INSTITUTO DE NEUROCIENCIAS DE ALICANTE

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

UNIVERSIDAD MIGUEL HERNÁNDEZ

MIGRATORY ROUTING DURING POSTNATAL HIPPOCAMPAL DEVELOPMENT: CELLULAR CONTRIBUTIONS AND TRAFFICKING MODULATIONS

TESIS DOCTORAL

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-2014-

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La presentación de la Tesis Doctoral titulada: "MIGRATORY ROUTING DURING POSTNATAL HIPPOCAMPAL DEVELOPMENT: CELLULAR CONTRIBUTIONS AND TRAFFICKING MODULATIONS", realizada por la Dª. Valentina Cuccioli, bajo su inmediata dirección y supervisión en el Instituto de Neurociencias (UMH-CSIC) y que presenta para la obtención del grado de Doctor por la Universidad Miguel Hernández.

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Agradecímientos

Resulta muy difícil resumir, en tan pocas palabras, lo agradecida que estoy a todas las personas que han contribuido en la realización de este trabajo y que me han apoyado y acompañado a lo largo de aquella que ha sido la más impactante experiencia de mi vida.

En primer lugar, agradecerle a mi director de tesis, Salvador Martínez, el haberme permitido realizar mi tesis doctoral en su laboratorio, abriéndome las puertas de la Neurociencia y mostrándome un mundo tan atractivo. Su laboratorio ha sido para mí como una segunda familia, donde he podido aprender y formarme gracias a él.

Quisiera agradecer especialmente al Dr. Constantino Sotelo su ayuda a través de sus críticas constructivas y el haberme enseñado, con su profundo conocimiento, a continuar estudiando para completar mis estudios.

Un infinito GRAZIE va para los doctores Eduardo de Puelles y Diego Echevarría, que han sabido aguantar mis "preguntitas" multidisciplinarias durante todos estos años hasta el final, teniendo la paciencia académica de ponerse conmigo hasta que el tema quedaba perfectamente entendido y zanjado.

Quiero agradecer a los doctores Emilio Geijo y Roberto Gallego por haber contribuido, con sus intervenciones, a que los conceptos de electrofisiología que tanto me costó aprender al principio, no quedaran totalmente en el olvido.

Quiero agradecer también al Dr Pierre Marie Lledo por haberme permitido hacer una grande experiencia en su laboratorio.

A todos mis compañeros de laboratorio quiero agradecer, ante todo, el hecho de hacer que el ambiente de trabajo sea tan agradable y relajado, pero productivo y dinámico a la vez; si algo tengo claro es que lo echaré mucho de menos y que no lo tendré en ningún otro sitio! A Ana le tengo que agradecer las numerosas conversaciones, las tantas veces que ha sabido devolverme el ánimo cuando pensaba haberlo perdido y todos sus preciosos consejos que tanto me han ayudado en los momentos de crisis durante la tesis. A Raquel, a Arancha y a Elena quiero darle las gracias por el gran apoyo que me habéis dado con vuestras atenciones, ayuda y

tiempo compartido entre muchos experimentos, que han sido momentos siempre enriquecedores e inolvidables.

Gracias a Jon, Jesús J, Mari Carmen, Diego Pastor y Carlos Bueno que me han apoyado mucho en mis primeros pinchazos y me han acogido en el nicho de las células compartiendo conmigo sus ideas y sus experiencias. Y a Mari Paz, que aunque hayamos coincidido poco tiempo, me alegro de que este doctorado se haya extendido tanto permitiendo nos conociéramos bien… quizás me hayas metilado mientras!

A María le tengo que agradecer tantas cosas que no sé ni por donde empezar. Hemos vivido tanto juntas durante estos años en el labo… hasta mudanzas, estancias y copachas! Eres una compañera y una amiga especial y he tenido mucha mucha mucha suerte de tenerte a mi lado durante mi doctorado, el cual no sé cómo habría podido acabar sin ti!

Al Puelles corner le tengo dar unas gracias especiales por haberme salvado en momentos de carencias de sitio y por haberme acogido en su rincón con tanto cariño! Ha sido un momento breve pero intenso y gracias a Jesús y a Juan Antonio no he echado en falta ná! Además, como olvidarme de los desayunos todos juntos con Pablo, Chispi, Almudena y Edu jr, gracias a los 4 por todas nuestras conversaciones en el corner, que han sido siempre de gran nivel, por haber hecho más ligeros y entretenidos esos momentos de trabajo, moltes gràcies!

Muchas son las gracias que tengo que dar al Cuerpo Técnico! Paqui, eres simplemente un cielo, nuestras conversaciones y tu apoyo me han ayudado muchísimo en varias situaciones de bajón. Carol y Olga, os debo dar las gracias también por tanto ánimo y ayuda técnica (y no solo técnica!) que me habéis sabido dar. Alicia, mi supermummy, eres mi héroe, eres una persona a la que siempre he podido contarle todo y que me ha dado mucho apoyo en todo momento durante estos años. Mónica, la referencia de todas mis inmunos; nena, todo lo que me has enseñado tú en estos años es algo que nunca podré agradecerte lo suficiente, y no hablo sólo de cortes!. Muchas gracias a Marusa, eres la persona más eficiente y energética que he conocido y me encanta tu contagiosa positividad, que es siempre necesaria en el labo. Agradezco también a Phil y Elisabetta para haberme acompañado en el laboratorio durante todos estos años.

Un súper gracias a "los peques" Abraham, Pili y Cosme que habéis sabido encajar tan estupendamente y con mucho interés para formar parte de este laboratorio. Espero que vuestro camino sea tan estimulante y positivo como ha sido el mío.

A mis compañeras de doctorado Deisy, Isabel y MariaJo por haber compartido conmigo lo malo y lo bueno, las risas y las lágrimas de este doctorado. Ha sido una suerte encontraros en mi camino y espero que nuestra amistad siga siempre tan fuerte y sincera a pesar de donde acabe cada una de nosotras.

Quiero agradecerle a Giovanna Expósito su amistad y su ayuda; todo lo que he podido hacer y aprender para obtener unas imágenes tan bonitas en esta tesis se lo debo a ella. Muchas gracias a Graciela por nuestros cafés y nuestras conversaciones en las que hemos hablado siempre de todo, me alegro mucho de tenerte como amiga.

Alle mie amiche Ceci, Giorgia y Elisa voglio fare un particolare ringraziamento. Ci siamo trovate un po' strada facendo, ma il vostro appoggio credetemi é stato fondamentale in piú di un momento. Mi avete dato tanto appoggio e tanti consigli ricreando un'atmosfera tutta italiana che mi ha fatto stare bene e mi ha dato come il vero calore di casa, complice chissá anche tanto buono spritz!

Grazie al mio emisfero destro, Eleonora Aquilini, senza di te tutto questo non sarebbe mai successo. Non trovo le parole per ringraziare il tuo continuo appoggio, la tua amicizia e il tuo essere tanto presente nella mia vita persino in questo momento cosí importante. Spero che la nostra connessione simbiotica non ci abbandoni mai!!!

Grazie mille a Paola Imperato, al piccolo Andrés y al grande Andre. Siete stati il mio rifugio lontano da tanti esperimenti e il sostegno nei momenti difficili. Sono contenta che abbiate fatto parte di questo pezzo della mia vita, YOU ARE THE BEST GUYS!!

Tantissimi ringraziamenti vanno anche alla mia famiglia. Da questa esperienza abbiamo capito che nessuna distanza sará mai troppo grande, che riusciamo a stare insieme e a condividere proprio tutto. Questo dottorato é stato anche per voi una grande avventura e vi ringrazio tantissimo per avermi sostenuto e appoggiato in ogni mia scelta in tutto questo tempo.

Muchísimas gracias a Hervé, no sé cuántas cosas tengo que agradecerte, no tengo suficientes palabras para ello! Me alegro infinitamente de haber decidido, por aquel

entonces, hacer el doctorado en este Instituto porque he podido conocer a una de las personas más importantes de mi vida! Esta tesis, que tanto ha marcado nuestra relación, ahora deja paso a algo mucho más importante… te quiero!

Al laboratorio de Embriología
Experimental y a Salvador Martínez,

LIST OF ABBREVIATIONS

Amy= amygdala

BDNF= brain derived neurotrophic factor

BNST=bed nucleus stria terminalis

CA= Cornu Ammonis

CA3alv= Cornu Ammonis 3 alveus

CB= calbindin

CC= corpus callosum

CGE= caudal ganglionic eminence

COUP TF= chicken ovalbumin upstream promoter-transcription factor

CNS= central nervous system

CMS=caudal migratory stream

CR= calretinin

Cx= cortex

DAPI= 4 ',6-diamino-2-fenilindol

DCX= doublecortin

DG= dentate gyrus

DMP= dorsal migratory pathway

DN= dentate notch

dpe=days post electroporation

dpi=days post injection

ECM= extra-cellular matrix

EGF= epidermal growth factor

EMP=external migratory pathway

Epha4= Ephryn 4

FDJ= fimbrio dentate junction

FGF= fibroblast growth factor

FGFR1= fibroblast growth factor receptor

Fi= fimbria

FISH= fluorescente in situ hybridization

GABA= gamma-aminobutyric acid

Gabrd= GABA A recptor d

Gad67=glutamate decarboxylase 67

GCL= granular cell layer

GDNF= glial cell line derived neurotrophic factor

GFAP= glial fibrillary acidic protein

GFP= green fluorescent protein

HF= hippocampal fissure

Hi= hippocampus

IPs= intermediate progenitors

LGE= lateral ganglionic eminence

MAP= microtubule associated proteins

MGE= medial ganglionic eminence

NAc= nucleus accumbens

Ngn2=neurogenin 2

NSC=neural stem cell

NYP=neuro-peptide Y

OB= olfactory bulb

PirCx= Pyriform cortex

POA= pre-optic area

PSA-NCAM= polysialic acid-neural cell adhesion molecule

PV= paravalbumin

RMS= rostral migratory stream

scSEZ=sub cortical subependimal zone

SE= septum

SGZ= sub-granular zone

SPZ= subpial zone

Str= striatum

TGFα= transforming growth factor alpha

Tuj1= III-beta tubulin

VIP=vasoactive intestinal peptide

VMP= ventral migratory stream

VZ=ventricular zone

Zbtb20= zinc finger and BTB domain-containing protein 20

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INTRODUCTION

1. Neural cell migration during telencephalic development

During brain development neural cell migration is a crucial process that led to the proper whole structural organization and is therefore highly regulated for the correct networks formation. Because both neuronal and non-neuronal cells are often generated in sites that differ from those in which they will eventually reside, most neurons during development has to migrate even long distances before to reach their final destinations. In the developing telencephalon, two main modes of migration have been identified on the basis of its orientation: radial migration, in which cells migrate from the progenitor pool towards the pial surface following the radial disposition of the neural tube; and tangential migration, in which cells migrate orthogonally respect to the radial glial fibers disposition (Rakic P., 1972a; Marin O. and Rubenstein JL, 2003). These are the two main mechanisms by which developing neurons arrive at their final position and constitute the essential cellular movements for the correct morphogenesis of cerebral cortex (Nadarajah B et al., 2001; Hatten M.E., 1999).

During radial migration, neural progenitors from the ventricular zone (VZ) of the telencephalon extend long processes from the ventricular wall to the pial surface of the cortex, providing an important substrate used by immature neurons to migrate into the cortex (Rakic P., 1972b; Schmechel D.E. and Rakic P., 1979; Marin O. and Rubenstein J.L., 2001). An example of radial migration inside the telencephalon is the migration of cortical excitatory projection neurons that are generated by the pallial neuroepithelium (Tan S.S. et al., 1998). In contrast, it has been proved in rodents that most if not all gamma-aminobutyric acid containing (GABAergic) cortical interneurons, which modulate through synaptic inhibition the cortical network, are born in the ventral forebrain in the ganglionic eminences. Those neurons perform a tangential migration parallel to white matter tracts before they turn and migrate radially to reach their final position into the cortex (Anderson S.A. et al., 1997; Porteus M.H. et al., 1994; Corbin J.L. et al., 2001).

These two ways of migration not only differ in their orientation but also in the mechanisms underlying their cellular movements (Fig.1A). In particular, during radial migration, a radial glial scaffold provides the primary guidance system for migrating

neurons, while in tangential migration cells do not coincide with the plane of radial glial fibers system and thus use distinct types of substrates to move (Rakic P 1972b; Rakic P 1974; Marin O. and Rubenstein J.L., 2001). Based on the observation of their migratory behavior, neurons can adopt different mechanisms to displace during radial migration. These cells can move through glial-guided locomotion, in which a short leading process not attached to the pial surface move forward displaying a unique saltatory pattern. Moreover, they are also capable of migrating by somal translocation, which appears largely independent of radial glial cells support and displays cells with long processes terminating at the pial surface (Nadarajah et al., 2001) (Fig.1A).

On the other hand, during tangential migration different types of substrates guide cell movements such as group of neurons which migrate supporting each other to promote their migration, as olfactory bulb interneurons, while in other cases can follow growing axons or vasophilic interactions with blood vessels (Bovetti S et al., 2007) to reach their destination. Finally, there are neurons that do not follow any specific cellular substrate and disperse in an individual manner, such as during subpallial to pallial migration. However, conversely to the cellular movements of neural progenitors in the VZ, cells moving tangentially do not seem to respect any regional telencephalic boundary thus cells move in the mantle layer across different subdivisions of the telencephalon (Heffron D.S. and Golden J.A., 2000, Letinic K and Rakic P, 2001; García-Moreno F et al., 2010; Pombero A et al., 2011) and even along axonal pathways (Spassky et al. 2002). In addition, tangentially moving cells are able to respond to some guidance molecules that control growing axons (Tessier-Lavigne M and Goodman 1996).

Several studies have been shown that multiple structures in the telencephalon including the neocortex, the hippocampus, the olfactory bulb, striatum and hypothalamus are generated by the integration of neurons moving both radially and tangentially (Nadarajah B et al., 2003) (Fig.1 B). The coexistence of these two different modes of migration in the developing telencephalon is probably an evolutionary strategy used to increase the cellular complexity of specific cortical circuits. In addition to that, the generation of certain populations of telencephalic neurons with distinct neurotransmitter phenotypes appears to be linked to specific progenitors located in different germinal regions of the forebrain. Furthermore, recent fate mapping analysis has suggested that formation of distinct classes of GABAergic interneurons in the subpallium is tightly linked to the existence of distinct classes of progenitor cells

(Flames N. and Marin O., 2005; Fishell G. et al., 2007). Thus, the telencephalon results one of the forebrain structures where all the ways of migration described above occur along its development and so constitute a good model for the study of cell migration throughout vertebrate brain development.

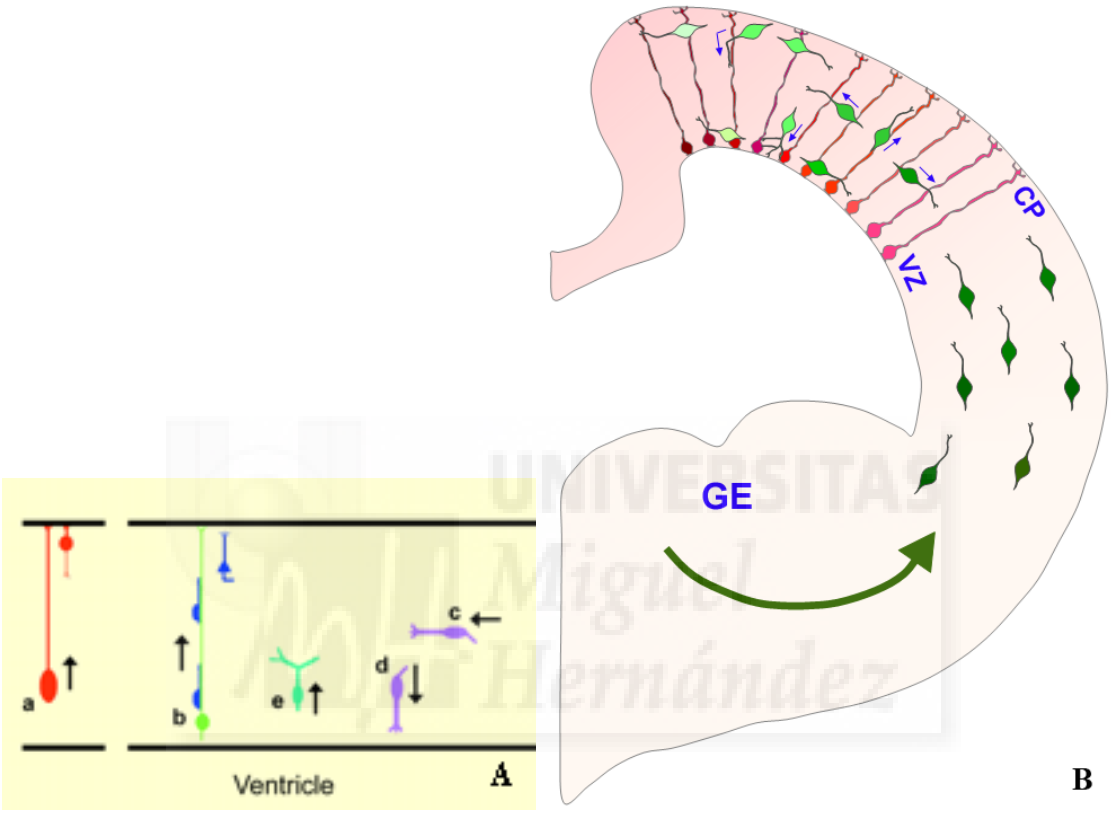


Fig.1 Schematic illustration of the various modes of neuronal migration in the developing cerebral cortex.
A) During early development the prevalent mode of radial migration is somal translocation (a). As development proceeds and the cortex thickens, the predominant mode of migration is glia-guided locomotion (b). Cortical interneurons that arise in the ganglionic eminence follow tangential migratory paths to reach the cortex (c) and seek the ventricular zone (ventricle-directed migration) (d). A subset of neurons that switch from radial to tangential modes of movement show active branching (branching cells) (e) Adapted from *Nadarajah B et al. Cereb. Cortex 2003;13:607-611*
B) Scheme resuming the migratory pathways during GABAergic interneuron production in the developing telencephalon: interneurons (green) are produced in the ganglionic emincences (GE) and move first tangentially and then radially following radial glial fibers (in red) in order to reach the cortical layers. In the ventricular zone (VZ) radial glial cells (red) not only are the scaffolding for radial migration but also produce neurons that migrate radially to the cortex. Adapted from *Yokota Y et al, Plos One 2007*.

1.1 Telencephalic germinative areas

Two main germinal regions in charge of producing the majority of neurons and glial cells can be identified in the developing telencephalon: the ventricular zone (VZ) and the subventricular zone (SVZ). In the VZ cortical cell precursors extend their processes across the width of the developing telencephalon and proliferate in accordance with cell cycle progression. As development proceeds, the VZ progeny converts into post-mitotic neuroblasts and they leave the VZ to migrate towards the pial surface using radial glial fibers scaffolding. Radially migrating cells settle in specific positions depending on the timing of their birth and thus generate an inside-out pattern of different layers with early born neurons laying in the deeper ones. Despite their role in radial migration, radial glial cells do not simply function as static supporting elements but are indeed dynamic components of the developing cortex, undergoing mitosis to develop new neurons and so representing an intermediate stage in the stem-cell lineage of the CNS (Alvarez-Buylla A. et al, 1990; Alvarez-Buylla A. et al., 2001, Götz M., 2003, Pilz GA et al., 2013).

Whereas the VZ proliferative fraction declines rapidly, the SVZ cell population expands exponentially during the latter third of prenatal development and achieves its highest peak in size during early postnatal development (Altman J. and Bayer S.A., 1991). During this period, over 90% of the SVZ population is dividing, while most of VZ cells are leaving the cell cycle (Takahashi et al., 1995). Cells generated in the SVZ start to migrate tangentially and then switch to move radially and reach their final destination.

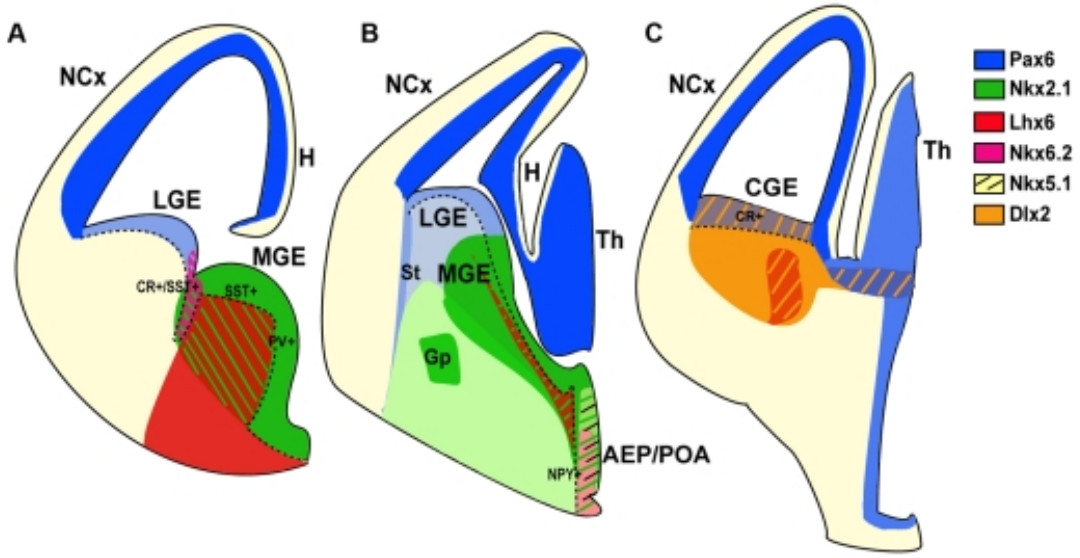
An example that best reflects the combination of both mechanisms of neural cell migration is the complex migratory pathways of migrating GABAergic interneurons in rodents originated in the ganglionic eminences (GE) in the ventral telecephalon or subpallium (Fig.1B) (Anderson et al., 1997 and 2002; Wichterle et al., 2001). Using vital dye migration assays, robust tangential subpallial-to-pallial migrations were identified during mouse embryonic development, leading to the GABAergic population insertion inside the neocortex (Anderson SA et al, 1997; Tamamaki N et al, 1997). While GABAergic interneurons populating these structures derive from the subpallium at the ventral telencephalon (Batista-Brito and Fishell, 2009; Gelman and Marín, 2010; Wonders and Anderson, 2006), glutamatergic neurons present in both the olfactory bulb

and the cerebral cortex are generated locally by progenitor cells residing in the developing pallium (Molyneaux et al., 2007; Rakic, 2007). Nevertheless, to date how these apparently disconnected processes are synchronized and regulated during development remains one fascinating question still poorly understood.

1.2 The embryonic subpallial telencephalon

During embryonic stages the subpallium consists of four main subdivisions with distinct molecular and morphological features: the lateral ganglionic eminence (LGE), the medial ganglionic eminence (MGE), the septum (SE) and the preoptic area (POA). The caudal part of the LGE and MGE lack a clear morphological demarcation leading to this region to be called the caudal ganglionic eminence (CGE) (Anderson S.A. et al., 2001; Nery S et al., 2002; Flames N et al., 2007).

As a matter of fact, the subpallium is described as the site of origin of multiple tangentially migrating interneurons that populate different distant areas in the pallium including the neocortex, the piriform cortex and the hippocampus (Pleasure S.J. et al., 2000). Experimental embryology studies based on different methods, including DiI-labeling techniques in slice-cultures (Lavdas A et al 1999; Wichterle H et al., 1999) and several transplantation approaches (Pleasure S.J. et al., 2000; Wichterle H et al., 2001), demonstrate the presence of different routes of migration that are very influenced by the place and the timing of interneuron production. According to that, the examination of interneurons distribution in mice lacking specific transcription factors regulating the regional specification of the subpallial telencephalon helped to clarify the anatomical origins of those different migratory routes (Sussel L. et al., 1999; Stoykova A et al., 2000; Toresson H et al., 2000; Corbin J.G et al., 2000). Many efforts mainly based on the study of transcription factor expression patterns are ongoing in order to identify how the gradual parcellation into several progenitor domains is generated giving rise to the LGE, the MGE, the SE and the POA subunits (Fig.2). For example, Gsh2 shows a stronger expression in the LGE, decreasing towards the MGE, while Nkx2.1 is expressed strongly in the pallidal and preoptic subdivisions including the MGE, while is absent in the LGE (Corbin et al, 2000; Sussel et al., 1999).



	LGE	MGE	CGE	POA
Dlx2	++	++	++	++
Gsh2	++	++	++	-
Couptf1	++	++	++	++
Lhx6	-	++	+	-
Lhx7	-	++	-	-
Nkx2.1	-	++	-	++
Nkx5.1	-	-	-	+
Nkx6.2	-	+	-	+
Olig2	+	++	+	+
Pax6	+	-	+	-

Fig2. Gene expression patterns in the subpallium

Schematic representation of medial (**A**), caudal-intermediate (**B**) and caudal (**C**) coronal sections of E13.5 mouse brain showing the expression of different genes in the subpallial domain which give rise to distinct interneuron subpopulations. Broken lines delineate the VZ. Sub-domains that express two or more genes are marked with stripes. (**A** and **B**) *Pax6* is expressed at high levels in the pallial proliferative zone and thalamic territory, and at a lower level in the subpallial proliferative zone of the LGE. *Nkx2.1* is specifically expressed in the VZ and mantle layer of the MGE. It is also expressed in the proliferative zone of the AEP/POA. *Nkx6.2* expression is confined to the LGE/MGE border and overlaps with the expression of *Pax6*/*Dlx2* in the LGE, and *Nkx2.1*/*Dlx2* in the MGE. CR+ (CR-positive) and SST+ (SST-positive) interneuron precursors arise specifically from this *Nkx6.2*/*Nkx2.1*-expressing territory. *Lhx6* expression is confined to the subventricular zone and the mantle layer of the MGE, and it specifies PV+ (PV-positive) interneurons. Its expression does not overlap with Nkx6.2 expression. AEP/POA, thought to give rise to NPY+ (NPY-positive) interneurons, expresses *Nkx2.1*, *Dlx2* and *Nkx5.1*, as well as *Nkx6.2* in its ventral domain. (**C**) The CGE is anatomically positioned at the most caudal part of the telencephalon where the LGE and MGE fuse together. Shown here is the expression of *Dlx2,* which characterizes all subpallial domains, *Pax6* confined to the proliferative zone, and *Lhx6*. (**D**) A summary of the expression of different genes in the LGE, MGE, CGE and POA. ++, High level of expression: +, lower level of expression; -, lack of expression. Abbreviations: Gp, globus pallidus; H, hippocampus; NCx, neocortex; St, striatum; Th, thalamus.
Adapted from *Hernández-Miranda LR et al., ASN Neuro 2010;2(2):e00031*

These early regulatory proteins not only are involved in the specification of the major subpallial subdivisions, but they also induce the expression of other transcription factors implicated in cell differentiation such as Dlx1/2, Lhx6 and Lhx7/8 and eventually regulate different aspects such as neuronal migration and neurite morphogenesis. In particular, Nkx2.1, Gsh2 and Pax6 have been identified as essentials proteins for the differentiation and specification of tangentially migrating subpallial interneurons and thus their lack produces severe defects in interneuron production and differentiation (Sussel et al., 1999; Toresson et al., 2000). Moreover, through mechanisms of mutually repressive interactions, these transcription factors establish boundaries between different subpallial progenitor zones thus generating different migratory pathways (Puelles L et al., 2000; Fogarty M et al., 2007; Flames N et al., 2007; Medina L et al., 2009).

MGE and POA seem to be the primary sources of tangentially migrating interneurons during early stages of mouse development (E11.5), including those of the striatum, cerebral cortex (neocortex, hippocampal formation and piriform cortex) and other parts of the pallium (claustroamygdaloid pallial complex). These streams begin their course superficially into the developing striatum and then continue and invade the cortical marginal zone and then the subplate. Both MGE and POA specification requires the expression of the homeobox transcription factor Nkx2.1, as demonstrated in the mutant mouse line in which its absence results in the reduction of the cortical and striatal interneuron population. Those data proved that the expression of this transcription factor is required for the generation of more than half of the GABAergic neural progenitors populating these two brain areas (Sussel L et al., 1999, Marín O et al., 2000). *In vitro* (Xu Q et al 2004; Wonders CP et al 2008) and *in vivo* (Flames N et al 2007; Butt SJB et al., 2005) experiments have revealed that the large majority of MGE-derived cortical interneurons are paravalbumin-containing cells (PV) and that MGE-progenitor molecular characteristics are linked to the expression of Nkx2.1, Dlx5, Dlx6, Lhx6 and Sox6 transcription factors. Interestingly, it has been described that immature MGE cells which maintain Lhx8 and not Lhx6 expression do not become cortical interneurons, but instead they give rise to subpallial telencephalic neurons such as globus pallidum (GP) GABAergic neurons and cholinergic interneurons of the striatum (Zhao Y et al., 2003; Fragkouli A et al., 2005). Additionally, between E12.5-E15.5 the MGE also contributes to the production of many interneurons in the

hippocampus, which are mostly restricted to the CA1 region (Pleasure et al., 2000; Wichterle H et al 2001), and to specific nuclei of the amygdala complex (Puelles et al., 2000; Nery S et al., 2002; García-Lopez et al., 2008). Recently, it has been reported that a novel small cortical population of GABAergic interneurons derives from the embryonic POA (Gelman D et al., 2009) expressing Nkx2.1 but shows different characteristics respect to the corresponding MGE derived population (Gelman D et al., 2009). This POA interneuron population shares similar features to another CGE derived neural population (Miyoshi G et al., 2010), and therefore this suggests that both this two areas may contribute to the generation of this specific population of interneurons in the neocortex (Gelman D et al., 2009).

