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S.I.S.S.A.-I.S.A.S.

Cortico-cerebral development in the gray short-tailed opossum *Monodelphis domestica*

PHD THESIS

Candidate: **Elisa Puzzolo** *Supervisor:* **Prof. A. Mallamaci**

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ABSTRACT

The marsupial South-American short-tailed opossum, Monodelphis domestica, is an appealing animal model for developmental studies on cortico-cerebral development, since the opossum cortex mainly develops after birth and newborns are particularly suitable for early ex-utero micro-surgical manipulations of this structure and the entire CNS. Opossum have been also largely employed as an ideal substrate for regenerative studies, since the pup is able to regenerate connections between neurons of the cerebral cortex and spinal cord upon experimental trauma. Moreover, branching from the common mammal ancestor about 180 My ago, Marsupials might provide a valuable tool for tracing evolutionary origins of key traits peculiar to the eutherian central nervous system (CNS).

Until recently, the cortico-cerebral marsupial development has been prevalently investigated by methods of classical histology, but several features of Monodelphis corticogenesis were still unknown. By taking advantage of molecular tools set up for developmental studies in Placentals and availability of Monodelphis domestica genomic sequence, we tried to fill gaps in our knowledge of opossum corticogenesis, studying in particular: origin of cortical neurons, their laminar differentiation and their migration profiles, from their birthplaces to their final layer positions.

We found many similarities between marsupial and placental corticogenesis, as for neuron generation, their laminar diversification and “inside-out” migration. This allowed us to establish a comparative time-table of mouse and opossum corticogenesis. One major difference emerged from our study. In the opossum, projection neurons are mainly born from apical progenitors and a basal proliferative compartment is hardly detectable.

INTRODUCTION

1. ORGANIZATION AND DEVELOPMENT OF THE NERVOUS SYSTEM

The development of the nervous system is a complex process that begins during early embryogenesis with the induction of neural cells, then the formation of the neural plate and afterwards the establishment of the primordial nervous system of the embryo.

The vertebrate nervous system has two anatomical distinct parts: the central nervous system (CNS) which consists of the brain and the spinal cord and the peripheral nervous system (PNS), which is composed of the sensory organs and of the autonomic and the enteric nervous system.

1.1. From neural plate to neural tube

The central nervous system (CNS) arises during early development from the neural plate, a cytologically homogeneous sheet of neuroepithelial cells (NE) (**Fig. 1**). The neural plate is induced from the underlying mesoderm. The neuroepithelial cells are thought to acquire distinct properties depending on the positions within the CNS primordium to yield enormously divergent neuronal cell types at specific locations. The neural plate is subdivided into molecularly distinct domains with characteristic locations.

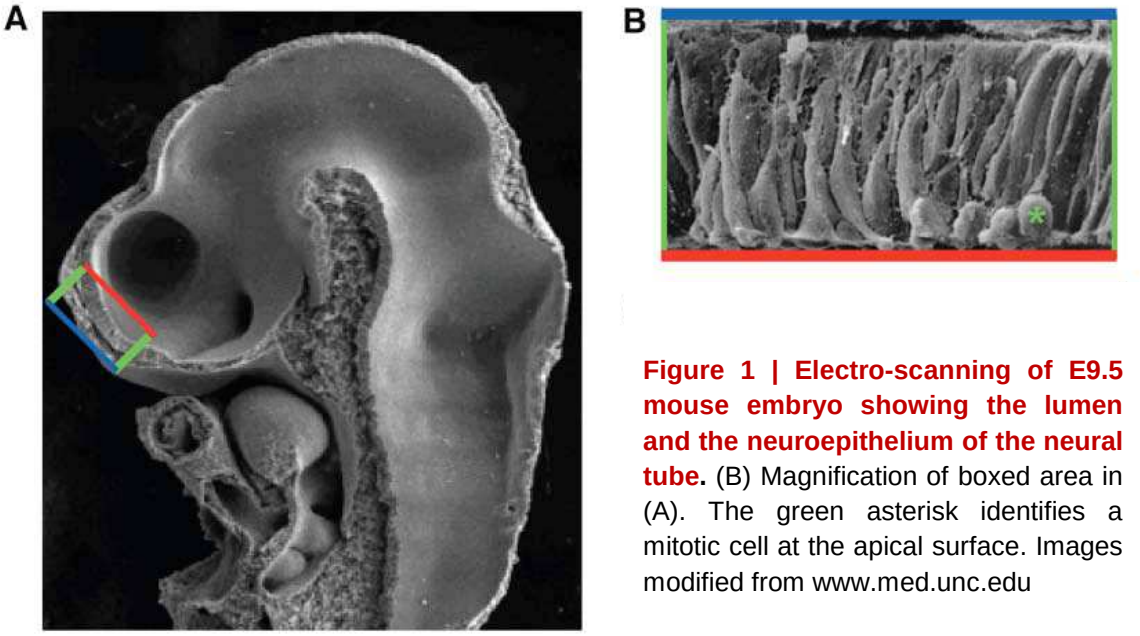

Figure 1 | Electro-scanning of E9.5 mouse embryo showing the lumen and the neuroepithelium of the neural tube. (B) Magnification of boxed area in (A). The green asterisk identifies a mitotic cell at the apical surface. Images modified from www.med.unc.edu

During neurulation, the neural plate folds up at the margins and forms the neural tube (**Fig. 2**).

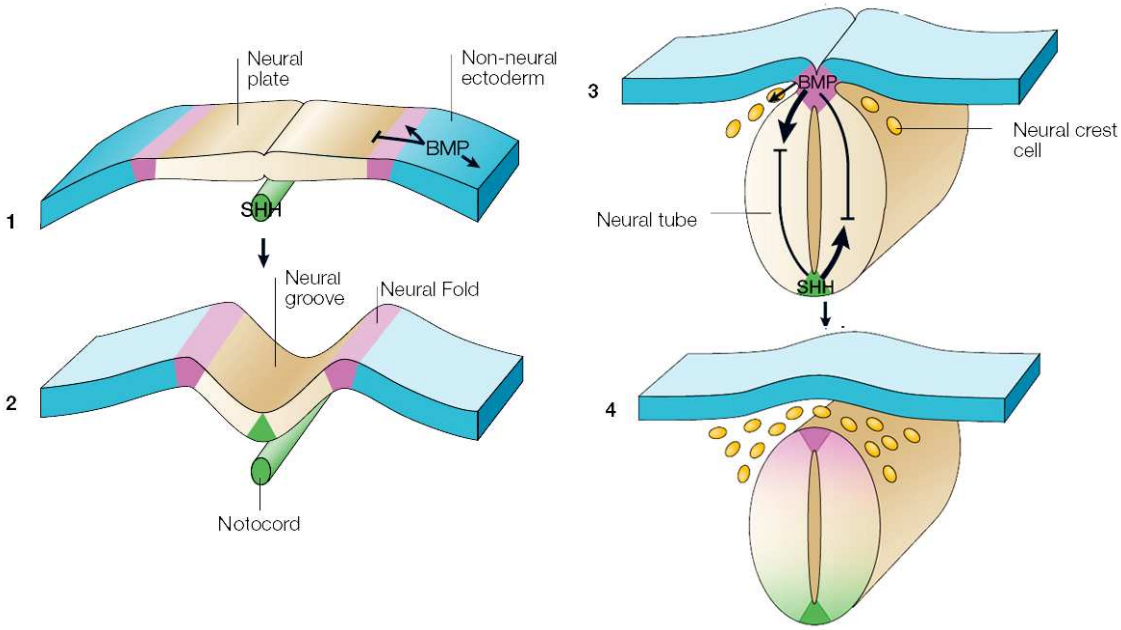

Figure 2 | Schematic view of the neural tube formation and the signaling sources involved. 1) opened neural plate; 2) neural grove formation; 3) closed the neural tube; 4) delaminating neural crests. Image modified from Nicholls *et al.*, 4th edition, 2001.

Before the closure of the neural tube, the neural plate becomes subdivided along the anteroposterior axis, into three distinct domains, corresponding to the three primary vesicles: the prosencephalon (the forebrain), the mesencephalon (midbrain), and the rhombencephalon (the hindbrain) (**Fig. 3A**). These initial regions become further subdivided as development proceeds: the prosencephalon will give rise to diencephalon and telencephalon; the rhombencephalon to metencephalon and mylencephalon (**Fig. 3B**).

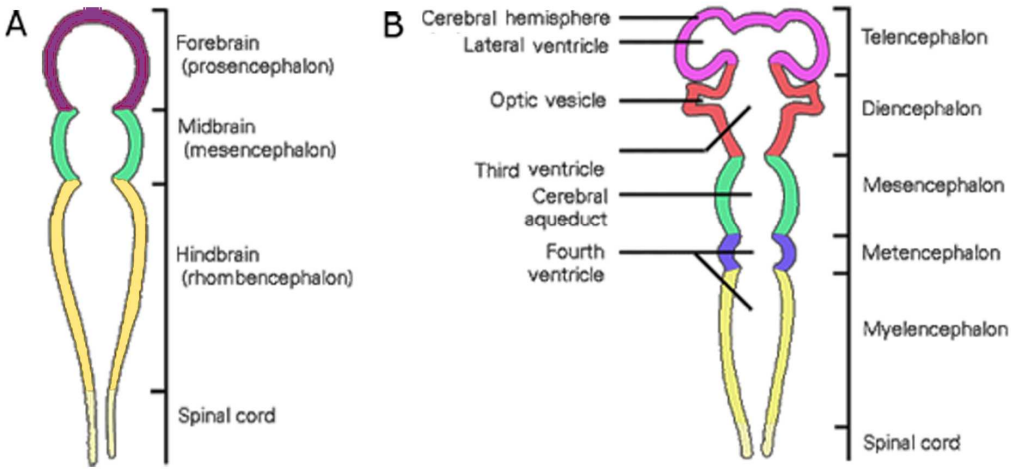

Figure 3 | Schematic view of the anterior neural tube. (A) three-vesicle stage; (B) five-vesicle stage. Image adapted from Nicholls *et al.*, 4th edition, 2001.

The telencephalic vesicles occupy the most rostral position of the neural tube and can be subdivided into a dorsal (pallial) and a ventral (subpallial) territory. The ventral telencephalon or subpallium is further subdivided into two main domains, called basal ganglia: the more ventrally located is the medial ganglionic eminence (MGE), precursors of the globus pallidum, the more dorsal is the lateral ganglionic eminence (LGE), which generates the striatum. A third eminence called caudal ganglionic eminence (CGE) supplies for the amygdala (**Fig. 4**).

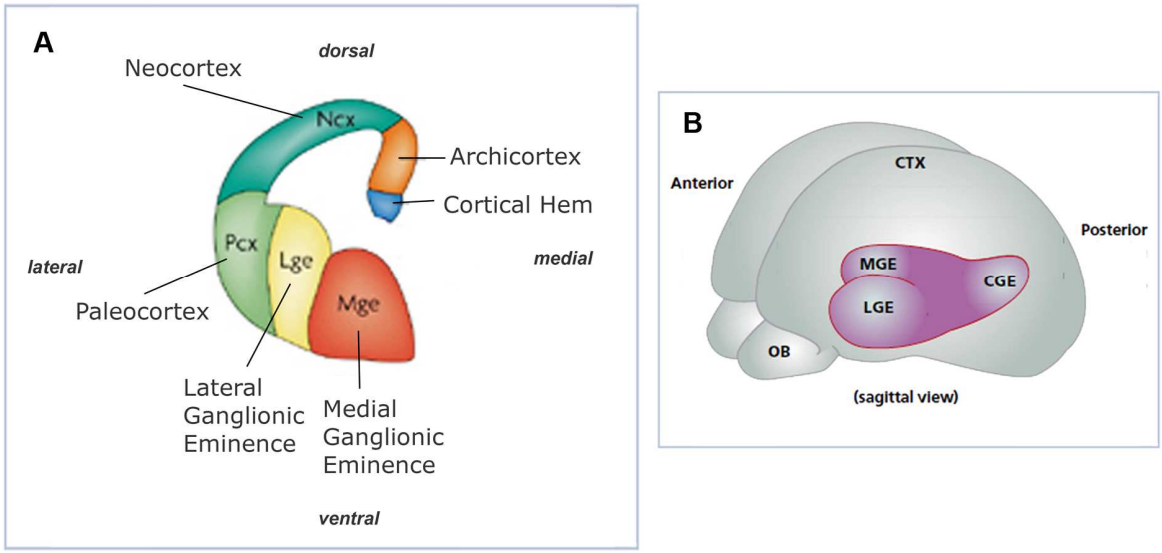

Figure 4 | (A) Schematic view of a coronal section through the developing mouse telencephalic vesicle at E12. (B) Sagittal view of the embryonic vertebrate telencephalon as a transparent structure to reveal the ganglionic eminences CGE, caudal ganglionic eminence; CTX, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; OB, olfactory bulb. Images modified from Molyneaux *et al.*, 2007 (A) and Corbin *et al.*, 2001 (B).

The cerebral cortex originates from the dorsal pallium. It includes four components, termed medial, dorsal, lateral and ventral pallium and corresponding to the hippocampus, the neocortex, the olfactory cortex and the claustroamygdaloid complex (Puelles *et al.*, 2000).

