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# *Emx2* and *Foxg1* inhibit gliogenesis and promote neuronogenesis

Thesis submitted for the degree of “Doctor Philosophiae”

Academic Year 2008/2009

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## Summary

# ABSTRACT

Neural stem cells (NSCs) give rise to all cell types forming the cortex: neurons, astrocytes and oligodendrocytes. The transition from the former to the latter ones takes place via lineage-restricted progenitors in a highly regulated way. This process is mastered by a large set of genes, among which some implicated in CNS pattern formation. Aim of this study was to disentangle the kinetic and histogenetic roles exerted by two of these genes, *Emx2* and *Foxg1*, in cortico-cerebral precursors.

For this purpose, we set up a new integrated in *vitro* assay design. Embryonic cortical progenitors were trasduced with lentiviral vectors driving overexpression of *Emx2* and *Foxg1* in NSCs and neuronal progenitors (NPs). Cells belonging to different neuronogenic and gliogenic compartments were labeled by spectrally distinguishable fluoroproteins driven by cell-type-specific promoters and by cell-type-specific antibodies and were scored via multiplex cytofluorometry and immunocytofluorescence.

A detailed picture of *Emx2* and *Foxg1* activities in cortico-cerebral histogenesis resulted from this study. Unexpectedly, we found that both genes inhibit gliogenesis and promote neuronogenesis, through distinct mechanisms, and *Foxg1* also dramatically stimulates neurite outgrowth. Remarkably, such activities, alone or combined, may be exploited to ameliorate the neuronal output obtainable from neural cultures, for purposes of cell-based brain repair.

# 1. INTRODUCTION

## 1.1 *Emx2* and *Foxg1* in the developing telencephalon

The neocortex is the most representative part of the mammalian telencephalon and the region which controls higher cognitive functions. It has a peculiar six-layered structure, well conserved in all mammals. Each layer is composed of projection neurons, which connect the neocortex to the subcortical structures (and different structures *within* the cortex itself), interneurons, which modulate the excitatory activity of projection neurons and glial cells (mainly astrocytes and oligodendrocytes), necessary for proper neocortical formation and functioning. In its tangential dimension the neocortex is organized in areas, that are functionally unique subdivisions of the brain, characterized by specific cytoarchitectures, wiring and gene expression profiles. During the evolution the neocortex has dramatically expanded its size and complexity, finally covering almost the entire brain in primates and humans.

The dynamical process leading the pseudostratified neuroepithelium to form such a complex structure encompasses different overlapping morphogenic processes: (1) Rostro-caudal (R-C) and dorso-ventral (D-V) specification of the telencephalic field (2) cortical arealization (3) cortical histogenesis of the six layered neocortical structure, (4) wiring of the neocortex. A pletora of genes are responsible for the molecular control of the overall process. Among these, developmentally regulated transcription factors (TFs) are particularly interesting, because they function as molecular hubs which harmonically co-regulate different morphogenetic aspects of cortical development, including cell death, self renewal, lineage fate choices, cell cycle control, differentiation, migration, wiring etc.. In the following paragraphs we will briefly describe the above mentioned morphogenic processes, focusing on the roles exerted by two of these transcription factors, *Emx2* and *Foxg1*.

## 1.2 Rostro-caudal (R-C) and Dorso-ventral (D-V) and identity specification

The embryonic Central Nervous System (CNS) develops starting from the neural plate. This is a region of dorsal ectoderm with a columnar cellular phenotype which, after gastrulation, thickens, rises at its borders, then invaginates, so forming a neural groove, and eventually closes at its latero-dorsal borders, resulting into a hollow cylinder, the neural tube (E7.5 in mouse). The unequal expansion of distinctive regions of the tube gives rise to a series of primary vesicles (prosencephalon, mesencephalon and rhombencephalon), separated by constrictions. In particular, the anterior-most neural plate gives rise to the prosencephalon, which is subsequently divided in telencephalon and diencephalon. The dorsal telencephalon (the pallium) will give rise to the archicortex (subiculum, hippocampus and dentate gyrus), the paleocortex (olfactory piriform cortex and enthorinal cortex) and the neocortex. The ventral telencephalon (the subpallium) will mainly generate the basal ganglia and a part of the amigdala (Fig. 1).

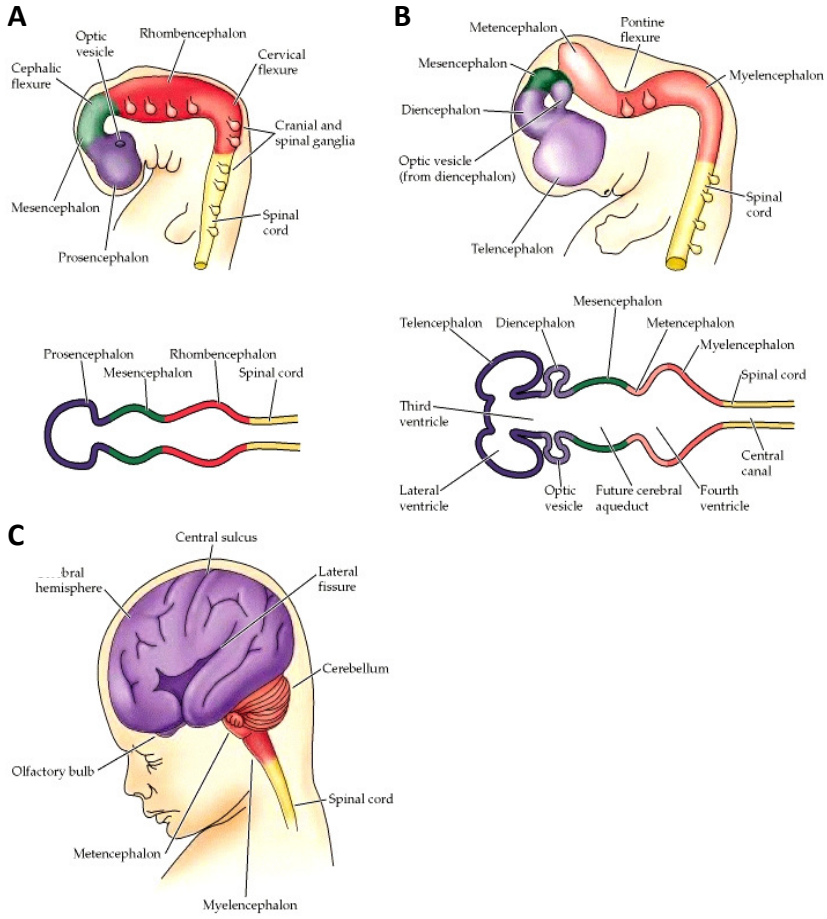



**Figure 1: Regional specification of the developing brain.**

Early in gestation the neural tube is split in prosencephalon, mesencephalon and rhombencephalon **(A)**. Further development distinguishes the telencephalon and diencephalon from the prosencephalon. These subregions give rise to the rudiments of the major functional subdivisions of the brain, while the spaces they enclose will form the ventricles of the mature brain. **(B)**. Several major subdivisions, including the cerebral cortex and cerebellum, are clearly seen from the lateral surfaces after birth **(C)**. Adapted from Purves et al. Neuroscience 2001 (Sinauer Associates)

The process leading the anterior neural plate to the specification of pallial and subpallial territories implies the determination and the specification of telencephalic identities along the R-C and D-V axis. At E7 the early R-C patterning of the anterior neural tissue **(anterior neural induction)** is mediated by antagonistic signals coming from the primitive node (Hensen's node in the chicken) and the anterior visceral endoderm (AVE), required for neural induction and maintenance (Thomas and Beddington, 1996). The AVE is an extra-embryonic tissue that underlies the neural plate and secrete molecules, like cerberus and dickkopf, which antagonize the effects of posteriorizing moleculesexpressed by the neural plate at this stage, including Wnt and fibroblast growth factor (Fgfs) family members as well as retinoic acid (RA) (Altmann and Brivanlou, 2001; Sasai and De Robertis, 1997) (Fig. 2). After the anterior neural induction, cells at the junction between neural and non neural tissue of the most rostral part of the brain, form the Anterior Neural Ridge (ANR), a secondary organizer, necessary for forebrain induction and maintenance. Ablation of the ANR in mice prevents the expression of the telencephalic markers *Foxg1* and *Emx1* (Shimamura and Rubenstein, 1997). ANR activity in zebrafish is mediated by Tlc, a frizzled related protein acting as a Wnt antagonist. This suggested a general model, in which the default forebrain identity is posterior (diencephalic) and anterior telencephalic identity is achieved through antagonization of Wnt signaling by the AVE and ANR patterning centers. Recent data suggest that in mammals also the Fgfs, secreted by the ANR, actively estabilish the telencephalic identity: when Fgf receptors are deleted the telencephalon does not form (Paek et al., 2009). At E8.0 the mouse telencephalic primordium lies within the anterior third of the paired, downward-folded leaves of the neural plate, and the two sides of the primordium meet at the anterior midline. At this stage a dramatic set of morphogenetic movements and extensive cell proliferation transforms the telencephalon into a set of paired vesicles.

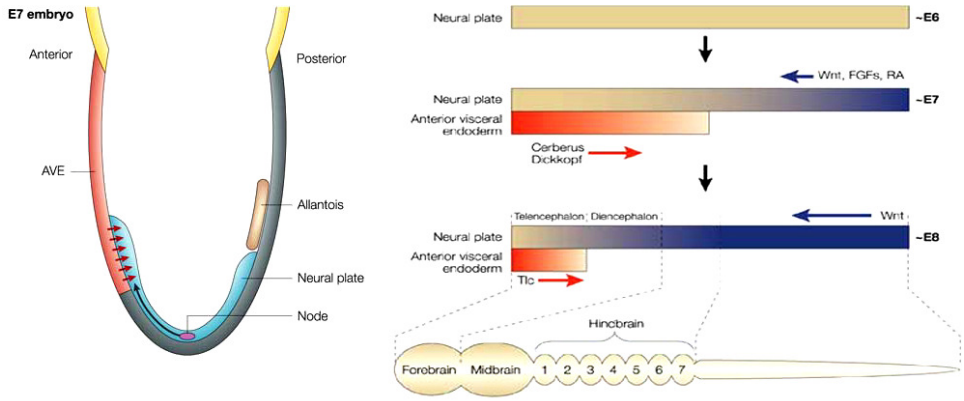



**Figure 2: Anterior- posterior patterning of the telencephalon, neural induction.**

Signals that come from the node establish gross anterior pattern (black arrow). The anteriorvisceral 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 neural plate (Wnts, FGF, RA). Adapted from Rallu et al. 2002.

The expression of transcription factors and secreted morphogenes along the D-V axis elicits the early partitioning of the telencephalon that will end up with the specification of pallial and subpallial structures. Among these factors major roles are played by the dorsalizing Zinc-finger transcription factor *Gli3* and the ventralizing Winged helix transcription factor *Foxg1* (Fig. 3A). In *Gli3* knockouts the plexus, the cortical hem and the hippocampus fail to form and the development of the neocortex is progressively compromised (Grove et al., 1998; Kuschel et al., 2003; Theil et al., 1999). Sonic hedgehog protein (Shh), secreted by the ventral midline, counteracts the dorsalizing effect of *Gli3*: the telencephalon of $Shh^{-/-}$ mice is reduced in size and ventral cell types lost. However rescue of $Shh^{-/-}$ phenotype in double $Gli3^{-/-}$ $Shh^{-/-}$ mice (Rallu et al., 2002) suggests that the Shh role in this process passes simply through inhibition of Gli3 activity. The ventralizing signal in the forebrain is probably mediated by a *Foxg1*/Fgf pathway. Fgfs secreted by the ANR serve as major telencephalic patterning signals throughout the forebrain development. Moderate inhibition of Fgf signaling pathway causes solely areal shift in the cortical field *(see below)*, whereas deletion of multiple Fgf receptors (Fgfr1 and Fgfr2) progressively involves the ventral telencephalon in addition (Fig. 3B). Remarkably, when three Fgf receptors are deleted, the telencephalon is no longer specified (Paek et al., 2009).

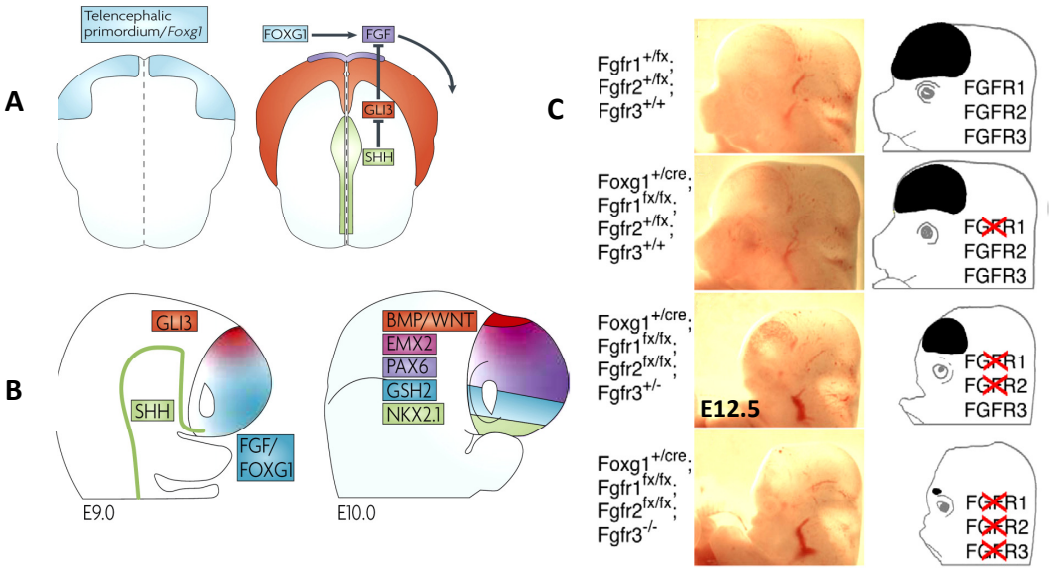



**Figure 3: Dorso-ventral patterning of the forebrain.**

The region that will become the telencephalon is defined by expression of *Foxg1*. *Foxg1* (directly) and Shh (indirectly, via *Gli3*) promote Fgfs expression in the ANR. This patterns the nascent telencephalon. Dorsal view E8,E9 **(A)**. Subsequently the dorsal telencephalon, expressing *Gli3* at E9. is split, by E10, into a BMP and Wnt expressing medial region and a more lateral cortical region expressing countergradients of *Emx2* and *Pax6.* The ventral telencephalon is subdivided into medial *Nkx2.1*-expressing domains and lateral *Gsh2*-expressing domains (partially overlapping at E10). Sagittal view E9, E10 **(B)**. Gradual loss of telencephalic tissue follows progressive Fgf receptors deletion **(C)**. **(A)** and **(B)** Adapted from Hèbert and Fishell, 2007. **(C)** Adapted from Paek et al. 2009.

*Foxg1* (Forkhead box G1, formerly known as Bf-1) is expressed in the anterior neural plate cells from E8.5 (Hébert and McConnell, 2000; Tao and Lai, 1992), slightly before the neural plate bends to form the head folds. It is necessary for the expression of Fgfs from the ANR (Martynoga et al., 2005) and in turn Fgf8 induces *Foxg1* expression (Shimamura and Rubenstein, 1997; Storm et al., 2006), forming a positive feedback loop. In *Foxg1*$^{-/-}$ mice the formation of the subpallium is strongly impaired (Xuan et al., 1995) (Fig 4A). Remarkably in double *Gli3* $^{-/-}$ *Foxg1*$^{-/-}$ mice this phenotype is far from being rescued: in fact in these mutants the telencephalon is mispecified as an hybrid telencephalic/diencephalic aberrant structure (Hanashima et al., 2007) (Fig. 4B). Wnt signaling from epidermal ectoderm of the dorsal telencephalon could also have a dorsalizing activity in both early and late D-V forebrain patterning (Backman et al., 2005; Gulacsi and Anderson, 2008; Gunhaga et al., 2003) Remarkably, recent data in zebrafish suggest that *Foxg1* could integrate signals from Shh, Wnt and Fgf8 pathways, so having a pivotal role in D-V forebrain specification. In fact,

according to these authors, *Foxg1* could also act as a Wnt/β-catenin antagonist, as well as downstream effector of Shh to specify the subpallial identities (Danesin et al., 2009).

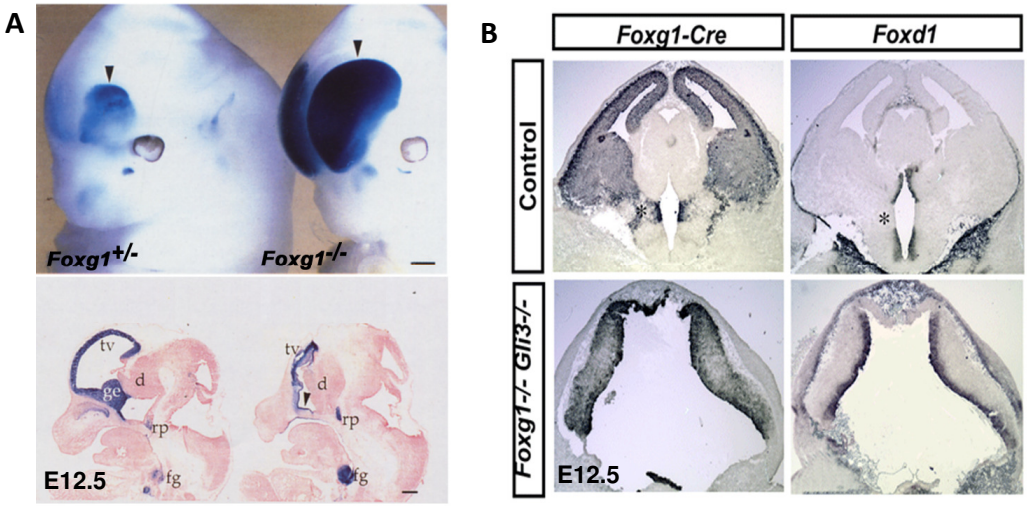



**Figure 4: Forebrain development impairment in Foxg1$^{-/-}$ and Foxg1$^{-/-}$ Gli3$^{-/-}$ mice.**

*Foxg1* knockouts display a smaller size of the telencephalic emispheres, if compared to heterozygous mice. The ventral telencephalon formation is heavily impaired, but also the dorsal telencephalon size is reduced. X-Gal histochemistry identifies structures that normally express *Foxg1* **(A)**. In double Foxg1$^{-/-}$ Gli3$^{-/-}$ mice an hybrid telencephalic/diencephalic aberrant structure is formed. This is revealed by the overlap of *Cre* expression driven from the *Foxg1* locus and *Foxd1* expression, a gene normally restricted to the diencephalon **(B)**. **(A)** Modified by Xuan et al.1995; **(B)** Modified from Hanashima et al 2007.

As a result of early R-C and D-V patterning events, the prosencephalon will be subdivided in pallial territories and subpallial territories, characterized by the expression of specific set of TFs. The subpallium will give rise firstly to the medial ganglionic eminence (MGE), and to the lateral ganglionic eminence (LGE) expressing respectively the TFs *Nkx2*.1 and *Gsh2*. In the the pallium *Gli3* expression will be flanked by the combined expression of the transcription factors *Pax6* and empty spiracles homeobox 2 (*Emx2*) (Fig. 3B). They are expressed early in the dorsal forebrain (E8.5) (Simeone et al., 1992; Walther and Gruss, 1991) along opposite gradient *(see below)* and are both necessary for dorsal telencephalon specification: *Emx2*$^{-/-}$ *Pax6*$^{-/-}$ mice exhibit an expansion of the choroidal roof and the subpallium at the expense of the cortex (Bishop et al., 2000; Kimura et al., 2005; Muzio et al., 2002)**.** Remarkably, in *Gli3*$^{-/-}$ mice *Emx2* is downregulated (Theil et al., 1999) and *Gli3*$^{-/-}$*Pax6*$^{-/-}$ mice have a similar phenotype *to Emx2*$^{-/-}$*Pax6*$^{-/-}$(Fuccillo et al., 2006), suggesting that *Emx2* is downstream to *Gli3*.

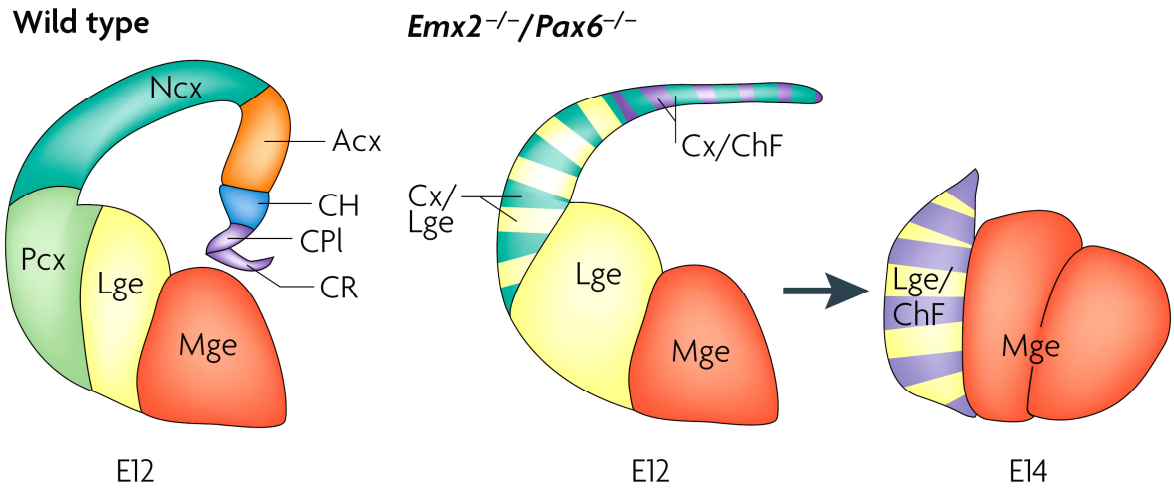



**Figure 5: *Emx2* involvement in dorsal forebrain specification.**

Loss of both *Pax6* and *Emx2* results in ventralization of cortical progenitors and the loss of the neocortical domain (Ncx), archicortex (Acx), cortical hem (CH) and choroid plexus (CPl), choroid field (choroid plexus and choroidal roof) (ChF) by embryonic day 14. Adapted from Muzio & Mallamaci 2003 and Molyneaux et al. 2007

The concerted activity of dorsal forebrain patterning centers and transcription factors expressed in the telencephalic field further subdivides the cerebral cortex in distinct anatomical and functional areas, treated in the next paragraph.

## 1.3 Cortical Arealization

In its tangential dimension the neocortex is subdivided in distinct areas, showing different structural and functional properties. Four primary areas can be distinguished: visual (V1), somato-sensory (S1), auditory (A1) and motor area (M1). An even slight affection of size, position and connectivity of these areas heavily affects brain functioning. The process leading to the specification of these areas is called cortical arealization. Cortical arealization starts at early stages (E9.5 in mouse), with the specification of a primitive molecular protomap, set up according to specific positional and temporal cues. The codification of these signals initiates intrinsically to the cortical field, resulting from the interplay between soluble factors, secreted at the borders of this field, and transcription factors expressed along tangential gradients within it. Subsequently, (E13.5 in mice) thalamo-cortical axons (TCA), relaying sensory information from distinct nuclei of the dorsal thalamus to different cortical regions, promote further inter-regional diversification, so leading to the properly called cortical arealization.

There are five main patterning centers around the cortical field involved in its patterning: (1) rostrally, the anterior neural ridge and commissural plate (ANR/CoP), secreting fibroblastic growth factors (Fgf 8,17,18); (2) ventrally, the precordal plate and the basal telencephalon, secreting Nodal and Sonic hedgehog (Shh), (3) dorsocaudally, the roof plate and the cortical hem, secreting bone morphogenetic proteins (Bmp2,4,6,7) and vertebrate orthologs of *Drosophila* wingless (Wnt 3a,5a,2b); (4) laterally, outside the neural tube, the olfactory placode (or mesenchimal cells within it), secreting Retinoic acid (RA); (5) lateroventrally, at the pallial subpallial border, the antihem, secreting epidermal growth factor family members (Tgfα, Nrg1 and Nrg3), Fgf7, as well as the Wnt signaling inhibitor Sfrp2 (Fig. 6).

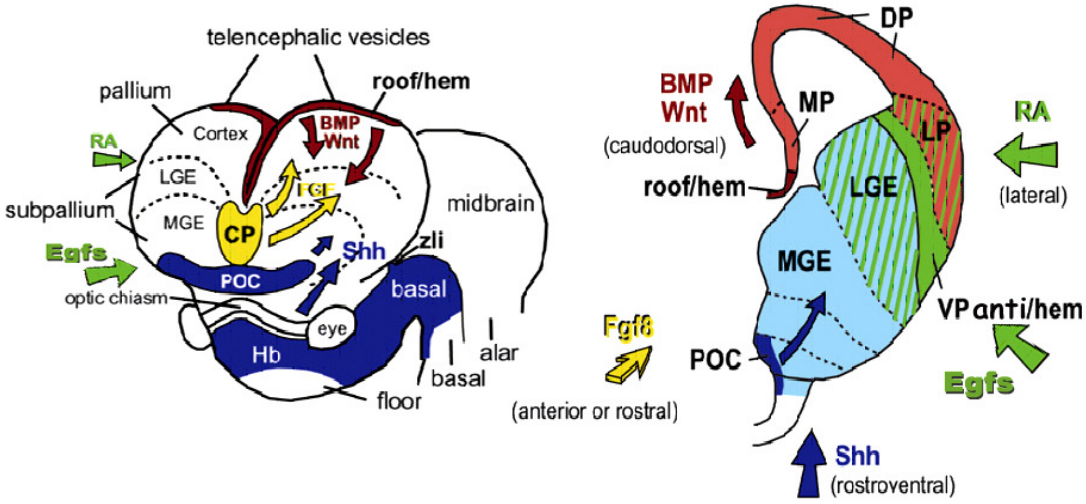



**Figure 6: Signaling proteins and patterning centers involved in pallial patterning, specification and area formation**.

Schematic diagrams of a mouse brain (left) or a frontal telencephalic hemisection (right) showing the signaling centers and proteins involved in pallial patterning. Modified from Medina and Abellan 2009.

Secretion of soluble cues starts at very early stages and alteration of dosages of these growth factors have dramatic consequences: as an example, Fgf8 secretion by the ANR begins very early (E8.5 in mice )(Crossley and Martin, 1995) and its overexpression leads to a posterior shift of cortical areas, on the other hand, overexpression of the soluble form for one of its receptors (Fgfr3) shifts areas anteriorly, by inhibiting Fgf8 activity (Fukuchi-Shimogori and Grove, 2001).

Transcription factors involved in the specification of cortical areas are expressed in proliferative layers of the developing neocortex, according to distinct spatial gradients . These TFs belong to different families and their gradient can be oriented in different ways: good examples of that are *Emx2, Sp8, Lhx2, Pax6, Foxg1* and *Coup-tf1* (Fig. 7)

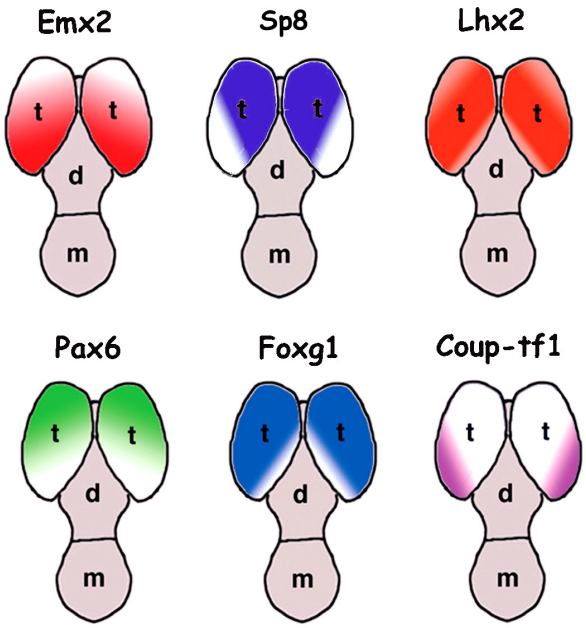



**Figure 7: Some of the transcription factors involved in cortical arealization.**

Patterns of graded expression of six transcription factors involved in cortical arealization. E12.5 brains, dorsal views: t, telencephalon; d, diencephalon; m, mesencephalon. Modified from Mallamaci and Stojkova 2006.