During mid-embryonic stages of mouse development (E14.5-E16.5), LGE-derived cells initiate a massive rostral migration directed to the olfactory bulb (OB). As described before, the LGE is specified dorsally by the expression of Gsh2 gene, responsible of the establishment of the boundary between LGE and the cortex; in addition, the distribution of Pax6 and Nkx2.1 expression contributes to the formation of the LGE-MGE boundary. The loss of expression of Gsh2 and Pax6 in mutant mice resulted in a reduction of OB interneurons during mid-embryonic stages and in a wrong establishment of the boundary between LGE and the adjacent cortex, confirming their specific roles in the specification of the LGE (Corbin J et al., 2000). Consistent with this, it has been subsequently reported that LGE-derived cells, but not MGE-derived cells, transplanted into the adult SVZ can give rise to neurons that migrate rostrally to the OB (Wichterle H et al., 1999). On the basis of these observations, the generation of olfactory interneuron precursors has been identified to occur in the embryonic subpallium, in the dorsal region of LGE, a process that continues throughout adulthood providing a continuous supply of GABAergic interneurons to the olfactory local circuitry (Lois C and Alvarez-Buylla A 1994). Moreover, the LGE appears to give rise to interneurons in the anterior nucleus accumbens (NAc), in the cortex (Anderson SA et al., 2001) and from its most caudal portion (caudal LGE-CGE) (Nery S et al, 2002; Xu Q et al., 2004) to the pallial amygdala and also becomes the source of projection neurons of the striatum (Olsson M et al, 1998).

Compelling evidences identified a posterior region where MGE and LGE fuse together into a single structure referred as the CGE (Nery S et al 2002) (Fig.3). The definition of CGE is mainly based on anatomical references due to the difficulties in consistently defining this region, which is known to produce between 30-40% of all

cortical interneurons. Although the CGE shares the expression of certain markers with the LGE or the MGE (Fig.2), the CGE is a progenitor region with a distinct identity (Nery S et al 2002). At present, no single gene exclusively marks the CGE and no genetic mutations specifically affect only the CGE. Nevertheless, different studies suggest that the CGE contains progenitor domains with unique molecular profiles implying the existence of distinct germinative nuclei in this area (Kanatani S et al., 2008; Willi-Monnerat S et al., 2008). Recently, it has been reported that two transcription factors CoupTFI and CoupTFII are highly expressed in progenitors within the CGE and experimental evidences suggest that CoupTFII promotes caudal tangential migration of interneurons to the cortex (Kanatani S et al., 2008). However, it seems that the expression of CoupTFII is not exclusively confined to the CGE, but shares some positive precursor population within the dorsal LGE and MGE (Yuqun Cai et al., 2013) further complicating the characterization of this structure.

CGE derived cells were found to migrate tangentially during a developmental period ranging between E12.5 and E15.5 directed to the cortex at its most caudal levels and to contribute to numerous structures such as the posterior NAc, the bed nucleus of stria terminalis (BNST), as well as to some nuclei of the limbic system including the hippocampus (most in the CA1 and CA3 regions) and selected amygdala nuclei (Nery S et al., 2002; Xu Q et al., 2004; Butt S.J. et al., 2005; Yozu M et al., 2005; Miyoshi G. et al., 2010; Tang Ke et al., 2012). In addition, although with lesser extent, some CGE cells were found in the posterior striatum and the posterior globus pallidus (Nery S et al, 2002). Further studies based on gene expression profiling, *in vitro* migration and *in vivo* fate mapping definitely confirmed that the CGE is indeed a different structure of the GE and is the source of specific subtypes of neocortical interneurons with bipolar and double-bouquet cell morphology. Additionally, CGE derived cells express GABA and/or other markers of interneurons (Calretinin CR, vasoactive intestinal peptide VIP and Reelin/neuropeptide-Y NPY expressing interneuron). However, the CGE is not only producing interneurons: in the striatum and the globus pallidus the majority of CGE derived cells are medium spiny projection neurons and CGE derived oligodendrocytes and astrocytes were observed also in many of the above cited structures (Nery S et al., 2002).

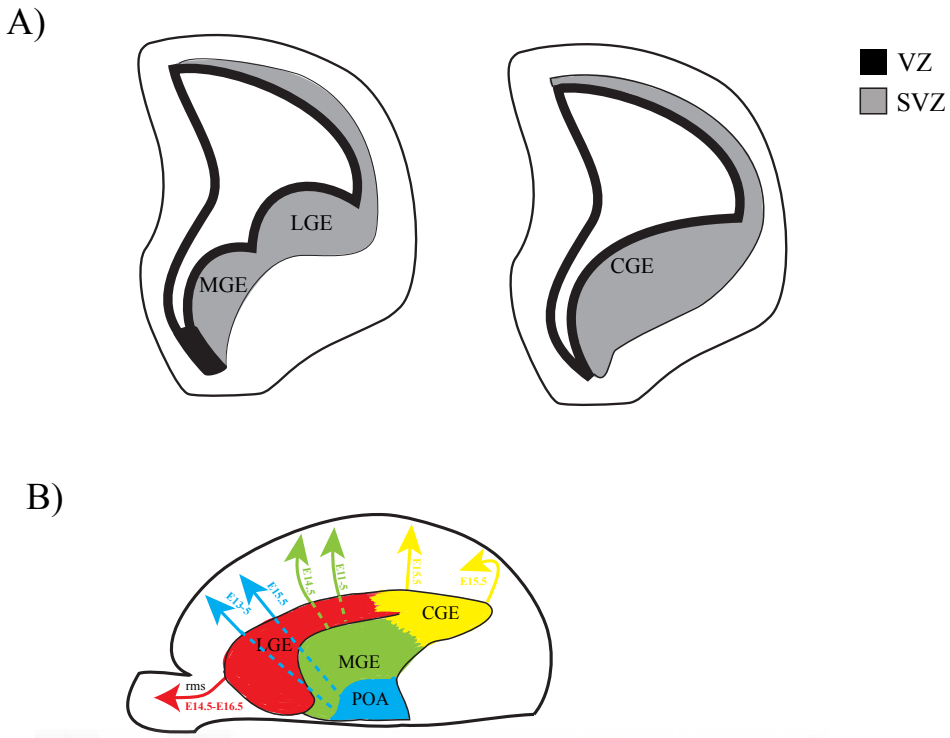


Fig.3 Contributions of the SVZ of the medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE), and caudal ganglionic eminence (CGE) during early postnatal brain development. (A) A schematic coronal view of the rodent ganglionic eminences in the postnatal telencephalon. The LGE and MGE are prominent structures in P0 brain with an extended SVZ (in grey). By contrast, at this age the neocortical VZ is almost unremarkable (in black). (B) Sagittal view of the telencephalon cut at the midline. Schematic representation of the different subpallial germinative domains LGE (red), MGE (green), CGE(yellow) and POA (blue) and their directional movements (colored arrows) as they migrate during mid-embryonic stages to the striatum, neocortex and nucleus accumbens. Cells from the MGE also may migrate through the LGE en route to the neocortex. LGE cells migrate mainly to the olfactory bulb (OB). Cells of the CGE migrate to the hippocampus, thalamus, pallidum and amygdala nuclei (OT). Recently, POA is also described to contribute with different GABAergic populations to the neocortex. Adapted from Gelman D et al., *Jasper's basic Mech.of Ep.* 2012.

2. Neural cell migration in the postnatal telencephalon

At birth neurogenesis is largely completed with few notable exceptions, such as the striatal SVZ and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), where an extensive amount of cell migration still occurs during the first few postnatal weeks and is maintained until adulthood.

Much of these postnatal cellular movements involve glial progenitors and most neural migration has been completed by this time. Nevertheless, during the first two postnatal weeks, a high degree of plasticity is maintained including axonal remodeling, synaptogenesis, neural cell migration and integration into synaptic circuits. Continuous neurogenesis in fact appears to be important in the striatal SVZ for the maintenance and renewal of the OB interneurons as well as in the SGZ for the refinement of DG circuitry involved in hippocampal-dependent memory function (Imayoshi I et al., 2008).

2.1 The postnatal SVZ

The postnatal SVZ appears as a large zone mainly composed of highly proliferative and migratory cells that extend rostrally from the striato-ventral pallium limit area of the lateral ventricle until the core of the OB. During perinatal stages, the SVZ can be subdivided into three main anatomical regions: the anterior SVZ (aSVZ) located rostral to the frontal tip of the lateral ventricle, the dorsolateral SVZ (dSVZ) a packed anlage of cells situated at the dorsolateral aspect of the lateral ventricles at the striato-callosal border, and the postnatal equivalent of the CGE that we considered in our study as the caudal SVZ (cSVZ). This last region appears as a specific cellular population residing in the dorsal/ventral axis of the lateral ventricles adjacent to the caudal striatum and corresponds to the ventricular domain of the amygdala complex (Brazel CY et al., 2003) (Fig.4).

The aSVZ has been identified as the major source of rostral tangentially migrating cells directed to the OB and can be considered as the postnatal remnant of the embryonic LGE, as confirmed by transplantation experiments of this embryonic domain into the adult SVZ (Wichterle H et al., 1999). In the postnatal aSVZ, neural progenitors

are continuously dividing and generating precursor cells and neurons that are able to migrate to the OB. In particular, many studies already described how aSVZ progenitors continue to generate large numbers of olfactory interneurons during postnatal development, a phenomena that lasts all along adulthood. Newly generated neuroblasts perform a long tangential journey towards the OB migrating in a well-defined pathway called the rostral migratory stream (RMS) (Lois C and Alvarez-Buylla A., 1994; Doetsch F and Alvarez-Buylla A., 1996). This migratory stream is described as a unique migratory route known as "chain migration" where migrating cells move through homophilic interactions and are organized in chains. Unlike radial migration, which is guided by glia (Rakic P., 1990), early postnatal RMS cells are associated with neither glial nor axonal fibers (Kishi K et al,1990) and showed a characteristic migration with chains formation (Wichterle H et al., 1997). However, staining for GFAP reveals a meshwork of GFAP positive cells encapsulating migrating neuroblasts and thus providing a neurophilic "tunnel" in which migrating cells move along one another to reach their target (Lois C et al., 1996). Once neuroblasts reach the bulk of the OB they detach from the chains and turn 90º to disperse radially inside the bulb, giving rise to periglomerular and glomerular interneurons (Lois C et al., 1996).

The dSVZ is characterized by the presence of a high number of multipotent cortical and striatal glial cells during postnatal period, able to give rise to both neurons and glia in vitro (Young KM et al., 2007). Most of these progenitors emigrate from the dSVZ into the striatum and white matter as well as to the medial, dorsal and lateral regions of the cerebral cortex, where some develop into oligodendrocytes (Levison S.W. and Goldman J.E., 1997; Parnavelas J.C. et al, 1999). Those cells are present at distinct states of fate commitment appearing as a mixed population of neuronal-glial clones in vitro (Levison S.W. and Goldman J.E., 1997). In general, all those migratory cells do not use chain migration whereas behave like other neocortical progenitors using radial glial cells to guide their dorsal migration towards the neocortex (Kakita A et al 1999). Because of their location adjacent to white matter tracts, those precursors are described to take advantage of those tracts using them as permissive migratory routes (Zerlin M et al., 1995).

The cSVZ is considered as the postnatal remnant of the embryonic CGE-SVZ and as its embryonic counterpart no single gene specifically labels this region, leaving its identification mostly based on morphology (Nery S et al., 2002). However, recent data reported that the majority of cells lining the lateral wall of the lateral ventricle,

including the dSVZ, were derived from the embryonic Gsh2 domain in the adult, indicating a possible correlation between this embryonic LGE-CGE domain and adult lateral wall descendants (Young K et al., 2007). Other transcription factors have been detected in the adult SVZ and/or RMS including Pax6 (Hack M.A. et al., 2005), Mash1 (Casarosa S et al., 1999), Dlx2 (Doetsch F et al., 2002) Er81 (Stenman J et al., 2003) and Sp8 (Waclaw R et al., 2006) arising important questions about whether any of these transcription factors (or specific combinations of them) are expressed in a subset of neuroblasts that can be traced back specifically for the identification of LGE or either CGE domains. Nevertheless, it remains poorly understood whether a conserved cSVZ is still present during early postnatal life and if caudal migratory currents are still maintained postnatally. Moreover, since recently the postnatal LGE has been also described as a source of cells moving to dorsal white matter structures generating new pools of neuronal production (Riccio O. et al., 2012), this opens the possibility that other SVZ regions different from the aSVZ compartment could contribute to hippocampal cell population during postnatal stages.

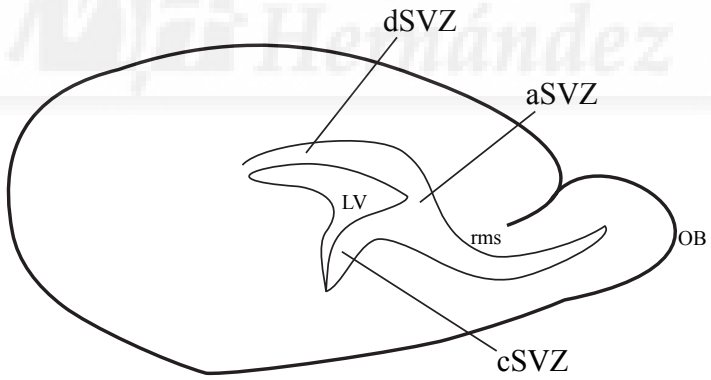


Fig.4 The postnatal SVZ subdivision in rodent brain.

Sagittal view of a cerebral hemisphere showing the postnatal subdivisions of the SVZ. The anterior SVZ (aSVZ) occupy the anterior anlage at the frontal tip of the lateral ventricle where dividing cells are present throughout postnatal life. From the aSVZ cells perform a tangential migration (rms) directed to the OB. The dorsal SVZ (dSVZ) is lining the dorsolateral aspect of the ventricle and represent the major source of forebrain neuroglia. The caudal SVZ (cSVZ) represents the remnants of the ganglionic eminences MGE-CGE and lines the lateral ventricles adjacent to the striatum.

2.2 The postnatal DG

The DG is another small forebrain area where neurogenesis continues throughout adult life and it has a prolonged developmental period that spans from embryonic until early postnatal stages involving large scale reorganization events of progenitors (Nowakowski R.S. and Rakic P., 1979, 1981; Eckenhoff and Rakic P., 1984; Altman J and Bayern S.A.,1990a,b).

Several studies using neuroanatomic labeling methods revealed key morphogenetic aspects of DG development starting during mid-gestation. Here, multi-potential dentate precursors are born in a specialized SVZ region and become apparent adjacent to a region that has been identified as the dentate notch, localized in the dorsal region of the medial pallium, between the pallium and the fimbria (the Hem) (Fig.5, A1) (Nowakowski R.S. and Rakic P., 1979; Altman J and Bayern S.A.,1990a,b). However, relatively little is known about factors controlling the transit of precursors and neurons to the developing DG and the mechanisms regulating neural precursors and future resident stem cells migration and differentiation here.

During early prenatal stages of mouse development (E13.5-E15.5), newborn dentate granule precursors, generated from the primary matrix of the nascent hippocampus, concentrate in an adjacent structure called the dentate notch (DN). From this region dentate precursors migrate forming the so called secondary matrix (along the primary CA3 alveus region) and seeding the nascent DG, providing the first dentate scaffolding (Rakic P., 1981; Altman J and Bayern S.A.,1990a) (Fig.5, A2). At the same time, dividing precursors destined to settle into the hilus and the SGZ start to migrate from the medial cortical neuroepithelium in a subpial stream at the boundary between the fimbria and the meninges (Altman J and Bayern S.A.,1990b; Bagri A et al, 2002) (Fig.5, A3). According to that, recent data reported the presence of a transient subpial neurogenic zone (SPZ), which is formed during late embryonic stages and persists until DG development is completed (Li G and Pleasure S.J., 2005; Li G et al., 2009). The SPZ seems to be regulated by Cajal-Retzius cells and Reelin that are described to have a special role in orchestrating DG morphogenesis (Forster E et al., 2002; Sibbe M et al., 2009). Nevertheless, the whole DG scaffolding is definitely formed only around the first two postnatal week when neurogenic precursors are settled at the border between the granule cell layer (GCL) and the hilus thus generating the proper neurogenic stem cell

niche known as the SGZ (Fig.5, A4) (Li G and Pleasure SJ 2007). During further postnatal stages then, neural stem cells in the SGZ continue to generate granule neurons that expand both the suprapyramidal and infrapyramidal blades, giving to the DG its characteristic arrowhead shape (Fig.5 B).

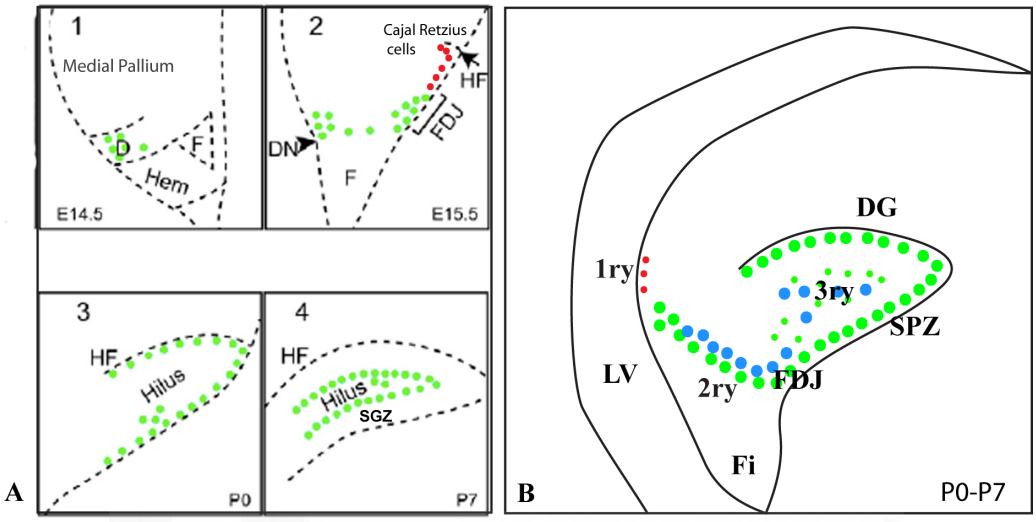


Fig.5 Schematic diagram summarizing the main steps during dentate gyrus morphogenesis.
A) Morphogenesis of DG during embryonic and postnatal stages: A1) At 14.5 the dentate neuroepithelium (D, in green) is forming adjacent to the cortical Hem in the medial pallium A2) Initial migration of dentate precursors begins around E15.5 from the dentate notch (DN) which seeds the forming fimbrio-dentate junction (FDJ) while at the hippocampal fissure (HF) initiate the DG shape controlled by Cajal Retzius cells movements (for details see text). A3) during early postnatal stages (P0) precursor cells and granule cells are mixing in the nascent DG and dentate precursors continue their migration along the subpial migratory route. A4) By P7 the cellular organization of DG layers are still ongoing with condensing movement of granule cells and radial positioning of precursors into the subgranular zone at the hilar border. Adapted from Li G et al., *Development* 2009
B) Scheme summarizing in a sagittal section the postnatal routes of dentate progenitors (in green) and granular cells (in blue) migration: 1ry=primary matrix (in red NSCs), 2ry=secondary matrix, 3ry=tertiary matrix.

The postnatal and adult SGZ niche includes quiescent multipotent stem cells (or B cells), transit amplifying precursor cells (or D cells) and immature neurons (or neuroblasts). B cells are astrocytes displaying a long radial process through the granular layer and a tangential extension lining the inner border of the granular layer, whereas D cells, produced by B cell divisions, divide and generate neuronal precursors. These precursors express Doublecortin (DCX) as well as PSA-NCAM (Seki T and Arai Y., 1993), two molecules highly associated with the migrating potential of immature neural cells. Once generated, neuronal precursors migrate short distances to penetrate into the base of granular cell layer (GCL) where they differentiate into granule neurons. All cellular transitions between these stages of neuronal maturation are carefully regulated

by signaling molecules from families of proteins that are also powerfully involved in dentate development (Pozniak C.D. and Pleasure S.J., 2006). Specific mutations of transcription factors such as Emx2 (Bishop K.M. et al., 2003), Lhx5 (Zhao C et al., 1999) and Lef1 (Galceran J et al., 2000) have shown a reduction in size of the hippocampal structure with a lack of a clearly distinguishable DG or a deficit in the expansion of early dentate precursors, elucidating fundamental processes of DG development. Moreover, further studies identified genes that are directly implicated in the control of either the migration of these cells or the proliferation of the radial glial network responsible for DG morphogenesis, as shown by the defects observed in the conditional FGFR1 mutant (Ohkubo Y et al., 2004). This mouse strain has diminished precursor proliferation in the DG and shows defects in neural migration, reflected by the failure to properly seed the dentate gyrus thus suggesting an important role of FGFR1 in DG development. Interestingly, the disruption of FGF signaling through the receptor FGFR1 was already demonstrated to be necessary for OB morphogenesis (Hébert JM et al., 2003), thus arising important questions on the implication of these molecules in mechanisms underlying the proper regulation of precursor cells as well as their maintenance and migration. Interestingly, although abundant data has been reported describing SGZ niche development in the DG, the migratory routes underlying the origin of dentate neural precursors (B cells) has not been elucidated and properly characterized yet.

2.3 Cell migration in the adult brain

In the normal adult brain only a limited amount of neuronal cell migration is observed, although its function is still debated and unclear. As described in early postnatal development, only few brain structures undergo neurogenesis in the adulthood: the striatal SVZ, lining the lateral ventricle and the DG in the hippocampus (Fig.6). Neural stem cells (NSC) in these discrete forebrain areas keep producing progenitors that give rise to both neurons and glia throughout life (Alvarez-Buylla A et al., 2001). As previously described, also during adult life these two sites of persisting neurogenesis and cell migration developed very differently from each other. For example, the SVZ largely exceeds the DG both in the number of cells generated and in

the distance travelled by cells during migration. Nevertheless in both cases, newborn neurons migrate and integrate into the pre-existing neuronal networks becoming functionally active (van Praag H et al., 2002; Carleton A. et al, 2003).

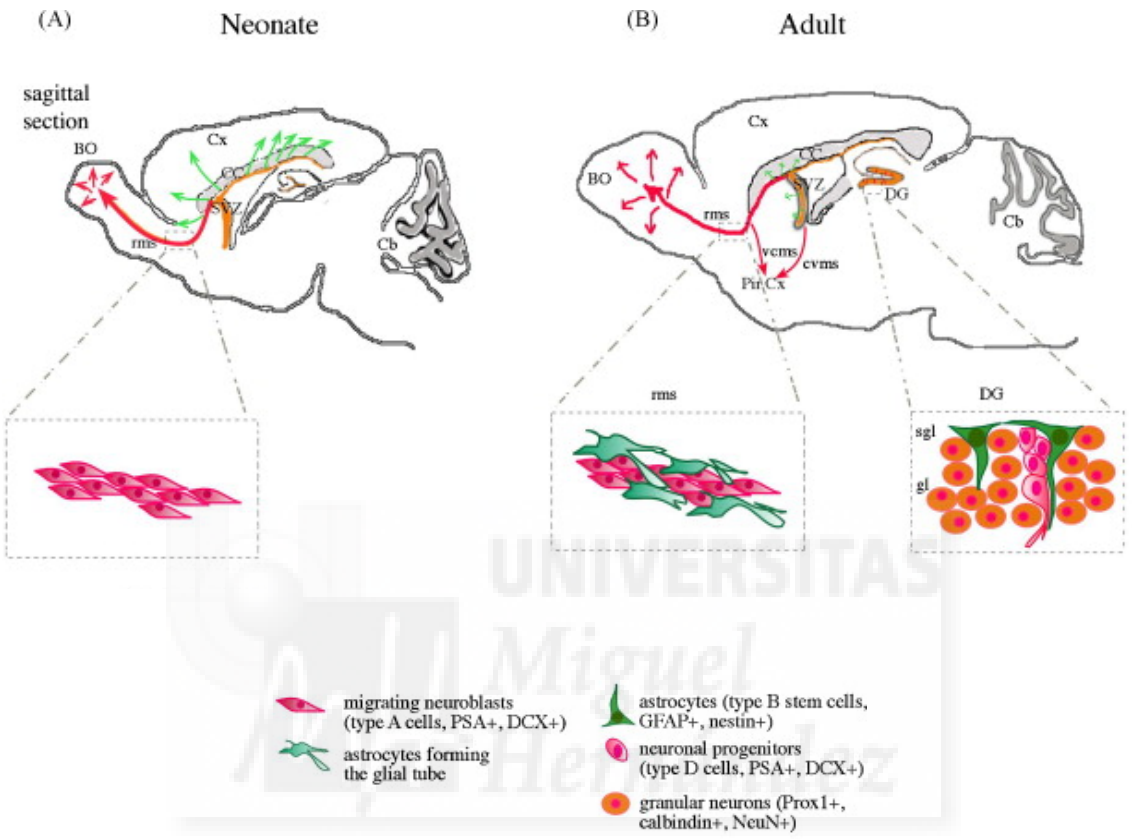


Fig.6 Schematic representation of neural cell migration in neonate and adult rodent brain
A) Sagittal section of newborn brain: neuronal progenitors (in red) travel from the aSVZ to the olfactory bulb (OB) along the rostral migratory stream (rms) and then migrate radially inside the OB. In green are represented the glial progenitor migrations toward the corpus callosum (CC) and the cortex (Cx). B) In the adult brain neuronal migration (in red) is maintained in the rms and also neuroblasts are described to migrate into other territories along white matter fiber tracts to the piriform cortex (Pir Cx) in the caudo-ventral or ventro- caudal migratory streams (cvms and vcms respectively). Glial progenitors are maintained inside the SVZ and can still migrate into the CC and striatum (in green). In the adult dentate gyrus (DG) very short migrations of both neuronal and glial progenitors perdure from the subgranular zone (SGZ) to the granular cell layer (GCL). Adapted from Cayre M et al., *Progress in Neurobiology* 2009.

Although the adult SVZ and adult DG are considered as the two specific niches where progenitors maintain their embryonic characteristics and their ability to continuously proliferate, substantial differences exist in the morphological and molecular aspects of their adult counterparts (Peretto P et al., 2005). For instance, the prenatal and early postnatal SVZ contains an open olfactory ventricle, which in rodents

closes only after birth giving rise to the RMS, the specialized migratory pathway of OB interneurons. Then, in adult SVZ and SGZ, radial glial cells identified as embryonic NSC, gradually disappear and are replaced by astrocytes in the adult SVZ with stem cell-like properties (Doetsch et al, 1999). Several studies indicate a direct link between radial glial cells and the adult SVZ-SGZ primary progenitors (Alvarez-Buylla 2001). In fact, in addition to their role as simply scaffold of neuronal activity, recent findings have suggested that radial glia cells behave as NSCs in the adult SVZ and DG (Doetsch et al 1999; Laywell E.D. et al, 2000; Garcia A et al., 2004). Several labeling methods using retroviruses helped to elucidate that NSCs have retained key astroglial characteristic and share common properties with the embryonic radial glial counterparts (Merkle F.T et al., 2004; Kriegstein and Alvarez-Buylla 2009). Moreover, depending on their location and developmental stage radial glia cells are able to generate both neurons and glia (Alvarez-Buylla et al., 2001).

As for embryonic and postnatal SVZ, neuronal progenitors (specifically expressing β-IIItubulin, DCX, mCD24 and PSA-NCAM) migrate along the RMS again following a peculiar chain migration in order to reach and populate the adult OB (Fig.6). As already mentioned, this migration continues to occur during all adult life and OB interneurons are continually replaced by newly generated neurons (Doetsch and Alvarez-Buylla, 1996). Migrating neuronal progenitors were already described to migrate without the aid of any radial glial substrate whereas ensheathed within the RMS by a meshwork of specialized astrocytes. In more details, those cells are able to organize the migratory stream forming glial tubes containing chains of neuroblasts or immature neurons migrating tangentially (Lois C and Alvarez-Buylla, 1994). For these cellular characteristics, the adult RMS is known as a homophilic migration that was described to be particularly efficient (Lois C et al, 1996) even though this cell migration is not constant, but rather irregular with periods of inactivity and exploratory behavior (Davenne M et al, 2005; Nam S.C. et al 2007). Interestingly, the presence of dorsoventral migration throughout the striatal SVZ is described as an example of the dynamicity of the caudorostral migration from the dorsal SVZ (Nam S.C. et al., 2007). However, once they arrive in the OB, migrating neural progenitors detach from the chain and migrate radially into the granule and periglomerular cell layers of the OB (Lois&Alvarez-Buylla 1994; Belluzzi et al 2003) where they differentiate into GABAergic and dopaminergic interneurons (Carleton A et al, 2003).