In the mouse forebrain the telencephalic vesicle appears at E9.0 and the cerebral cortex can be distinguished from E9.5 The development of the telencephalic hemispheres does not proceed uniformly. Anterior-lateral regions are more advanced than posterior-medial regions. Regional specification becomes visible after the telencephalic hemispheres are formed.

1.2. Organization of the adult mammalian cerebral cortex

The mammalian cerebral cortex is a complex, highly organized structure, containing hundreds of different neuronal and glial cell types, which has undergone a dramatic expansion during evolution (Rakic, 2007; Abdel-Mannan *et al.*, 2008). It is composed of a phylogenetically older region called allocortex and a younger region called isocortex or neocortex (NCx) that represents an evolutionary acquisition unique of mammals. The neocortex is the largest part of the mammalian brain and has a six-layer organization (**Fig. 5**). By contrast, the allocortex is a three-layered structure and is further subdivided into paleocortex (PCx), corresponding to the pirimiform cortex, and archicortex (ACx), corresponding to the hippocampus, dentate gyrus and cingulate cortex.

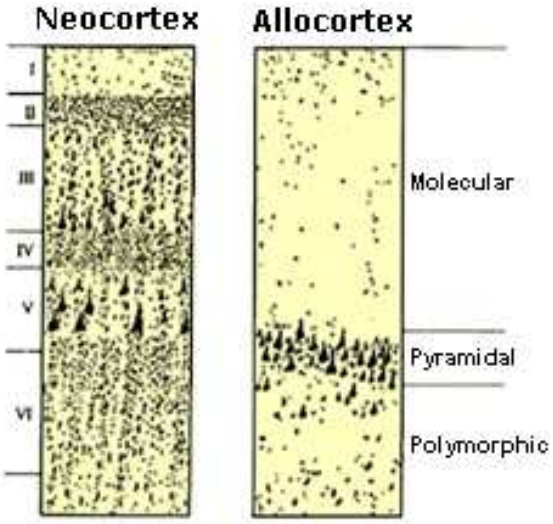

Figure 5 | Cortical representation of the Neocortex and the Allocortex.

According to different functions and cytological architecture, it was divided into distinct areas (Brodmann, 1909). Each cortical layer contains two distinct neuronal types: glutamatergic and GABAergic neurons, using glutamate and gamma-aminobutyric acid (GABA) as neurotransmitters, respectively. Glutamatergic neurons mainly include neurons with pyramidal morphology. They project to close and/or distant targets and they account for 75 to 85% of the total neuronal population in diverse mammalian species. The remaining 15 to 25% of cortical neurons are GABAergic neurons. They display diverse non-pyramidal morphologies, make short inhibitory “local circuit” connections and are commonly referred to as interneurons (Meinecke and Peters, 1987; Hendry *et al.*, 1987).

Cerebral cortical functions depend on the accurate construction of neural circuits, which begins very early during development in pallial progenitor cells.

1.3. Determination of the pallial field

In the mouse, the specification of the dorsal telencephalic field is progressively specified from E7.5 onward, thanks to a complex cascade of events involving secreted ligands released by specific signaling centers at the borders of the neural field and at specific positions within it, as well as transcription factors (TFs), gradually expressed within primary proliferative layers of the field itself.

Signaling centers are formed by regionally restricted groups of cells releasing signaling molecules that specify different neuronal cell types at precise positions along the anterior–posterior (A/P) and dorsal–ventral (D/V) axes of the neural tube during development. Among signaling molecules, a special role is played by morphogens. A morphogen is a secreted factor form a localized source, that induce more than two different cell fates over a sheet of cells in a concentration-dependent manner, forming a concentration gradient across a developing tissue. Secreted ligands regulate the expression of cortical TFs encoding for positional information peculiar to distinctive region of the cortical field.

Positional identities in the early cortical sheet are generally defined by the interaction of different signaling pathways in the patterning centers. Principal signaling molecules involved in antero-posterior patterning, as well as in regional specification of the dorsal, ventral and later telencephalon, are summarized in **table 1**.

Signalling pathways	Receptors	Major intracellular effectors	Antagonists
Wnt	Frizzeled 1-10, LRP	β-Catenin, Tcf	Dkk, Sfrp
FGF	FGFR1-4	MAPK, Ras	Sprouty, Pyst
BMP	BMPR-type-I (Alk2, 3&6) & type-II	Smad1,5,8, Smad4, p38	Smad6,7 Bambi
Shh	Patched, smoothened	Gli1-3	Gli3
RA	RAR, RXR	CRABP	Cyp26

Table1 | Overview of the main signaling molecules and their effectors involved in the mammalian telecephalon's patterning and regionalization.

1.4. Antero-posterior patterning

The first and most evident process occurring in the mouse developing nervous system from E8.5 is the regionalization along the antero-posterior axis (A/P). By E10.0 forebrain midbrain, hindbrain and spinal cord domains are formed. The patterning of this region is associated with precise antero-posterior expression domains or gradients of several regulatory genes coding for transcription factors.

The early patterning of anterior and posterior neural tissues is mediated trough signals released by the primitive node or organizer, known as Hensen's node in chick, and Spemann organizer in frog. In general, the so called neural-plate organizers are signaling centers located in different positions and established to maintain and further refine positional cell identities along the A/P axis of the neural plate (Rhinn *et al.*, 2006). They produce signals that influence cellular fate, histogenic organization and growth of adjacent tissue in a position-specific manner.

Patterning starts when markers expressed throughout the early neural plate ultimately become restricted to anterior domains of the central nervous system and molecules, including the Wnts, fibroblast growth factors (FGFs) and retinoids (RA), start to function at this stage of development to induce posterior character in the neural plate (Gamse and Sive, 2000). Conversely, antagonists of Wnt factors, including Cerberus and Dickkopf, are expressed in the anterior visceral endoderm and act to maintain and stabilize the anterior neural plate character (Ciani and Salinas, 2005) (See **Fig. 6**).

As a consequence of the Wnt signalling, two different domains are defined along the anteroposterior axis by the expression of two homeobox genes: *Otx2* and *Gbx2*. The Otx-expressing region, rostrally located, will give rise to the forebrain and midbrain, whereas the Gbx2-expressing region, at caudal position, will develop into hindbrain and spinal cord. The boundary between them corresponds anatomically to the isthmus, a narrowing of the neural tube at the border between mesencephalon and metencephalon. Canonical Wnt signaling represses directly *Otx2* expression, whereas it induces *Gbx2* (**Fig. 7I**). Wnts induce also the expression of other two genes, *Irx3* and *Six3,* confining *Six3* to the anteriormost neural territory and promoting posterior expression of *Irx3,* at levels caudal to the presumptive zona limitans intrathalamica (ZLI), subsequently placed between thalamus and prethalamus.

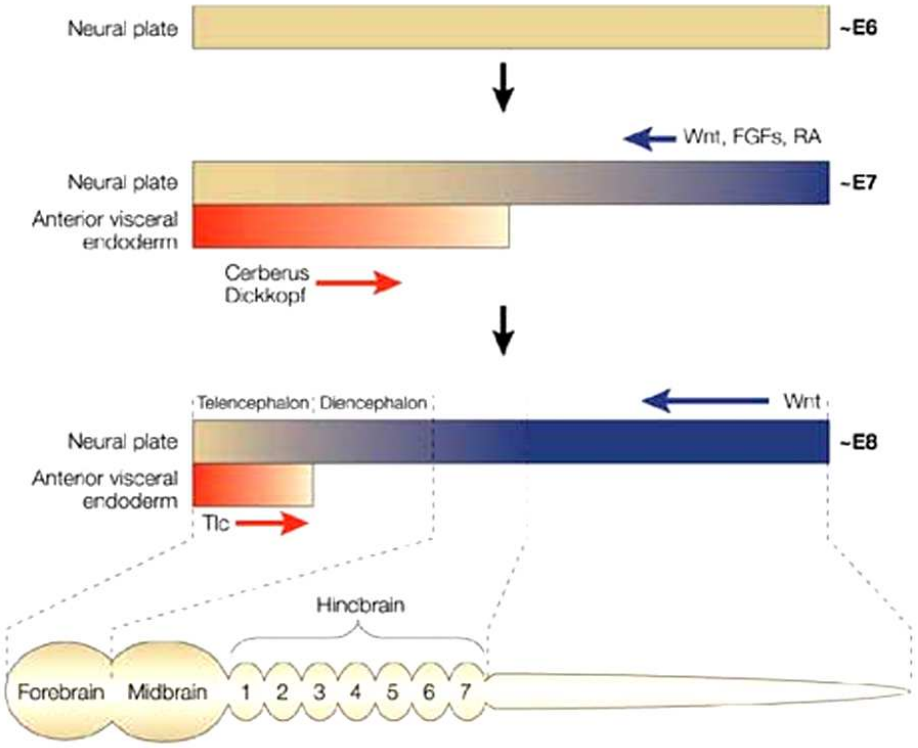

Figure 6 | Antero/posterior patterning of the early mouse neural tube. Image adapted from Rallu *et al.*, 2003

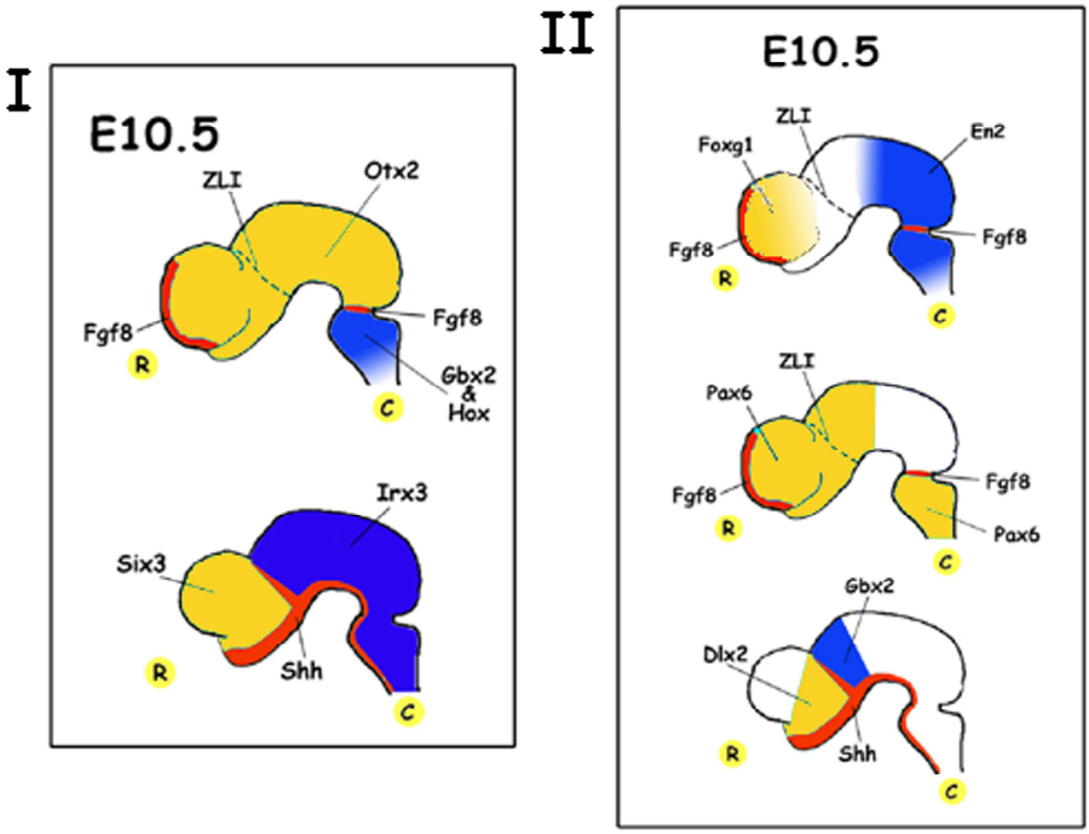

Figure 7 | Schematic expression domains of the principal transcription factors involved in the antero/posterior patterning of the mouse central nervous system at E10.5. Images adapted from Mallamaci A., unpublished.

Upon anterior neural induction, two sources of Fgf molecules are established at the borders of the anterior neural field. One is at the junction between the anterior neural and non-neural ectoderm – that is the anterior neural ridge (ANR) or anterior neural boundary (ANB), the other at the boundary between midbrain and hindbrain fields, i.e. at the isthmus. Both are crucial to subsequent patterning of the anterior brain. The former stimulates the expression of *Foxg1*, a key transcription factor implicated in R/C specification of the telencephalic field, the latter promotes the expression of *En2*, in the anlage of mesencephalon and anterior rhombencephalon.