*Emx2* is highly expressed in progenitors of the posterior-medial areas of the telencephalic field (as V1) and much less in anterior-lateral areas (as M1). *Emx2* constitutive knockout elicits a dramatic enlargement of anterior cortical areas, at expenses of caudal-medial ones (Bishop et al., 2000; Mallamaci et al., 2000) (Fig. 8A). This phenotype strongly suggests the *Emx2* capability to impart a posterior medial identity to the neocortical progenitors. Possible drawbacks to this interpretation is the activity exerted by *Emx2* in repressing Fgf8 signaling from the ANR (Fukuchi-Shimogori and Grove, 2003) and in TCA pathfinding (López-Bendito et al., 2002). 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. 8B).

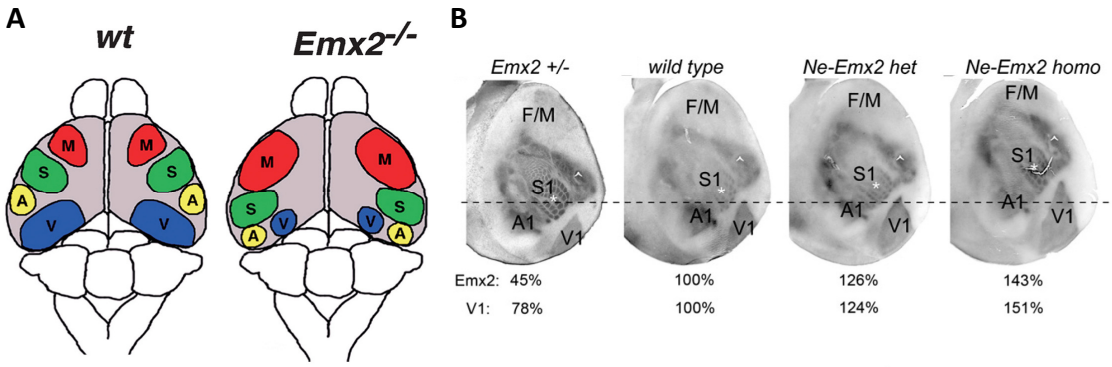



**Figure 8: Area identity shifts in cerebral cortices of perinatal and adult *Emx2*$^{-/-}$**

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).(A)** Adapted from Muzio and Mallamaci 2003; **(B)** Adapted from Hamasaki et al. 2004.

It has to be stressed that a much more complex gene network implying feed-back and feed-forward mechanism among TFs and signaling centers, lies beyond the specification of cortical areas. As an example, rostral Fgf8 signaling from the ANR/CoP is maintained thanks to the Shh signaling from ventral telencephalon as well as by *Sp8* transcription factor, which is more expressed in anterior-medial areas. The Fgf8 signaling might be confined in more caudal areas by the concerted activity of Bmp secreted by the cortical hem, as well as by Sp8 protein sequestering by *Emx2* (Sahara et al., 2007) (Fig. 9).

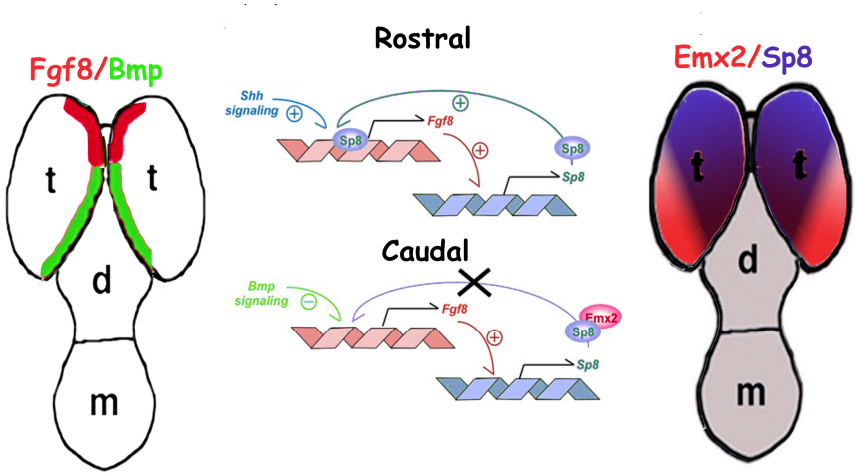



**Figure 9: Secreted protein from patterning centers and graded transcription factors interactions.**

Morphogenes secreted from the patterning centers and transcription factors are involved in a complex web of positive and negative feedbacks. As an example Fgf8 expression could be restricted to the anterior forebrain by the concerted activity of Bmp proteins and Emx2 mediated sequestering of Sp8. Modified from Mallamaci and Stojkova 2006 and O'Leary et al. 2007

*Foxg1* is highly expressed in progenitors of the anterior-lateral areas of the telencephalic field and progressively less in posterior-medial areas. As previously stated, it is necessary to estabilish ventral identity of the telencephalon. In cortical arealization it helps Fgfs to the partition of antero-lateral and posterior medial territories. In *Foxg1* null mice there is an aberrant areal differentiation, leading to ectopic expression of the Cajal-Retzius marker Reelin throughout the cortical field (Hanashima et al., 2004), moreover, this field is abnormally specified as a hippocampal anlage (Muzio and Mallamaci, 2005).

## 1.4 Cortical Histogenesis

### *-Extra cortical sources of cortical neural cell types*

Mammalian cortical histogenesis is tightly regulated both in time and in space. During embryonic life neurons are generated first, followed by astrocytes and then by oligodendrocytes, in partially overlapping waves. The process is further complicated by the fact that not all the cell types which will populate the cortex are generated within the cortex itself. In particular, it has been shown that projection glutamatergic neurons belonging to the II-VI layers, astrocytes and a subset of oligodendrocytes are generated in the dorsal pallium (*Emx1* expression domain) (Gorski et al., 2002; Kessaris et al., 2006). On the other hand, glutamatergic Cajal-Retzius (CRs) cells (populating layer I), interneurons (all in rodents, only a subset of them in primates) and another subset of oligodendrocytes are generated outside the pallium. They reach their final position within the cerebral cortex via tangential migration across the telencephalon (Fig. 10).

CRs are a transient population of glutamatergic neurons (Derer and Derer, 1990), localized at the surface of the cerebral cortex during the entire cortical development (in the layer I) and born before the formation of the cortical plate. After birth, CR die massively: in rodents after the first postnatal week more than 95% of these neurons are eliminated (Derer and Derer, 1990; del Río et al., 1995). These cells are the main source of Reelin, a large extracellular protein strictly necessary for the proper formation of the cerebral cortex

(Tissir and Goffinet, 2003). In fact, newborn cortical projection (layer II-VI) neurons, need the Reelin signal to radially migrate from progenitor layers toward the pia and to detach from the radial glia cells scaffold (see below) (Soriano and Del Río, 2005). Recently it has been shown that CR have three extracortical sources: the cortical hem (Meyer et al., 2002; Takiguchi-Hayashi et al., 2004; Yoshida et al., 2006) the ventral pallium and the septum from which they migrate all over the cortex (Bielle et al., 2005; Meyer et al., 2002) (Fig.10A).

Interneurons constitute about the 35% of total neurons present in cortex and use γ-aminobutirric acid (GABA) as neurotransmitter to modulate the function of cortical projection neurons. Different subtypes of interneurons have been distinguished based on origin and expression of different molecular markers (somatostatin, parvalbumin and calretinin): the vast majority of them are born in the subpallium and migrate towards the cortex after terminal mitosis of their progenitors (Fig 10B). Remarkably, also some interneurons secrete Reelin and could therefore contribute to master radial migration of projection neurons, together with CRs. (Soriano and Del Río, 2005; Wonders and Anderson, 2006). There is little evidence of interneurons born in the cortex in rodents (Xu et al., 2004). Conversely, retroviral labeling of human cortical slices showed that the vast majority of GABA positive cells are originated by the cortex itself (Letinic et al., 2002). It is noteworthy that cells in the human cortical proliferative layers strongly express *Nkx2.1*, a marker that is absent in the rodent cerebral cortex and is specifically expressed by the MGE instead (Rakic and Zecevic, 2003).

As for oligodendrocytes, their origin has been controversial for a long period (Richardson et al., 2006), but, recently, Kessaris et al. have clearly shown that there are a temporal ventral to dorsal gradient in the their production (Kessaris et al., 2006). The first wave comes from the MGE, then a second wave from the LGE-CGE and finally a third wave is generated within the pallium, in the *Emx1*-expressing domain. So, it seems that oligodendrocytogenesis is not regional restricted, but proceed in a “Mexican wave” fashion, from the most ventral to dorsal areas (Fig. 10C). However, only oligodendrocytes derived from the last cortical wave populate the adult neocortex. It is not clear if this is due to dilution of the oligodendrocytes coming from the subpallium, or because of their progressive replacement by those generated within the pallium (Kessaris et al., 2006).

Since the vast majority of cells which populate the adult cortex comes from cortical neural precursors, we will mainly focus on cortical precursors and mechanisms of radial migration, lamination and gliogenesis which takes place within the cortex.

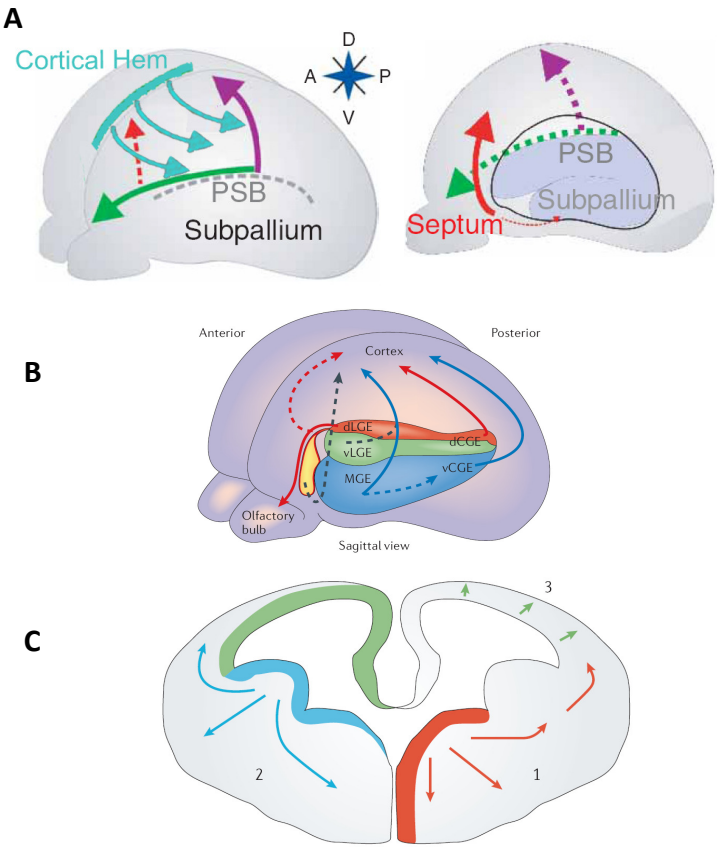



**Figure 10: Extra-cortical sources of cortical neural cell types: tangential migration.**

Cajal-Retzius cells are generate in three main locations, the cortical hem, the pallial-subpallial boundary and the septum. From these points they will cover all the uppermost cortical layer (A). The vast majority of interneurons are born in the subpallium in rodents in (MGE, CGE, and LGE) (B). Three waves of oligodendrocytes from different sources MGE, LGE and cortex reach the cortex: however only cortical derived oligodendrocytes are retained after birth (C). (A) Modified from Bielle et al. 2005;(B) modified from Wonders and Anderson 2006

## *-Cortical neural precursors*

At the onset of neurogenesis (E9.5) neuroepithelial (NE) cells forming the cortico-cerebral wall undergo proliferative and differentiative divisions, giving rise both to the first post-mitotic neurons and to different subset of progenitors. Some of these progenitors delaminate from the VZ giving rise to a secondary proliferative layer, the subventricular zone (SVZ), that lies above the VZ (Mérot et al., 2009). The earliest born neurons migrate from the VZ forming a transient structure, called preplate (PP), which is subsequently split into a superficial marginal zone (MZ) and a deeply located subplate (SP), by the radial migration of newborn neurons coming from the proliferative layerswhich will form the so-called cortical plate (CP) (Fig. 11A).

Neural precursors are a complex mix of distinct progenitor populations, which differ both in nature and position. A first positional classification can distinguish two subpopulations: apical progenitors, lining the ventricular wall, and basal progenitors delaminating from the VZ duringearly steps of corticogenesis. A summary of the different neural precursor populations and their principal features is summarized is summarized in Fig. 10B,C.

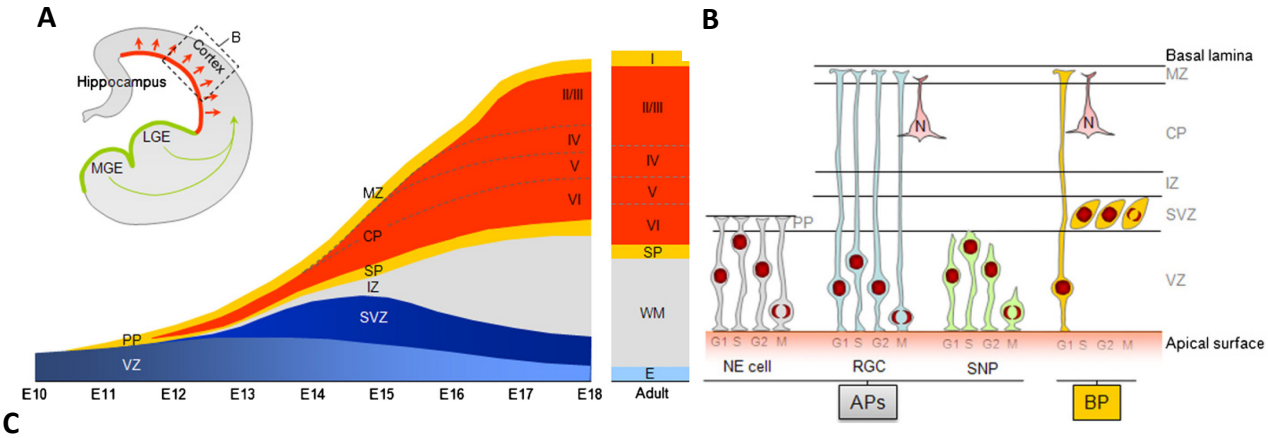



**Table 1.** Neurogenic progenitor cells in embryonic rodent cerebral cortex (mouse)

| | NE | RG | SNP | IPC |
|---|---|---|---|---|
| Ages | E9.5–E13.5 | E11.5–P0.5 | E12.5–E16.5 | E10.5–P0.5 |
| Morphology | radial | radial | radial (VZ only) | multipolar (few radial) |
| Migration | IKNM | IKNM | IKNM | multipolar |
| Mitosis | apical | apical | apical | basal (rare apical) |
| Mitotic cycles | unlimited | many (≥10) | n.d. | 1–3 |
| Daughter cell fates | NE, RG, IPC, N | RG, IPC, N | SNP, N | IPC, N |
| Molecular expression | prominin 1 (CD133), nestin | Pax6, tenascin-C, BLBP, vimentin, nestin, GLAST | tubulin $\alpha_1$ promoter | Tbr2 (some low-level Pax6) |
| Abundance | 0–100% depending on age | approx. 50% of VZ progenitors (E13.5–E16.5) | approx. 50% of VZ progenitors (E13.5–E16.5) | 10–50% of all progenitors (E10.5–P0.5) |
| References | 1, 6, 7 | 6–8 | 9 | 1–3, 10–12 |

BLBP = Brain lipid-binding protein; GLAST = astrocyte-specific glutamate transporter; IKNM = interkinetic nuclear migration; N = neuron; n.d. = not determined; P = postnatal day; VZ = ventricular zone.

**Figure 11. Cortical neuronogenis: the heterogeneity of proliferative layers.**

Projection neurons arise from progenitors in the dorsal–lateral ventricular wall (red line) and migrate radially to populate the nascent cortex (red arrows) **(A).** Morphological, cellular and molecular properties of apical and basal progenitors subpopulations are summarized In **(B)** and **(C). (A)** and **(B)** are adapted from Mérot et al. 2009; **(C)** is adapted from Pontious et al. 2008

NE cells are the founder population of cortical precursors and appear in the developing telencephalon at the onset of cortical neuronogenesis. They can give rise to all the histotypes which form the mature cortex (neurons, astrocytes and oligodendrocytes). At early stages these cells undergo a symmetrical self-renewing pattern of cellular divisions, so leading to an increase of the surface area of the VZ. NE cells are characterized by a peculiar

radial morphology, with an apical process pointing to the ventricular lumen and a basal one which reaches the PP. Mitosis in these cells occurs when their nuclei are close to the apical lumen, whereas DNA synthesis occurs when they reach the most basal position lining the PP. This process is called interkinetic nuclear migration (IKNM).

Around E10 in mouse, the NE division pattern progressively switches to a more asymmetric one, giving rise to the first neurons and to Radial Glial Cells (RGCs). RGCs share with NE cells many histological, morphological and molecular properties, including full histogenetic potentials, radial morphology and IKNM. At a molecular level, both populations express the transcription factor *Pax6* and the intermediate filament protein Nestin (Hartfuss et al., 2001) (and are labeled by the RC2 antibody, recently shown to be directed against an epitope encoded by the *Nestin* itself (Park et al., 2009)). However RGCs selectively express the astrocyte specific glutamate transporter (GLAST), as well as the brain lipid binding protein (BLBP) (Hartfuss et al., 2001; Pinto and Götz, 2007). Remarkably, the basic-helix–loop–helix (bHLH) Hes transcription factors seem to be important for the NE-to-RGC transition: mice deficient in *Hes1* and *Hes5* show normal NE cells at E8 but impaired RGC differentiation at E9.5 (Hatakeyama et al., 2004). Thus, Notch signalling mediated by Hes transcription factors seems not to be required by NE cells. Alternatively Notch signaling may be mediated via molecular mechanisms independent of Hes-mediated transcription prior to E9, as no defects are obvious in the *Hes1*, 3, 5 triple mutants at this stage (Hatakeyama et al., 2004). It is only at the onset of neurogenesis, when RGCs appear, that Hes-mediated Notch signaling becomes essential for RGCs maintenance.

Recently, the strong heterogeneity of the VZ precursors has been further underlined by the identification of a new neuronal restricted progenitor population, the Short Neural Precursors (SNP). These precursors are similar to RGCs, however, they have a basal process which does not reach the MZ, so showing a “pin-like” morphology. They undergo IKNM similarly to NE and RGC cells, but can be distinguished from other apical progenitors by the activity of the alpha1 tubulin promoter (pTα1) (Gal et al., 2006; Sawamoto et al., 2001).

Basal progenitors, also called Intermediate Progenitor Cells (IPC), originate from apical progenitors and undergo a symmetric neuronogenic pattern of divisions away from the VZ (1-3 mitoses). Even if they are easily distinguishable at later developmental stages in the SVZ, they are born at early stages of the cortical neuronogenesis in the VZ and contribute to neurons belonging to both deep and superficial layers (Miyata et al., 2004,

Noctor et al., 2004, (Haubensak et al., 2004; Noctor et al., 2008). They show a multipolar morphology and do not undertake INKM. From the molecular point of view, they are characterized by the expression of the transcription factor *Tbr2* (Englund et al., 2005) and a fading expression of *Pax6*. Notably, IPCs precursors are a minority within the progenitor pool of the cortex (only at later stages reaching 25%), while they are the majority in the ventral telencephalon (Martínez-Cerdeño et al., 2006; Pinto and Götz, 2007).

Summarizing, cells with NSC properties (NE and RGC) and neuronal restricted progenitors (SNP and IPC) have been identified and characterized until now in the mammalian cerebral cortex; however the precise relationship between these different precursor classes, their relative contribution the cortical histogenetic output and the evo-devo significance of this heterogeneity are still extensively debated.

The identification of distinct telencephalic glial restricted progenitors (GRP) has revealed much more complicated. Recently, Strathmann et al. have identified by a combined *in vivo/in vitro* approach a telencephalic precursor population which is capable to originate both astrocytes and oligodendrocytes, but not neurons (Strathmann et al., 2007). This population expresses the c-series ganglioside specific A2B5 antigen and is conversely negative for Neural Cell Adhesion Molecule (PSA-NCAM). GRPs seem the earliest population along the gliogenic lineage, since they still express NSC molecular markers (Nestin and Sox2), and are present *in vivo* before the appearance of oligodendrocyte restricted progenitor population (OPC) (Strathmann et al., 2007). Telencephalic OPC progenitors, are characterized by expression of *Sox10* and platelet-derived growth factor receptor alpha (PDGFR-alpha) or *Olig2* (not expressed by GRP). The presence of astrocyte restricted progenitors (ARP) in the telencephalon has not been demonstrated yet. However, as many analogies have been found between the telencephalic GRPs and spinal cord GRPs, it could be reasonable the model represented in Fig. 12. In the spinal cord ARP are generated from GRP and can give rise only to astrocytes both *in vivo* and *in vitro.* They express CD44 and are negative for *Sox2*, *Olig2* and PDFGR-alpha (Liu et al., 2004). However morphological identification of the above mentioned telencephalic GRP, ARP and ORP is still lacking.

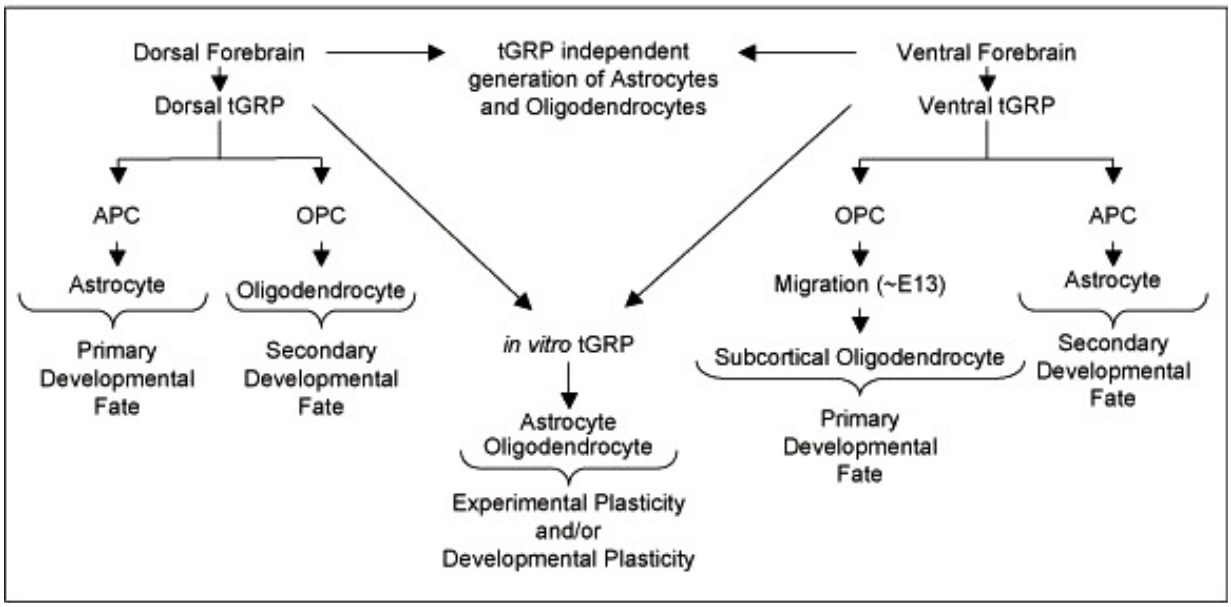



**Figure 12: A model for glial cell generation in the telencephalon.**

The dorsal telencephalon and ventral telencephalon would give rise to glial restricted progenitors (GRPs) with a primary developmental fate towards astrocyte and oligodendrocyte restricted progenitor (OPC) generation, respectively. As the ventral and dorsal telencephalon continues through development, each GRP population would have the potential to participate in a secondary developmental fate towards astrocytes ventrally, or OPCs dorsally. Adapted from Strathmann et al 2007.

## *-Self renewal vs differentiation of cortical progenitors*

Neural precursors pools assure the correct number of neurons and glial cells populating the cortex at the end of embryonic life. A complex process, involving self renewal/expansion of the pools and lineage fate choice/differentiation is responsible for the final outputs coming from the cortical progenitors. Control of cell cycle progression is a major key point in this balance (Fig. 13).

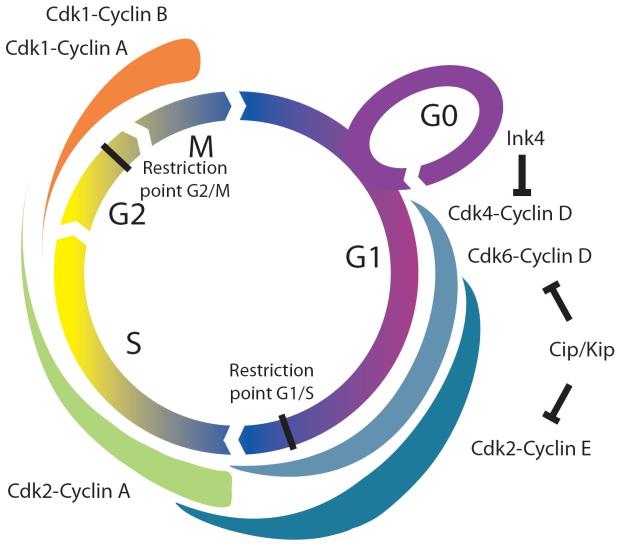



**Figure 13. Cell cycle control**.

Progression through the cell cycle is regulated by cyclin/CDK complexes and their inhibitors (Ink4 family and Kip/Cip family). Two main restriction points have been described, that control whether cells enter a new round of DNA replication (G1/S check point), and whether DNA replication has been correctly performed before the cell divide (G2/M check point). Adapted from Letasa et al. 2009

It has been demostrated that during corticognesis progression both the length of the cell cycle and the fraction of cells exiting from cell cycle (Q) increase. In particular cell cycle lengthening is to be ascribed to a prolonged time of G1 phase (TG1) (Takahashi et al., 1995) (Fig. 14A). The increase of Q is mainly due to a switch in division patterns of progenitors, from a self-renewing one to a more differentiative (Fig.14A). It has been proposed that lengthening of G1 phase and cell division pattern are linked: during cell division each daughter cell receives an unequal amount of a neurogenic cell-fate determining factor, which, during the G1 phase, sets the probability of the daughter cell will undergo neuronal differentiation. As G1 length increases, the neuronogenic activity exerted by this factor goes up as well and the probability for both the daughter cells to pass the neuronogenic activity threshold augments, so leading to the shift from a symmetrical self-renewing division pattern to an asymmetrical neuronogenic one and, further, to asymmetrical, fully pro-differentiative one (Calegari and Huttner, 2003; Götz and Huttner, 2005) (Fig.14B).

