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Emx2 expression levels in NSCs modulate astrogenesis rates by downregulating *EgfR* and *Fgf9*

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A te che hai reso la mia vita bella da morire

I, Carol Filippis, declare that the experimental data reported in this thesis are original and generated by me myself during my PhD work at SISSA. The experimental data employed for the compilation of this thesis have been supplemented with additional data kindly provided by:

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Abstract

Generation of astrocytes within the developing cerebral cortex is a tightly regulated process, which bursts after neuronogenesis completion and precedes the bulk of oligodendrogenesis. Astrocytic outputs depend on two primary factors: progression of multipotent precursors towards astroglial lineages and proliferation of astrocyte-committed progenitors. Molecular mechanisms controlling lineage choice have been investigated and reconstructed to large extent. On the contrary, very little is known about control of astroblast population kinetics.

In this study we show that Emx2 inhibits astrocyte progenitors proliferation by repressing *EgfR* and *Fgf9*, and report details of molecular mechanisms mediating such repression. Indeed we demonstrated that *Emx2* overexpression in cortico-cerebral stem cells decreased proliferation within the astrogenic lineage, resulting in a severe reduction of the astroglial outcome. We also showed that this was caused by *EgfR* and *Fgf9* downregulation. The former originated from exaggerated Bmp signalling. The latter was due to *Emx2*-dependent suppression of the *Sox2* activation elicited by *Brn2.*

Finally, we provided evidence that temporal progression of Emx2 levels in neural stem cells confines astrogenesis to postnatal life.

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INTRODUCTION

1. Cerebral cortex Development: main features

The development of the Central Nervous System (CNS) is a complex process that begins in the early phase of the vertebrate embryogenesis. The CNS arises from a simple sheet of ectoderm. Diffusible signaling proteins (like Follistatin, Noggin and Chordin) released from a particular "organizer" region of the gastrula called the Spemann node (in amphibian eggs) or Hensen node (in chick and mammalian embryos) promote the formation of the neural plate: a cytologically homogeneous sheet of neuroephitelial cells (NE) from which the nervous system will develop.

The edges of neural plate thicken and move dorsalward along the entire length of the plate, forming the neural folds, which fuse at the midline giving rise to the neural tube (**Fig. 1**).

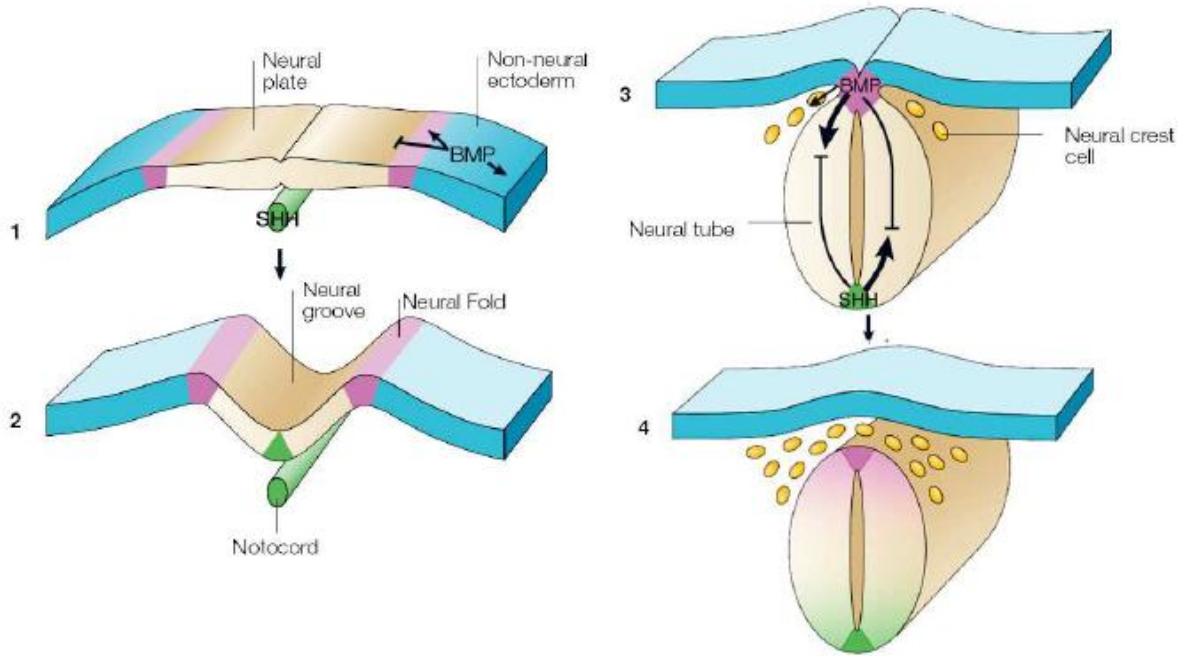

Fig 1: Schematic view of the neural tube 1) opened neural plate 2) neural grove formation 3) closed neural tube 4) delaminating neural crests. Modified from Gammill *et al.*;2003.

The process by which the neural plate is formed and converted into a neural tube is called neurulation.

Some of the cells close to the neural folds aggregate between the neural tube and overlying ectoderm. These cells compose the neural crest. Neural crest cells will migrate

away from the neural tube and will generate a wide variety of peripheral tissues, including neurons and satellite cells of sensory, sympathetic and parasympathetic nervous system, cells of the adrenal medulla, pigmented cells of epidermidis and bones and connective tissue in the head.

As the neural tube develops, the anterior (cephalic or rostral) portion of the neural tube undergoes a series of swellings , constrictions and flexures that will form anatomically defined regions of the brain; while the posterior (caudal) portion of the neural tube retains a relatively simple tubular structure and will form the spinal cord.

Before the closure of the neural tube, the neural plate becomes subdivided along the anteroposterior axis into three distinct domains, corresponding to the three primary vesicles: the prosencephalon (the forebrain), the mesencephalon (midbrain) and the rhombencephalon (the hindbrain) (**Fig. 2A**). As development proceeds, the prosencephalon will divide into two parts - the telencephalon and the diencephalon. The rhombencephalon will also divide into two parts - the metencephalon and the myelencephalon (**Fig. 2B**).

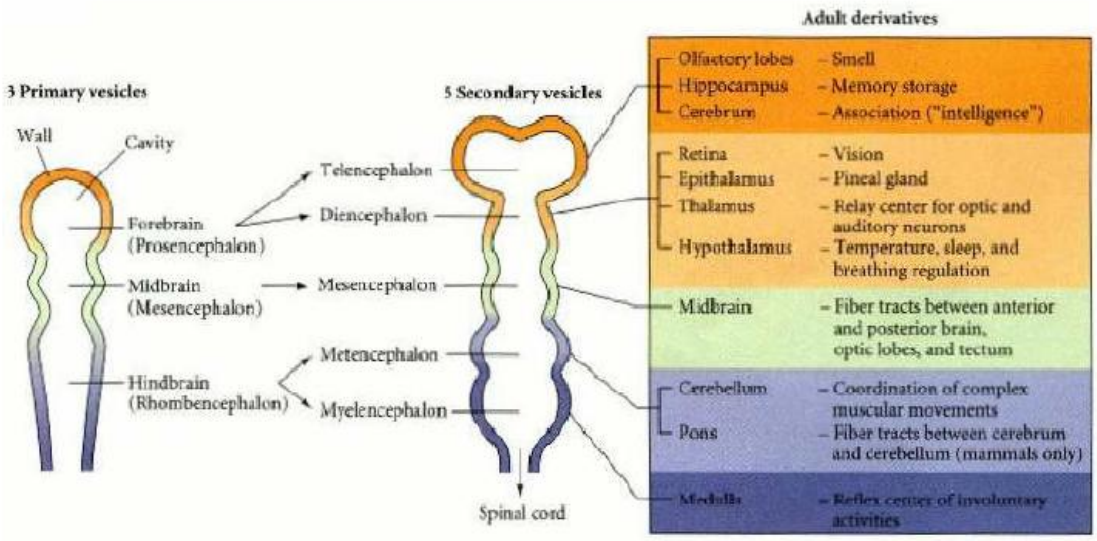

Fig 2: Schematic view of the anterior neural tube. (A) three-vescicle stage: (B) five-vescicle stage. Adapted from Gilbert Developmental Biology

The wall of the neural tube is initially composed by a single layer of cells. Each cell extends from the luminal, or ventricular, edge to the external, or pial, surface. As each cell progresses through the cell cycle, its nucleus migrates up and down between the

ventricular and pial surfaces, performing the so-called intermitotic nuclear migration (INM). Soon after cell division, one or both the daughter cells may lose their contact with the ventricular surface and migrate away. This is the point at which they become committed progenitors, neurons or glial cells. Once they migrate away from the ventricular zone, cells destined to become neurons are *often* postmitotic: they will never divide again. On the other hand, glial cells precursors can divide even after they have reached their final locations. As more and more postmitotic cells are produced, the neural tube thickens and assumes a three-layer configuration:

- an innermost ventricular zone (where proliferation continues) (VZ)
- an intermediate mantle zone containing the cell bodies of the migrating neurons
- and a superficial marginal zone composed of the elongating axons of the underlying neurons (MZ)

This three-layered structure persists in the spinal cord (Nicholls, Martin, Wallace, Fuch). The telencephalic vesicles occupy the most rostral portion of the neural tube and can be subdivided into a dorsal (pallial) and a ventral (subpallial) territory. The ventral telencephalon (or subpallium) generates the basal ganglia: the more ventral located part is the medial ganglionic eminence (MGE) of the the globus pallidum, the more dorsal one is the lateral ganglionic eminence (LGE), which generates the striatum, and a third caudal part is called caudal ganglionic eminence (CGE) and supplies for the amygdala (**Fig. 3**).

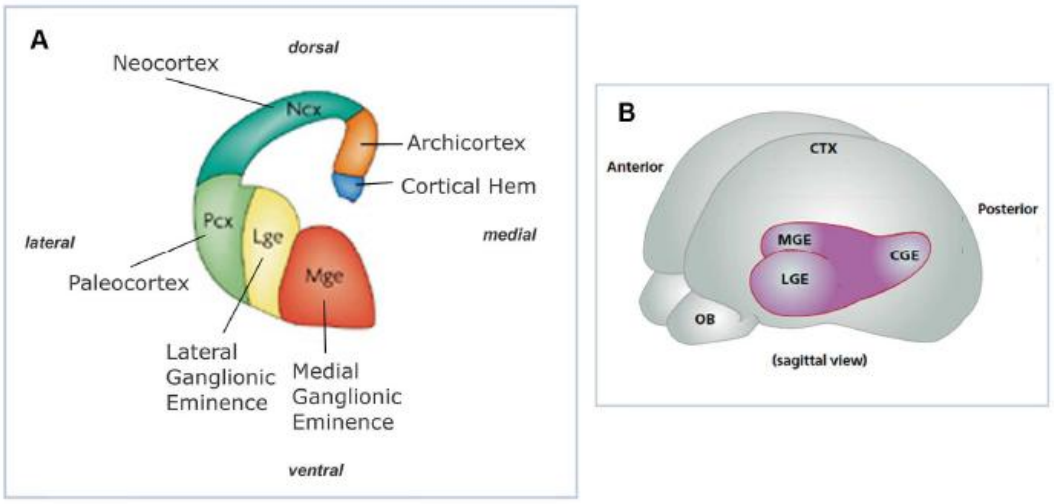

Fig.3 (A) Schematic view of a coronal section through the developing mouse telencephalic vesicle at E12. Image modified from Corbin *et al*., 2001. (B) Sagittal view of the embryonic vertebrate telencephalonb as transparent structure to reveal the ganglionic eminences CGE (Caudal Ganglionic Emince); CTX (Cortex); LGE (Lateral Ganglionic Eminence); OB (Olfactory Bulbs). Image modified from Molyneaux *et al*., 2007

The dorsal telencephalon (pallium) gives rise to the archicortex (subiculum, hippocampus and dentate gyrus), the neocortex and the paleocortex, from medial to lateral (Puelles *et al.*, 2000).

1.1 Antero-posterior (Rostro-caudal) patterning

Rostro-caudal (RC) patterning is the process that leads to the generation of distinct transverse domain at different axial position in the central nervous system. It begins during early gastrulation. Indeed the first and most evident process occurring in the mouse developing nervous system from E8.5 on is the regionalization along the antero-posterior axis (A/P). From E10.0 forebrain, midbrain, hindbrain and spinal cord domains are formed. The patterning of these regions are associated with precise antero-posterior expression domains or gradients of several regulatory genes coding for transcription factors.

The early patterning of both anterior and posterior neural tissue is mediated by signals emanated from the primitive node or organizer, known as Hensen's node in chick and mammalian embryos and Spemann organizer in amphibian eggs. Studies in mammals indicate that, in addition to the organizer, the Anterior Visceral Endoderm (AVE) is required for head induction and maintenance (de Souza & Niehrs 2000) (P Thomas &

Beddington 1996) (**Fig 4)**. The AVE is an extraembryonic tissue that underlies the future neural plate.

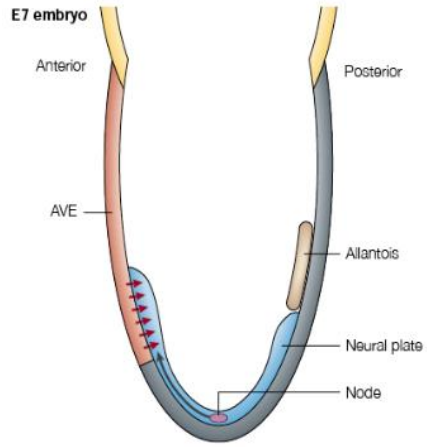

Fig.4 . Signals and tissues involved in inducing anterior neural character (Rallu et al. 2002)**.** Signals that come from the node establish gross anterior pattern (black arrow). The anterior visceral endoderm (AVE), together with the node, acts to induce and/or maintain anterior neural character. The AVE is located beneath the future neural plate and expresses molecules, such as Cerberus and dickkopf (red arrows), that inhibit factors that would otherwise act to posteriorize the anterior neural plate (Rallu et al. 2002).

Removal of the AVE from mouse embryos at early stage of gastrulation leads to a loss or reduction of forebrain markers expression (P Thomas & Beddington, 1996). On the other hand, transplantation of the mouse AVE in the chick embryos results in the expression of the forebrain markers (CD Stern, 2001).

The main molecules which induce posterior identity in the neural plate are:

- Wnts: are mainly secreted in the posterior territory, where they inhibit *Otx2* and *Six3*, which will co-specify the most anterior part of the brain. Conversely, Wnts induce the expression of the *Gbx2* and *Irx3*, determining more caudal fates.
- Fgfs (Fibroblast Growth Factors)*: Fgf8* promotes *Foxg1* expression in the rostral brain, while activating *Engrailed2* in the midbrain field
- Retinoic Acid (RA)

Conversely, antagonists of these factors are released from AVE, are expressed in the anterior visceral endoderm and act to maintain and stabilize the anterior neural plate identity (**Fig 5**). Cerberus and Dickkopf are only two of the many proteins that block the signaling pathway of the "posteriorizing" factors. Among these proteins there are also chordin, noggin follistatin and the Frizzled-related protein, Frzb (Thomsen 1997)

(Wessely & De Robertis 2002), which all seem to act in the specification of the forebrain.

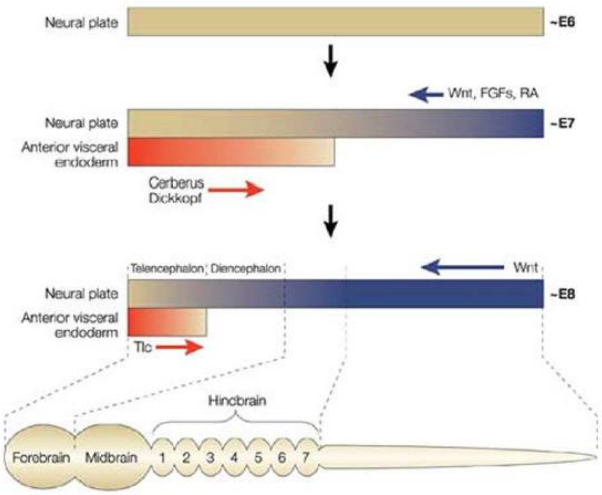

Fig 5 Antero/posterior patterning of the early mouse neural tube. Image adapted from Rallu *et al.* 2003

Morphogenetic controlling processes at specific locations of the developing neural primordium have led to the concept of secondary organizers, which regulate the identity and regional polarity of neighboring neuroepithelial regions (Ruitz i Altaba 1998) (Figdor & C D Stern, 1993) (Echevarria et al., 2003). These organizers usually develop within the previously broadly regionalized neuroectoderm at given genetic boundaries and their subsequent activity refines local neural identities along the AP and DV axes, patterning the anterior neural plate and neural tube (Meinhardt 1983)(Figdor & C D Stern 1993)(J L Rubenstein et al. 1998) (Joyner et al. 2000).

Along the AP axis, secondary organizers are: the anterior neural ridge (ANR), at the anterior end of the neural plate/tube; the zona limitans intrathalamica (ZLI) and the isthmic organizer (IsO) at the mid-hindbrain boundary.

Soon after neural induction is initiated, the anterior neural ridge (ANR) play an important role in promoting telencephalic development within the forebrain territory (Shimamura & J L Rubenstein 1997)(Houart et al. 1998)(Tian et al. 2002)(Echevarría et al. 2003). It begins to express *Fgf8* which in turn promotes the expression of *Foxg1* in the early anterior plate cells that are destined to form the telencephalon (Shimamura & J L Rubenstein 1997). *Foxg1* is expressed in early telencephalon along a caudomedial^{low} – rostrolateral ^{high} gradient. It appears to be crucial for basal ganglia morphogenesis, cortical

neuroblast differentiation (Dou *et al.*, 2000; Hébert and McConnell, 1995 Seone *et al.*,2004; Martynoga *et al.*, 2005), as well as for the proper laminar histogenetic progression of cortical progenitors. In the absence of *Foxg1*, paleo and neocortex are undersized or even absent and the telencephalon becomes similar to an enlarged and distorted hippocampus (Muzio and Mallamci, 2005).

1.2 Dorso-ventral patterning

The specification of longitudinally aligned regions within the CNS involves patterning along the Medio-Lateral dimension of the plate. This ML patterning in the neural plate is topologically equivalent to Dorso-Ventral (DV) patterning in the neural tube.

The polarity of the neural tube is induced by signals coming from its immediate environment **(Fig 6).**

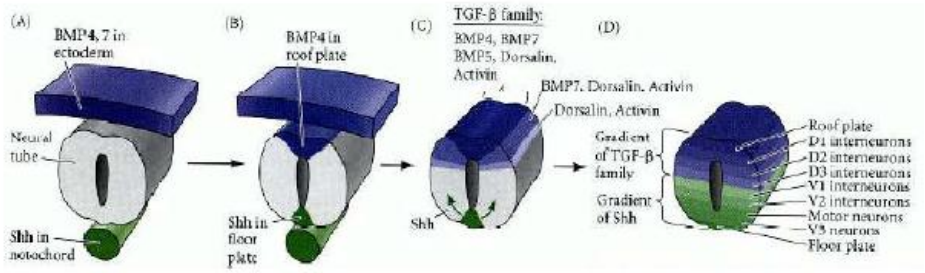

Fig 6. Dorsal-Ventral specification of the neural tube. (A)Two signalling centers influence the newly formed neural tube : the roof of the neural tube is exposed to BMP4 and BMP7 from the epidermis, while the floor is exposed to Shh protein from the notochord. (B) Secondary signaling centers within the neural tube. The roof plate cells express and secrete BMP4, the floor plate are a source of Shh protein . (C) BMP4 establishes a cascade of TGF factor, diffusing from the roof plate to the ventral neural tube. Sonic hedhog proteins spread dorsally as a gradient from the floor plate cells. (D) The several spinal cord neurons identities are established by the exposure to BMP4/Shh gradients of paracrine factors. (Adapted from *S. Gilbert*).

The DV patterning relies initially on external signals: at the earliest stages of neural tube formation, the neural epithelium is constrained between BMP-secreting tissue, defining the edge between neural and non-neural epithelium; at the same time, an external, mesodermic source, the notochord, secretes the morphogen *Sonic Hedgehog (Shh*), which is in turn also expressed in the adjacent midline neural tissue (Patten and Placzek 2002)(Lupo et al. 2006).

Then the most important signaling involved in the DV patterning are:

- *Shh* signaling is crucial for ventral patterning at all levels of the nervous system (Briscoe & Ericson 1999*). Shh* is first produced by the notochord (E7.5), and later its expression is induced in the overlying medial neural plate. Such early *Shh* expression commits the whole anterior neural plate to express *Nkx2.1,* a transcription factor specifying ventral identity (Ericson et al. 1995).
- *BMP* signaling is required for the formation of the dorsal midline, which gives rise to the choroid plexus and the cortical hem. *BMP* function is further regulated by inhibitors, such as *noggin* and *chordin*, secreted by ventrally-placed mesoderm (Patten & Marysia Placzek 2002)(Lupo et al. 2006).
- In addition to *BMPs, Wnts* are also involved in dorsal telencephalic development. *Wnts* factors, secreted at the border between the neural folds and the ectodermal field, activate dorsal, i.e. cortical, genes (Gunhaga et al. 2003) and at the same time antagonize *Shh* effects, so inhibiting *Nkx2.1* expression in the dorsal neural field.
- After the closure of the neural tube, retinoic acid (RA) synthesized by the adjacent lateral ectoderm, has a crucial role in specifying telencephalic cells of intermediate character. In particular it activates striatum-specific genes.
- Later, FGF signals derived from dorsal midline cells act together with *Wnt* signals to induce definitive dorsal/precortical character in early dorsal cells (Gunhaga et al. 2003).

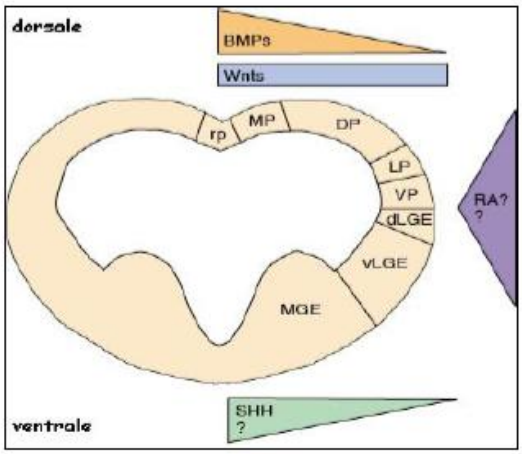

Fig 7.specification of distinct dorso-ventral territories within the E10.0 mouse telencephalon (adapted from Campbell K. 2003).
Four main classes of diffusible ligands partition the telencephalic field along the dorso-ventral axis: *BMPs* and *Wnts*, more abundant in dorsal-most territories, promote pallial identity; retinoic acid, released from the ectoderm which covers the lateral part of the vesicle, stimulates striatal programs; *Shh*, more abundant in ventral-most territories, promotes pallidal morphogenesis. Abbreviations: dLGE, dorsal lateral ganglionic eminence; DP, dorsal pallium; LP, lateral pallium; MP, medial pallium; rp, roof plate; vLGE, ventral lateral ganglionic eminence; VP, ventral pallium; RA, retinoic acid. Morphogens expressed in early patterning centers help establish the expres

Morphogens expressed in early patterning centers help establish the expression patterns of individual transcription factors (TFs) or combinations of TFs which play a prominent role in regionalization of the telencephalon, including establishing and maintaining the identities of the ventral and dorsal telencephalon and the general characteristics of specific cell types generated within them (Rallu et al. 2002).

Among the most important TFs involved in the DV specification there are *Emx2* and *Pax6* which are expressed in opposing and overlapping gradients in the dorsal telencephalon .

Emx2 is expressed along a caudomedial- rostrolateral decreasing gradient (Simeone et al., 1992; Mallamaci et al., 1998). This suggests the possibility that it could act as a promoter of caudomedial fates, which has been demonstrated by the shrinkage of the caudomedial areas in *Emx2*$^{-/-}$ mutans (Bishop *et al., 2000;* Cecchi *et al.*, 2000).

Pax6 is transcription factor which has been strongly conserved during evolution. It possesses two DNA binding domains and a paired-like homeodomain (Callaerts *et al.*,1997). It begins to be expressed in mouse since E8.0, only on the anterior surface ectoderm and neuroephitelium in the regions of the spinal cord, forebrain and hindbrain (Walther and Gruss, 1991; Grindley *et al*. 1995). Within the telencephalon its expression is confined in the dorsal part. This suggests *Pax6* may contribute to pallial *vs* subpallial

specification (Stoykova *et al*. 1997). *Pax6* seems to play a role which is complementary to that of *Emx2* in the determination of cortical area size and distribution along the rostrocaudal axis (Bishop *et al*., 2000)

Emx2 and *Pax6* are the key determinants of the proper development of cortical areas and are also required for establishing the identity of dorsal progenitors (Luca Muzio et al. 2002b). In mice deficient in either *Pax6* or *Emx2*, cortical gene expression patterns shift along the A/P axis, and the shifts are complementary in two mutants (Bishop et al. 2000). At least one functional allele of *Emx2* or *Pax6* is necessary and sufficient to activate cortical fate and suppress ventral telencephalic fate. In the absence of both genes, the cortex does not form and ventral progenitor domains expand across the entire dorsal telencephalon (Luca Muzio et al. 2002a).