On the other hand in the hippocampal germinative niche, progenitors remain inside the DG in the SGL, defined as a narrow band of about 50 μm in depth between the inner border of the granular layer and the hilus. Once inside the DG germinal zone, the SGZ layer, neural precursors migrate a short distance to penetrate into the base of the granular cell layer (GCL). During this migration, migratory neuroblasts are described to first move tangentially through the SGL, leaving a trailing process in the proliferative area and then extend radially orientated dendrites and performing radial migration (Seki T et al., 2007). Once in the GCL, neuronal precursors differentiate into granule neurons, establish synaptic contacts and contribute to the functional plasticity of the hippocampus. New neurons in the adult DG have been implicated in learning and memory (Zhao C.S et al., 2008) and aberrant neurogenesis in this region has been related to several diseases (Parent J.M et al., 2006).

These continuous events of neurogenesis in the adult brain appears to be very important for the maintenance and renewal of OB interneurons as well as for the refinement of DG circuitry involved in hippocampal-dependent memory. Thus, a deeper understanding of SGZ progenitors' origin and trafficking during hippocampal formation is necessary to be addressed.

3. Postnatal alternative routes of migration

As already mentioned, classical neurodevelopmental studies led to the view that the generation of neocortical neurons occurs during embryonic development in regions considered sites of primary neurogenesis in the VZ and SVZ of the subpallial ganglionic eminences (Marin O and Rubenstein JL et al., 2003). Interestingly, further neuronal proliferative processes are described to continue postnatally in specific sites called of secondary neurogenesis restricted to the telencephalic regions of the SVZ and DG. Although the RMS and the SGZ migratory pathways were introduced so far as the main neuronal migrations occurring in the postnatal telencephalon, emergent observations of alternative routes of migration are currently being reported, opening new important questions related to brain plasticity and repair.

Different studies carried out in primates and rodents already suggested the existence of newly generated neurons in other telencephalic areas including neocortex, piriform cortex, olfactory tubercle and amygdala (Gould E et al, 1999; Bedard A et al., 2002; Bernier P.J et al., 2002) and the existence of SVZ-derived cell migrations through the subcortical parenchyma (Luzzati F et al., 2003) (Fig.6B). In fact, although the vast majority of cortical interneurons originate in the embryonic subpallium and migrate as postmitotic cells toward the cortex, postnatal sources of cortical interneurons have been reported. One of these sources has been identified in the dorsal white matter and comprises what seems to be an expanding pool of progenitor cells possibly derived from the LGE and/or CGE (Riccio et al., 2012; Wu et al., 2011). Interestingly, these interneurons appear to follow a unique specification program and differentiate later than interneurons born in the embryo. So, in addition to that, the postnatal SVZ has also been described to generate neuronal precursors able to populate different forebrain structures other than the OB, including neocortex, caudoputamen and nucleus accumbens, and to differentiate into distinct GABAergic phenotype (Inta et al., 2008).

Intriguingly, at different postnatal stages, diverse alternative neuronal routes of migration originated from the SVZ were described. For example, the secondary ventrocaudal migratory stream, which originates at the elbow between the vertical and the horizontal limbs of the RMS and crosses the nucleus accumbens, was detected during first postnatal week and gives rise to the subcortical neuronal structures named the Islands of Calleja (De Marchis S et al., 2004a). In particular, in this study three different pathways were identified based on their location: the dorsal migratory pathway (DMP) extended above the hippocampus and directed to the occipital cortex, the ventral migratory pathway (VMP) containing many labeled cells dispersing from the SVZ to the striatum and nucleus accumbens and finally an external migratory pathway (EMP) emerging from the anterior SVZ and extending along the external capsule toward latero-dorsal brain regions (Inta D et al., 2008) (Fig.6B). Moreover, experiments using BrdU labeling and DiI tracing have suggested the presence of newly generated neurons inside the adult piriform cortex derived from SVZ precursors that have migrated through the caudoventral migratory stream (Shapiro L.A et al., 2007). These data are consistent with emerging observations of extensive dorsoventral migration in ventral regions of the lateral ventricle (Nam et al., 2007). While previous studies have shown that migration from the dSVZ occurs mainly towards rostral direction, two-photon microscopy studies

suggest that neuroblasts are orientated dorsoventrally and ventral migration can occur throughout the entire striatal SVZ (Nam S.C et al., 2007).

Additionally, *in vitro* studies using neurosphere assays (Gritti A et al., 2002) as well as more recent *in vivo* analysis (Alonso M et al., 2008) suggest that progenitors with stem-like characteristics are not only confined to the SVZ, but they are also present all along the RMS. Furthermore, these studies elucidate different functional characteristics such as that depending on neuroblasts' position at different rostrocaudal levels of the SVZ-RMS pathway they are able to generate more oligodendrocytes or periglomerular interneurons (Hack M.A et al., 2005).

Thus, the RMS appears as a very dynamic migratory stream where neuroblasts can move bidirectionally all along the rostrocaudal extent even turning 180º as confirmed by confocal imaging (Suzuki S.O. and Goldman J.E., 2003). However, migration inside the RMS is characterized by highly directed movements mediated by the presence of chemotactic agents such as Slits and Robo which contribute to avoid cell dispersion into the adjacent structures and have an important role in neuroblasts convergence to the OB (Kaneko N et al., 2010). In the adult RMS, specialized astrocytes form with their processes an additional barrier arranging tubular structures typically known as glial tubes, ensheating migratory neuroblasts (Peretto P et al., 1997). Interestingly, these glial tubes do not appear to be present in newborn brain whereas are formed only 2-3 weeks after birth (Kishi K et al., 1990). According to these observations, RMS neuroblast migration seems to switch to an adult less permissive mature context of migration, harboring the possibility of a more plastic RMS for neonate neuroblasts.

4. Factors and molecules involved in postnatal migration

A fundamental aspect concerning postnatal cell migration is to understand the intrinsic and extracellular cues that guide neural progenitors during their migration towards their final targets. Increasing evidences argue for the implication of developmental signals that are maintained in restricted postnatal neurogenic areas and are essentials for the proper neuroblasts migration. Interestingly, the persistence of these signals during postnatal and further adult life appears to be an important developmental strategy used by the brain for the maintenance of a high degree of plasticity, necessary for several brain activities such as migration, survival and repair.

4.1 PSA-NCAM

Polysialylated NCAM (PSA-NCAM) was almost the first molecule found to regulate chain migration in the RMS (Chazal G et al., 2000) and to be implicated in the regulation of cell-cell interaction during development (Kiss and Rougon 1997). In newborns, PSA-NCAM expression is down regulated and at this time chain migration in the RMS is inhibited, which led progenitors to migrate as single cells (Hu H. 2000). According to this, PSA-NCAM is not necessary per se for cell migration whereas allows and promote chain migration. In the adult, PSA-NCAM persists only in discrete areas, such as type A cells in the SVZ and RMS and newborn neurons inside the DG, that keep the ability to undergo structural and functional plasticity during all postnatal life.

4.2 Microtubule associated proteins: Lis1 and DCX

Two major microtubule-stabilizing proteins (MAPs) with a strong causative relation with the human lissencephaly disorder are known to have a crucial role in cortical development: doublecortin (DCX) and Lis1.

DCX protein is expressed by migrating neuroblasts in the adult brain (Brown J.P et al., 2003; Yang HK et al., 2004). Multiple evidences suggest that this MAP functions in the stabilization of the microtubule network (Francis et al., 1999). Accordingly, DCX is required for neuronal migration and its absence during development leads to severe defects such as the X-linked lissencephaly syndrome (des Portes et al 1998; Francis F et al 1999). Moreover, postnatal migrating progenitors lacking DCX are misplaced and exhibit anomalous migration with defects in polarity and directionality with frequent pauses due to defects in nuclear translocation (Belvindrah R et al, 2011). However, not all migrating progenitors in the SVZ are DCX-positive suggesting that DCX is not indispensable for rostral cell migration (Nam SC et al., 2007). It has been described that null mutations of DCX gene in mice do not affect cortical laminar organization probably due to compensatory effects (Corbo JC et al., 2002). Interestingly, DCX has been also reported to interact with another MAP, the Lis1 protein, which appears to be co-expressed and involved in the same protein complex, coordinating similar processes during neuronal migration, such as nuclear translocation and other soma contents in cell movements (Caspi et al., 2000) (Fig.7).

The Lis1 gene (the non catalytic β1 subunit of the platelet-activating factor acetylhydrolase, *Pafah1b1*) encodes for a protein involved in multiple protein-protein interactions and mutations in human Lis1 cause a severe form of lissencephaly named Miller-Dieker syndrome (Hattori et al., 1994; Reiner et al., 1993). While Lis1 homozygous null mutant mice die soon after implantation, genetic reduction of Lis1 expression causes cortical malformations, mainly due to neuronal migration defects, resembling the human lissencephaly (Hirotsune S et al., 1998). Moreover, RNA interference (RNAi) experiments revealed the implication of Lis1 in multiple steps of corticogenesis, transformation of multipolar cells into locomoting cells, nuclear translocation and proliferation of neural progenitors (Tsai JW et al., 2005). Lis1 forms a complex with cytoplasmic dynein and dynein-associated proteins (Fig.7), such as Nde1 (known as mNudE) and Ndel1 (known even as Nudel), which control the localization of

dynein and the microtubule network at the centrosome, respectively (Feng Y et al., 2000).

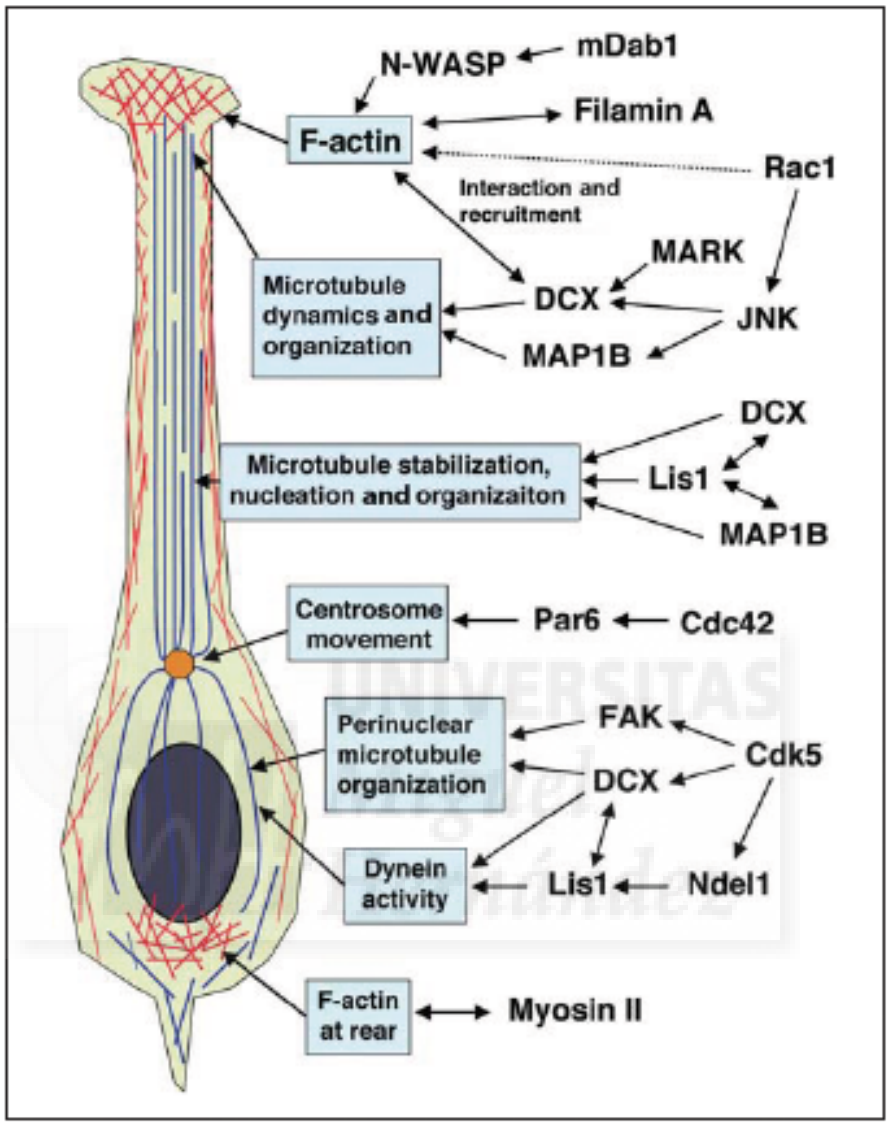


Fig.7 Summary of proteins involved in subcellular regulation of neuronal migration
Schematic representation of the intracellular microtubule machinery and the main proteins implicated in different phases of microtuble cellular dynamics. In the boxes are summarized the diverse cytoscheletal compartments in which each protein complex is involved .
From *Kawauchi T et al., 2007.*

Because the centrosome plays a prominent role in cell division, it is possible that Lis1-mNudE-dynein interactions also contribute to mitosis and cell-cycle progression (Faulkner N.E. et al., 2000; Liu G et al., 2000). Neuronal migration is delayed in the cortex of transgenic mice with only one active *Lis1* allele and in haploinsufficient transgenic mice with further genetic reduction of the wild-type protein, which displayed

severe cortical organization (Hirotsune S et al., 1998). However, the strongest phenotype is described in the hippocampus (Fleck et al., 2000). Different studies showed that heterozygous deletion of *Lis1* gene led to aberrant disposition of immature granule cells with an abnormal cellular morphology (Wang Y and Baraban SC 2008).

These roles of Lis1 and DCX proteins in the developing cortex illustrate the prominent influence that microtubule dynamics have on radial and tangential migration during CNS development (Fig.7) (Kawauchi T et al., 2007).

4.3 Extracellular matrix molecules and interactions

In addition, migration in the relatively non-permissive postnatal brain requires also modifications of the microenvironment surrounding the migrating cell and the cooperative action of attachment, repellant and guidance signals together with proteins conferring to the cell the intrinsic capacity to migrate. For that, numerous extracellular matrix (ECM) molecules such as tenascin-C, chondroitin sulfate proteoglycans and laminin are present in the RMS as well as metalloproteases (MMPs), which are important components for individual neuroblast migration found all along the whole SVZ-OB pathway (Bovetti S et al., 2007a). Moreover, the diverse extracellular environment promoting neuroblast migration includes integrins, an important family of heterodimeric cell surface proteins able to mediate migration not only recognizing various other structural proteins but also acting directly as receptors for MMPs (Belvindrah R et al., 2007).

Many cell surface molecules such as Ephrins tyrosine kynase receptors, EGF receptor and ErbB4 receptors and several secreted substances regulate the activity of cell-surface receptors that control cell-to-cell and cell-environment interactions thus contributing to the adhesion during chain migration processes (Anton E.S et al., 2004; Aguirre A et al., 2005). Concentration gradients of chemoattractant and chemorepulsive molecules also actively participate in the directionality of the orientated rostral migration in neuronal progenitors in the postnatal RMS. Repellant signals from Slits (Slit1/2) and Robo (Robo2/2) receptors appear to be necessary for the maintenance of stem cells inside the adult SVZ and neuroblasts migration inside the RMS (Wu W et al., 1999). Interestingly, in contrast with initial data in the adult reporting that the OB is not essential for proliferation and directional migration of SVZ progenitors (Kirschenbaum

B et al., 1999), different attractant molecules such as chemokines, Netrin or Neuregulin-1 need the presence of the OB structure for the correct migration inside the RMS (Murase S and Horwitz AF, 2002). Nevertheless, Netrin expression tends to disappear early after birth thus other signals must be in charge of the regulation of migration in the adult.

Recently, many studies revealed that even trophic factors have a motogenic role and can exert a chemotactic action promoting neuroblasts migration as for example GDNF and BDNF (Paratcha G et al., 2006; Chiaramello S et al., 2007). In addition, morphogenetic factors such as FGFs were found to have a prominent role in neuronal migration and the blocking of the FGF signaling leads to defects of migration (Hasegawa H et al., 2004). In particular, FGF8 was found to have a strong attractive role in the tangential migration of a specific pallial cell population thus suggesting a key role as long distance chemo-attractant molecule (Pombero A et al., 2011).

Altogether, the analysis of progenitor cell migration in the postnatal brain reveals that most mechanisms involved in migration (repulsive, attractant, permissive or motogenic factors) are shared during both developmental and adult stages, emphasizing the concept that proper migration relies on the integration of all the environmental cues signaling in a cooperative manner.

5. Implications of migration in brain repair and hippocampal dependent memory

Recent findings of neurogenesis persistence together with NCS migratory potential maintenance inside the adult brain arise several questions about their possible implications as potential therapeutic strategies.

A number of trophic factors and cytokines with known implications in neurogenesis and NSC migration have been tested in pathological animal models reporting promising results. The first evidence that endogenous progenitors could respond to extrinsic factors or promote directed migration and neuronal replacement in a lesioned brain was given by Fallon et al., (2000). These results showed that TGF alpha infusion into the putamen attracted neuronal progenitors toward the lesion with

improved functional deficits. Moreover, the infusion of FGF and EGF in the same animal model also proved increasing direct neuroblast migration towards the lesioned striatum (Winner B et al. 2008) and the infusion of the same trophic factors into the ventricle enhanced neuron replacement and hippocampal recovery (Nakatomi H et al.2002). Further studies showed that EGF signaling plays a critical role in enhancing SVZ cell mobilization as shown by the focal demyelination model with intranasal EGF subministration (Cantarella C et al., 2008) or by genetic approaches (Aguirre A et al., 2007) that contribute to elucidate its role in proliferation, migration and survival of both SVZ and white-matter endogenous progenitors. Inside healthy brains, intraventricular administration of BDNF triggers increased olfactory neurogenesis and also induces migration of newborn neurons from the SVZ to non-neurogenic regions such as the neostriatum (Benraiss A et al., 2001).

All these experimental procedures point out the need to associate different strategies targeting different steps of the migration process: proliferation, inhibition of glial differentiation, neuronal differentiation and survival. According to that, the right mobilization and migration of neural cells is essential in order to reach the correct final destination for a proper integration into the preexisting neuronal network (Kempermann G et al., 2000).

Another important aspect related to the importance of maintenance of adult neurogenesis is related to brain plasticity. A broad number of studies suggest that environmental enrichment and exercise enhance brain plasticity and, together with running, they increase the levels of growth factors such as BDNF, NGF and FGF (Carro et al., 2001; Ding et al., 2004; Ickes et al., 2000) stimulating DG neurogenesis (Kempermann and Gage 1999; van Praag et al., 1999). Recent studies showed that such enrichment could be used to promote SVZ cell mobilization and directed migration of progenitors toward brain lesions. In fact, it was observed that environmental enrichment coupled with voluntary exercise stimulates SVZ reactivity, increased the number of SVZ-derived cells within the lesions and accelerated motor recovery (Magalon K et al., 2007). Recruitment of endogenous neural progenitors for their mobilization and direct migration towards different brain areas represents an important strategy to be solved especially for its implications in brain repair.

All these arguments demonstrate that a vigorous effort has been dedicated for better understanding neural stem cell biology, to decipher why stem cells reside in specific niches (Ninkovic and Götz, 2007) and to determine which signaling cascades

control their proliferation, migration and differentiation. However, their potential use in therapies, together with enrichment strategies and exercise as complementary approaches need to be further explored as they might clarify the behavior of neural progenitors outside the neurogenic niches as well as their survival into a pathologic brain.

OBJECTIVES

The main objective of this work is to study the postnatal cellular contributions and the principal migratory routes present during postnatal hippocampal development. In particular, our study is focused on the analysis of one of these contributions coming from the cSVZ, a novel source of hippocampal interneurons. Moreover, we explored the dynamic early phases of DG development in order to elucidate the origin of subgranular progenitors, specific cellular migratory routes and the implications of Lis1 protein during key steps of dentate precursors niche formation.

In particular, these objectives have been organized as the follows:

Section I:

1. **To study of the postnatal CGE (cSVZ) analyzing the distribution of different expression patterns.** The embryonic CGE was already described as a different structure of the ganglionic eminences resulting not simply as the fusion of the MGE and LGE structures as demonstrated by the distribution of different transcription factors (Nery et al., 2002). In fact, diverse transcription factors as for example COUP-TF II were found to be important for CGE specification during embryonic mouse telencephalic development. However, the maintenance of these expression patterns during postnatal life has not been elucidated yet. Thus, using both *in situ* hybridization and immunohistochemistry techniques the postnatal cSVZ will be analyzed early on during perinatal and postnatal stages of mouse brain development.

2. **To investigate the migratory potential of cSVZ cells using *ex vivo* organotypic slice culture grafts.** As classical neurobiology experiments of slice culture already showed (Anderson et al., 1997), this approach is suitable for the identification of tangentially migrating currents in the telencephalon. However, the majority of those experiments were limited to mouse or chick embryos and less adopted later on in development. Postnatal telencephalic slice preparations

grafts will be adopted to better elucidate the presence of SVZ derived migratory currents moving caudally toward the hippocampus. Donor GFP SVZ will be dissected and homotopically-isochronically transplanted into wild type slices.

3. **To study the migratory behavior of cSVZ derived cells *in vivo* and follow their postnatal migration and integration into the DG structure.** Several data of rostrally migrating cells starting from the anterior SVZ (Lois C and Alvarez-Buylla A 1994) described massive migratory events that are maintained postnatally. In addition, recent evidences elucidate the presence of alternative routes of migration opening several questions about their final destination and integration (Inta et al, 2008). However, further studies are needed to better understand these postnatal migratory contributions. Thus, *in vivo* homotopic transplantation of cSVZ derived GFP cells, *in vivo* electroporation and lentiviral infection will be used to label the cSVZ and thus characterize the caudal migratory current at different postnatal stages.

4. **To explore the molecular mechanism acting on this caudal migration and its possible implications in DG development.** In particular, different data have shown the involvement of Fgf8 in migratory processes in the central nervous system (CNS) (Sun et al., 1999; Smith et al., 2006; Pombero A. et al., 2011) and its implication both in radial and non-radial migration during diencephalic vertebrate development (Golden et al., 1997; Ortino et al.,2003). Moreover, Fgfr1 mutant mice showed a strong phenotype specifically affecting the hippocampus thus arguing for a possible involvement of Fgf signaling during hippocampal development (Smith et al., 2006). However, the role of Fgf8 in postnatal caudal migration has not yet been investigated and its role during migration still poorly understood. *In vitro* migratory assays with the Fgf8 protein

will be set up to study its attractive potential and dissect an attractive mechanism acting in cooperation with its receptor Fgfr1 on caudally migrating cells.

Section II

1. **To study the trafficking of postnatal migratory streams generated from medial pallium ventricular and subventricular zones during postnatal hippocampal development.** As recently described, DG development entail a wide multi-step process that pass through different cellular conformations and migratory pathways re-organization (Li G and Pleasure, 2005). However, the maintenance of the postnatal dentate pool and the disentangling of all the phases during which the DG structure is generated is a process still poorly understood. In order to clarify dentate precursors migratory pathways organization, we analyzed different expression patterns of the Allen Brain project database and we followed their expression during different postnatal stages.

2. **To characterize through immunohistochemistry neuronal migration of DG subgranular progenitors during key steps of postnatal DG development.** The identification of different migratory currents during key moments of DG development constitute together with the classical studies (Altman and Bayer, 1990; Li G and Pleasure, 2005) an important aspect in the understanding of SGZ niche origin. Although the immature dentate progenitors subpial route of migration was already described (Li G et al., 2007), few studies focused on the role of radial glial scaffolding in regulating DG migratory progenitor trafficking were reported. Thus, we performed the analysis of radial glial cells using different markers that will give important insights to follow dentate precursors migration.

3. **To analyze the radial glial scaffolding and the persistence of the secondary matrix during DG development of Lis1/sLis1 mutant mice.** Radial glial scaffolding is the fundamental structure used by neurons to move in radial migration and for that the proper scaffold formation is necessary for the right layer organization and proper circuitry formation (Rakic 1972). According to that, we will study using *in vivo* elecroporation how in Lis1/sLis1 mutant mice radial glial cells are affected and their implications in secondary matrix cell trafficking during DG development.

MATERIALS and METHODS

1. Animal models

All animal experiments were performed in agreement with the Spanish and European Union laws on animal care in experimentation (Council Directive 86/609/EEC) and they have been analyzed and approved by the Animal Experimentation Committee of the University Miguel Hernàndez.

1.1 Mouse strains maintenance

A control mouse line (ICR, *Mus musculus*) was used for the *in vivo* injections and electroporation experiments, while a transgenic mouse line of CD1-GFP was bred for the organotypic slice culture grafts and for *in vitro* culture studies (Hadjantonakis A.K. et al., 2002). Another transgenic mouse line Lis1/sLis (background ICR) was used for this experimental study in which an inserted point mutation with Cre-loxP strategy generate a truncated shorter protein (Cahana et al., 2001) respect to the wild type. Only heterozygouse animals were adopted for our experimental work because homozygous deletion of *Lis1* results in post-implantation lethality.

1.2 Lis1 mice genotyping

For identification of heterozygous Lis1/sLis1 animals carrying the mutant allele, we performed genomic DNA isolation from adult or pups tail specimens as follows: each tail was digested with 300µl Tris 1M solution (60.6 gr Tris base Sigma for 500ml milliQ water) at 98ºC during 1 hour with vigorous shaking. Then the digesting solution was neutralized with 30µl of 1M Tris HCl pH8 (Sigma), vortexed for 3 minutes and centrifuged at 14000 rpm during 8 minutes. The surnatant was transferred into a new tube and stored at -20ºC until used for PCR reaction.

For PCR amplification we used the following primers: oligonucleotides for allele specific genotyping for the mutant allele 5' GCT GGC AGT GTT GAG ATG CCT AGC C3' (sense) and 5' GCA TTC CTG TAA TCC AGT ACC TGG 3'(antisense). Afterwards 12 µl of a master mix solution for PCR (Ready Mix Thermo Scientific) were added in an eppendorf tube together with 1 µl of each primer (forward and reverse at 25µM) and 1 µl of DNA (100ng/µl). Amplifications were carried out as follows: 5 min at 94ºC; 35 cycles with 1 min at 94ºC, 40 sec at 60ºC and 3 min at 72ºC; 10 min at 72ºC. PCR amplification products were separated through electrophoresis on a 1% agarose gel and identified by their size (800 bp).

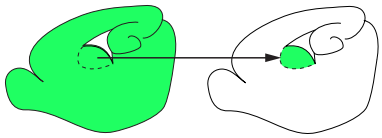
2. Experimental Embryology

2.1 Organotypic slice culture SVZ grafts

Organotypic slice cultures of postnatal mouse forebrain were prepared as described in from Anderson et al., (Anderson S.A et al, 1997) with some modifications. Briefly, postnatal day 4 (P4) pups were anesthetized in ice and subsequently sacrificed by decapitation. Brains were removed in ice-cold Krebs buffer (126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM glucose, and 25 mM $NaHC0_3$) and embedded in 4% low-melting agarose for fresh tissue inclusion. 250 μm sagittal sections were then cut on a vibratome into cold Krebs buffer, and transferred to enriched Krebs buffer (Krebs supplemented with 10 mM HEPES, Penicillin-Streptomycin, and Gentamycin) on ice. Grafts of the SVZ area were performed as described from Marin et al. (Marin O et al, 2001) using GFP donor mice to ICR recipients (Fig.8) operating on polycarbonate culture membranes (diameter, 13 mm; pore size, 8 μm; Millipore) in Falcon organ tissue culture dishes containing 1 ml of medium (Dulbecco's modified Eagle's medium DMEM- Sigma, culture medium supplemented with 2mM L-glutamine, 10% fetal bovine serum, and 100U/ml-100μg/ml Penicillin-Streptomycin antibiotics). After incubation period of about 30 minutes in DMEM medium, operated slices medium were subsequently replaced by serum free Neurobasal medium (Gibco Neurobasal medium supplemented with B-27 factor, 50% glucose Sigma, 100U/ml-100μg/ml Penicillin-Streptomycin antibiotics) and maintained in culture 48 hours under controlled conditions (sterile culture incubator 37°C and 5% CO_2). After the incubation period, slices were fixed in 4% paraformaldehyde (PFA) at 4°C over night. Pictures were taken with fluorescence dissection microscope (MZ16FA Leica).

To quantify the migration distance in slice cultures, the longest distance covered by GFP SVZ cells from the graft-host boundary was measured in each experiment trough Image J software.

cSVZ



Section I

Fig.8 Schematic representation of organotypic slice culture experiments
For the organotypic slice culture grafts the whole SVZ was dissected from 300μm GFP sagittal slices and placed maintaining its orientation homotopically and isochronically into recipient wild-type slices as represented in this scheme.