Moreover the division pattern and G1 phase length could also have a major role on the neuronal subtype specification during neuronogenesis. The causative link between G1 phase and cortical lamination has been recently addressed, by overexpression of cyclin D1 and cyclin E1 by in utero electroporation in mouse embryos. Remarkably overexpression of each of these cyclins promove: (a) early apical progenitors self renewal, followed by increased transition of this compartment to the IPCs. (b) IPCs expansion at the expenses of neuronogenesis. Moreover laminar fates is heavily altered in this case, with an increase in the number of late generated neurons (layer II & III), probably due to the delay of the last mitosis of the progenitor pools (Pilaz et al., 2009).

Therefore the control of the restriction point from the G1 to S phase transition seems to be critical for self renewal and differentiation choices. Cdk-cyclin complex inhibitors of the Kip/Cip family ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$ )and Ink4 ($p16^{Ink4}$, $p15^{Ink4}$, $p19^{Ink4}$ $p18^{Ink4)}$ could exert major roles in the balance between the two process. $p21^{Cip1}$ knockout lead to exhaustion of proliferative pools in embryonic cortical progenitor *in vitro* culture (Kippin et al., 2005).

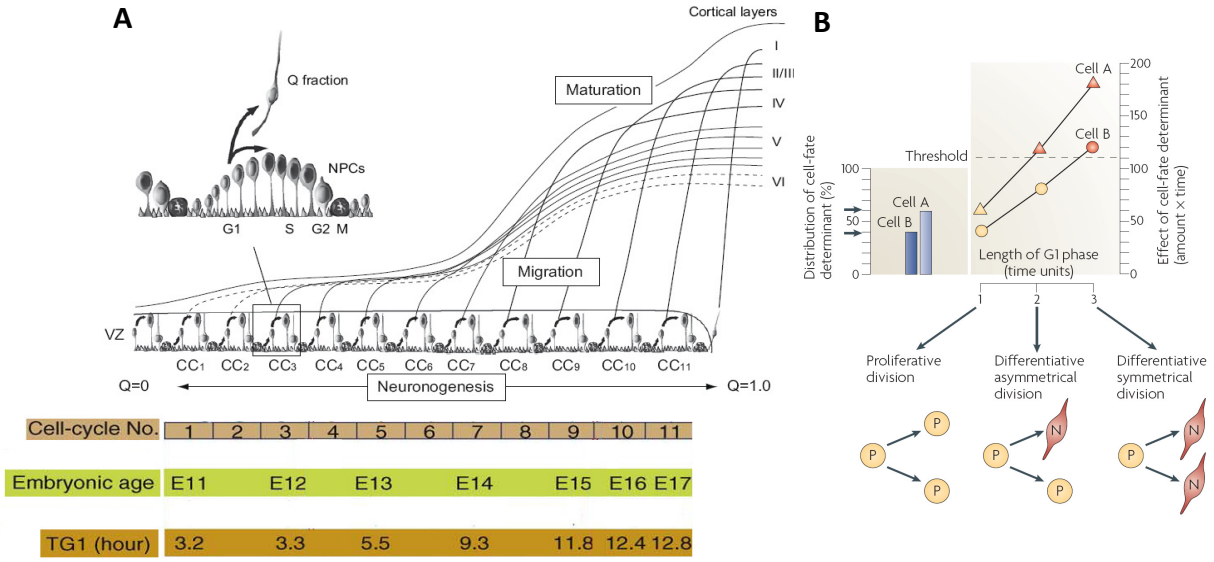



**Figure 14: Cell cycle and division pattern control in cortical histogenesis.**

During the neuronogenic period (E11-E17) the velocity of the cell cycle is not constant, but there is an increase of time of G1 phase (TG1). This time is important for correct lamination of the cerebral cortex. At the same time, the fraction of cells leaving the cell cycle (Q) increases from 0 to almost 1 **(A)**.TG1 has been linked to the progression a symmetric proliferative division pattern to differentiative one **(B). (A)** Modified From Caviness et al. 2009 and Mitsuhashi and Takahashi 2009; **(B)** Adapted from Dehay and Kennedy 2007.

*Foxg1* is highly expressed in proliferative layers throughout the neocortical development. *Foxg1*$^{-/-}$ mouse cortexes are hypoplastic and previous studies demonstrate reduced progenitor cell proliferation, increased rate of differentiation, cell cycle lengthening and increased cell death (Hanashima et al., 2002; Martynoga et al., 2005; Xuan et al., 1995). However the observed mis-specification of the neocortical domain towards a medial fate in knockout mice (Muzio and Mallamaci, 2005) and the Fgf8 inducing activity exerted by *Foxg1* (described above) make the interpretation of the biological meaning of this complex phenotype challenging. Moreover, *Foxg1* overexpression in chicken elicits overproliferation of cortical neuroblast without affecting cell cycle length, but by increasing cell survival (Ahlgren et al., 2003). Recently Fasano et al. have shown that *Foxg1* is necessary to mediate *Bmi-1* promotion of cortical precursors self renewal; however, the same authors also claim that *Foxg1* overexpression is not sufficient to promote NSCs self renewal, but only to slightly protect them from cell death (Fasano et al., 2009). Studies from *Foxg1*$^{+/-}$ heterozygous mice have further complicated this already intricate landscape, since they show no alterations in the apical progenitor compartment size, but a reduced SVZ, possibly due to a blockade of

VZ-to-SVZ transitions. Remarkably, this activity could be due to *Foxg1* suppression of $p21^{Cip1}$ expression (Siegenthaler et al., 2008). Little is presently known about the role of *Foxg1* in the transition from RGCs to SNPs.

*Emx2* is specifically expressed in the proliferative layers during neocortical development. Proliferation rates in the medial cortical territories of $Emx2^{-/-}$ mice are reduced (Bishop et al., 2000; Mallamaci et al., 2000; Tole et al., 2000). This is associated to $p27^{Kip1}$ and $p57^{Kip2}$ increased expression and Delta-Notch signalling alterations (Muzio et al., 2005). A lengthening of cell cycle length may be found in these mutants, however it is restricted to earliest phases of pallial development (Mallamaci et al., 2000). High density *in vitro* cultures derived from $Emx2^{-/-}$ cortexes show decreased proliferation, whereas *Emx2* overexpressing cultures show enriched symmetrical divisions (Heins et al., 2001). Strikingly, when *Emx2* GOF and LOF manipulations are performed in perinatal and adult neural stem cells, they elicit an exactly opposite effect, compared to embryo-derived cortical cultures (Galli et al., 2002; Gangemi et al., 2001). Finally, all *Emx2* GOF and LOF studies published so far agree on one point: there is no statistically relevant change in neural lineages differentiation, with no significant variations in numbers of neurons, astrocytes and oligodendrocytes (Galli et al., 2002; Heins et al., 2001).

## -*Neuronogenesis*

Progenitors residing in the VZ and SVZ produce the projection neurons of the different neocortical layers in a tightly controlled temporal order from embryonic day E11.5 to E17.5 in the mouse. Newborn neurons migrate radially from the proliferative layers and finally detach from the RGC scaffold to reach their final position. They are settled down in an inside-outside fashion, with the later born neurons migrating post the early born ones. In this manner a six layers structure is formed, in which deeply located neurons (Layers V and VI) mostly project to subcortical structures and upper layers (Layers II and III) mostly project to targets within the ipsilateral and the controlateral hemisphere (Angevine and Sidman, 1961; Rakic, 1974) (Fig. 15).

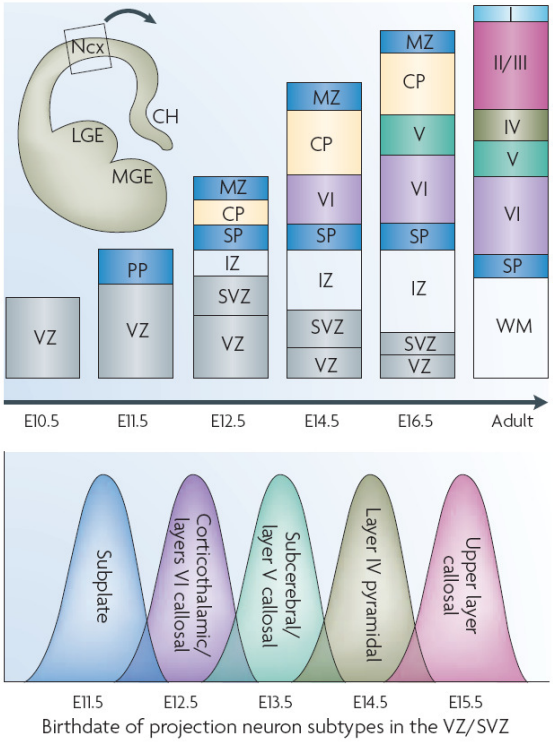



**Figure 15: Timing of cortical neuronogenesis.**

Neoortical neurons are born according to a precise time schedule in an inside-out fashion. First neurons to be generated are the subplate neurons, followed by layer VI, V, IV and upper layers III and II. Layer I is cell sparse and mainly composed of Cajal-Retius cells reaching the cortex by tangential migration. Adapted from Molyneaux et al. 2007.

The precise relationships between neural precursors and the identities to each projection neuron type are still largely unknown. Lineage tracing of clonally related populations shows that, at the earliest stages of neurogenesis individual progenitors are able to give rise to pyramidal neurons belonging to each layer (Reid and Walsh, 2002). As development goes on, progenitors become progressively restricted in their competence states, even if late born progenitor can be still forced to produce neurons of deep layers (Frantz and McConnell, 1996; Fukumitsu et al., 2006; McConnell and Kaznowski, 1991). It has been proposed that apical progenitors might contribute to generation of deep layers post-mitotic neurons, whereas basal progenitors might contribute for upper-layer neurons. This idea came from the identification of markers expressed in the SVZ during upper-layer neurogenesis and in upper-layer postmitotic neurons, as well as of markers expressed by apical progenitors and persistently present in deep layer neurons, as they mature. (Chen et al., 2008; Molyneaux et al., 2005). As an example, *Svet1* and *Cux2* are expressed in a subset of dividing cells in the SVZ during the generation of upper-layer neurons and postnatally in neurons of layers II–IV, suggesting that they might be markers for upper-layer progenitors within the SVZ. In particular, *Cux2* expression is detected in the basal VZ starting at E11.5 in

mice, suggesting that IPCs could be committed to the generation of upper-layer neurons early in cortical neurogenesis (Nieto et al., 2004; Tarabykin et al., 2001; Zimmer et al., 2004). However, as previously stated, IPCs are abundant during all stages of cortical neurogenesis, including early stages when lower layers are generated, and before a distinct SVZ has formed. Moreover time-lapse imaging of neuronogenic divisions has shown that IPCs produce the majority of neurons even in the murine E11-13 time window, when lower layers are generated (Haubensak et al., 2004). A great effort has been made to identify layer specific markers of post-mitotic neurons, however it is not still clear whether the same markers can be used to identify progenitors of each neuronal subtype, or whether such lineage-committed progenitors even exist (Molyneaux et al., 2007).

Remarkably, it has been reported that *Foxg1* knockdown in cortical E12 derived neural precursors (which have already acquired cortical identity) is sufficient to revert them to a Cajal-Retius phenotype, so suggesting that *Foxg1* would be necessary to set the proper timing of neocortical plate histogenesis (Shen et al., 2006). However, the absence in this study of clonal trees showing the presence of all the neocortical laminar markers (Shen et al., 2006), as well as the above mentioned extracortical origin of Cajal-Retzius cells and the capability of *Foxg1* to confine hippocampal and cortical hem programs to the dorsomedial-most telencephalic primordium are major drawbacks to interpretation of these data (Muzio and Mallamaci, 2005).

As for an *Emx2* involvement in neocortical lamination, actually this gene is not expressed by the vast majority of neocortical neurons (Mallamaci et al., 1998; Simeone et al., 1992), but is selectively expressed by Cajal-Retzius cells, seeming to be important for their maturation. In fact, Cajal-Retzius cell formation is progressively impaired in *Emx2*$^{-/-}$ mice, resulting in aberrant cortical plate layering and pronounced radial glia abnormalities (Mallamaci et al., 2000).

### *-Neuronogenis/Gliogenesis switch*

Similarly to other vertebrates, mouse gliogenesis is a temporally restricted process that occurs mostly in the first month of post embryonic life. The switch that turns cortical precursors to make glial cell instead of neurons originates from both extrinsic and intrinsic factors. Feedbacks coming from the neighboring developing cortical plate promote

gliogenesis against neuronogenesis at mid developmental stages. Differentiated cortical neurons feedback on precursors, via the cardiotrophin 1-gp130-JAK-STAT pathway, to activate gliogenesis (Barnabé-Heider et al., 2005). Committed neuronal precursors and young neurons express Notch ligands which activate Notch signalling in cortical precursor, so inducing the expression of the transcription factor nuclear factor I, which binds to astrocytic gene promoters, resulting in demethylation of astrocyte-specific genes (Namihira et al., 2009). Also long range signals coming from the patterning centers could play a role: BMP2/4-Smad signalling has been shown to be neuronogenic at early stages and gliogenic at later stages of development (Gross et al., 1996; Li et al., 1998; Nakashima et al., 2001). However, even if extracellular cues are important for the proper regulation of the neuronogenic/gliogenic switch, cell intrinsic mechanisms seem to play major roles in this fate choice. In fact, at early stages of cortical development, cells of the proliferative layers are refractory to external gliogenic stimuli, strongly suggesting that they have still not acquired the competency to respond due to DNA methylation (Fan et al., 2005; Takizawa et al., 2001). Downregulation of proneural transcription factors of the bHLH (helix–loop-helix) family, like Neurogenin-1 (Neurog1), attenuates neuronogenesis and contributes to timely activation of gliogenesis. In fact Neurog1 mediates a tonical inhibition of the gliogenic pathway, by sequestering the Smad/CBP/p300 complex and making it unavailable to interact with the pSmad1,3 complex. That inhibits the latter from synergizing with the former, so preventing promotion of gliogenesis at early neuronogenic stages (Sun et al., 2001). Remarkably, expression of proneural genes is mainly restricted to committed IPCs and even combinatorial deletions of them do not lead to precocious glial differentiation (Nieto et al., 2001; Tomita et al., 2000); this make unlikely the possibility that modulation of these genes acts as a main trigger of the gliogenic pathway. Interestingly, Coup-TF1/2 TFs, master genes involved in cerebral cortex arealization, cell migration, differentiation and axon growth (Armentano et al., 2006; Armentano et al., 2007; Tripodi et al., 2004), are capable to reset the timing of neuronogenesis/gliogenesis switch. In fact, it has been recently shown by Naka et al. that double knockdown of *Coup-tf1/2* causes prolonged neuronogenesis and a strong impairment of gliogenesis both *in vitro* and *in vivo,* due to persistent retention of early DNA and histone H3 methylation profiles, by astrocyte specific promoters (Naka et al., 2008). Remarkably, microarray analysis of tertiary neurospheres, coming from *Coup-tf1/2* knockdown shows not only a predictable upregulation of bHLH genes (*Neurog1 & 2, Mash1,*

*NeuroD*), but also a strong *Foxg1* overexpression. In such a context, conversely, *Emx2* and *Pax6* expression are only slightly upregulated (Naka et al., 2008).

## 1.5 Wiring

The astonishing complexity of the neocortex has to be reconducted directly to the function of this telencephalic structure: the integration of signals coming different sensory pathways and the elaboration of adequate responses to these stimuli. Cortical projection neurons can connect different areas of the cortex within the same hemisphere (corticocortical connections) or in the opposite one (callosal connections) or connect the cortex to subcortical structures (corticofugal connections). Projection neurons show lamina specificity: callosal and corticocortical projection neurons are mainly localized in layer II and III; corticofugal projection neurons lie in layer VI (corticothalamic) and layer V (to other subcortical structures, including striatum, midbrain, hindbrain and spinal cord). As for inputs coming from the environment to the neocortex, virtually all of them pass through the thalamus to reach their specific area target in the cortex. As an example, somatosensory stimuli from the whiskers reach the ventrobasal complex (VB) in the dorsal thalamus; neurons present in VB send axons to to layer IV of the S1 area. Both the the VB and the S1 area in the neocortex contain a faithful bi-dimensional topological representation of the facial whisker pad (Woolsey and Van der Loos, 1970). Similar processing pathways also convey visual and auditory modalities (Fig. 16A). Thalamocortical axons (TCAs) show not only area but also lamina specificity: most of the thalamic input terminates in cortical layer IV (although some project also to layer I,II/III and VI).

As previously stated, information for the specification of the cortical protomap is encoded within the cortex, well before the arrival of TCA (E13 in mouse). However TCA activity is responsible both for differential citoarchitectonic area properties and topographic and features maps (Katz and Shatz, 1996).

The development of the thalamus runs parallel to the cortical neuronogenesis and signals coming from the cortex are involved in axon outgrowth in the thalamus (Barbe and Levitt, 1992; Fukuchi-Shimogori and Grove, 2001; Gao et al., 1998; Molnár and Blakemore, 1995), however these cues are not well characterized yet. It seems that pioneer axons of subplate neurons projecting to the internal capsule are important in this process. Both thalamocortical and corticothalamic axons have to pass through the pallial-subpallial and

the telencephalic-diencephalic boundaries to reach their targets, so having to interact with a variety of well defined molecular territories during this journey (Puelles et al., 2000).

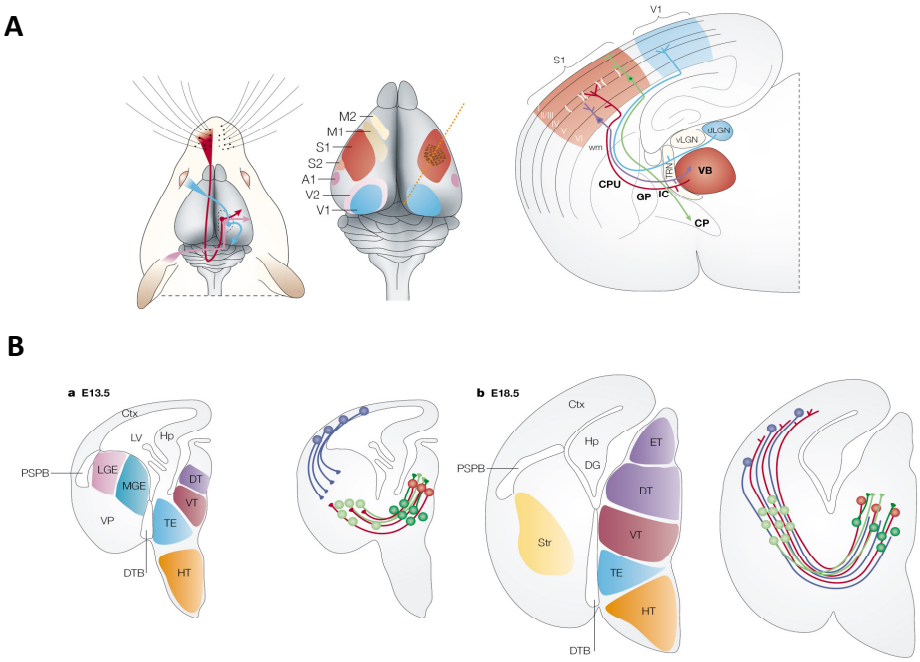



**Figure 16: Thalamocortical and corticothalamic connections of the brain.**

Somatosensory information from the whiskers (red line) reaches the ventrobasal complex (VB) through the brainstem and is relayed to the S1 layer IV; visual input from the retina (blue line) is relayed through the dorsal lateral geniculate nucleus (dLGN) to reach the visual cortex in (A).Thalamocortical and corticothalamic trajectories during forebrain development. PSPB: Pallial-subpallial boundary; DTB telencephalic-diencephalic boundary (B). Adapted from Lopez-Bendito and Molnar 2003.

Many of developmentally regulated TFs, expressed along this route of migration have been shown to heavily affect the corticothalamic pathway.

*Emx2* is expressed in subset of post-mitotic cells at the borders of the ventromedial pallium, as well as at the telencephalic-diencephalic boundary. In *Emx2*$^{-/-}$ mice the areal shift is flanked to TCA misrouting at the telencephalic-diencephalic boundary (Bishop et al., 2000; Mallamaci et al., 2000). Approximately half of the fibres descend into the ventral telencephalon, instead of entering the internal capsule and extending towards the cortex (López-Bendito et al., 2002) (Fig 17A).

*Foxg1* is highly expressed in the ventral telencephalon, but is not expressed in the diencephalon. In *Foxg1*$^{-/-}$ mice the telencephalon is highly compromised, but the diencephalon appears normal (Xuan et al., 1995). In these mice TCAs fail to turn laterally to

enter the ventral telencephalon and are misrouted deeply into the hypothalamus at E15.5 (Pratt et al., 2002) (Fig 17B).

*Foxg1* is also expressed in sense organs (Hwang et al., 2009; Kawauchi et al., 2009; Pauley et al., 2006; Picker et al., 2009; Tian et al., 2008). In particular, *Foxg1* is expressed in the nasal retina and the anterior optic chiasm (Hatini et al., 1994; Huh et al., 1999). In Foxg1$^{-/-}$ mice there is a significative increase in the number of retinal ganglion cells axons joining the ipsilateral optic chiasm (Pratt et al., 2004). Recently Tian et al. provided functional evidences that *Foxg1* promotes contralateral projections through actions in nasal retina; moreover its expression by optic chiasm also stimulate retinal ganglion cell axons growth (Tian et al., 2008).

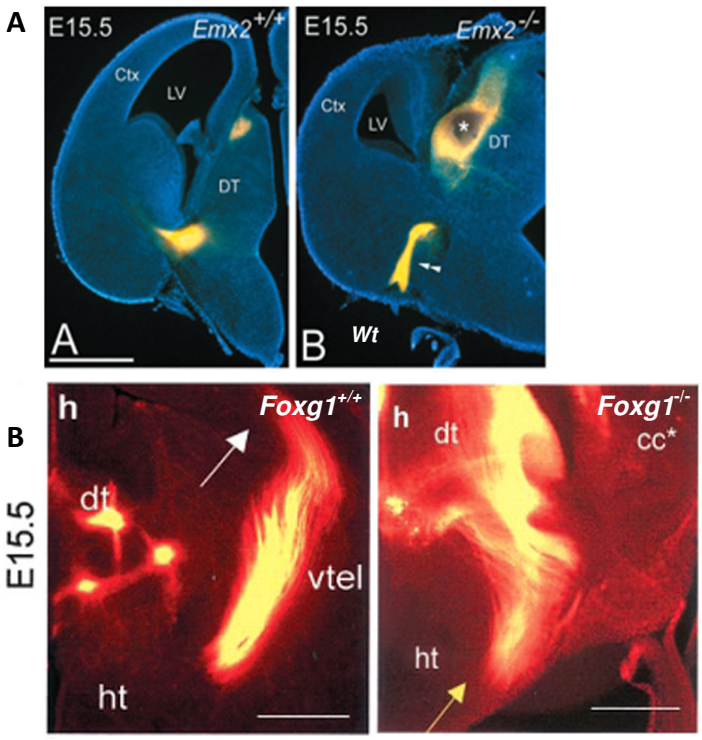



**Figure 17: *Emx2* and *Foxg1* knockout mice show altered thalamocortical connections.**

In *Emx2* $^{-/-}$ mice many TCAs reach the cortex through an aberrant route. They descend from the dorsal thalamus, but enter the telencephalon abnormally, at the ventral-most point of the diencephalon–telencephalon boundary. However, a subset of thalamic axons entered the internal capsule normally and continues to grow through the striatal anlage toward the cerebral cortex **(A).** In *Foxg1*$^{-/-}$ mice TCAs can no more enter the pallium and are misrouted to the hypothalamus **(B). (A)** Adapted from Lopez-Bendito et al. 2002. **(B)** Adapted from Pratt et al. 2002

## 1.6 *Emx2* and *Foxg1* in the adult brain

At the end of the developmental period the proliferative layers of the telencephalon lose most of their neurogenic properties. Most RGCs cells loose the apical process, which kept them in contact with the ventricles, and transform into astrocytes (Misson et al., 1991; Noctor et al., 2008; Schmechel and Rakic, 1979; Voigt, 1989). However, it has been demonstrated that the capability to produce newborn neurons and glial cells is retained both in neonatal and in adult mammalian telencephalon, in specific regions. There are two major sites of *de novo* neuronogenesis in physiological conditions: the subventricular zone of the lateral ventricle wall (aSVZ) (Lois and Alvarez-Buylla, 1994; Luskin et al., 1993) and the subgranular zone of the dentate gyrus in the hippocampus (SGZ) (Altman and Das, 1965; Gould and Cameron, 1996; Kempermann et al., 1997)(Fig.18).

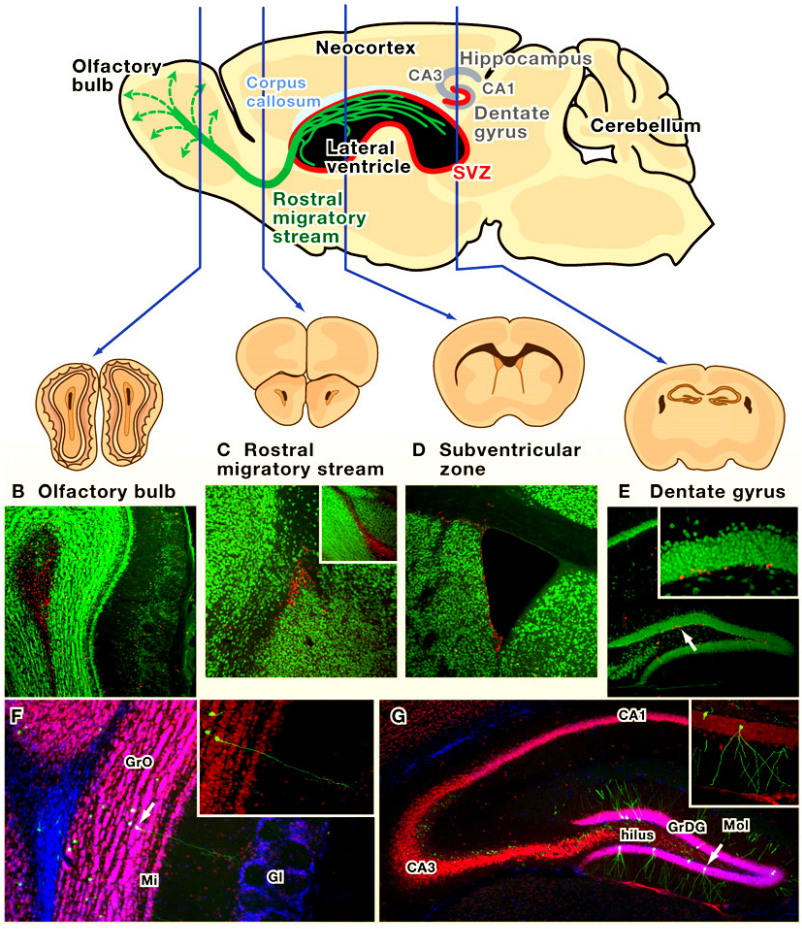



**Fig. 18: Adult neuronogenic niches**

In physiological conditions there are only two sites of neuronogenesis in the adulthood: the aSVZ and SGZ (in red). Sagittal and frontal view at different rostrocaudal levels, show BrdU incorporation by proliferating cells. Colors indicate the following: red, BrdU; green, NeuN. Newborn neurons in the olfactory bulb and dentate gyrus labeled by retrovirus-mediated expression of green fluorescent protein (GFP)(B). Colors indicate the following: red, NeuN; green, GFP; blue, DAPI. Adapted from Zhao et al. 2008.