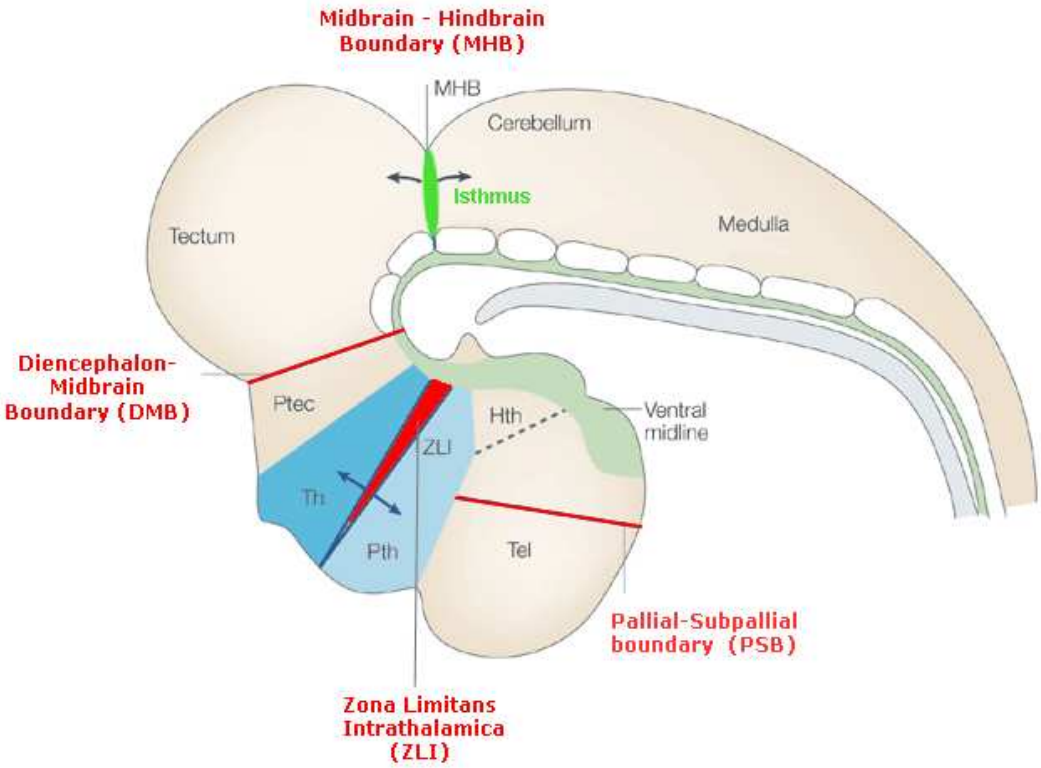

Figure 8 | Schematic view of antero/posterior boundaries along the mouse neural tube. Abbreviations: Ptec, pre-tectum; Pth, pre-thalamus; Tel, telencephalon; Th, thalamus; ZLI, zona limitans intrathalamica. Modified from Kiecker and Lumsden, 2005.

The ZLI, deriving from the collapse of the region between Six3 and Irx3 domains, releases molecules of the Sonic hedgehog (Shh) family (Fuccillo *et al.*, 2006) and splits the anterior neural plate into two distinct domains, able to differentially respond to *Fgf* signaling, expressing either Foxg1 or En2 (Garcia-Lopez *et al.*, 2004). Remarkably, signals coming from the ZLI induce expression of *Gbx2* and *Dlx2* in the thalamus and the prethalamus, respectively.

1.5. Dorso-ventral patterning

Mechanisms responsible for the dorso-ventral (D/V) patterning are well characterized within posterior region of the neuraxis, but many similarities are also found in the telencephalic D/V patterning.

A detailed analysis of molecular mechanisms by which secreted molecules of Shh (Sonic hedgehog), Wnt, RA and Fgf families and transcription factors *Nkx2.1*, *Pax6* and *Gsh2* and *Emx1* sequentially and coordinately control DV regionalization of the rostral neural field was performed by Gunhaga and colleagues in the developing chick embryo (Gunhaga et al, 2000 and 2003; Marklund *et al.*, 2004) (**Fig. 9**). Results of this analysis provide a valuable paradigm for the comprehension of the homologous process in mammals.

Shh is the main signaling molecule involved in ventral patterning at all levels of the nervous system. In the spinal cord, Shh is released by the ventrally located floor plate, whereas at dorsal position the roof plate secretes BMP (bone morphogenetic protein) and proteins of the Wnt family. Corresponding signaling centers are found in the prospective forebrain: BMP molecules (BMP2, BMP4, BMP6, BMP7) and Wnts (Wnt3a, Wnt5a and Wnt2b) are released by the dorsal midline, (which later will give rise to the hippocampal primordium and the choroid plexus) and the paramedial neuroectoderm.

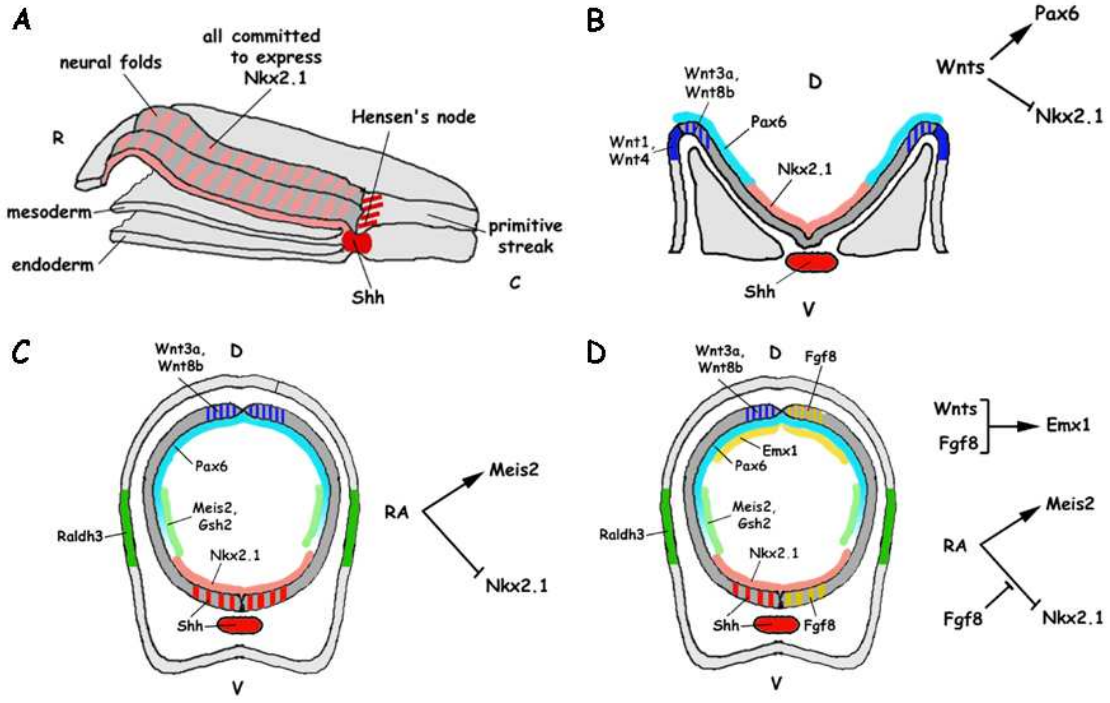

Figure 9 | Schematic view of early phases in dorso/ventral patterning of the rostral neural plate in the chick embryo. Image adapted from Mallamaci A, unpublished.

The initial subdivision that defines what will later become the dorsal and ventral telencephalon is regulated at least in part by the dorsalizing effect of *Gli3* expression and ventralizing influence of Shh. The earliest site of Shh expression appears at E7.5; as neurulation progresses it is initially expressed by both prechordal plate and anterior mesoderm (Fig. 9A), then from the ventral hypothalamus and finally by the ventral telencephalon itself, from the medial ganglionic eminence together with the preoptic area (reviewed in Hoch *et al.*, 2009). Shh specifies ventral identity in the telencephalon by the repression of *Gli3,* a zinc-finger transcription factor crucially involved in dorsal patterning (Rallu *et al.*, 2002). Gli3 is induced by BMPs and is initially expressed broadly throughout the telencephalic anlagen and then is progressively downregulated in the ventral portion of it. Shh signaling neutralizes the repressive form of Gli3, blocking the conversion from the activator (Gli3) to the repressor (Gli3R) and, as a consequence, promoting Fgf expression. In absence of *Gli3,* the development of the dorsal telencephalon is disrupted (reviewed in Herbert and Fishell, 2008). Hence, Shh promotes ventral identity by preventing dorsalization of the telencephalon, rather than by directly promoting ventral cell character. Ventral specification also requires the inhibition of dorsal signals by BMP antagonists, such as noggin (NOG) and chordin (CHRD).

The region that will become the telencephalon is defined by the expression of the forkhead box G1 (*Foxg1*) that promotes Fgf expression, necessary for forming all regions of the telencephalon. Disruption of *Foxg1* expression results in a loss of ventral cell types (Martynoga *et al.*, 2005). Following the *Foxg1* expression the telencephalon becomes subdivided into several distinct territories. Wnt and BMP expression (promoted by Gli3) are required for Empty-spiracle expression (*Emx1,2*), confined to the primary proliferative layer of the cortex. Other transcription factors act subsequently to form specific subdivisions, such as *Pax6, Gsh2* and *Nkx2.1*, crucial for the proper morphogenesis of the lateral cortex, striatum and anlage of globus pallidus respectively (**Fig. 9, 10**). In the absence of any of them, the corresponding morphogenetic field is shrunken and the adjacent ones substantially enlarged (Sussel *et al.*,1999, Toresson *et al.*,2000, Stoykova *et al.*,2000).

Pax6 and *Gsh2* play complementary roles reciprocally compartmentalizing and establishing pallial and subpallial identities. In *Pax6* null mice, there is a dorsal expansion of markers of ventral progenitors, such as *Mash1*, *Gsh2* and *Dlx2*, whereas in $Gsh2^{-/-}$ is the opposite (Yun *et al.*, 2001).

Mash1 and *Ngn1/Ngn2* are proneural genes, that play important roles in the development of the ventral and dorsal telencephalon, respectively, However they do not act as "master genes", but simply link regional patterning to activation of specific neuronogenetic pathways in these structures (Fode *et al.*,2000, and Yun *et al.*, 2002).

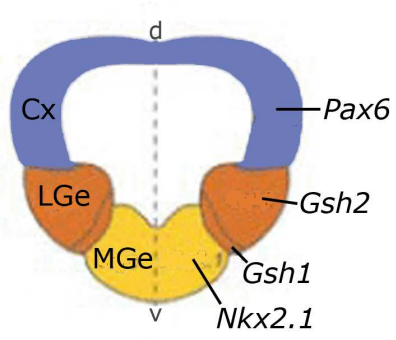

Figure 10 | Schematic representation of main transcription factors involved in regionalization of the early cortical primordium. Coronal section of mouse telencephalon at E10. Image adapted from Lupo *et al*., 2006

Retinoic acid (RA) has a crucial role in specifying telencephalic progenitor cells of intermediate character and in controlling lateral ganglionic eminence specification. Its action is opposed by FGF signaling, involved in maintaining ventral progenitor character and medial ganglionic eminence specification (Marklund *et al.*, 2004).

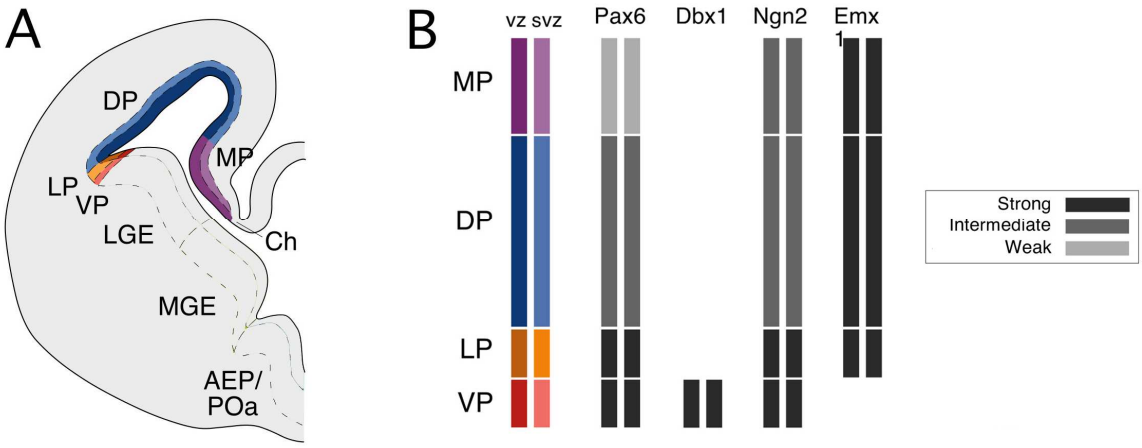

Figure 11 | Schematic representation of main genes expressed in the pallial domains of mouse telencephalon at E16. Abbreviations: AEP, anterior entopeduncular area; DP, dorsal pallium; LP, lateral pallium; MP, medial pallium; dLGE, dorso-lateral ganglionic eminence; MGE, medial ganglionic eminence, POA, anterior preoptic area; vLGE ventro-lateral ganglionic eminence; VP, ventral pallium.