2.2 SVZ cells dissection and injection

SVZ cells were obtained by dissecting SVZ area of transgenic GFP heterozygous E14.5-E15.5 pregnant mice. Mice were sacrificed by cervical dislocation and GFP embryos were extracted and placed in a Petri dish containing sterile cold 1X PBS (137mM NaCl, 2.7mM KCl, 80.9mM Na_2HPO_4, 1.5mM KH_2PO_4 in ddH_2O). Embryonic brain dissection was performed in fresh Hank's Basic Salts enriched medium (HBSS) (HBSS 10X, Glucose 50%, Hepes 1M, Penicillin-Streptomycin) and SVZ cells taken from the two brain hemispheres were collected into tubes containing HBSS enriched medium. In order to obtain a single cell suspension, cells were dissociated using Trypsin 0.005% in EDTA and DNase at 37°C for 15 minutes pipetting gently. Cells were subsequently washed in HBSS through centrifugation steps at 1200 rpm at 4°C. The cell pellet was then suspended in DMEM medium and maintained on ice some minutes before the injection.

For the injection, newborn mice were anesthetized by hypothermia and immobilized into the platform of a stereotaxic apparatus. SVZ injections of 1.5 µl of cell suspension were injected unilaterally using a Hamilton needle stabilized to the stereotaxic apparatus (injection coordinates: antero-posterior AP1.4, medio-lateral ML0.45, dorso-ventral DV1.8). After injections, animals were returned to their mother and monitored every 10 minutes until they resumed nursing. Pups were sacrificed and transcardially perfused at different postnatal stages using PFA 4%, postfixed overnight in 4% PFA and then processed for immunohistochemical analysis.

2.3 Implantation of Fgf8- and PBS-soaked beads on telencephalic slice cultures

P4 vibratome slices were generated following the same procedure described before (Matherials and Methods 2.1) and placed in polycarbonate culture membranes (Millipore) with DMEM culture medium. Heparin beads (Sigma-Aldrich H-5263) were rinsed in PBS1x and then soaked with an Fgf8b containing solution (1 µg/µl; R&D Systems) for 1 hour at 4ºC. Fgf8-soaked beads were implanted in the hippocampus. PBS beads were prepared and implanted as well to generate control conditions.
Slices were maintained in culture with supplemented Neurobasal medium (Gibco) under controlled culture conditions (sterile culture incubator 37°C and 5% CO_2).

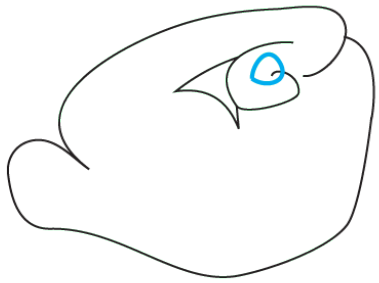


Fig.9 Schematic representation of beads insertion

FGF8 soaked beads or PBS soaked beads were implanted in organotypic vibratome slices as showed in this scheme using a tungsten needle.

3. *In vivo* labeling manipulations

3.1 Postnatal GFP electroporation

In vivo electroporation was performed in newborn mice (P0) as described from Boutin and colleagues (Boutin C et al., 2008): 2µl of pCX-GFP plasmid solution at the concentration of 5 µg/µl (in PBS1X containing 1% FastGreen) was injected into pups unilaterally inside the lateral ventricle using an Hamilton siringe and following as references lambda-eye axis (Fig.8). Electroporation was performed using CUY21 edit device and 10 mm tweezers electrodes (CUY650P10) (Nepagene, Chiba, Japan) oriented with the positive pole in contact with the injected ventricle (Fig.10). Successfully injected animals were subjected to five electrical pulses (95mV, 50 ms, separated by 950 ms intervals) and electroporated animals were reanimated for several minutes on a 37°C heating plate before being returned to the mother. Finally, electroporated animals were sacrificed at different stages and perfused with 4% PFA before of operating with any immunohistochemical analysis.

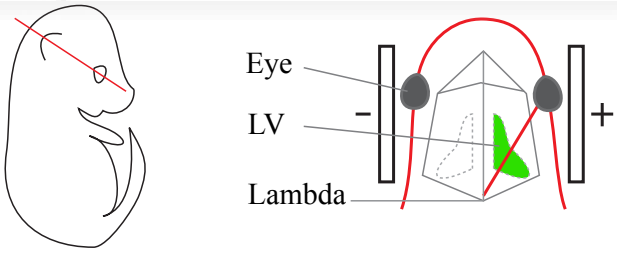


Fig.10 Schematic illustration of the GFP *in vivo* electroporation injection site

In the scheme is illustrated in sagittal view the reference line used for the injection and on the right a top view of the animal head in which the same reference orientated with lambda is used to inject directly in the lateral ventricle.

3.2 Postnatal lentiviral injection

Lentiviral vectors

Lentiviral vectors were generated in the lab of Pierre Marie Lledo (Laboratoire du Perception et Mémoire, Institute Pasteur Paris) as follow: a custom-built lentivirus containing the GFP gene under the control of the PGK promoter was used to transduce newborn neurons as previously described (Grubb MS, et al 2008). Viruses (2.2 × 1010 transducing units (TU)/ml) were stored at -80°C. Immediately before injection, the lentiviral vectors were diluted in phosphate-buffered saline (PBS) to a final concentration of 15 ng of p24 protein per microliter.

Virus injection

ICR pups were deeply anesthetized in ice and immobilized in a stereotaxical apparatus and bilaterally injected using Bregma as reference (AP +1.4 mm; ML ±0.45 mm; DV −2.5 mm from the pial surface). Viral vector particles (150 nl) were injected using nanoliter injector Nanoject II (Drummond Scientific) with a pulled glass micropipette at a rate of 23 nl/s. Animals were sacrificed at the indicated survival times after virus injection (in which dpi is days post-injection): 7dpi, 14 dpi, 30 dpi.

4. Tissue culture

4.1 Neurosphere assay

Neural stem cells (NSCs) were isolated from the dorsal forebrain of E16 GFP mouse embryos. Cells were cultured in serum-free Neurobasal medium (Gibco) with B27 supplement (Gibco), EGF (Sigma; 20 ng/ml), bFGF (Sigma; 10 ng/ml), mitogens and heparin (Sigma; 0.7 U/ml) in non-coated and non-treated petri dishes. Cells were passaged every 4 days by complete cluster disintegration into a single cell culture. For differentiation assays, we used whole neurospheres after the third passage on pre-coated culture plastic dishes treated with laminin (Sigma; 0.5 mg/ml) for 5 hours. Cells were cultured for periods of 48 hours. Differentiation medium consisted of Neurobasal medium (Gibco) with 10% FBS (Sigma), without EGF, bFGF and heparin.

4.2 Cell culture

Control HEK293T cells disposable in the lab were used in this Matrigel assay and were maintained in culture in 10% fetal bovine serum (FBS)-supplemented DMEM (Sigma), 100 units/ml penicillin-streptomycin (Sigma) and 2 mM L-glutamine (Sigma).

4.2.1 Mammalian expression vectors

pCMV-eGFP-IRES1hyg was generated by subcloning eGFP cDNA as a *Bam*HI-*Not*I fragment from pEGFP-N1 (Clontech, Cambridge, UK) into *Bam*HI and *Not*I digested pIRES1hyg (Clontech, Cambridge, UK).

The mouse Fgf8b coding region without stop codon was subcloned into the *Eco*RI site of a pEGFP-N1 vector to obtain pCMV-Fgf8b-eGFP-an.

4.2.2 Fgf8-expressing cells generation

One day before transfection (with Lipofectamine 2000, Invitrogen), the cells were seeded at a density of 0.5×10^5 cells/cm^2 in multi-well (12- or 24-well) plates. Cells were incubated with DNA-lipid complexes for 4 hours (following the supplier's instructions), after which the lipofection mix was removed and replaced with fresh medium. Drug selection of stable transfectants was performed with 50-100 μg/ml hygromycin B (hyg; Calbiochem) and G418 disulfate salt solution (Sigma).

4.3 Co-cultures in Matrigel

Co-cultures of SVZ or RMS tissue with transfected HEK293T cells were performed as described by Marin et al. (Marin et al., 2001). P4 mice were processed as described previously in order to obtain vital vibratome brain slices. Small pieces of SVZ or RMS were dissected and placed on the top of pipetted Matrigel drops (BD Biosciences, Franklin Lakes, NJ, USA) together with transfected HEK (Fig.9). Onced positioned, the small pieces of tissue were covered with Matrigel diluted 1:1 with Neurobasal (Gibco Life Technologies). Co-cultures of SVZ or RMS were separated by 400 μm and incubated under controlled conditions (sterile culture incubator 37°C and 5% CO_2) with Neurobasal medium for 24 to 48 hours.

To quantify cell migration in Matrigel cultures, the SVZ tissue was subdivided into four sectors and then migrating cells were counted inside the proximal (P) sector, as well as from the distal (D) sector on the opposite side. A P/D ratio was obtained and a statistical

analysis was performed by Student's *t*-test using SigmaPlot software.

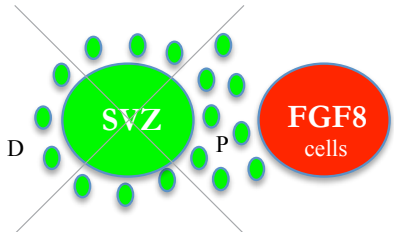


Fig.11 Scheme showing Matrigel assay

Dissected SVZ explantes from GFP mice were exposed as showed in the scheme with transfected HEK cells in a Matrigel matrix. Different control conditions were prepared in order to see the different migratory potential of SVZ vs RMS explants exposed to FGF protein. Perpendicular lines were drawn for the quantification and the statistical analysis.

5. Histological Analysis

5.1 Immunohistochemistry on vibratome slices

Floating sections (80μm) were obtained using a vibratome (Leica) apparatus and processed for the immunostaining. Sections were rinsed in PBS1X with TritonX-100 0,1% (PBT0,1%) and blocked in 10% Goat Serum (GS) diluted in PBT0,1% and incubated over night at 4°C with different primary antibodies (Table.1).

After washings in PBT0,1%, slices were then incubated with the appropriate fluorescent labeled secondary antibody (Table). Before mounting with Mowiol-NPG solution, cell nuclei staining was performed with 4',6-diamidino-2-phenylindole (DAPI, Life Technology) to 1:1000 dilution in PBS1X. All immunofluorescence pictures were taken with an inverted confocal microscope (Laser Scanning Spectral Microscope Leica TCS SP2 AOBS, Leica Microsystems Heidelberg GmbH).

Primary Antibody	Dilution	Company
anti human CoupTFII mouse IgG	1:500	PPMX Perseus Proteomics
anti GFP chicken IgY	1:1000	Aves
anti Calretinin rabbit IgG	1:1000	Swant
anti Calbindin (D28K) rabb IgG	1:2000	Swant
anti Paravalbumin Rabbit IgG	1:1000	Swant
anti GFAP rabbit IgG	1:300	Sigma

anti Doublecortin rabbit IgG	1:500	Abcam
anti BrdU mouse monoclonal IgG	1:300	Dako
anti Tuj1 mouse monoclonal IgG	1:1000	Covance
anti Gad67 mouse monoclonal IgG	1:500	Chemicon
anti GABA rabbit polyclonal IgG	1:250	Sigma
anti-Tbr2 rabbit polyclonal IgG	1:500	Abcam
anti-Nestin mouse monoclonal	1:300	Chemicon
anti PSA-NCAM (clon2B) mouse IgM	1:250	Chemicon
Secondary antibody	**Dilution**	**Company**
Donkey-anti-mouse Rodamine Red	1:500	Jackson Immuno Research
Goat-anti-mouse Cy5	1:500	Molecular Probes
Goat-anti-mouse Alexa 488	1:1000	Molecular Probes
Goat-anti-mouse-biotinilated	1:200	Vector
anti-rabbit Alexa 594	1:1000	Molecular Probes
anti-rabbit Alexa 488	1:1000	Molecular Probes
Goat-anti-rabbit-biotinilated	1:200	Vector
Goat-anti-rabbit Cy5	1:1000	Molecular Probes
Goat-anti-chicken Alexa 488	1:1000	Molecular Probes

Table.1 List of primary and secondary antibodies used during the histological analysis of vibratome sections

5.2 *In situ* Hybridization on vibratome slices

5.2.1 Antisense mRNA probe preparation

Circular DNA plasmids kindly provided by several laboratories (Table.2) were used for RNA probe preparation. In order to obtain a high amount of plasmid, competent E.Coli DH5α cells were defrosted 20-30 minutes on ice and transformed by heat-shock at 42ºC for 1 minute in contact with 100-300ng of plasmid. After 2 minutes of ice transformed cells were added to an eppendorf containing 1 ml of Luria-Bertani (LB)-Agar and let it grow for at least 1h a 37ºC at 200rpm shaking. After growing incubation, they were were plated into an LB-Agar petri dish with Ampicillin (50mg/ml Sigma-Aldrich) for clone selection and incubated overnight at 37ºC.

Thereafter a single colony was selected and grown into 100ml of LB medium with Ampicillin at 37ºC for colony amplification. Plasmid extraction and purification was

performed using Genopure plasmid midi Kit (Roche) and then stored at -20ºC in a concentration of 2mg/ml.

Once purified plasmids were linearized using specific restriction enzymes (Table.2) and for that 10µg of DNA was mixed with 5µl of linearization buffer (Fermentas), 2µl of enzyme (Fermentas) and H_2O Sigma up to 50µl and was incubated at 37ºC for 2 hours.

For in vitro transcription, 1µg of linear DNA plasmid previously obtained was incubated at 37ºC for 2 hours together with 2µl of transcription buffer (Roche), 2µl of a mixture of labeled dNTPs (Roche), 2µl of RNase inhibitor (Takara), 2µl of RNA-Polymerase enzyme (Roche) and H_2O Sigma up to 20µl. The labeled dNTPs mix was composed of 10mM ATP, 10mM CTP, 10mM GTP, 6.5mM UTP and 3.5mM of digoxigenin- of biotin-labeled UTP in RNase-free H_2O (Sigma). Thereafter 1µl of DNase enzyme (Roche) was added to the synthetized probes and incubated for 15 minutes at 37ºC together with 1µl of RNase inhibitor for DNA elimination. Probes were further purified using RNAeasy Mini Protocol for RNA Cleanup (Quiagen) and finally probes were stored at -20ºC ready to use.

Probe	Antisense	Lab	Size
mDlx-1	HindIII/T3	J Rubenstein	1180bp
mDlx-2	HindIII/ T3	J Guimera	1752bp
mFgfr1	XbaI/T3	J Partanen	500bp
mMash1	XbaI/T7	F Guillemot	1400bp
mPax6	EcoRI/T3	C Walther	260bp

Table.2 In situ hybridization probe conditions and characteristics

5.2.2 Tissue preparation

In situ hybridization was carried out according to Shimamura et al, 1995 preparing all solutions with autoclaved H2O distilled water treated to inactivate all Rnases. Briefly, 50 µm vibratome slices of post-fixed brain tissue were mounted on superfrost slices and let it dry on air overnight at room temperature (RT). Mounted slides once dehydrated in an ascending scale of Methanol solutions (25%-50%-75%-100% Methanol) were bleached with a 5:1 solution of Methanol and 30% hydrogen peroxide for 1 hour at room temperature (RT) and then rehydrated through a descending series of Methanols (100%-75%-50%-25%). Then samples were treated with 10µg/ml of proteinase K for 5 minutes at RT.

After treatment with 4%PFA and 0,25% gluteraldehyde (Sigma-Aldrich) for 20 minutes at RT, slides were washed several times in PBT0.1% and subsequently incubated with pre-hybridization buffer (Deionized Formamide 50%, 5X SSC buffer pH=7, Heparin 50µg/ml, Tween20 0.1%, tRNA 50µg/ml, salmon sperm DNA 50µg/ml, completed with distilled DEPC treated milliQ water) at 65ºC for 3 hours. After that slides were incubated with different probes (Table.2) at a final concentration between 100-300ng/ml which were previously denaturalized at 80ºC before the overnight hybridization at 65ºC. Further, after the hybridization overnight several washing steps were performed with different washing buffers (Solution 1:Formamide 50%, 4X SSC pH=5, SDS 20%, Tween20 1%, completed with autoclaved water. Solution 2:Formamide 50%, 2X SSC pH=4.5, Tween20 1% and autoclaved water) of 45' each at 65ºC. Then slides were washed with MABT1X 0.1% solution pH= 7.5 (MAB 5x: 21.75gr NaCl, 29gr $C_4H_4O_4$, 19gr NaOH; Tween20 0.1%) at RT before the incubation with 10% sheep serum (SS) (Sigma Aldrich) for 2 hours and subsequently incubated overnight at 4ºC with a 1.3500 dilution of alkaline-phosphatase coupled anti-digoxigenin Fab fragment antibody (Roche Applied Science).

After washing thoroughly with MABT 0,1%, all samples were washed several time with NTMT (NaCl 0.1M, TrisHCl pH=4.5 0.2M, $MgCl_2$, Tween20 0.1% and autoclaved water) and then a NBT/BCIP (Fermentas/Roche) solution was used as a chromogenic substrate (0.45µl NBT + 3.4µl BCIP per ml of NTMT solution) to detect the digoxigenin-labeled probes.

Several washings with PBT0.1% were used to stop the chromogenic reaction and after a rinse in distilled water slides were mounted with a glycerol-gelly (BDH Microscopy).

5.2.3 Fluorescent *in situ* hybridization (FISH) on vibratome slices

For FISH technique, vibratome slices (80µm) were processed following the same protocol (Shimamura et al., 1994) with some modifications. Mounted vibratome sections on superfrost slides were treated as usual (Material and Methods.5) and hybridized with biotin synthesized RNA labeled probes (Table.2). After washings with different rinsing buffers (Solution 1 and 2 at 65ºC and then MABT1X at RT) slides were incubated with the streptavidin-Cy3 complex for biotin fluorescence revelation (1 hour each at RT separated by MABT0.1% washings for fluorocrome excess elimination). DAPI (Life Technology) staining for cell nuclei was performed at 1:1000 dilution in PBT0.1% for 10 min RT.

RESULTS

Section I:

1. The postnatal expression pattern of the caudal SVZ (cSVZ) revealed similar molecular characteristics compared with its embryonic counterpart (CGE)

Taking advantage of recent molecular and anatomical data that have revealed the genoarchitecture regulating neurogenic patterns in the neuroepithelium and the importance of cellular migrations, we focused our attention on the analysis of the postnatal SVZ area of the CGE, which we will describe as the caudal subventricular zone (cSVZ). The CGE structure was classically referred as to be the caudal fusion of the LGE and MGE nuclei, the other two subpallial derivatives. However, different studies reported that, although lacking a clear morphological demarcation, the CGE is indeed a different territory with distinct intrinsic properties and which express a combination of diverse transcription factors (Wichterle H et al., 2001; Nery S et al., 2002; Yozu M et al., 2005). Actually, the difficulty to precisely localize the CGE structure resides almost in the fact that no single gene is able to mark solely and precisely the whole CGE region, thus leaving multiple unsolved questions about its exact limits in developing and mature brain, as well as the distribution of its derivatives. Our first proposal was focused on the study of the main morphological characteristics of the cSVZ during early postnatal stages of mouse brain development.

For that, we performed *in situ* hybridization of Dlx-2 and Mash1 transcription factors in order to study their expression patterns at birth (P0) and to follow their distribution in the postnatal subpallium. Dlx-1 and Dlx-2 (Dlx-1/2) transcription factors are expressed in the subpallial derivatives and are involved in the specification of the ganglionic eminences: LGE, MGE and CGE. The expression of Dlx genes in the subpallium is implicated in the generation of interneurons and oligodendrocites for the whole prosencephalon (Anderson S.A.et al, 1997; Casarosa S et al.,1999; Marin O et al., 2001). First, we decided to follow Dlx-1/2 distribution at medial levels of sagittal sections and we found that at P0 Dlx1/2 is widely distributed in the whole anterior SVZ (aSVZ), derived by the embryonic LGE domain, as well as in the angle between aSVZ and the dorsal (callosal) neuroepithelium (Fig.12 A). Intriguingly, we could appreciate

that ventral and caudally this transcription factor is also present with a disminished distribution in the transition between LGE and MGE (Fig.12 A'-A''). Further, we checked at the same sagittal level for the expression of another proneural gene Mash-1 and we observed that this gene has a continuous and homogeneus distribution in the neuroepithelium of both the aSVZ and the cSVZ at P0, confirming that this pattern of expression is maintained in the transition LGE-CGE (Fig.12 B-B'').

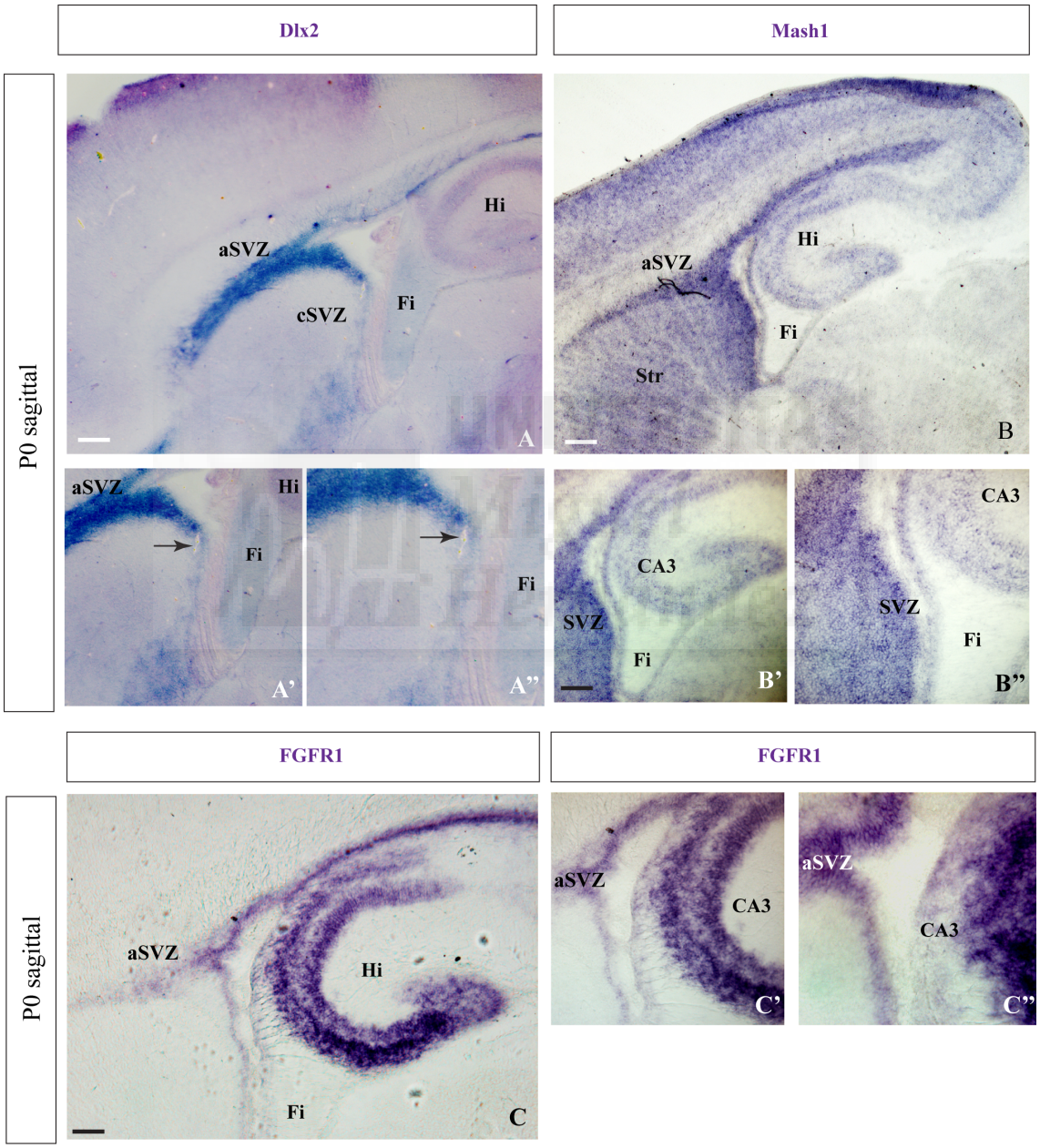
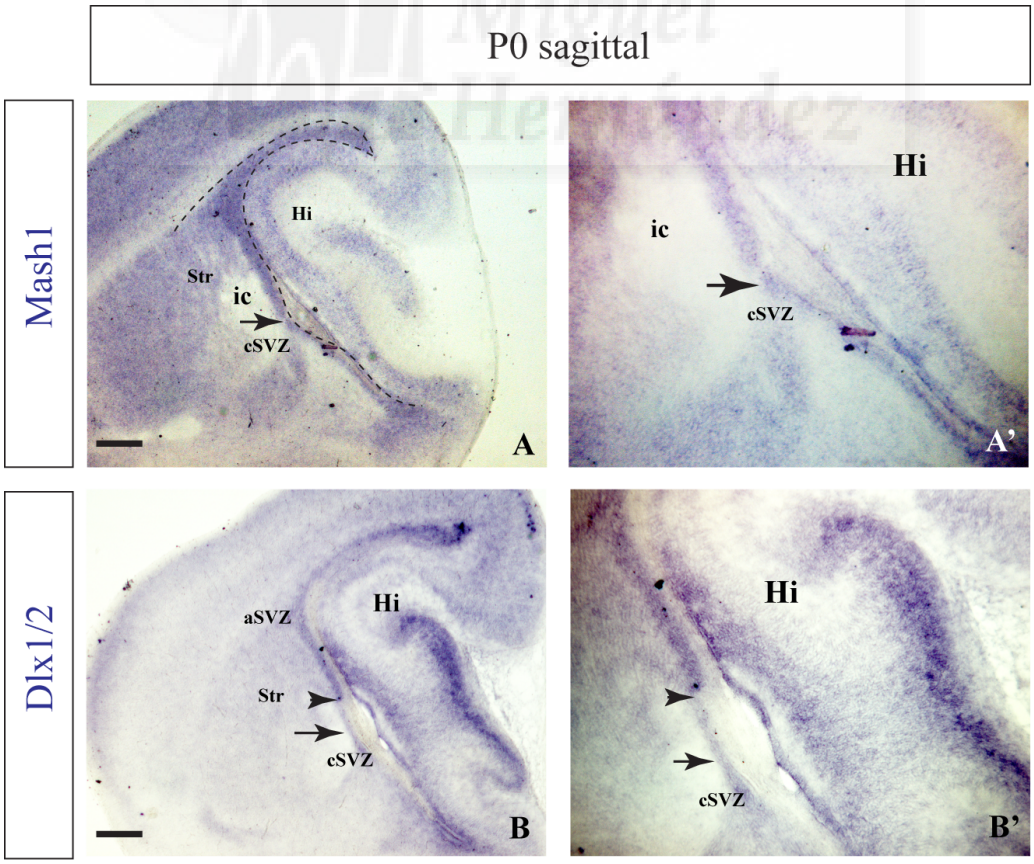


Fig.12 Distribution of Dlx2 and Mash1 proneural genes in the postnatal LGE-CGE transition (aSVZ-cSVZ)
A) In situ hibridization showing Dlx-2 expression at P0 in blue labeling the whole aSVZ and the aSVZ-cSVZ transition. A' and A'') higher magnifications (10x-20x) of labeled transition aSVZ-cSVZ. B) distribution of Mash1 expression labeling the lateral walls of lateral ventricles B' and B') higher magnifications (10x-20x) showing Mash1 expression in the aSVZ-cSVZ transition. C) FGFr1 distribution

in the aSVZ, cSVZ, the dorsal neuroepithelium and hippocampus (purple) C' and C'') higher magnifications (10x-20x) of the aSVZ-cSVZ transition positive for Fgfr1 expression. (sagittal view) Scale barr: 100μm

Moreover, we found that the fibroblast growth factor receptor 1 (FGFr1) expression pattern appeared heterogeneusly distributed in the transition between aSVZ and cSVZ ventricular epithelium (Fig.12 C-C'''). While dorsal (callosal) and aSVZ ventricular epithelium strongly express this gene, the cSVZ ventricular epithelium developed a dorso-ventral progressive decreasing of Fgfr1 expression pattern (Fig.12 C-C'').

In order to better clarify their distribution inside the postnatal CGE we followed the same gene expression patterns in more lateral sagittal sections (Fig.13). Here the aSVZ and the cSVZ appeared widely separated by a ventricular sulcus (Fig.13 A-C black arrows). Dlx-1/2, Mash1 and Fgfr1 genes were expressed in both aSVZ and cSVZ ventricular epithelium, with a rostro-caudal (dorso-ventral) decreasing expression gradient (Fig. 13 A',B',C').