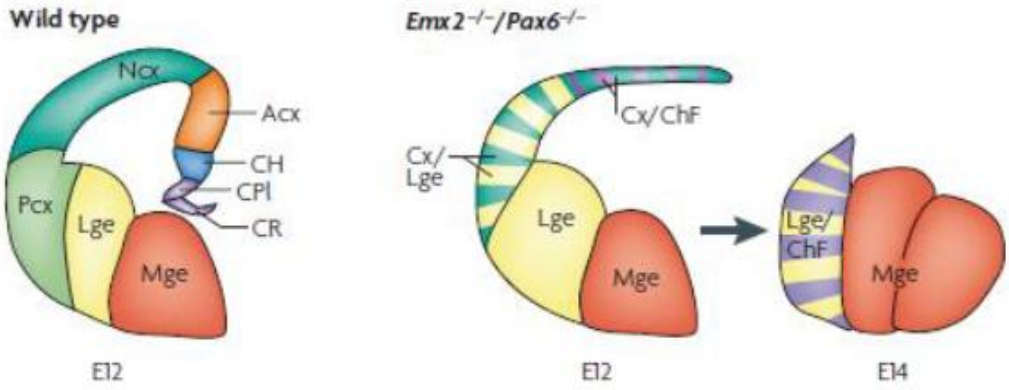

Fig 8. *Emx2* and *Pax6* have crucial roles in the specification of neocortical progenitors. Loss of both empty spiracles homologue 2 *(Emx2*) and paired box 6 *(Pax6)* results in ventralization of cortical progenitors and the loss of the neocortical domain (Ncx), archicortex (Acx), cortical hem (CH) and choroid plexus (CPl) by embryonic day (E) 14. CR, choroidal roof; ChF, choroid field (choroid plexus and choroidal roof); Cx, cortex; Lge, lateral ganglionic eminence; Mge, medial ganglionic eminence; Pcx, paleocortex. Modified from Muzio et al., 2002.

1.3 Arealization: specification of cortical areas identities

As corticogenesis goes ahead, distinct regions of the cortical primordium get more and more diversified in terms of neuronal packaging profile, neurocircuital blueprint and high-order computational capabilities.

Form a functional point of view, the neocortex can be subdivided in subdomains, called areas. Mature cortical areas differ by their location within the cortex, molecular properties, histological organization, patterns of connectivity and function (Sur and

Rubenstein, 2005). This process of inter-areal diversification is commonly referred to as cortical arealization and its cellular and molecular control has been subject of a hot scientific debate. Two main models were proposed: the protomap model (Rakic 1988) and the tabula rasa (or protocortex model (Van der Loos and Woolsey, 1973)(O'Leary, 1989).

According to the protomap model, cortical arealization occurs on the basis of molecular cues intrinsic to the cortical primordium. Spatial information, encoded by the patterned expression of the area-specific genes along the tangential axes of the early cortical proliferative sheet, would dictate temporal profiles of proliferation, apoptosis and differentiation, peculiar to different regions of this sheet. In this way, axial (i.e. tangential) values encoded by neural precursors of distinct regions dictate the final positional identity of areas originating from these regions, as well as the thickness of distinctive layers within such areas. The same information would also be transferred- in a more elaborated and stable format- to newborn neurons, which would retain it, while migrating along rails of radial towards their final laminar locations. They would rely on this information while executing distinct area- specific differentiation programs (chemoelectrical, cytoarchitectonic and neurocircuital ones).

Conversely, according to the tabula rasa model, distinct regions of the cortical primordium would not display any areal bias at all. Arealization would occur on the basis of information carried to the developing cortex by cortical afferents. This information would be used to "write" distinctive areal programs onto this blank cortical sheet, just like onto a *tabula rasa.*

Protomap and tabula rasa are not intrinsically antithetic and, as presently accepted, both catch key aspect of cortical arealization. According to the modern synthesis, the early steps of arealization take place on the basis of information intrinsic to the cortical primordium, as in the protomap model. That predisposes the different regions of such primordium to be differentially targeted by axons coming from distinct parts of thalamus. Then after the arrival of these axons (from E13 onward) and as postulated in tabula rasa model, cortical arealization is refined, based on distinct patterns of information brought by these projections (Sur and Rubestein, 2005).

Special relevance to the whole process is given to a particular development window, from E 10.5 to E 12.5 when cortical neuroblasts are areally committed or determined, i.e. their areal potencies become restricted in a progressively less reversible way.

At the moment, two main classes of molecules are supposed to be crucial for early arealization of the cortical primordium:
-Secreted ligands, released around the borders of the cortical field by signaling center.

-Transcription factors, gradually expressed within primary proliferative layers of this field.

Moreover, there are three candidate signaling centers lying at the borders of the cortical field, which are relevant for its arealization:

-The commissural plate (at the rostromedial pole of telencephalon). From earlier than E 10 to ~ E 12.5, the commissural plate (which derives from the ANR at the closure of the anterior neural folds), and its surrounding regions release *Fgf8, 17* and *18*, which promote rostral vs. caudal areal programs (Bachler & Neubüser 2001).

-The cortical hem (which forms between the cortical and the choroidal fields, at the caudiomedial edge of the cortical neuroepithelial sheet). From E10 it is a source of Wnts (Wnt2b, 3a, 5a, 7b, 8b) and bone morphogenetic proteins (Bmp2, 4, 6, 7), expressed in nested domains which also span the adjacent dorsomedial cortical field (Furuta et al. 1997)(Lee S M et al. 2000).

-The cortical antihem (which forms on the lateral side of the cortical field, at the pallial-subpallial boundary). Around E12.5 and afterwards, neural progenitors within the antihem specifically express five secreted signaling molecules: Fgf7, the Wnt-secreted inhibitor *Sfrp2* and three Egf-related ligands, *Tgf-α, Nrg1* and *Nrg3* (Assimacopoulos S., et al. 2003).

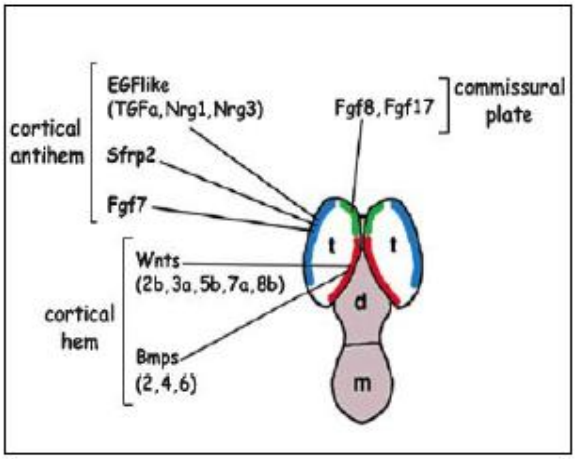

Fig. 9: Expression patterns of secreted ligands (A. Mallamaci & A. Stoykova 2006) Abbreviations: t, telencephalon; d, diencephalon; m, mesencephalon.

Secreted ligands, diffused trought the cortical morphogenetic field, regulate the expression of cortical TF genes in a dose-dependent manner. Graded and transient expression of these factors would finally encode for positional values, peculiar to distinctive regions of the cortical field. Among these transcription factors genes the most important are: LIM homeobox 2 (*Lhx2*), Forkead box G1 (*Foxg1*), Empty spiracles homologue 2 (*Emx2*) and Paired box 6 (*Pax6*). Each of these genes has crucial roles in specifying the progenitors in neocortex. Together these for genes establish the neocortical progenitor domain by repressing dorsal midline (*Lhx2* and *Foxg1*) and ventral (*Emx2* and *Pax6*) fates.

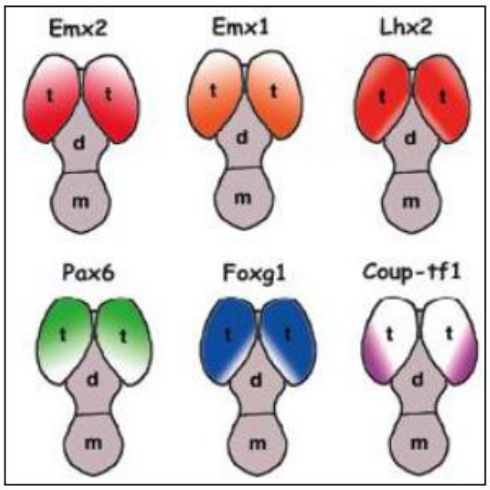

Fig.10: Graded transcription factor genes in the early cortical primordium E12.5. Abbreviations: t, telencephalon; d, diencephalon; m, mesencephalon.

Foxg1, expressed in the early telencephalon, is relevant for basal ganglia morphogenesis and cortical neuroblasts differentiation. Loss of Foxg1 causes agenesis of the basal ganglia, elimination of neocortical progenitor domains and expansion of archicortical and cortical hem progenitors (Muzio and Mallamaci, 2005) (**Fig. 11).** Remarkably, *Foxg1* removal as late as E13.5 from progenitors that already have a neocortical identity, results in the production of cells with characteristics of Cajal–Retzius cells (Hanashima *et al.*, 2004; Shen *et al.*, 2006), indicating that the persistent expression of *Foxg1* throughout neurogenesis is required for the maintenance of neocortical progenitor identity and repression of layer I programs.

Lhx2 is expressed in the whole telencephalic neuroepithelium except the cortical hem. It commits neuroblasts within the dorsal telencephalon to cortical fates and, within the cortical field, it promotes hippocampal vs neo- and paleocortical programs. In the absence of *Lhx2*, neocortcaical progenitors of medial cortex and the hippocampus are lost, whereas the cortical hem and choroid plexus (structures normally limited to the dorsal midline) are expanded (Monuki *et al.*, 2001) (**Fig. 11**).

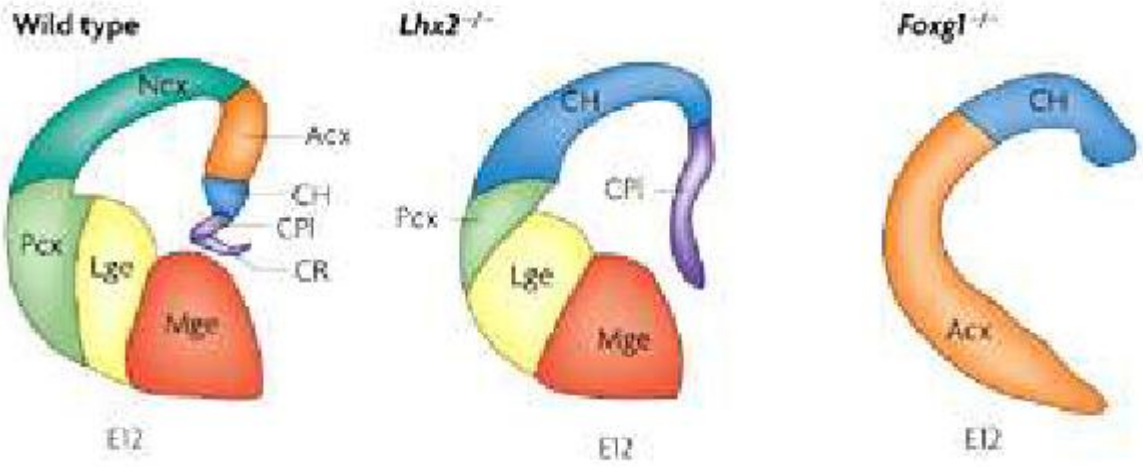

Figure 11 | Mutant phenotypes of mice knock-out for the Lxh2 and Foxg1, transcription factor involved in cortical specification. Abbreviations: CH, cortical hem; CR, choroidal roof; ChP, choroid plexus; Cx, cortex; Lge, lateral ganglionic eminence; Mge, medial ganglionic eminence; Pcx, paleocortex. Adapted from Molyneaux *et al.*,2007

The two genes *Emx2* and *Pax6* are expressed in opposing and overlapping gradient along the A/P and D/V axes of the cortical primordium, and are key determinants of the proper development of cortical areas. Loss of both *Emx2* and *Pax6* results in ventralization of cortical progenitors and the loss of the neocortical domain (Ncx), archicortex (Acx),

cortical hem (CH) and choroid plexus (CPl) by E14 (Muzio and Mallamaci, 2002). In particular, *Emx2*, restricted to the primary proliferative layer of the cortex along rostral/lateral low-to-caudal/medial high gradients (**Fig. 12**), is more intensely expressed in V1 and less in frontal/motor areas. In *Emx2* knockout mice, occipital cortex and hippocampus are shrunken and frontal cortex is enlarged (**Fig. 13**). Moreover, the areal distribution of the thalamo-cortical radiation is perturbed, coherently with such areal disproportions. *Pax6* opposes the pattern of *Emx2* expression, showing a low posterior-medial to high anterior-lateral gradient.

So within the neocortical field, *Emx2* and *Pax6*, have a direct role in arealization determining size and position of cortical areas (Reviewed in O'Leary *et al.*, 2007) (**Fig. 13**).

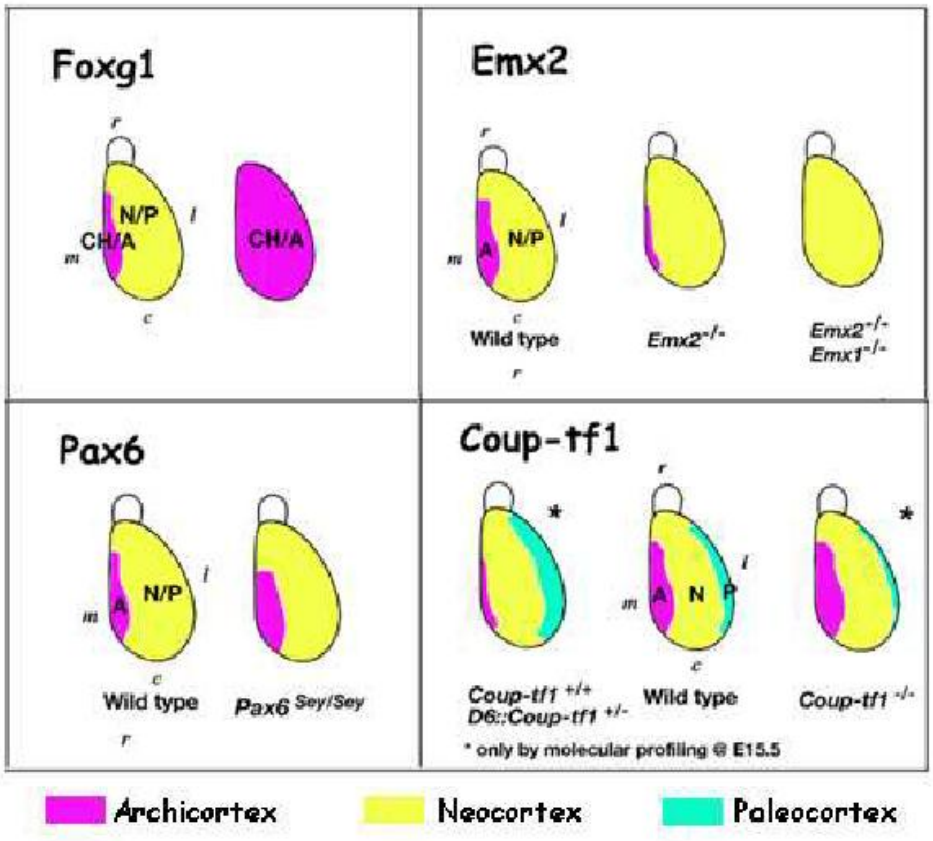

Figure 12 | Key transcription factors involved in main repartitions of cortical field and loss of-function or gain-of-function mice mutant phenotypes. Image taken from Mallamaci, unpublished.

Other key TFs involved into cortical arealization are *Coup-Tf1* (Chick Ovalbumin Upstream Transcription Factor I) and *Sp8*.

Coup-tf1 is expressed in the ventricular zone, subplate and cortical plate along a high-caudal/lateral to low-rostral/medial gradient across the neocortex (**Fig. 13**). It is

necessary to make the cortical field responsive to *Emx2* and *Pax6* patterning activities. Recent analysis of conditional knockout mice reveals a massive expansion of frontal/motor area in absence of this TF, paralleled by a reduction in size of the three primary sensory areas (Armentano *et al.*, 2007) (**Fig. 13**).

Sp8 is expressed in a high anterior-medial to low posterio-lateral gradient by cortical progenitors. It is a direct transcriptional activator of *Fgf8* expression (Sahara *et al.*, 2007). Analysis of conditional *Sp8* knockout mice at late embryonic ages show an anterior shift of cortical markers, suggesting *Sp8* preferentially specifies identities associated with frontal/motor areas (Zembrzycki *et al.*, 2007) (**Fig. 13**).

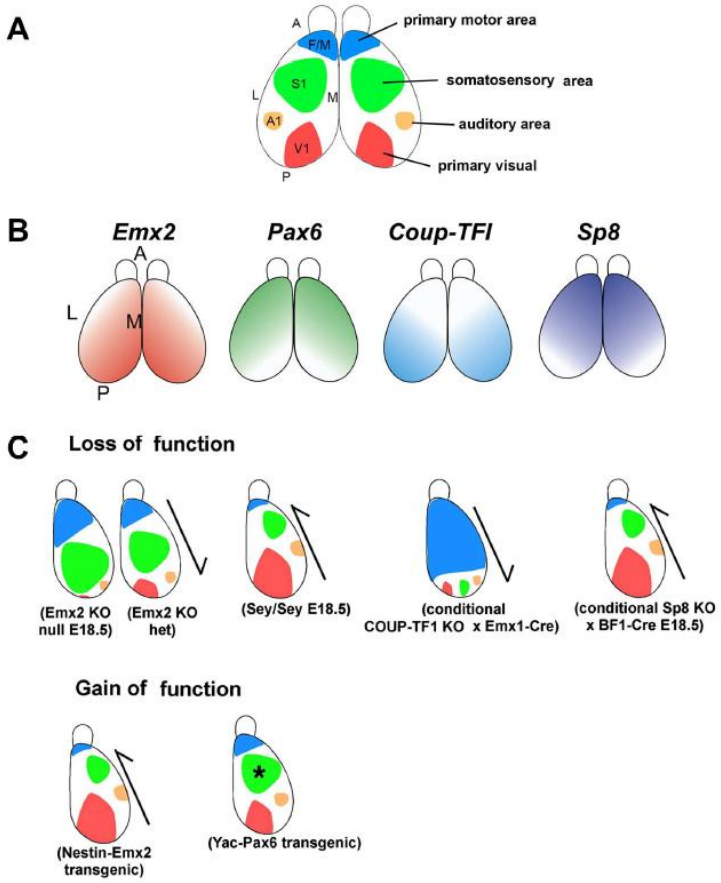

Figure 13 | Summary of area patterning and mutant phenotypes. (A) Schematic diagram of anatomically and functionally distinct areas in the mouse. (B) Graded expression of transcriptor factors along the anterior-postirior and lateral-medial axes (C) Summary of all reports of loss-of function or gain-of-function mice mutant for TFs that regulate area patterning. Image modified from O'Leary *et al.*, 2007.

2. Neocortex neuronogenesis

The first postmitotic cortical neurons form a transient structure called the preplate (PP). The PP persists until embryonic day (E) 13 in mice, when the earliest cortical plate cells reach the upper part of the neuroepithelium and divide the PP into two regions: the superficial marginal zone (MZ) (future layer 1) and the lower subplate (SP) (Marin-Padilla, 1971, 1972). The cortical plate (CP), which will become the mature six-layered neocortex, is formed between these two layers according to an "inside-out" neurogenetic gradient, with later generated neurons bypassing early generated cells to settle at the top of the cortical plate, forming the upper layers of the cerebral cortex. As cortical development proceeds, an additional proliferative zone, called subventricular zone (SVZ), appears on top of the VZ. It will initially give rise to projection neurons and subsequently to glia. In the mouse, cortical neurogenesis begins around E10.5 and last up to E17 (**Fig.14**).

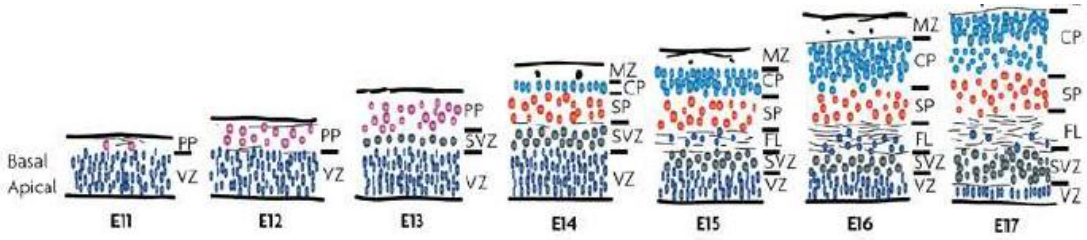

Figure 14 | Mouse cortical neurogenesis. Abbreviations: CP, cortical plate; FL, intermediate zone, PP, preplate; MZ, marginal zone; SP, subplate; SVZ, subventricular zone; VZ, ventricular zone

First generated neurons are the Cajal-Retzius cells (CR), which secrete Reelin, an extracellular matrix protein that plays a fundamental role for the formation of cortical layers during development and its maintenance in adulthood (Frotscher *et al.*, 2009). CR neurons arise from restricted locations at the borders of the developing pallium, the hem, the antihem and the septum (Takiguchi-Hayashi *et al.*, 2004; Yoshida *et al.*, 2006), and spread into the cortex by tangential migration. Subplate cells have a role in directing the first thalamic axons to the pallium (Allendoerfer and Shatz, 1994).

Cortical neurons can be divided into: interneurons, which make local connections; and projection neurons, which extend axons to distant intracortical, subcortical and subcerebral targets.

Projection neurons are glutamatergic neurons, often characterized by a tipical pyramidal morphology. They transmit information among different regions of the neocortex and to other regions of the brain. During development, they are generated from progenitors of

the neocortical germinal zone located in the dorsolateral wall of the telencephalon (Anderson et al. 2002).

By contrast, at least in rodents, GABA (y-aminobutyric acid)-containing interneurons are generated primarily from progenitors in the ventral telencephalon and migrate long distances to their final locations within the neocortex (Wonders & Anderson 2006). Almost all glutamatergic neurons (amounting to about 80% of the neuronal compartment) originate from the neural stem cells located in the cortical VZ directly or- much more frequently- via intermediate neuronal progenitors lying in the cortical VZ and SubVentricular Zone (SVZ) (Martinez-Cerdeno et al 2006)(Kowalczyk et al 2009) . These neurons radially migrate towards their final location into the outer cortical wall: here they settle, according to defined rules, giving rise to a tri- (in archi- and paleocortex) or hexalaminar (in neocortex) structure. In the mouse embryo, the generation of glutamatergic neurons takes place from embryonic day 11(E11) to E17 and, in the rat, their radial migration is virtually completed by postnatal day 2 (P2) (Bayer and Altmann. 1991).

Conversely, cortical gabaergic interneurons are generated within ganglionic eminences in ventral telencephalon and anterior hypothalamus, reach the developing cortex by tangential and radial migration and share laminar destination of their isochronically generated glutamatergic partners (Marin and Rubenstein, 2001). While migrating and also after migration, cortical neurons undergo a complex cytoskeletal, chemical and electrical maturation (Heng et al 2010). Moreover they establish an intricate network of synaptic connections with others neurons, cortical (homolateral and heterolateral) as well subcortical (thalamic and subthalamic) (Molyneaux et al. 2007). Finally, after neuronogenesis completion, two waves of gliogenesis take place in the cortex. Details will be elucidated below.

3. Neocortex gliogenesis

Glial cells constitute 10-20% of the cells in the Drosophila nervous system and at least 50% of the cells in the human brain (Rowitch and Kriegstein, 2010). This means that glial cells have acquired increasing importance for the complexity of the system during evolution.

During mammalian cerebral cortical development, 3 main cell types have to been generated: neurons and the 2 macroglial cell types, astrocytes and oligodendrocytes. Astrocytes and oligodendrocytes play diverse roles in the maintenance of neurological functions.

Oligodendrocytes provide the myelin sheath to ensure insulation for neuronal axons so allowing faster conduction of electric stimuli.

Astrocytres contribute to genesis and function of the blood-brain barrier (Tao---Cheng et al., 1987; Abbott et al., 2006; Alvarez et al., 2011; Bozoyan et al.,2012), provide structural and metabolic support to neurons (Allen and Barres, 2009; Belanger et al., 2011; Prebil et al., 2011), and modulate synaptic transmission and information processing (Nedergaard et al., 2003; Eroglu and Barres, 2010; Sasaki et al.,2011; Min and Nevian, 2012). Moreover, they react to pathological conditions, by upregulating specific gene products (intermediate filament glial fibrillary acidic protein (GFAP) and inhibitory extracellular matrix (ECM) proteins among them), and demarcate the damaged site from surrounding tissue with an ECM-rich scar, poorly permissive for axonal regeneration (Silver and Miller, 2004; Schachtrup et al., 2010, 2011).

In the rodent cerebral cortex most neurons are generated between embryonic day 12 (E12) and birth (around E20), whereas glial cells are mostly generated at late embryogenesis (from E16 on) and first postnatal weeks (Angevine and Sidman 1961; Berry and Rogers 1965; Lent *et al.* 1990; Bayer and Altmann 1991; Jacobson 1991) (**Fig 15**).

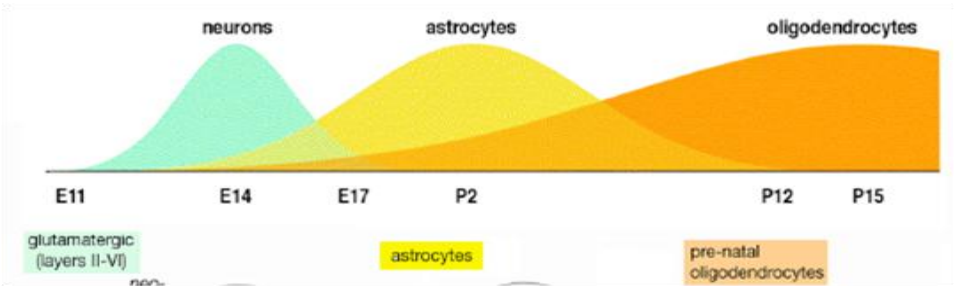

Fig 15 Cortico-cerebral histogenesis

The temporal separation of cell production allows the neuronal population to be well established before the glial system develops. This ensure, for example, that most axons are elaborated before the appearance of the oligodendrocytes that wrap them and that the number of glial cells generated can be appropriately balanced to the extant neuronal population (Burne et al., 1996; Calver et al., 1998; Barres and Raff, 1999).