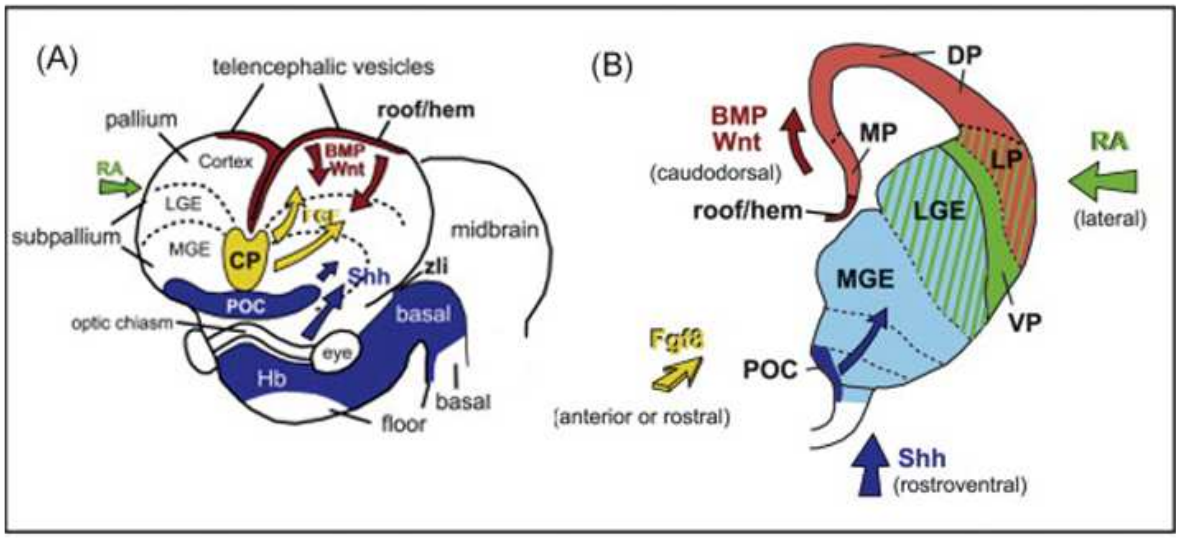

Figure 12 | Schematic view of the main signaling centers and signaling proteins involved in mouse pallial patterning. ANR, anterior neural ridge; CP, commissural plate, DP, Dorsal pallium; LGE, lateral ganglionic eminence; LP, lateral pallium; MP, medial pallium; MGE, medial ganglionic eminence; RA, retinoic acid; VP, ventral pallium. Image taken from Medina and Abellan, 2009.

In conclusion, the coordinate action of different signals including BMP/Wnt, RA, Fgf8 and Shh and the differential activation of their targets in space and time establish the side where the pallium will develop and regulates its size. Remarkably, as reported below, some of these signalling systems and their targets are subsequently involved in further subdivision of the pallial anlage, in a process termed cortical regionalisation and arealization.

1.6. Specification of cortical area identities

Arealization of the developing cortex is a very complex process beginning in the mouse at mid-gestational ages with areal commitment of neuronal progenitors and completed after birth. Form a functional point of view, the neocortex can be subdivided in subdomains, called areas. Mature cortical areas differ by their location within the cortex, molecular properties, histological organization, patterns of connectivity and function. Within adult the neocortex, rostral regions regulate motor and executive functions, whereas caudal regions process somatosensory, auditory, and visual inputs (**Fig. 13A**). These different cortical areas have a precise connectivity, particularly with nuclei within the dorsal thalamus, which provides some of the principal inputs to the cerebral cortex (Sur and Rubenstein, 2005).

Two models have been proposed to explain how cerebral cortex gets organized into distinct areas: the protocortex model (O'Leary. 1989) and the protomap model (Rakic. 1988). The first model suggests that the early cortical primordium would be like a "tabula rasa" and each region composing it would not display any areal bias at all. In such a model, cortical arealization is primed by information born by afferents coming from different thalamic nuclei, each preferentially projecting to a different presumptive cortical area. According to the second model, the protomap model, cortical arealization would take place on the basis of information intrinsic to the early cortical primordium. Positional values would be encoded by the graded expression of specific genes within the cortical proliferative epithelium. This positional information would be epigenetically transferred from neuroblasts to neurons in distinct cortical regions, eventually leading to the activation of different areal morphogenetic programs.

Presently, it is accepted that arealization is controlled by both intrinsic and extrinsic factors: early phases of the process occur before the axons coming from the thalamus reach the cortex, on the basis of cortex-autonomous cues (in accordance to the protomap model); late phases occur after the arrival of first thalamocortical afferences and are partially influenced by them (Ragsdale and Grove, 2001; O'Leary and Nakagawa, 2002).

Genetic control of this process is very sophisticated and is based on a complex interplay among diffusible ligands, released by signalling centres at the perimeter of the cortical morphogenetic field, and transcription factors genes, expressed by periventricular neuronal progenitors, gradually along the main coordinate axes of this field. Diffusible ligands may spread a large distance through cortical field and generate concentration gradients, promoting graded expression of transcription factors genes, able to activate subsequently distinctive area-specific programs (O'Leary and Nakagawa, 2002).

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1.6.1 Patterning centers involved in cortical arealization

Three complex signalling centres at the borders of the cortical field are crucial for the arealization process (and previously for regionalization of the early forebrain):

1) the cortical hem, corresponding to the medial margin of the cortical primordium, between the cortical and the choroidal fields. It expresses multiple *Wnt* and *Bmp* genes (Grove *et al.*,1998) and disappears by the time of birth. Many evidences support the hypothesis that it is necessary for proper development of caudal-medial cortical areas,

in particular for hippocampal formation, presumably by expanding an already specified population of cells through proliferative signals (Grove and Fukuchi-Shimogori, 2003). In mice deficient of *Wnt3a*, one of *Wnt* genes expressed at the hem, the hippocampus is nearly absent, whereas neighboring neocortical areas appear grossly normal (Lee *et al.*,2000). The formation of the cortical hem is dependent on LIM-homeodomain factors, in particular *Lhx2* and *Lhx5*; loss of *Lhx2* expands dramatically the hem and choroid plexus at the expense of the cortex (Monuki *et al.*, 2001), targeted deletion of *Lhx5*, leads to loss of choroid plexus and cortical hem, and impaired development of the hippocampus (Zhao *et al.*, 1999).

2) the commissural plate (CoP), placed at the rostromedial pole of telencephalon, is the derivate of the anterior neural ridge (ANR), formed by fusion of the neuralplate folds at the anterior margin of the forebrain. It is an anterior patterning center for arealization and is a source of FGF proteins, in particular Fgf8, previously shown to regulate early forebrain patterning, and Fgf17.
3) the cortical antihem, which forms on the lateral side of the cortical field, at the pallial–subpallial boundary. It releases members of the EGF family, Tgfα, Ngr1 and Ngr, and signaling molecules such as Fgf7, Fgf15 and sFrp2, antagonizing Wnt signaling coming from the hem (Assimacopoulos et al, 2003). These signals serve as guidepost signals for axons and interneurons migrating through this boundary.

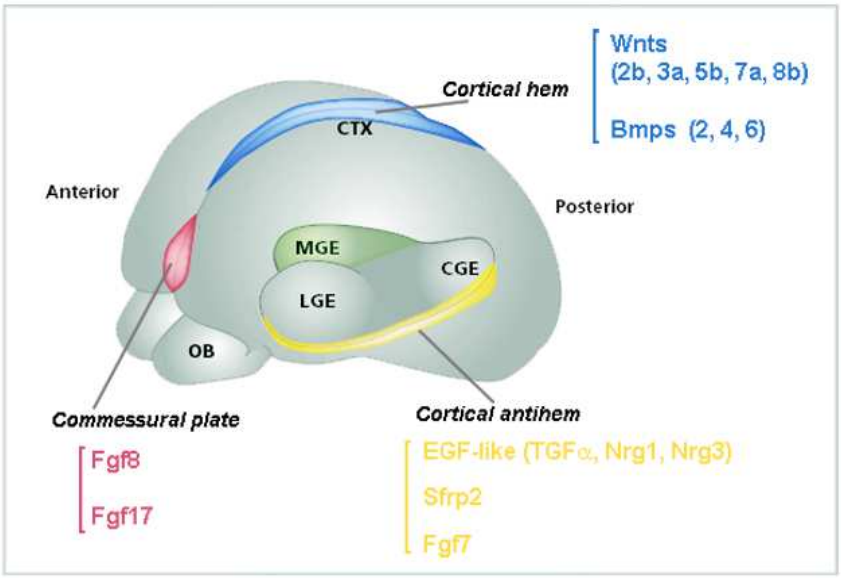


Figure 13 | Localization of signaling centers involved in cortical arealization. Abbreviations: Ctx, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; CGE, caudal ganglionic eminence; OB, olfactory bulb. Adapted from Corbin *et al*, 2001.

1.6.2 Transcription factors involved in areal identities

Before the onset of neurogenesis, transcription factors TFs in the cortical neuroephithelium function mainly to promote forebrain regionalization (establishment of distinctions and boundaries between dorsal and ventral telencephalon), areal patterning, and progenitor proliferation and suppress neuronal differentiation. Areal patterning depends on setting up rostrocaudal and mediolateral gradients of TF expression, which are modulated by diffusible signals released by patterning centers at the edges of the cortical field. By experimental perturbation of their gradients, some genes have been shown to be particularly important in areal pattering, and they are: *Emx2*, *Emx1*, *Pax6*, *Lhx2*, *Foxg1*, *Coup-tf1* and *Sp8* (**Fig. 14**).

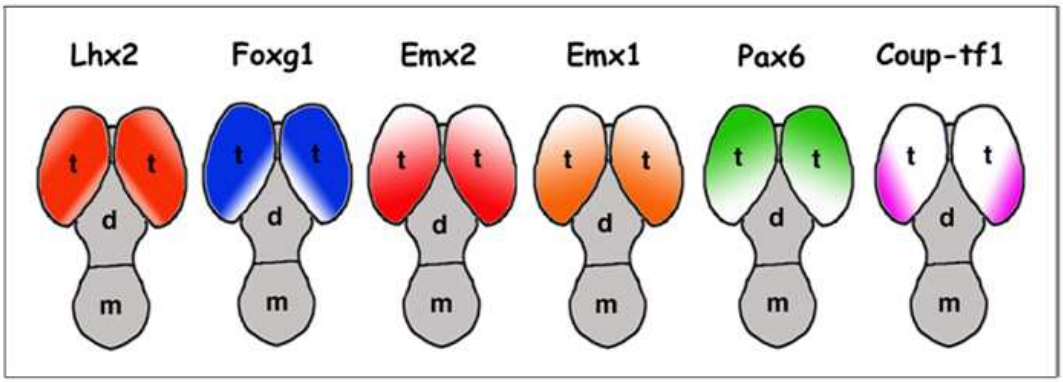


Figure 14 | Graded transcription factors genes expression in the early cortical primordium. Schematic representation of E10 mouse brains. Abbreviations: t, telencephalon; d, diencephalon; m, mesencephalon. Image taken from Mallamaci and Stoykova, 2006.

The onset of neocortical neurogenesis is directed by a number of genes expressed across the dorsolateral wall of the telencephalon. These include LIM homeobox 2 (*Lhx2*), forkhead box G1 (*Foxg1*), empty spiracles homologue 2 (*Emx2*) and paired box 6 (*Pax6*), each of which has crucial roles in specifying the progenitors that give rise to the projection neurons of the neocortex. Together, these four genes establish the neocortical progenitor domain by repressing dorsal midline (*Lhx2* and *Foxg1*) and ventral (*Emx2* and *Pax6*) fates.

Foxg1, expressed in the early telencephalon, is relevant for basal ganglia morphogenesis and cortical neuroblast differentiation. Loss of Foxg1 causes agenesis of the basal ganglia, elimination of neocortical progenitor domains and expansion of archicortical and cortical hem progenitors (Muzio and Mallamaci, 2005) (**Fig. 15**). Remarkably, *Foxg1* removal as late as E13.5 from progenitors that already have a neocortical identity, results in the production of cells with characteristics of Cajal–Retzius

cells (Hanashima *et al.*, 2004; Shen *et al.*, 2006), indicating that the persistent expression of *Foxg1* throughout neurogenesis is required for the maintenance of neocortical progenitor identity.

Lhx2 is expressed in the whole telencephalic neuroepithelium except the cortical hem, it commits neuroblasts within the dorsal telencephalon to cortical fates and, within the cortical field, it promotes hippocampal vs neo- and paleocortical programs. In the absence of Lhx2, neocortical progenitors of medial cortex and the hippocampus are lost, whereas the cortical hem and choroid plexus (structures normally limited to the dorsal midline) are expanded (Monuki *et al.*, 2001) (**Fig. 15**).