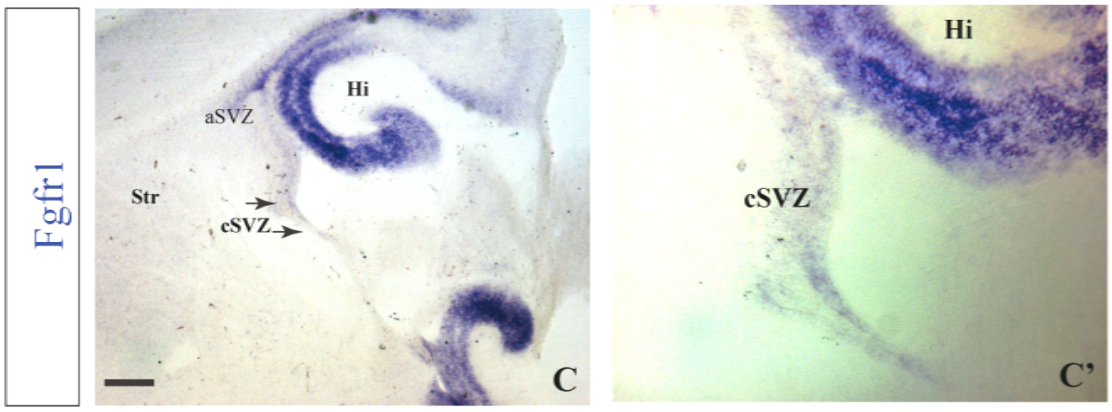


Fig.13 In situ hibridizations of Mash1, Dlx1, Fgfr1 and Pax6 during early postnatal development in the postnatal CGE. A-A') Sagittal sections showing Mash1 expression pattern (in violet) into the dorsal neuroepithelium and in the both aSVZ and cSVZ (black arrows) as well as in neocortical and hippocampal layers. B-B') Dlx-1 distribution is detectable into the neuroepithelium of the postnatal LGE (aSVZ), into the cSVZ (black arrow), and granular layers of the developing hippocampus (in violet). C) distribution of FGFr1 into the a SVZ, the dorsal neuroepithelium, the cSVZ (black Arrows) and into hippocampal granular layers. C') details in higher magnification showing the cSVZ lightly positive for Fgfr1 (violet).

All these expression patterns data suggest that the CGE maintains its specific molecular characteristics postnatally. Moreover, the presence of these proneural gene expression patterns suggests that, such have been demonstrated for the aSVZ, the cSVZ may represent a continuous source of interneurons during postnatal development.

In order to define more specifically the extension of the CGE, we studied the postnatal distribution of the protein Coup-TFII (Chicken ovalbumin upstream promoter transcription factor II), a transcription factor already described to be preferentially expressed in the CGE domain during mid-embryonic stages (Kanatani S et al., 2008). Although Coup-TFII was already reported labeling the embryonic CGE and CGE derived cells in the caudal migratory stream, its role and pattern of expression during perinatal and early postnatal stages is quite poorly described. With the aim to investigate better its postnatal distribution, we decided to study the presence of the Coup-TFII protein in the cSVZ cells during embryonic (E16.5-E18.8) and postnatal (P0-P4) stages using immunohistochemical techniques.

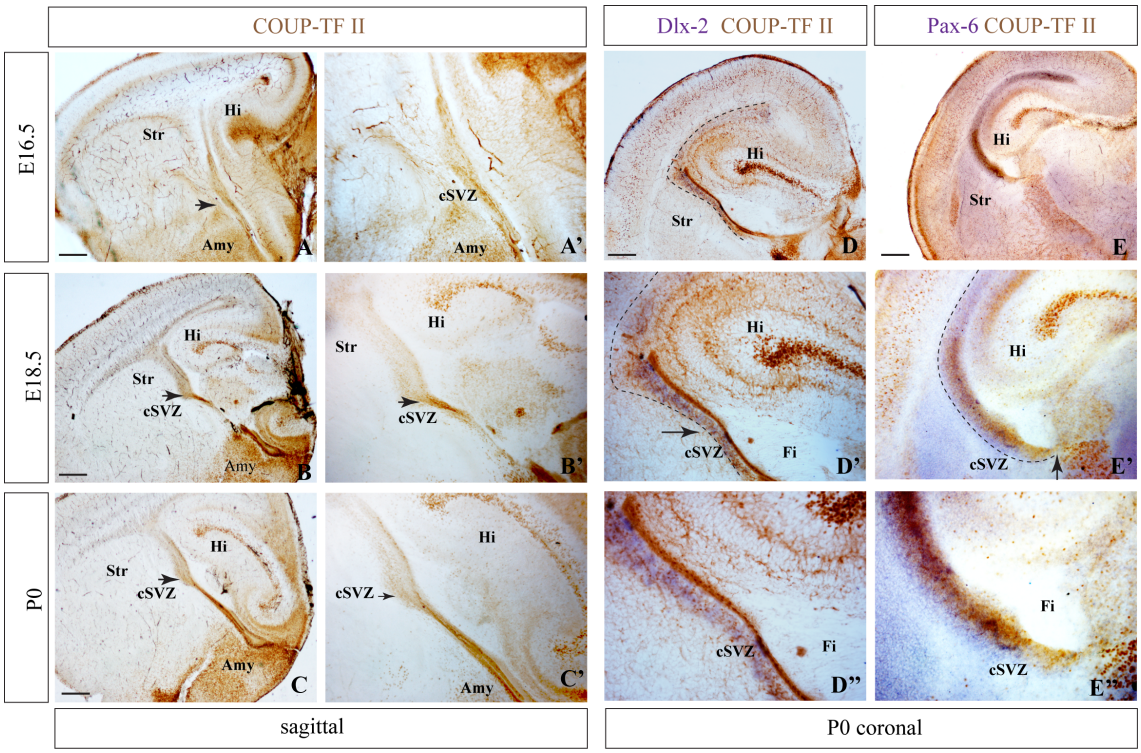


Fig.14 Immunohistochemistry showing the distribution of CoupTF-II during perinatal and early postnatal development
Immunohistochemistry with anti-COUP-TFII antibody (sagittal sections): A) E16.5, B) 18.5 and C) P0. Labeled cells are detectable in the cSVZ (black arrows), inside the hippocampus, the amygdala and in the neocortex (in B and C in DG upper blade). In A'-C' are respresented in higher magnification th cSVZ cells positive for Coup-TFII antibody.
Scale barr:100µm Hi=hippocampus, Fi= fimbria, Amy= amygdala, Str=striatum

During embryonic stages (E16.5 and E18.5), Coup-TFII is present in the whole CGE (Fig.14 A and B) and in particular we could detect this protein also in the cSVZ region (Fig.12 A-B' indicated by arrows) as well as in the amygdala, in the developing hippocampus (CA1 and DG) and in deep layers of dorsal cortex (Fig.14 A and B).

Interestingly, at P0 Coup-TF II protein distribution is maintained in the same structures, in the cSVZ of the postnatal CGE. In addition, we followed Coup-TFII protein signal in combination with both Dlx-2 and Pax6 gene expression patterns in caudal postnatal coronal sections, which let us to investigate how Coup-TFII is distributed postnatally in the LGE and MGE boundary. In fact, Dlx-2 is known to be present in all the SVZ neuroepithelium of both aSVZ and cSVZ and thus overlap with Coup-TFII antibody in the CGE domain (Fig.14 D-D'-D''). Moreover, the gene expression pattern of Pax6, an homeobox transcription factor important for telencephalic patterning and for the establishment of the boundary between LGE and

MGE, was found to be expressed in a caudal territory positive for Coup-TFII (Fig.14 E-E'-E'') suggesting that Dlx2, Pax6 and Coup-TFII are all expressed in the postnatal cSVZ consistent with reported evidences that the CGE represent the caudal fusion of both LGE and MGE territories. In addition to that, studying Pax6 expression pattern later on during postnatal development (P4) confirmed that the aSVZ and the cSVZ were both positive for Pax6 antisense probe and that these two germinative areas share similar molecular features (Fig.15 A-A''). However, a double staining with anti Coup-TFII antibody clearly identify the cSVZ as a specific caudal territory of the caudal subpallial germinative areas (Fig.15 B-B').

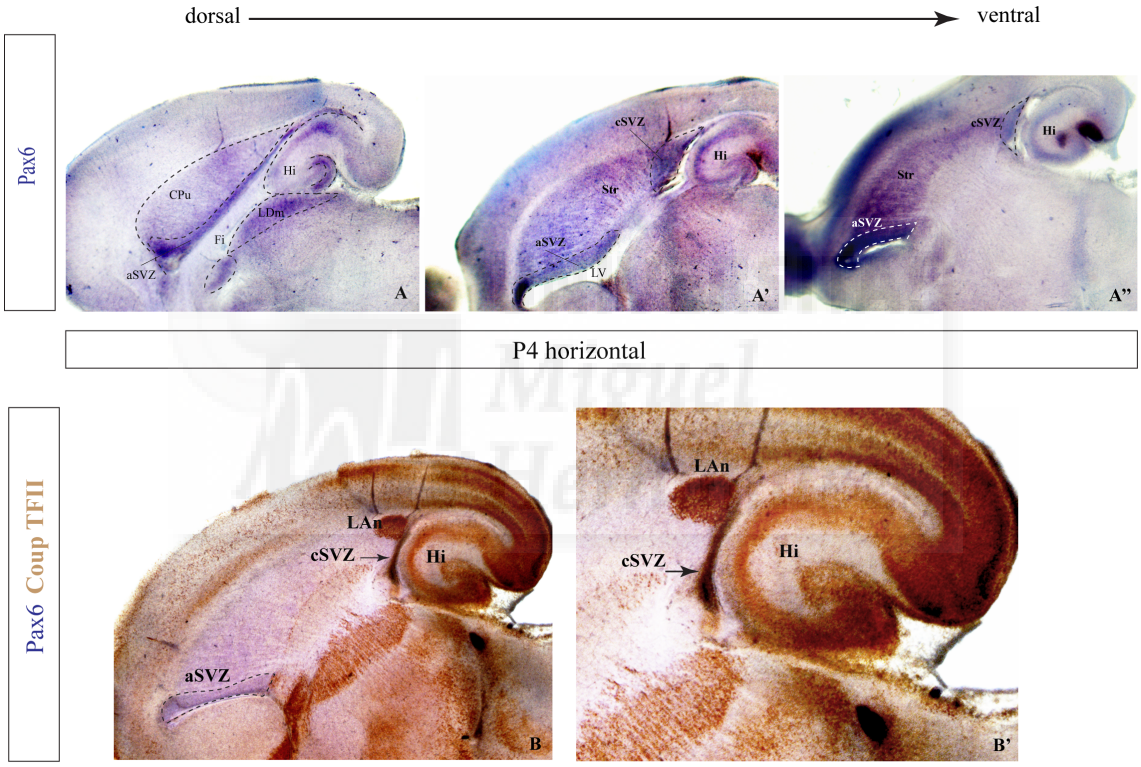


Fig.15 Pax6 and Coup-TFII distribution in horizontal P4 sections
A-A'') horizontal postnatal day 4 (P4) sections showing Pax6 distribution in the SVZ all along the lateral ventricle in its antero-posterior axis and in A'-A'' cSVZ becomes progressively more evident as long as reaching more ventral sections (A''). B-B') CoupTFII immunohistochemistry in horizontal P4 ventral section. Positive CoupTFII cells (in brown) are found in different telencephalic areas, in the layers of neocortex and hippocampus, in different amigdala nuclei and in the cSVZ of the postnatal CGE.

Altogether, these expression data confirmed that the postnatal CGE maintains a cSVZ, showing the expression of specific proneural genes and common molecular characteristics with embryonic stages. Therefore, the cSVZ could represent a germinal subventricular epithelium generating newborn neural cells at postnatal stages. These

cells could be incorporated following specific migratory routes into caudal derivatives of the telencephalic pallium (hippocampus) and/or subpallium (amygdala), as occurs with the aSVZ for anterior telencephalic structures (olfactory bulb and striatum).

2. *Ex vivo* cSVZ grafts revealed the presence of caudal tangentially migrating cells from the postnatal CGE

In order to elucidate the presence of conserved caudal migratory currents from the CGE during postnatal life, we first decided to realize *ex vivo* slice culture graft experiments of the cSVZ. In particular for the visualization of a possible caudal and dorsal pathway connecting the cSVZ with the hippocampus, GFP donor newborn brains were dissected and the SVZ was homotopically-isocronically transplanted into wild type recipient telencephalic slices (Fig.16). After 48 hours of incubation under controlled conditions, we could observe few GFP labeled cells leaving the borders of the grafted tissue and some of them migrating from its most caudal angle (cSVZ) (n=4) (Fig.16 A, B). Most of the migrating GFP cells observed in caudal territories were attracted by the subcallosal subependimal zone (scSEZ) (Fig.16 B, D) and were approaching and invading the hippocampal structure near the CA3 layers. Additionally, we focused our attention on the distance covered by GFP cells from the explant borders both in rostral and in caudal directions (Fig.16 D). As expected, the rostral trajectory resulted as the most viable journey for SVZ cells with more μm-covered (number of GFP cells: 236.7±36.12) respect to the caudal side (number of GFP cells: 154.6±16.8) (Fig.16 D). Nevertheless, the observation of caudal migrating cells reaching the CA3 layers showed the presence of a conserved postnatal migration, different from the classical rostral one, which could have an important contribution during postnatal development in hippocampal morphogenesis.

Altogether these data show the presence of viable caudally GFP migrating cells derived from the subpallial cSVZ, which are able to reach the hippocampus and thus

demonstrate the persistence during postnatal stages of caudal migratory currents possibly implicated in hippocampal development.

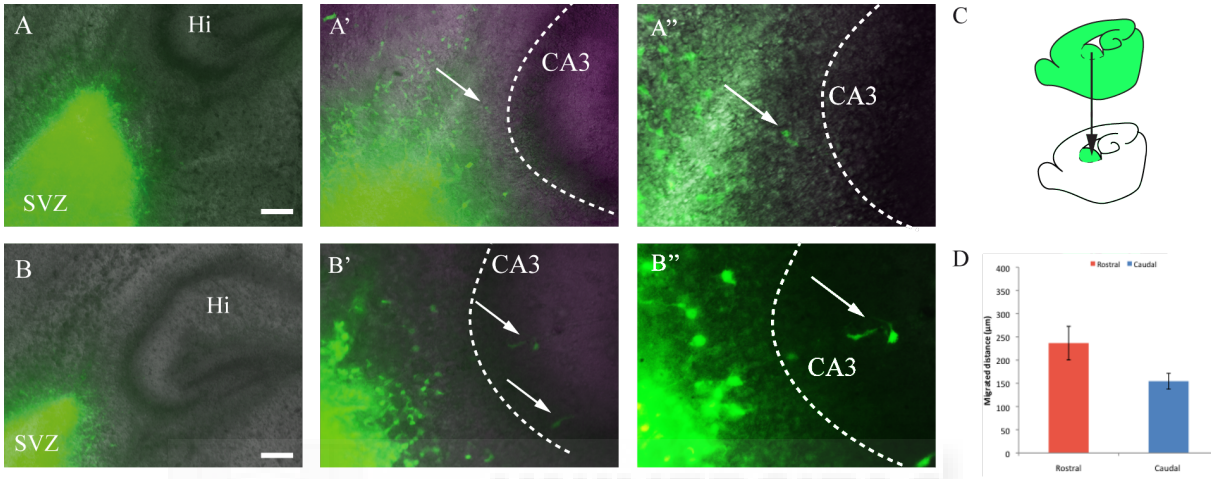


Fig.16 Organotypic slice culture grafts of GFP postnatal SVZ
A and B) GFP grafted tissue (in green) in the SVZ area of wild type slices (sagittal view). Arrows indicate migrating cells leaving the caudal angle of the transplanted tissue. A',A''-B',B'') higher magnifications showing dispersed GFP cells reaching the hippocampal CA3 layers. C) Schematic representation of operated slice culture grafts. D) Quantification of the main distance covered by GFP cells from the explant border towards both rostral and caudal directions expressed in migrated µm (average±SEM). Scale barr= 200µm CA3= Cornu Ammonis 3, Hi= hippocampus.

3. *In vivo* cell transplantation of GFP SVZ-derived cells confirmed their caudal migratory potential towards the hippocampus

In order to confirm the persistence during development of the caudal migratory stream, we decided to follow the behavior of SVZ-derived cells *in vivo* by injecting a single cell suspension of dissected GFP SVZ neural progenitors into the lateral ventricle of recipient P0 wild type mice. These SVZ cells were obtained from the embryonic SVZ of GFP transgenic mice and were transplanted into recipient wild type newborns. After the transplantation procedure pups were analyzed at different postnatal stages and thus we could observe the perfect integration of GFP cells inside the telencephalic ventricular epithelium after anti-GFP immunostaining (Fig.17 A and B). As showed in these pictures, SVZ cells were mainly grafted in the caudal angle of the lateral ventricle.

Then the caudal migratory stream was analyzed after 7 and 14 days post injection (dpi) using different immunological markers in order to elucidate cellular movements and their lineage characteristics.

As showed in Fig.17 (A-B) we found that at 7 dpi the majority of transplanted GFP cells were still confined inside the graft derived cell mass (gcm) and that few of them were migrating outside in the surrounding brain areas (n=3, 9± 3,1 total number of GFP cells). However, by this time no massive migratory currents or streams of GFP cells were observed approaching the hippocampus, conversely scattered cells leave the graft and travel caudally to reach the hippocampal structure in analyzed post grafted stages (Fig.16 A, C and E).

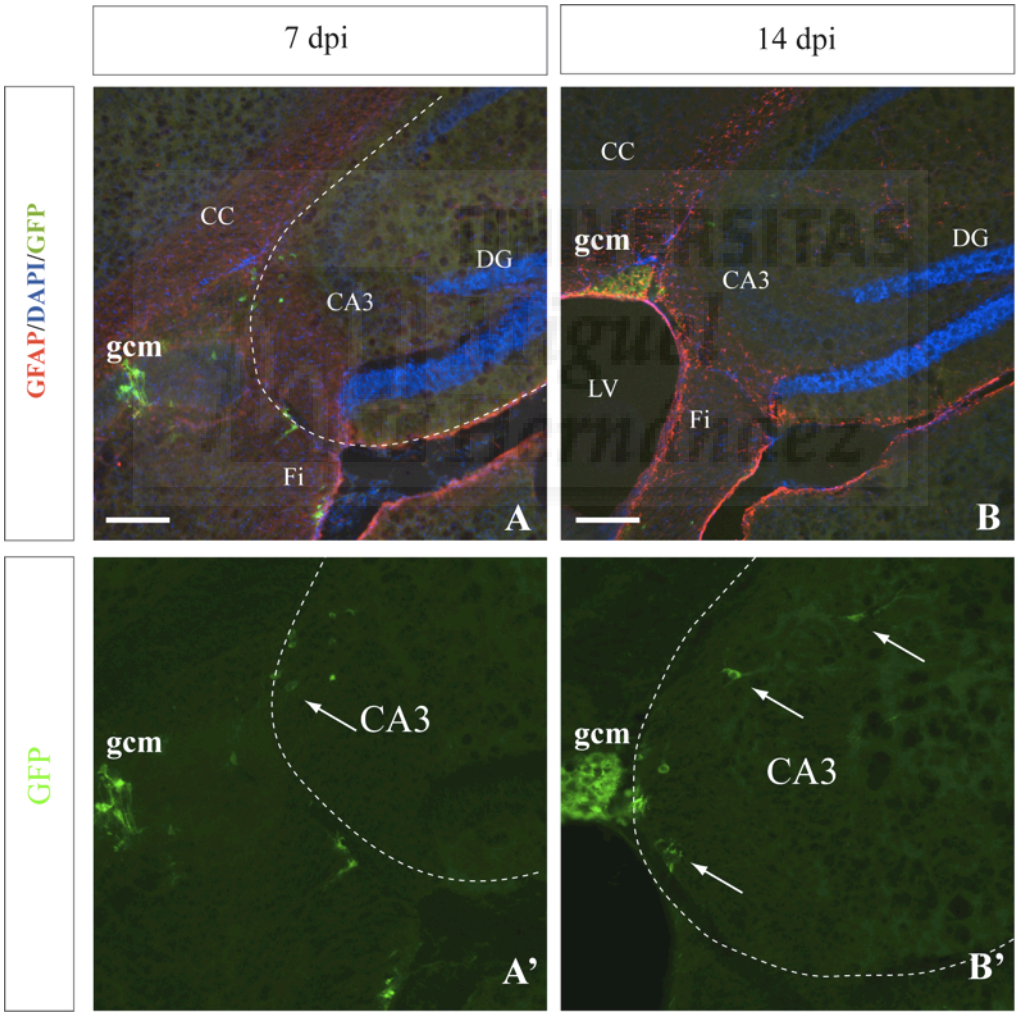


Fig.17 *In vivo* cell transplantation of cSVZ GFP cells in the caudal migratory stream

Immunostaining for GFAP (red) and GFP (green) showing the injection site and the integration of GFP injected cells *in vivo* both at 7 (A) and 14 (B) dpi. In blue is the DAPI staining of cell nuclei.

In A') and B') is represented in higher magnification the same area in which GFP cells are leaving the graft (white arrows). Abbreviations:CA3= Cornu Ammonis 3, CC= corpus callosum, Fi= fimbria, LV=lateral ventricle, gcm=grafted cell mass. Scale bar:100μm, Magnifications: A,B) 10x; A',B') 20x.

To further clarify the main characteristics of injected GFP cells, we performed immunohistochemical analysis for both neuronal or glial cell markers. We observed that inside the graft GFP cells were co-localizing with the glial marker GFAP (glial-fibrillary-acidic-protein) and DCX (doublecortin) (Fig.18 A and C) whereas GFP cells that were leaving the graft and approaching the CA3 layers of the hippocampus were not positive for these markers (Fig.18 B, D and F white arrows). Moreover, we performed the same analysis at 14 dpi when some cells had already reached the molecular layer of DG (n=5, 13±1.26 total number of GFP cells). These cells showed a very heterogeneous morphology but predominantly a fusiform soma and a migratory morphology. The immunohistochemical analysis for the determination of their cellular phenotypes revealed that fusiform cells in the possible migratory pathway from the SVZ were co-expressing GFAP-GFP or DCX-GFP, while migrated cells inside the DG granular layer were found Tuj1-GFP positive (Fig.18 H, L and N white arrows).

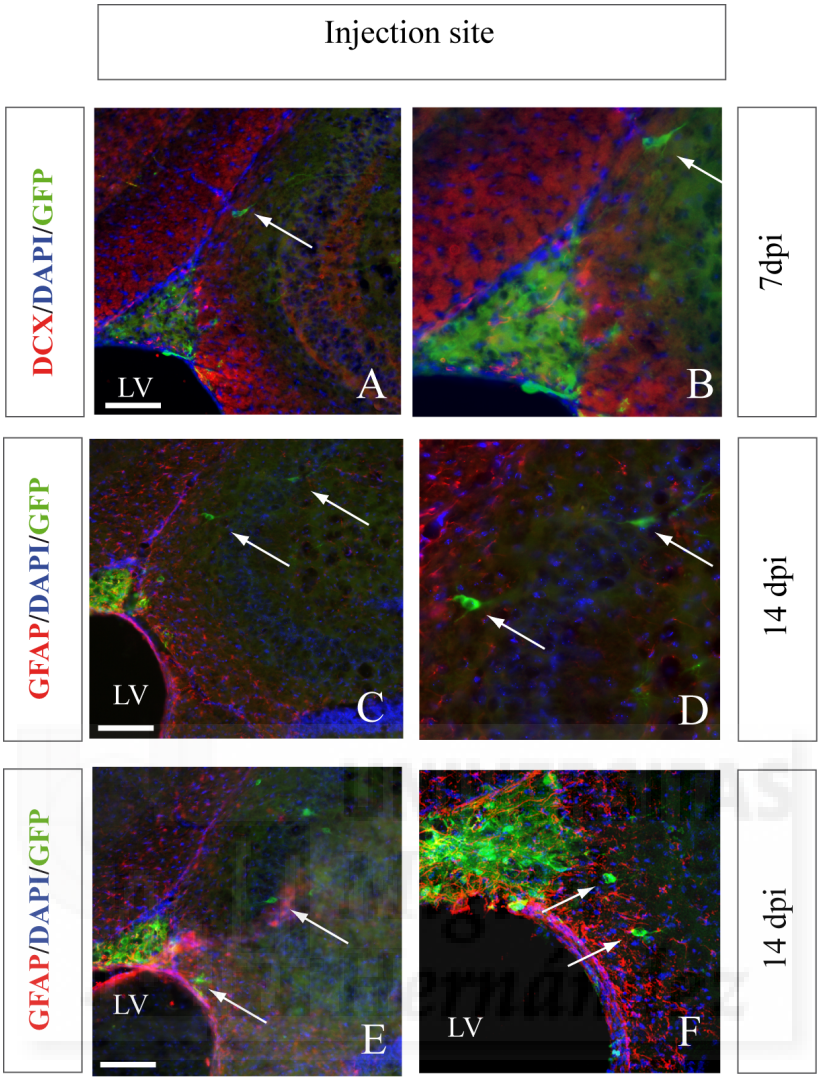


Fig.18 Immunohistochemical analysis of in vivo GFP transplants
A) Inmunohistochemistry showing transplanted GFP cells (in green) at the beginning of the CMS and DCX positive cells (in red) inside the graft. B) higher magnification. White arrows indicate single cells leaving the transplant and approaching caudally the hippocampal layers. C) GFAP inmunostaining (in red) and single GFP cells crossing CA3 layers. D) higher magnification of single GFP cells. E) GFAP cells (in red) inside the graft and GFP cells (green) inside the CA3. F) white arrows indicate GFP cells not GFAP (red) positive leaving the graft (in green). Scale bar: 100μm

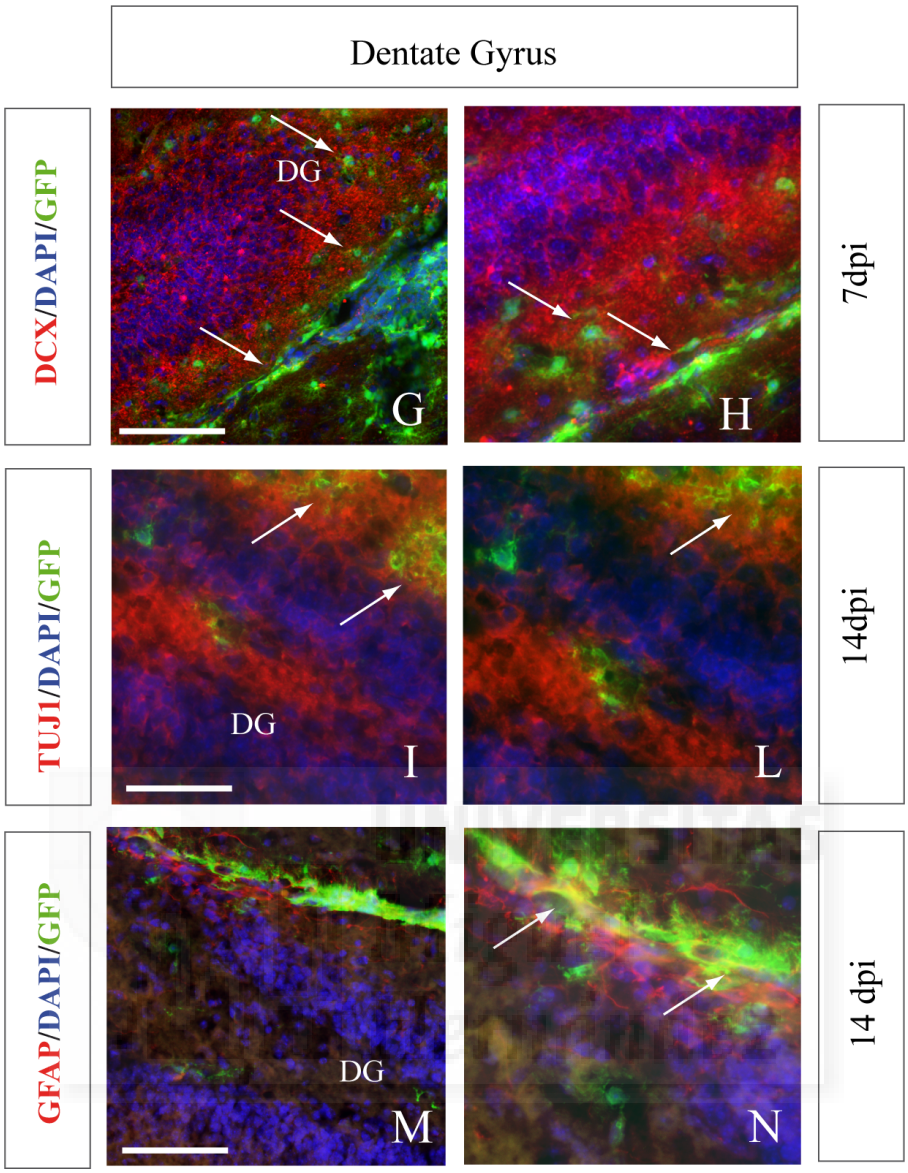


Fig.18 Immunohistochemical analysis of in vivo GFP transplants
G) GFP cells reached the DG and positive for DCX (in red; white arrows). I) GFP cells inside the DG double positive for Tuj1 (in red; white arrows). M) GFP cells next to DG layers. N) higher magnification (40x), white arrows indicate GFAP positive-GFP cells.
Scale bar= 200 μm LV=lateral ventricle; DG= dentate gyrus;

At the same time, part of the single cell suspension of GFP SVZ derived cells was tested in culture for its pluripotency using the neurosphere assay, an important aspect related to the adult neurosphere-forming activity already described (Merkle et al., 2004). We could observe that the same GFP SVZ derived cells that we were injecting *in vivo* were able to form neurospheres *in vitro* once cultured upon controlled free-floating conditions (Fig.19 A). Neurospheres at the third passage cultured with a differentiation media supplemented with EGF and bFGF were able already at 24 hours to the produce neuron like cells (Fig.19 B). However, only after 48 hours in the differentiation media

SVZ derived cells differentiate giving raise both to neurons and glial cells such as demonstrated by immunocytochemistry for both neuronal (Tuj1) and oligodendrocyte (NG2) markers, thus confirming their multipotent properties *in vitro* (Fig.19 C and D).