The presence of such well defined loci of neuronogenesis have raised many questions about the functions of these newly generated neurons in the adult brain physiology, as well as the cellular and molecular determinants, making these two sites able to make new neurons.

Imayoshi et al. have demonstrated that continuous neurogenesis is required for the maintenance and reorganization of interneurons of the olfactory bulbs and to modulate and refine the existing neuronal circuits in the dentate gyrus (Imayoshi et al., 2008). Moreover, ablation of adult neural stem cells by gene manipulations shows that only spatial learning is heavily impaired with no impairment of contextual fear conditioning, locomotion or diurnal rhythmic (Imayoshi et al., 2008; Shi et al., 2004; Zhang et al., 2008). This suggests that neuronogenesis in the hippocampus is important for specific cognitive functions.

But what makes the aSVZ and SGZ apparently the unique regions in which NSCs are still present and able to generate neurons? Many researchers agree that these loci are neuronogenic niches, retainining some embryo-specific environmental and cellular cues, responsible for the maintenance of NSCs. This opens up the possibility to exploit them to increase neuronogenic properties of the telencephalon, after damage induced by ischemia or neurodegenerative diseases. It is remarkable that other parts of the brain, similarly to the cerebral cortex, show neuronogenic potencies too, under some pathological conditions. This makes the idea to manipulate developmentally regulated transcription factors so to exploit the dormant cortical neuronogenic potencies even more appealing.

*Emx2* and *Foxg1* are still present in the adult brain and are particularly expressed in the adult neuronogenic niches. In the next paragraphs we will briefly (1) describe the progenitor cells of the neuronogenic niches and their molecular properties; (2) summarize evidences for *de novo* neuronogenesis in different pathological models and discuss evidences for *Emx2* and *Foxg1* involvement in regulation of these processes, both in physiological and pathological conditions.

## 1.7 The neuronogenic niches

### *-The subventricular zone of the lateral ventricle wall*

The aSVZ is located next to the ependima, which is a thin layer of cells lining the ventricle wall. In this region there are three progenitor cell populations: A,B,C (Fig. 19). The type B is considered to be the adult NSC of the aSVZ for its cellular, molecular, morphological and functional properties. It has been demonstrated that type B cells directly descend from embryonic RGC (Merkle et al., 2004). B cells are located just underneath the ependimal layer and have a small apical process (Mirzadeh et al., 2008; Shen et al., 2008) that ends in the ventricle and a process which ends on blood vessels of the niche (Mirzadeh et al., 2008). This morphology is highly reminiscent of embryonic RGCs. Moreover, B cells share many molecular markers with embryonic RGC, like GLAST, BLBP, Nestin, Sox2 (Colak et al., 2008; Doetsch et al., 1997; Platel et al., 2008). They are relatively quiescent. However, after a 1 week treatment with arabinoside C (Ara-C), which kills fast proliferating C and A types progenitors, B cell are able to regenerate all the cells of the niche (Doetsch et al., 1999).

Type C cells are also called transient amplifying population, because they are a rapidly proliferating population, giving rise to A cells, that are migratory neuroblasts. Type A cells migrate across the brain along a specific pathway, the rostral migratory stream (RMS), formed by chains of specialized astrocytes, to reach the olfactory bulbs (Jankovski and Sotelo, 1996; Lois and Alvarez-Buylla, 1994). After detaching from these chains and migrating from the RMS into the olfactory bulb, adult-born cells from the SVZ mature into olfactory inhibitory interneurons of two main types: granule cells and periglomerular interneurons.

It has been proposed that also a subset of ependymal cells that are $CD133^{+}$ $CD24^{-}$ could have stem cell properties (Coskun et al., 2008; Johansson et al., 1999). However many studies have found that ependymal cells are post-mitotic after development (Capela and Temple, 2002; Spassky et al., 2005) and cannot generate neurons in *in vitro* cultures. Moreover, Mirzadeh et al have subsequently show that all the ependymal cells are $CD133^{+}$ $CD24^{+}$ and negative for BrdU uptake: the only $CD133^{+}$ $CD24^{-}$ cells are type B cells, strongly suggesting a misinterpretation of ependymal cell identity (Mirzadeh et al., 2008).

Interestingly it has been demonstrated that aSVZ has heterogeneous sources, in fact it contains cells deriving from different counterparts of the embryonic telencephalon. As an example, progenitors present in the dorsal part of the aSVZ derive from cortical progenitors (Ventura and Goldman, 2007; Young et al., 2007). These cells remain in positions reminiscent to the one of their ancestors and contribute to distinct types of olfactory bulb interneurons (Merkle et al., 2007; Ventura and Goldman, 2007; Young et al., 2007).

A very recent breakthrough in the field is the discovery that dorsal aSVZ can also give rise to iuxtaglomerular glutamatergic neurons of the olfactory bulbs. Progenitors of these cells sequentially express *Pax6*, *Neurog2*, *Tbr2*, *Tbr1*, like glutamatergic neurons progenitors during cortical development (Brill et al., 2009). It is really remarkable that after chemo-photo-ablation induced cortical damage (see below), some of these progenitors are rerouted from the RMS to the site of cortical damage and also integrate in cortical layers (those going in upper layer show *Cux1* expression) (Brill et al., 2009).

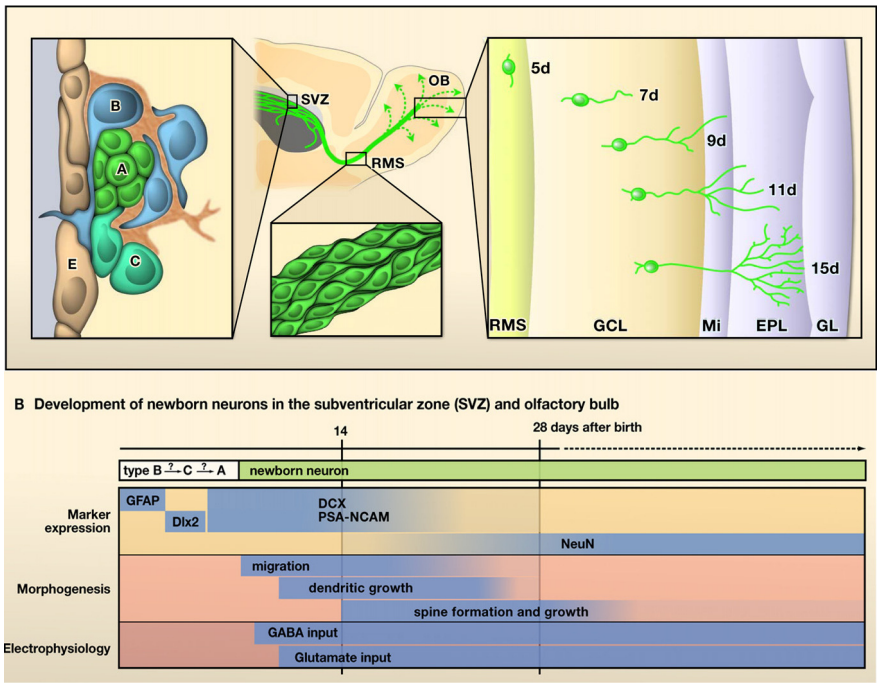



**Figure 19: Neurogenesis in the aSVZ**

Progenitor cells (B,C,A) in the aSVZ lie adjacent to the ependymal layer (E) lining the lateral ventricles and interact with basal lamina. Newborn neurons reach the olfactory bulb (OB) through chain migration in RMS and go through morphological and physiological changes before integrating in the OB. Mi, mitral cell layer; EPL, external plexiform layer. Adapted from Zhao et al. 2008

## *-The subgranular zone of the dentate gyrus (SGZ)*

Neuronogenesis in the SGZ is of great interest, not only for the above mentioned role in spatial learning, but also because it has been implied in different neurologic disorders, like depression (Dranovsky and Hen, 2006) and epilepsy (Parent et al., 2006).

The SGZ is located at the interface between the hilus and the granule cell layer (Fig. 20). Also in this niche three types of progenitors can be distinguished: type 1, type 2a and type 2b. Type 1 are thought to be the neural stem of this niche. There is no direct evidence of their descent from embryonic RGCs, although classical anatomical studies by Altmann suggest that RGCs of the dentate neuroepithelium give rise to the type 1 cells, which show a radial phenotype, with a long process crossing the granule cell layer and a smaller one oriented horizontally to the SGZ itself (Altman and Bayer, 1990; Seri et al., 2004). Also type 1 cells, like B cells, are GFAP, GLAST, BLBP, Nestin, Sox2 positive (Steiner et al., 2006) and are relatively quiescent, showing a long lasting cell cycle. (Seri et al., 2004). They seem not to give rise directly to neurons in vivo, but pass through a type 2a progenitor state. Type 2a cells show no radial phenotype and are a transient amplifying population, positive for *Tbr2,* giving rise to type 2b cells. These last cells will express with maturation early and late neuronal markers and finally will be integrated in the hippocampus circuitry as glutamatergic granule cells (Fukuda et al., 2003; Seri et al., 2004).

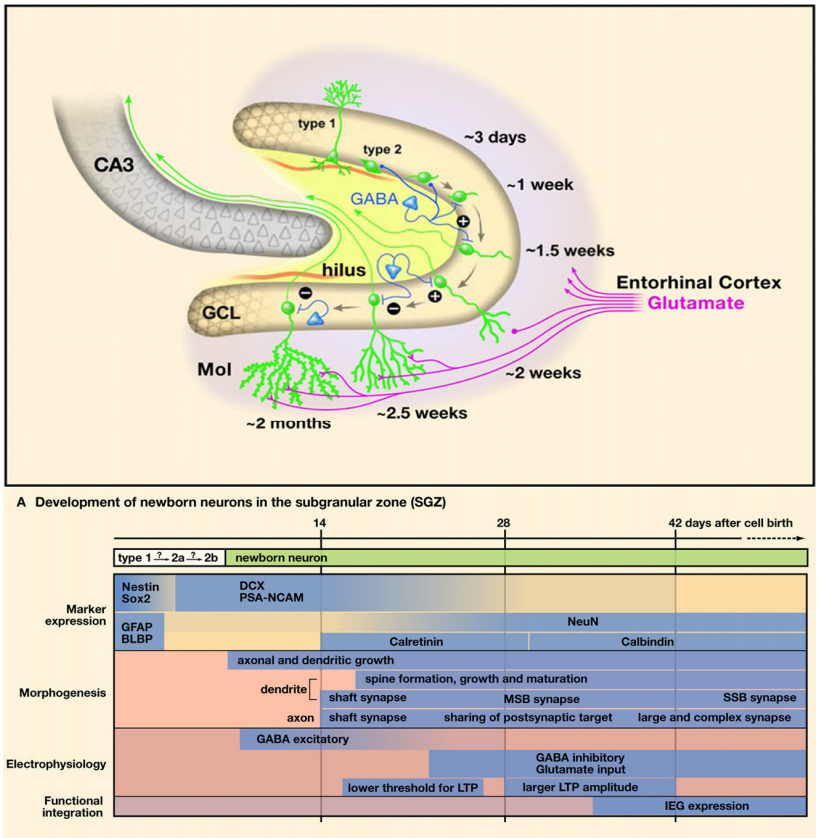



**Figure 20: Neurogenesis in the SGZ**

Type 1 and type 2 progenitor cells in the SGZ show distinct morphologies and express specific molecular markers. Newborn neurons in the DG of the hippocampus pass through different stages of morphological and physiological development. Specifically, the transition from GABA excitatory to GABA inhibitory and glutamate excitatory inputs to newborn neurons occurs during the third week after cell birth, concomitant with the growth of dendritic spines. GCL, granule cell layer; Mol, molecular layer. Adapted from Zhao et al. 2008

## *-Neuronogenic niches resemble the embryonic molecular environment*

The structural and anatomical features, suggesting that neuronogenic niches retain some features of embryonic proliferative layers, is further confirmed by evidences showing that many soluble cues and transcription factors and involved in forebrain development.

As an example, Shh signalling is fundamental for neuronogenic niches maintenance through activation of the transcription factor *Gli1;* systemic administration of the Shh inhibitor cyclopamine strongly impairs aSVZ proliferation *in vivo* and *in vitro* (Palma et al., 2005). Other morphogenes implied in adult stem cell renewal are Bmp, Fgf, Il-6 gp130, Wnt, Egf families (Basak and Taylor, 2009).

Also the Notch signalling seems (Mumm and Kopan, 2000) to be still at work during adult neuronogenesis: mice heterozygote mice for presenilin 1, a mediator of Notch signalling, show impaired adult neuronogenesis. Moreover the Notch ligand Jagged1 is expressed in adult niches, also by ependimal cells, and infusion of the this ligand in the ventricle enhances proliferation (Androutsellis-Theotokis et al., 2006; Basak and Taylor, 2009).

Among transcription factors *Pax6* is still expressed in subsets of immature $GFAP^{+}$ $Nestin^{+}$ adult neural stem cells, both in aSVZ and in SGZ. Its function is required for specification of periglomerular cells and for granule neurons (Hack et al., 2005; Kohwi et al., 2005). The number of periglomerular cells are increased by *Pax6* overexpression in the adult neuronal progenitors (Hack et al., 2005) and decreased in $Pax6^{+/-}$ mice (Kohwi et al., 2005).

*Emx2* is still expressed during adulthood in the progenitors of the SGZ, aSVZ and RMS both in rodents (Galli et al., 2002) and in primates (Tonchev et al., 2006). Its overexpression *in vitro* culture elicits an increase in differentiative asymmetrical divisions, its reduction an opposite phenotype (Galli et al., 2002)(Fig. 21).

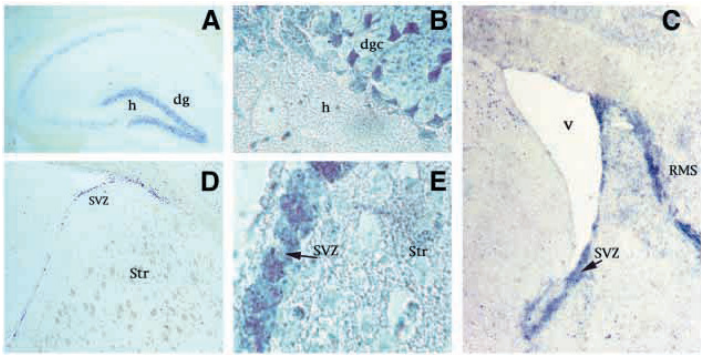



**Figure 21: *Emx2* expression in the adult forebrain**

In situ hybridization reveals *Emx2* expression in progenitors of both the SGZ (A,B) and aSVZ (D,E). Strong expression of the gene can be also observed along the RMS (C). V, ventricle; Str, striatum; SVZ,subventricular zone; dg, dentate gyrus; h, hilus; dgc, dentate granule cells.

In the adult brain, *Foxg1* is expressed at high levels in the neuronogenic niches (Fasano et al., 2009; Shen et al., 2006) and in the olfactory bulbs; *Foxg1* heterozygous mice have a 60% decrease in the total number of hippocampal dentate granule cells, due to impaired proliferation and reduced cell survival. Moreover this is associated behavioural abnormalities (hyperlocomotion, impaired habituation in the open field, deficit in contextual fear conditioning) and resistance to antidepressants (Kinsler et al., 2010; Shen et al., 2006)(Fig. 22) .

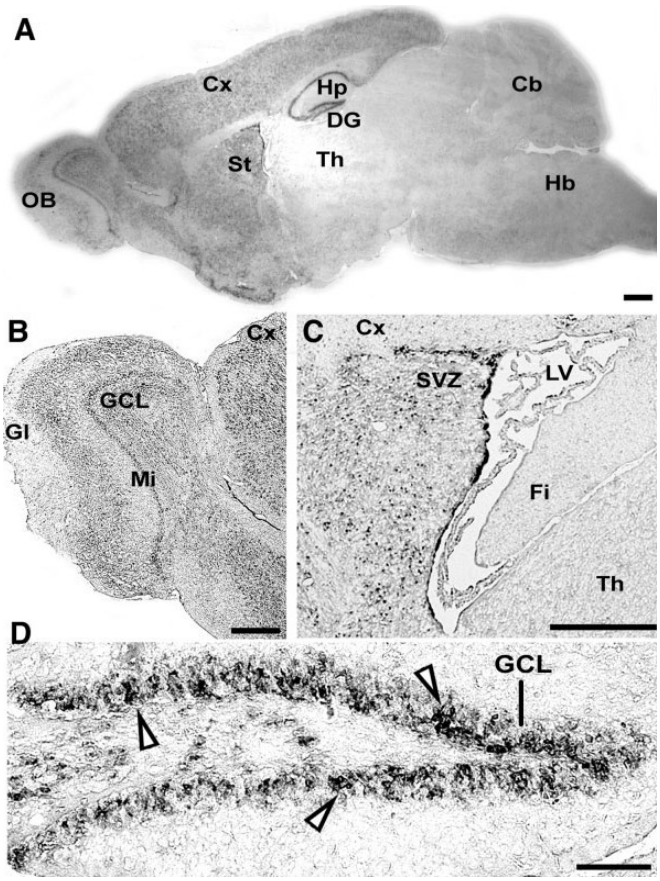



**Figure 22: *Foxg1* expression in the adult forebrain**

In situ hybridization reveals *Foxg1* expression in a sagittal section in the RMS **(B)**, aSVZ **(C)**, and the DG **(D)**. Within the DG, cells with the highest level of Foxg1 mRNA are present in small clusters within or near the SGZ (arrowheads in D). In the rest of telencephalon Foxg1 transcript is present at low levels. GCL, granular cell layer; Gl, glomerular layer; H, hilus; Hb, hindbrain;Hp, hippocampus; LV, lateral ventricle; Mi, mitral cell layer; OB, olfactory bulb. Adapted from Shen et l. 2006.

It has been demonstrated that *Bmi1*, a transcriptional repressor of the polycomb family is required for postnatal self-renewal of neural stem cells (Molofsky et al., 2003). Recently Fasano et al have shown that *Bmi1* stimulates specifically adult NSC self-renewal from telencephalon and that this effect requires *Foxg1* activity (Fasano et al., 2009). However, according to these authors, *Foxg1* would be *necessary,* but not sufficient to stimulate NSCs self-renewal: its constitutive overexpression only elicits slightly protective effects in neurospheres cell culture (Fasano et al., 2009) .

## 1.8 Neuronogenesis in pathological conditions

Although there is a general agreement about the aSVZ and SGZ production of new neurons in the adult brain, it remains controversial if other regions of the brain, especially the neocortex, retain neuronogenic potentials. Several groups have reported evidences for this, both in rodent and in primates (Dayer et al., 2005; Gould et al., 1999), but many others were not able to reproduce the data (Bhardwaj et al., 2006; Kornack and Rakic, 2001). Recently it has been proposed that lack of evidences could be to false negatives (Gould, 2007).

However there are strong evidences that in some pathological conditions, like ischemia, or focal lesions, new neurons are born in different parts of the brain. It is not clear if this arises from the plastic recovering of dormant potentialities of these regions, or simply by an enhancement of a physiological ongoing neuronogenesis.

Moreover several studies show that in different pathological conditions progenitors and neuroblasts normally originating from the neuronogenic niches can be rerouted to repair brain damage due to ischemia (Faiz et al., 2008; Goings et al., 2004), chromophore-activated synchronous apoptosis, (Brill et al., 2009; Chen et al., 2004; Magavi and Macklis, 2002) or neurodegenerative diseases (Curtis et al., 2003).

In particular, the most striking evidences of neocortical neurons replacement has been provided by Macklis group: selective ablation of specific cortical projection neurons, via retrograde uptake of a toxic laser-activated chromophore, resultes in robust replacement of the specific neural subtypes ablated. These newborn neurons, derived from progenitors located in the aSVZ and RMS reach the right neocortical layer and project to appropriate targets in the thalamus and spinal cord (surviving for up to one year) (Chen et al., 2004; Magavi and Macklis, 2002).

Also in clinical relevant mice models of global ischemia, endogenous progenitors have revealed to be effective for brain repair: it has been demonstrated that, after bilateral vertebral arteries occlusion, hippocampal neurons of the CA1 region can regenerate (Nakatomi et al., 2002). Moreover intraventricular infusion of growth factors markedly augments this response and the functional recovery. It is remarkable that in this model an increase of *Emx2* expression slightly precedes *de novo* neuronogenesis (Nakatomi et al., 2002).

The endogenous capacity to replace neurons, although showing that the adult brain is permissive to neuronogenesis, seems to be quantitatively limited: a lot of newborn neurons fail to integrate in pre-existing circuitry and finally die. Nevertheless, it is hypothesizable that ameliorating differentiation, survival and functional integration properties of transplanted elements may allow in the future to override this drawback, so making cell-replacement-based therapies a feasible therapeutic approach.

Different strategies are being presently followed to obtain cell replacement in the brain (Kokaia, 2009) (Fig. 23)

| | NSCs from embryonic and adult tissues | Embryonic stem cells | PGD-embryonic stem cells | Induced pluripotent stem cells |
|---|---|---|---|---|
| Advantages | Only moderate ethical concerns about derivation process | Unlimited expandability<br>Broad patterning potential | Unlimited expandability<br>Broad patterning potential<br>Intrinsic expression of disease-associated genes | Derivation without ethical concerns<br>Unlimited expandability<br>Broad patterning potential<br>Intrinsic expression of disease-associated genes<br>Possibility to include sporadic forms of the disease |
| Disadvantages | Limited access<br>Limited expandability<br>Limited patterning potential<br>Transgene overexpression mostly required<br>Bias towards glial differentiation<br>Limited to candidate gene approaches | Ethical concerns about derivation process<br>Transgene overexpression required<br>Genetic and epigenetic instability<br>Limited to candidate gene approaches | Ethical concerns about derivation process<br>Genetic and epigenetic instability<br>Limited to candidate gene approaches | Genetic and epigenetic instability |

PGD=preimplantation genetic diagnosis.

*Table:* **Advantages and disadvantages of the different donor sources for the establishment of cell-based models of neurodegenerative diseases**

**Figure 23: Strategies actually tested for cell replacement based brain repair purposes.**

Adapted from Kokaia et al 2009

In particular one of the major drawbacks of embryo and adult derived NSCs is that their prolonged *in vitro* proliferation under standard growth factors mix is associated with a decrease of their neurogenic potential and a concomitant increase of gliogenic properties (Herrera et al., 1999; Winkler et al., 1998; Wright et al., 2006). Probably this effect has to be attributed to a cell-autonomous mechanism, involving epigenetic regulation of the chromatin state. It is noteworthy that artificial manipulation of *Coup-tf1* and *Coupt-tf2* expression levels (these are two key transcription factors involved in forebrain specification and patterning) can apparently reset this timer, allowing a robust neuronal differentiation, even in tertiary neurospheres (Naka et al., 2008).

However the suitability of embryonic progenitor for brain repair has been widely assessed: as an example, it has been demonstrated that foetal cortical human stem cells generate region specific neurons *in vitro* and, if transplanted in rats after stroke, can survive and differentiate into neurons. Remarkably, *Pax6* strongly promotes this phenotype (Kallur et al., (Darsalia et al., 2007; Kallur et al., 2006; Kallur et al., 2008).

Taken all together, these data suggest that *in vitro* genetic manipulations of embryonic and adult NSCs could be useful not only to dissect the complex functions exerted by developmentally regulated TFs. In fact, simple analytical manipulations of these transcription factors, by "waking up" multiple dormant pro-neuronogenic activities of neural progenitors, could also result advantageous for *ex vivo* cell-replacement approaches to brain diseases. In the next paragraphs we will briefly discuss the neurosphere culture system, one of the most employed *in vitro* model, used in this study.

## 1.9 The neurosphere culture system

Neurospheres are free floating spherical clusters of cells, usually originating from a single cell suspension of stem cells and/or neural progenitor cells isolated from the embryonic (Reynolds et al., 1992) or adult CNS tissues (Reynolds and Weiss, 1992) and allowed the proliferate and/or aggregate in serum free medium supplemented with Fgf2 and Egf growth factors.

Neurosphere composition is heterogenous: they contain neural stem cells, as well as proliferating progenitor cells of different lineages (Suslov et al., 2002). Neural precursors grown as neurospheres have been shown to retain NSC properties (i.e. indefinite self-renewal and capability to give rise to neurons and glial cells, if placed in differentiative conditions). However, it has been demonstrated with different methods that only a very small fraction of cells within the neurospheres are *bona fide* stem cells and that, as neurospheres are passaged (Louis et al., 2008; Reynolds and Rietze, 2005), the percentage of lineage restricted progenitors (presumably glial) increases. Traditionally, methods based on counting neurospheres generated in clonal conditions have been used to estimate the number of neural stem cell present in a neural precursor suspension. However, as neurospheres may also largely derive from lineage committed progenitors (even after repeated *in vitro* passages), dedicated approaches had to be developed to account (and to compensate) for that. Unfortunately, such approaches are cumbersome and also the most accurate ones, like the neural colony-forming cell assay (NCFCA), based on counting >2mm large neural colonies adhering onto a collagen matrix (Louis et al., 2008), may have serious drawbacks. In fact, experimental gene manipulations, altering neural precursors kinetics may impact on colony growth rates, so making even this assay poorly reliable. Such methodological problems urge to be fixed.

Modelling proliferation and differentiation kinetics of neural precursors by neurosphere cultures has some technical pitfalls, being particularly sensitive to a variety of parameters describing the culturing method used. In particular, variations in cell density (Caldwell et al., 2001; Hack et al., 2004; Tropepe et al., 1999) different constituents or concentrations of growth factors in the media (Arsenijevic et al., 2001; Caldwell et al., 2001; Irvin et al., 2003) and frequency of passaging (Caldwell et al., 2001) strongly affects reproducibility of results. So, data from neurosphere culture analysis have to be tightly and carefully normalized on controls used in the study.

Remarkably, many evidences demonstrate that regional restriction of developmentally regulated genes is maintained in these cultures, even after many passages, and that cells grown as neurospheres retain the ability to differentiate into neural subtypes characteristic of the region of origin (Hitoshi et al., 2002; Kelly et al., 2009; Klein et al., 2005; Ostenfeld et al., 2002; Parmar et al., 2002; Zappone et al., 2000). However, Machon et al.

have shown that Fgf2 and Egf present in the neurosphere culturing medium would be able to alter the genetic programs of neural cells. Cells isolated from the dorsal telencephalon would downregulate and the expression of dorsal markers (*Emx2/1, Pax6, Ngn1/2, Dach1*) and upregulate that of ventral markers *(Mash1, Nkx2.1, Shh, Oligo1/2)* (Machon et al., 2005). Downregulation of *Pax6* and *Emx2* in cortical progenitors grown as neurosphers had also been previously reported by (Hack et al., 2004). It is possible that discrepancies between Machon et al. and Hack et al. studies, on one side, and other reports cited above, on the other, may arise - partly or completely - from the different densities at which NSC were cultured in different studies. Community effects could encourage retention of initial regional identity in high density cultures, clonal growing conditions would favor loss of cell identities.

Notwithstanding the caveats, neurospheres can be a very powerful model for *in vitro* study of neurogenesis and neural development.

They can be useful to estabilish the degree of intrinsic specification present in neural precursors at various developmental time-points. They can allow studying the full potential of neural precursor cells, when removed from extrinsic cues provided by their normal environment, thanks to the possibility to easily vary the culture medium composition.

The intrinsic neurosphere heterogeneity, usually seen as major limitation in the usage of this model, is probably its most relevant point of strength. In fact, neurospheres can be considered close to neuronogenic niches, in which a basal lamina as well as contacts among distinct precursor types are preserved. This probably allows NSC and progenitors within neurospheres to behave like their *in vivo* correlates in several respects, as for example proliferative capacity and differentiation after exposition to soluble cues (Irvin et al., 2003).