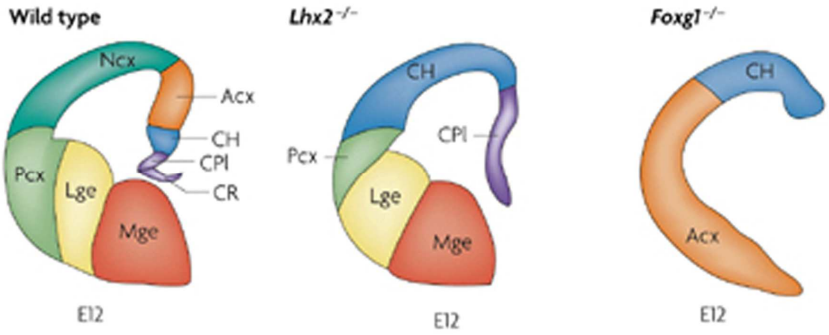


Figure 15 | Mutant phenotypes of mice knock-out for *Lxh2* and *Foxg1* transcription factors involved in cortical specification. Abbreviations: CH, cortical hem; CR, choroidal roof; ChP, choroid plexus; Cx, cortex; Lge, lateral ganglionic eminence; Mge, medial ganglionic eminence; Pcx, paleocortex. Adapted from Molyneaux *et al.*, 2007

The two genes *Emx2* and *Pax6* are expressed in opposing and overlapping gradients along the A/P and D/V axes of the cortical primordium, and are key determinants of the proper development of cortical areas. Loss of both *Emx2* and *Pax6* results in ventralization of cortical progenitors and the loss of the neocortical domain (Ncx), archicortex (Acx), cortical hem (CH) and choroid plexus (CPl) by E14 (Muzio and Mallamaci, 2002). In particular, *Emx2* is expressed in the primary proliferative layer of the cortex along rostral/lateral low-to-caudal/medial high gradients (Gulisano *et al.*, 1996) (**Fig. 16**), being more expressed in V1 and less in frontal/motor areas. In *Emx2* knockout mice, occipital cortex and hippocampus are shrunken and frontal cortex is enlarged (**Fig. 17**). Moreover, the areal distribution of the thalamo-cortical radiation is perturbed, coherently with such areal disproportions. *Pax6* opposes the pattern of *Emx2* expression, showing a low posterior-medial to high anterior-lateral gradient, mostly expresses in frontal/motor cortex

(Stoykova *et al.*, 1994). As assessed by molecular profiling, in the absence of either *Emx2* or *Pax6*, the full repertoire of areal identities is still encoded.

Within the neocortical field, *Emx2*, *Pax6*, *COUP-TFI* and *Sp8* have a direct role in arealization determining size and position of cortical areas (Reviewed in O'Leary *et al.*, 2007) (**Fig. 17**). In general, TFs confer different area identities to cortical cells within distinct parts of the field, allowing for proper expression of axon guidance molecules that control the area specific targeting of thalamo-cortical afferents.

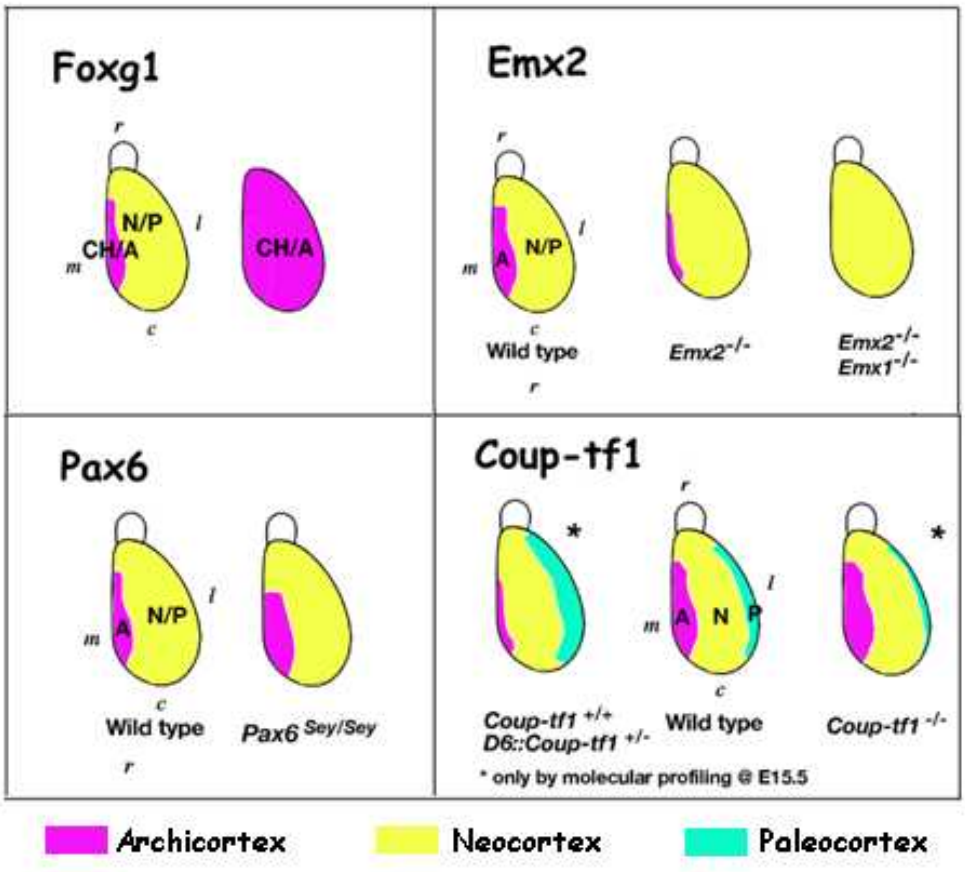


Figure 16 | Key transcription factors involved in main repartitions of cortical field and loss-of-function or gain-of-function mice mutant phenotypes. Image taken from Mallamaci A., unpublished.

Coup-Tf1 (Chick Ovalbumin Upstream Transcription Factor I), is expressed in the ventricular zone, subplate and cortical plate along a high-caudal to low-rostral graded expression across the neocortex (Liu *et al.*, 2000) (**Fig. 17**). It acts downstream of *Emx2* and *Pax6*, being necessary to make the cortical field responsive to their patterning activity (Zhou *et al.*, 2001). Recent analysis of conditional knockout mice reveals a massive expansion of frontal/motor area in the absence of this TF, paralleled by a reduction in size of the three primary sensory areas (Armentano *et al.*, 2007) (**Fig. 17**).

Sp8 is expressed in a high anterior-medial to low posterio-lateral gradient by cortical progenitors. It is a direct transcriptional activator of *Fgf8* expression within the CoP (Sahara *et al.*, 2007). Analysis of conditional *Sp8* knockout mice at late embryonic ages show an anterior shift of cortical markers, suggesting *Sp8* preferentially specifies identities associated with frontal/motor areas (Zembrzycki *et al.*, 2007) (**Fig. 17**).

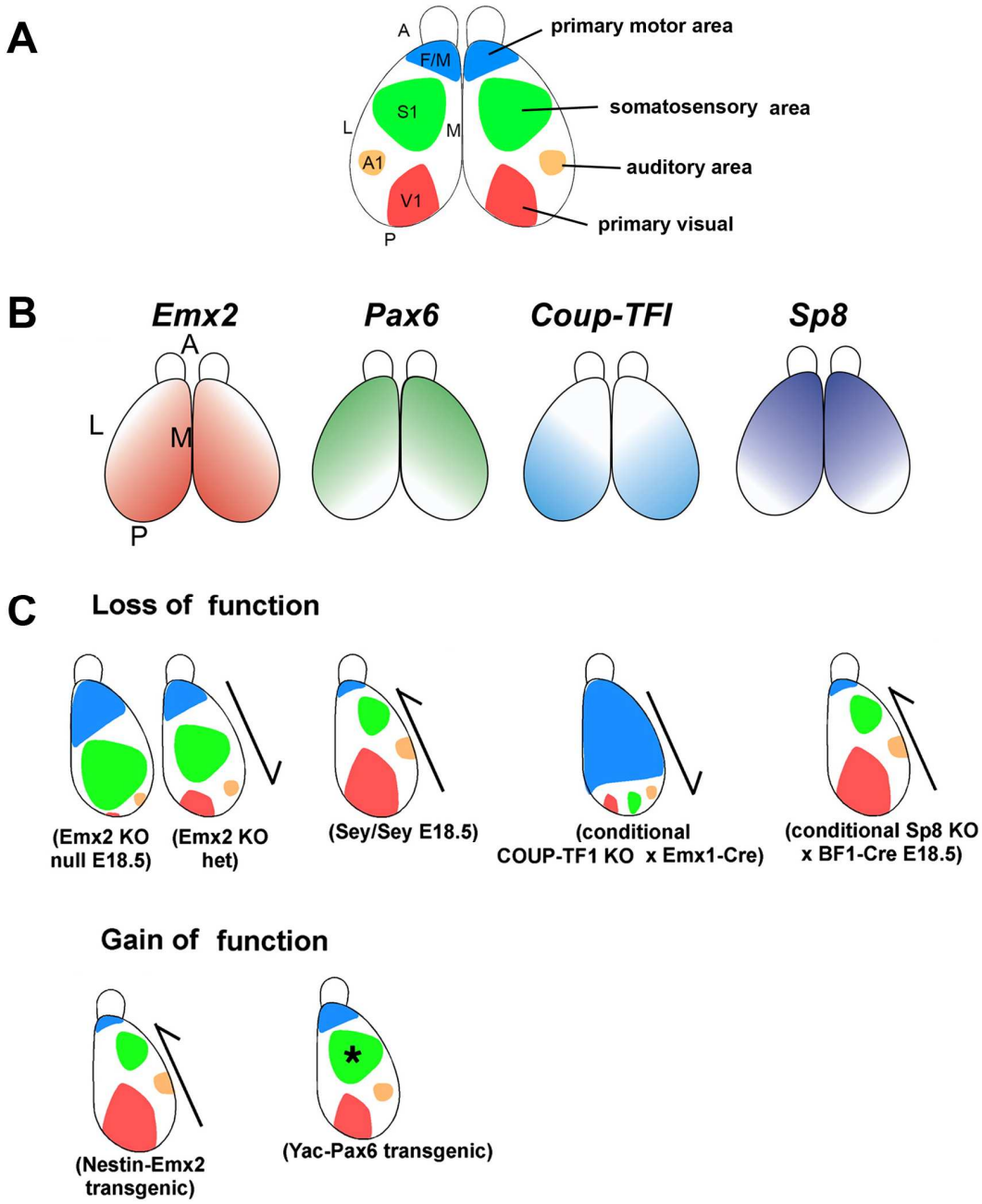


Figure 17 | Summary of area patterning and mutant phenotypes. (A) Schematic diagram of anatomically and functionally distinct areas in the mouse. (B) Graded expression of transcriptor factors along the anterior-postirior and lateral-medial axes (C) Summary of all reports of loss-of-function or gain-of-function mice mutant for TFs that regulate area patterning. Image modified from O'Leary *et al.*, 2007.

2. MOUSE CORTICAL DEVELOPMENT

During mammalian cerebral cortical development, neural stem cells present within periventricular generative zones of the pallium first extensively proliferate, so allowing for an impressive tangential expansion of the structure, then give rise to successive waves of neurons and radial glia, followed by oligodendrocytes and astrocytes.

Neural progenitors are initially generated in a proliferative layer adjacent to the lateral ventricles called the ventricular zone (VZ). The first postmitotic cortical neurons form a transient structure called the preplate (PP). The PP persists until embryonic day (E) 13 in mice, when the earliest cortical plate cells reach the upper part of the neuroepithelium and divide the PP into two regions: the superficial marginal zone (MZ) (future layer 1) and the lower subplate (SP) (Marin-Padilla, 1971, 1972). The cortical plate (CP), which will become the mature six-layered neocortex, is formed between these two layers according to an "inside-out" neurogenetic gradient, with later generated neurons bypassing early-generated cells to settle at the top of the cortical plate, forming the upper layers of the cerebral cortex. As cortical development proceeds, an additional proliferative zone, called the subventricular zone (SVZ), appears on top of the VZ. It will initially give rise to projection neurons and subsequently to glia (Baltman SA and Altman J, 1991).

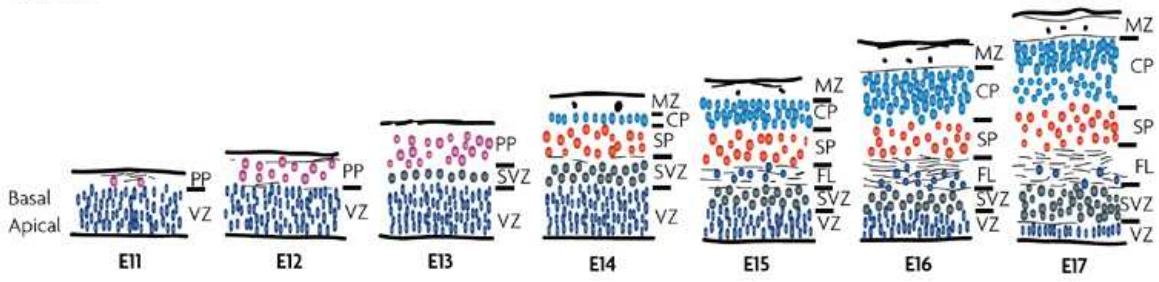


Figure 18 | Mouse cortical neurogenesis. Abbreviations: CP, cortical plate; FL, intermediate zone, PP, preplate; MZ, marginal zone; SP, subplate; SVZ, subventricular zone; VZ, ventricular zone.