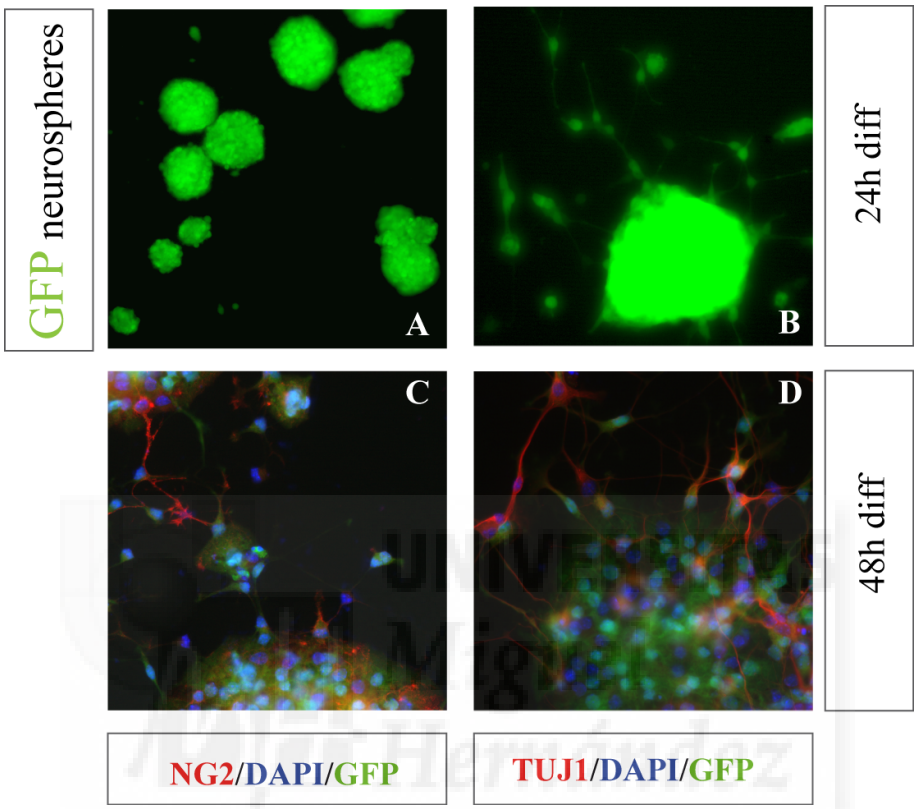


Fig.19 Neurosphere assay with SVZ derived GFP cells.
A) Free-floating culture of generated neurospheres from GFP SVZ cells B) higher magnification of GFP neurosphere in adherent culture after 24h in differentiation medium. C) and D) Immunostaining for using anti-NG2 and anti-Tuj1 antibodies respectively (in red) of GFP neurosphere in adherent culture after 48h.

In summary, consistently with our previous results with slice cultures grafts, SVZ derived cells transplanted in the caudal migratory stream were able to migrate caudally *in vivo* and to reach the hippocampus settling both inside CA3 and DG layers displaying very heterogeneous morphological and lineage characteristics. Moreover, we found that the same cells were able to generate *in vitro* both neurons and glia, thus confirming their ability to act as neural progenitors.

4. *In vivo* electroporation of the cSVZ visualized the caudal migratory stream postnatally and labeled migrating cells inside the DG

For a better visualization and characterization of the caudal migratory stream during postnatal stages *in vivo*, we decided to use the in vivo electroporation approach at P0 as described from Boutin C et al., 2008. This technique helped us to increase the labeling inside the proper SVZ, and more specifically in the cSVZ, and to obtain more cues about the migratory route between the cSVZ and their final destination in the DG. Moreover, as suggested from the expression patterns analyzed at the beginning of this study present in the CGE, the postnatal cSVZ could be the source of a specific postnatal population of hippocampal circuitry neurons. Thus, in order to identify the specific interneuron population that is born postnatally from the cSVZ we analyzed at 7, 14 and 28 days post electroporation (dpe) the injected animals for the expression of both markers of migrating cells such as DCX and PSA-NCAM and different interneurons markers such as Paravalbumin (PV), Calretinin (CR) and Calbindin (CB).

As showed in Fig.20 by confocal mosaic reconstruction (Fig.20A), at 7 dpe we observed that the whole SVZ of the electroporated lateral ventricles was GFP positive and that three main migratory streams were visible: the rostral migratory stream (RMS), the radial glial fibers extending to the cortex and the caudal migratory stream (CMS). Single migrating cells were visible and by this time the olfactory bulb was invaded by newly generated olfactory interneurons.

As mentioned before, the cSVZ can be visualized by the CoupTFII transcription factor thus we used the anti-CoupTF II antibody to ensure that the cSVZ was correctly electroporated. As expected, we performed the immunohistochemistry for CoupTFII and we found a strong co-localization at 24 hours post electroporation (hpe) confirming that the electroporation was successfully labeling the cSVZ as well as the hippocampal ventricular epithelium (Fig.20 B and C). After 7 dpe, GFP cells invaded the postnatal CMS and were detected until the most caudal tip of the SVZ-hippocampal boundary (Fig.19 A).

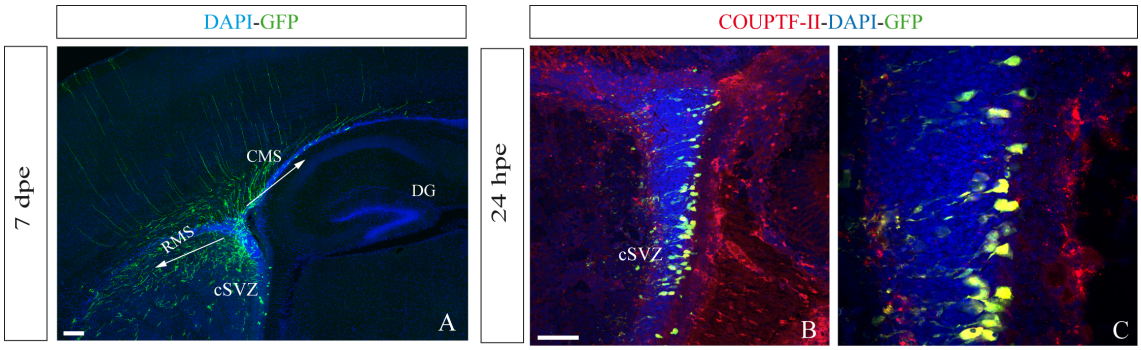


Fig.20 In vivo postnatal electroporation of GFP in the cSVZ
A) Confocal mosaic reconstruction: at 7 dpi the RMS (white arrow) and the CMS (white arrow) in green resulted labeled after GFP plasmid electroporation. In blue DAPI staining for cell nuclei. Scale bar: 200μm. B) 20x and C) 40x confocal images showing immunostaining of CoupTF-II at 24h from the electroporation (in red) showed high colocalization (yellow stained cells) in the electroporated cSVZ. Scale bar: 100μm, Magnifications: A) 20x reconstruction, B) 40x and C) 60x.

In order to characterize the GFP electroporated cells when they reached their final destination inside the DG, we checked for co-expression with the glutamate-decarboxylase 67 (GAD67) and different calcium binding proteins typical of interneuron populations such as calretinin (CR), calbindin (CB) and paravalbumin (PV). As espected from previous findings about the embryonic CGE derived cells (Nery S et al., 2002), we found that at 7dpe during the migration inside the CMS few GFP cells were able to coexpress calretinin (CR) and the analysis at further stages (14 dpe and 28 dpe) revealed that very few GFP cells (n= 4) were able to settle inside the DG. These cells seemed to be integrated in the DG granular layer, developed a typical neuronal morphology, with long dendrites distributed in both granular and molecular layer, suggesting to be DG interneurons, whereas showed negative immunoreaction for Gad67+, PV and CB (Fig.21). Thus, since most of the GFP+ observed cells were integrated in the granular layer and showed typical granular cell morphology during the process of neurite growth (Zhao et al., 2006), these postnatal generated neurons in the DG may be matured as dentate circuitry neurons.

Finally, the *in vivo* electroporation experiments helped us to observe that the postnatal CGE appeared implicated in the production of small number of CR-positive interneurons that are able to settle into the postnatal hippocampus. However, the low number of GFP positive cells that reached the hippocampus made complex the characterization analysis thus further *in vivo* studies became necessary.

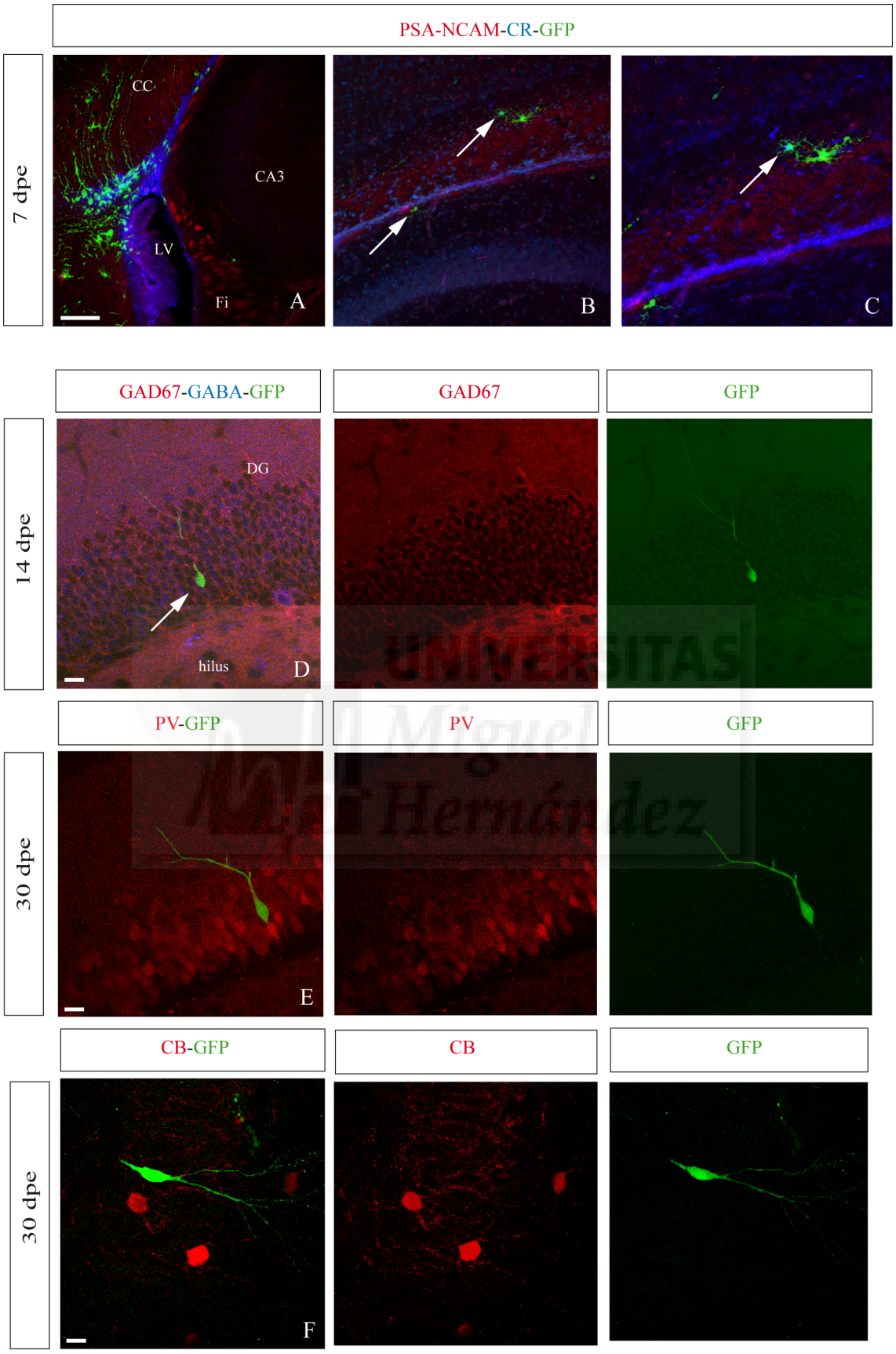


Fig.21 Immunohistochemical analysis of in vivo GFP electroporated brains at different postnatal stages

A) Sagittal view at 7dpe showing GFP positive cells (in green) inside the aSVZ and in callosal neuroepithelium. B) another view of CR+ cells in the dorsal hippocampus, C) higher magnifications (40x) of the same section indicating GFP cells inside the CMS and co-localizing with calretinin (in blue). D) a single GFP cell inside the DG immunostaining for Gad67 (in red) and GABA (in blue) at 14 dpe, E) and F) other GFP cells (in green) that reached and settled into the DG negative for PV (red) and CB (red), adjacent panels show individual fluorescence channels.
Scale bar:100μm, Magnifications: A) and B) 20x, C) 40x, D),E),F) 40x

5. *In vivo* lentiviral infection of the cSVZ revealed the presence of GFP-cells integrated in the DG and showing phenotype of CR positive neurons

For a more accurate labeling of the cSVZ area we decided to perform a clonal analysis of cSVZ derived cells by infecting newborn wild type mice with lentiviruses carrying a GFP construct. With this approach we aimed to a more specific and permanent labeling restricted to the cSVZ population, as lentiviruses were demonstrated by *in vitro* assays not to have a replicative behavior and so no daughter cells were expressing the GFP vector. In this way we assured that all the GFP positive cells (GFP+) we found migrating and reaching the hippocampus were belonging to the infected cSVZ germinative region and we could perform a tracing of infected cells during their migration from the SVZ to their final destination. The study of the experimental brain was performed after 7, 14 and 28 dpi, a time-window in coherence with previous experiments of cell transplantation and *in vivo* electroporation in which caudal postnatal migration was occurring.

After 7 dpi the analysis of the injection site showed the presence of several GFP cells in the cSVZ and few migrating cells caudally directed with the typical migratory morphology (Fig.22 A-A'-A''). The immunohistological characterization at 7 dpi of the injection site showed the presence of few GFAP+ cells (7±1.16) and some CR+ cells (27±6.4). The quantification of three different areas involved in the caudal migration (injection site=1, migratory stream=2 and final destination=3) as showed in Fig.22 (Fig.22H) reproduce previous data observed using *in vivo* electroporation in which the migration rate towards the hippocampus was very low, probably related to the fact that cSVZ cell migration represent a strongly restricted cellular contribution to medio-pallial derived areas at postnatal stages.

Nevertheless, the clonal analysis of the migrating GFP+ cells that reached the DG granular and molecular layers confirmed their neuronal characteristics as shown by immunohistochemistry with the co-localization with DCX and almost no cells were found double-positive for GFAP glial marker (Fig.22 E and F). Those GFP+ cells were found to run on the way of the caudal migratory stream and settling in DG layers, giving further evidences of the persistence of postnatal cellular insertion of cSVZ and into the DG circuitry.

In relation with the molecular regulation of the previously described migratory behavior of cSVZ-derived cells, we performed the analysis of fluorescent *in situ* hybridization (FISH) for the expression of the Fgf -receptor-1 (Fgfr1) in SVZ cells. It has been recently reported that DG granular cells express basic FGF at these perinatal and postnatal stages (Bueno C et al.,2013), that could act as a chemoattractive signal for the activation of the caudal migration of postnatal derived progenitors. Our results confirmed that SVZ and VZ cells (Fig.22 I-L') and migrating GFP+ cells prior to reach the DG were expressing this receptor (7±1.13). Those cells seems to express the receptor just during their caudal migration, while once settled into the DG layers they down regulated its expression (Fig.22 M and M'), an observation that opens important questions about a possible implication of Fgfr1 in this process.

In conclusion, the clonal analysis with GFP-lentivirus confirmed our previous *in vivo* data in which we observed a caudal migration conserved from the cSVZ during early postnatal life. Those data strongly support the hypothesis that the postnatal cSVZ is maintained as a postnatal source of newborn neurons for the hippocampus, although very little number of cells has been identified. However, a further understanding of the mechanisms that regulate this current is needed in order to elucidate how to improve the migration in the CMS of newly generated neurons in case of pathologic circumstances and to provide more information related to the function of this pathway especially for its possible implications on brain repair and hippocampal plasticity.

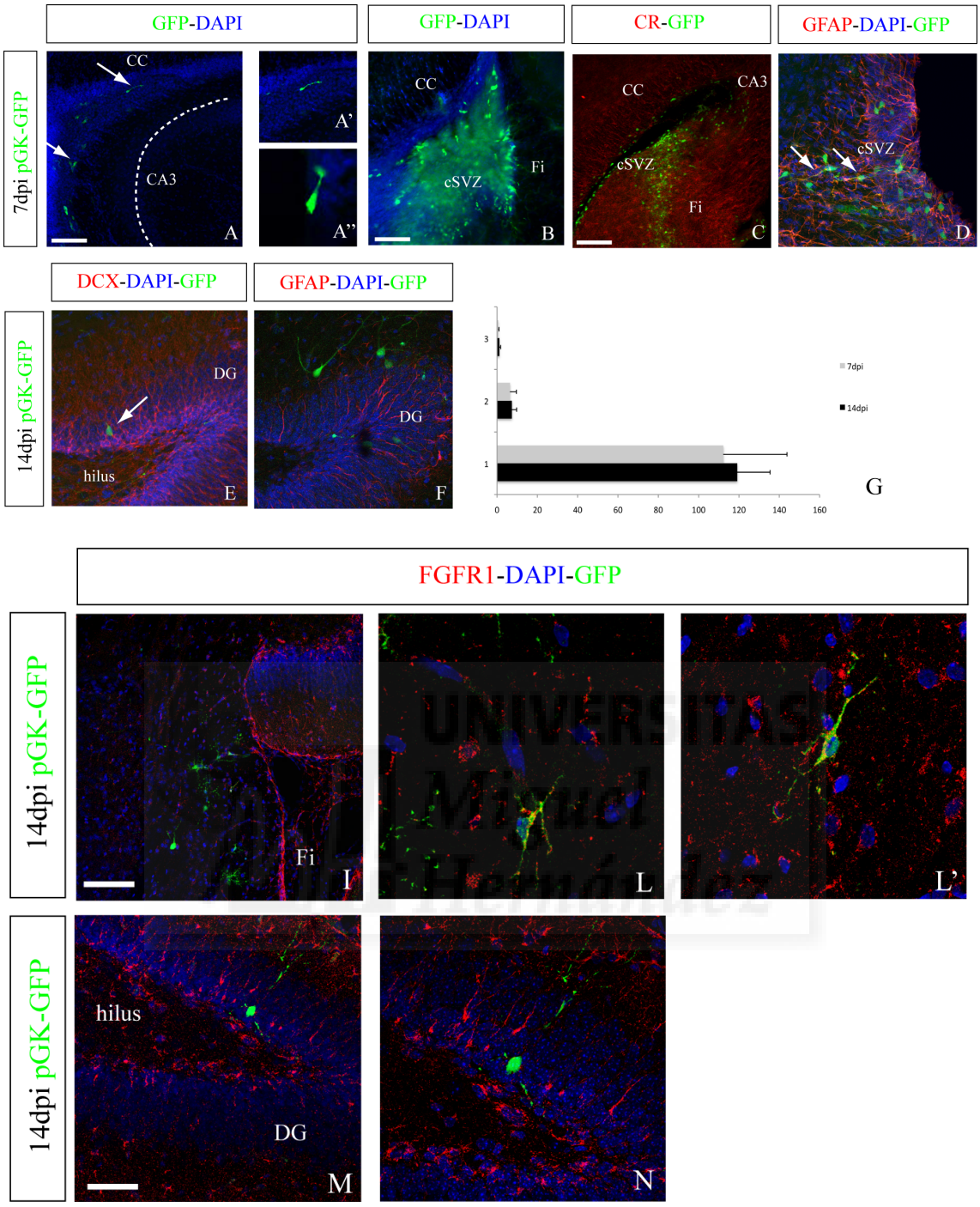


Fig.22 Characterization of lentiviral in vivo infection of cSVZ cells

A) Immunostaining at 7dpi with single GFP cells migrating caudally (in green). A'-A'') higher magnification showing the typical migratory morphology B) injection site at 7dpi in which the cSVZ is labeled with GFP (in green). C) another view of the cSVZ with double positive GFP (green) and CR cells (red) in the injection site and in D) with GFAP cells (in red). White arrow indicates a GFP cell migrating to the hippocampus. In E) and F) GFP cells inside the DG at 14 dpi (magnification 40x). White arrow show co-localization with DCX (in red). G) graphical representation of the number of GFP cells distributed as in the injection site (1), caudal migration (2) and final destination (3) at 7 and 14 dpi.
Scale barr= 100µm. Magnifications: A),B),C),D) 20x; A')40x,A") 63x; E),F) 40x.
I) Fluorescent in situ hybridization for Fgfr1 at 14 dpi, white arrows indicate GFP cells approaching the hippocampus and expressing Fgfr1. L) and L') higher magnification of GFP migrating cells expressing Fgfr1. M) Fish expression for Fgfr1 inside the DG and a GFP cell (in green) settled into the granular layer and N) higher magnification. Scale bar: 100µm; Magnifications: I) 20x; L-L') 63x; M) 20x; N) 40x.

6. FGF8 is able to exert an attractive role on caudally postnatal migrating cells acting trough its receptor FGFr1.

As showed previously by FISH on infected 14 dpi animals, GFP+ cells were able to express the receptor FGFr1 during their migration towards the hippocampus. Additionally, we carefully confirmed the same expression pattern in GFP+ caudally migrating cells at the same stage (14 dpi) by performing *in situ* hybridization as shown in Fig.23 (Fig.23 A-A'). Interestingly, we could demonstrate that by P0 FGF8, an important FGF molecule, was present in DG cells and it's probably maintained postnatally in caudal telencephalic structures (Fig.23 B-B'), consistent with our previous observation into the adult mouse brain (Bueno et al., 2013).

Starting from these observations, we tried to elucidate a possible mechanism suggested by the FGFr1 receptor expression and we decided to study the interaction with FGF signal. Since FGF8 was detected by this age into hippocampal cells (CA and DG), we decided to investigate if could act as a good candidate in promoting cSVZ cell migration. The FGF family is already know to be implicated in several biological pathways during mammalian development and has already been described having a role in neural migration especially during embryonic development (Pombero A et al., 2011). Thus, in order to study the migratory potential induced by FGF8 and in particular its possible implications in postnatal caudal migration, slice cultures from *in vivo* electroporated mice were prepared and heparin FGF8-soaked beads were implanted inside the hippocampus, to increase the signaling activity in a precise focus. After 48h of incubation, GFP+ migrating cells were observed migrating towards the hippocampus attracted by the FgF8-bead (n=8) (Fig.23 C') and showing a strong attraction exerted from the FgF8 implanted bead. Comparison with control slices in which PBS-beads were inserted in the same slice preparations showed normal migration of GFP+ cells that followed their caudal pathway (n=5) (Fig.23 D-D").

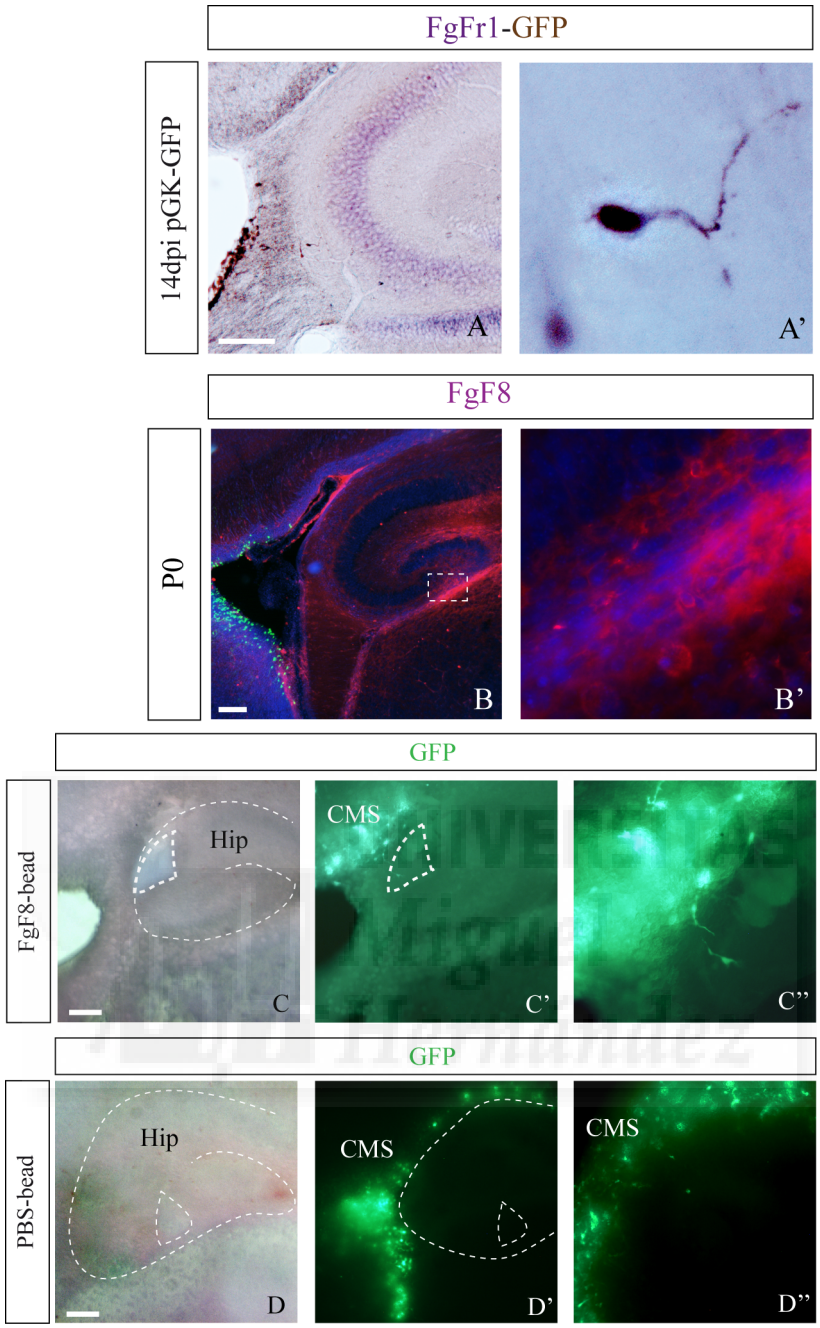


Fig.23 Fgf8-beads implantation in electroporated organotypic slice cultures
A) In situ hybridization coupled with anti-GFP immunostaining showing a caudal migrating cell positive for Fgfr1 approaching the hippocampus. A') higher magnification of a GFP cell in which the co-localization with the *in situ* and the immuno signals B) and B') immunostaining anti-Fgf8 (in red) at P0 in a sagittal view of the hippocampus. C) and D) sagittal view of GFP electroporated slices with implanted Fgf8-and PBS-beads respectively. C'-C''-D'-D'') higher magnification showing GFP cells (in green). Hi=hippocampus; CMS=caudal migratory stream.

To better elucidate those data on the attraction potential exerted by Fgf8 on migrating GFP+ FgFr1 cells, we outlined an experiment based on an invasion assay using Matrigel matrix. For this experiment cSVZ explants were put in contact with

Fgf8-transfected HEK cells, a cell line that was already tested for the appropriate release of Fgf8 outside the cells inside the culture medium (Crespo-Enriquez I et al, 2012). As displayed in Fig.24 (Fig.24 A), cSVZ explants showed a strong attraction when exposed to Fgf8-transfected cells (n=8) as detectable by the presence of several migrating cells (90±8,90) proximal to the Fgf8-cells (in yellow), whereas a different situation was observed with the control HEK cells (in red) as demonstrated by almost no attraction (n=4). The quantification of cell migration was expressed using the proximal/distal ratio (P/D ratio) taking into account even explants derived from rostral areas of migration, the RMS, as a positive control of migration. As showed in the graph (Fig.24 F) cSVZ in contact with Fgf8-transfected cells showed a significant strong attraction phenotype even respect to the RMS situations (t-test: p-value<0.05). A FISH was performed on cSVZ explants to confirm the presence of the receptor Fgfr1 on migrating GFP cells and thus giving a further confirmation for our model of Fgf8 acting trough its receptor Fgfr1 to promote neural migration on cSVZ derived cells.