Moreover, genetic manipulation of neurospheres can be easily accomplished via retroviral (Falk et al., 2002; Heins et al., 2002) and lentiviral gene delivery (Capowski et al., 2007). Finally, recent advances in fluorescent protein usage and cell-type specific promoters further allow to shed new light on neurospheres, often considered as “thin black boxes” (Jensen and Parmar, 2006), and follow up their composition dynamically, in real time, both in normal or perturbed conditions.

Following these considerations and in order to systematically dissect biological activities exerted by *Emx2* and *Foxg1* in distinct cortico-cerebral precursors, we developed a new integrated perturbative-analytic system, by combining lentiviral gene delivery in free-floating neural cultures, spectrally distinguishable fluoroproteins driven by cell-type-specific promoters, Tet-ON technology, multiplex immuno-profiling and multichannel fluorocytometry. By this system, we overexpressed *Emx2* and *Foxg1* specifically in NSCs and/or NPs and assayed consequences of such manipulations on these precursors and their progenies. This allowed us to get a detailed insight into genetic control of cortical histogenesis, unmasking some previously unnoticed functions exerted by both genes. It further pointed to a possible exploitation of *Emx2*/*Foxg1* overexpression, for purposes of gene/cell therapy of brain diseases.

# 2. RESULTS

## 2.1 Developing molecular tools for dissecting Emx2 and Foxg1 regulatory functions in cortico-cerebral precursors.

In order to get an enduring overexpression of *Emx2* or *Foxg1* and a stable fluorescent labeling of neural precursors, we relied on lentiviral vectors. As already described (Capowski et al., 2007), in fact, this delivery system allowed us to stably trasduce almost the totality of cortical precursors (>97%, Fig. 24).

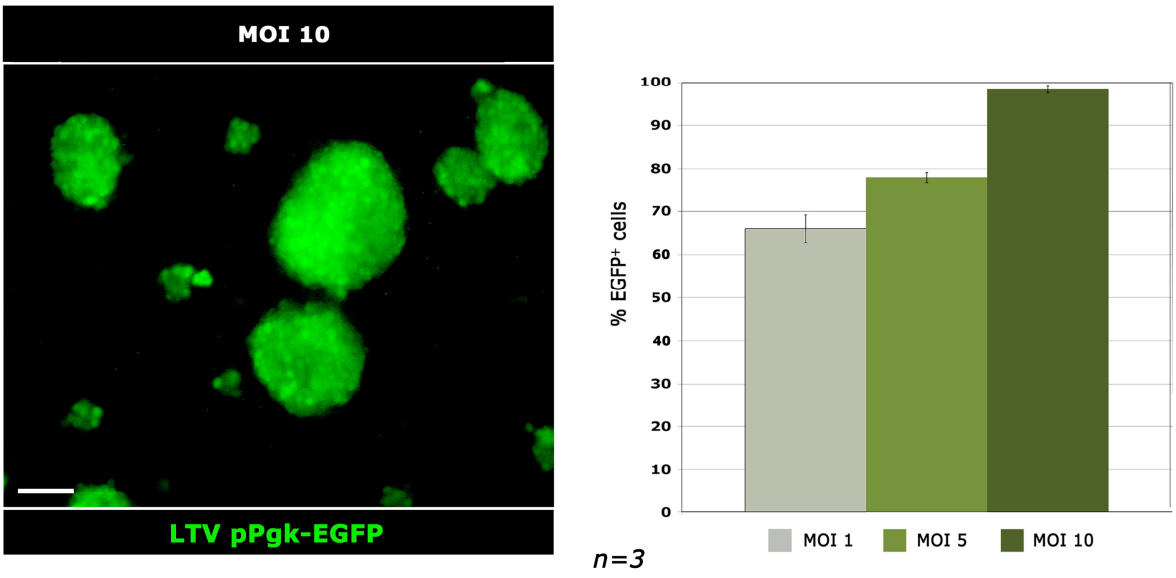



**Figure 24. LTV dose dependent infection of neural precursors**.

Correlation between Multiplicity of Infection (MOI) and infection rate, upon infection of dissociated E11.5 cortico-cerebral precursors by the constitutive EGFP expressor LTV pPgk-EGFP, as assessed 4 days upon infection. Scalebar, 50 µm.

To specifically label distinct neural compartments with different fluoroproteins, we selected four promoters, firing in selected neural types: pNes/hsvtk, pNes/hsp68, pTα1 and pSGC10/S2/2N. The first two share the neural enhancer from the II intron of the rat nestin gene, alternatively conjugated to the minimal promoters of the murine hsp68 heat shock gene (Yamaguchi et al., 2000) or the herpes virus simplex thymidine kinase gene (Lothian and Lendahl, 1997), and mainly fire in NSCs (Kawaguchi et al., 2001; Lothian and Lendahl, 1997; Sawamoto et al., 2001). pTα1, promoter of the rat α1 tubulin (Gloster et al., 1994), is

specifically active in neuron-restricted progenitors and in their young progenies (Gal et al., 2006; Mizutani et al., 2007; Sawamoto et al., 2001). pSCG10/S2/2N includes the 2kb rat stathmin promoter plus two neuronal restricted silencer elements (NRSEs) and specifically fires in post-mitotic neurons (Namikawa et al., 2006).

We tested the specificity of these promoters, by comparing their firing patterns with the expression domains of established markers of the neuronogenic lineage. We electroporated a pTα1-EGFP plasmid into the E14.5 murine cortex and scored the EGFP distribution 12 hours later. As expected (Gal et al., 2006), the fluoroprotein was mainly restricted to a subset of ventricular zone (VZ) precursors, colocalizing to some extent with the basal progenitor marker Tbr2 and the neuronal marker β-tubulin (Fig. 25A). Colocalization of pTα1-driven EGFP with Tbr2 and β-tubulin was quantified on E12.5 cortical precursors, transduced with LTVs and kept under anti-differentiative medium for 3.5 days: among $EGFP^+$ cells, about 4% coexpressed Tbr2, 30% β-tubulin, 10% both of them (Fig. 25B). Significant pTα1-driven mCherry/β-tubulin colocalization (about 17%) could still be found upon prolonged in vitro culture of cortical precursors, followed by 7 days differentiation under 5% serum (Fig. 25C). Finally, we scored E12.5 cortical precursors infected with LTV pSGC10/S2/2N-mCerulean and kept 3.5 days under anti-differentiative medium. Upon this treatment, 97% of $mCerulean^+$ cells expressed β-tubulin, whereas 80% of $β\text{-}tubulin^+$ cells were $mCerulean^+$, suggesting that pSGC10/S2/2N firing is limited to more mature post-mitotic neurons. Finally, we assessed the suitability of the four promoters, pNes/hsvtk, pNes/hsp68, pTα1 and pSGC10/S2/2N, for multichannel fluorescence profiling. We infected E11.5 cortical precursors with two lentiviral mixes, each driving the expression of three different fluorescent protein genes in pNes-, and pSGC10/S2/2N-firing domains, and profiled the resulting neurospheres after 7 days of anti-differentiative in vitro culture (Fig. 25E,F).

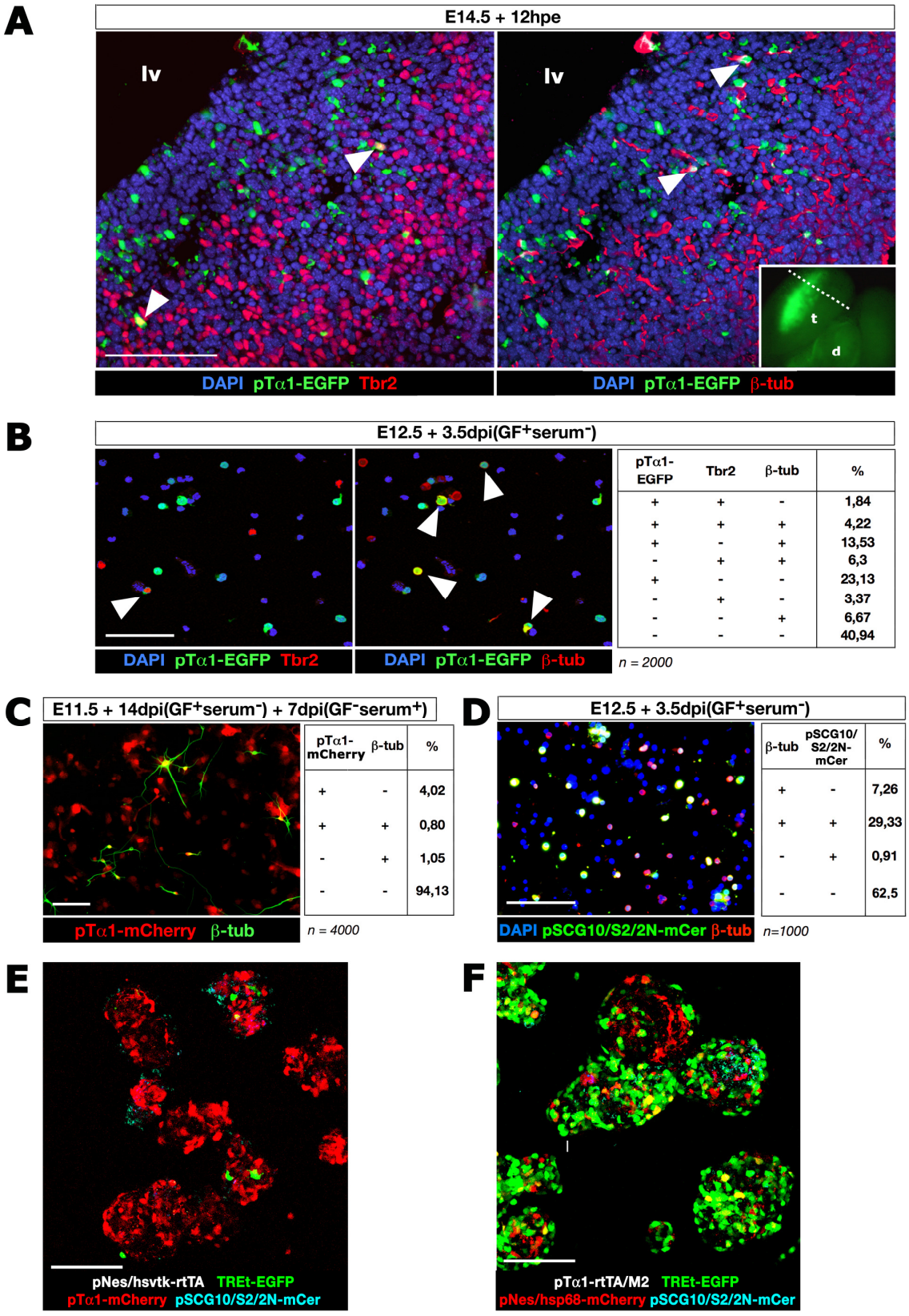



**Figure 25. Firing domains of the pTα1 and pSGC10/S2/2N promoters.**

**(A)** Comparison among pTα1-driven EGFP and endogenous neuronal lineage markers Tbr2 and β-tubulin, on frontal sections of E14.5 rostro-lateral cortex, 12 hours after electroporation of a pTα1-EGFP plasmid. **(B-D)** Comparison of pTα1-driven EGFP **(B)**, pTα1-driven mCherry **(C)** and

pSGC10/S2/2N-driven mCerulean **(D)** with Tbr2 and β-tubulin, in dissociated E11.5/12.5 cortical precursors, infected by LTVs pTα1-rtTA/M2 and TREt-luc-IRES2EGFP **(B)**, LTV pTα1-mCherry **(C)** and LTV pSGC10/S2/2N-mCerulean **(D)**, and cultured for different times under antidifferentiative and pro-differentiative conditions ($GF^+serum^-$ and $GF^-serum^+$, respectively). **(E,F)** Free-floating neurospheres generated by cortico-cerebral precursors from E11.5 pNes-rtTA-IRESbgeo$^{+/-}$ **(E)** and their wild type littermates **(F)**, infected with the LTV-sets "TREt-luc-IRES2EGFP; pTα1-mCherry; pSGC10/S2/2N-mCerulean" **(E)** and "pTα1-rtTA/M2; TREt-luc-IRES2EGFP; pNes/hsp68-mCherry; pSGC10/S2/2N-mCerulean" **(F)**, and kept 7 days in vitro under GFs. Arrowheads point to $EGFP^+Tbr2^+$ and $EGFP^+$b-tub$^+$ cells. Abbreviations: hpe, hours post electroporation; lv, lateral ventricle; t, telencephalon; d, diencephalon. Scalebars, 100 µm.

To achieve reversible overexpression, we selected the TetON technology (Gossen and Bujard, 1992; Gossen et al., 1995). We put the rtTA gene under the control of the state-specific promoter and the TF gene under the "Tetracycline-Response Element, tight" (TREt) (Fig. 26). As for rtTA expression in NSC cells, it was achieved by performing the experiments in neural precursors from pNes-rtTA-IRESbgeo$^{+/-}$ mutants (Mitsuhashi et al., 2001), harboring the rtTA cassette under the pNes/hsvtk promoter. A pTα1-rtTA/M2 expressing lentivector was conversely used to engineer the neuronal progenitor (NP) compartment. The TF-overexpressing compartment was generally labelled by an IRES2EGFP module, placed downstream of the TREt-TF cassette.

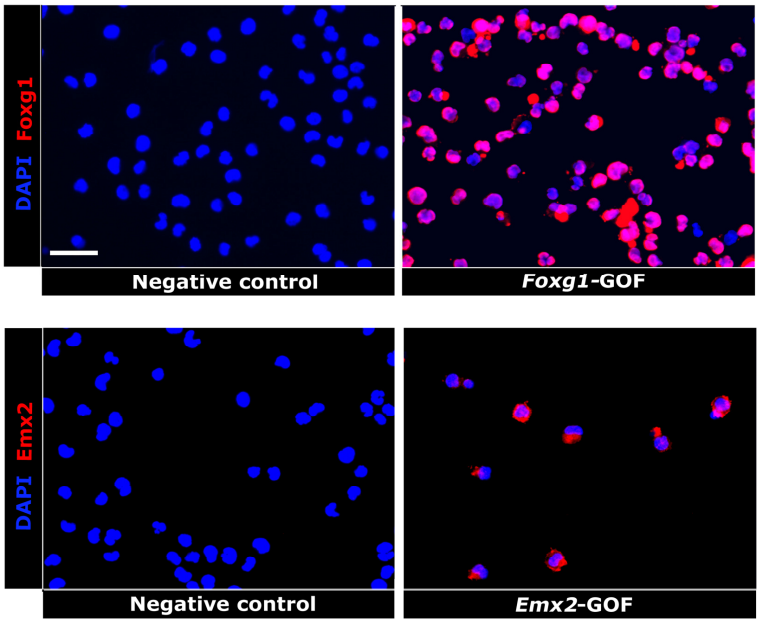
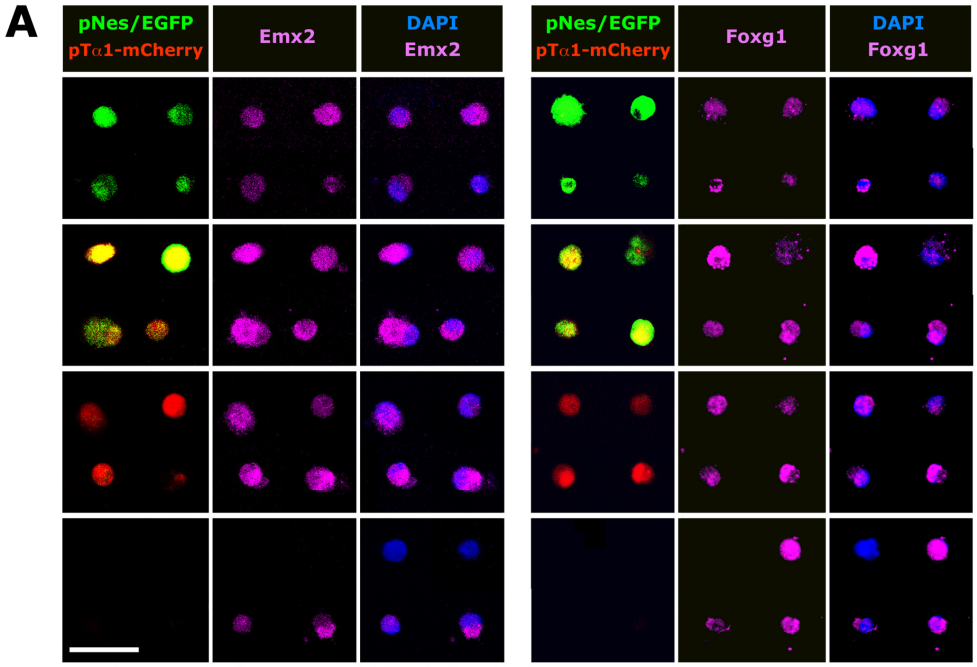



**Figure 26. Testing LTVs TREt-Emx2-IRES2EGFP and TREt-Foxg1-IRES2EGFP**

Immunofluorescence detection of the Foxg1 and Emx2 proteins in tTA-expressing Hela cells infected with LTVs TREt-Emx2-IRES2EGFP and TREt-Foxg1-IRES2EGFP at MOI = 15. Scalebar, 50µm

### *2.2 Endogenous Emx2 and Foxg1 expression in NSCs and NPs*

To investigate the *Emx2* and *Foxg1* expression patterns in NSCs and NPs, we performed immunofluorescence analysis of E12.5 cortical precursors, previously labelled with pNes-driven-EGFP and pTα1-driven-mCherry and kept 7 days in culture, under anti-differentiative conditions. *Emx2* and *Foxg1* were expressed in all of $pNes^+$ and the $pT\alpha1^+$ cells, displaying heterogeneous intracellular distributions. They were absent in a significant fraction of $pNes^-$ $pT\alpha1^-$cells, presumptively including glial lineage cells and mature neurons (Fig. 27A,B). The two proteins colocalized in a large fraction of cells, where they displayed different mutual distribution profiles. $Emx2^-Foxg1^+$ and $Emx2^-Foxg1^-$ cells were detected as well, but we could not find any $Emx2^+Foxg1^-$ elements (Fig. 27C). This specific expression of *Emx2* and *Foxg1* in NSC and NP points to a likely involvement of both genes in fine regulation of key aspects of their dynamic




**B**

| pNes/EGFP | pTa1/mCherry | Emx2+ % | Emx2+ pattern* | Foxg1+ % | Foxg1+ pattern* |
|---|---|---|---|---|---|
| + | - | 100 | U,C | 100 | U,N,C |
| + | + | 100 | U,C | 100 | U,N,C |
| - | + | 100 | U,C | 100 | U,N,C |
| - | - | 47 | U,C | 55 | U,N,C |

* U, ubiquitous; C, prevalently cytoplasmic/perinuclear; N, prevalently nuclear

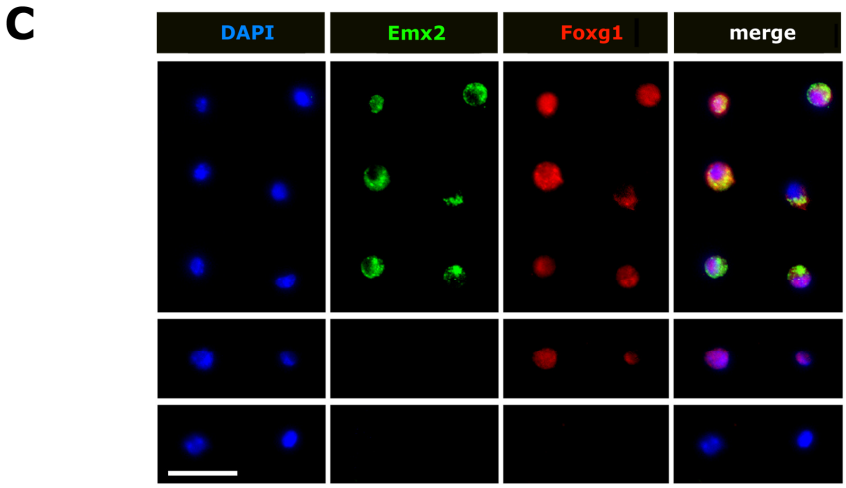



**Figure 27. Expression of endogenous Emx2 and Foxg1 proteins in embryonic cortico-cerebral precursors.**

**(A,B)** Immunofluorescence detection of Emx2 and Foxg1 in E12.5 cortical precursors, harboring the EGFP and mCherry genes under the control of the pNes/hsvtk and  promoters, respectively, and kept in vitro for 7 days under anti-differentiative conditions. **(C)** Emx2 and Foxg1 colocalization in E12.5 wild type cortical precursors, kept in vitro for 7 days under anti-differentiative conditions. Scalebars, 25 µm.

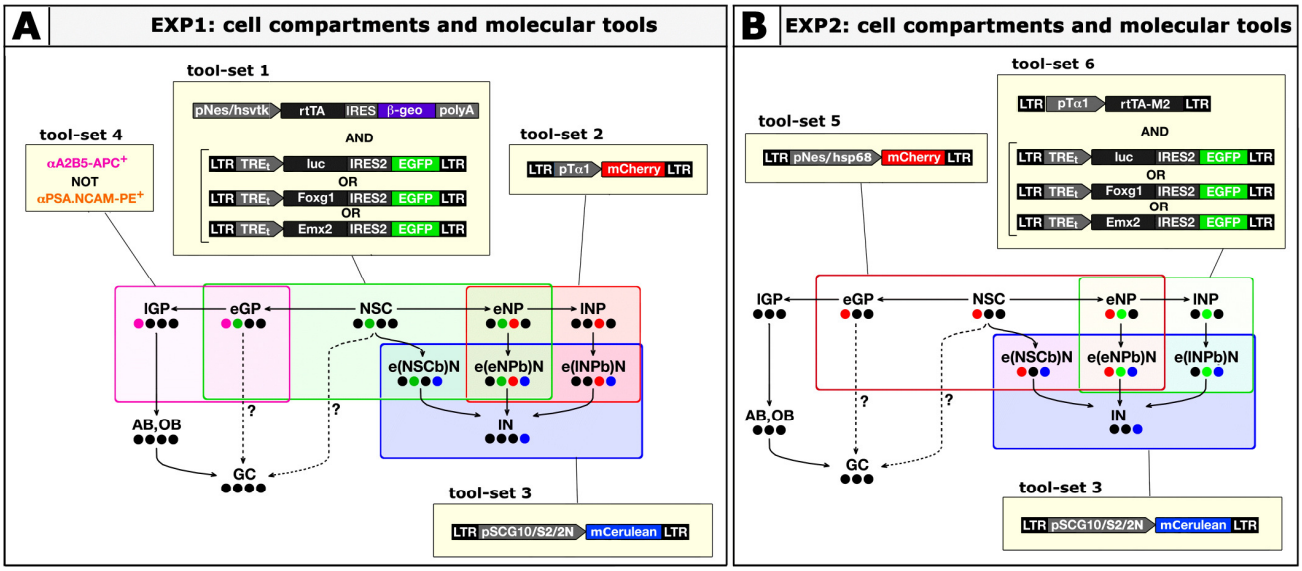
### *2.3 Assessing the roles of Emx2 and Foxg1 in the NSC and NP compartments: the experimental design*

To investigate consequences of gene manipulations in NSCs, we developed two complementary experimental designs: EXP1a, to monitor the neuronogenic lineage (Fig. 28A,C); EXP1b, to monitor the glial lineage (Fig. 28A,D). [Selection/validation of cell-type specific promoters used in this study, set-up of protocols for lentivector-mediated transgene delivery, doxycycline-controlled TF overexpression and immunoprofiling of endogenous Emx2 and Foxg1 in cortical precursors are reported in SUPPLEMENTARY RESULTS].

In EXP1a, we infected E11.5 cortical neural precursors from pNes-rtTA-IRESbgeo$^{+/-}$ mutants with a common mix containing LTVs pTα1-mCherry and pSGC10/S2/2N-mCerulean plus, alternatively LTV TREt-*Emx2*-IRES2EGFP or TREt-*Foxg1*-IRES2EGFP. LTV TREt-luc-IRES2EGFP, encoding for luciferase (*luc*), was used as a control. Under these conditions, the TF in order is mainly overexpressed in NSCs, but also - to a minor extent - in their immediate direct progenies. Similarly, the fluoroproteins EGFP and mCherry label NSCs and NPs, respectively, but also their early descendants, which may be so distinguished from more mature elements thanks to their peculiar fluorescence patterns. In such a way, several compartments of the neuronogenic lineage, downstream of NSCs and up to late neurons (lNs), may be distinguished and followed up in living cultures (Fig. 28A,C). As for the glial lineage, it has been previously reported that a large fraction of glial progenies obtainable *in vitro* from embryonic cortico-cerebral precursors derives from bipotent glial progenitors (GPs), generating astrocytes and oligodendrocytes but not neurons. This population is distinguishable thanks to its A2B5$^{+}$PSANCAM$^{-}$ immunological profile (Strathmann et al., 2007). In EXP1b, we overexpressed again TREt-*Emx2*-IRES2EGFP or TREt-*Foxg1*-IRES2EGFP within NSCs and used antibodies conjugated with different fluorophores to label the bipotent GP population. So, similarly to EXP1a, we were able to follow early transitions from NSCs, through GPs, up to more mature glial elements (Fig. 28A,D).

In EXP2, we investigated the role played by *Emx2* and *Foxg1* along the neuronogenic lineage, by overexpressing them in NPs and early neurons (eNs) of the pTα1$^{+}$ compartment (Fig. 28B,E). We infected E11.5 cortical neural precursors from wild type mice with a common mix containing the LTVs pTα1-rtTA/M2, pNes/hsp68-mCherry, pSGC10/S2/2N-mCerulean and, alternatively, TREt-*Emx2*-IRES2EGFP or TREt-*Foxg1*-IRES2EGFP. LTV TREt-luc-

IRES2EGFP was used as a control. Under these conditions, the TFs and EGFP are overexpressed in NPs and their early neuronal progenies, mCherry labels NSCs and their immediate derivatives, including eNPs, mCerulean labels more mature neurons. In this way, we can distinguish early(NSC-born)-NPs from late NPs, as well as early NP-born neurons from more mature ones.