In the mouse, cortical neurogenesis begins around E10.5 and last up to E17 (**Fig.18**). First generated neurons are the Cajal-Retzius cells (CR), which secrete Reelin, an extracellular matrix protein that play a fundamental role for the formation of cortical layers during development and its maintenance in adulthood (Frotscher *et al.*, 2009). CR neurons arise from restricted locations at the borders of the developing pallium, the hem, the anti-hem and the septum (Takiguchi-Hayashi *et al.*, 2004; Yoshida *et al.*, 2006), and spread into the cortex by tangential migration. Subplate cells have a role in directing the first thalamic axons to the pallium (Allendoerfer and Shatz, 1994).

2.1. Cortical Progenitors in Rodents

Different types of progenitors, characterized by the expression of different genes, contribute to cortical neurogenesis. Two principal classes have been identified on the basis of their nucleus position during the M-phase of the mitotic cycle:

(1) *apical progenitors*, so called because dividing at the ventricular (apical) surface of the VZ and expressing Pax6 gene (**Fig.20**) (Englund *et al.*, 2005). They include neuroepithelial cells (NE), radial glia cells (RGCs) and short neuronal precursors (SNPs) **(Fig.19a)** (Götz and Huttner, 2005; Gal *et al.*, 2006).

(2) *basal* or *intermediate progenitors* (IPC), that undergo division away from the ventricular surface, often at the VZ/SVZ border **(Fig.19b)** (Götz and Huttner, 2005; Kriegstein *et al.*, 2006) and express the transcription factor Tbr2 (**Fig.20**) (Englund *et al.*, 2005).

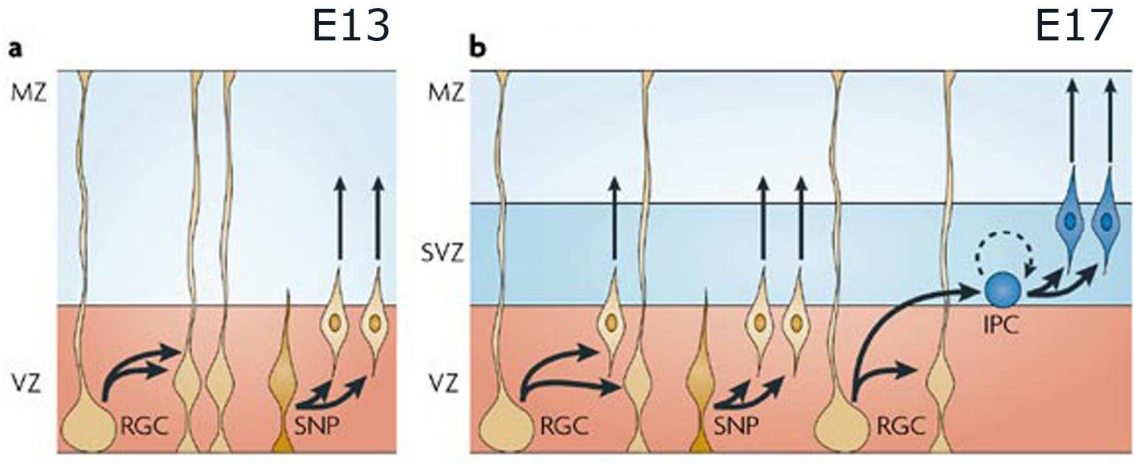


Figure 19 | Schematic overview on different types of mouse cortical precursors. Abbreviations: IPCs, intermediate progenitor cells; RGCs, radial glia cells; SNP, short neural precursors. Image modified from Dehay and Kennedy, 2007.

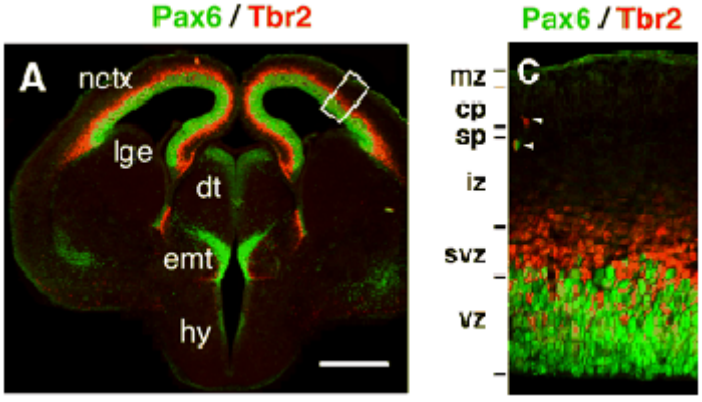


Figure 20 | Pax6 and Tbr2 protein expression in E14.5 mouse coronal section. Tbr2^{+} cells are expressed throughout corticogenesis and detectable also in the VZ and in double positive Pax6/Tbr2 cells represent the transition from glia to IPC, where Pax6 is substantially downregulated Image adapted from Englund *et al.*, 2005.

Neuroepithelial cells are the primary stem/progenitor cells, initially expanding via symmetric division and then undergoing asymmetrical divisions with the onset of neurogenesis, producing preplate neurons (reviewed in Götz and Huttner, 2005) and generating distinct types of secondary neural stem cells and progenitor cells.

An important characteristic of apical progenitors is that they undergo interkinetic nuclear migration (INM), in which the nucleus moves along the apico-basal axis of the cell in concert with the cell cycle (Sidman and Rakic, 1973; Takahashi *et al.*, 1993; Götz and Huttner, 2005). Cell nuclei migrate from the apical surface to a basal position during the G1 phase of cell cycle, replicate their DNA in S-phase far from the ventricle, and during G2 return to the apical surface, where mitosis finally takes place (**Fig.21**).

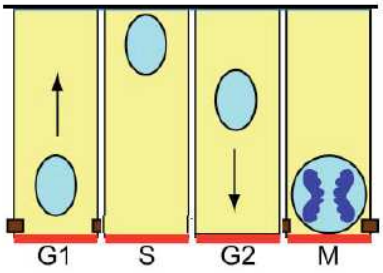


Figure 21 | Schematic cartoon representing interkinetic nuclear migration. Cell nuclei, in light blue, move during the cell cycle along the apico-basal axis. The apical surface corresponds to the red line.

After the closure of the neural tube, and in particular with the onset of neurogenesis, neuroepithelial cells start to express glial markers and transform into the related radial glial cells (RGCs). Like neuroepithelial cells, RGCs exhibit apical-basal polarity and span the entire cortical wall, with an apical end-foot (apical process) at the ventricular surface, and a basal end-foot (basal process) at the pial surface. RGCs not only act as migratory guides for newly generated neurons, but also give rise to the majority of cortical neurons (Noctor *et al.*, 2001; Miyata *et al.*, 2004). Imaging studies have shown that mouse RGCs undergo symmetrical and asymmetrical divisions, including self-renewing and neurogenic divisions, producing either a neuron or a further type of neuronal progenitor (SNP or IPC) (**Fig.19**) (Noctor and Kriegstein, 2004).

Short neuronal precursors (SNPs) are dedicated neuronal progenitors that posses a short basal process (not reaching the basal lamina), populate the VZ and SVZ zones, divide at the apical surface of the VZ and are molecularly identifiable by the specific firing of the α1-tubulin promoter (pTα1^{+} cells) (Gal *et al.*, 2006; Ochiai *et al.*, 2009).

Basal progentors (BPs), also called non-surface or subventricular zone or intermediate progenitors (IPCs), constitute the second class of neural progenitors in rodents (Noctor *et al.*, 2004; Götz and Huttner, 2005). They derive from asymmetrical division of

RGCs, migrate basally and retract both apical and basal process, before division. IPCs do not exhibit interkinetic nuclear migration and mitosis are randomly located in the basal VZ and SVZ. Most IPCs divisions are symmetric, self-consuming, producing two to four neurons (Noctor *et al.*, 2004; Miyata *et al.*, 2004; Kriegstein *et al.*, 2006; Noctor et al, 2008). However, a small fraction of them (≈10%) appears to be capable of self-renewal, undergoing symmetrical proliferative divisions to expand the IPCs pool in the SVZ (see dotted circular arrow in **Fig.19b**), (Noctor et al, 2004; Wu *et al.*, 2005). Basal progenitors produce only neurons and not glial cells. Until recently, it was thought that they would have given rise mostly to upper layer neurons (Zimmer *et al.*, 2004). Presently, the reported prevalence of basal mitosis during early stages, before SVZ formation and the recent work of Kowalczy and collaborators (Kowalczyk *et al.*, 2009) suggests that IPCs produce pyramidal projection neurons of all cortical layers and not specialized type of neurons.

Hypotheses concerning the role of basal progenitors and the significance of having two distinct pathways for cortical neurogenesis support the idea that the indirect pathway may provide a mechanism to increase the number of neurons generated by a single radial stem cell, so contributing to the lateral expansion of the cerebral cortex (**Fig.22**) (Kriegstein *et al.*, 2006; Cerdeño *et al.*, 2006). The tendency in corticogenesis is amplifying the neuronal production by an increase in the rate of neurons per stem cell production and not by an increase in the frequency of differentiative divisions (Polleux *et al.*, 1997). Moreover, the slowing down of neuron production during the later phases of corticogenesis is not mainly due to the slowing down of cell-cycle progression but rather reflects the exhaustion of the precursor pool (Dehay and Kennedy, 2007). So, a possible two step model of neurogenesis has been proposed: in the first step, asymmetric radial glial cell divisions generate neuronal diversity, in the second step, symmetric intermediate cell division produce large numbers of neurons of the same subtype.

Temporally ordered changes in gene expression patterns have been observed in radial glia cells and in their progeny during stages of cortical layer formation. RGCs and IPCs make the commitment to neurogenesis during G1 phase (Haubensak *et al.*, 2004) and laminar fates are selected around S/G2 transition (McConnell *et al.*, 1991). Asymmetric division of RGCs allows genes governing cell identity to be differentially inherited in basal progenitor cells generated in different cell cycles. Symmetric division of IPCs produced in a given cell cycle permits a stable inheritance of identity genes by both of their progeny, producing large number of neurons of an identical subtype appropriate for that particular

stage of development, but different from those produced by basal progenitors cells that where generated in previous or subsequent cell cycles (**Fig.22c**).

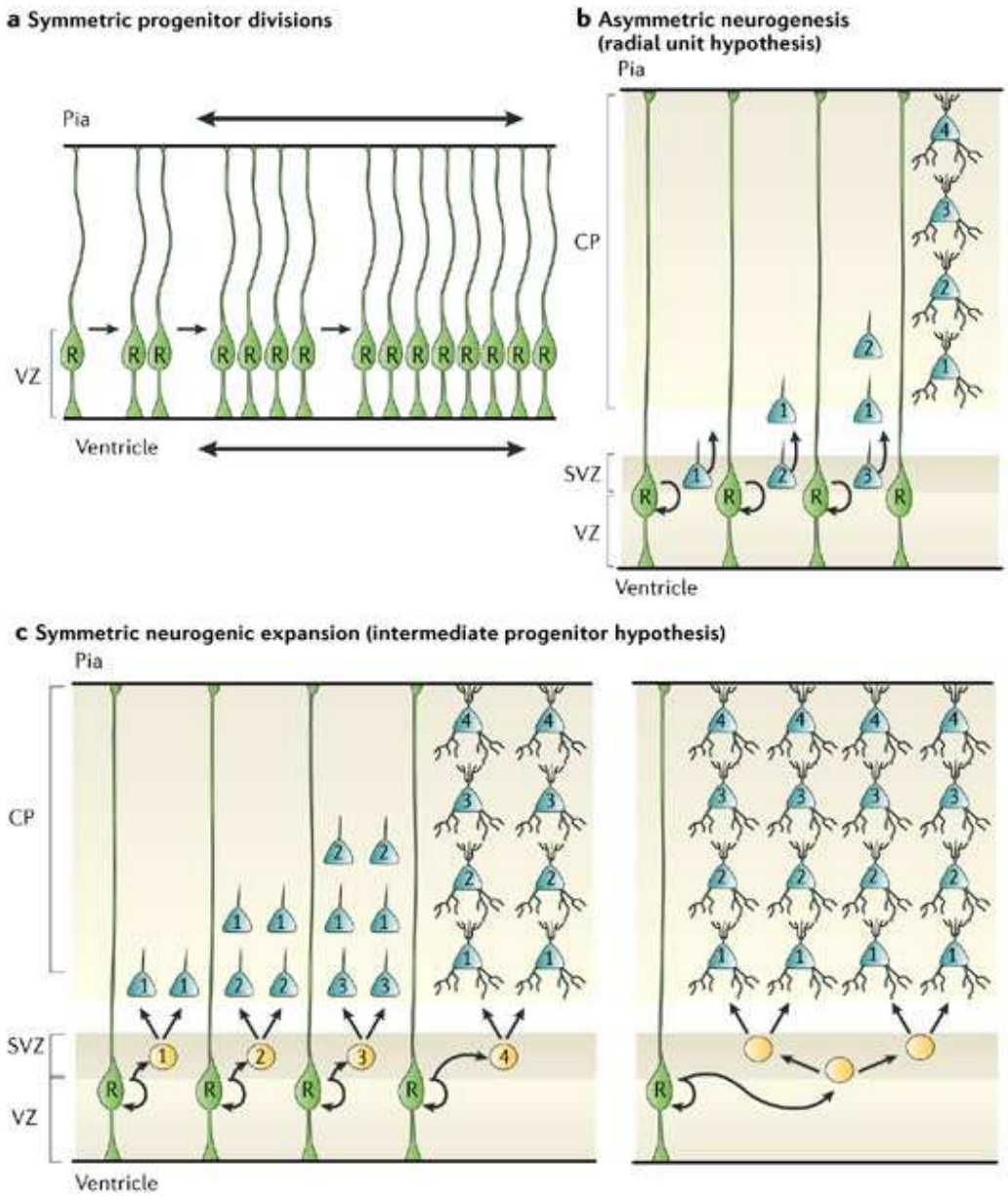


Figure 22 | Modalities of progenitors cells division observed in the embryonic cortex during development. a) Symmetric progenitor divisions increase the number of radial cells. b) Asymmetric neurogenic divisions give rise to self-renewed radial glial cells and neurons (blue) destined for different layers in the cortical plate. c) Symmetric neurogenic divisions of intermediate progenitor cells (yellow) in the SVZ resulting in the amplification of cells of the same type that have the same birth dates and occupy the same cortical layer. Progenitors can undergo additional symmetric divisions in the SVZ before terminal neurogenic divisions (right panel). Abbreviations: CP, cortical plate; R, radial glia; SVZ, subventricular zone; VZ, ventricular zone. Image taken from Kriegstein *et al.*, 2006.

