niches, their high neuronal differentiation potential together with novel knowledge about immune system roles in LeSC regulation will open novel studies in regenerative medicine. Infact a more deeper characterization of SCID leptomeninges and understandings about immune system regulation will drive further in vitro studies. Comparison of in vitro cultured LeSC dissected from SCID vs wt mice will address questions about immune system roles in LeSC proliferation and differentiation. These future findings together with an extensive characterization of specific LeSC receptors involved in immune system interaction will be helpful in findings novel pharmacological target to modulate LeSC proliferation/differentiation *in vivo* in both physiological/pathological conditions.

Finding factors that may enhance *in vivo* migration, replication and differentiation properties of leptomeningeal stem/progenitors cells may be relevant for the improvement of regenerative therapies for brain diseases.

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