At some point in the developing embryo, neural stem cells must face the option to remain in a pluripotent self-renewing cell state, enter neurogenesis and become new neurons, or shift into gliogenesis. Complex genetic mechanisms underlie such big decision. Indeed, one of the main goals of the studies undertaken in the developmental genetics in recent times is to understand which specific molecular mechanisms (cell-autonomous or not), signaling molecules and effector pathways are involved in the regulation of this hard decision so finely restricted in time and space.

For long time, glial cells and neurons were considered to take their origin from separated precursor pools diverging early during development. Conversely, the process is more elaborate and elegant than previously thought.

Even if committed progenitors may emerge very early, recent studies in the developing brain have given evidence of the fact that one single population called *radial glia* (RG) operate as primary precursor pool, which is now considered as *neural stem cells population* (NSCs).

Moreover, NSCs give rise to transit amplifying *intermediate progenitors* (IPCs) before generating one of the extraordinary different specific neural subtypes (**Fig. 16**).

These intermediate progenitors are lineage restricted and coexist not only in the embryo but also in some germinal regions in the adult (Kriegstein and Alvarez-Buylla, 2009).

Everything begins in the *ventricular zone* (VZ),where RG cells have the potential to give rise directly to neurons, or, more frequently, to different pools of intermediate

progenitors (IPCs), which continue to proliferate but are already addressed to one specific differentiated progeny (**Fig16**).

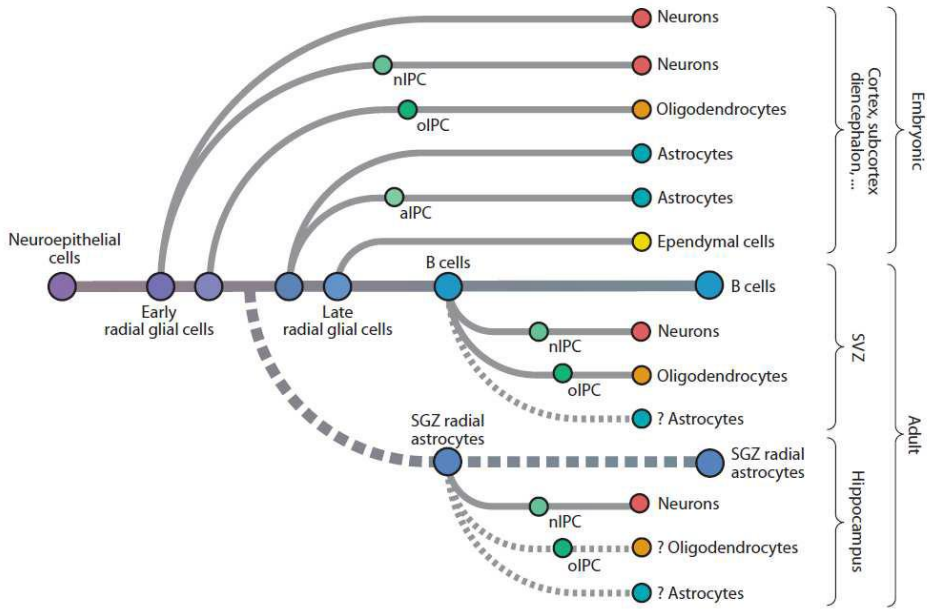

Figure 16. Different cell lineages originating from neuroepithelial cells in the developing CNS (Kriegstein and Alvarez-Buylla, 2009).

Around E11 early radial glial cells within dorsal telencephalon proceed to generate *neuronal intermediate progenitors* (nIPCs) which in turn will originate the almost totality of corticocerebral *glutamatergic* neurons.

The second output of *dorsal* telencephalic RG cells is represented by astrocytes, which are generated to a large extent after neuronogenesis completion, by differentiation of RG cells via committed progenitors. In this respect, some astrocytes, still locally dividing before terminal differentiation are often referred to as aIPCs. Although there is a peak in the production of astrocytes just after birth and a global fading of this phenomenon around the end of the second week of postnatal life, the process of astrocytic amplification may happen also in the postnatal murine cortex (Hajós et al., 1981; Ichikawa et al., 1983).

The situation is more complex for cortico-cerebral oligodendrocytes, as their progenitors b (oIPCs) are born in different telencephalic regions. Since E12, oIPCs expressing the oligodendroglial marker Nkx2.1 may be found within the medial ganglionic eminence (MGE). Whereas starting from E14, oIPCs expressing another ventral gene, Gsx2, may be detected within lateral and caudal ganglionic eminences (LGE and CGE).

Progenies of both these subpopulations migrate towards the cortex after their generation and contribute to the oligodendrocytic complement of specific pallial regions, according

to a well defined schedule (Kessaris et al., 2006). At E18 and around birth, additional oIPCs are produced in dorsal cortex, starting from local stem elements expressing Emx1. Their contribution to the cortical oligodendrocytic pool will become more and more relevant, as postnatal CNS maturation proceedes (Kessaris et al., 2006). Finally, residual, late radial glial cells (lRG) get largely converted into ependymal cells of the neonatal individuals (**Fig. 17**).

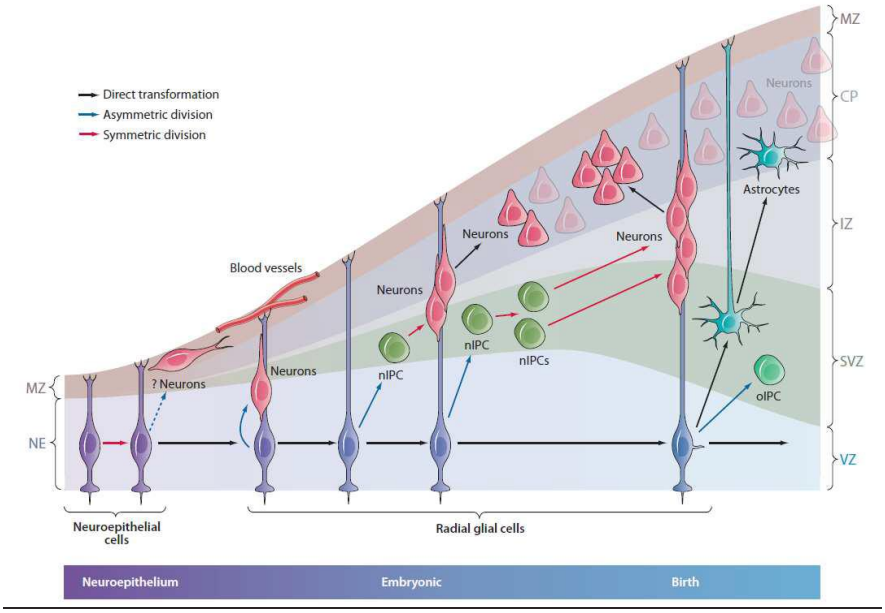

Figure 17. Neurogenesis during cortical development (Kriegstein and Alvarez-Buylla, 2009)

However, NSCs cells are retained in some restricted regions of postnatal and adult brain, where not only glial cells keep being produced, but also neurons. Remarkably, in the adult, part of the RG cells are converted to adult SVZ cells (B cells), that are also identified as SVZ astrocytes but continue to play a NSC-like role, by generating neurons and glial cells through other IPC-like subpopulations. In particular, B cells, usually quiescent, develop into C cells (similar to embryonic nIPCs) which are designed to become differentiated neurons (A cells). B cells also give rise to distinct C cells, contributing to postnatal oligodendrocytogenesis, as oIPCs (**Fig. 18**).

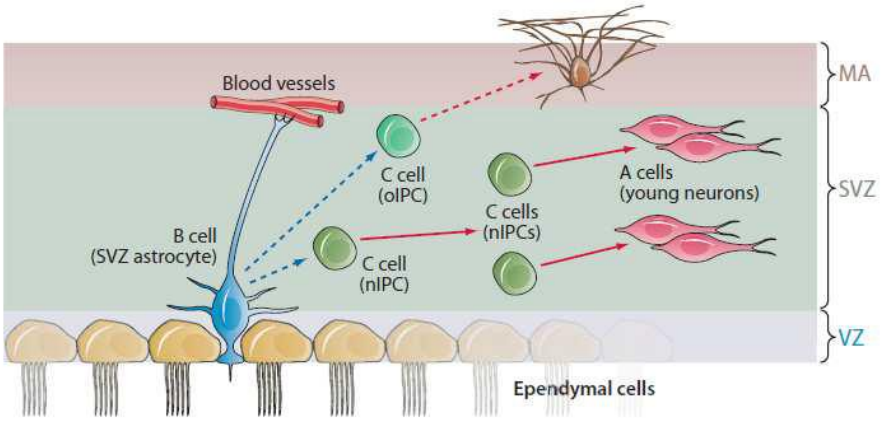

Figure 18. Progenitor types and lineages in the adult brain SVZ (Kriegstein and Alvarez-Buylla, 2009).

Of course, a fine regulation is required to modulate the commitment of NSC towards one fate or another.

Many lines of study indicate that temporal changes of differentiation of CNS stem cells are driven by a combination of intrinsic temporal programs and extracellular signals from the changing environment of the developing brain: combination of transcription and secreted factors in the embryonic and adult SVZ result in the differentiation of diverse subtypes of mature cells.

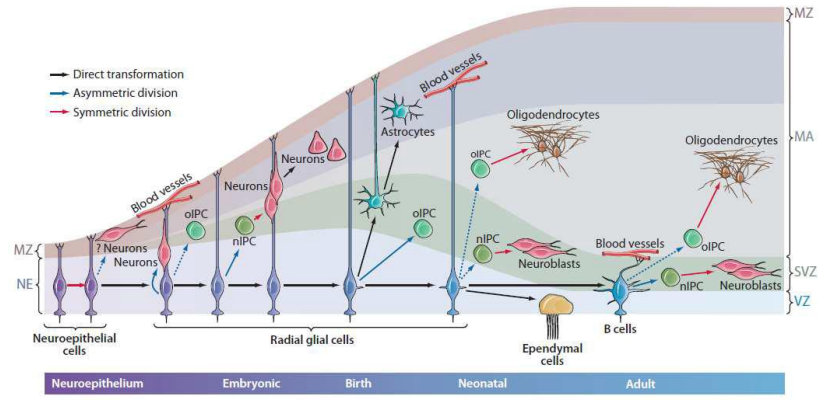

Figure 19. Summarizing cartoon of glial nature of neural stem cells (NSCs) in development and in the adult (Kriegstein and Alvarez-Buylla, 2009).

4. Astrogenesis

The astrogenesis is the most characterized process. The switch from neuronogenesis to astrogenesis, taking place in the rodent cerebral cortex around birth, is the result of two key concurrent factors: the advancement of an intrinsic "developmental clock" hardwired in neural multipotent precursors, and the activity of paracrine regulatory signals, impinging on these precursors from their surroundings.

This means that astrocyte-specific gene expression is regulated by specific extracellular ligands secreted by surrounding cells, acting on multipotent neural precursors and modulating their histogenetic properties. Information carried by these ligands is generally conveyed to the nucleus of neural precursors via dedicated receptors and transducers.This ultimately results in differential nuclear availability of transcription factors, which interact with chromatin of astroglial genes and may regulate their transcription.

Collectively, expression levels of these receptors and transducers as well as the epigenetic state of this chromatin dictate precursors ability to respond to astrogenic stimuli, namely the other key determinant of astrogenesis progression.

4.1 Main pathways modulating astrogenesis

It has been shown that at least 5 main trans-active pathways regulate transcription from astrocytic promoters (*Gfap* and *S100*β the best characterized ones): *CT1/IL-6/Jak/Stat pathway*, Delta/Notch/RbpJk, Nrg1/ErbB4, Tgfβ1/Tgfβ1RI,II, and Pacap/Pac1/Dream.

An overview of these pathways follows.

4.1.1. CT1/IL-6/Jak/Stat pathway

The evidence that embryonic cortex sequentially generate neurons and glial cells strongly suggests that neurons could act as the main promoter of gliogenesis. Indeed several experiments report that neurons secrete gliogenic cytokines, in particular members of the Interleukin-6 (**IL- 6**) family, including ciliary neurotrophic factor (**CNTF**), leukemia inhibiting factor (**LIF**), cardiotrophin-1 (**CT-1**), neuropoietin and CT-like factor.

These secreted factors bind to a receptor complex that is made up by the α-subunit of the LIF receptor (**LIFR**) and **gp130**, activating the **gp130-JAK-STAT** pathway in cortical

precursor cells, therefore inducing the onset of gliogenesis (Rowitch and Kriegstein, 2010).

Stat1 and Stat3, once phosphorylated, translocate into the nucleus and take contacts with astrocyte-specific gene promoters (like GFAP and S100β promoters). In fact it was shown pStat- responsive elements are located within S100β and GFAP promoters (**Fig.20**).

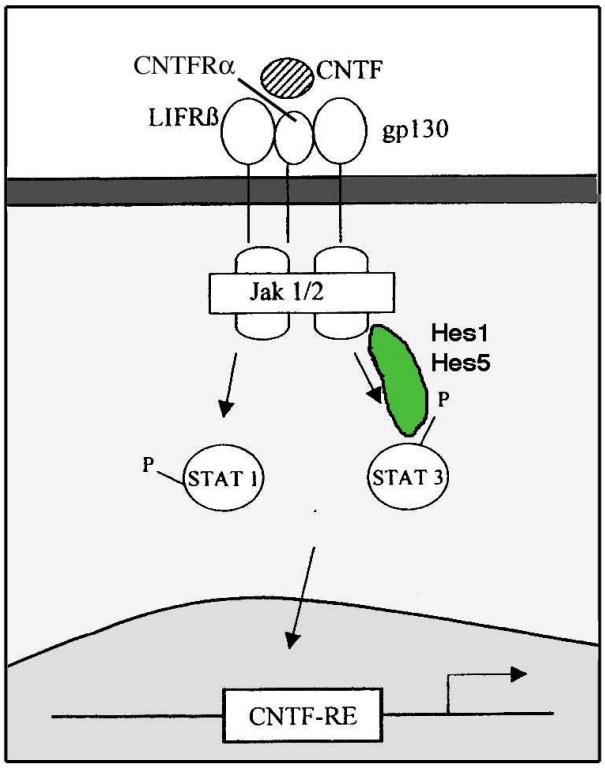

Figure 20. CT1/IL-6/Jak/Stat pathway

It was also demonstrated that production of GFAP+ astrocytes had almost completely abolished in LIFRβ$^{-/-}$ and gp130$^{-/-}$ mutants, reduced by 50 to 75% in CT-1$^{-/-}$ mice and only slightly affected in CNTF$^{-/-}$ and LIF$^{-/-}$ mutants (Ware et al., 1995; Bonni et al., 1997; Koblar et al., 1998; Ochiai et al., 2001; Barnabé-Heider et al., 2005).

All these data seems to drive people to the reasonable conclusion that these molecules may be among the factors playing the lead roles in inducing the astrocytogenic genes expression (Rowitch and Kriegstein, 2010).

These results support the concept that neuronal feedback may help to regulate the developmental switch to gliogenesis. Thus this reflects the necessity of restricting gliogenesis in time: it must not occur until neurogenesis is complete. Indeed *gp130,Jak1,stat1* and *stat3* expression levels are very low in E11 cortico-cerebral

precursors, they arise more and more during neuronogenesis progression and finally peak around birth. This upregulation is promoted by astrogenic cytokines through the gp130-LifR/Jak/Stat axis.

Numerous modulatory plugins act on this axis.

First of all, two autocatalytic loops are known to upregulate the process;

- CNTF is produced by newborn astrocytes and it mimics the action of CT1, by synergyzing with it (**Fig.21**);
- pStat1/pStat3 upregulates transcription of gp130, Jak1, Stat1 and Stat3, therefore empowering the responsiveness of the system.

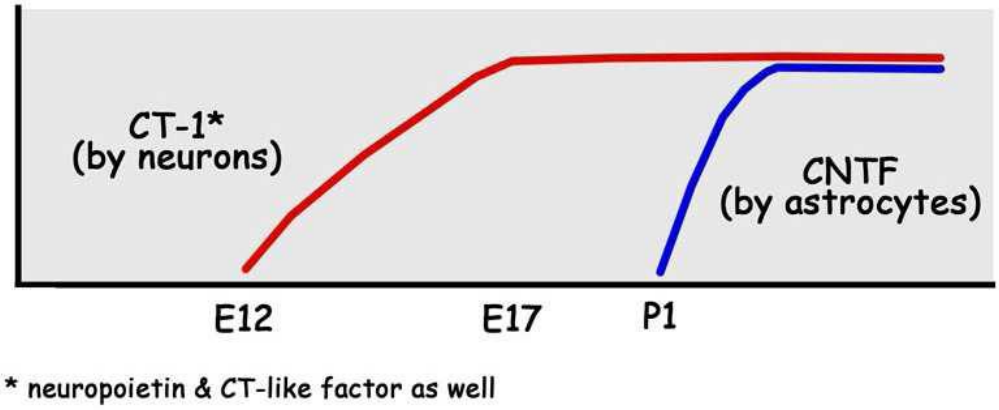

Figure 21. Differential production of CT-1 and CNTF during mouse development

There are also extrinsic modulatory afferences.

Among negative modulators, some mechanisms are noteworthy:

- during neuronogenic phases, unknown factors promote DNA-methyl transferase 1-(Dnmt1)- hypermethylation of Stat1 and Stat3 promoters: it results in the inhibition of their transcription;
- neurogenic Neurogenins/NeuroD1 inhibit the *gp130*, *jak1*, *pStat1* and *pStat3* expression;
- Neurogenins compete with pStat1/pStat3 for the binding to the CBP/p300-pSmad1/pSmad4 complex. Indeed Neurog1 and Neurog2 peak during neurogenic period and so contribute to postponing aastrgenesis to perinatal and postnatal phases.

In this way molecular mechanisms supporting neuronogenesis and astrocytogenesis reciprocally cross-inhibit and the "winner" is promoted by BMP signalling (**Fig. 22**).

The Jak/Stat signalling promoting astrogenesis is stimulated instead by other afferences:

- Notch/Hes5 facilitate the Jak2/Stat3 interaction

- EGFR modulate Stat3 expression, phosphorylation and activity on astroglial targets (see later).

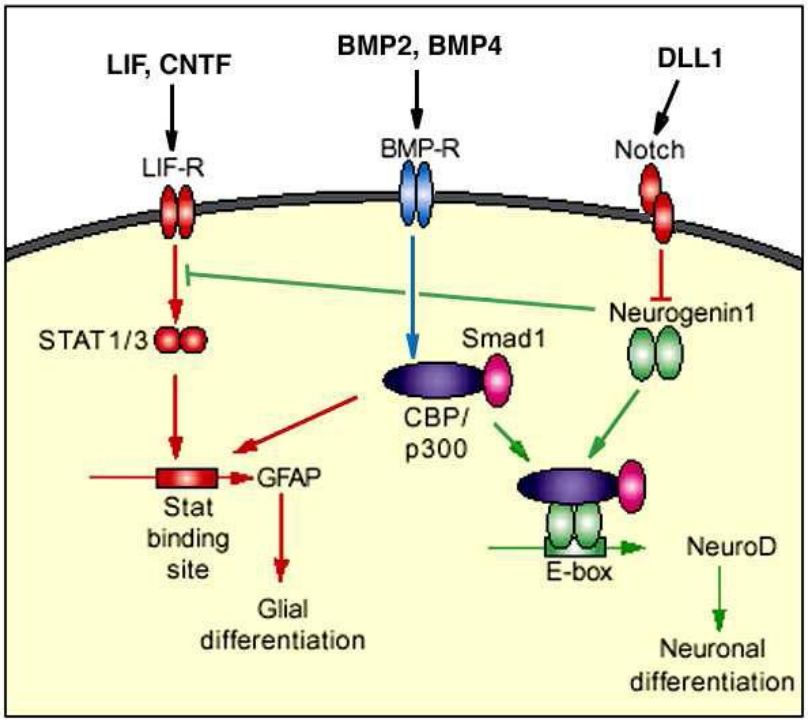

Figure 22. BMP signalling in neuronogenesis and gliogenesis

4.1.2 Nrg1/ErbB4: pathway

A major role in setting up the proper onset of astrogenesis is played by the ligand Neuregulin1 (Nrg1), expressed by neurons and neural precursors, and its ErbB4 receptor, transducing its signalwithin cortico-cerebral precursors. ErbB4 is a member of the EGF receptor family. Upon its neuregulin–induced activation, it undergoes two sequential cleavages, by TACE (TNF-α-converting enzyme) and presenilin/γsecretase, respectively. That allows the release of its intracellular domain (EICD), which, complexed by TAB2 and the corepressor N-CoR, translocates to the nucleus and inhibits the transcription of astrocytic genes, such as GFAP and S100β (**Fig.23).**

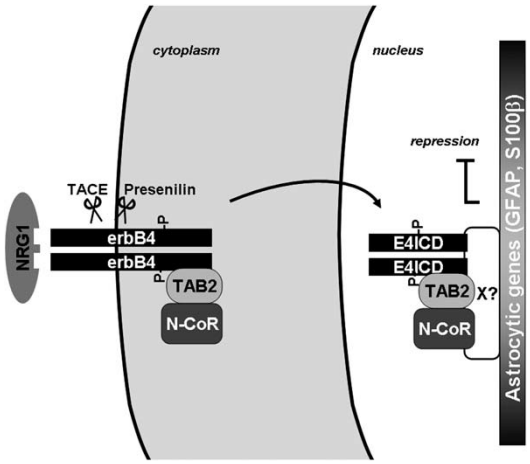

Figure 23. ErbB4 intracellular pathway

Remarkably, mice knock-out for ErbB4 exhibit a precocious cortical astrogenesis, a phenotype which may be rescued by re-expression of this gene (Sardi et al., 2006). No doubt, this pathway represents one of the most prominent brakes impinging on the astrogliogenesis onset.

4.1.3 Delta/Notch/RBPJk pathway

Delta/Notch signalling is a well-known highly conserved cell signalling system present in most multicellular organisms. This pathway is essential for the maintainance of NPCs in the developing brain.

Furthermore, it appears to possess an instructive role in order to directly promote the differentiation of several glial cell subtypes (Louvi and Artavanis-Tsakonas, 2006) (**Fig.24**). Remarkably, although Notch signaling is already active within neurogenic cortico-cerbral precursors, astrogenesis initiates 7 days later. This suggests that Notch signaling is not sufficient to trigger this process and additional conditions have to be fulfilled in order to unveil its pro-gliogenic activity. Moreover, the astrogenic activity of Notch signaling requires Jak/Stat signaling, but not vicerversa (Ge et al.2002).

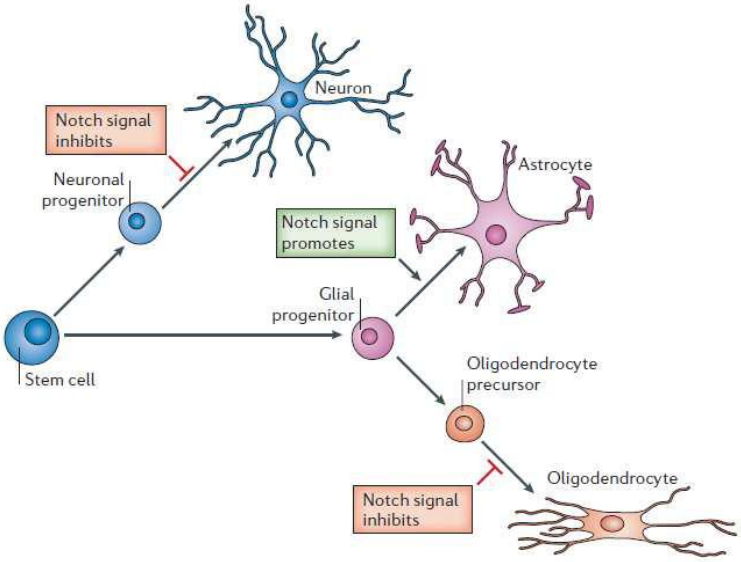

Figure 24. Notch signalling throughout glial differentiation

In the developing central nervous system (CNS), Notch signaling preserves progenitor pools and inhibits neurogenesis

Upon binding to Notch ligands (Delta and Jagged), the heterodimeric Notch undergoes two sequential proteolytic cleavage events, first by tumor necrosis factor-α-converting enzyme (TACE) and then by γ-secretase. These cleavages cause the Notch intracellular domain (NICD) to be released from the membrane, allowing its translocation to the nucleus. Through its RAM domain and ankyrin repeats, NICD associates with the DNA binding protein CSL (RBPJk) and the CSL partner SKIP, turning CSL-SKIP from a transcriptional repressor into an activator, stimulating the transcription of target genes, such as Hes1 (for hairy and enhancer of split) and Hes5, that function to inhibit neurogenesis

It has recently been postulated that Notch instructively drives astrocyte differentiation. Astrogliogenic role of Notch is mediated by RBPJk. Indeed it can directly bind to an element within the GFAP promoter and promote its transcription.

During neurogenic phase, in the absence of CT1/Jak/Stat signalling, RBPJk recruits the powerful transcriptional co-repressor NCoR to the GFAP promoter. This repressor is usually available within the nucleus. This results in transcriptional inhibition of the GFAP gene expression (Ge et al., 2002).

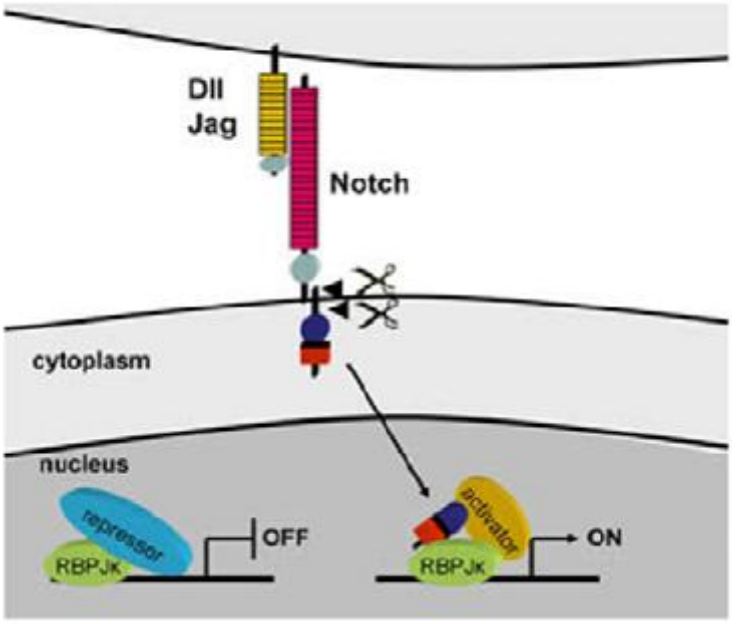

Figure 25. Molecular details of Notch signaling

This regulation is crucial for the proper timing of the onset of astrocytogenesis. Thus 70% of brain from $NCoR^{-/-}$ mice showed prominent GFAP expression at E14.5-15.5, the time at which wild-type brains virtually never express GFAP.