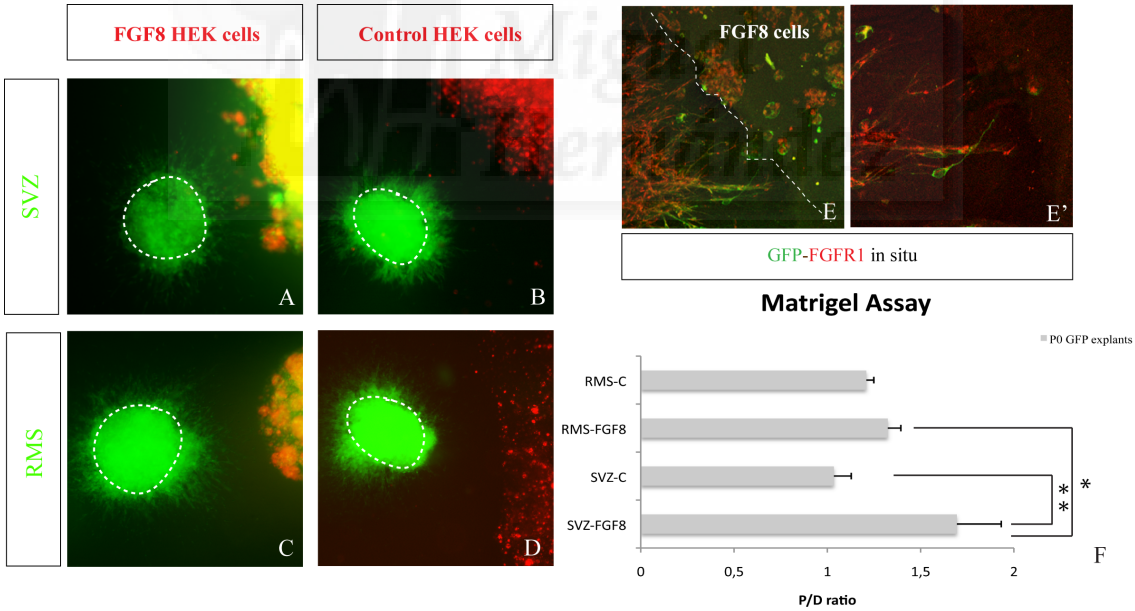


Fig.24 Matrigel assay with SVZ and RMS explants in contact with Fgf8-HEK cells

A) cSVZ GFP explant (in green) in contact with Fgf8-transfected HEK-cells (in red). B) cSVZ GFP explant (in green) in contact with control HEK-cells (in red). C) RMS GFP explant (in green) in contact with Fgf8-transfected HEK-cells (in red). D) RMS GFP explant (in green) in contact with control HEK-cells (in red). E) FISH for Fgfr1 receptor (in red) on SVZ explants (in green) in contact with Fgf8-cells after the assay E') particular of a cSVZ cell (in green) positive for Fgfr1 (in red). F) Graph summarizing the migration rate expressed as P/D ratio in all the assay conditions.(p-value<0.05)

Altogether those data showed that in our *in vitro* conditions in the presence of its receptor Fgfr1, Fgf8 is able to play an important mobilization and likely attractant role on migrating cSVZ derived cells. Nevertheless, further analysis and *in vivo* experiments will be required to elucidate this mechanism and to identify exactly the molecular interactions and the implication of other molecules that may be present modulating this caudal cellular migration.

Section II

1. Trafficking dynamics during postnatal DG development

The initial formation of the DG and the hippocampal structure are unlikely to be considered as separated events from the ones regulating the neocortex formation, of which the hippocampus constitute the most medial and dorsal portion. However, the DG neurogenic morphogenesis follows a very peculiar developmental program that spans from mid-embryonic stages (E15.5) until several days after birth (P7-10) in order to achieve its final morphogenetic conformation which is maintained throughout mammalian brains (Rakic P., 1981; Altman and Bayer 1990a,b ; Gage F.H., 2002).

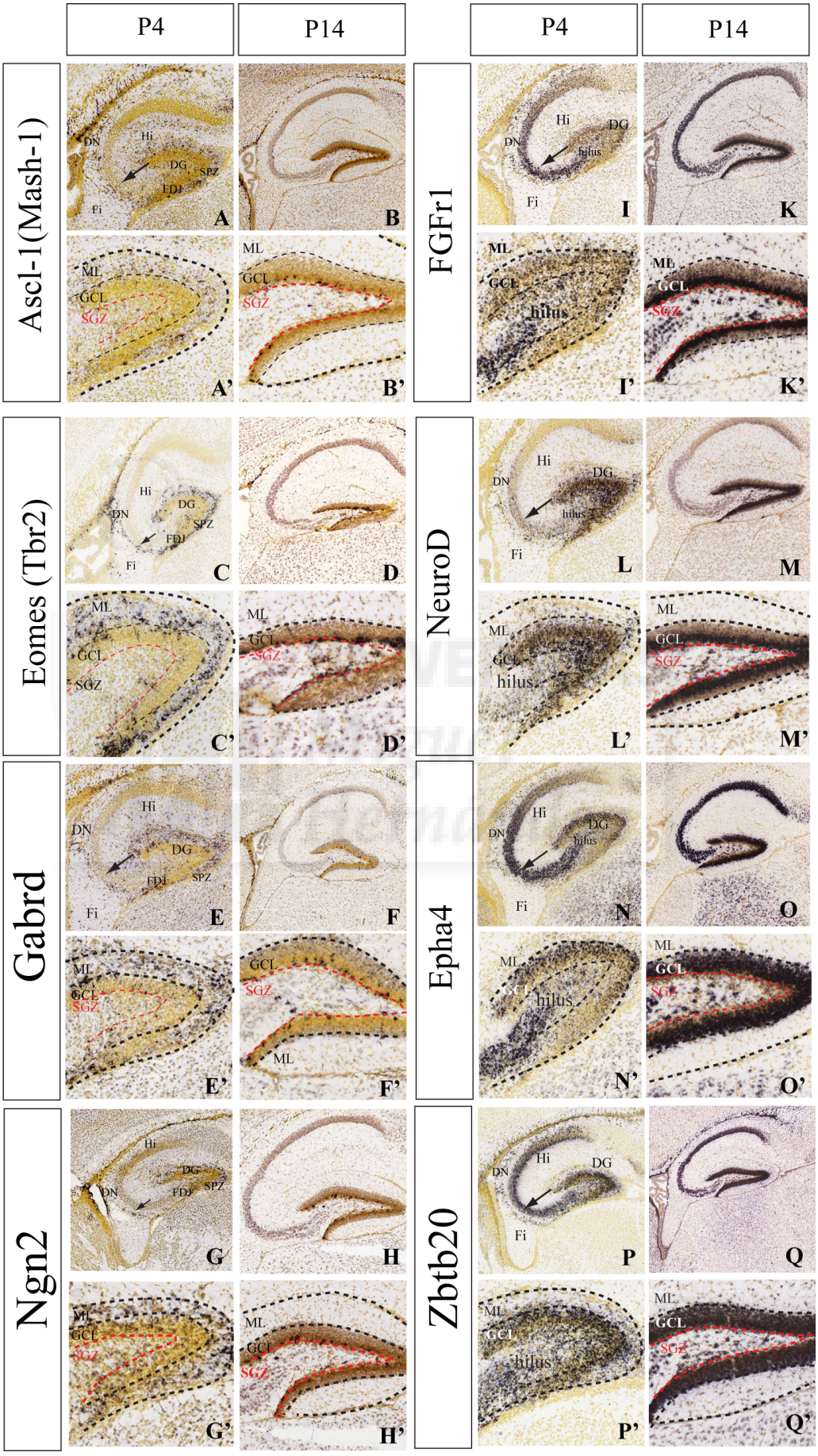
To elucidate the main cellular pathways and to identify the key migratory events present during DG postnatal development, we decided to perform a wide transcription patterns analysis taking advantage of the Allen Brain Atlas database (Fig. 25; www.brain-map.org). Thanks to this study, we could identify a list of transcription factors and other developmental genes that were expressed specifically in distinct populations of the hippocampal formation during DG development and follow them through different postnatal stages. Interestingly, these expression patterns allowed us to follow dentate neural stem cells (NCSs), intermediate precursors and granule cells at different stages of maturation localized in the diverse cellular currents generated in the described hippocampal cell matrices (Altman and Bayer 1990 a,b). From this analysis we could resume the patterns into two groups of genes, each one outlining a different migratory route: one from the mediopallial VZ and SVZ following the migratory stream in which intermediate progenitors and NCSs follow a radial migration, passing through a narrow migratory pathway in the CA3 alveus domain (CA3alv), underneath the fimbria, to the subpial zone (SPZ) in the proximal pole of the DG (Fig. 25 A, C, E, G arrow). Then, these migratory cells invaded the DG molecular layer (ML) (Fig.25 A', C', E', G'). And a second radial migratory current, in which a mixed population of dentate granule neurons at different maturation state follows radial glial fibers moving inwards and settle at the subgranular layer and the hilus (Fig.25 I, L, N, P).

In Fig. 25 are summarized the subset of genes that were included in our study as the most descriptive of these two pathways: Ascl-1, Eomes (Tbr2), gamma-aminobutyric acid (GABA) A receptor subunit delta (Gabrd) and Neuregulin2 (Nrg2) are expressed in the SVZ and cells migrating trough the CA3alv and following the subpial route reaching the DG molecular layer (Fig.25 A, C, E, G). Conversely, Fgfr1, NeuroD, Ephrin4 (Epha4), zinc finger and BTB domain containing 20 (Zbtb20) are

expressed in CA3 pyramidal layers, DG hilus and granular layer cells (GCL) (Fig.25 I, L, N, P). In particular, the comparison of all these different mRNA expression patterns was performed at two key postnatal stages of DG development: at P4 when the dentate migratory streams are still occurring and the DG structure is still incompletely mature, and at P14 when cellular movements in the hippocampus are completed, the granule cell layer (GCL) is clearly defined and the SGZ layer of the DG is detectable by specific markers.

In more details, the *in situ* hybridization patterns analyzed revealed the presence of Ascl-1 positive cells present all along the SVZ of the hippocampus, in the precursors niche (also identified as the primary matrix or dentate notch [Altman and Bayer 1990a,b]) and in the CA3alv migratory stream, which have been classified in rat brain as the secondary matrix migratory current by Altman and Bayer (Altman and Bayer 1990a,b) (Fig.25 A and A' in higer magnification). In addition, Ascl-1 positive cells were detected also in the ML of the DG by P4 (Fig.24 A'). This expression pattern is similar to Tbr2, Nrg2 and Gabrd gene patterns both at P4 and P14 (Fig.25 C, C', E, E', G, G'). Ascl-1, Tbr2, Nrg2 and Gabrd are expressed at P4 in migrating and progenitor cells, which originate in the SVZ, migrated through CA3alv into the DG molecular layer, where by inward directed radial migration across the DG progressively populate the GCL in order to generate the SGZ around P14, where they retain stem cell characteristics (Fig.25 B,B',D,D', F, F', H, H').

Other genes such as Fgfr1, NeuroD, Eph4 and Zbtb20 are expressed in CA3 pyramidal and DG granular layers and constitute the main cellular populations present in these structures (Fig.25 I, L, N, P). Moreover, they reveal how these cells are generated in a more extended SVZ in the medial pallium (hippocampus SVZ). Cells transcribing these genes migrated through more evident radial migratory routes at perinatal stages, P4 and P14. In the DG the Fgfr1, NeuroD, Eph4 expression pattern reveal clearly the progressive maturation of granular cells following first an inside-out and later an outside-in gradient of molecular maturation, as suggested by previous studies using mutant mice for those molecules (Ohkubo et al., 2004; Miyata et al., 1999; Liu et al., 2000; Hara Y et al., 2010) (Fig.25 I-Q'). The comparative analysis of all these gene expression patterns has been fundamental for the characterization of the different migratory dynamics underlying the diverse origins of SGZ and the granular cells of the DG (Fig.25 A-Q').


P4
P14
P4
P14
Ascl-1(Mash-1)
FGFr1
Eomes (Tbr2)
NeuroD
Gabrd
Epha4
Ngn2
Zbtb20
Hi
DG
DN
SPZ
FDJ
Fi
hilus
ML
GCL
SGZ
A
B
I
K
A'
B'
I'
K'
C
D
L
M
C'
D'
L'
M'
E
F
N
O
E'
F'
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O'
G
H
P
Q
G'
H'
P'
Q'

Fig.25 Analysis of different expression patterns along DG development

In situ hybridization expression patterns from the Allen Brain Project database (www.developingmouse.brain-map.org) Sagittal sections of: A,A') Mash1 C,C') Tbr2 E,E') Gabrd G,G') Ngn2 expression patterns recapitulating dentate progenitor migration at P4. Black arrows indicate the secondary matrix stream of migration. A', C', E',G') higher magnification showing the distribution of migrating cells in the different DG layers: molecular layer (ML), granular cell layer (GCL) and sub-granular zone (SGZ, in red). In B), D), F) and H) the same expression patterns are represented at 2 weeks of mouse development (P14). B'), D'), F'), H') higher magnifications in which are illustrated the reorganized layers of formed DG. In I,I') Fgfr1 L,L') NeuroD N,N') Ephrin 4 (Epha4) P,P') Zbtb20 expression patterns resuming the migratory route of the immature granule cells populating the tertiary matrix in the hilus. In K), M), O), Q) the same expression patterns are shown at P14 and in K'), M'), O') and Q') the higher magnification with the details of each reorganized layer (ML, GCL and SGZ in red).

Additionally, the same expression patterns showed important morphological modifications in DG layers conformation and organization occurring in between the first two postnatal weeks. As appreciable in higher magnification in P4 panels (Fig.25 A', C', E', G') in correspondence of the fimbria-dentate-junction (FDJ) the presence of a meshwork of cells positive for Tbr2, Gabrd and Nrg2 seems to create a direct communication between the secondary matrix stream and the forming granular-molecular layers. This situation is completely different after 2 postnatal weeks (P14) where those layers appear structurally separated and a cellular communication with the FDJ and the layers looks totally interrupted (Fig.25 B', D', F', H'). Fatherly, when the DG structure is almost completed, a profound reorganization of the GCL and the hilar structure occurs inside the dentate: between P4-P14 migrating precursors present in the outer ML migrated radially into the hilus and contribute to generate the SGZ. Thus, those movements confirmed an outside-in radial migration of dentate progenitors to reach their final position inside the proliferative DG niche.

At the same time, other expression patterns labeling the granular cell present in the tertiary matrix were analyzed in order to follow another fundamental radial migratory pathway, which is necessary for SGZ formation. Positive labeled Fgfr1, NeuroD, Ephrin4 and Zbtb20 cells were detected in migrating granule cells performing a radial migration from the radial migratory cellular streams directly oriented to occupy the presumptive GCL passing through the hilar region (Fig.25 I, L, N, P). As confirmed by the *in situ* hybridizations patterns in Fig.21 (Fig. 25 K-T'), following labeled granular cells during DG development between P4 and P14 delineate how a precise regulation of the radial glial scaffolding has to be present in order to orchestrate properly all the DG specific compartments of cells. By P4 granular cells appear

dispersed into hilus and started to move filling the GCL (Fig.25 I',L', N' P'). However, the comparison of all those genes at P14 showed again a positional rearrangement of granular cells condensing in the GCL that occur following a radial inside-out pattern and that profoundly change the organization of this layer (Fig.25 K', M', O', Q').

Altogether these observations gave more insights about DG trafficking during early postnatal development opening several questions about the fundamental role of radial glial scaffolding regulating all this cellular migrations and about the organization of different dentate precursors during their routing before to find their final destination.

2. Radial glial scaffolding in the secondary matrix dentate stream is altered in Lis1/sLis1 mutant mice

As already described, the CA3alv (secondary matrix) stream begins during mid-gestation stages and seeds the nascent DG (Allen and Bayer 1990). This radial migration is important for the formation of the so called tertiary matrix and dentate precursors, so a proper radial glial scaffolding is necessary for the right establishment of all those dentate routes during DG development. Interestingly, the protein Lis1 was already described as directly involved in radial and tangential migration defects (McManus MF et al, 2004; Tsai JW et al, 2007), nevertheless the role of Lis1 in radial glial scaffolding organization in the DG neurogenic matrices have been poorly investigated.

In order to study the radial glial organization inside the developing hippocampus, we performed immunohistochemical analysis for GFAP, a specific marker that label radial glial cells and astrocytes throughout development and postnatal stages. We compared the distribution of GFAP into the dentate matrix streams at P4 both into wild-type and Lis1/sLis1 mutant mice in which one allele of the Lis1 gene is mutated and produce a truncated shortened protein (sLis1). As showed in Fig.26 (Fig.26 A) the GFAP positive immunostaining in the wild type revealed the presence of many radial glial cells packed at the borders of the hippocampus; that is, in the SVZ, the proximal fimbria and the fimbrio-dentate junction (FDJ); whereas much less dense

radial glia fibers intermingled with abundant fibrillar astrocytes along the CA3alv and in the ML of the DG. In more details, in the CA3alv migratory route we could appreciate the presence of fibrillar astrocytes distributed all along the migration substrate (Fig.26 B arrows, C,D). These astrocytes seem to be arranged in cellular rows along the route, leaving empty spaces between them, parallels to the radial glial fibers (Fig. 25 B-D). Astrocytic rows and radial glial fibers converge in the subpial zone at the FDJ, a strategic point during DG morphogenesis in which migratory CA3alv progenitors converge before invade the DG molecular layer (Fig. 26, E,F; arrows).

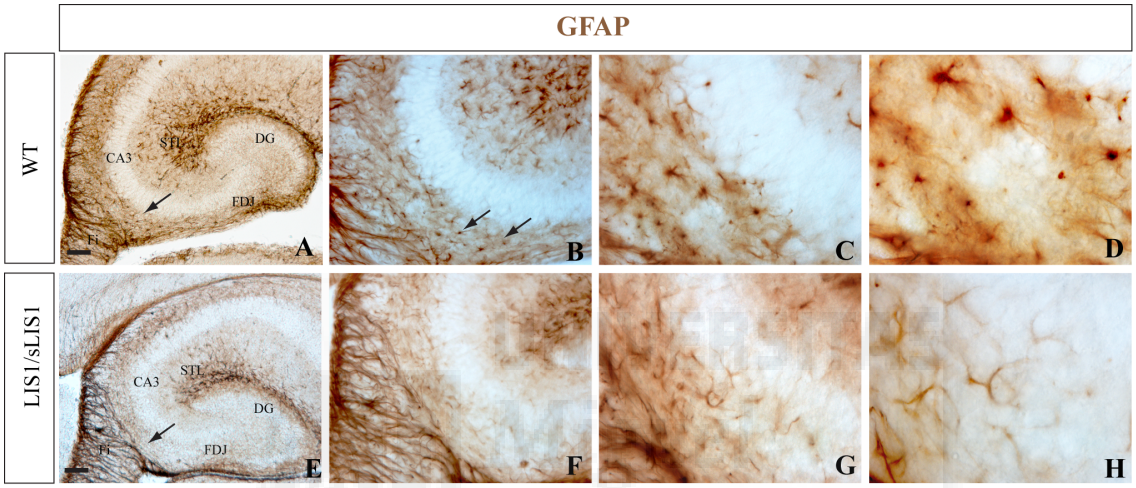

Fig.26 Immunohistochemistry with DAB GFAP showing affected radial glial cells in Lis1 mutant
Sagittal sections of P4 wild type (WT) and Lis1 mutant (+/-) mice. A) GFAP immunostaining inside the postnatal hippocampus. Arrow indicates the secondary matrix B-D) Higher magnifications showing morphological details of GFAP cells in the wild type. Arrows indicate GFAP cells in the secondary matrix E-H) GFAP immunostaining at different magnification steps in the Lis1 mutant. Arrows indicate the secondary matrix. CA3= Cornu ammonis 3; DG=dentate gyrus; Fi= fimbria; FDJ= fimbrio-dentate junction STL=stratum lacunoso moleculare
Scale barr: 250µm. Microscope magnifications: A,E) 5x B,F)10x C,G) 20x D,H) 40x.

Interestingly, in Lis1/sLis1 mutant it is clearly present a reduction in GFAP signal in almost all the hippocampal structure and especially in the CA3alv migratory route where few scattered fibrillary astrocytes GFAP+ are observed (Fig.26 E arrow) (n=4/4). As appreciable in higher magnification not only we observed a reduction in the number of GFAP cells in this region, but also GFAP cells have a different gross morphology from the star-like cells present in the wild type and appear with a lesser and thinner expansions (Fig. 26 G-H). In addition these sparse astrocytes were heterogeneously distributed in the CA3alv, without any distinguishable arrangement (Fig. 26 F-H).

In order to investigate more precisely how are affected radial glial cells in the Lis1 mutant, we performed the same analysis using Nestin antibody, a specific marker of both SVZ neural precursor cells and radial glial cells during development. As illustrated in Fig.27 Nestin positive cells are distributed and organized in a similar way as the GFAP immunostaining (Fig.27 A), showing an overall more compacted radial glial scaffolding in the wild type than in the mutant (Fig.27 A and E). In particular, in the wild-type radial glial fibers are clearly detected following a parallel distribution and occupying the CA3alv route, organized as forming cellular corridors (Fig.27 A and B indicated by arrows), while in the Lis1/slis1 mutant they seems to be more defasciculated and disorganized, with a lacking of this radial corridor distribution (Fig.27 E-H) (n=4/4).

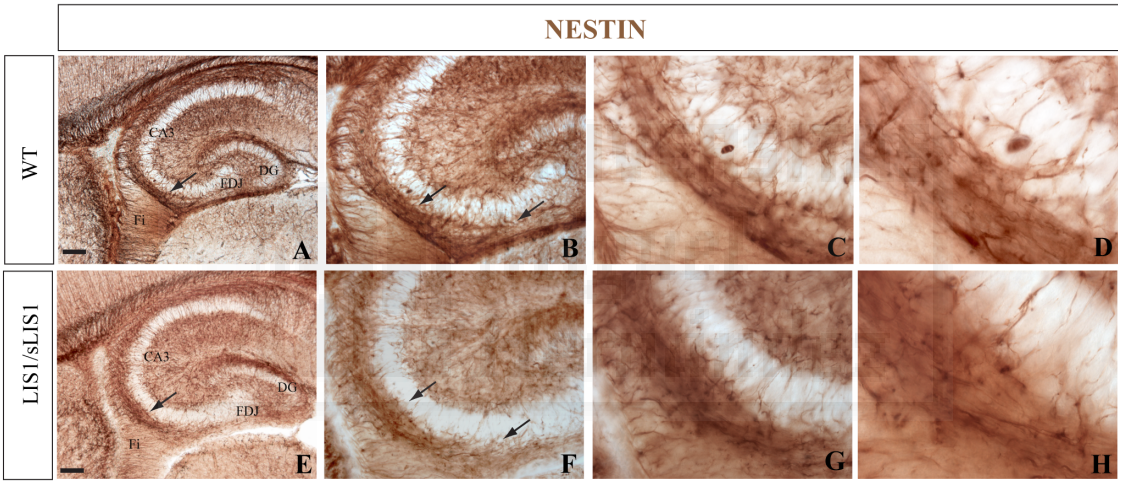

Fig.27 Nestin immunohistochemistry indicates radial glial cells defects in the hippocampus of Lis1 /sLis1mutant
Sagittal sections of P4 wild type (A-D) and Lis1/sLis1 mutant mice (E-H) hippocampus with Nestin-Dab immunostaining. In A) Black arrow indicates the radial glial Nestin cells in the secondary matrix scaffolding B-D) higher magnifications showing detailes of Nestin positive cells in the secondary matrix showing compacted Nestin fibers. E) Lis1/sLis1 secondary matrix Nestin fibers. Black Arrows indicate the secondary matrix radial glial scaffold defasciculated. F) Arrows indicate Nestin fibers dispersion. G-H) higher magnifications with Nestin positive cells in details.CA3= Cornu ammonis 3; DG=dentate gyrus; Fi= fimbria; FDJ= fimbrio-dentate junction
Scale barr: 250μm, Microscope magnifications:A,E)5x. B,F)10x. C,G)20x. D,H) 40x.

All these data indicate that in Lis1/sLis1 mutant mice radial glial cells are defective and that the radial glial scaffolding is unproperly formed especially inside the CA3alv migration route (the secondary matrix described from Altman and Bayer, 1990a,b) of the developing DG where the packaging conformation of radial glial fibers appear affected. Analyzing those fibers in more details we could observe that radial

glial cells and astrocytes in Lis1/sLis1 mice suffer profound changes in their structural morphology thus loosing their parallel distribution and star-like characteristic shape, respectively. In order to reveal the consequences of those defects, further investigations were settled up to elucidate how the dentate migratory pathways can result compromised.

3. In vivo electroporation of Lis1/sLis1 secondary matrix revealed migratory defects of dentate granule cells and progenitors

To further elucidate the possible implications of Lis1 mutation of our mouse model on dentate precursors migration during postnatal stages, we decided to electroporate *in vivo* the hippocampic SVZ, which is known to be the germinative niche origin of many subgranular dentate precursors (Altman and Bayer 1990a,b). During early postnatal development (P0-P4) the CA3alv migratory stream is visible leaving the SVZ of the hippocampus (Fig 28 A-A' green cells) directed to seed the proximal hilus of the nascent DG and to continue the subpial route into the molecular layer, surrounding the external layers of the DG. As shown in Fig.28 (Fig.28 A-A''), these precursors' migration follows radial glial fibers during its routing until reaching a specific structure, the FDJ, at the subpial zone, which seems to be important for their trafficking before reaching the molecular region of the DG (Fig.28 A'').

Intriguingly, GFP electroporation in the Lis1/sLis1 hippocampal SVZ at equivalent postnatal stages revealed the presence of dramatically compromised radial glial cell morphology and distribution. In fact, GFP+ subgranular precursors were accumulating in the SVZ and seemed to be delayed in their migration toward the DG appearing blocked in this region into an extended SVZ due to an abnormal radial glial scaffolding (n=3) (Fig.28 B-B'). In Fig.28 are also illustrated in higher magnification two sites of the CA3alv matrix, which appeared as the most descriptive evidence of this affected migration (Fig.28 boxes C, D for the wild type and E, F for the mutant respectively). All these data about GFAP radial glial cells defects were also quantified

in order to specify the reduced amount of multipolar GFAP+ cells observed inside the secondary matrix stream (Fig.28 G).

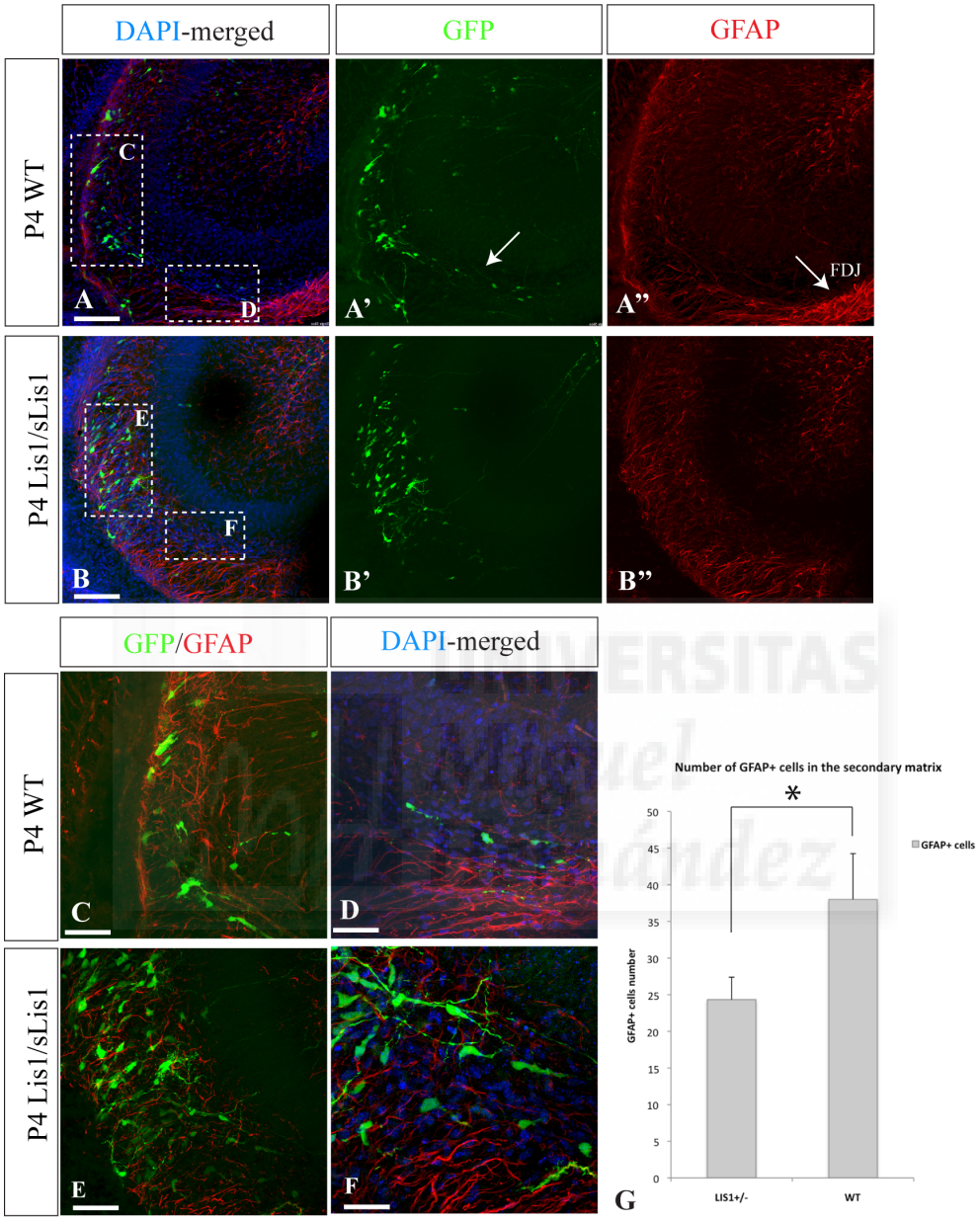

Fig.28 In vivo electroporation of the postnatal primary dentate germinative niche in wild type and Lis1/sLis1 mice

Confocal images of immunofluorescence in sagittal sections of the postnatal hippocampus electroporated with GFP (in green): A) merged channels for nuclei staining with DAPI (in blue), GFP cells (green) and GFAP (in red) showing the electroporated primary matrix in the wild-type after 4 days of electroporation (4dpe) at P4. A') details of GFP cells (in green) in the secondary matrix (arrow) A'') normal conformation of radial glial GFAP+ cells (in red) creating the scaffolding. The arrow indicates the fimbrio-dentate junction (FDJ). B) merged channels for DAPI (blue), GFP (green) and GFAP (red) in the mutant Lis1/sLis1. B') GFP cells are shown blocked in the hippocampal SVZ and are delayed in their migration to the secondary matrix. B'') GFAP+ cells (red) in the Lis1/sLis1 mutant built a defective scaffolding. The FDJ is not properly formed. C-F) represent in higher magnification the white dashed boxes present in A and B respectively with details of failed GFP cells migration (in E) and unproper

radial glial scaffolding (in F). G) graph showing the quantified reduction in GFAP+ stellate cells present in the Lis1/sLis1 mutant. Scale bars: 100 μm in A-B''); 50μm C-F).