A large number of transcription factors regulate the choice between proliferation and differentiation, inhibiting or promoting the exit from the cell cycle. In particular, *Emx2* and *Tlx* genes favor progenitor proliferation (Heins *et al.*, 2001; Muzio *et al.*, 2005; Roy *et al.*, 2004), *Pax6* promotes the maintenance of the size of the cortical progenitor pool (Quinn *et al.*, 2007). Proneural genes (*Ngn1* and *Ngn2*) promote neuronal fate commitment, whereas members of the *Hes* and *Id* families are important inhibitors of neurogenesis. RGCs cells are maintained in the proliferative state by the simultaneously action of different genes (such as *Emx2*, *Hes1*, *Hes5*, *Id3*, *Id4*) **(Fig. 23)**. The direct transition from radial glia to newborn neurons is regulated by *Ngn1* and *Hes5* genes and correlates with downregulation of radial glia marker *Pax6* and upregulation of postmitotic neuronal markers *Tbr1*, *Math2*, and *neuroD2* (Englund *et al.*, 2005; Schuurmans *et al.*, 2004; Schwab *et al.*, 1998).

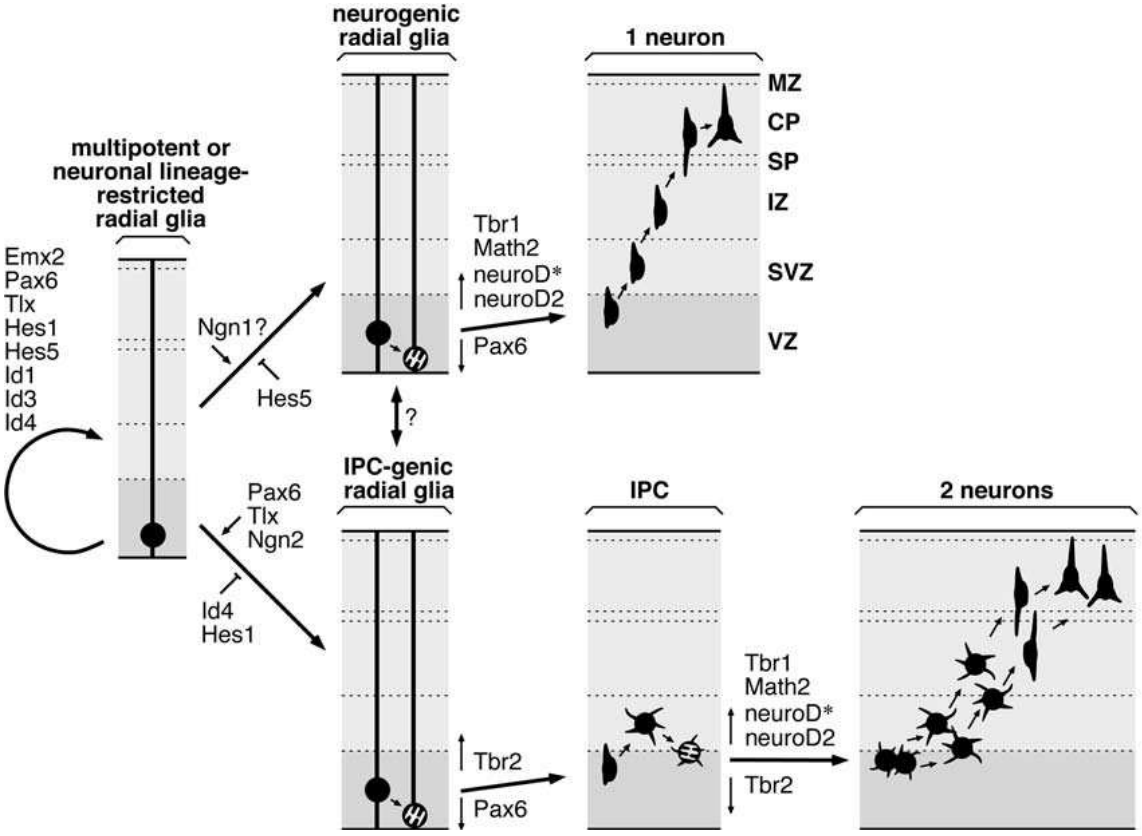


Figure 23 | Model for TF regulation of direct and indirect pathways of cortical neurogenesis. Image taken from Hevner, 2006.

In the case of indirect neurogenesis, the transition from radial glia to basal progenitors involves upregulation of *Tbr2* and downregulation of *Pax6* (Englund *et al.*, 2005) (**Fig.24**). The subsequent transition from IPCs to neurons correlated with downregulation of *Tbr2* and upregulation of *Tbr1*, *Math2*, and *NeuroD2*, *NeuroD* (which are all expressed by newborn cortical projection neurons, at least transiently). So, the following TF sequence *Pax6* → *Tbr2* → *Tbr1* can be established in the transition from RGC → IPC → postmitotic neuron (Englund *et al.*, 2005).

In Pax6 $^{-/-}$ embryos, radial glial progenitors present defects in their mitotic cycle, molecular phenotype and morphology (Götz *et al.*, 1998). Moreover, a loss of Tbr2^{+} cells corresponding to basal progenitors can be identified, indicating that Pax6 is necessary for the activation of Tbr2 expression (Quinn *et al.*, 2007). The expression of Pax6 protein in cortical progenitors determines also the expression of the proneural gene Ngn2, providing evidence of a direct regulatory link between neural patterning and neurogenesis (Scardigli *et al.*, 2003).

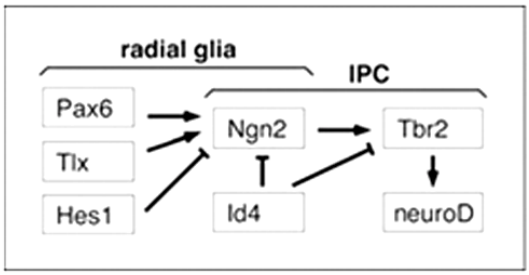


Figure 24 | Transcription factors implicated in regulating IPC production from radial glia. A balance of TFs promotes (Ngn2, Pax6 and Tlx) or inhibits (Hes1, Id4) IPCs production from radial glia. Image taken from Hevner, 2006.

2.2. Neuronal subclass specification

Neocortical neurons are organized in six layers and are generated sequentially over time. The pattern of projection of pyramidal neurons is layer-specific. Layers II–III contain pyramidal neurons which form cortico-cortical connections, including projections to the contralateral hemisphere across the corpus callosum. Layer VI neurons project to the thalamus. Layer V neurons mainly project to several subcortical targets including the spinal cord, pons and superior colliculus; a subset of layer V neurons contributes to cortico-cortical connections. The thalamocortical axons make direct synaptic contact neurons of layer IV.

Once the commitment to neurogenesis is made by cortical precursors, additional transcription factors consolidate differentiation of projection neurons phenotype and begin to specify projection neurons subtypes related to the laminar fate. Laminar fate is tightly linked to cell birthday (Takahashi *et al.*, 1999) and is determined in progenitor cells during their final mitotic cycle (McConnell and Kaznowski, 1991). They then continue as postmitotic neurons and migrate to their destination within the cortical plate. Birth-dating experiments in rodents coupled with manipulation of the cellular environment suggested that cell fate is determined prior to migration (Caviness, 1982). Both extrinsic and intrinsic factors cooperate in determining the fate of cortical precursors and different neuronal phenotypes. Lineage studies of isolated cortical precursors shown a cell-intrinsic program (mainly TFs) (Shen *et al.*, 2006) that is influenced by extrinsic factors from neighboring cells (i.e. Notch signalling) (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996).

A series of neuronal sublineage genes, in particular transcriptions factors, have been described that are expressed by subsets of neurons in specific cortical layers, as well as by neuronal precursor cells during the specific developmental periods when those neurons are generated. For example, *Otx1* and *Fezf2* are expressed early in cortical development by neuronal precursors cells in the VZ and SVZ and later, by subsets of deep cortical neurons (Molyneaux *et al.*, 2007), whereas *Cux2*, *Satb2*, *Nex* and the non-coding RNA *Svet1* (Zimmer *et al.*, 2004; Nieto *et al.*, 2004; Britanova *et al.*, 2005; Tarabykin *et al.*, 2001) are selectively expressed in both upper layer neurons and SVZ progenitors.

A number of specific molecular markers for neurons of distinct cortical layers have been recently identified (**Fig.25**) and these represent a very useful tool for the elaboration

of a more comprehensive classification of the cortical neurons. Many of these genes are not expressed uniformly within a given layer, and often their expression demarcates boundaries between different cortical areas.

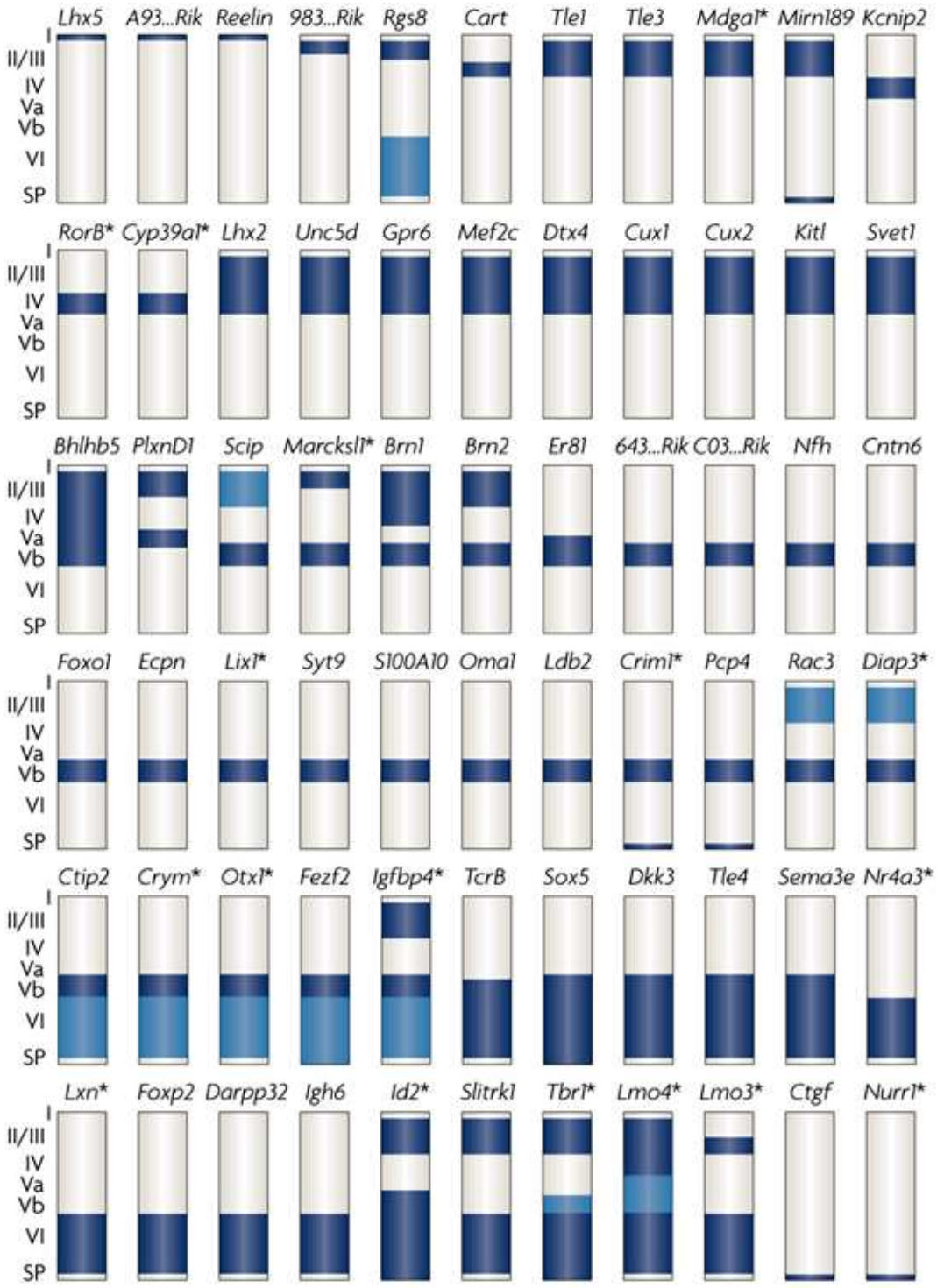


Figure 25 | Gene expression patterns in the developing mouse cerebral cortex during mid-neurogenesis and early postnatal life. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. From Leone *et al.*, 2008.