Starting from E15.5, the activation of the Jak/Stat axis normally leads to progressive exclusion of NCoR from the nucleus and its translocation to the cytoplasm. In these conditions, RBPjk already bound to the GFAP promoter possibly recruits NICD and starts to transactivate its target.

This mechanism shows how CT1/Jak/Stat signalling is tightly necessary to achieve Notch promoted stimulation of astrocytogenesis.

Conversely, conditional RBPJk-KO in neural stem cells does not suppress astrocytogenesis.

It can be explained because CT1/Jak/Stat signalling is on. Under these conditions, astrocytogenesis is simply delayed but, when activated, it proceeds at normal speed.

4.1.4 PACAP/…/ Dream pathway

This pathway has been discovered and characterized by the team of Vallejo (Vallejo 2009; Cebolla et al., 2008) and represents a fundamental biphasic regulation of astrocytic promoters (**Fig 26**). This pathway controls cortico-cerebral astrogenesis by Pituitary Adenylate Cyclase-Activating Polypeptide 1 (PACAP1). PCAP1 activity leads to an

intracellular calcium concentration increment. Calcium is the last intracellular transducer that triggers enhanced transcription of astroglial genes, in response to Pacap (Cebolla et al. 2008).

Indeed, in the absence of the secreted factor called PACAP, Dream (Downstream Regulatory element Antagonist Modulator) transcription factor stays on the GFAP promoter. When PACAP binds to the PAC1 receptor (the short isoform of the PCAP specific receptor), cytoplasmic cAMP is upregulated and Ca^{2+} massively dashes into the cell. The calcium wave triggerd by PACAP induces Dream to undergo a profound conformational change. Thanks to it, Dream, still attached to the Gfap promoter, gets converted into a transactivator and starts to promote Gfap transcription (**Fig.26**).

The PACAP/PAC1/cAMP/Dream pathway works in parallel to the CT1/IL6R/Jak/Stat pathway illustrated above. That is the reason why, if Jak/Stat axis is efficient, Dream-KO only determines a transient shrinkage of the astrocytic compartment between E17 and P7, associated to an enlargement of the neuronal one (Cebolla et al., 2008).

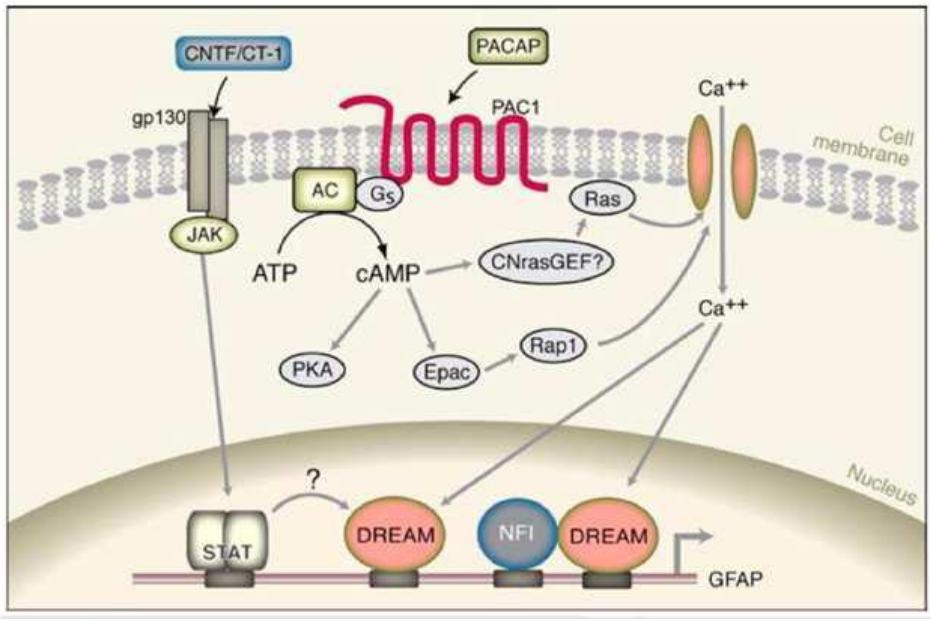

Figure 26. PACAP/…/ Dream pathway

4.1.5 Tgfβ1/Tgfβ1RI,II

Transforming factors beta (TGF-βs) are multifunctional growth factors which act in key events of development and cell repair (Shi and Massagué, 2003).

TGF-β signalling involves mainly 2 threonine kinase receptor, TGFRI and TGFRIII which in turn activate Smad 2/3 and Smad4 transcription factors. These proteins are

phosphorylated, activated and complexed in Smad 2/3-4 complexes, which translocate to the nucleus and regulate transcription (Shi and Massagué, 2003) of the astrocyte-lineage marker *Gfap*.

The first piece of evidence of TGF-β1 important role in development came from the fact that cortical neurons activate the *Gfap* gene promoter in astrocytes by secreting this factor (de Sampaio e Spohr et al., 2002) (**Fig.27**) and so stimulating the astrocytes maturation.

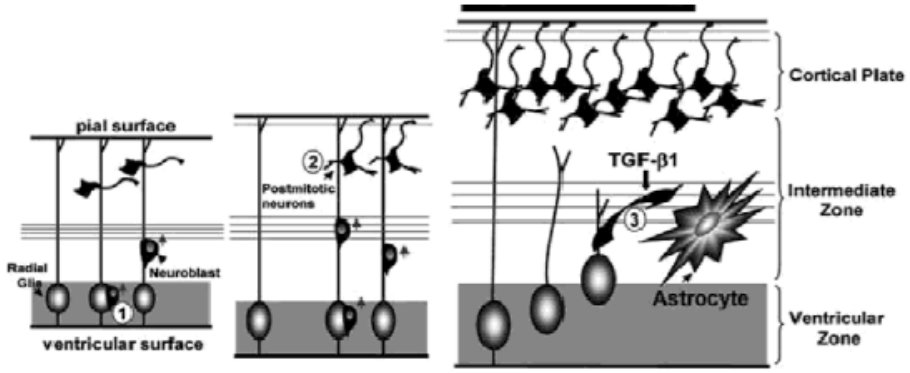

Figure 27. Role of TGF-β1 in astrocyte differentiation

Moreover, neurons can modulate the differentiation from RG cells to astrocytes too (Stipursky and Gomes, 2007) (**Fig.28**) also thank to this secreted factor.

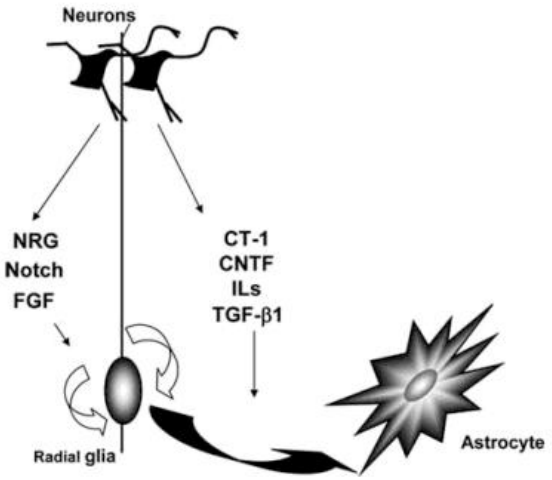

Figure 28. Summarizing draft of molecular signalling promoting astrocytic differentiation

In fact, when conditioned medium derived from pure neuronal culture or astrocyte-neuronal coculture (which both contain active TGF-β1) is added to E14 cortical cultures, the *Gfap* gene promoter is strongly activated and the fraction of RG marker-expressing

cells is increased. This testifies that TGF-β1 can drive progenitor cells (RG) towards an astrocytic phenotype (Stipursky and Gomes, 2007).

4.2 Accessibility of chromatin to transcription

It has been shown that, in addition to several pathways described before, astrogenesis requires an appropriate temporal regulation of chromatin accessibility to transcription.

It has been proven that a major pre-condition for the switch from neuronogenesis to astrocytogenesis is the progressive "opening" of astrocytic chromatin. This is a complex process which takes place while neuronogenesis is on, from E11.5 to E14.5 and beyond, and essentially consists in the acquisition by the astroglial genes of the capability to get bound by their specific transactivators.

Indeed precursor cells change in their competence to respond to gliogenic cytokines over development. Among mechanisms mediating such changes in cytokine-sensitivity, there is DNA methylation. The first demonstration of this involved the finding that *Gfap* promoter was preferentially methylated in neurogenic cortical precursors, and that this methylation inhibited STAT3 association and *Gfap* transcription, so preventing the transcription of this gene (Takizawa et al., 2001) (**Fig 29**).

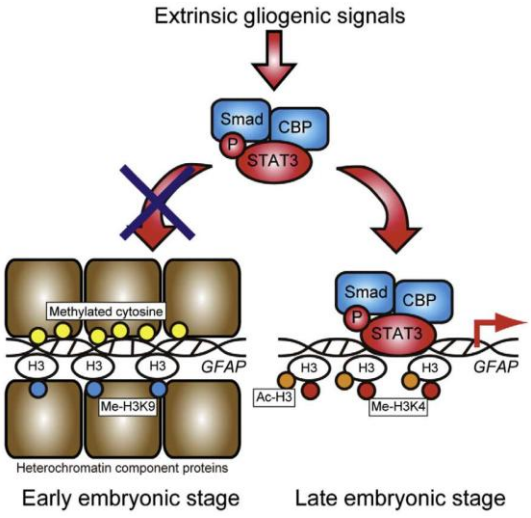

Figure 29. Temporal regulation and epigenetic modification of *gfap* promoter: temporal changes in the epigenetic status of glial genes are necessary for initiating gliogenesis. For example, A STAT3 binding site in the *gfap* promoter, which is responsible for JAK-STAT pathway-dependent expression of GFAP, undergoes epigenetic silencing in NSC during the early neurogenic phase through DNA methylation at CpG sites and K9-methylation of Histone H3. However, during late embryonic stages (gliogenic phase) , this site becomes free from epigenetic silencing, adopting a euchromatic histone code, including acetylated and K4-methylated Histone H3 and reduction of methylation of this STAT3-binding site

Gfap promoter methylation varies from high in early telencephalic progenitors to lower and lower as the progenitors develop in vivo. Furthermore, high methylation is found in neurons, whereas low levels are present in astrocytes (**Fig.30**).

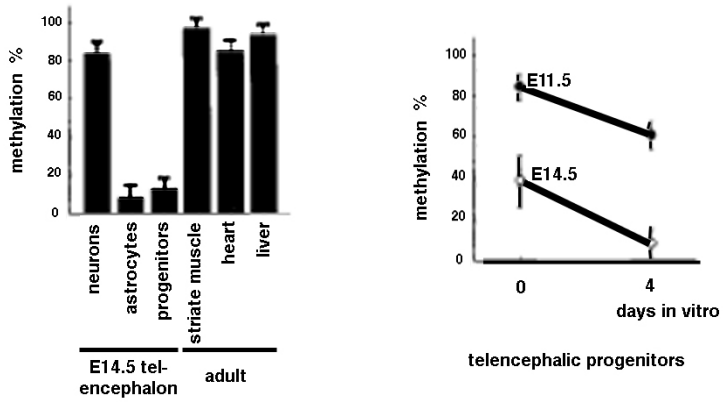

Figure 30. Levels of DNA methylation associated to glial genes during development

However, the preparation of astrocytic genes to transcription is not limited to proper regulation of their DNA methylation levels. It encompasses also a fine modulation of the covalent modification profile of histones associated to them, as well as a conformational rearrangement of chromatin (Matsumoto et al., 2006). Indeed, in addition to DNA methylation, a second crucial mechanism controlling chromatine remodeling is represented by differential methylation of Histone 3.

It has been recently shown that various factors are responsible for chromatine opening. At the moment, at least 3 main players have been shown to master this control:

(1) Notch via Nuclear Factor Ia (NFIa is capable to mediate DNA demethylation); (2) CoupTfI and CoupTfII (promoting the H3K9me2→H3K4me2 switch, H3 acetylation and DNA demethylation); (3) Retinoic Acid (which leads to an increase of acetylation of specific astroglial genes).

(1) It was demonstrated that Notch signaling is sufficient and necessary to promote demethylation of the main pStat3-Binding Site located in the *Gfap* promoter. That normally happens between E11.5 and E14.5 and it is a pre-condition for subsequent activation of this gene by Lif-like ligands (Namihira et al., 2009). Indeed the Notch effector RBPJk binds to the Nuclear factor Ia (Nf1a) promoter and stimulates its transcription (Namihira et al., 2009). In turn, Nf1a binds to the *Gfap* promoter (at least four Nf1a binding sites were found within the Gfap promoter (Cebolla and Vallejo,

2006; Piper et al.,2010)). This is necessary and sufficient to detach Dnmt1 from this region and get it demethylated, so paving the way to the astrogenic program (Namihira et al., 2009).

Thus, Nf1a mediates the pro-astrogenic function of the Delta/Notch axis, being transcribed in response to Notch signaling and possibly competing with Dnmt1 for binding to the *Gfap* promoter (Namihira et al., 2009). Remarkably, overstimulation of the Delta/Notch/NfIa axis is not sufficient *per se* to enhance astrogenesis. The pro-astrogenic activity of this pathway only emerges when the system is co-stimulated by Lif (Namihira et al., 2009) and/or Pacap (Cebolla and Vallejo, 2006).

(2) CoupTfI and CoupTfII are agents promoting the opening of astrocyte genes chromatin. *Coup-tf*s are widely expressed in the telencephalic ventricular zone, where they display a transient peak around E12.5. Their impact on gliogenesis is mediated by H3 acetylation and DNA demethylation at the LIF-RE of the *Gfap* promoter, allowing LIF-dependent *Gfap* transcription.

(3)Retinoic acid (RA): Upon binding of RA to RARα (RA receptor α), this receptor makes a complex (the RARαβ/RXR complex) which leads to an increase of histone acetylation at the pStat3-BS level. This makes pStat3-BS suitable to be bound by pStat3 and leads to LIF-dependent transcriptional activation (Asano et al., 2009).

4.3 Regulation of committed progenitors proliferation

Our knowledge of such regulation is still very poor and only a few genes have been demonstrated to be implicated in it. Among these there are:

- Fgf2 and Egf stimulate proliferation of neonatal rodent astroblasts (Mayer et al., 2009; Riboni et al., 2001).
- Fgf9 promotes a substantial expansion of the perinatal astrogenic proliferating pool (Seuntjens et al., 2009) and delays terminal differentiation of mature astrocytes (Lum et al., 2009).
- Emx2 induces a reduction of late astrocyte progenitors compared to early ones, suggesting in this way that this TF may reduce early astrocyte progenitors proliferation (Brancaccio et al.2010).

4.3.1 Role of Fgf2

Fgf2, stimulating proliferation, possibly contributes to the "dilution" of the 5-methylcitosines originally clustered at the *Gfap* and *S100*β promoters, which occurs upon the Notch-induced detachment of Dnmt1 from these promoters (Namihira et al., 2009). In these ways, Fgf2 allows pStat3 to bind to the astrocytic promoters and stimulate transcription (Song and Ghosh, 2004; Namihira et al., 2009). Moreover Fgf2, is a known stimulator of Ras/Raf/Mek/Erk axis (Dorey and Amaya, 2010). The effects of the Mek/Erk machinery on astrogenesis are largely mediated by the Ets transcription family member Etv5/Erm. It is detectable in the VZ at E14.5-E18.5 and is necessary and sufficient for activation and progression of astrogenesis (Li et al., 2012).

4.3.2 Role of Fgf9

Like Fgf2, this protein is a member of the huge fibroblast growth factors (FGF) family. In general, members of this family hold mitogenic and cell survival properties and are involved in various biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion.

In particular, Fgf9 is a secreted factor that shows its main effect as a growth-stimulating effect on cultured glial cells. In nervous system it has been shown to be produced mainly by neurons and may be fundamental for glial cell induction and development (Santos-Ocampo et al., 1996). In particular it has been thought to be produced by neurons, as neurogenesis is termined, in order to induce the onset of production of glial cells.

4.3.3 Role of Egf

The Egf/EgfR pathway promotes cortico-cerebral astrogenesis.

Already at the end of 90's, it was reported that mice lacking EgfR suffer of delayed astrocyte development (Kornblum et al., 1998; Sibilia et al., 1998).

Concerning molecular mechanisms underlying EgfR promotion of astrogenesis, Egf signaling upregulates astrogenesis rates, mainly by facilitating transmission of the Ct1 signal through the Jak2/Stat3 axis via EgfR-dependent upregulation of Stat3 expression. Indeed, among the various pathways interfering with the IL-6/Jak/Stat transduction machinery, the EgfR one plays a master role as a positive regulator.

Lillien et al. performed an elegant experiment in which they demonstrate that EGFR (induced by Fgf2) is necessary and sufficient to make cortical progenitors differentiate to astrocytes, under high LIF (Viti et al., 2003). This EgfR has been shown to increase Stat3 levels and, even more important, to render Stat3 more easily phosphorylable under high LIF. However the transfection of a constitutively activated, phospho-mimetic variant of Stat3 into $EGFR^{-/-}$ progenitors was not able to rescue GFAP expression. This suggests that the full astrocytogenic activity of EgfR requires a wider range of still unknown effects which are independent of Stat3 activation.

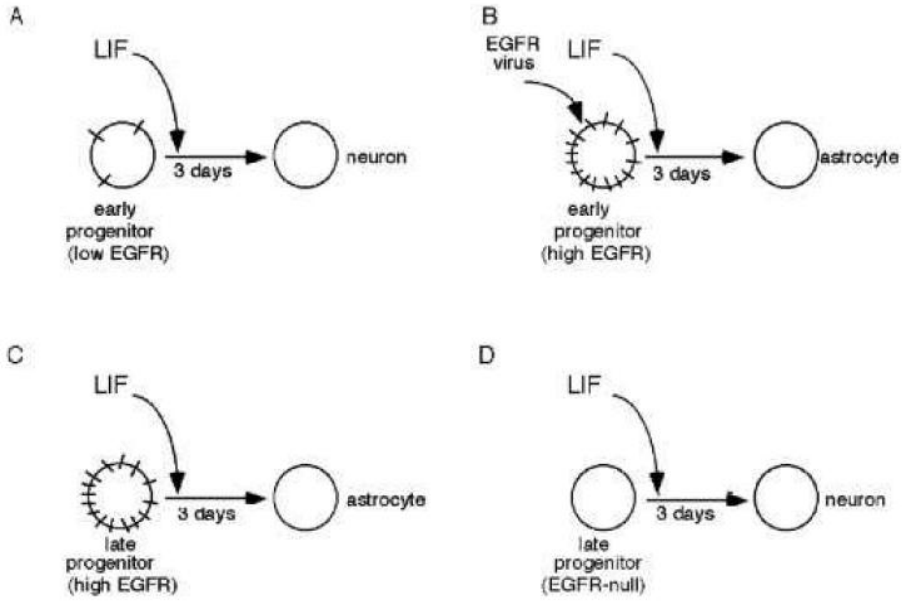

Figure 31. Relationship between EGFR expression and responsiveness to LIF. Regardless of their age, progenitors that express low (*A*) or no (*D*) EGFRs generate neurons when stimulated with LIF, whereas progenitors that express a high level of EGFRs (*B, C*) generate astrocytes in response to LIF (adapted from Viti et al., 2003).

4.4 Egf Receptor (EgfR)

EgfR is expressed at low level in the ventricular zone of the neuronogenic pallium and, at higher and higher levels in basal proliferative layers of the developing cortex (Caric et al., 2001).

The epidermal growth factor receptor (EgfR; ErbB1, HER1 in humans) is a cell-surface receptor. EgfR is a member of the ErbB family of receptors, which includes four strictly related receptors tyrosine kinases (RTKs): Egf (ErbB1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4). The genes encoding for these four receptors can be alternatively spliced to generate partial protein products (Bazley and Gullick, 2005).

EgfR exerts several roles in normal development, differentiation, migration, wound healing and apoptosis, which are of course essential for cell survival of multicellular organisms. It is confirmed by the fact that aberrant functions has been observed in dramatic disease such as cancer (Bazley and Gullick, 2005).

There are 11 ligands which have been identified to bind these receptors in mammals, such as: EGF, transforming growth factor-α (TGF-α), HB-EGF (heparin binding), betacellulin, amphiregulin, epiregulin, epigen and neuregulins (NRGs) (Olayioye et al., 2000). Many of them are located in plasmamembrane and require protelytic cleavage to be secreted in the extracellular milieu and bind receptors.

As all the members of ErbB family, EgfR comprises a conserved protein tyrosine kinase domain that stays within the cytoplasm, a transmembrane domain that makes a single pass through the plasma membrane and a glycosylated, extracellular ligand-binding domain.

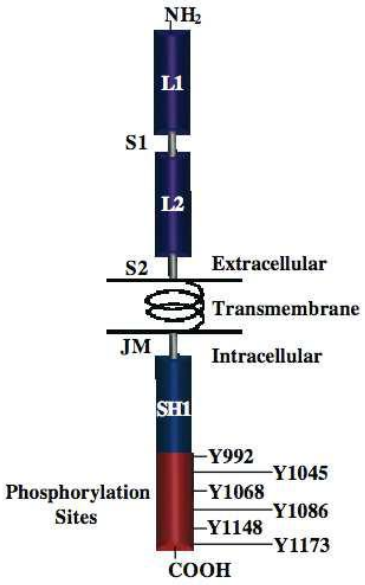

Figure 32. Structural motifs and regulatory elements in the EGF receptor.

In details, the extracellular domain is composed by four subdomains called L1, S1 (CR1), L2 and S2 (CR2). S1 and S2 are homologous regions rich in cysteine, whereas L1 and L2 form the ligand-binding site (Garrett et al., 2002; Ogiso et al., 2002).

Conversely, the cytoplasmic region of EGFR comprises three well-defined domains: (1) the juxtamembrane domain, which is required for feedback by protein kinase C; (2) the noncatalytic carboxy-terminal tail, which possesses the six tyrosine transphosphorylation

sites responsible for recruitment of second messenger proteins, containing SH2 domains or PTB domains, plus the motifs necessary for internalization and degradation of the receptor; (3) the central tyrosine kinase domain, responsible for mediating transphosphorylation of the six carboxyterminal tyrosine residues (Bazley and Gullick, 2005).

4.4.1 EgfR: pathways and functions

Prior to ligand binding, the receptor resides within the cell membrane and the activation loop that is contained in the cytoplasmic PTK domain adopts an inactive conformation, which is inaccessible to both substrate and ATP.

As the ligand binds the receptor, each S1 domain projects an extracellular "dimerization loop", which interacts with the other one and triggers dimerization.

Dimerization may also include a direct contact between the helical transmembrane domains of the receptors, association permitted by the aminoacids dimerization motifs.

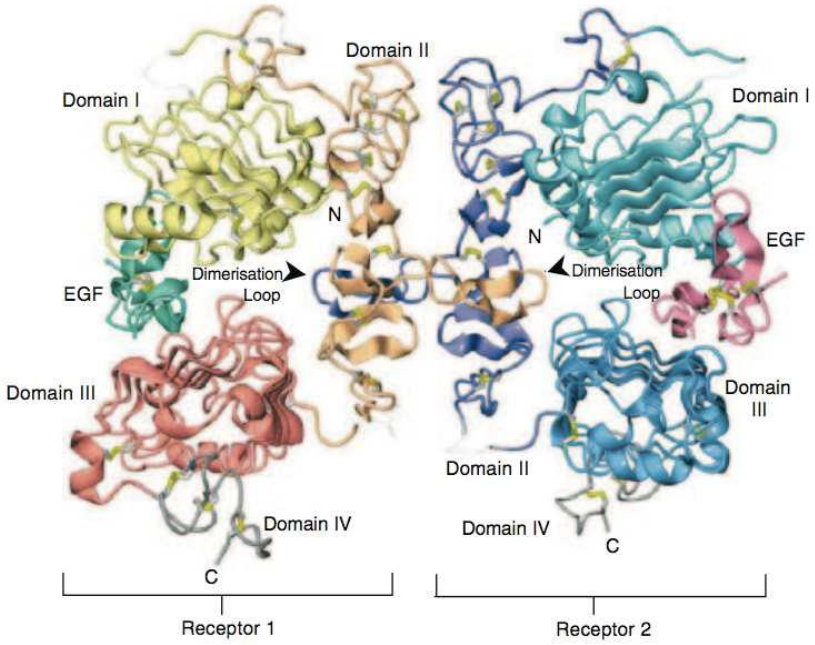

Figure 33. Crystal Structure of the 2:2 EGF-EGFR Complexes. Ribbon diagram with the approximate two-fold axis oriented vertically. One EGF chain in the 2:2 EGF•EGFR complex is pale green, and the other EGF chain is pink. Domains I, II, III, and IV in one receptor in the dimer are colored yellow, orange, red, and gray, respectively. Domains I, II, III, and IV in the other receptor are colored cyan, dark blue, pale blue, and gray, respectively. Most of domain IV is disordered. The disulfide bonds are shown in yellow. The intervening parts that were not assigned are transparent. (adapted from Ogiso et al.,2002)

Moreover, the transmembrane domain is thought to be involved in the regulation of receptor association, spatial arrangement and consequently signalling dynamics (Alroy and Yarden, 1997).

EgfR can form homodimers or heterodimers with the other members of its family. Furthermore, an oligomerization has been demostrated to occur after ligand binding and second messenger recruitment.