**C** **EXP1a (toolsets 1, 2, 3)**

| fluorescent profile: pNes/hsvtk-Egfp | pTa1-mCherry | pSCG10/S2/2N-mCerulean | cell type |
|---|---|---|---|
| + | - | - | NSC, eGP |
| + | + | - | eNP |
| - | + | - | INP |
| - | - | - | IGP, AP, OP, GC |
| + | - | + | e(NSCb)N |
| + | + | + | e(eNPb)N |
| - | + | + | e(INPb)N |
| - | - | + | IN |

**D** **EXP1b (toolsets 1, 4)**

| fluorescent profile | cell type |
|---|---|
| pNes-Egfp(+) NOT [αA2B5-APC(+) AND αPSA.NCAM-PE(-)] | NSC, eNP, e(NSCb)N, e(eNPb)N |
| pNes-Egfp(+) AND [αA2B5-APC(+) AND αPSA.NCAM-PE(-)] | eGP |
| pNes-Egfp(-) AND [αA2B5-APC(+) AND αPSA.NCAM-PE(-)] | IGP |
| pNes-Egfp(-) NOT [αA2B5-APC(+) AND αPSA.NCAM-PE(-)] | AP, OP, GC, INP, e(INPb)N, IN |

**E** **EXP2 (toolsets 3, 5, 6)**

| fluorescent profile: pNes/hsp68-mCherry | pTa1-Egfp | pSCG10/S2/2N--mCerulean | cell type |
|---|---|---|---|
| + | - | - | NSC, eGP |
| + | + | - | eNP |
| - | + | - | INP |
| - | - | - | IGP, AP, OP, GC (G) |
| + | - | + | e(NSCb)N |
| + | + | + | e(eNPb)N |
| - | + | + | e(INPb)N |
| - | - | + | IN |

**NSC**, *neural stem cell*
**eNP**, *early neuron-restricted progenitor*
**INP**, *late neuron-restricted progenitor*
**e(NSCb)N**, *early, NSC-born, neuron*
**e(eNPb)N**, *early, eNP-born, neuron*
**e(INPb)N**, *early, INP-born, neuron*
**IN**, *late neuron*
**eGP**, *early glia-restricted progenitor*
**IGP**, *late glia-restricted progenitor*
**AP**, *astrocyte progenitor*
**OP**, *oligodendrocyte progenitor*
**GC**, *glial cells*

**Figure 28. Monitoring early consequences of Emx2 or Foxg1 overexpression in neural stem cells (NSCs) or neural precursors (NPs): the experimental designs EXP1 and EXP2.**

In **(A,B)** molecular tools used in the different designs are grouped in six sets (1,2,3,5,6, including lentiviral vectors, and 4, including fluorolabelled antibodies), to be employed in different combinations. The overlapping firing domains of distinct fluorescent markers are represented by

colored frames and the fluorescent profile of each neural cell type is symbolized by a four circles code. Fluorescent patterns of different cell types are summarized in **(C-E).**

### *2.4 Kinetics of precursor-compartment sizes upon Emx2 or Foxg1 overexpression in NSCs.*

Neural cultures set up according to the EXP1a design were kept as floating neurosphere cultures under anti-differentiative conditions (Rietze and Reynolds, 2006) and were FACS-profiled each week, starting from 7 days post infection (dpi) up to 28 dpi. Based on state-specific promoter-driven fluorescence, eight compartments (corresponding to defined cell-types or cell-type sets, Fig. 28A,C), were distinguished and their relative sizes evaluated each week (Fig. 29A). In control cultures transduced with *luc*, NSC frequency fell below 6%, i.e. near the value estimated in embryonic telencephalic cultures by neural colony-forming cell assay (NCFCA) (Louis et al., 2008). In these cultures, cells belonging to the neuronogenic lineage became progressively less frequent: NPs declined from 22% to 8%, neurons (Ns) from less than 5% down to 1%. Conversely the frequency of non-fluorescent cells, presumptively belonging to the glial lineage, increased from 7 dpi onwards, from 68% to 90%. Overexpression of *Foxg1* or *Emx2* led to an expansion of the $pNes^{+}pT\alpha1^{-}$ $pSCG10/S2/2N^{-}$ compartment (corresponding to NSCs and early glial-progenitors (eGP)), more pronounced for *Foxg1*, as well of the NP compartments, both early and late (eNP, lNP).

In EXP1b, neural cultures were set up and followed similar to EXP1a. In this case, based on pNes-driven EGFP fluorescence and A2B5-PE and PSA-NCAM-APC immunofluorescence, four compartments (corresponding to defined cell-types or cell-type sets, Fig. 28A,D), were distinguished and their sizes evaluated (Fig. 29B). In control cultures transduced with *luc*, frequency of $pNes^{+}NOT(A2B5^{+}PSA/NCAM^{-})$ elements, corresponding to NSCs and their immediate neuronal-lineage progenies, decreased from 7 through 28 dpi. The early GP (eGPs) compartment size was very small and relatively stable over time. The frequency of late GPs (lGPs) dramatically increased (from 10 up to almost 70%). Overexpression of *Foxg1* or *Emx2* led to an expansion of the $pNes^{+}NOT(A2B5^{+}PSA/NCAM^{-})$ compartment (NSCs and their early neuronal-lineage descendants), progressively increasing for *Foxg1*, progressively diminishing for *Emx2*. Moreover, under Foxg1, the eGP compartment was more and more enlarged, the lGP shrunken; under Emx2, the eGP compartment was moderately enlarged.

Remarkably, the size of the whole pNes$^{+}$ compartment (including NSCs and their immediate progenies), while not changing between EXP1a and EXP1b *luc*-GOF cultures ($p<0.91$), resulted much larger in *Foxg1-GOF* ($p<3.4*10^{-9}$) and *Emx2*-GOF cultures ($p<6.5*10^{-4}$) set up according to the EXP1b design (Fig. 29C). As the only technical difference between 1a and 1b designs was the higher total MOI used in the former (35 vs 10), this suggests that the exacerbated toxicity originating from higher lentiviral loads may partially mask the effects of *Emx2* or *Foxg1* overexpression. This suggests that the beneficial effects of *Foxg1* overexpression on the size of the pNes$^{+}$ compartment could have been even more pronounced, by delivering the transgene by less genotoxic technologies.

As both EXP1a and EXP1b did not provide a direct estimation of the NSC compartment size, we calculated its presumptive value, assuming that the partition of pNes$^{+}$ elements among stem, neuronal lineage and glial lineage elements did not change between the two experimental designs (Fig. 29D). The effects elicited by *luc*, *Emx2* and *Foxg1* on the NSC compartment resulted qualitatively consistent between the two designs. In control cultures, NSCs decreased from 4% to lower values. *Foxg1* overexpression led to a progressive enlargement of the NSC compartment ($p<0.001$). *Emx2* overexpression elicited a similar effect at 7 dpi, while inducing an opposite effect at later stages ($p<0.03$, EXP1a, 14-28 dpi). However, the size of the NSC-only compartment upon the same *Emx2/Foxg1* gene manipulation varied between EXP1a and EXP1b, similar to what described for total pNes$^{+}$ elements. So the above-mentioned considerations about the detrimental effects of high lentiviral loads, also presumably apply to NSCs.

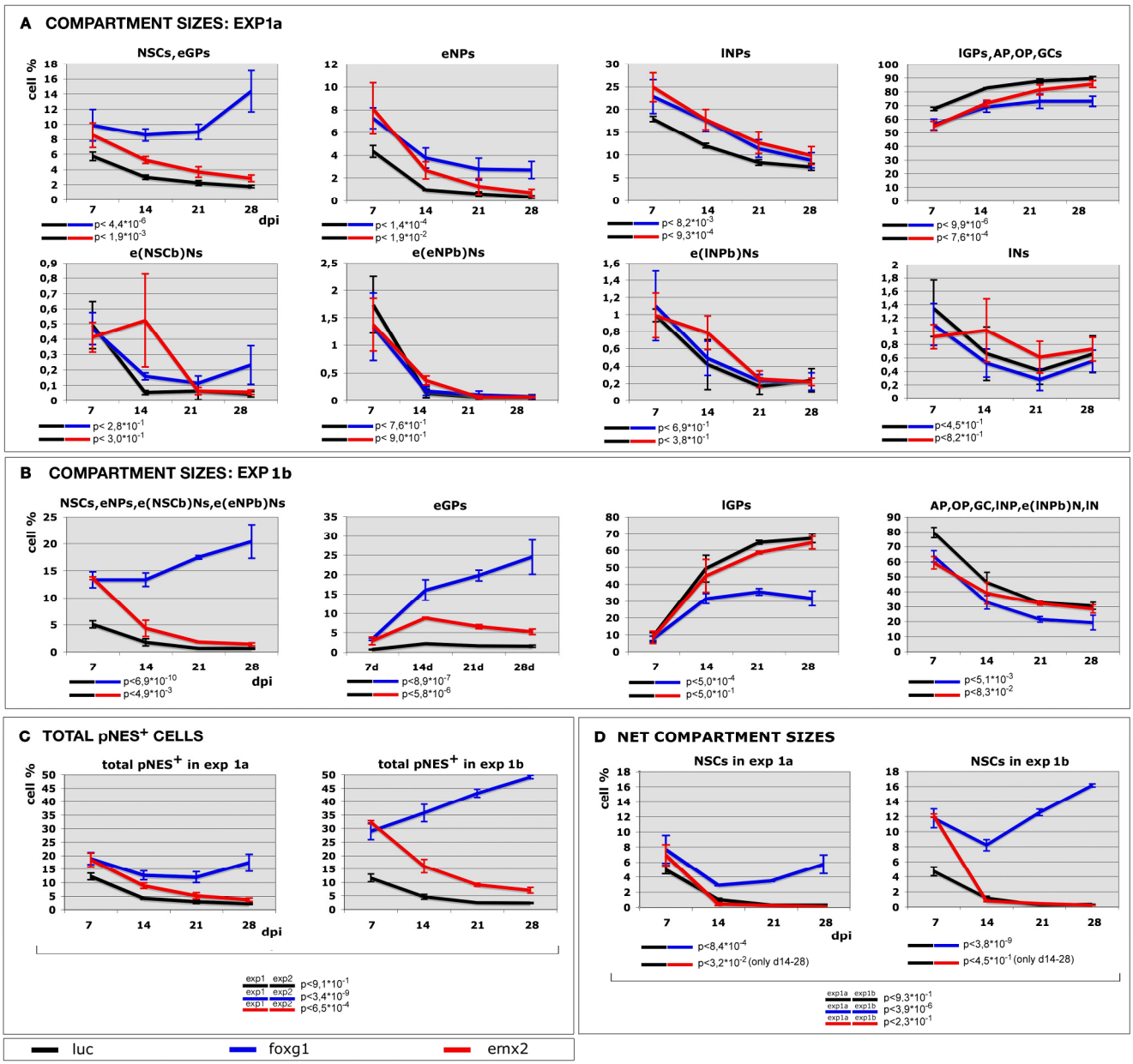



**Figure 29. Kinetics of absolute neural cell type compartment sizes, upon Emx2, Foxg1 or control gene (luc) overexpression in NSCs.**

Kinetics of neuronogenic **(A)** and gliogenic **(B)** lineage compartment sizes upon delivery of the experimental designs EXP1a and EXP1b, respectively. **(C,D)** Time course of the total pNes/hsvtk/EGFP$^{+}$, the net NSC- and the cumulative glial compartment sizes, upon delivery of the experimental designs EXP1a and EXP1b. In **(A-D)**, EXP1a and 1b designs were delivered to E11.5 pNes-rtTA-IRESbgeo$^{+/-}$ cortico-cerebral precursors. Acronym meanings are as in Fig. 28; dpi, days post infection. Bars represent s.e.m.'s. Statistical significance of differences was evaluated by one-way ANCOVA.

### *2.5 Dynamics of precursor-compartment sizes upon Emx2 or Foxg1 overexpression in NSCs.*

Altered sizing of engineered neural compartments described above can result from distinct kinetic mechanisms, including altered frequency of transition from the NSC-

compartment to the downstream ones, altered NSC proliferation rate, altered NSC death rate. 7AAD staining of engineered NSCs showed that death rates of these cells were unaffected (Fig. 30D). On the other hand, it has been previously shown that proliferation rates are not responsive to altered *Emx2* gene dosage, starting from E15 (Mallamaci et al., 2000; Muzio et al., 2005) (A. Mallamaci, unpublished data), as well as to up-regulation of *Foxg1* (Ahlgren et al., 2003). So the kinetic profiles described above are likely to originate from changes of NSC differentiation and expansion rates.

To get an index of the rates at which elements belonging to the perturbed compartment X transit to adjacent downstream compartments Yi's (hereafter referred to as "X-to-Yi transition indexes"), we calculated, for each gene manipulation and each time point, the Yi/X size ratio. Indexes of *Emx2*-GOF and *Foxg1*-GOF cultures were normalized against *luc*-transduced cultures and all data were plotted against time (Fig. 30A,B). As for the neuronal lineage, the NSC-to-early(NSC-born)neuron [NSC-to-e(NSCb)N] transition index was slightly down-regulated by *Foxg1* ($p<0.05$) and strongly upregulated by *Emx2*, specifically from 14 dpi onward ($p<0.01$). A similar pattern was displayed by the NSC-to-eNP index, poorly affected by *Foxg1* but still upregulated by *Emx2*, from 14 dpi onward ($p<0.002$). (Fig. 30A). As for the glial lineage, *Foxg1* slowed down the NSC-to-eGP transition at 21-28 dpi, a sustained promotion of such transition was conversely elicited by *Emx2*, at every time point ($p<1.2*10^{-4}$). Instead, both genes dramatically antagonized the subsequent eGP-to-lGP progression ($p<4.3*10^{-25}$ for *Foxg1* overexpression, $p<8.8*10^{-25}$ for *Emx2*), so prefiguring a possible blockade of gliogenesis upon these manipulations.

As for the NSC expansion rate, we found that it was increased by *Foxg1* ($p<2.4*10^{-2}$), not affected by *Emx2* (Fig. 30F).

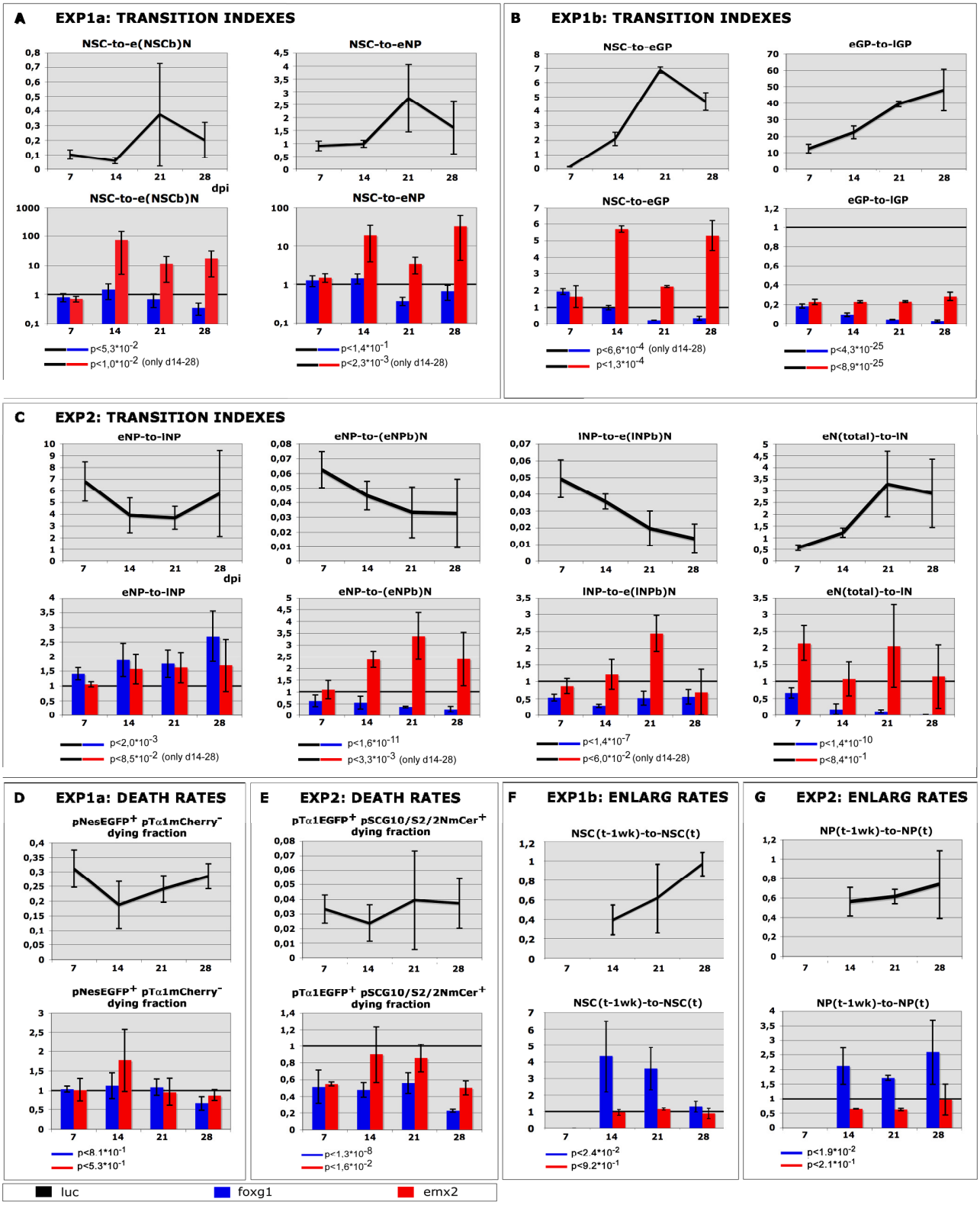



**Figure 30. Dynamics of neural cell type compartment sizes, upon Emx2, Foxg1 or control gene (luc) overexpression in NSCs or NPs.**

**(A-C)** Time course of compartment-to-compartment transition indexes, calculated by dividing the size of the downstream compartment by the size of the upstream one. **(D,E)** Time course of the pNes/hsvtk/EGFP$^{+}$pTα1/mCherry$^{-}$ and the pTα1/EGFP+ pSCG10/S2/2N/mCerulean+ compartment death rates, as evaluated by 7AAD staining. **(F,G)** Time course of the NSC and the NP compartment

enlargement rates, as evaluated by dividing the size of the compartment at time = t by the size of the same one week before. Indexes shown in **(A-C)** and **(F,G)** were calculated based on primary data reported in Figures 2 and 3. Absolute indexes are provided for the control gene *luc*, relative indexes, i.e. indexes normalized on *luc*-specific values, for both *Emx2* and *Foxg1*. Acronym meanings are as in Fig. 28; dpi, days post infection. Bars represent s.e.m.'s. Statistical significance of differences was evaluated by one-way ANCOVA.

### *2.6 Kinetics and dynamics of precursor-compartment sizes upon Emx2 or Foxg1 overexpression in NSCs.*

Neural cultures transduced according to the EXP2 design were grown and followed as in EXPs 1a,b. Based on state-specific promoter-driven fluorescence, eight compartments (corresponding to defined cell-types or cell-type sets, Fig. 28B,E), were distinguished and their sizes evaluated (Fig. 31). Frequencies of NPs, both eNPs an lNPs, were up-regulated under *Foxg1* ($p<0.02$ and $p<0.0001$, respectively) and down-regulated under *Emx2* ($p<0.03$ and $p<0.006$, respectively). Changes of their immediate neuronal progenies were not statistically relevant. As for more mature neurons, their frequency was more than halved upon *Foxg1* overexpression ($p<3.8*10^{-11}$), poorly affected by *Emx2* overexpression. Relative abundances of other elements not originating from engineered NPs were reduced following *Foxg1*-GOF, possibly as a consequence of NP compartment oversizing.

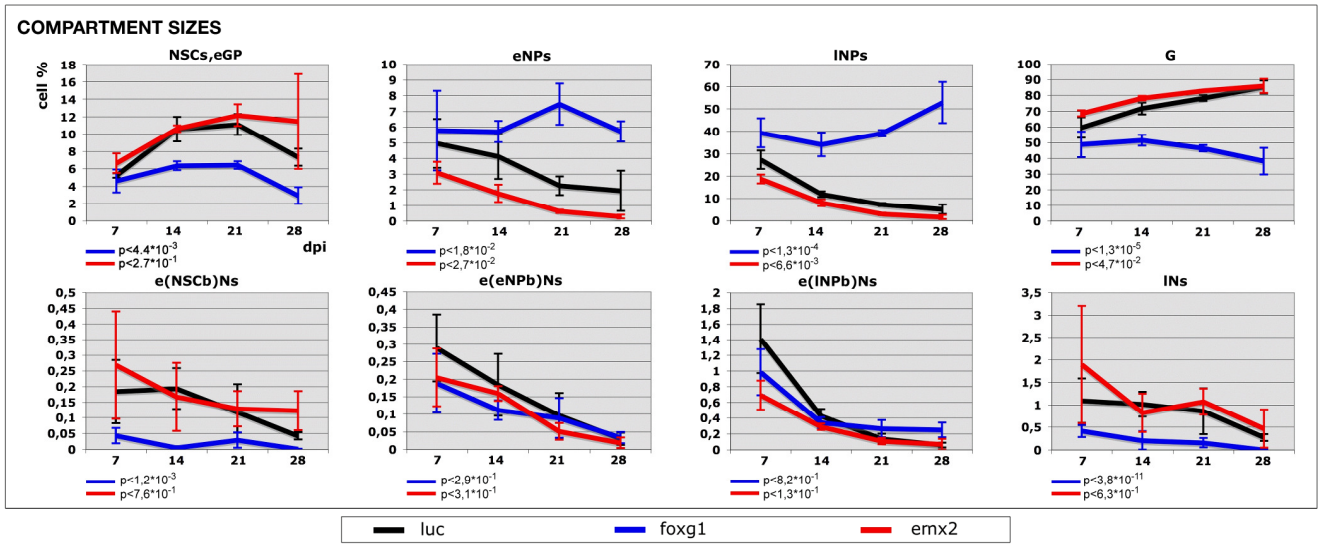



**Figure 31. Kinetics of absolute neuronogenic lineage compartment sizes, upon Emx2, Foxg1 or control gene (luc) overexpression in NPs.**

Compartment sizes were measured according to the experimental design EXP2 on cultures of E11.5 wild type cortico-cerebral precursors. Acronym meanings are as in Fig. 28; dpi, days post infection. Bars represent s.e.m.'s. Statistical significance of differences was evaluated by one-way ANCOVA.

The eNP-to-lNP transition index was augmented in *Foxg1*-GOF cultures all over the culturing time ($p<0.002$); *Emx2* exerted a similar effect, however without reaching statistical significance ($p<0.07$). *Foxg1* counteracted the eNP-to-e(eNPb)N and lNP-to-e(lNPb)N transitions ($p<1.6 *10^{-11}$ and $p<1.3*10^{-7}$, respectively); *Emx2* pushed eNPs to differentiate ($p<3.3*10^{-3}$, at 14-28 dpi). Finally, *Foxg1* inhibited the eN-to-lNs transition (p<1.4*10-10) (Fig. 30C).

Interestingly, pTα1-driven overexpression of both TFs increased the viability of NPs, as assessed by 7AAD exclusion assay (Fig. 30E). This effect was particularly strong and consistent for *Foxg1,* which halved the fraction of dead cells at each time point ($p<1.3*10^{-8}$), less pronounced for *Emx2* ($p<0.02$). Such protective effects may lower the apparent NPs-to-Ns transition indexes (Fig. 30E), so leading to underestimation of pro-differentiative *Emx2* abilities and overestimation of anti-differentiative *Foxg1* properties.

Generally, *Foxg1* promoted a progressive enlargement of the NP compartment ($p<1.9*10^{-2}$), *Emx2* possibly contributed to its shrinkage (Fig. 30G).

### *2.7 Neuronal and glial outputs from neural cultures overexpressing Emx2 or Foxg1 in the NSC or the NP compartment*

To assay the histogenetic output of the NSC manipulations described above, we transferred E11.5 pNes-rtTA-IRESbgeo$^{+/-}$ cortical precursors, transduced with EXP1 toolset 1,2 lentivectors (Fig. 28A) and kept for 14 dpi under doxycycline/GFs, to a differentiation-permissive, doxycycline/GFs-free medium, containing 5% serum; 7 days later, we immunoprofiled the resulting cultures for lineage-specific markers. Overexpression of *Emx2* or *Foxg1* almost halved the fraction of S100$^{+}$ mature astrocytes ($p<0.03$ for *Foxg1*, $p<0.04$ for *Emx2*) and reduced CNPase$^{+}$ oligodendrocytes by at least one third ($p<0.004$ for *Foxg1*, $p<0.03$ for *Emx2*), while not affecting neuron frequency (Fig. 32A and Fig. S1A). When the duration of growth under doxycycline/GFs was doubled, the reduction of glial frequencies

was less pronounced; however, in such case, neuron frequency was upregulated by about one half, specifically upon *Foxg1* overexpression (Fig. 32B and Fig. S1B).

We also quantified the histogenetic output of NP manipulations, by immunoprofiling E11.5 wild type cortical precursors, transduced with EXP2 lentivectors (Fig. 28A) and grown *in vitro* according to the "GFs-doxycycline/7days; serum/14 days" schedule. Neuron frequency was more than triplicated upon *Foxg1* overexpression ($p<0.03$), increased by almost 40% under *Emx2* ($p<0.04$) (Fig. 32C and Fig. S2A). Similar effects were still observed, when the length of the not-differentiative phase of the test was doubled (Fig. S2B). Unexpectedly, upon 14+7 days culture, *Foxg1* also induced a prominent increase of neurite outgrowth. As quantified by NeuriteTracer software, the mean neurite length resulted 61.9±6.3 µm and 84.4±4.9 µm, for control and *Foxg1*-treated cultures, respectively ($p<0.01$) (Fig. 32D). Remarkably, the relative amplitude of this phenomenon became even more prominent (+36.6% vs +57.0%) when the length of the differentiation-permissive phase of the test was doubled (Fig. 32E).

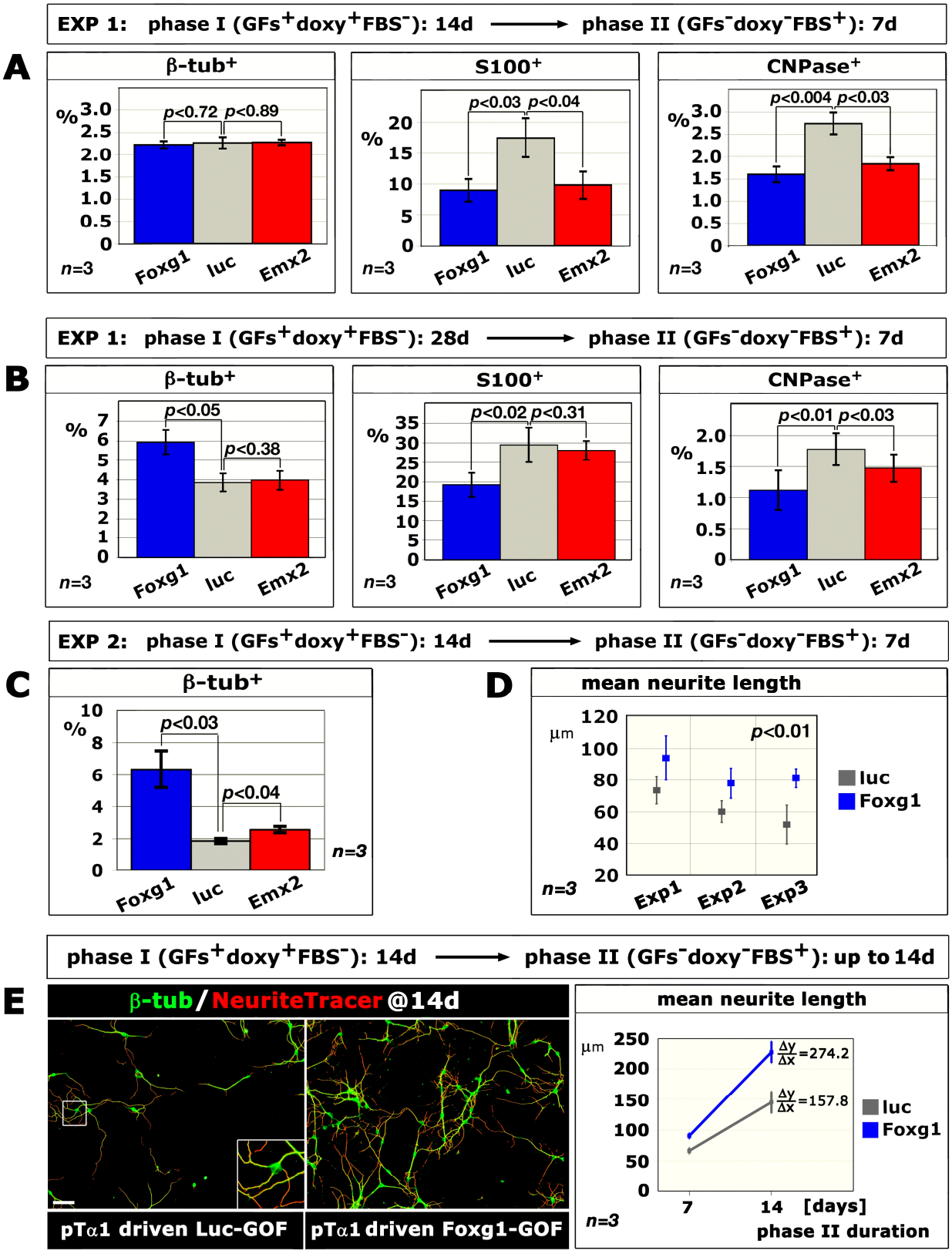



**Figure 32. Quantifying late histogenetic consequences of Emx2 or Foxg1 overexpression in NSCs or NPs, following the experimental designs EXP1 and 2.**

(A,B) Expression of the neuronal (β-tubulin), astrocytic (S100) and oligodendrocytic (CNPase) markers, upon delivery of molecular tool-sets 1 and 2 (Figure 1) to dissociated E11.5 pNes-rtTA-IRESbgeo$^{+/-}$ cortico-cerebral precursors. (C) Expression of the neuronal marker β-tubulin, upon

delivery of the molecular tool-sets 3, 5 and 6 (Figure 1) to dissociated E11.5 wild type cortico-cerebral precursors. Infected cells were kept for 14 (A,C) or 28 (B) days under anti-differentiative conditions and in the presence of the transgene inducer doxycycline (phase I: $GFs^{+}doxy^{+}FBS^{-}$). They were subsequently transferred to pro-differentiative conditions, where they were kept for 7 more days, in the absence of doxycycline (phase II: $GFs^{-}doxy^{-}FBS^{+}$), and finally profiled. (D) Mean neurite length of luc-GOF and Foxg1-GOF neurons quantified in (C), evaluated in three independent experiments by β-tubulin immunofluorescence and NeuriteTracer analysis. (E) Mean neurite length of neuronal progenies originating from E12.5 wild type cortico-cerebral precursors, overexpressing luc or Foxg1 under the control of the pTα1 promoter. LTV-transduced cells were grown according to the (A,C) schedule, except phase II, prolonged up to 14 days. Evaluation of neurite length was as in (D). Bars represent s.e.m.'s. Statistical significance of differences was evaluated by one-way ANOVA. Scalebar, 50 µm.