As already observed, in the Lis1/sLis1 mutant the radial glial marker Nestin also showed defective radial glial scaffolding particularly pronounced in the CA3alv migratory route (Fig. 29 A'-A'' and B'-B''). Consistently with these observations, we observed differences in Nestin radial glial fibers disposition and in the dentate migration also in electroporated wild type animals when compared with the Lis1/sLis1 (Fig.29 A-A''and B-B''). In the mutant subgranular precursors' migratory matrix, the Nestin positive corridors formed by radial glial fibers are severely affected and appear disorganized in the orientation of the fibers. (Fig.29 A'' and B'' white arrow). Moreover, we could confirm with double staining experiments our previous results in which GFP+ dentate progenitors migration was slowed (n=3) and GFP+ cells were found accumulated in the hippocampal SVZ instead to leave it towards the subpial migration trough the CA3alv corridors (Fig.29 A, A' and B, B').

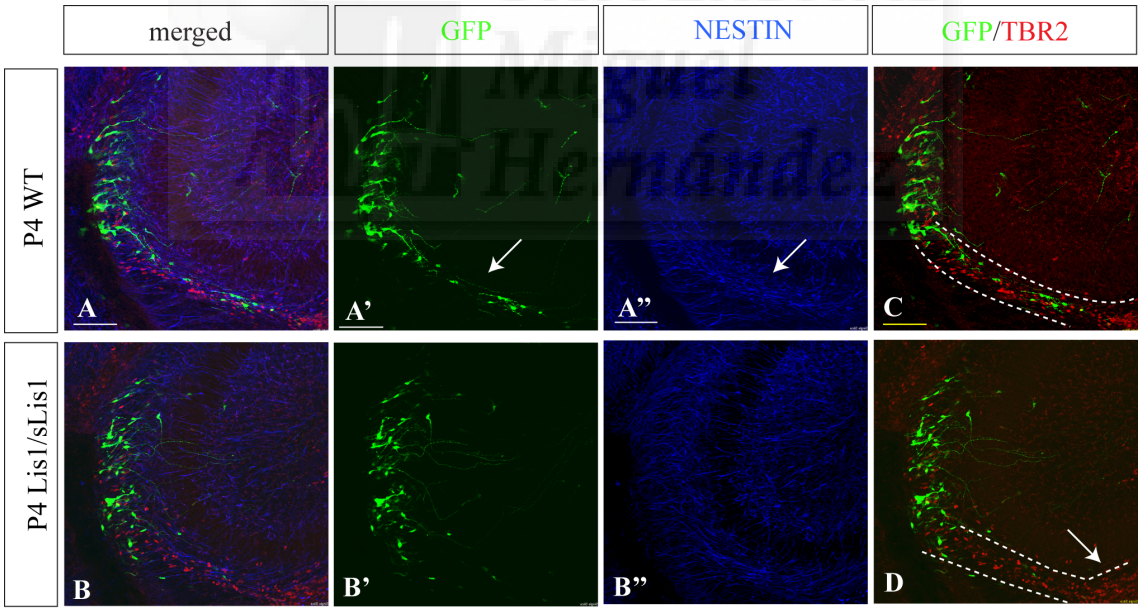

Fig.29 Nestin and Tbr2 positive cells defective distribution in postnatal Lis1/sLis1 mice
Confocal images of sagittal hippocampal sections analyzed through immunofluorescence at P4 (4dpe) using anti-GFP (green), anti-Tbr2 (red) and anti-Nestin antibodies. A-A') wild-type electroporation with GFP labeling the secondary matrix stream (arrow). A'') radial glial fibers appear compacted in the wild type secondary matrix (arrow). B-B') GFP cells are delayed in Lis1/sLis1 mice their migration to the secondary matrix. B'') Nestin + fibers are more defasciculated compared to the wild-type (blue).

Interestingly, the analysis of intermediate subgranular dentate progenitors detected by Tbr2 immunoreaction along the CA3alv migratory matrix, revealed further defects of migration. This study confirmed that the defective disposition of the GFAP and Nestin radial glial fibers previously analyzed in the mutant Lis1/sLis1 may be correlated also with an impaired migration of Tbr2+ cells (Fig.28 C and D). In more details, Tbr2+ cells were found dispersed inside this migratory stream, occupying a wider area inside the migratory route (n=3) (Fig.29 E and F). Moreover we have detected a greater accumulation of precursors in the FDJ (Fig.29 C and D) probably indicating an aberrant production in the transit amplifying precursors pool.

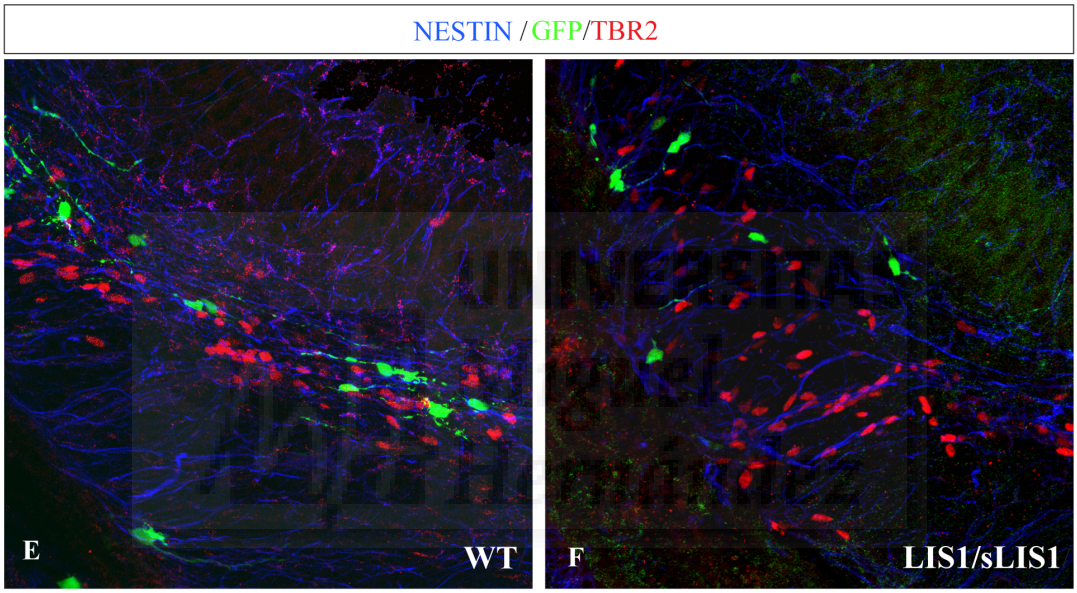

Fig.29 Nestin and Tbr2 positive cells defective distribution in postnatal Lis1/sLis1 mice
C) merged Tbr2+ (red) and GFP cells (green) migrating in the secondary matrix. D) in the Lis1/sLis1 mutant hippocampus the Tbr2+ population is augmented and dispersed in a wider stream, cumulating in greater amount in the FDJ (arrow). E) and F) higher magnifications (40x) of the wild-type and mutant secondary matrix respectively. Scale bar: 100μm

Altogether those data demonstrate that in Lis1/sLis1 mutant mice subgranular dentate progenitors trafficking is impaired and slowed due to a disorganization of the radial glial fiber scaffolding. Moreover, those defects arise important questions about the possibility that subgranular dentate precursors amplification in the migration stream could be also defective and impaired in these mutant mice. However, further studies at older stages of DG development are necessary in order to investigate how those affected

migrations can led to an abnormal development of the DG progenitor niche and affect to the subsequent adult hippocampal neurogenesis.

DISCUSSION

Section I

During brain development, neurogenesis and cell migration are crucial processes for brain structural organization and therefore these processes are highly regulated for the correct neuronal networks formation. During all postnatal life, neurogenesis and neural migration appear conserved restricted to specific forebrain areas of secondary neurogenesis: the SVZ and the DG. Those germinative areas are thought to be necessary to ensure specific functional properties of olfactory bulb and hippocampus that require the maintenance of new neuron production and for their correct integration into pre-existing neuronal networks (van Praag et al., 2002; Carleton et al., 2003; Mizrahi 2007). The presence of preserved migratory routes such as the RMS in developing and postnatal rodent brain, as already described in previous works (Doetsch F et al., 1999; Temple 2001; Alvarez-Buylla and Lim 2004), is known to be fundamental for olfactory interneurons production throughout life (Lledo PM et al., 2006). Thus, the identification and the study of different postnatal migratory pathways is an important question to be addressed especially for its implications in brain repair and plasticity.

In the first part of this work, we reported evidences about the persistence of a caudal migratory stream connecting the cSVZ with the hippocampus and the maintenance of this pathway results in a postnatal cellular contribution.

1. The early postnatal cSVZ showed conserved characteristic of the embryonic CGE

As development proceed, the neuroepithelial germinal zone is progressively reduced and neural precursors persist confined in the SVZ. However, there are intrinsic regional characteristics of specific transcription factors expression defining different progenitor cell specification that are maintained even postnatally, suggesting that some aspects of the embryonic patterning are conserved in the adult SVZ (Young K et al., 2007).

In the present work, we decided to analyze the main morphological characteristics of the early postnatal cSVZ respect to its embryonic counterpart, the CGE (Nery et al., 2002). Our results showed that the expression patterns of the main CGE transcription factors are maintained postnatally inside the cSVZ as confirmed by Dlx1/2 and Mash1 mRNA distribution; which correlates with migration-permissive populations and interneuron specification properties (Marin and Rubenstein 2001, 2003). In addition, we confirmed that the postnatal cSVZ was positive for the COUP-TFII transcription factor, a marker described in Cajal-Retzius cells (Tripodi et al., 2004) and in some caudally migrating interneurons derived from the CGE (Kanatani et al., 2008), thus showing conserved postnatal CGE characteristics. Although the MGE is known to be the primary source of hippocampal GABAergic interneurons, different works described that also CGE cells were migrating directed to the hippocampus and the cortex passing through the SVZ/lower intermediate zone and generating mostly calretinin interneurons (Nery et al., 2002; Xu et al., 2004; Butt et al., 2005). Based on these observations, we propose that the postnatal cSVZ acts postnatally as a continuity of the embryonic CGE maintaining a caudally migrating cell population.

2. The postnatal cSVZ showed a strong caudal migratory potential

With *ex vivo* experimental embryology experiments we firstly demonstrated that the postnatal grafting of the SVZ not only showed GFP cells migrating rostrally (RMS) but also the presence of a caudal migration directed to the corpus callosum, subcortical white matter tracts and the hippocampus (Fig.16). These results are consistent with previous works describing the presence of a caudal migratory stream during mid-embryonic stages (Yozu M et al., 2005; Kanatani et al., 2008; Lopez-Bendito G et al., 2004), nevertheless little is known about the maintenance of this stream during postnatal stages.

The presence of secondary neurogenic migratory currents have already been reported by different studies demonstrating how in the postnatal/adult brain a strong level of plasticity is maintained allowing the presence of migratory pathways starting from the SVZ and directed to different brain regions involving caudal and ventral forebrain structures (Kakita and Goldman, 1999; Suzuki and Goldman, 2003; De

Marchis S et al., 2004; Navarro-Quiroga I et al., 2006; Inta D et al., 2008). According to these observations, our data suggests the existence of a conserved postnatal migration that starts from the cSVZ niche and is directed to the hippocampus generating an important cellular contribution with important consequences on hippocampal plasticity and function.

These observations were widely confirmed even *in vivo* using grafting experiments of cSVZ derived cells and we were able to reproduce the caudal journey during the two first postnatal weeks and to find integrated GFP-cSVZ derived cells inside the hippocampal DG. Similarly, it has been already described that selected FACS-sorted SVZ population NG2-positive cells (Aguirre A et al., 2004) were able to migrate and integrate in the postnatal hippocampal network supporting our observations about the presence of backward permissive cues of migration inside the postnatal brain in the opposite direction respect to the classical rostral ones. Related to this, we could demonstrate that cSVZ GFP grafted cells were able to generate neurons and glial cells in vitro (Fig.19) but when injected caudally migrating cells were a different migrating population from the one described by Aguirre and colleagues, as showed by negative staining for NG2 marker. In fact, the immunohistochemical analysis of grafted cSVZ cells showed their belonging to the neuronal lineage as confirmed by DCX and Tuj1 positivity (Fig.18).

3. Postnatal cells in the caudal migratory stream are migrating neurons

The immunohistochemical characterization of transplanted SVZ derived cells revealed their neuronal fate as showed by their preferential expression for DCX marker (Fig.18) instead of GFAP marker. However, few GFAP+ cells were found in the injection site and into immature hippocampal migratory currents probably due to the manipulation of the graft. Nevertheless, further characterization experiments using in vivo electroporation (Fig.21) and in vivo lentiviral infection of the cSVZ (Fig.22) confirmed the neuronal phenotype and the ability to express proneural markers.

Several reportings on embryonic CGE cells fate (Nery et al., 2002; Xu et al., 2004; Butt et al., 2005; Kanatani et al., 2008) described caudally migrating cells as

calretinin interneurons (CR) positive for the CoupTFII transcription factor, suggested as a determinant factor in the generation of GABAergic diversity (Fuentealba P. et al., 2010). Consistent with those data, our analysis after *in vivo* electroporation of the cSVZ confirmed the co-localization of CoupTFII and GFP in cSVZ GFP-electroporated cells (Fig.20). In addition, we could confirm the co-expression with CR whereas other markers for distinct interneuron populations, such as PV or CB, resulted negative as expected (Fig.21). Therefore, our postnatal integrated neurons in the DG could represent immature interneurons that did not yet showed the typical GABAergic phenotype or the typical DG granular neurons although being integrated inside the DG circuitry. This discrepancy about GFP integrated cell phenotype can be explained with recent data proposing that the CGE can generate both type of neurons (inhibitory and excitatory) depending on the timing of neurogenesis and the differential combinatorial gene expression in the cells, as occurr for example for some CGE cellular contributions to the amygdala complex (Tang K et al., 2012).

However, lentiviral labeling of cSVZ gave lineage informations related to CR co-localizing cells and thus confirmed that late migrating neurons from the cSVZ are preferentially indentified as to be the postnatal contribution of CR CGE derived interneurons (Nery S. et al., 2002; Inta et al., 2008).

4. FGF signaling acts as an attractive cue on postnatal cSVZ cells

From our *in vivo* data emerged that FGFr1 was expressed on caudally migrating cells thus suggesting a possible implication of the FGF signaling in regulation of the caudal migratory stream (Fig.22 I-L' and Fig.23 A-A').

FGFs and their receptors constitute an elaborate signaling system that participates in many developmental and repair processes in mammal central nervous system including migration, neurogenesis, differentiation, axonal branching and neuron survival. Different studies in mutant mouse lines of Fgfr1 described alterations in hippocampal development and dysgenesis of dorsal telencephalic commissures including the CC and the hippocampal commissure (OhkuboY et al., 2004) and the complete lack of OB (Hebert JM et al., 2003) suggesting an antero-posterior structural

perturbation affecting both rostral and caudal regions. Those data together with other reported evidences about FGF signaling action during migration (Osterhout DJ et al., 1997; Pombero A et al 2011) were in agreement with our observations, which firstly describe FGF8 in promoting migration on cSVZ cells. Accordingly, we showed *ex vivo* experiments of FGF8-bead implantation where FGF8 signal promotes a strong migration acting as a guidance attractive cue. Moreover, those data were confirmed *in vitro* by an assay of migration demonstrating the attraction exerted by FGF8 on cSVZ cells expressing the receptor Fgfr1 as revealed by FISH staining (Fig.22 I-L'' and Fig.24 E-E'). However, this strong attraction that we reported in postnatal migration has to be further analyzed due to the complex cascades and pathways that can be generated by the interaction of FGFs and their differential binding affinity for many FGFrs. Interestingly, this signaling pathway seems to be recapitulated as attractant into the DG region by grafted neural crest stem cells from adult human periodontal ligaments (Bueno C et al., 2013).

Section II

The DG is one of the two forebrain locations in which neurogenesis continues until adult life and its development is characterized by a prolonged developmental program starting from embryonic and ending only until late postnatal stages (Altman and Bayer 1990a,b; Pleasure 2000; Li and Pleasure 2005). As described by classical neuroanatomical studies during mid-gestation the dentate neuroepithelium constitute the embryonic niche where dentate precursors start to proliferate and then migrate seeding the nascent granular cell layer of the DG (Nowakowski and Rakic, 1981; Eckenhoff and Rakic, 1984; Altman and Bayer 1990a,b). Different studies described the presence of transient cellular migrations during DG development reporting the important role of specific factors guiding the proper migration of dentate precursors (Bagri et al., 2002a; Lu et al., 2002; Li G et al, 2009; Hodge et al., 2013), however little is known about the origin of SGZ precursors, weather their postnatal maintenance may involve different cellular mechanisms, including ventricular origin and migration.

1. Molecular characteristics of postnatal DG morphogenesis

Different studies have suggested that the presence of morphogenes and different transcription factors are instructive signals for the microenvironment of the neurogenic niche to be maintained, for an appropriate cellular differentiation and for DG proper formation (Furuta Y et al., 1997; Grove EA et al., 1998, Lee SM et al., 2000; Bagri A et al., 2002a). From our analysis of different expression patterns of the Allen Brain Atlas Database we could conclude that at least two different cellular populations are migrating during DG development following distinct pathways in order to reach different positions into the DG layers: the dentate precursors and immature granule cells. In particular, the study of Tbr2 transcription factor clarified the presence of another population of intermediate progenitors (IPs), which perform a specific subpial

current which starts in the secondary matrix route and migrate all along the molecular layer of the forming DG around P0-P4.

Tbr2 was already mentioned as a critical regulator of neurogenesis in the developing and adult DG (Machon O et al., 2007; Hodge RD et al., 2008; Roybon L et al, 2009; Hodge RD et al., 2012) thus confirming that its expression pattern correlates with dentate intermediate progenitors migration and maturation (Li G et al, 2009, Hodge RD et al., 2013). During early postnatal stages, those intermediate immature cells were found to generate a transitory layer during their migration, the SPZ, with cells occupying the most external layers of the developing DG (Fig.25 C, black arrow). All these observations, together with our analysis of other gene expression patterns related to dentate progenitors neurogenesis and migration, gave us more cues about the existence of heterogeneous populations of precursors (NSCs), intermediate amplifying progenitors (IPs) and immature neurons in the CA3alv region that during these postnatal stages are migrating following different routes and at different immature states. In agreement with that, the Nrg2 positive dentate population was demonstrated to express this proneural gene transiently before to differentiate into granule neurons (Galichet C et al., 2008). Accordingly, although the secondary matrix and the subpial streams were already described as fundamental steps during DG formation, the postnatal characterization of dentate precursors routing and the presence of different immature states of those migrating precursors are crucial aspects of DG development that remain to clarify. Beside that, we showed that different transcription factors included in our study reproduced Tbr2 distribution (Ascl-1, Gabrd, Ngn2 in Fig.25 A-H') confirming the presence of an heterogeneous population of precursors inside these dentate migratory routes and reflecting a complex machinery which fine tune this trafficking of migrating precursors. Related to that, it was already described that dentate precursors migrate along different routes while seeding the nascent DG, also forming a cellular transitory stream in the subpial zone (Galichet C et al., 2008; Li G et al., 2009), however the CA3alv radial migration and all its cellular postnatal contributions which led to the final SGZ conformation have been poorly investigated.

Interestingly, the patterns of expression analyzed trough different postnatal stages of DG development showed clearly that cells in between P4 to P14 pass through outside-in and inside out radial movements in order to find and settle into their final destinations inside the different DG layers. In particular, the comparison of the pattern distribution at P4 of dentate progenitors (Fig.25 A', C', E', G' in higher magnification)

inside the different layers of the forming DG revealed that the reorganization process is still ongoing and that the majority of precursor cells are positioned mainly in outer DG layers. Concomitantly, other gene expression patterns (Fig.25 I-Q') related to granular immature cells condensed in the hilar region (tertiary matrix) and migrate are radially inside-out to generate the presumptive granular layer (GCL). Clearly, by P14 all migratory pathways are reorganized and showed a different distribution inside the different DG layers and the SGZ appear now as a completely formed structure (Fig.25 K',M', O', Q').

In summary, the study of all those expression patterns helped to better describe the presence of two different migratory populations, dentate progenitors and immature granule cells, and opens important questions on their implications in the formation of the SGZ during fundamental steps of DG development.

2. The postnatal radial glial scaffolding in the CA3alv is severely compromised in Lis1/sLis1 mutant mice

Since the secondary matrix stream is generated first during mid-embryonic development but its migration proceeds until early postnatal stages contributing to produce other important cellular structures, we decided to focused our attention on the deep understanding of its main characteristics after birth. In particular, this secondary matrix was defined as an important source of different dentate precursors and granule immature neurons responsible of the correct germinative matrices organization (Nowakowski and Rakic 1979, 1981; Altman and Bayer 1990a,b), however the consequences of defective radial migration of the matrix during postnatal stages has been poorly studied.

In this study we analyzed the organization of the CA3alv during hippocampal postnatal development in a mutant mouse line with a genetic reduction of Lis1 protein (short Lis1, sLis1), which is already known to be implicated in neuronal migration defects (Hirotsune S et al., 1998; Gambello MJ et al., 2003; McManus MF et al., 2004). Our data illustrate that the radial glial scaffolding is severely compromised in the Lis1/sLis1 mouse hippocampus, especially where the secondary matrix takes place as suggested by the GFAP and Nestin immunostainings in Fig.26 (Fig.26 E-H) and Fig.27 (Fig.27 E-H). In this heterozygous mutants one copy of the Lis1 protein is truncated and

not functional and furnish a good animal model for the study of the migration defects derived from this mutation as well as acting as a good model for the study of the human lissencephaly syndrome. Cellular lamination defects were already described in another adult Lis1 transgenic model in which was generated a haploinsufficiency for the protein (Wang Y and Baraban SC 2008), suggesting the presence of migration defects responsible of this cellular disorganization. However, our model resulted closer to the human disease because generated from just a genetic reduction of the wild type protein instead of a knock out, thus reproducing better the pathologic situation. In addition, in our mutant we could describe a strong phenotype affecting the radial glial scaffolding organization during hippocampal development. In particular, in the postnatal study of the secondary matrix radial glial scaffolding conformation, we could observe dramatic morphological defects affecting radial glial cells shape and fiber organization (Fig. 26 D, H and Fig.27 D, H). These characteristical changes in morphology that we observed are in agreement with the results obtained years ago using RNA interference (RNAi) experiments performed by Tsai and colleagues (Tsai JW et al., 2005, 2007), which contribute to reveal that Lis1 is important for corticogenesis, including transformation of multipolar cells into locomoting cells, nuclear translocation and proliferation of neural progenitors (Tsai JW et al., 2005).

3. Postnatal *in vivo* labeling of dentate progenitors revealed migratory defects due to unproper radial glial scaffolding

Lis1 protein was already described to be associated to both radial and tangential migration defects (Hirotsune S et al., 1998; Cahana A et al., 2001; Tsai JW et al., 2005, 2007), however its role during hippocampal development, in which different radial movements such as in the secondary matrix dentate stream formation are occurring, its still poorly understood.

Using in vivo electroporation we studied the migratory defects present in our Lis1/sLis1 mutant mice during early phases of DG development. Our results indicate that the secondary matrix dentate stream formation was compromised in P4 Lis1/sLis1 mice and the defective radial glial scaffolding seemed to slow this migration forcing dentate precursors in the hippocampal SVZ (Fig.28 B, B'). Together with these cell-autonomous effects exerted by Lis1 protein in neuronal migration, as previously

demonstrated using *in vitro* experiments (Hirotsune et al., 1998; Gambello MJ et al., 2003), this protein was described to have also a non-cell-autonomous effect as described for example for tangential migration (McManus et al., 2004). In fact, using embryonic brain slice transplants, from wild-type into a *Lis1* mutated context, they were able to demonstrate alterations in interneuron migration due to the migration substrate, suggesting that *Lis1* mutation in our mutant could affect radial glial scaffolding also in a non-cell-autonomous manner.

In agreement with our data on affected radial glial cells, the presence of granule cells dispersion and GFAP-cells with aberrant orientation were already described in adult hippocampus of haploinsufficient Lis1/sLis1 mice (Wang Y and Baraban SC 2008) with defects in cell proliferation, migration and differentiation in the SGZ of adult DG (Wang Y and Baraban SC 2007) thus reflecting that alterations occurring during DG morphogenesis can led to defects that are maintained in the progenitor DG niche until adult life.

According to this, further analysis of mutant Lis1/sLis1 electroporated mice revealed that the intermediate progenitor population Tbr2+ was also affected which probably will have consequences in the progenitor pool amplification. Here in this work we reported that the secondary matrix stream Tbr2+, which connects with the subpial dentate route, is wider in the Lis1/sLis1 with more dispersed IPs in the whole route of migration (Fig.29 E and F). These data are in agreement with other studies in which an abnormal proliferation was detected in the presence of *Lis1* mutation (Yingling J et al., 2008; Pramparo et al., 2010) and consistently with our hypothesis that the transit amplifying pool of intermediate progenitors is also affected.

CONCLUSIONS

Section I

1. The cSVZ represent the postnatal CGE and is a conserved germinative structure that maintains the same embryonic molecular characteristics of Dlx-2 and Mash-1 expression patterns and the specific CGE marker CoupTF-II, thus suggesting a common molecular signature.

2. During early postnatal life cSVZ derived cells are able to perform a caudal migration trough the caudal migratory stream (CMS), a conserved pathway of neuronal migration, which is conserved postnatally, and constitute an external cellular contribution directed to the hippocampus.

3. cSVZ cells display a neuronal phenotype as suggested by the presence of DCX and CR markers and are able to reach the dentate gyrus and settle into the pre-existing hippocampal cytoarchitectony.

4. Fgf signaling appears to exert an important attractant role in the caudal cSVZ postnatal migration as widely explored *in vitro*. In fact, the interaction between Fgfr1 present in cSVZ migrating cells and the Fgf8 expressed in the hippocampus suggests an important mechanism of neuronal attraction.

Section II

1. The analysis of different gene expression patterns in the hippocampus revealed the presence of different postnatal migratory radial routes contributing to the generation of the DG granular neurons and subgranular progenitors.

2. The study of the secondary matrix radial glial migration in mutant Lis1/sLis1 mice revealed the presence of defective radial glial scaffolding in the developing hippocampus.

3. *In vivo* electroporation of newborn Lis1/sLis1 mice showed defective dentate migration, revealing that CA3alv migratory route is necessary for proper dentate precursors migration.

4. Intermediate progenitor cells Tbr2+ in *Lis1/sLis1* mice were abnormally distributed along the secondary matrix route always due to a defect of the radial glial scaffolding and probably to proliferation defects of the transit amplifying pool.

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