Remarkably, dimerization is required to enhance catalytic activity: in the case of EgfR it is sufficient to induce the cross-phosphorylation between the two receptors in specific tyrosine residues.

The tyrosine residues which become phosphorylated recruit specific second messenger, such as SH2- and PTB-containing proteins, which in turn can take contacts with phospholipids or nucleic acids and/or possess intrinsic enzymatic activity.

So finally, downstream signalling pathways, such as the MAPK or PI3K/Akt pathways, promote cell proliferative and survival/antiapoptotic signals, via activation of specific transcription factors. Undoubtedly, as this class of receptors is so deeply implicated in such complicated mechanisms fundamental for cell development and survival, an intricate and fine regulation of them is required for proper working of the whole system (**Fig. 34**).

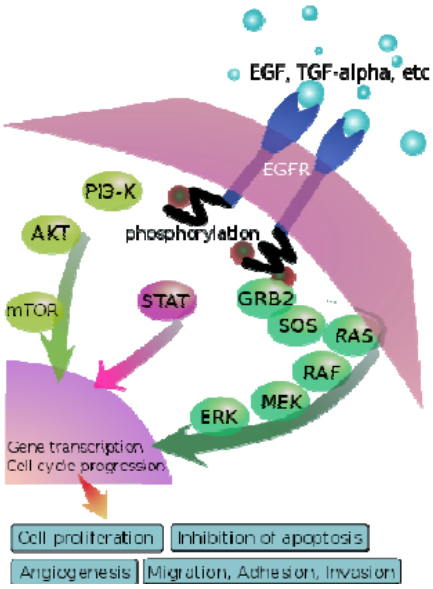

Figure 34. Epidermal growth factor receptor (EGFR) signaling pathway

4.5 Emx2: main features

The Empty spiracles homolog gene-2 (*Emx2*) is localized on murine chromosome 19, includes three exons and encodes for a homeodomain transcription factor, EMX2, playing multiple roles in the developing mouse embryo.

Emx2 is expressed in cephalic regions of mouse embryos including the developing cerebral cortex (Simeone et al., 1992a). *Emx2* is expressed also in primordial of the urogenital system and limb buds (Pellegrini et al., 1997; Simeone et al., 1992a).

The lack of *Emx2* is fatal. *Emx2* homozygous mutant mice ($Emx2^{-/-}$) die soon after birth due to the absence of kidneys (Miyamoto et al., 1997; Pellegrini et al., 1996; Yoshida et al., 1997). They also suffer specific skeletal defects, including the absence of scapulae and ilia (Pellegrini et al., 2001). Knock-out of *Emx2* also deeply affects central nervous system (CNS) development. The cerebral hemispheres, olfactory bulbs and hippocampus are reduced and the dentate gyrus is absent, largely due to decreased cell proliferation (Pellegrini et al., 1996; Yoshida et al., 1997). There are also defects of migration, differentiation and innervations in specific neuronal populations (Mallamaci et al., 2000a and 2000b).

Heterozygous null mice ($Emx2^{-/+}$) suffer a minor morphological defect between the incus and malleus within the middle ear and they have a small but significantly greater number of hair cells in the apical region of the cochlea (Rhodes et al., 2003).

4.5.1 Emx2 in cortical development

Emx2 is one of the earliest markers of cerebral cortex. Indeed, it is activated in the mouse central nervous system at around E8.0-E8.5 (Gulisano et al., 1996). From this stage, its transcript becomes more and more abundant in the anterior dorsal neuroectodermal regions of the embryo. At E9.5, its expression domain includes part of presumptive diencephalon, the coelomic epithelium which will generate the urogenital system (**Fig 35**).

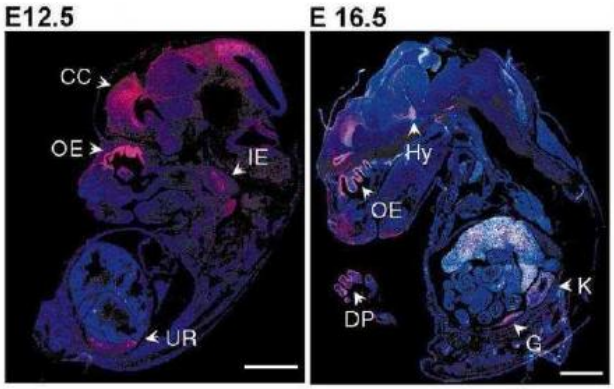

Figure 35. *Emx2* expression profile. In situ analysis on sagittal sections of embryonic day 12.5 (E12.5) and E16.5 mouse embryo. *Emx2* is highly expressed in telencephalon, especially in cerebral cortex (CC) and olfactory epithelium (OE). At E16.5 a strong signal of *Emx2* is detected in hypothalamus (Hy), digit primordia (DP) and gonads (G). Scale bars: 1 mm in E12.5 embryo and 4 mm in E.16.5 embryo. UR,uro-genital ridge. (Adapted from Simeone et al., 1992).

From E10 *Emx2* expression is confined to the neuroephitelium while is absent in most postmitotic neurons of the transitional field of the cortical plate (Giulisano et al., 1996). At E12.5 *Emx2* mRNA becomes restricted to the ventricular zone (VZ), following a gradient from the posterior dorsal telencephalon (where there is higher expression) to the anterior boundary. This expression pattern becomes more pronounced from E14.5 onwards (Simeone et al., 1992; Giulisano et al., 1996). Furthermore, the distribution of the *Emx2* product (EMX2) displays the same antero-posterior and medio-lateral gradient. Until E17 *Emx2* domain of expression remains confined to the proliferative layers of the cortex plus the pioneer neurons of Cajal-Retzius. **(Fig 36).**

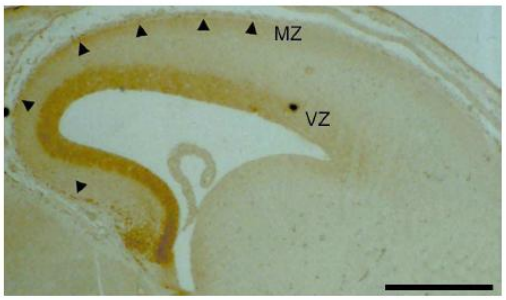

Figure 36. Immunohistochemical analysis of a coronal section of E15.5 mouse cerebral cortex. *Emx2* distribution covers the ventricular zone (VZ) and marginal zone (MZ) where Cajal-Retzius cells lie (arrowheads). Scale bar: 0.5 mm (Adapted from Mallamaci et al., 1998).

The peculiar patter of expression of *Emx2* suggests that this gene is involved in many key aspacts of the cortical development as:

1. cortical specification

Emx2 is implicated in early pancortical specification of dorsal telencephalon, playing this role in concert with *Pax6*. In particular, in the absence of both genes, dorsal telencephalon is mis-specified and rapidly acquires molecular striatum-like properties (Muzio et al., 2002b).

2. cortical regionalization and arealization

Moreover, *Emx2* is expressed in the primary proliferative layer of the cortex along rostral/lateral low-to-caudal/medial high gradients (Gulisano et al., 1996)(**Fig.37**). This pattern is crucial to proper specification of different areal identities within the developing cortical primordium. Indeed in *Emx2* knockout mice, occipital cortex and hippocampus are shrunken and frontal cortex is enlarged. Moreover, the areal distribution of the thalamo-cortical radiation is perturbed, coherently with such areal disproportions.

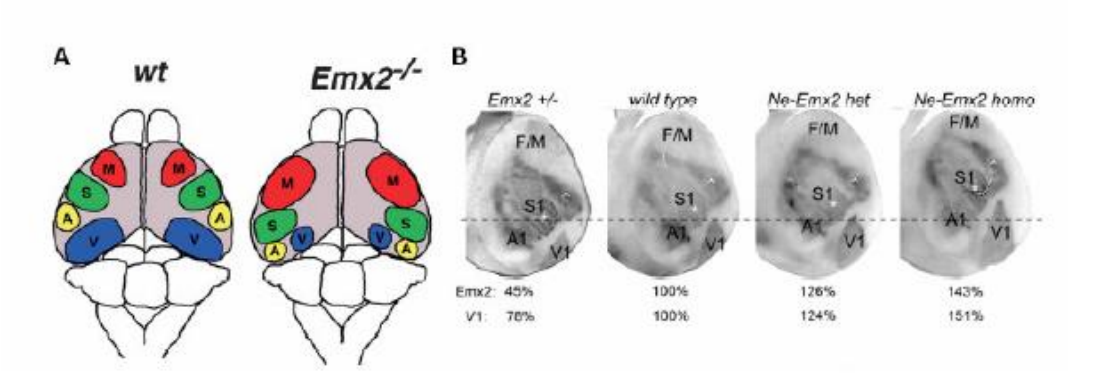

Fig 37 Area identity shifts in cerebral cortices of perinatal and adult Emx2-/-. Pioneering work on Emx2 null mice showing cortical area shift with enlargement of anterior areas and shrunkage of posterior and medial areas (A). Direct involvement of Emx2 in cortical arealization has been later confirmed by conditional gain of function and loss of function studies (B).

Molecular mechanisms mediating cudo-medializing properties of Emx2 are very complex. In particular, it has been shown that *Emx2* imparts a posterior medial identity to the neocortical progenitors repressing *Fgf8* signaling from the ANR (Fukuchi-Shimogori and Grove, 2003), However pioneer works, claiming a direct implication of *Emx2* in cortical arealization, have been later confirmed by conditional loss of function (LOF) and gain of function (GOF) experiments (Hamasaki et al., 2004). These authors show that overexpression of *Emx2* by the nestin promoter is sufficient to cause anterior

area shift in a dosage dependent manner, when compared to wild type and heterozygous mice. Since in pNes- *Emx2* and in *Emx2*$^{+/-}$ mice no effects on *Fgf8* expression and no aberrant TCAs are detectable, the direct implication of *Emx2* in cortical area specification has been confirmed (**Fig.13**).

3. ***in cortical lamination***

Emx2 is expressed in the VZ, but not in the vast majority of neocortical neurons (Mallamaci et al., 1998;Simeone et al., 1992a), except Cajal-Retzius cells within the marginal zone. Cajal-Retzius cell formation is progressively impaired in *Emx2*$^{-/-}$ mice, resulting in aberrant cortical plate layering and pronounced radial glia abnormalities (Mallamaci et al., 2000a).

Abnormal radial migration displayed by cortical plate neurons in *Emx2*$^{-/-}$ mice is similar to that observed in *reeler* mutant mice. In *reeler* mice, early cortical plate neurons do not penetrate the preplate, which is not split in marginal zone and subplate and give rise to the so called super-plate. Moreover, late born cortical plate neurons do not overcome earlier ones, so that the classical inside-out rule is not followed. In *reeler* mutants, these migratory defects originate from constitutive functional ablation of the *Reln* gene, whose expression in the cortical marginal zone is necessary and sufficient to properly orchestrate neocortical neuron layering (D'Arcangelo et al., 1995). In the *Emx2*$^{-/-}$ marginal zone, *Reln* mRNA expression is apparently normal at E11.5, it is reduced at E13.5 and completely absent since E15.5. In the same mutants, early phases of cortical plate radial migration are poorly affected whereas late phases are impaired in a *reeler* like way (Mallamaci et al., 2000a). It is reasonable to hypothesize that the same Cajal-Retzius neurons do not require the products of *Emx2* at the very beginning of their life and only subsequently become dependent on them for surviving and/or retaining their proper differentiation state. However, the increase of the absolute total number of *Reln* expressing cells taking place in wild-type animals between E11.5 and E15 (Alcántara et al., 1998), as well as E10.5-E19 and E12-E19 birth dating survival data (Mallamaci et al., 2000), suggest that at least two different populations of *Reln* expressing cells do exist, which can be operationally distinguished on the basis of their dependence on *Emx2* function. There is an early transient population, prevalently generated before E11.0 and not dependent on the *Emx2* function, and a later one, still detectable at approximately

birth, prevalently generated after E11.5 and dependent on the *Emx2* function for crucial steps of its development.

Remarkably, the caudomedial telencephalic wall and the cortical hem are one of the main sources of neocortical *Reln*-positive Cajal-Retzius (CR) cells (Takiguchi-Hayashi et al., 2004), being the other two the ventral pallium and the septum (Bielle et al., 2005). Cortical hem-born CR cells tangentially migrate beneath the pia mater, in an overall posterior anterior direction, and finally distribute throughout the entire neocortex, along a caudomedial-high to rostrolateral-low gradient. Embryos lacking *Emx2* display an impaired development of their cortical caudomedial region. Thus, absence of CR cells in these mutants may be a subset of this regional phenotype, possibly arising from dramatic size-reduction of the caudal-medial proliferating pool that generates them.

4.5.*2 Emx2* and regulation of cortical precursors kinetics

Initial studies on cortical precursors expressing abnormal *Emx2* levels indicated that the impact of this TF on their histogenetic properties is quite complex. Acute inspection of embryonic brains knock-out for *Emx2* and short term analysis of embryonic neural precursors harboring abnormal dosages of this gene showed that *Emx2* promotes cell cycle progression and inhibits premature neuronal differentiation (Heins et al., 2001; Muzio et al., 2005). Conversely, *Emx2* overexpression in more advanced neural stem cells (long-term passaged perinatal ones or adult ones) turned out to force neural precursors to stop proliferating and undergo neuronal differentiation (Galli et al., 2002; Gangemi et al., 2006). Molecular and cellular mechanisms mediating pro-proliferative activity of *Emx2* have been addressed in vivo to some extent. It has been shown that in *Emx2*$^{-/-}$ mice there is an elongation in neuroblast cycling time (TC) due to lengthening of TS (DNA synthesis phase), particularly pronounced in the early caudal-medial cortical primordium. Due to TC elongation, the proliferating pool of the mutant caudal medial cortex “loses” one cell cycle out of four/five, with respect to its wild type counter part. Moreover, cortical progenitors leave cell cycle more frequently. So, because of exaggerated neuronal differentiation, the caudal-medial proliferating pool is deprived of its components at even doubled rates. Those kinetic changes are associated to increased pro-neural/anti-neural gene expression ratio, down-regulation of lateral inhibition machinery and depression of canonical Wnt signalling (Muzio et al., 2005). Remarkably, by pharmacologically reactivating Wnt signaling in *Emx2*$^{-/-}$ mutants, the neurogenic rates

are rescued. Wnt and Bmp signaling synergically promote *Emx2* transcription, through a betacatenin/Smad1,4 binding module located within the *Emx2* telencephalic enhancer (Theil et al., 2002). In turn, *Emx2* up-regulates the final output of the canonical Wnt-signaling machinery, thanks to concerted modulation of ligands (*Wnt3a*, *Wnt8b*, *Wnt5a, and Wnt2b*), surface receptors (*Fzd9*, *Fzd10*), intracellular beta-catenin agonists (*Lef1*) and intracellular beta-catenin antagonists (*Groucho*) (Muzio et al., 2005). In this way, near the cortical hem, a positive regulatory loop establishes between *Emx2* and Wnt signaling, crucial for proper sizing of occipital cortex and hippocampus. All these phenomena are much more pronounced in caudal-medial than in rostral pallium, substantially contributing to selective hypoplasia of occipital cortex and hippocampus in the late gestation *Emx2* null embryos.

A more recent study (Brancaccio et al., 2010), confirmed the bimodal impact of *Emx2* on neural stem cells (NSC). In fact, lentivirally-mediated overexpression of *Emx2* in dissociated E11.5 cortico-cerebral stem cells initially promotes their self-renewal and then addresses them to neuronogenesis, directly or via neuronal progenitors (NPs). This study also disclosed three new previously unknow biological activities played by Emx2 in cortical precursors. *Emx2* promotes the commitment of NSCs to glial fates, while inhibiting further maturation of early bipotent glial progenitors; it protects NPs fom cell death; it strongly accelerates neuronal differentiation of NPs (Brancaccio et al., 2010). Molecular mechanisms underlying bimodal activity of Emx2 in NSCs (earlier pro-self-renewing, later prodifferentiating) are presently unknown. It is possible that, depending on the stage, embryonic or post-natal, Emx2 may undergo different post translational covalent modifications or also may be bound by different age-specific cofactors.

That might modify its ability to be recruited to distinct chromatin loci and/or its transcription trans-modulating properties, so making it alternatively able to promote neural precursors self-renewal or neuronal differentiation. Remarkably, it has been shown that temporally structured overexpression of Emx2 (alone or in combination with the TF Foxg1) may promote the expansion of the neural stem pool and ameliorate the neuronogenic output of engineered cultures, while reducing their gliogenic one (Brancaccio et al., 2010). That might allow to circumvent two of the most basic problems arising in cell-mediated therapy of brain disease, the "collapse" of the proliferating stem pool and its exaggerated glial differentiation, so making the histogenetic activities of Emx2 of paramount interest for purposes of gene-assisted therapy of brain diseases.

4.5.3 .*Emx2* extraneural expression

Apart from being expressed in CNS, *Emx2* is also active in other tissues, resulting crucial to their proper morphogenesis. At E12.5 it is expressed in the developing olfactory epithelium, the kidneys and the gonads. Knocked-down mutants die soon after birth because they defects of development of the urogenital system.

Kusaka et al. (Kusaka et al., 2010) recently dissected cellular and molecular mechanisms leading to dismorphologies of the urogenital system occurring in *Emx2*-LOF mutants

The gonadal primordium emerges as a thickening of the embryonic coelomic epithelium, which then migrates mediodorsally to form the primitive gonad. Epithelial cell polarization results in specialized surfaces, such as apical, lateral and basal ones. These cells adhere one with the other through tight and adherens junctions positioned at the apical border of their lateral surfaces. These junctions are formed by adhesion molecules and intracellular membrane associated proteins (β-catenin, zonula occludens) (Shin et al., 2006).

Interestingly, in *Emx2*$^{-/-}$ mice the early stages of gonad development are disrupted, with a dramatic enlargement of the surface of epithelial cells hosting tight juntions and migration of the epithelial cells significantly affected (Kusaka et al., 2010).

Microarray analysis of the epithelial cells of the embryonic gonad displays a dramatic upregulation of EgfR in *Emx2* KO mice. Based on previous demonstration of an EgfR involvement in regulation of tight junctions assembly, such ectopic expression of EgfR was strongly suggested to act as *trait-d'union* between *Emx2* ablation and junctional anomalies of *Emx2*-LOF mutants.

As EgfR has been deeply implicated in early promotion of gliogenesis, this mechanism of action demonstrated in gonad development provided us with a valuable suggestion about key mechanisms linking altered *Emx2* expression and astrogliogenic abnormalities. The possibility that *Emx2* depresses gliogenesis by down-regulating the *EgR* gene will be just the starting hypothesis of the present work.

MATERIALS AND METHODS

5. Lentiviral vectors packaging

Gene transfer mediated by vectors derived by lentiviruses, such as Human immunodeficiency virus (HIV-1), is an interesting tool in gene therapy and basic research fields. Remarkably, lentiviral vectors (LV) provide effective means for the delivery, integration and expression of exogenous genes in mammalian non-dividing cells. (Vigna and Naldini, 2000).

Lentiviral vectors have a complex genome. Indeed lentiviruses contain three main structural genes, encoding for the viral proteins (in order 5' gag pol env 3') common to all other retroviruses. They also contain additional genes (called accessory genes), depending on the virus (e.g. for HIV:Vif, Vpr, VPu, tat, rev, nef), whose products are involved in modulation of viral gene expression, assembly of viral particles, and structural and functional alterations in the infected cells.

Viral replication is partially modulated by *cis*-active non-coding sequences. They include:

- the 5' long terminal repeat (LTR) which, in the DNA form found in the provirus acts as a transcriptional promoter, and in the RNA (genomic) form contains sequences important for reverse transcription of the genome;
- the psi (ψ) sequence which directs packaging of the genomic RNA into the virion;
- the polypurine tract (ppt) which is the primer binding site for second strand DNA synthesis during reverse transcription and the 3' LTR which, in the DNA form (in the provirus) acts as a polyadenylation signal, and in the RNA (genomic) form contains sequences important for the reverse transcription process

The general strategy to produce lentiviral vectors relies on the segregation of *cis*-active sequences from *trans* active ones, which are strictly required for the production, infection and integration, as well as the removal of all the unnecessary genes from HIV-1 genome (Delenda, 2004).

Most of *cis*-active sequences (essential for LV functioning) are usually included in the transfer construct (the part of LV which integrates in the host cell genome and encodes the gene of interest).

Conversely, *trans*-active sequences encode for three groups of proteins (structural, regulatory and accessory) are inserted in one or more constructs distinct from the transfer one (**Fig.38).**

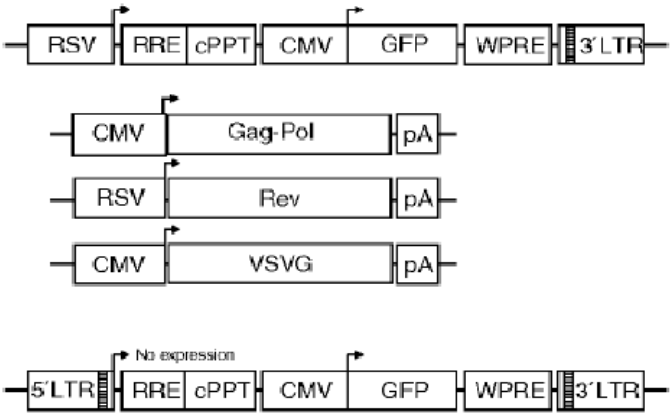

Fig. 38 Schematic representation of third generation lentiviruses (Tiscornia et al., 2006).Packaging plasmids (pMDL, pREV, pVSVG) provide trans factors, such as Gag-Pol, Rev and the envelope protein VSVG, respectively. The plasmid pMDL encodes for the protein Gag-Pol that is subsequently processed into integrase, reverse transcriptase and structural proteins. These latter proteins are absolutely required for the production of viral particles, while the integrase and the reverse transcriptase (which are already present in the viral particle) are involved in the events following the infection. Rev interacts with a *cis* sequence (RRE) in the transfer vector in order to increase the efficiency of the transport of non-spliced genomic transcripts from the nucleus to the cytoplasm. The presence of VSVG in the envelope confere the ability to infect a large variety of cells, such as primary cells and stem cells (Vigna and Naldini, 2000; Lois et al., 2002; Pfeifer et al., 2002).

Remarkably, 3rd generation lentiviral vectors are replication-defective, this means that they retain the capability of executing only the former passages of their life cycle (**Fig. 39**): attachment, entry, reverse transcription, nuclear transport and integration.

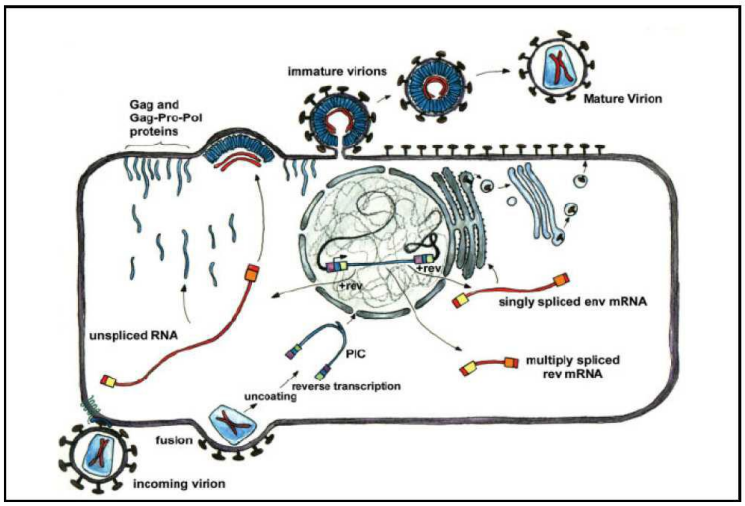

Fig 39.**The lentiviral life cycle**

The system safety is granted by the providing of the functions in *trans* which are required for the packaging by three distinct and additional plasmids (pMDL, pREV, pVSVG); besides, by the deletion in the enhancer region in the 3' LTR (Long Terminal Repeat) on the transfer vector. The first measure drastically reduces the accidental formation risk of viral genomes ready for replication, thanks to casual recombination. The second one, since the 3'LTR is used as a template to generate both copies of the LTR in the integrated proviral form of the vector, the deletion results in transcriptional inactivation of both LTRs and prevents its mobilization and recombination in transduced cells (SIN vector: self inactivating vector)(Bukovsky et al. 1999). The expression constructs used for LV production are maintained in the form of bacterial plasmids and can be transfected into mammalian cells to produce replication-defectice virus stocks. To improve the efficiency of gene delivery and expression in target cells some modifications have been made. One of these modifications involved inserting the posttranscriptional regulatory element from the genome of the woodchuck hepatitis virus (Wpre) into the 3' end of the transfer vector. The Wpre acts at the posttranscriptional level, by promoting nuclear export of transcripts and/or increasing the efficiency of polyadenilation of the nascent transcript, thus increasing the total amount of mRNA in cells (Zufferey et al. 1999). LV are traditionally produced by transient cotransfection of human embryonic kidney 293T cells (a continuous human embryonic kidney cell line transformed by shared Type 5 adenovirus DNA, by transfection with the tsA 1609 mutant gene of SV40 large T antigen and the Neo gene of *E.coli*). Indeed these cells are good DNA recipients in transfection procedures and the backbones of the vector construct contain SV40 origin of replication. Third-generation lentiviral vectors are produced by cotransfection of four types of plasmids into 293T cells (Dull et al. 1998a). The four plasmids used are:

1. *pMDLg/pRRE*, encode for proteins GAG-POL;
2. *pRSV.REV*, encode for protein Rev;
3. *pCCL-SIN18PPT.Prom.EGFPWpre*, transfer vector containing the gene of interest (selfinactivating)(A Follenzi & L Naldini 2002);
4. *pMD2 VSV.G* encode for the envelope protein VSVG (L Naldini et al. 1996)(Dull et al. 1998b) (Zufferey et al. 1998)(A Follenzi et al. 2000);

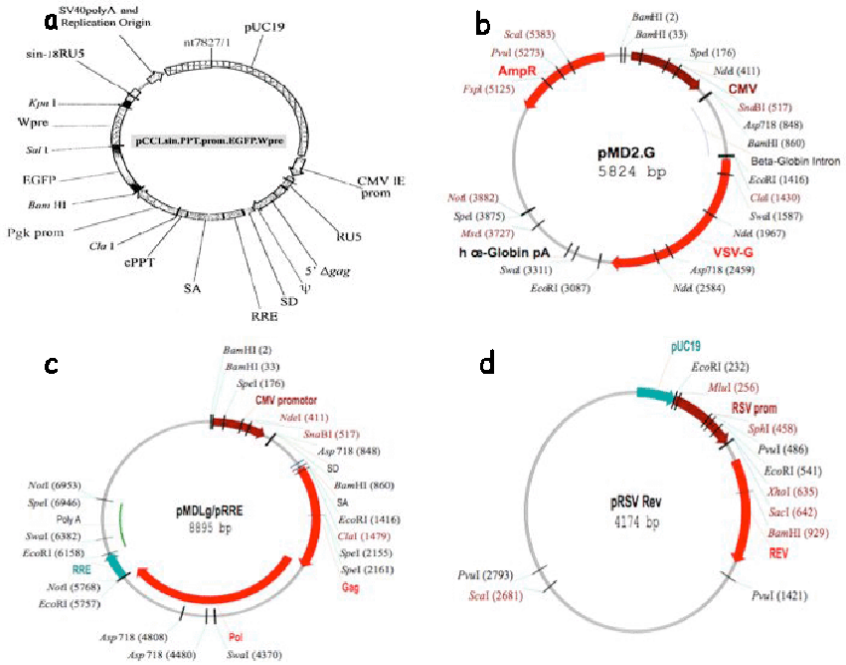

Fig. 40 Schematic drawing of the four constructs required for the production of self-inactivating vectors (SIN). a)The selfinactivating (SIN) transfer construct containing HIV-1 cis-acting sequences and expression cassette for the transgene (enhanced green fluorescent protein or EGFP) driven by the internal promoter. b)The second construct encoding the heterologous protein of the envelope to pseudotype the vector, the protein G of the vesicular stomatitis virus (VSVG) under the control of the CMV promoter. c)The construct expressing the *gag* and *pol* genes driven by the CMV promoter. d)The construct for the expression of Rev protein under the RSV promoter.