### *2.8 Enhancing the neuronal output of engineered neural cultures by combined gene manipulations.*

Some of the histogenetic activities displayed by *Emx2* and *Foxg1* (Fig. 33) are of paramount interest, as their artificial exploitation might be useful to ameliorate the neuronal output obtainable from neural precursor cultures, for purposes of cell-based brain repair. We sought to further enhance such output, by conveniently combining *Emx2* and *Foxg1* GOF manipulations. Two designs were tested: (1) *Foxg1* overexpression in both NSCs and NPs (EXP3); (2) chronic *Foxg1* overexpresson in NPs, followed by terminal overactivation of *Emx2* in the same progenitors (EXP4).

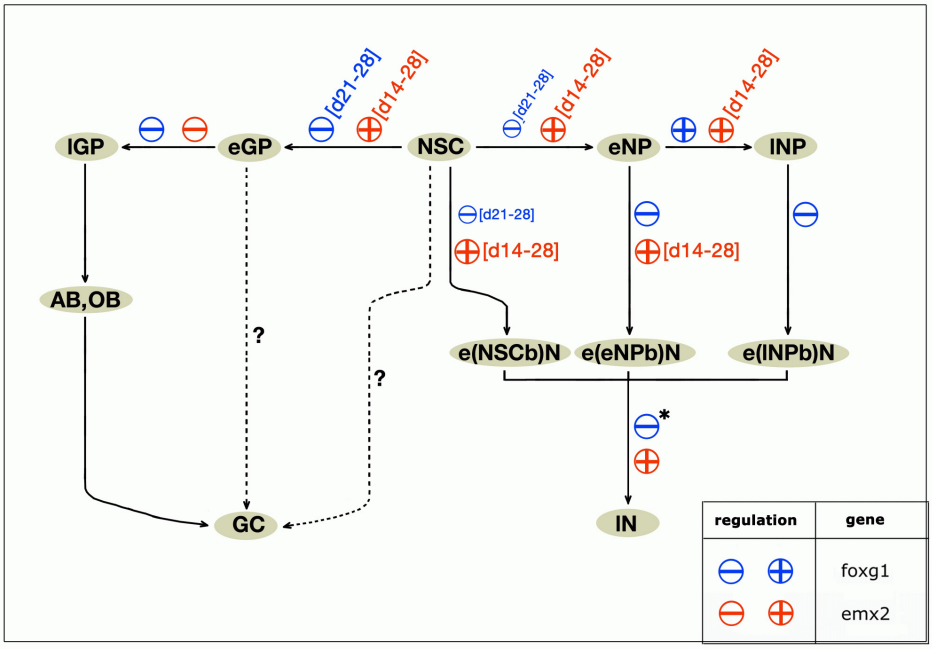



**Figure 33. Synopsis of Emx2 and Foxg1 effects on neural precursors dynamics.**

Synthesis of the main effects exerted by Emx2 and Foxg1 on neural cultures of E11.5 cortico-cerebral precursors, under anti-differentiative conditions. Acronym meanings are as in Fig. 28B,C and 7B. "d14-28" and "d21-28" refer to time intervals (days post infection 14-28 and 21-28), during which

such activities have been assessed. The asterisk recalls the capability of Foxg1 to stimulate neuronal morphological maturation, upon its previous expression in NPs and/or early neurons.

As for EXP3 rationale, we already showed that *Foxg1* overexpression in NSCs for 28 days leads a 1.6-fold increase of NPs (Fig. 29A), associated to a 1.5-fold increase of the neuronal output (Fig. 32B) plus a 2-fold decrease of lGPs (Fig. 29B). Moreover, when overexpressed in NPs for the same time, *Foxg1* increases their frequency by 7.2 (Fig. 31), resulting in a 1.7-fold raise of their neuronal output (Fig. S2B and data not shown). So, we reasoned that combined upregulation of *Foxg1* in both NSCs and NPs should lead to a multiplicative neuronogenic effect, in front of reduced gliogenesis. Therefore, we overexpressed *Foxg1* in both stem cells and neuronal progenitors originating from E12.5 cortexes, for 28 days, keeping them under GFs/doxycycline. Then, we washed out doxycycline and transferred cells under 5% serum for additional 14 days. The neuronal output obtained by this treatment was 20% higher, as compared to that obtained overexpressing *Foxg1* in pTα1$^{+}$ elements only ($p<0.05$, N=3). This effect, despite of its moderate amplitude, is noteworthy. In fact, compared with pTα1-driven *luc*, pNes/pTα1-driven *luc* expression leads to a 20% *reduction* of the neuronal output ($p<0.05$, N=3) (Fig. 34A and Fig. S3A), suggesting that our lentiviral overexpression system is *per se detrimental* to the neuronal outcome. Remarkably, if the pNes/pTα1-*Foxg1*-GOF neuronal output is compared with the pNes/pTα1-*luc*-GOF one, the increase is about 2.4-fold (Fig. 34A), near the product of the neuronogenesis gains elicited by single pNes-*Foxg1* (Fig. 32B) and pTα1-*Foxg1*-GOF treatments (Fig. S2B and data not shown). Moreover such pNes/pTα1-driven *Foxg1* overexpression did not impair the enriched neurite morphology peculiar to pTα1-driven *Foxg1*-GOF cultures (Fig. S3A). Finally, when the EXP3 was repeated by halving the duration of the anti-differentiative phase of the culture, then similar results were obtained (Fig. S4).

As for EXP4, our design was inducing a preliminary expansion of the NP compartment, by pTα1-driven *Foxg1* upregulation, and then forcing them to differentiate *en masse* as neurons, by subsequent pTα1-driven *Emx2* overexpression. Therefore, we transduced E12.5 cortical precursors with LTVs pTα1-rtTA/M2 and TREt-*Foxg1* and kept them under anti-differentiative medium in the presence of GFs/doxycycline for 21 days. We further infected these precursors with LTV TREt-*Emx2* (or its control LTV TREt-*luc*), keeping

them under the same medium for 4 more days. Finally, we washed out doxyxcycline and transferred infected cells under 5% serum. Interestingly, an even brief *Emx2* overexpression strongly impaired proliferation of *Foxg1*-GOF neural precursors (Fig. S5). Moreover, the neuronal output of *Foxg1*/*Emx2*-GOF precursors, assessed upon exposition to serum for 7 days, was increased by about one third as compared to *Foxg1/luc*-GOF precursors (Fig. 34B and Fig. S3B).

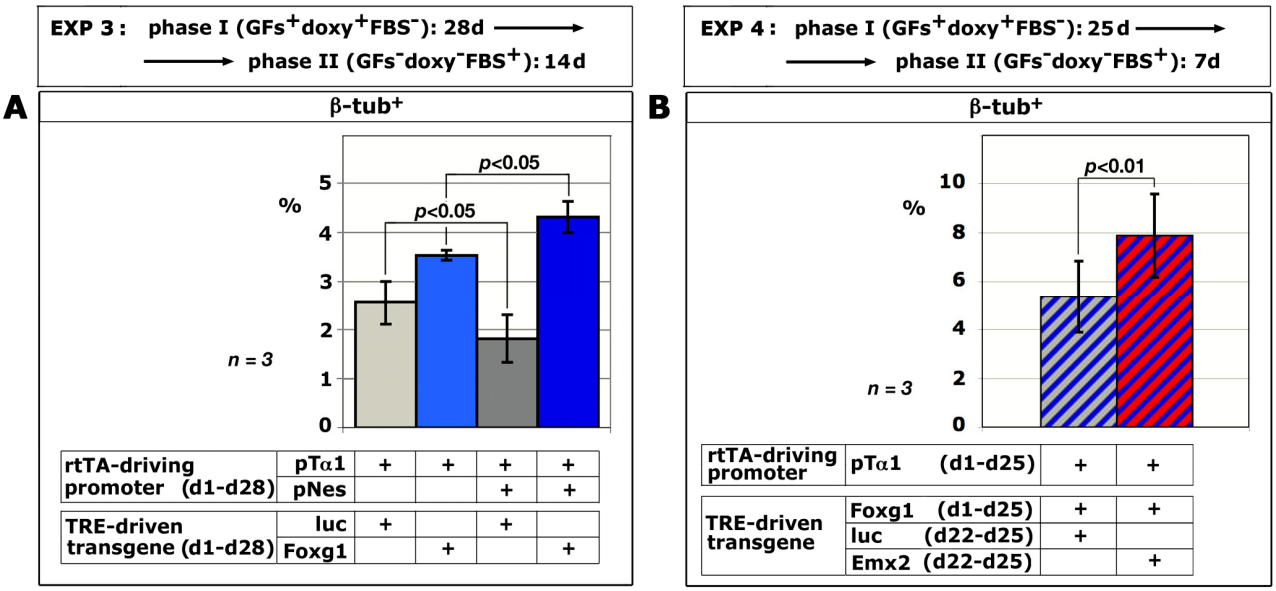



**Figure 34. Quantifying late histogenetic consequences of Foxg1 and/or Emx2 overexpression in NPs and/or NSCs, following the experimental designs EXP3 and EXP4.**

**(A)** Expression of β-tubulin, upon delivery of tool-set 6 LTVs (Figure 1) to dissociated E12.5 wild type and pNes-rtTA-IRESbgeo$^{+/-}$cortico-cerebral precursors, according to the combinations shown below the histogram. Infected cells were kept for 28 days under anti-differentiative conditions and in the presence of the transgene indexes doxycycline (phase I: GFs$^{+}$doxy$^{+}$FBS$^{-}$). They were subsequently transferred to pro-differentiative conditions, where they were kept for 14 more days, in the absence of doxycycline (phase II: GFs$^{-}$doxy$^{-}$FBS$^{+}$), and finally profiled. **(B)** Expression of β-tubulin, upon delivery of tool-set 6 LTVs (Figure 1) to dissociated E12.5 wild type cortico-cerebral precursors, according to the combinations shown below the histogram. Infected cells were kept for 25 days under anti-differentiative conditions and in the presence of the transgene inducer doxycycline (phase I: GFs$^{+}$doxy$^{+}$FBS$^{-}$). They were subsequently transferred to pro-differentiative conditions, where they were kept for 7 more days, in the absence of doxycycline (phase II: GFs$^{-}$doxy$^{-}$FBS$^{+}$), and finally profiled. Bars represent s.e.m.'s. Statistical significance of differences was evaluated by one-way ANOVA.

# 3. DISCUSSION

### *3.1 Methodology and main findings*

To investigate the roles of *Emx2* and *Foxg1* in cortico-cerebral histogenesis, we developed a novel, perturbative-analytic approach, based on integrated usage of different state-of-art technologies. We overexpressed either gene in NSCs or NPs originating from primary precursors, by lentivectors, under the control of precursor-type-specific promoters. We investigated immediate kinetic consequences of these manipulations, by genetically and/or immunologically labelling distinct precursor types, and then monitoring the fluorescence time course profile of the culture, kept under anti-differentiative conditions. We evaluated late histogenetic consequences of such manipulations, scoring neuron and glia-specific antigens, upon transfer of the culture under pro-differentiative conditions.

Main consequences of our *Emx2*- and *Foxg1*-GOF manipulations were as follows. During the first week of *in vitro* culture, *Emx2* enlarged the NSC compartment. Later, it promoted neuronogenesis, directly from NSCs or via NPs. At the same time, *Emx2* committed NSCs to glial fates, but inhibited further maturation of early bipotent GPs. *Foxg1* gave conversely rise to an enduring enlargement of the NSC compartment, possibly due to increased NSCs self-renewal, in the absence of overt pro-survival effects on these cells. It counteracted glial commitment of NSCs and further maturation of glial progenitors. It enlarged the NP compartment as well, by promoting progenitor survival and delaying neuronogenesis. All that led to a remarkably increased neuronal output. Furthermore, neurons derived from *Foxg1*-GOF NPs showed a prominent neurite overgrowth. Finally, combined overexpression of *Foxg1* and/or *Emx2* according to appropriate schemes allowed to further ameliorate neuron production, already increased by single gene gain-of-function manipulations.

### *3.2 Foxg1 inhibits gliogenesis and potentiates neuronogenesis, stimulating neuronal differentiation*

We have shown that Foxg1 overexpression in NSCs leads to an expansion of this compartment accompanied by a blockade of glial fate choice. Remarkably, this expansion is at odd with what recently reported by (Fasano et al., 2009). According to these colleagues, *Foxg1*, while being *necessary* to the enlargement of the NSC compartment promoted by Bmi1, is not *sufficient per se* to stimulate NSC self-renewal. This discrepancy might depend on the different cell type subject of analysis (Fasano et al., 2009). Alternatively, it might originate from the different methodology employed for evaluating the NSC frequency: counting $pNes^+$ cells not expressing neuronal or glial lineage-markers in our case, counting $>2^{nd}$-order neurosphere derivatives in the other. In fact, it has been shown that a largely predominant fraction of *wild type* neurospheres does not originate from NSCs but from lineage-restricted progenitors. Among them, glial progenitors are prominent (Louis et al., 2008) and more frequent as the culture goes ahead (Fig. 29B). Therefore, the reduced GP frequency peculiar to *Foxg1*-GOF cultures (Fig. 29B) could compensate for their increased NSC frequency (Fig. 29D), so resulting in similar numbers of neurospheres and masking the "increased self-renewal" phenotype. Interestingly, the smaller size of *Foxg1*-GOF spheres reported (Fasano et al., 2009) might arise just from reduced gliogenesis in these mutants, so further supporting this hypothesis.

Moreover, the *Foxg1*-promoted enlargement of the NSC compartment was enhanced by the reduced gliogenic commitment of NSCs and the dramatically impaired progression of early born GPs toward more mature glial profiles (Fig. 30B and Fig. 32A,B). Actually, no antigliogenic activity of *Foxg1* has been described until now in vertebrates. However, such activity is not phylogenetically novel. Two *Drosophila Foxg1* orthologs, *Sloppy paired-1* and *-2* (*Slp1* and *Slp2*), promote neuronal specification at expenses of glial specification (Bhat et al., 2000), by downregulating *G*lial *cell missing* (*Gcm*), binding to its protein product and inhibiting its transactivating abilities. Furthermore, Foxg1 rescues the *Slp1&2 null* phenotype and binds to Gcm, Slp binds to the murine Gcm homolog, mGcm2 (Mondal et al., 2007). This suggests that a *Foxg1/Gcm* pathway might control vertebrate gliogenesis as well. Consistent with this hypothesis and despite of the absence of any CNS developmental abnormalities in *mGcm-1* and *mGcm2 null* mice (Günther et al., 2000; Schreiber et al., 2000), murine

embryonic brain precursors, infected with *Gcm*- or *mGcm*-encoding retroviruses, are massively induced to express the astrocyte-specific S100 protein (Iwasaki et al., 2003) and Gcm induces human DEV medulloblastoma cells to astro/oligodendroglial differentiation (Buzanska et al., 2001).

Remarkably, following the expansion of the NSC compartment and the shrinkage of the glial one, the *Foxg1*-GOF NP compartment became accordingly wider and wider. As a consequence of that, the neuronal output of the culture, still unchanged at 14+7 dpi (Fig. 32A), was increased by more than 50% at 28+7 dpi (Fig. 32B).

NP-restricted Foxg1 overexpression elicited a progressively more prominent enlargement of this compartment (Fig. 31), reasonably originating from reduced cell death (Fig. 30E), doubled expansion rate (Fig. 30G), diminished neuronal differentiation (Fig. 30C). Reduced NP death is compatible with increased precursor survival reported in (Fasano et al., 2009), therefore to be ascribed only to lineage-restricted progenitors. *Foxg1* ability to promote precursor self-renewal and inhibit their cell cycle exit is prefigured by a number of LOF studies on the embryonic (Hanashima et al., 2002; Xuan et al., 1995) or the post-natal brain (Shen et al., 2006).

Until *Foxg*1 is overexpressed under the control of the pTα1 promoter, not only are NPs forced to keep proliferating, but also newborn neuron maturation is dramatically impaired (Fig. 30C). However, if the transgene is shut down, then NPs exit cell cycle and massively differentiate as neurons, regardless of the age of the culture (Fig. 32C, Fig. S2A and Fig.S3). In this case, newborn neurons mature remarkably faster. Their average neurite length is about 40% higher than controls after 14+7 dpi (Fig. 32D) and subsequently becomes even higher, possibly as a consequence of an almost doubled elongation rate (Fig. 32E). As pTα1 fires not only in NPs but also in early neurons, we cannot say whether is this neurite overgrowth only “primed” by *Foxg1* in NPs or is it further sustained by transgene expression in early post-mitotic neurons. Neurite outgrowth is reduced in *Foxg1*-LOF sense organs, both eye and ear (Pauley et al., 2006; Tian et al., 2008); however, the capability by *Foxg1* to stimulate neurite outgrowth in the cortical field is a novel finding.

### *3.3 Emx2 channels neural precursors to neuronal differentiation*

The early enlargement of the NSC compartment elicited by *Emx2* overexpression (Fig. 29A,B,D), not associated to any reduced differentiation and death rates (Fig. 30A,B,D), is puzzling. It might be due to a *very early Emx2* activity, promoting NSC self-renewal and inhibiting their neuronal differentiation. Actually, such activity did not result from our evaluation of NSC-to-NP and NSC-to-N indexes at 7 dpi, reasonably because these indexes were measured too late. It was conversely documented in short term cultures of E14.5 cortical precursors (Heins et al., 2001) and later confirmed in v*ivo* (Muzio et al., 2005).

Later *Emx*2-induced shrinkage of the NSC compartment conversely reflects a pronounced channelling of stem cells to neuronal and glial differentiation (Fig. 29A,B,D and Fig. 30A,B,D). The *Emx2* ability to promote neuronal differentiation of *older* cortical precursors has been already documented in both *Emx2*-GOF and *Emx2*-LOF neurosphere cultures, originating from *perinatal* and *adult* cortical precursors (Galli et al., 2002; Gangemi et al., 2001). How the same gene may regulate the same process in opposite ways, depending on the NSC age, is an interesting issue. Several mechanisms, including differential post-translational covalent modifications and differential availability of key cofactors, may account for that.

The capability of *Emx2* to jeopardize further differentiation of eGPs (Fig. 30B) is a novel finding. It contrasts with what reported by Galli et al. (Galli et al., 2002), who did not observe any change of frequencies of GFAP+ and GalC+ elements, within progenies of *Emx2*-GOF/LOF cultures. It is apparently at odd with Heins et al. (Heins et al., 2001), who conversely reported a doubling of mixed neuronal-glial clones and a reduction of pure neuronal clones, in E14.5 *Emx2*-GOF cortical precursors. Several reasons may underlie such discrepancies, including distinct temporal schedules of the experiments and different readouts employed to monitor gliogenesis.

Finally, overexpression of *Emx2* in NPs elicited a pronounced shrinkage of their compartment, from 7dpi to 28 dpi (Fig. 31). This happened despite of reduced NP death rates (Fig. 30E) and as a consequence of their exaggerated neuronal differentiation (Fig. 30C). Moreover, postmitotic neuronal maturation was enhanced (Fig. 30C) and the neuronal output was increased (Fig. 32C).

### *3.4 Potential therapeutic implications*

Thus, *Foxg1* promotes NSCs and NPs self-renewal as well as NPs survival, inhibits gliogenesis and primes neuronal maturation; *Emx2* channels NSCs to neuronogenesis, inhibits gliogenesis, promotes NPs survival and accelerates their neuronal differentiation (Fig. 33). These activities are particularly appealing, as potentially useful to overcome two of the main drawbacks of cell-based brain repair: exaggerated death of transplanted cells, especially if differentiating to neurons; their preferential astrocytic rather than neuronal differentiation (Burns et al., 2009). It is noteworthy that the enhanced neuronal output, obtainable upon overexpression of a single TF in a single precursor compartment (Fig. 32A,B,C and Fig.7A), may be further ameliorated upon combined gene manipulations, such as overexpression of *Foxg1* in both NSCs and NPs (Fig. 34) as well as by sequential overactivation of *Foxg1* and *Emx2* in NPs (Fig. 34B). That points to pursuable combined gene therapy designs for improving cell-based brain repair. Moreover, neither beneficial effects of these manipulations require irreversible transgene activation (Fig. 32, Fig. 34), especially bio-hazardous in the case of *Foxg1* (Adesina et al., 2007; Li et al., 1995), nor do these manipulations induce any transformation foci (data not shown), so making them even more appealing for therapeutic approaches. Hopefully, delivering these treatments by molecular tools different from lentivectors, besides preventing genotoxicity of multiple insertional mutations (Nienhuis et al., 2006), will fully unveil the neuronogenic potential of such manipulations (Fig. 29D and Fig. 34A).

### *3.5 Conclusion*

The overexpression of *Foxg1* and *Emx2* channels cortico-cerebral NSCs to neuronogenesis and antagonizes the activation of gliogenic programs. *Foxg1* stimulates NP self-renewal/survival and induces neurite overgrowth. Such activities may be exploited to ameliorate the neuronal output obtainable from neural cultures, for purposes of cell-based brain repair.

# 4. MATERIALS AND METHODS

### *4.1 Animal handling*

Wild type mice (strains CD1 and FVB/N, purchased from Harlan-Italy) and pNes-rtTA-IRESβgeo$^{+/-}$ mutants (Mitsuhashi et al., 2001) (obtained from EMMA, Monterotondo, Italy) used in this study were maintained at the SISSA-CBM mouse facility. Embryos were staged by timed breeding and vaginal plug inspection.

### *4.2 Primary cortical precursors (cPCs) isolation*

pNes-rtTA-IRESβgeo$^{+/-}$ FVB/N males (Mitsuhashi et al., 2001) were mated to wt FVB/N (EXP1a, EXP1b, EXP2) or CD1 (EXP3 and EXP4) females. E11.5/12.5 heterozygous transgenic embryos (which express β-galactosidase in the neural tube) were distinguished from their wild type littermates by X-gal staining [in: 1X PBS, 5mM $K_3Fe(CN)_6$, 5mM $K_3Fe(CN)_6$*3 $H_2O$, 2mM $MgCl_2$, 0.01% Na deoxycholate, 0.02% NP40; 1mg/ml 5-Bromo-4-Cloro-3-Indolilb-D-Galactopiranoside (X-Gal)].

pNes-rtTA-IRESβgeo$^{+/-}$ embryos were used for EXP1a, EXP1b, wild type embryos for EXP2, and EXP4, embryos with both genotypes for EXP3.

cPCs were isolated from E11.5 (EXP1a, EXP1b, EXP2) or E12.5 (EXP3 and EXP4) embryonic cortices and plated onto uncoated 24 multiwell (BD Falcon) after gentle mechanical dissociation to single cells. 2.5*$10^5$ cPCs were plated for each well in 350 µl of 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).

### *4.3 Long term cPCs culture maintenance*

For long term maintenance, fresh GFs (20 ng/ml bFGF and 20 ng/ml EGF) 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 $10^5$ 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.

### *4.4 cPCs differentiation*

For differentiation experiments neurospheres were harvested after 14 days or 28 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 $5*10^5$ 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 or 14 days.

### *4.5 Lentiviral transfer vector construction*

Basic DNA manipulations (extraction, purification, ligation) as well as bacterial cultures and transformation, media and buffer preparations were performed according to standard methods. DNAs were transformed in the E.Coli Xl1-blue strain.

All the plasmids used in the present study were built up starting from the pCCL-SIN-18PPT.Pgk.EGFP-Wpre lentiviral transfer vector backbone (Follenzi and Naldini, 2002) (gift from L. Naldini), by replacing the pPgk-EGFP cassette with specific modules:

(A) transgene expressing vectors (tool-sets 1 and 6) were obtained by replacing the pPgk-EGFP cassette with the TREt promoter (from the pTRE-Tight plasmid, Clontech) and a bicistronic cassette alternatively containing *Foxg1* (GeneID: 15228), *Emx2* (GeneID: 13797) or *Luciferase* (from the pGEM®-*luc* plasmid, Promega) coding sequences, plus the IRES2EGFP module (from the pIRES2-EGFP plasmid, Clontech).

(B) pSGC10/S2/2N-mCerulean vector (tool set 3) was obtained by replacing the pPgk-EGFP cassette with the pSGC10/S2/2N promoter, taken from the pSGC10/S2/2N-luciferase plasmid (Namikawa et al., 2006) (gift from H.Kiyama) and the mCerulean coding sequence (Rizzo et al., 2004) (from the Cerulean plasmid, Addgene).

(C) pTα1-mCherry (tool-set 2) vector was obtained by replacing the pPgk-EGFP cassette with the pTα1-mCherry cassette (gift from E.S.Ruthazer). Starting from the pTα1-mCherry vector, pTα1-rtTA/M2 (tool set 6) was obtained by replacing the mCherry sequence with the rtTA/M2 cassette (from the pTet-ON Avanced plasmid, Clontech).

(D) to obtain the pNes/hsp68-mCherry vector (tool set 5), the neural enhancer from the second intron of the rat nestin gene was taken from the pE/Nestin-EGFP plasmid (Kawaguchi et al., 2001) (gift from H.Okano) and cloned upstream of the minimal hsp68 murine promoter, in the pHPS.sis plasmid (Choi et al., 1991). The resulting pNes/hsp68 promoter was then used to replace the pTα1 promoter in the pTα1-mCherry vector.