The following 3rd generation SIN lentiviruses were used for this study: LV:*pPgk1-rtTA2^S-M2* (Spigoni et al., 2010), LV:*pNes/hsp68-rtTA2^S-M2*, LV:*TREt-Emx2-IRES2-EGFP*, LV:*TREt-luc-IRES2-EGFP* and LV:*pTα1-mCherry* (Brancaccio et al., 2010); LV:*TREt-IRES2-EGFP* and LV:*TREt-luc* (derived from LV:*TREt-luc-IRES2-EGFP*, via deletion of the *luc* and the *IRES2-EGFP* cassettes, respectively); and LV:*pCCLsin.PPT.hPGK.EGFR.pre* (Mazzoleni et al., 2010). LV_*TREt-Sox2*, aka TetO-FUW-sox2 (Brambrink et al., 2008), corresponding to plasmid #20326 of the Addgene collection; LV_*TREt-Brn2* aka Tet-O-FUW-Brn2 (Vierbuchen et al., 2010), corresponding to plasmid #27151 of the Addgene collection.

All lentiviruses were generated as described below:

293T cells were co-lipofected (LipoD, SignaGen) with the transfer vector plasmid plus three auxiliary plasmids (pMD2 VSV.G; pMDLg/pRRE; pRSV-REV).

The conditioned medium was collected after 24 and 48hs, filtered and ultracentrifuged at 50000 RCF on a fixed angle rotor (JA 25.50 Beckmann Coulter) for 150 min at 4°C. Viral pellets were resuspended in PBS without BSA (Gibco) and stored at -80°C.

5.1 Lentiviral vectors titration

The titer, TU/μL (Transducing Units/Volume Unit) is a measure of virus concentration per 1 μL. Lentiviral vectors used in this work have been titered using two methods:

- EGFP-expressing lentiviral vectors were titrated on HeLa TET-off cells (Clontech), by end point fluorescence titration. Fluorescent titration allows to estimate the number of productive integrations of a lentiviral vector containing a fluorescent tracing detectable by microscope. The titer was expressed as transducing units per mL (TU/mL) and it is obtained estimating the number of tranduced cells/total cells, in the appropriate serial dilution (positivity in the range of 1-15%).
- Other LTVs were titrated by Real time quantitative PCR after infection of HEK293T cells. One end point fluorescence-titrated LTV was included in each PCR titration session and PCR titers were converted into fluorescence-equivalent titers. This kind of titration involves the amplification of ϕ region (packaging sequence) in the lentiviral vector genome and of the housekeeping gene *Otx2*. Data obtained by the amplification of the ϕ region are normalized on data obtained with the housekeeping gene. The number of lentiviral amplicons found in the infected cells, that represents the number of integrated pro-virions, is estimated through the standard titrating curve. The number of genomic DNA insert in each well is calculated with the formula: Titer (TU/μL) = (2*number of infected cells*number of LV amplicons detected)/(assayed volume of LV suspension*number of *Otx2* amplicons detected). Then, the ratio of titer between the examined LTV and a second LTV at known concentration is calculated. By multiplying the titer of a LTV obtained by real time PCR for the fluorescence titer of the second LTV, the real titer is thus given.

The protocols used for this kind of titration are:

for ψ region:

Incubate at 95°C for 00:04:00

Incubate at 94°C for 00:00:10

Incubate at 60°C for 00:00:15

Incubate at 72°C for 00:00:20

Plate Read at 72°C hold 00:00:01

Plate Read at 78°C hold 00:00:01
Go to line 2 for 39 more times
Melting curve from 58°C to 98°C, read every 0.5°C, hold 00:00:01 END

Protocol used for *Otx2* is the follows:
Incubate at 95°C for 00:04:00
Incubate at 94°C for 00:00:10
Incubate at 63°C for 00:00:15
Incubate at 72°C for 00:00:20
Plate Read at 72°C hold 00:00:01
Plate Read at 78°C hold 00:00:01
Go to line 2 for 39 more times
Melting curve from 58°C to 98°C, read every 0.5°C, hold 00:00:01

Primer sequences are the follow:
ψ region /F2 5' GGCAAGCAGGGAGCTAGAACGATTCGCAG 3'
ψ region/R2 5' CTTCTGATCCTGTCTGAAGGGATGGTTGTAGCTGTCC 3'
h*Otx2*/F 5' CTCAGGCTTCAGGTTATAGTCAAGGATATGCTG 3'
h*Otx2*/R 5' CTGATTGAGATGGCTGGTGACTGCATTGG 3'

In both kind of titration (at 9 µg/mL) to increase the efficiency of infectionwas used Polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide, hexadimethrine bromide(sigma)). Polybrene is a cationic polymer which acts by neutralizing the charge repulsion between virions and sialic acid on the cell surface.

6. Animal handling and Embryo dissection

Animal handling and subsequent procedures were in accordance with European laws [European Communities Council Directive of November 24, 1986 (86/609/EEC)] and with National Institutes of Health guidelines. Wild type mice (strains CD1 purchased from Harlan-Italy) and *Emx2*$^{+/-}$ mutants (Pellegrini et al., 1996), moved to a CD1 background, were maintained at the SISSA mouse facility. Wild type and *Emx2*$^{+/-}$ embryos were staged by timed breeding and vaginal plug inspection. *Emx2*$^{+/-}$ embryos were genotyped by PCR.

Pregnant females were killed by cervical dislocation. Embryonic cortices were dissected out in cold PBS, under sterile conditions.

6.1 Genotyping

The terminal portion of the tail was incubated in a lysis buffer solution (10 mM Tris HCl pH=8.3, 5 mM EDTA, 300 Mm nAaCETATE,1% Triton X-100) containing proteinase K at the final concentration of 1mg/mL, overnight at 55°C. The morning after, the samples were kindly vortexed and then centrifugated at 3000 rpm for 2 minutes. Next, the samples were diluited 1:20 in H2O.

Polymerase Chain Reaction (PCR)

The reagents used were:

	Final concentration
Buffer GoFlexi	1X
MgCl2	1,5 mM
dNTPs	0,2 mM
Primer E2F	0,8 µM
Primer E2R/wt	0,8 µM
Primer E2R/KO	0,8 µM
Taq	0,05U/µL
H2O	until the final volume of 50 µL.

Protocol used:

98°C/5’ 1X

98°C/1’ ; 72°C/2’ 5X

94°C/1’ ; 72°C/2’ 30X

72°C/10’ 1X

Primer sequences:

E2F CACAAGTCCCGAGAGTTTCCTTTTGCACAACG

E2R/wt ACCTGAGTTTCCGTAAGACTGAGACTGTGAGC

E2R/KO ACTTCCTGACTAGGGGAGGAGTAGAAGGTGG

7. Neocortical sampling

For time course analysis of *Emx2* in the developing cortex, neocortical explants were taken from E12.5, 14.5, 16.5 and 18.5 embryos and processed for RNA extraction as described below.

8. Primary cortical precursors (cPCs) isolation

Cortical primordia were dissected from E12.5 mouse embryos and mechanically dissociated to single cells. Neural cells were initially cultured onto uncoated 24 multiwell (BD Falcon) , as floating neurospheres in serum free anti-differentiative medium [1:1 DMEM-F12, 1X Glutamax (Gibco), 1X N2 supplement (Invitrogen), 1 mg/mL BSA, 0.6% w/v glucose, 2 µg/mL heparin (Stemcell technologies), 20 ng/mL bFGF (Invitrogen), 20 ng/mL EGF (Invitrogen), 1X Pen/Strept (Gibco), 10 pg/mL fungizone (Gibco)] added with 2µg/µL doxycycline (Clontech).

When required, they were acutely infected, at a concentration of 1,000 cells/µL, by a mix containing lentiviral vectors, each one at a multiplicity of infection (m.o.i) = 8. TetON-controlled transgenes were switched on by 2µg/mL doxycyclin (Clontech).

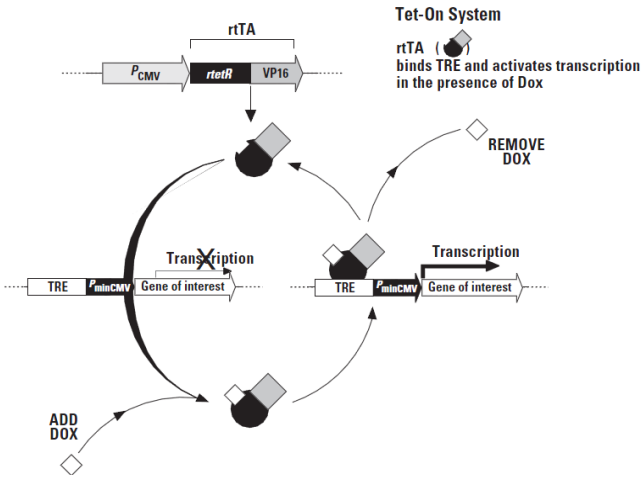

Fig 41 :TEt-On technology Gene expression is regulated by the presence or absence of tetracycline or one of its derivatives such as Doxycycline. Tetracycline binds directly to the transcription factors. In the Tet-On system: the "reverse" Tet repressor (rTetR) can bind the TRE-promoter and activate transcription in the presence of Dox.

Tet-On systems has several advantages over other regulated gene expression systems that function in mammalian cells:

- Extremely tight on/off regulation. Background, or leaky, expression of Gene X in the absence of induction is extremely low with pTRE
- No pleiotropic effects. When introduced into mammalian cells, the prokaryotic regulatory protein (rtTA) acts very specifically on their target sequences, presumably because these regulatory DNA sequences are nonexistent in eukaryotic genomes
- High inducibility and fast response times. With the Tet Systems, induction can be detected within 30 minutes using nontoxic levels of inducer. Induction levels up to 10,000-fold have been observed.

9. Long term cPCs culture maintenance

For long term maintenance, fresh GFs (20 ng/ml bFGF, 20 ng/ml EGF and when required was added 20ng/mL Fgf9 (Sigma)) plus 2µg/µl doxycycline were added 2 days after each cell dissociation. 1.5 more days later, cPCs were harvested, dissociated to single cells by trypsin (Gibco), followed by addition of DNase1 (Sigma) and soybean trypsin inhibitor (Sigma) (Rietze and Reynolds, 2006). cPCs were then replated at 250 cells in 400 µl of anti-differentiative medium/well. These culture conditions lead to free floating neurospheres formation (Reynolds and Weiss, 1992) and effective cPCs propagation.

10. cPCs differentiation

For differentiation experiments neurospheres were harvested after 14 days propagation in serum-free, anti-differentiative culture conditions and dissociated to single cells. cPCs were plated onto 200µg/mL poly-L-lysine-coated 24-wells, at 5*105 cells/well in 500µL

of doxycycline-free differentiative medium [1:1 DMEM-F12, 1X Glutamax (Gibco), 1X N2 supplement (Invitrogen), 1X B27 supplement (Invitrogen), 1 mg/mL BSA, 0.6% w/v glucose, 2 µg/mL heparin (Stemcell technologies), 1mM N-acetylcysteine (Sigma), 5% tetracycline-free serum (Clontech), 1X Pen/Strept (Gibco), 10 pg/mL fungizone (Gibco)]. Differentiative medium was replaced every 3-4 days with fresh medium and cPCs were allowed to differentiate for 7 days.

In specific cases, anti-differentiative medium was supplemented by 0.7 µM LDN (Stemgent#130-096-226), or 20 ng/ml Fgf9 (Sigma#SRP4057-10UG), pro-differentiative medium was supplemented with GFs (20 ng/ml bFGF, 20 ng/ml EGF, Invitrogen) or Lif (30 ng/ml, Millipore).

11.Immunocytofluorescence

For immunocytofluorescence on differentiated cPCs, cells were fixed directly on poly-L-lysine coated 24 multiwell plates, with 4% PFA for 15 min at RT. Wells were then washed in PBS 1X, and blocked in a solution of: 10% fetal bovine serum; 0,1% Triton X-100;1mg/mL BSA and PBS 1X. Incubation with primary antibody was performed overnight at 4°C. The day after, cells were washed in PBS and 0,1% Triton X-100 4 times and incubated with a secondary antibody for 2 hours at RT.

For Emx2 protein quantification, neocortices collected from 10 E12.5 embryos and 5 P0 neonates were pooled by age, mechanically dissociated to single cells and allowed to acutely attach on polylysin-coated SuperFrost N^+ slides.

As for BrdU, its unmasking was performed by 0.2M HCl for 15' at RT.

The following antibodies were used: anti-BrdU, mouse monoclonal, B44 antibody (Becton-Dickinson#347580), at 1:50; anti-S100 rabbit polyclonal (DAKO#Z0311), at 1:1,000 -1:800, anti-GFAP rabbit polyclonal (DAKO#Z0334), at 1:600; anti-Sox2 rabbit polyclonal (Abcam#97959), 1:1,000; anti-Emx2 mouse monoclonal, M06-4F7 antibody (Abnova#H00002018-M06), at 1:200.

Secondary antibodies were conjugates of Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 546, Alexa Fluor 633 (Invitrogen, 1:600).

DAPI (4', 6'- diamidino-2-phenylindole) was used as nuclear counterstaining.

Immunos were photographed on a Nikon Eclipse *TI* microscope equipped with a 20X objective and a Hamamatsu camera. Images were imported and analyzed by Photoshop CS 8.0 (Adobe) software.

Where not otherwise stated, each experiment was performed at least in biological triplicate. In general, for each experimental condition, 2,000-3,000 cells (from 20 randomly assorted photographic fields) were scored, by an operator blind of sample identity.

Frequencies of immunoreactive cells were averaged and s.e.m.'s were calculated. Results were normalized against controls. Their statistical significance was evaluated by the t-test (one-tail; unpaired).

In the case of Emx2 protein quantification, photos were taken from ≥4 slides per age. $Sox2^{+}$-containing fields were selected by an operator blind Emx2-fluorescence and sample age. 30-40 randomly picked $Sox2^{+}$ cells per slide were analyzed. Emx2-levels were evaluated by Photoshop-Histogram plugin, per each single cell.

Data were analyzed by Excel and their significance was evaluated by the t-test (one-tail;unpaired).

12.Cytofluorometry

To prepare FACS analysis, cells were labelled as follows. Dissociated cortico-cerebral precursors, obtained as described above, were acutely infected with the following lentiviruses: LV:*pNes/hsp68-rtTA2^{S}-M2*; LV:*TREt-Emx2-IRES2-EGFP* (or, alternatively, LV:*TREt-Luc-IRES2-EGFP*, as a control); LV:*pTα1-mCherry*. 96 hours prior to analysis, transgenes were activated by doxycyclin addition. Moreover, 72 hours prior to analysis, standard Egf was replaced by biotinylated, Alexa Fluor 555 streptavidin-complexed Egf (equally concentrated), which was refreshed 48 hours later. Finally, just prior to fluorimetric profiling, cells were mechanically dissociated and further labelled with anti-A2B5 mouse monoclonal antibody, APC conjugated (MACS Miltenyi), at 1:10 concentration, according to manifacturer instructions.

Labelled cells were analyzed on a Cyan ADP flow cytometer (Dakocytomation, Denmark). Forward scatter (FSC) and side scatter (SSC) were used to exclude debris and cell aggregates (live gate). Cells belonging to the live gate were evaluated for their EGFP±/mCherry±/Egf-Alexa555±/A2B5-APC± fluorescence profile. Data analysis was performed by Flowjo^{TM} software (Tree Star, Ashland, OR).

This experiment was performed in biological septuplicate. For each experimental condition, 80,000 cells were scored. Frequencies of immunoreactive cells were averaged

and s.e.m.'s were calculated. Statistical significance of results was evaluated by the t-test (one-tail; unpaired).

13. RNA profiling

13.1 Total RNA extraction

Aliquots of $3*10^5$ E12.5 wild type murine cortico-cerebral precursors were infected with a lentivector mix containing LV:*pPgk1-rtTA2^S-M2*, at m.o.i. 8, and, alternatively, LV:*TREt-IRES2-EGFP* or LV:*TREt-Emx2-IRES2-EGFP*, at m.o.i. 8 as well. 7 days after infection, 2μg/mL doxycycline was further added. 4 more days later, cells were centrifuged at 300g for 10 min and processed for RNA extraction by Trizol™ (Invitrogen). The RNA extraction from tissues was performed in Trizol™ (Invitrogen) according to manufacturing instructions.

Agarose gel electrophoresis and spectrophotometric measurements (NanoDrop ND-1000) were employed to estimate quantity, quality and purity of the resulting RNA.

13.2 Reverse transcription (RT)

cDNA was produced by retrotranscription. 1.5 μg RNA were retrotranscribed by SuperScriptIII™ (Invitrogen) according to manufacturer's instructions, in the presence of random hexamers. Then the RT reaction was diluted 1:5 for the subsequent Real-Time PCR.

13.3 qRT-PCR

Gene expression or messenger RNA (mRNA) analysis is the most commonly-used application for qPCR. This technique involves the reverse transcription of the RNA and then the cDNA is used as a template in qPCR reactions to detect and quantitate gene expression products.

With qPCR, fluorescent dyes are used to label PCR products during thermal cycling. Indeed the real-time PCR instruments measure the accumulation of fluorescent signal during the exponential phase of the reaction for fast, precise quantification of PCR products and objective data analysis. Moreover real-time quantitative PCR, also known as qPCR, combines PCR amplification and detection into a single step.

The SYBR Green technique makes use of a cyanine dye able to complex with double-stranded DNA and emit strong green fluorescence (max = 522 nm) after excitation from blue light (max = 488 nm). The fluorescence quantifies the amount of double stranded DNA within the reaction which, if the primer pair has been chosen correctly, will represent target amplification.

Fluorescence readings are then plotted against cycle number on a logarithmic scale; a threshold for detection of fluorescence above background is determined. The cycle at which the fluorescence from a sample crosses the threshold is called the cycle threshold, Ct. Its values can be transformed into absolute values comparing the results to a standard curve produced by real-time PCR of serial dilutions of a known amount of DNA. To accurately quantify gene expression, the measured amount of RNA from the gene of interest is normalized by dividing the amount of RNA from a housekeeping gene measured in the same sample to normalize possible variations in the amount and quality of RNA between different samples. This normalization permits accurate comparison of expression of the gene of interest between different samples, given that the expression of the reference (housekeeping) gene used in the normalization is very similar across all the samples.

1/100 of the cDNA obtained from reverse transcription was used as substrate of any subsequent qPCR reaction. PCR reactions were performed by the SybrGreen™ platform (Biorad), according to manifacturer's instructions.

Primers were as follows:

-*Gapdh*, Gapdh5/F, 5'ATCTTCTTGTGCAGTGCCAGCCTCGTC3', and Gapdh5/R, 5'GAACATGTAGACCATGTAGTTGAGGTCAATGAAGG3';

-*EgfR*, EgfR/F, 5'AGACCCACAGCGCTACCTTGTTATCCA3', and EgfR/R, 5'CAACTACATCCTCCATGTCCTCTTCATCCA3';

- *Sox2*, Sox2/FEXT, 5'CGGCACGGCCATTAACGGCACACTG3', and Sox2/REXT, 5'GAGCATTATCAGATTTTTCCTACTCTCCTCTTTTTG3';

-*Emx2*, E2S/N2F, 5'GGAAAGGAAGCAGCTGGCTCACAGTCTCAGTCTTAC3', and E2S/N2R, 5'GTGGTGTGTCCCTTTTTTCTTCTGTTGAGAATCTGAGCCTTC3';

- *Fgf9*, Fgf9/F4, 5'CGAGAAGGGGGAGCTGTATGGATCAGAA3', and Fgf9/R4, 5'AGAGGTTGGAAGAGTAGGTGTTGTACCAGT3';

-*Id3*, Id3/F1, 5'CGGTCCGCATCTCCCGATCCAGACA3', and Id3/R1, 5'CGGGCGCCAGCACCTGCGTTC3';

-*Msx1*, Msx1/F1, 5'GACTCCTCAAGCTGCCAGAAGATGCTC3', and Msx1/R, 5'GTCCTGGGCTTGCGGTTGGTCTTGTG3';
-*Brn2*, mBrn2/F1, 5'CAACAACAGCGACCGCCACATCTGGTG3', and mBrn2/R1, 5'GTGAAGCTCGGCTGCGAATAGAGCAAAC3';
- *Sip1*, mZeb2/F2, 5'CGAGAGGCATATGGTGACGCACAAG3', and mZeb2/R1, 5'CACTGTGAATTCTCAGGTGTTCTTTCAGGT3';

The reagents used were:

	Final concentration
IQ SYBR Green Supermix	2X
Forward primer	0,25 μM for both *Gapdh* and *Egfr* 0,25 μM *Sox2*, 0,25 μM *Fgf9*
Reverse primer	0,25 μM for both *Gapdh* and *Egfr* 0,25 μM *Sox2*, 0,25 μM *Fgf9*
DNA template	1μl (corresponding to 15 ng of RNA)
Sterile deionized water	to 20 μl final volume

The reactions were run on a Bio-Rad Mini Opticon MJ Mini thermocycler.

Each experiment was performed in biological triplicate. Where not otherwise stated, normalization was against *Gapdh*. Results were averaged and evaluated by the t-test (one-tail; unpaired).

Aim of the work

Generation of astrocytes within the developing cerebral cortex is a tightly regulated process, which bursts after neuronogenesis completion and precedes the bulk of oligodendrogenesis. Astrocytic outputs depend on two primary factors: progression of multipotent precursors towards astroglial lineages and proliferation of astrocyte-committed progenitors. Molecular mechanisms controlling lineage choice have been investigated and reconstructed to large extent. On the contrary, very little is known about control of astroblast population kinetics.

We previously reported that Emx2 overexpression in cortico-cerebral NSCs, while promoting their commitment to astrogenesis, severely reduces the number of advanced astrocyte progenitors. In the absence of overt signs of altered survival of Emx2-GOF astroblasts, we suspected that this could be due to defective proliferation and premature exit of these progenitors from cell cycle. Therefore, main aim of this study was to understand if and how Emx2 inhibits astrocyte progenitors proliferationas well as toreconstruct molecular mechanisms which possibly mediate such inhibition.

Results

Rodents cortico-cerebral astrogenesis mainly takes place during the first postnatal week, following neuronogenesis and prior to the major oligogenic wave (Kessaris et al., 2006) (**Fig 42**).

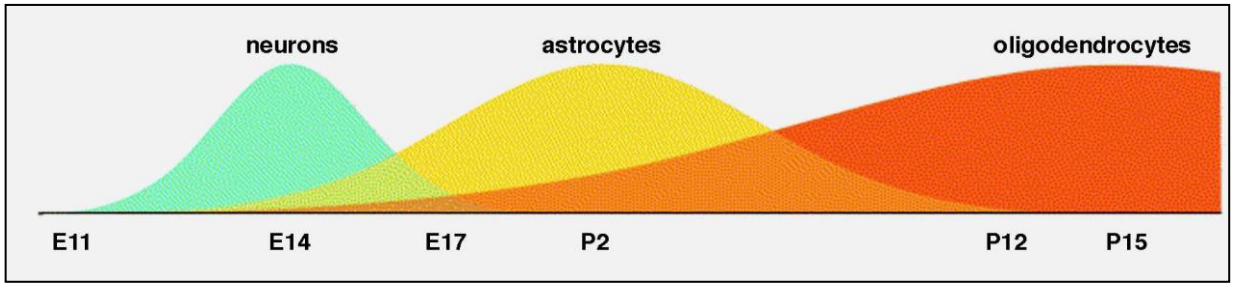

Fig 42: Cortico-cerebral histogenesis

Astrogenic rates are the outcome of two concurrent regulatory processes:

1) progressive commitment of neural precursors to macroglial fates;
2) proper tuning of astroblasts proliferation.

The former process has been largely investigated (reviewed in Mallamaci, in press). Viceversa, molecular mechanisms modulating astroblasts proliferation have been poorly characterized (Bani-Yaghoub et al., 2006; Lum et al., 2009; Mayer et al., 2009; Seuntjens et al., 2009).

It was shown that, in the very middle of neuronogenesis, the mouse cortex undergoes a profound chromatin reconfiguration, endowing it with the latent capability to generate astrocytes (Fan et al., 2005). Despite of that, astrogenic rates remain still very low for three-four more days, even after this deadline. Mechanisms concurring to this scenario are complex ans still subject of intensive investigation (reviewed in Mallamaci, in press).Among factors cooperating to achieve this effect, there is just the limited proliferation of astrocyte-committed progenitors (Seuntjens et al., 2009).

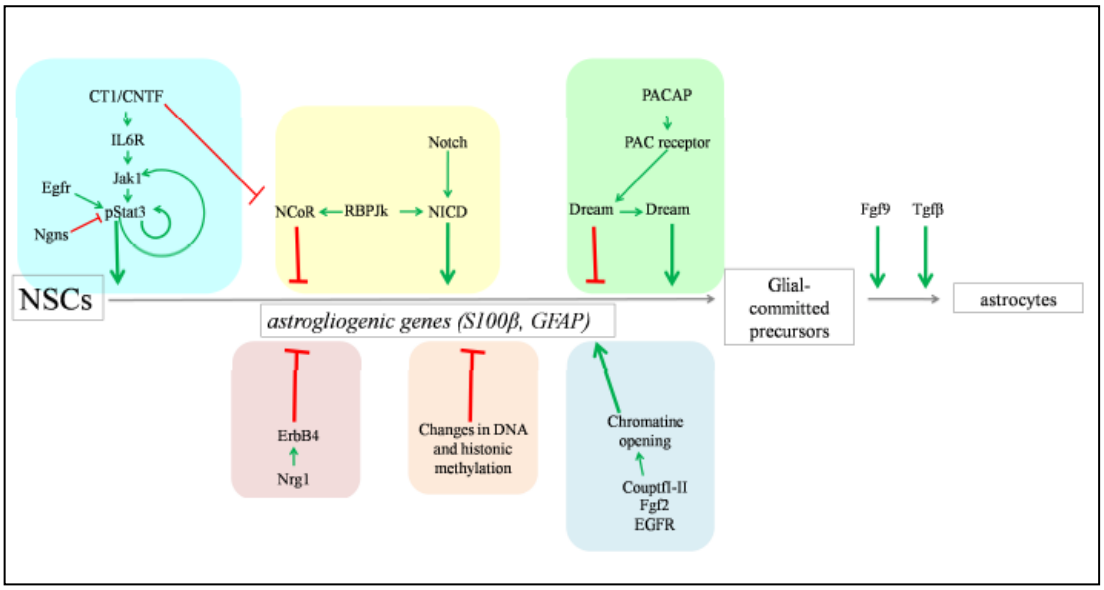

Fig. 43: Schematics summarizing molecular mechanisms regulating gliogenesis

Emx2, controlling a number of cortico-cerebral developmental subroutines (Muzio et al., 2002; Mallamaci et al., 2000b; Muzio et al., 2005; Mallamaci et al., 2000a), is strongly expressed in the early neuronogenic pallium, while fading out concomitantly with neuronogenesis decline (Mallamaci et al., 1998). Additionally it has previously reported that *Emx2* overexpression in cortico-cerebral neural stem cells (NSCs) results in a decrease of their astroglial output. The opposite phenotype was conversely found in cultures heterozygous for an *Emx2*-null allele (Brancaccio et al, 2010) **(Fig 44).**

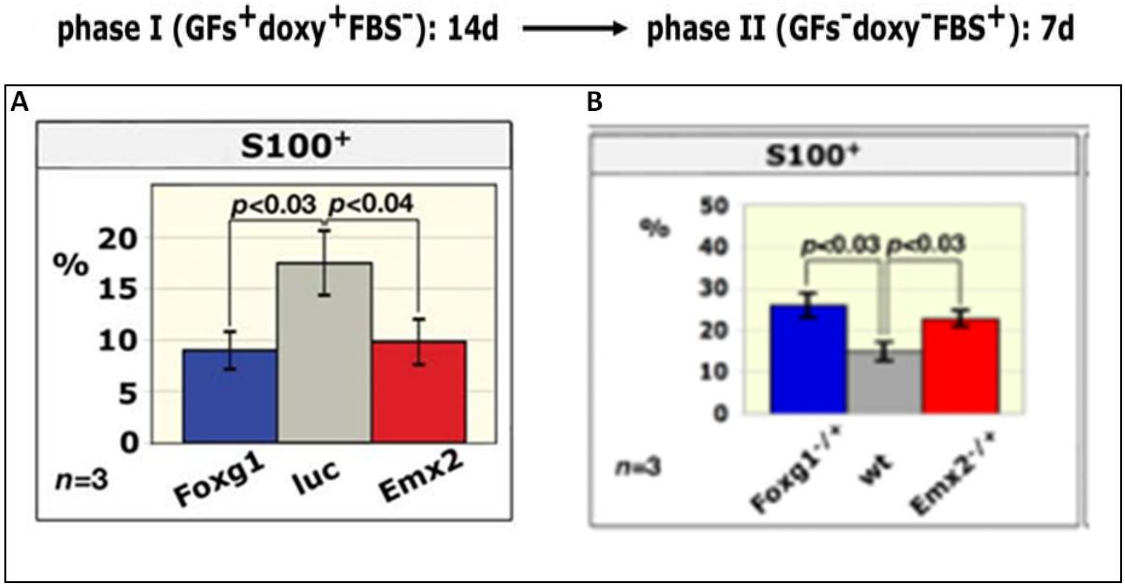

Fig 44: Quantifying late histogenetic consequences of Emx2 or Foxg1 overexpression/reduction. A: Infected cells were kept for 14 days under antidifferentiative conditions and in the presence of the transgene inducer doxycycline (Phase I: GF^+, $doxy^+$, FBS^-). They were subsequently transferred to prodifferentiative conditions, where they were kept for seven more days, in the absence of doxycycline (Phase II: GF^-, $doxy^-$, FBS^+). Finally these cultures were profiled for the expression of astrocytic (S100β). **B:** Differentiation profiles of gliogenic lineage precursors from $Emx2^{-/+}$ and $Foxg1^{-/+}$ neural cultures derived from E12.5 cortices were cultured like in A and then profiled for the expression of astrocytic (S100β).

Therefore we suspected that *Emx2* might dampen prenatal astrogenic rates, possibly mastering astroblasts proliferation.

To address if *Emx2* might dampen prenatal astrogenic rates, by mastering astroblasts proliferation, we engineered E12.5 cortico-cerebral precursors for conditional *Emx2* overexpression. Then we activated the transgene at in vitro equivalent of perinatal ages and finally pulsed them by BrdU. This test showed that the $BrdU^+S100\beta^+/S100\beta^+$ cell ratio was almost halved upon *Emx2* overexpression (**Fig. 45**), suggesting that our hypothesis was correct.

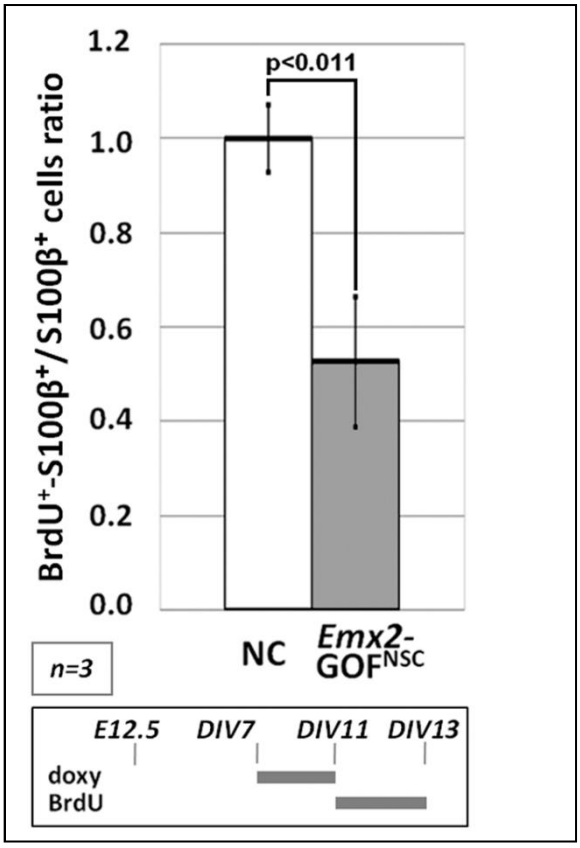

Fig 45: Reduced proliferation of astrocyte progenitors upon *Emx2* overexpression in embryonic neural stem cells. Frequencies of $BrdU^+$ elements among $S100\beta^+$ astroglial cells originatingfrom E12.5 cortico-cerebral precursors, acutely infected with LV:*pNes/hsp68--rtTA2S--M2* and, alternatively, LV:*TREt--IRES2--EGFP* (NC) or LV:*TREt--Emx2--IRES2--EGFP* (*Emx2--GOFNSC*). Cells kept for 11 days under anti-differentiative medium and 2 more days under GF-supplemented pro-differentiative medium; doxycyclin administered at DIV8-11, BrdU at DIV12-13.
Data normalized against sample (NC); absolute frequency of NC $BrdU^+S100\beta^+$ cells, 58.44±4.15%. Scalebar=s.e.m.

Molecular mechanisms mediating Emx2 astrogenesis reduction

To get hints on molecular mechanisms mediating astrogenesis reduction promoted by Emx2, we monitored a few established genes implicated in controlling proliferation of astrocyte-committed progenitors:

- *EgfR*. It is expressed in neural precursors starting from circa E14.5, a progressively higher and higher levels. Egf signaling upregulates astrogenesis rates, mainly by facilitating transmission of the Ct1 signal through the Jak2/Stat3 axis via EgfR-dependent upregulation of Stat3 expression. In this respect, Lillien et al. demonstrated that EGFR (induced by Fgf2) is necessary and sufficient to make cortical progenitors differentiate to astrocytes, under high LIF (Viti et al.,

2003). Moreover, it was shown that EGF signallig upregulates astrogenesis, by inducing proliferation of astrocytes via fast ERK activation (Mayer et al.2009)

- *Fgf9*. It encodes for asecreted factor promoting proliferation of cultured glial cells (Santos-Ocampo et al., 1996). Within the nervous system, it is produced mainly by neurons and may be fundamental for glial cell induction and development (Seuntjens et al., 2009 and references therein).
- *Sip1* (Seuntjens et al., 2009) Highly expressed in postmitotic neurons troughout neocortical development, Sip1 regulates the production of signals from postmitotic cells back to the germinal zone to ensure sequential generation of appropiate numbers of different neuronal cell types and glia. In a specific way at E17.5, Sip1 controls the level of Fgf9 production, which reaches its threshold only when neurogenesis is completed and *Sip1* expression is downregulated. At this point Fgf9 can induce proliferation of glial precursors.

To test if Emx2 reduces astrogenesis through these genes, we firstly quantified their mRNA levels upon experimental manipulation of *Emx2* expression levels. Interestingly, we found that *Emx2* downregulated *EgfR* and *Fgf9* in Gain Of Function samples (GOF) samples by about 30% and 50%, respectively, while not affecting *Sip1* **(Fig. 46 A**). Consistently, *EgfR*-mRNA and *Fgf9*-mRNA were upregulated in isochronic $Emx2^{+/-}$ cultures, by circa 75% and 30%, respectively (**Fig. 46 B).**

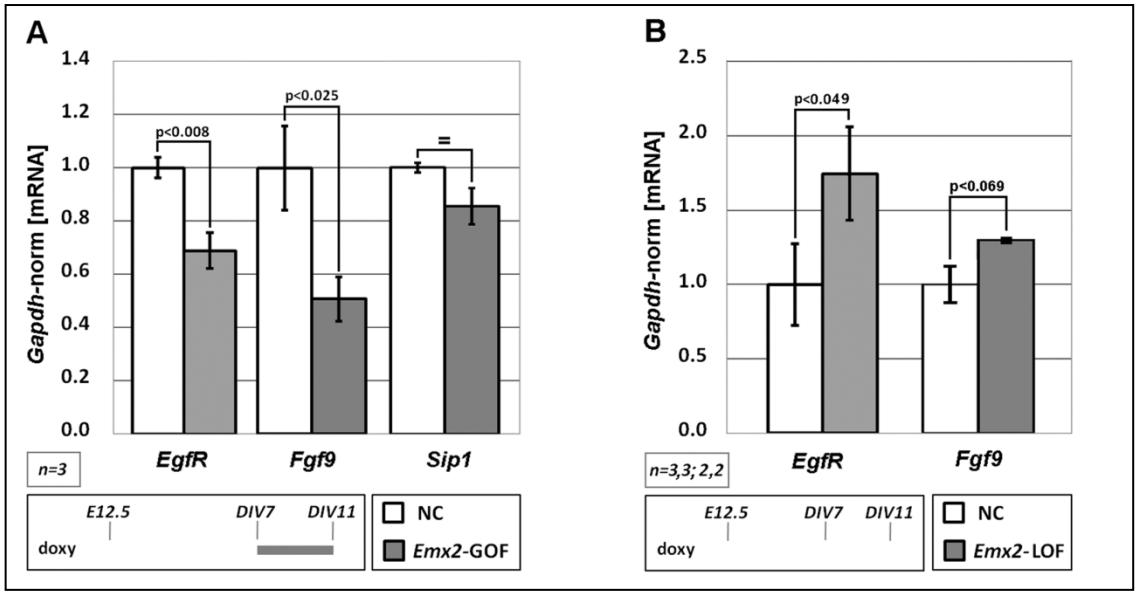

Fig 46: *EgfR* and *Fgf9* independently mediate antiastrogenic properties of *Emx2* in derivatives of E12.5 cortico--cerebral precursors. (A) *EgfR*-, *Fgf9*- and *Sip1*-mRNA levels in derivatives of precursors acutely infected with LV:*pPgk1--rtTA2S--M2* and, alternatively, LV:*TREt--IRES2--EGFP* (NC) or LV:*TREt-- Emx2--IRES2-EGFP* (*Emx2--GOF*). Cells kept for 11 days under anti-differentiative medium; doxycyclin administered at DIV8-11.Data double--normalized, against endogenous *Gapdh*-mRNA and NC values. **(B)** *EgfR*-and *Fgf9*-mRNA levels in derivatives of wild type (NC) and $Emx2^{+/-}$ (*Emx2--LOF*) precursors. Culture conditions and data normalization as in (A).

In particular, to cast light on celular and molecular mechanisms mediating Emx2-dependent regulation of *EgfR*, we evaluated the frequencies of EgfR-expressing cells within the NSCs (neural stem cells), eGPs (early glial progenitors) and lGPs (late glial progenitors) compartments, after the overexpression of Emx2 in the only NSC compartment. Intriguingly, FACS profiling of NSC-restricted-*Emx2*-GOF cultures revealed no significant change in frequencies of $EgfR^+$ NSCs, but a delayed and pronounced reduction of EgfR levels within the astrogenic compartment (**Fig. 47 A,B**), pointing to an indirect regulation of *EgfR* by *Emx2*.

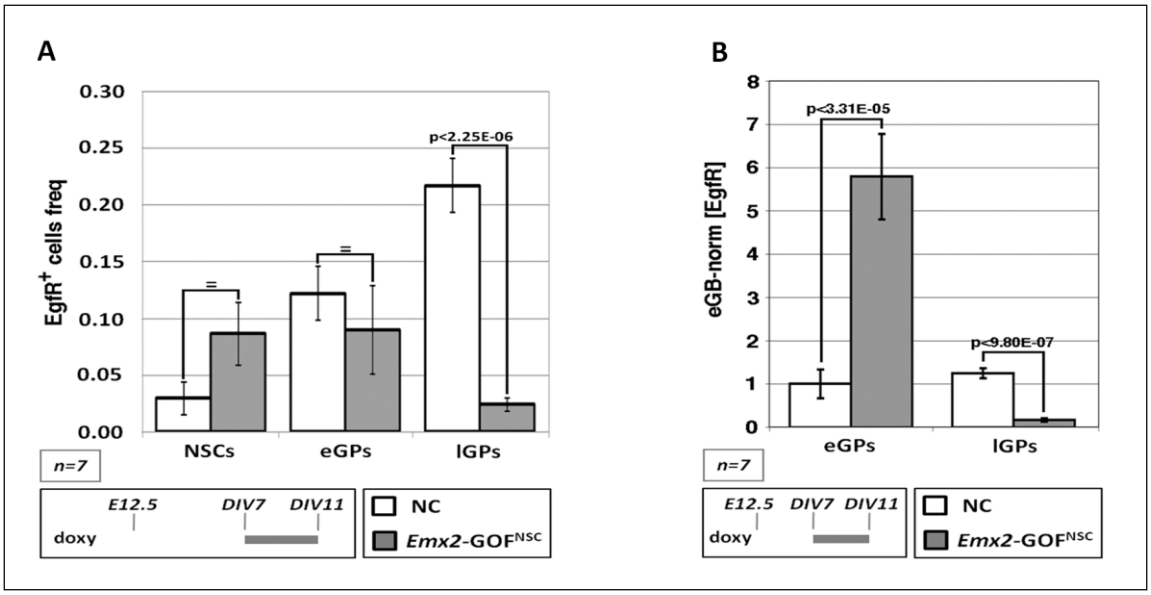

Fig 47: (**A)** Frequencies of EgfR-expressing elements in derivatives of precursors acutely infected with LV:*pNes/hsp68-rtTA2S--M2*, LV:*pTα1-mCherry* and, alternatively, LV:*TREt-IRES2-EGFP* (NC) or LV:*TREt-Emx2-IRES2-EGFP* (*Emx2-GOFNSC*). Cells kept for 11 days under anti-differentiative medium; doxycyclin administered at DIV8-11 but standard Egf replaced by Alexa 555-tagged Egf at DIV9-11. FACS-profiling performed after terminal antiA2B5-APC labelling. Frequencies of EgfR-expressing cells evaluated within the NSCs (neural stem cells), eGPs (early glial progenitors) and lGPs (late glial progenitors) compartments. **(B)** EgfR cytofluorescence levels in $EgfR^+$ glial progenitors, normalized against eGPs. In (A,B), different precursor types recognized according to the following profiles: NCS = pNes-$EGFP^+$/antiA2B5-APC^-/pTα1-$mCherry^-$, eGPs=pNes-$EGFP^{+/}$antiA2B5-APC^+/pTα1-$mCherry^-$; lGPs= pNes-$EGFP^-$/antiA2B5-APC^+/pTα1-$mCherry^-$).

Then, to rigorously assay functional relevance of *EgfR* downregulation to *Emx2* depression of astrogenesis, we delivered a lentivector driving constitutive *EgfR* expression to *Emx2*-GOF cultures at DIV8. As a control, this *EgfR*-expressing lentivirus was also administered to*Emx2-wt* cultures at the same time. First we found that the delivery of this lentivector to *Emx2*-GOF cultures restored the normal astrocytogenic rate. Secondly we reported that when the *EgfR* lentivirus was administered to *Emx2-wt* cultures, such parameter was not affected **(Fig 48A).**

Then, to corroborate functional relevance of Egf signalling to *Emx2*-dependent modulation of astrocytogenesis, we compared consequences of Emx2 overexpression or Egf withdrawal from the culturing medium. We had previously found that *Emx2* overexpression restricted to DIV8-14 elicits an antigliogenic effect highly similar to that elicited by DIV1-14 overexpression (not shown). This suggests that Emx2 overexpression impacts astrogenic rates mainly at DIV8-14. We observed that Egf removal from cultures at the beginning of the 2nd week reduced the astroglial output by

40%. This is consistent with previous results of Lilien and coll. Similarly, the astrogenic output was reduced by circa 50 % when Emx2 was overactivated at DIV1-14 (**Fig. 48 B**). All that suggests that dampening of Egf signalling elicited by Emx2 overexpression may be a major determinant of the astrogenesis impairment caused by this manipulation.

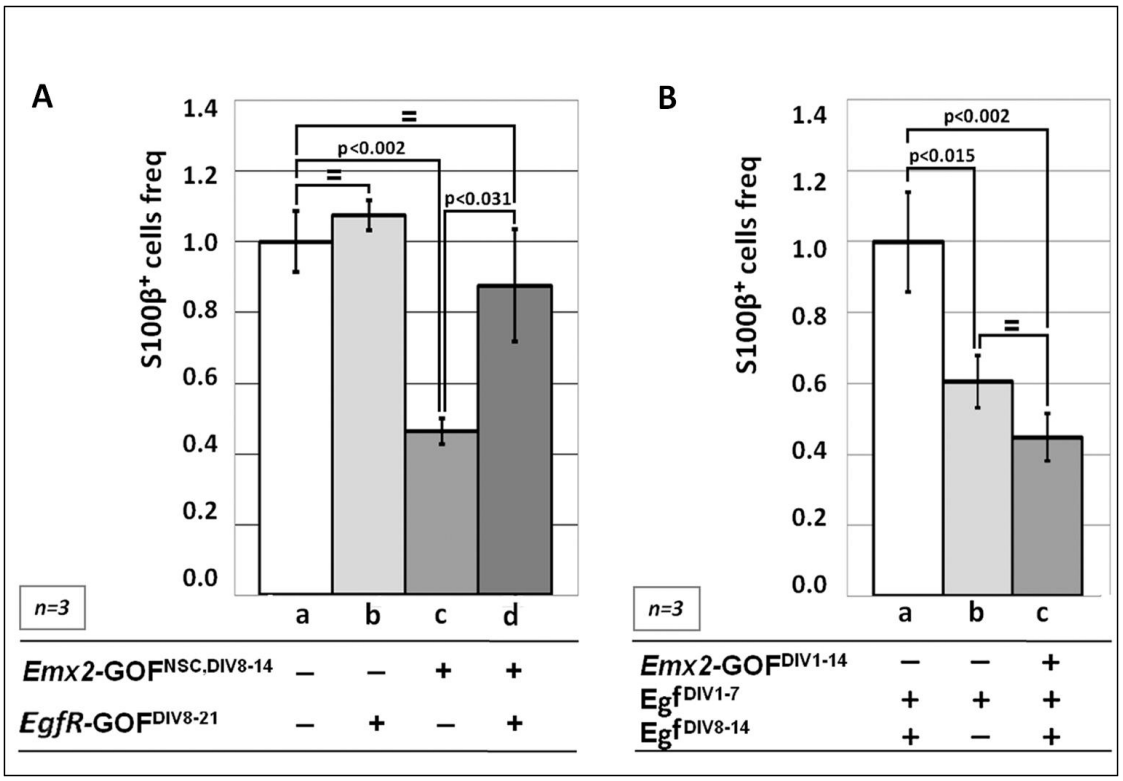

Fig 48: (A) Frequencies of S100β$^+$ derivatives of cortical precursors, acutely infected with LV:*pNes/hsp68-rtTA2S-M2* (a-d), LV:*TREt-Emx2-IRES2-EGFP* (c,d) and LV:*TREt-IRES2-EGFP* (a,b), and subsequently (DIV8) superinfected with LV:*pPgk--EgfR* (b,d) and LV:*TREt-luc* (a,c). Cells kept for 2 weeks under antidifferentiaive medium and one more week under pro-differentiative medium; doxycyclin administered at DIV8-14. Data normalized against sample (a); absolute frequency of S100β$^+$ cells in (a), 19.95±1.72%. **(B)** Frequencies of S100β$^+$ derivatives of cortical precursors, acutely infected with LV:*pNes/hsp68-rtTA2S-M2* and, alternatively, LV:*TREt-IRES2-EGFP* (a,b) and LV:*TREt-Emx2-IRES2-EGFP* (c). Cells kept for 2 weeks under anti-differentiative medium and one more week under pro-differentiative medium; doxycyclin and EGF temporally restricted according to the table. Data normalized against sample (a); absolute frequency of S100β$^+$ cells in (a), 17.70±2.49%.

Then, we assayed functional relevance of *Fgf9* downregulation to *Emx2* depression of astrogenesis. To do this, we supplemented *Emx2-GOF* and *Emx2-wt* neural cultures with exogenous Fgf9 from DIV 8 onwards. We observed that also this treatment:

- rescued normal astrocytogenic rate in *Emx2-GOF* neural cultures
- did not affect astrocytogenic rate in *Emx2-wt* neural cultures **(Fig. 49).**

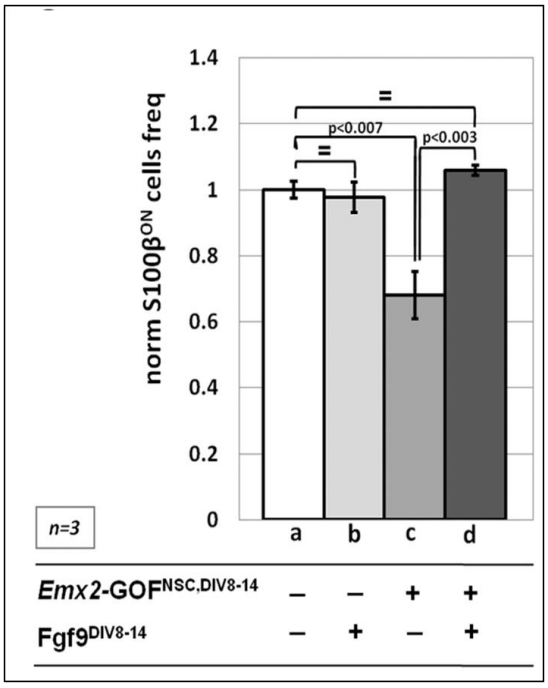

Fig 49: Frequencies of S100β$^+$ derivatives of cortical precursors, acutely infected with LV:*pNes/hsp68rtTA2S-M2* (a-d), LV:*TREt-Emx2-IRES2-EGFP* (c,d) and LV:*TREt-IRES2-EGFP* (a,b), and subsequently (DIV8) exposed to Fgf9 (b,d). Cells kept for 2 weeks under antidifferentiaive medium and one more week under pro-differentiative medium; doxycyclin administered at DIV8-14. Data normalized against sample (a); absolute frequency of S100β$^+$ cells in (a), 17.07 ± 0.45%.