### *4.6 Lentiviral vectors packaging and titration*

Third generation self-inactivating (SIN) lentiviral vectors were produced as previously described (Follenzi and Naldini, 2002) with some modifications. Briefly, 293T cells were co-lipofected (Lipofectamine 2000, Invitrogen) 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).

EGFP-expressing lentiviral vectors (tool-sets 1, 6) were titrated on HeLa TET-off cells (Clontech), by end point fluorescence titration, as previously described (Follenzi and Naldini,

2002) and titer expressed as transducing units per ml (TU/ml). Other LTVs were generally titrated by Real Time quantitative PCR after infection of HEK293T cells, as previously reported (Sastry et al., 2002). One end point fluorescence-titrated LTV was included in each PCR titration session and PCR-titers were converted into fluorescence-equivalent titers throughout the study.

### *4.7 Lentiviral infection of cPCs*

At least 7.5 *$10^5$ cPCs were dissociated to single cells and plated onto a 24 multiwell, at 2.5*$10^5$ cells/350µl, in anti-differentiative doxycycline-containing medium, and infected with the appropriate LTV mix. Lentiviral vector conditioned medium was washed away 3.5 dpi and neurospheres were dissociated and plated as already described. The following lentiviral vector mixes were used for the different experimental setups:

EXP1a: LTV pTα1-mCherry, at MOI 10; LTV pSGC10/S2/2N-mCerulean, at MOI 10; alternatively, LTV $Tre_t$-Luc-IRES2EGFP, $Tre_t$-*Foxg1*-IRES2EGFP or $Tre_t$-*Emx2*-IRES2EGFP, at MOI 15.

EXP1b: alternatively, LTV $Tre_t$-Luc-IRES2EGFP, $Tre_t$-*Foxg1*-IRES2EGFP or $Tre_t$-*Emx2*-IRES2EGFP, at MOI 15.

EXP2: pTα1-rtTA/M2, at MOI 8; LTV pNes/hsp68-mCherry, at MOI 8; LTV pSGC10/S2/2N mCerulean, at MOI 8; alternatively, LTV $Tre_t$-Luc-IRES2EGFP, $Tre_t$-*Foxg1*-IRES2EGFP or $Tre_t$-*Emx2*-IRES2EGFP, at MOI 8.

EXP3: LTV pTα1-rtTA/M2, at MOI 10; alternatively, LTV $Tre_t$-Luc-IRES2EGFP or $Tre_t$-*Foxg1*-IRES2EGFP, at MOI 10.

EXP4 - primary infection: LTV pTα1-rtTA/M2, at MOI 10; LTV $Tre_t$-*Foxg1*-IRES2EGFP, at MOI 10.

EXP4 - secondary infection: alternatively, LTV $Tre_t$-Luc-IRES2EGFP or $Tre_t$-*Emx2*-IRES2EGFP, at MOI 10. In this case, the two LTVs were delivered to the two halves of the same primarily infected EXP4 preparation.

### *4.8 FACS analysis of cPCs*

Every 7 days, an aliquot of 100,000-200,000 dissociated cPCs, prepared as described above, were analyzed on a three lasers-equipped Cyan ADP flow cytometer (Dakocytomation, Denmark). Multivariate data analysis was performed by using FlowjoTM software (Tree Star, Ashland, OR).

Forward scatter (FSC) and side scatter (SSC) were used to gate nucleated cells and to exclude debris and cell aggregates (live gate) in every analysis. Cells belonging to the live gate were then further evaluated for the expression of the fluorochromes in use.

In case of EXP1a and EXP2, cells were categorized on the basis of their EGFP, mCherry and mCerulean fluorescence profiles. To assess their viability, an aliquot of them was further labelled with a 7-Amino-actinomycin D (7-AAD) which is excluded by viable cells, but can penetrate cell membranes of dying or dead cells and intercalate into double-stranded nucleic acids.

In the case of EXP1b, cells were previously decorated with anti-A2B5-APC (Miltenyi Biotec, Germany) and anti-PSA-NCAM-PE (Miltenyi Biotec, Germany) antibodies, according to manufacturer's instructions, and subsequently categorized, based on their EGFP, APC and PE fluorescence profiles.

### *4.9 Immunocytofluorescence*

For immunocytofluorescence analysis on proliferating cPCs, neurospheres were dissociated to single cells as already described and transferred on poly-D-lysine (20µg/µl) coated SuperFrost ® microscope slides (Menzel-Gläser). After 1 hour, cells were fixed in 2% paraformaldehyde (PFA) for 20 min at RT and then washed in PBS, blocked in 10% fetal bovine serum and 0,1% Triton X-100 . 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. Finally, microscope slides were washed and mounted in Fluorescent Vectashield™ Mounting Medium

For immunocytofluorescence on differentiated cPCs, cells were fixed directly on poly-L-lysine coated 24 multiwell plates, with 4% PFA for 10 min at RT. Wells where then treated as described above.

The following primary antibodies were used: anti-β-tubulin mouse monoclonal, (clone Tuj1, Covance, 1:600), anti-CNPase mouse monoclonal (clone 11-5B, Millipore, 1:400), anti-S100 rabbit polyclonal (DAKO 1:1000) anti-EGFP chicken polyclonal (AbCam, 1:800), anti-RFP/DsRed rabbit polyclonal (antibodies-online GmbH 1:1000), anti-Tbr2 rabbit polyclonal (AbCam, 1:600), anti *Foxg1* rabbit polyclonal (gift from G. Corte, 1:200), anti-*Emx2* mouse monoclonal (clone 4F7, Abnova, 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.

### *4.10 Images acquisitions and quantifications*

Acutely poly-D-lysine-attached cPCs were photographed on a TCS SP2 Leica confocal microscope with a 20X objective and generally collected as 6µm Z-stacks of 1024*1024 pixel images. Images were averaged and cells were imported and counted with Adobe Photoshop CS3 software™. At least 8 fields from 3 independent biological replicates were counted for each experimental condition.

Differentiating cPCs were photographed on a Nikon Eclipse TS100 fluorescence microscope equipped with a DS-2MBWC digital microscope camera with a 20X objective. At least 8 fields from 3 independent biological replicates were counted for each experimental condition. Images were imported and counted with Adobe Photoshop CS3 software™. DAPI stained nuclei images were imported and counted with MacBiophotonics Image J nucleus counter plug-in (software available at http://www.macbiophotonics.ca/).

Analysis of mean neurite length was performed with the NeuriteTracer ImageJ plugin (Pool et al., 2008). 7 to 15 fields were evaluated for each biological replicate.

### *4.11 Statistical analysis*

*FACS data: compartment sizing.* Frequencies of cells with different fluorescence-profiles, evaluated on samples of at least 100,000 elements, were averaged over three biological replicates and plotted against time. Statistical significance of differences was evaluated by one-way ANCOVA.

*FACS data: X-to-Yi transition indexes.* This index was calculated for each gene manipulation and each culturing time point, by dividing the size of the "downstream" Yi compartment by the size of the "upstream" X compartment. *luc* data were averaged over three independent biological replicates and plotted against time. For each biological replicate and each time, indexes of *Emx2*-GOF and *Foxg1*-GOF cultures were normalized against *luc*-transduced cultures. The resulting normalized indexes were averaged over the three biological replicates and plotted against time. Statistical significance of differences was evaluated by one-way ANCOVA.

*FACS data: cell death rates and enlargement rates.* The first parameter was calculated as the percentage of cells with an NSC- or an NP-specific fluorescent profile further stained by 7AAD. The second parameter was evaluated by dividing the size of compartment in order at time = t by the size of the same compartment one week earlier. *luc*, *Emx2* and *Foxg1* data, collected over three independent biological replicates, were further processed like X-to-Yi transition indexes.

*Immunocytofluorescence data.* Frequencies of cells expressing specific antigens, evaluated on samples of at least 10,000 elements, were averaged over three biological replicates and plotted against the gene manipulation in order. Statistical significance of differences was evaluated by one-way ANOVA.

# 5. SUPPLEMENTARY FIGURES

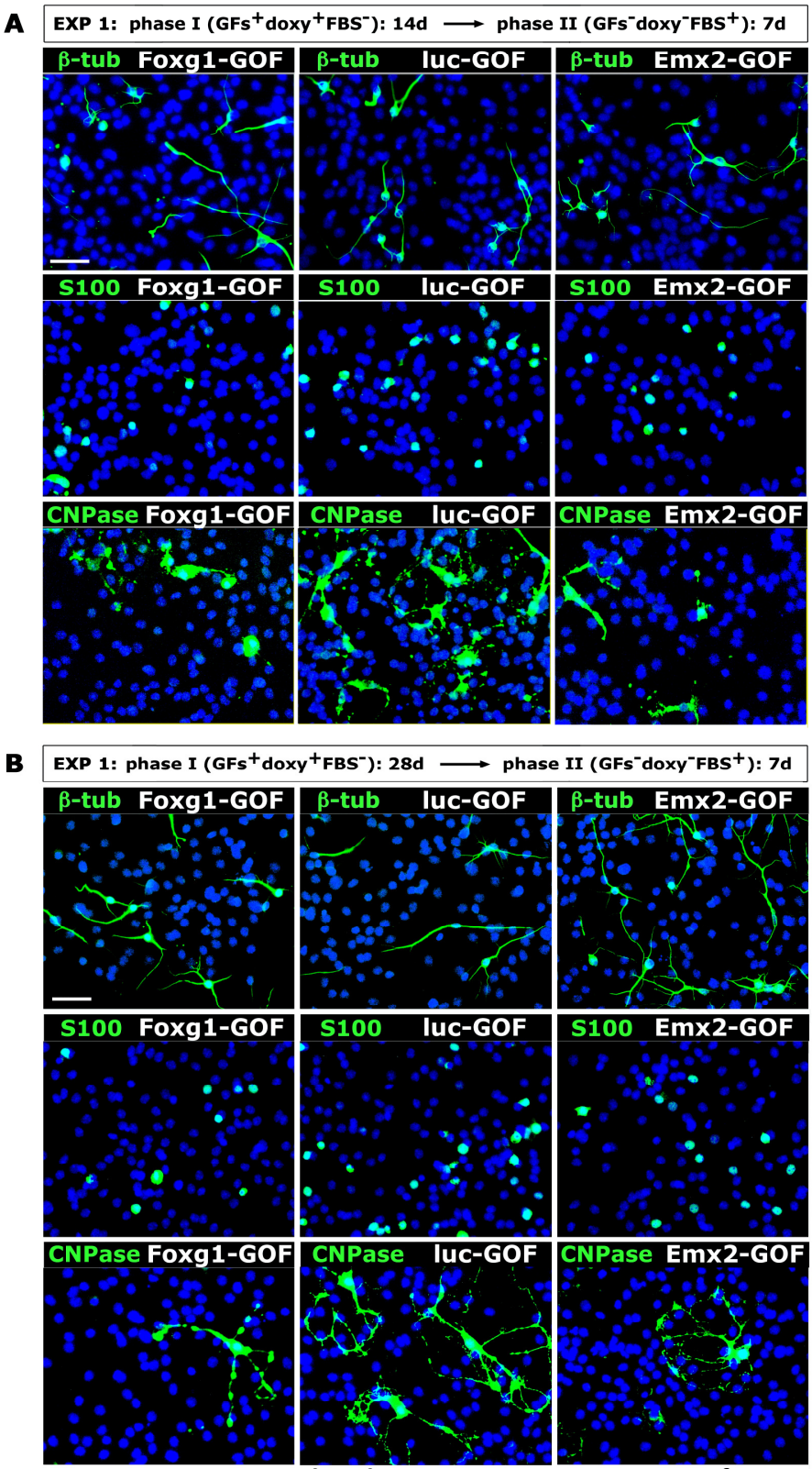



**Figure S1. Monitoring late histogenetic consequences of Emx2 or Foxg1 overexpression in NSCs, following the experimental design EXP1.**

(**A,B**) Examples of neural cultures immunoprofiled for neuronal (β-tubulin), astrocytic (S100) and oligodendrocytic (CNPase) markers, quantified in Figures 5A and 5B, respectively. Scalebars, 50 µm.

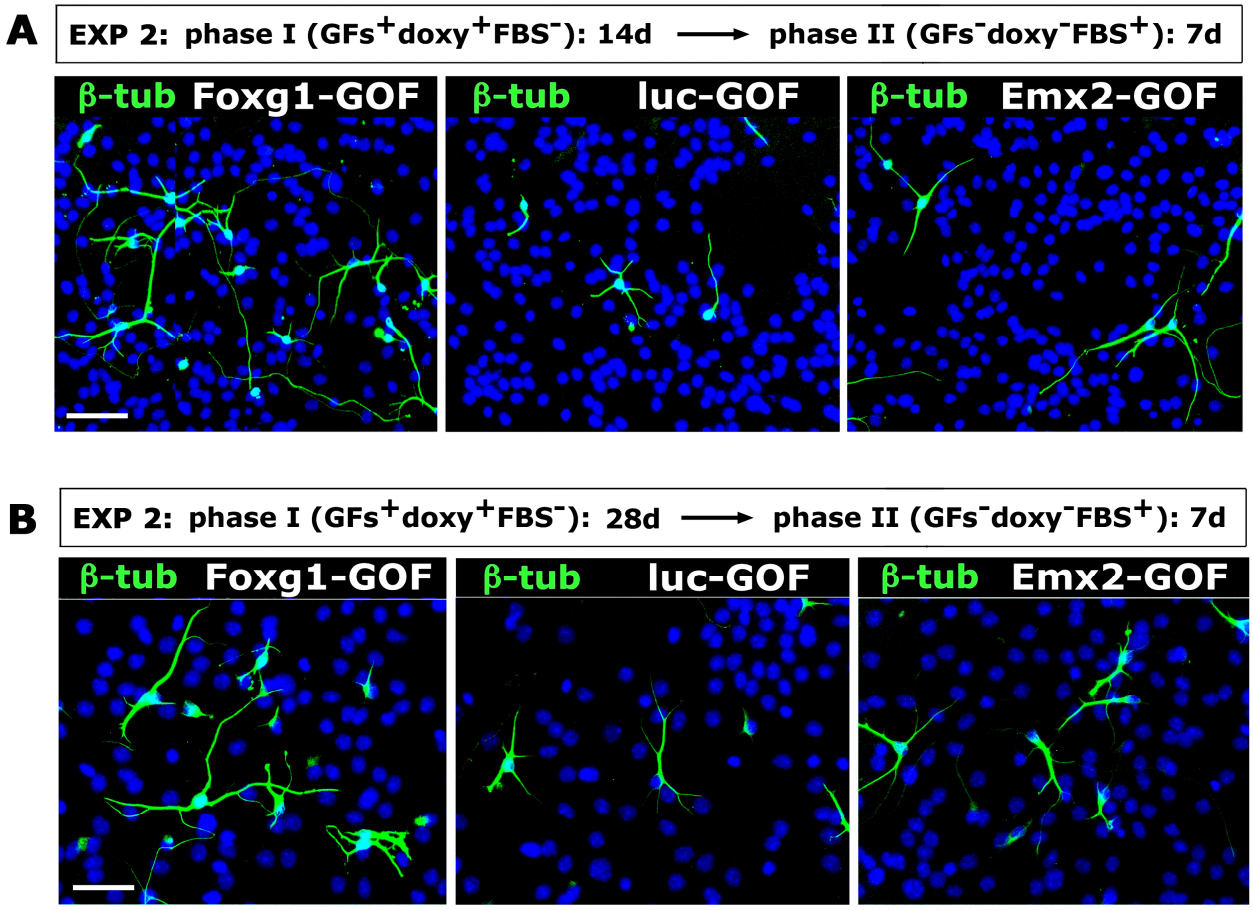



**Figure S2. Monitoring late histogenetic consequences of Emx2 or Foxg1 overexpression in NPs, following the experimental design EXP2.**

(A) Examples of neural cultures immunoprofiled for the neuronal marker β-tubulin, quantified in Figure 5C. (B) Distribution of β-tubulin on neural cultures set-up as in Figure 5C, except for lengthening of phase I to 28 days. Scalebars, 50 µm.

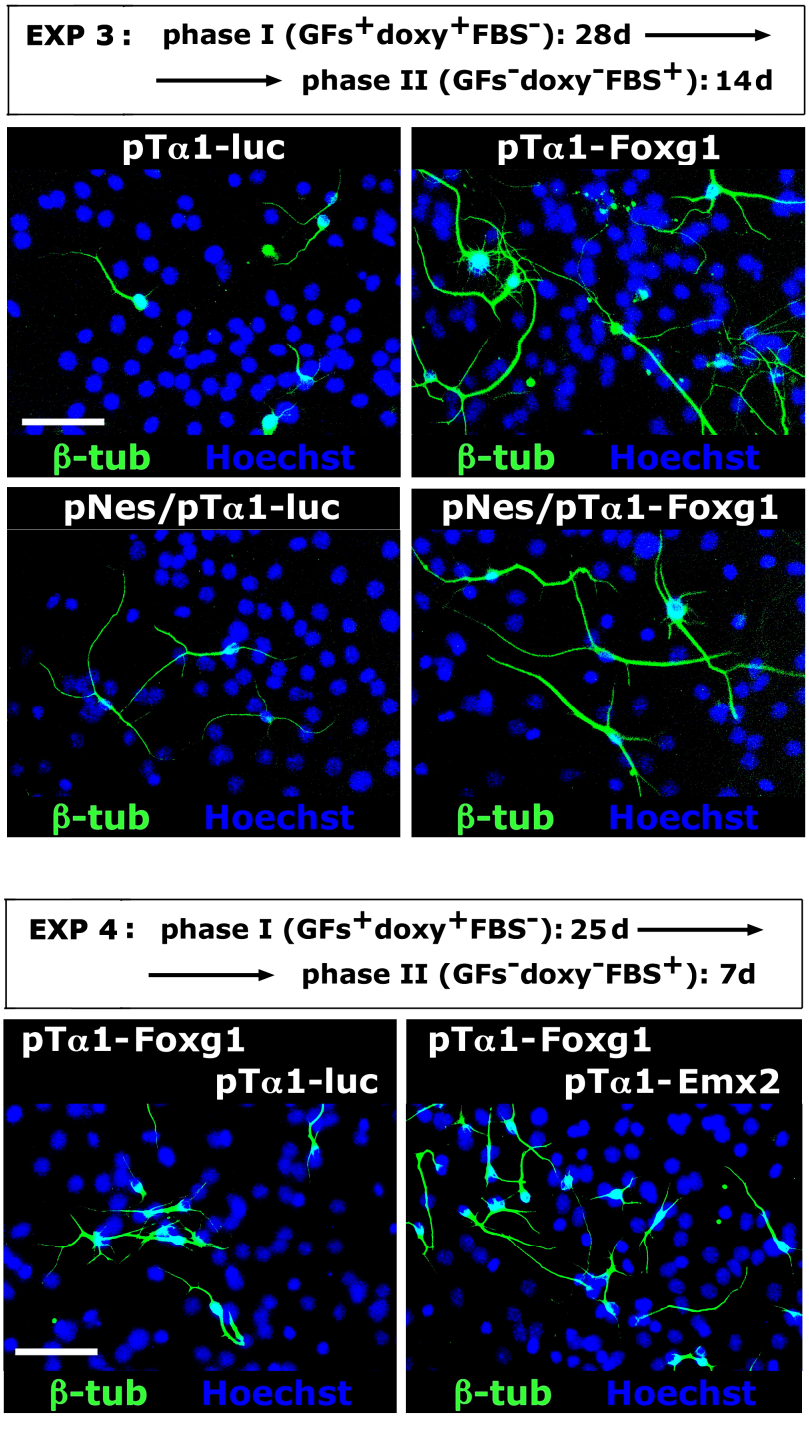



**Figure S3. Monitoring late histogenetic consequences of Foxg1 and/or Emx2 overexpression in NPs and/or NSCs, following the experimental designs EXP3 and EXP4.**

(**A,B**) Examples of neural cultures immunoprofiled for the neuronal marker β-tubulin, quantified in Figures 6A and 6B, respectively. Scalebars, 50 µm.

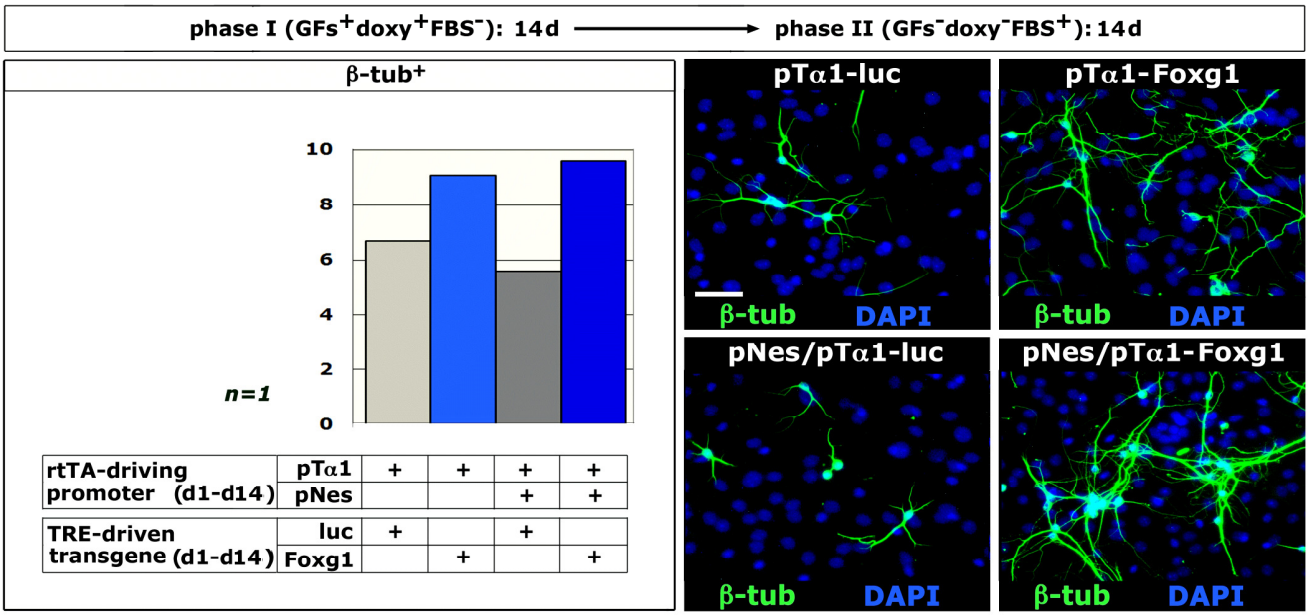



**Figure S4. Monitoring late histogenetic consequences of Foxg1 overexpression in NPs and/or NSCs, following the experimental design EXP3.**

Expression of the β-tubulin neuronal marker, upon delivery of tool-set 6 LTVs (see Fig. 32A) to dissociated E12.5 wild type and pNes-rtTA-IRESbgeo$^{+/-}$cortico-cerebral precursors, according to the combinations shown below the histogram. Infected cells were kept for 14 days under anti-differentiative conditions and in the presence of the transgene inducer doxycycline (phase I: GFs$^{+}$doxy$^{+}$FBS$^{-}$). They were subsequently transferred to pro-differentiative conditions, where they were kept for 14 more days, in the absence of doxycycline (phase II: GFs$^{-}$doxy$^{-}$FBS$^{+}$), and finally profiled. Scalebar, 50 µm.

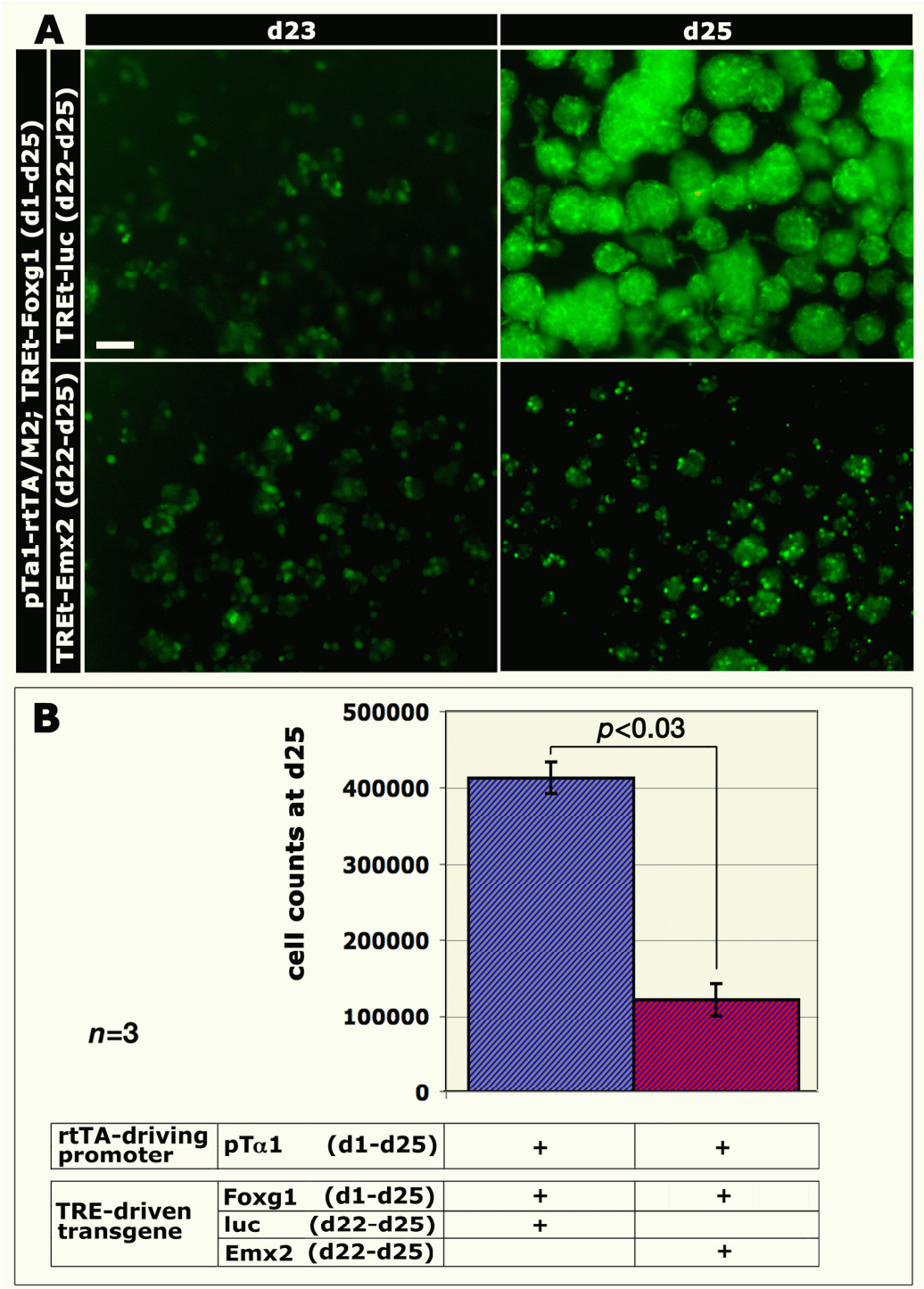



**Figure S5 Emx2 inhibits neurophere growth in pTα1 Foxg1 treated neural precursor cultures.**

(A) Neurospheres derived from E12.5 wild type cortico-cerebral precursors, infected by tool-set 6 LTVs (see Fig. 28A), according to the combinations shown aside, and kept under anti-differentiative conditions up to 25 days. (B) Number of cells derived from dissociation of the above neuropheres at d25. d, days post infection. Bars represent s.e.m.'s. Statistical significance of differences among graphs was evaluated by one-way ANOVA. Scalebar, 50 µm.

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