Altogether, these data strongly support the hypothesis that both *EgfR* and *Fgf9* downregulation mediate *Emx2* impact on astrogenesis.

At this point we wondered if Emx2 regulation of astrogenesis mediated by EgfR and Fgf9 repression proceeds along two different pathways or only one. To clarify this point, we exposed *Emx2-GOF* and *Emx2-wt* neural cultures to exogenous Fgf9 or alternatively infected them by a lentivirus consitutively overexpressing EgfR. Then, wemonitored *EgfR*- and *Fgf9*-mRNA levels, respectively.

Remarkably neither of these levels were significatively affected (**Fig. 50 A,B**), suggesting that *Emx2* regulation of astrogenesis proceeds along two different pathways.

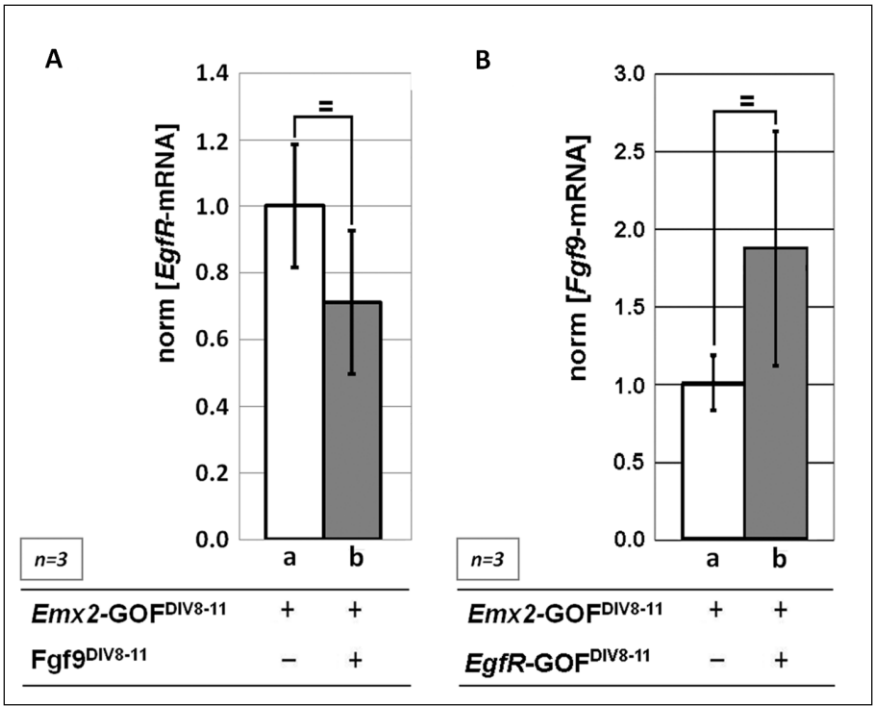

Fig 50: (A,B) *EgfR*- and *Fgf9*-mRNA levels in preparations of E12.5 cortico-cerebral precursors, acutely infected with LV:*pPgk1p-rtTA2-M2* and LV:*TREt-Emx2-IRES2-EGFP* (a,b), grown in anti-differentiative medium and subsequently (DIV8) exposed to doxycyclin. In (A), the (b) sample was also exposed to Fgf9, since day 8. In (B), sample (a) and (b) were further superinfected at day 8, with LV:*TREt-IRES2-EGF* and LV:*pCCLsin.PPT.hPGK.EGFR.pre*, respectively. In both (A) and (B), data double-normalized, against *Gapdh* and samples (a). Scalebar = s.e.m.

Bmp signaling links *Emx2* and *EgfR*

Concerning molecular mechanisms linking *Emx2* overexpression to *EgfR* misregulation, we speculated that a key role might be played by Bmp signalling. In fact, first, Emx2 promotes this signalling, by downregulating its established inhibitors Noggin and Fgf8 (Fukuchi-Shimogori and Grove, 2003; Shimogori et al., 2004; Bilican et al., 2008). Second, Bmps inhibit *EgfR* expression (Lillien and Raphael, 2000).

To test our hypothesis we monitored two well known endogenous reporters of Bmp signalling, *Id3* and *Msx1* (Hollnagel et al., 1999) in preparations of E12.5 *Emx2-GOF* and *Emx2-wt* neural cultures. We observed upregulation of bothr reporters in *Emx2-GOF* neural cultures (**Fig. 51)**, confirming the *Emx2* capability to enhance Bmp signalling also in our system.

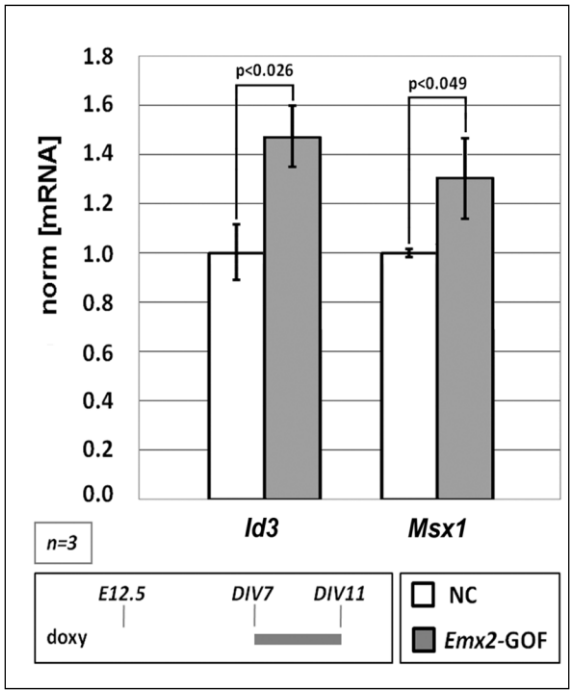

Fig 51: *Emx2* represses *EgfR* by enhancing Bmp signalling.Upregulation of the Bmp targets *Id3* and *Msx1* in preparations of E12.5 cortico-cerebral precursors, acutely infected with LV:*pPgk1p--rtTA2S--M2* (*Emx2-GOF*, NC), LV:*TREt-Emx2- IRES2-EGFP* (*Emx2-GOF*) and LV:*TREt-IRES2-EGFP* (NC), grown in anti-differentiative medium and subsequently (DIV8) exposed to doxycyclin

Then, to assay functional relevance of Bmp signaling upregulation to *EgfR* misregulation, we performed a pharmacological rescue in which we treated *Emx2-GOF* and *Emx2-wt* neural cultures with the Bmp-inhibitor LDN193189**.**

Consistently with our expectations, Bmp inhibition by LDN193189 restored *EgfR* expression levels in an *Emx2*-GOF environment, while not affecting them in controls **(Fig. 52).**

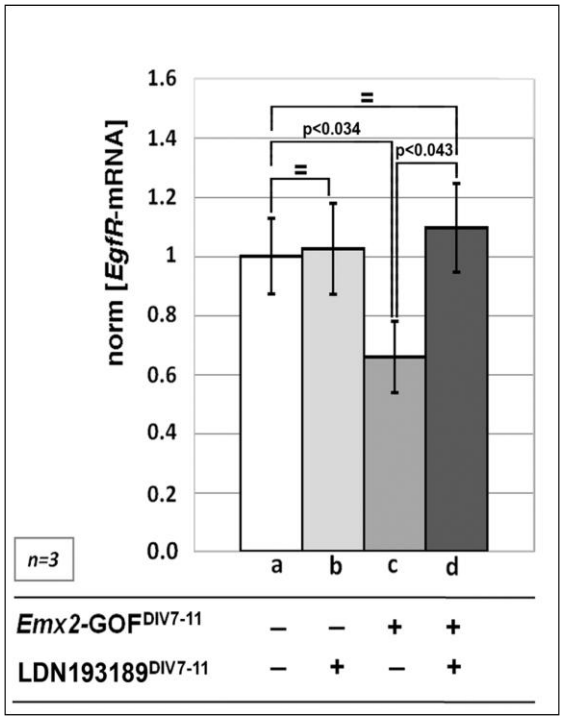

Fig52:**Bmp inhibition by LDN193189 restored *EgfR* expression levels in an *Emx2*-GOF environment.** *EgfR*-mRNA levels in preparations of E12.5 cortico-cerebral precursors, acutely infected with LV:*Pgk1p-rtTA2S-M2* (a-d), LV:*TREt-Emx2-IRES2-EGFP* (c,d) and LV:*TREt-IRES2-EGFP* (a,b), grown in anti-differentiative medium and subsequently (DIV8) exposed to doxycyclin (a-d) and the Bmp-inhibitor LDN193189 (b,d).

Emx2 might downregulate Fgf9 inhibiting the Sox2 activation

Concerning *Fgf9*, its expression might rely on *Sox2*, which is specifically promoted by signals triggering astrocytic proliferation (Bani-Yaghoub et al., 2006). *Emx2* might downregulate *Fgf9* just inhibiting the Sox2 activation elicited by Brn2 (Mariani et al., 2012), which is expressed in a variety of neural cells including the astrogenic lineage (Abe et al., 2012).

To test if *Fgf9* downregulation could be mediated by Brn2, we performed a Brn2-rescue of *Emx2*-dependent *Fgf9* downregulation. We measured *Fgf9*-mRNA levels in preparations of E12.5 *Emx2-GOF* or wt cortico-cerebral precursors, in basal conditions or upon further Brn2 overexpression. We found that *Brn2* overexpression rescued *Fgf9* levels in an *Emx2*-GOF environment, while not affecting them in controls **(Fig. 53).**

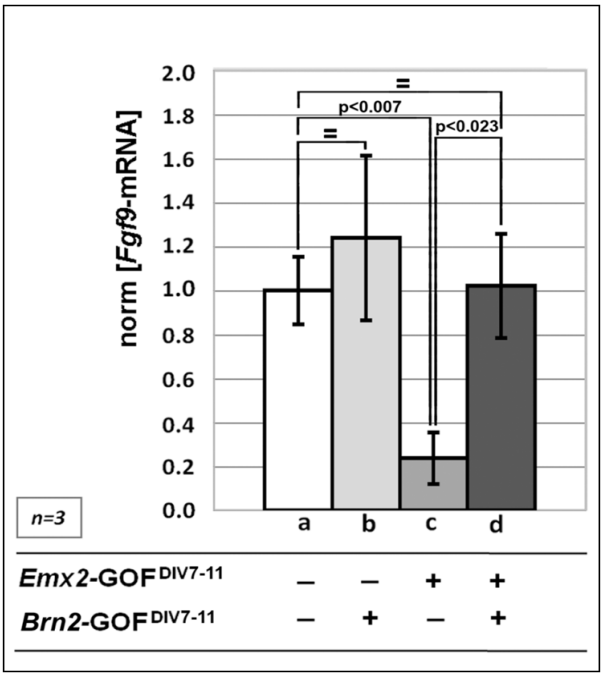

Fig 53: Brn2 rescue *Emx2*-dependent *Fgf9* downregulation. *Fgf9*-mRNA levels in preparations of E12.5 cortico-cerebral precursors, acutely infected with LV:*Pgk1p-rtTA2S-M2* (a-d), LV:*TREt-Emx2-IRES2-EGFP* (c,d), LV:*TREt-IRES2-EGFP* (a,b), LV_*TREt-Brn2* (Cb,d) grown in anti differentiative medium and subsequently (DIV8) exposed to doxycyclin (a-d). Data double-normalized against *Gapdh* and samples (a). Scalebar=s.e.m.

To confirm functional involvement of *Sox2* in *Emx2*-dependent *Fgf9* downregulation we tried to rescue *Emx2*-dependent *Fgf9* downregulation, by overexpressing this gene.

As expected, *Sox2* overexpression restored normal *Fgf9* levels in an *Emx2*-GOF environment. However, differently from Brn2, Sox2 triplicated *Fgf9* levels when delivered to *Emx2-wt* cells.

All that confirms that *Brn2* and *Sox2* may be implicated as key mediators of *Emx2*- dependent *Fgf9* regulation **(Fig. 54).**

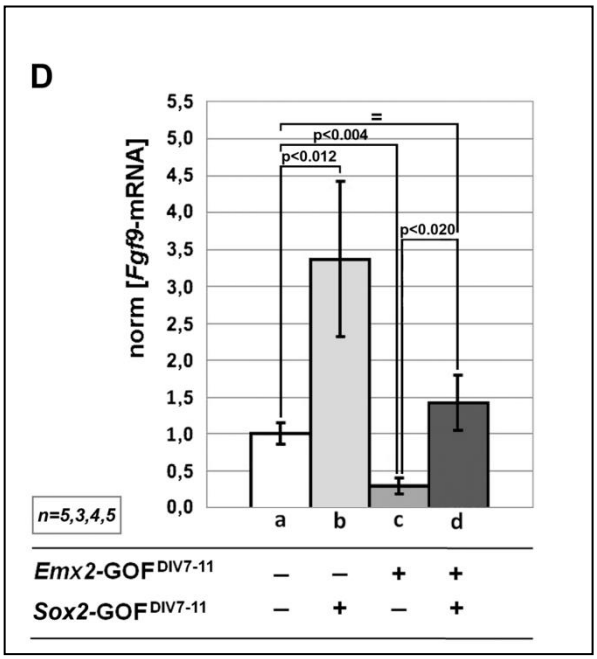

Fig. 54: Sox2 rescue *Emx2*-dependent *Fgf9* downregulation *Fgf9*-mRNA levels in preparations of E12.5 cortico-cerebral precursors, acutely infected with LV:*Pgk1p-rtTA2S-M2* (a-d), LV:*TREt-Emx2-IRES2-EGFP* (c,d), LV:*TREt-IRES2-EGFP* (a,b), LV_*TREt--Sox2* (b,d),grown in anti differentiative medium and subsequently (DIV8) exposed to doxycyclin (a-d). Data double-normalized against *Gapdh* and samples (a). Scalebar=s.e.m.

Emx2 levels in NSCs preprogram proliferative abilities of astrocyte-committed progenitors.

Based on our results as well as on previously reported dynamic Emx2 levels in multipotent precursors of the apical compartment (Gulisano et al., 1996), we hypothesized that Emx2 may indirectly modulate astrogenesis rates, by preprogramming proliferative abilities of astrocyte-committed progenitors and dampening rodent astrogenesis within the neuronogenic window.

To better assess if Emx2 expression levels are compatible with this hypothesis, we performed an accurate time course profile of *Emx2*-mRNA in the embryonic neocortex. As expected, we found that *Emx2*-mRNA levels, normalized against the NSC marker *Sox2*, progressively decreased from E14.5 to E18.5, concomitantly with the arousal of astrogenesis **(Fig. 55).**

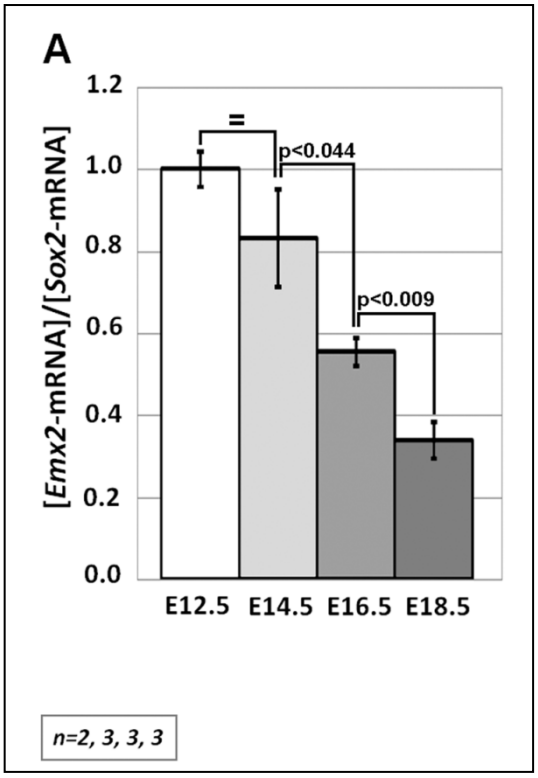

Fig 55: High *Emx2* expression in embryonic cortical NSCs dampens astrogenesis. Time course profile of *Emx2*-mRNA in the embryonic neocortex. qRT-PCR results normalized against the NSC marker *Sox2* and further normalized against E12.5 data.

To corroborate this result, we evaluated the temporal distribution of Sox2-normalized Emx2 immunofluorescence levels in acute, single $Sox2^+$ precursors originating from E12.5 and P0 neocortices.

As expected, Sox2-normalized Emx2 immunofluorescence levels in acute, single $Sox2^+$ precursors originating from P0 neocortices was almost halved with respect to their E12.5 counterparts (**Fig. 56 A,B**).

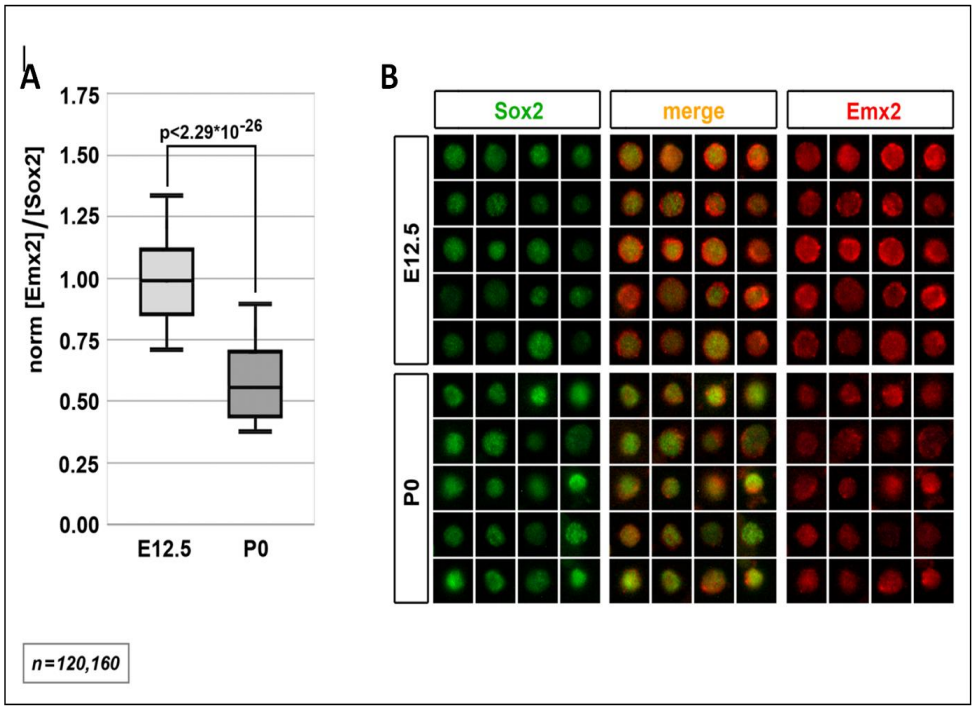

Fig 56: Sox2-normalized Emx2 immunofluorescence levels (A) Distribution of Sox2-normalized Emx2 immunofluorescence levels in acute, single Sox2$^+$ precursors originating from E12.5 and P0 neocortices. Per each box-plot,bars represent 1st decile, 1st quartile, median, 3rd quartile and 9th decile, respectively. **(B)** Example of Sox2$^+$ cells referred to in (B).

Then, to measure the actual *Emx2* capability to dampen rodent astrogenesis *within the embryonic neuronogenic window*, we evaluated the astrogenic output upon short term *Emx2* overexpression in *embryonic* NSCs, turned to astroglial fates by Lif treatment.

In particular, we calculated frequencies of GFAP$^+$ and S100β$^+$ elements originating from E12.5 *Emx2-GOF* or *Emx2-wt* cortico-cerebral precursors kept under pro-differentiative medium for 6 days and treated with Lif (which directly contribute to the generation of astroglia by instructing a glial fate) at DIV3-6.

We observed that *Emx2* exerted a pronounced antigliogenic effect, reducing their GFAP$^+$ and S100β$^+$ progenies by about 75% and 40%, respectively (**Fig. 57 A,B)** also in this window.

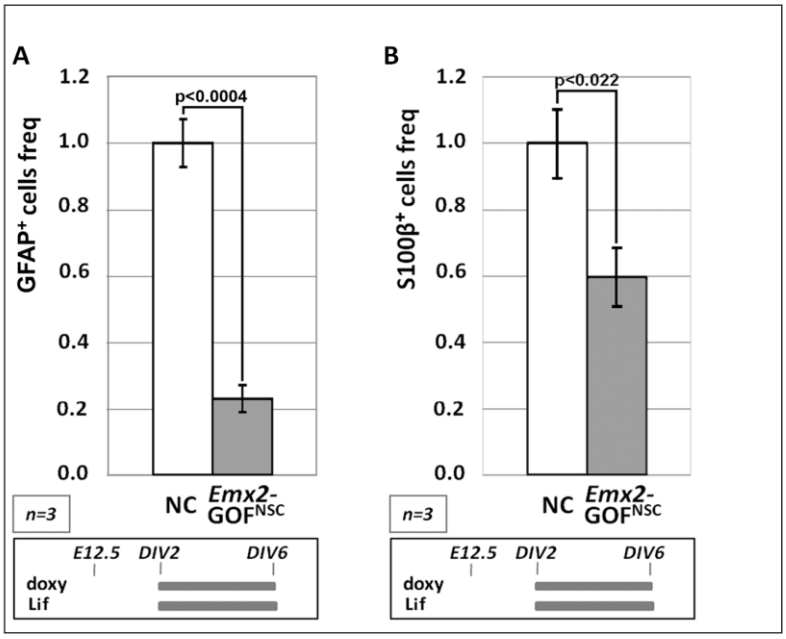

Fig. 57: Frequencies of GFAP$^+$ and S100β$^+$ elements originating from E12.5 cortico-cerebral precursors, acutely infected with LV:*pNes/hsp68-rtTA2S-M2* and, alternatively, LV:*TREt-IRES2-EGFP* (NC) or LV:*TREt-Emx2-IRES2-EGFP* (*Emx2*-GOFNSC). Cells kept under pro-differentiative medium for 6 days; Lif and doxycyclin administered at DIV3-6. Data normalized against NC samples; absolute frequencies of GFAP$^+$ and S100β$^+$ cells in NCs, 36.55±2.62% and 46.32±4.86%, respectively. Scalebar=s.e.m.

DISCUSSION

In this study we showed that *Emx2* overexpression in cortico-cerebral stem cells decreased proliferation of astrocyte-committed progenitors, resulting in a severe reduction of their ultimate astroglial output. We found that this was due to downregulation of *EgfR* and *Fgf9*. The former stemmed from increased Bmp signaling promoted by *Emx2*. The latter apparently originated from *Emx2*- dependent suppression of the *Sox2* activation elicited by Brn2. Finally, we provided evidence that in vivo temporal progression of Emx2 expression levels in neural stem cells contributes to restrain astrocyte generation during the neuronogenic phase.

Generation of astrocytes within the developing cerebral cortex is a tightly regulated process, which bursts after neuronogenesis completion and precedes the bulk of oligodendrogenesis. Astrocytic outputs depend on two primary factors: progression of multipotent precursors towards astroglial lineages and proliferation of astrocyte-committed progenitors. Molecular mechanisms controlling lineage choice have been investigated and reconstructed to large extent (reviewed in Mallamaci, in press). On the contrary, very little is known about control of astroblast population kinetics.

A key determinant of cortico-cerebral astrogenic rates is the accurate tuning of proliferation within the astrogenic lineage. In this context, a special role is played by the Egf signaling axis and Fgf9, as a key promoters of astrogenesis.

- Egf signalling has a dual impact on astrogenesis. About ten years ago, Lillien et al. demonstrated that EGFR (induced by Fgf2) is necessary and sufficient to make cortical progenitors differentiate to astrocytes, under high LIF (Viti et al., 2003). This is achieved by facilitating transmission of the Ct1 signal through the Jak2/Stat3 axis, via EgfR-dependent upregulation of Stat3. Moreover, Egf signalling enlarges the astrocytic outputs, by inducing proliferation of astroblasts via a rapid ERK activation (Mayer et al.2009).
- Fgf9 is a secreted factor that shows its growth-stimulating effect on cultured glial cells. In nervous system it has been shown to be produced mainly by neurons and may be fundamental for glial cell induction and development (Santos-Ocampo et al., 1996). In particular it has been thought to be produced

by neurons, as neurogenesis is termined, in order to induce the onset of production of glial cells

Despite of saturating levels of Tgfα/Egf ligands throughout the embryonic life (Burrows et al., 1997; Assimacopoulos et al., 2003), EgfR expression, very low in the early pallium, progressively increases as neuronogenesis proceeds (Burrows et al., 1997; Viti et al., 2003). This limits the early pallial competence to activate the Egf signaling axis, at least up to birth (Burrows et al., 1997). Similarly, Fgf9 expression, prominent after birth (http://developingmouse.brain-map.org/), is very poor during prenatal life, when it results limiting for glioblasts proliferation (Seuntjens et al., 2009).

Here we demonstrated that *Emx2* acts as a master gene coordinating proper temporal progression of *EgfR* and *Fgf9*. In particular:

- An *Emx2*-dependent repression of EgfR occurs within the astrogenic lineage, where it is mediated by promotion of Bmp signalling. This phenomenon is not peculiar to the neural lineage, as it was previously documented within the developing urogenital apparatus (Kusaka et al., 2010)
- Emx2 also represses Fgf9 expression, which conversely is a fully novel finding. Here we provided evidence that it takes place via dampening of Sox2.

Finally, we showed that in vivo time course progression of Emx2 levels in late-embryonic / early postnatal cortico-cerebral NSCs is a key determinant contributing to postpone the bulk of astrogenesis to postnatal ages.

Emx2 regulation of astrogenesis adds to a number of earlier developmental processes mastered by this gene (Muzio et al., 2002; Mallamaci et al., 2000b; Muzio et al., 2005; Mallamaci et al., 2000a).

It points to *Emx2* as a new powerful molecular tool, suitable to limit the undesidered gliogenesis which may follow transplantation of neural precursors for purposes of experimental brain repair (Burns et al., 2009) and selectively channel such precursors towards neuronogenesis.

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