Fate determination of neurons generated later in time, might not only be a consequence of combinatorial patterns of gene expression, but can also depend on the suppression of early fate signals, as in the case of *Foxg1*, which suppress the fate of early born neurons (Cajal Retzius cells) in order to permit the generation of later born neurons (Hanashima *et al.*, 2004).

An example of how an elegant genetic mechanism can control the neuronal identity is represented by the specification of subcortical projection neurons versus corticocortical neurons, the two major classes of pyramidal neurons in the mammalian cerebral cortex.

The determination of subcortical neurons is linked to the expression of *Fezf2* that acts upstream of *Ctip2*, whereas *Satb2* is required for the development of callosal projection neurons (layers II-III), and represses the expression of *Ctip2* in these cells (**Fig.26**). In the absence of *Satb2*, callosal projection neurons extend axons subcortically. Conversely, in the absence of *Fezf2*, *Satb2* expression is derepressed, enabling cells to take on a callosal projection neuron fate (Leone *et al.*, 2008).

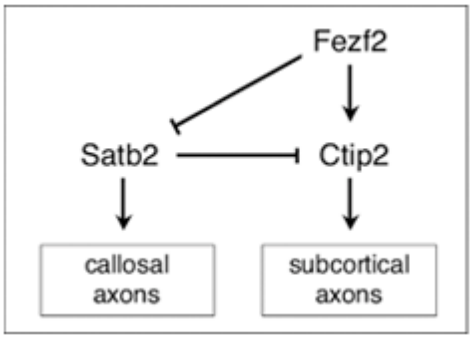


Figure 26 | A working model for the specification of callosal versus subcortical projection neuron identity during the development of the cerebral cortex. Image taken from Leone *et al.*, 2008

2.3. Migration in Rodents

During development of the mammalian telencephalon, cells migrate via diverse pathways to reach their final destinations. Projection neurons of the developing cerebral cortex are generated in the cerebral proliferative layers and subsequently move to the developing cortical plate by radial migration. Conversely, the vast majority of inhibitory interneurons originate in the ventral telencephalon (almost entirely, in the case of Rodents), and invade the cortex by tangential migration.

2.3.1 Radial migration

Once excitatory projections neurons have been generated in the telencephalic proliferative zone, they radially migrate to the developing cerebral cortex, where they settle according to the "inside-out rule": the earlier the deeper, the later the more superficial. Two principal modalities of radial migration can be distinguished.

During early stages of cortical development, when the cerebral wall is relatively thin, neurons generated from RGC move from proliferative layers to the preplate as well as to the forming cortical plate by "somal translocation". Later, when the cerebral wall is thicker, they proceed by "glia-guided locomotion" (**Fig.27**) (Nadarajan, 2003; Kriegstein and Noctor, 2004).

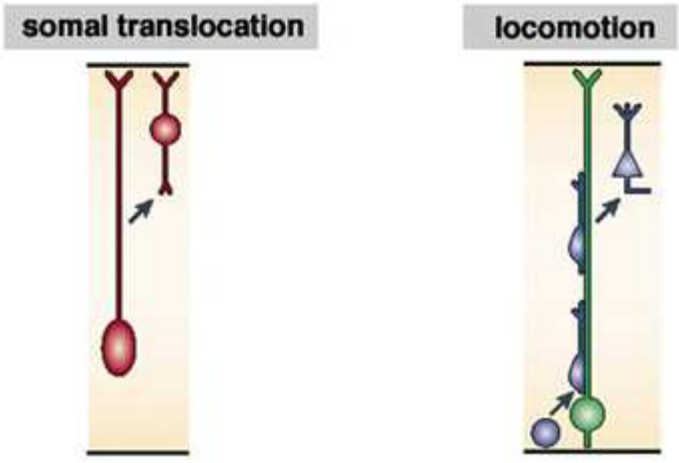


Figure 27 | Model illustrating the stage-dependent role of radial glia in the developing cerebral cortex. During corticogenesis, radial glia generate daughter neurons and radial glial cells. In the early stages, the daughter neuron use somal translocation as their mode of migration. Later in development, neurons locomote along the radial fiber of the parent glia.

Cells that undergo somal translocation typically have a long, radially oriented basal process that terminates at the pial surface, and a short, transient trailing process. The migratory behavior is characterized by continuous advancement that results in a faster rate of migration. Translocation has been proposed to be the major mode of migration during the earliest stages of cortical development, (i.e. early-generated preplate neurons or deep layer neurons).

By contrast, cells that adopt glia-guided locomotion have a shorter radial process that is not attached to the pial surface. Migration of these cells does not progress smoothly from ventricle to cortical plate, but is instead characterized by distinct migratory phases in which neurons change shape, direction and speed of movement (Kriegstein *et al.*, 2004). In particular most of newborn neurons exhibit four distinct phases of migration, including a phase of retrograde movement toward the ventricle before migration to the cortical plate (**Fig.28**). During this kind of migration, neurons that arrest in the SVZ, acquire a multipolar morphology, pausing for approximately one day or more (Noctor *et al.*, 2004). Multipolar daughter cells show dynamic behavior: they frequently change orientation, and extend and retract processes, while moving in the tangential plane.

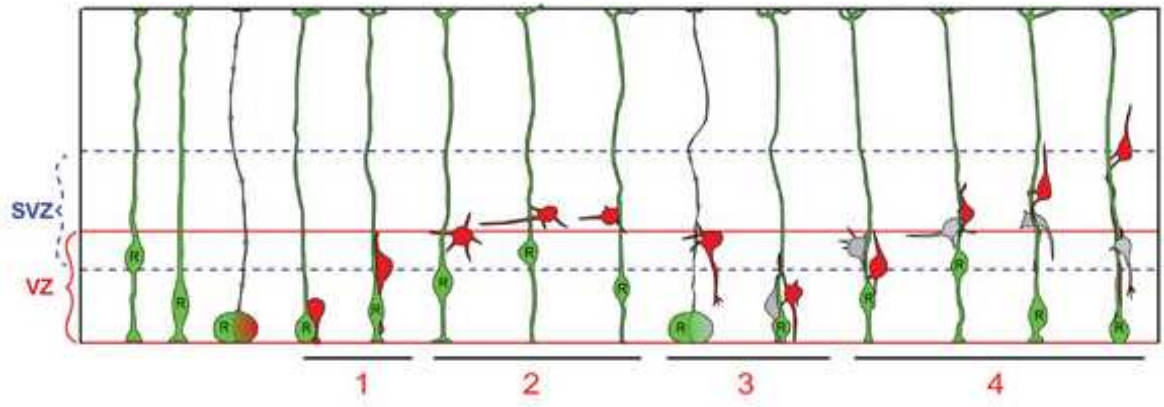


Figure 28 | Distinct phases of radial migration. CP neurons migrate by locomotion and follow different phase of migration: (1) rapid movement from the ventricle to the SVZ. (2) migratory arrest in the SVZ (24 h), where neurons become multipolar. (3) retrograde migration toward the ventricle. (4) migration to the cortical plate. Many, but not all neurons exhibit phase 3, and some of them do not translocate the cell body toward the ventricle but only extend a process towards the ventricle. Images taken from Noctor *et al.*, 2004.

Numerous transcriptor factors and signaling molecules are implicated in correct cortical migration and lamination. In particular, the extracellular matrix molecule Reelin plays a crucial role in the correct positioning of neurons during the development of the cerebral cortex (D'Arcangelo *et al.*, 1995; Frotsher 1998; Frotsher *et al.*, 2009).

Reelin, synthesized and released by Cajal-Retzius cells, induces neurons to migrate bypassing their predecessors, but also provides a stop signal, which tells neurons to detach from their radial guide and prevents them from invading the marginal zone (Frotscher *et al.*, 1998; Trommsdorff *et al.*, 1999).

Lack of Reelin in the mouse mutant *reeler* results in neuronal migration defects in the neocortex, hippocampus and cerebellum. The six-layered neocortical structure results disorganized: radially migrating neurons fail to split the subplate and get arranged inversely to wild-type, with late born neurons occupying the deep layers of the cortical plate (according to an outside-in gradient), giving rise to a grossly inverted cortex (Lambert de Rouvroit and Goffinet, 1998) (**Fig.29**).

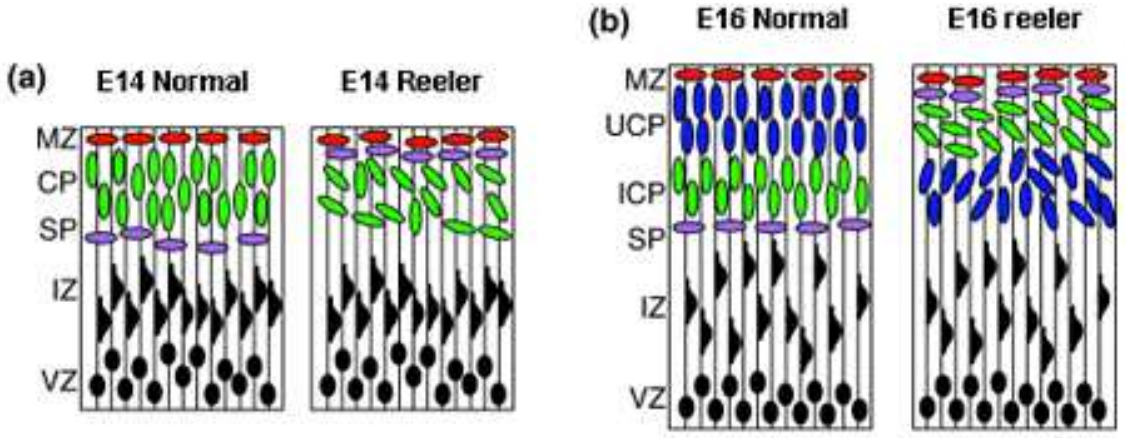


Figure 29 | Cortical layering in normal and reeler mutant mice. Wild-type mouse cortex (left panels) and reeler mutant cortex (right panels) at E14 (A) and E16 (B) respectively. In reeler mice at E14, CP cells are not as densely packed and less strictly radial as in normal animals, preplate cells are sparse in the MZ. At E16, late generated neurons in reeler mice, do not follow the inside-out rule, but migrate from outside to inside settling in the inner cortical plate. Abbreviations: CP, cortical plate; IZ, intermediate zone; ICP, inner cortical plate, MZ, marginal zone; SP, subplate; UCP, upper cortical plate; VZ, ventricular zone. Image adapted from Bar *et al.*, 2000.

Reelin signaling involves two lipoprotein receptors, the VLDLR (very low density lipoprotein receptor) or ApoER2 (apolipoprotein E receptor type-2) expressed by migrating neurons in the CP. Binding to this receptors results in the phosphorylation of the intracellular adaptor Dab1 (Disabled 1) that binds to the cytoplasmic tails of the lipoprotein receptors and initiates the signal transduction pathway that control the cytoskeleton reorganization (Reviewed by Bar *et al.*, 2000). Recently, it has been shown that Reelin cascade leads to phosphorylation of cofilin (Chai *et al.*, 2009), an actin-depolymerization protein that promotes the disassembly of F-actin, thereby stabilizing the cytoskeleton of

migrating neurons in their terminal phase of radial migration (Frotscher *et al.*, 2009) (**Fig.30**).

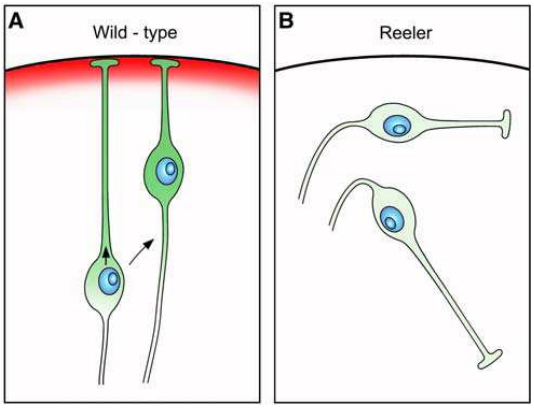


Fig. 30 | Schematic diagram summarizing the effects of Reelin (red) in wildtype animals (A) and in Reeler mouse (B). Phoshorylation of cofilin in the leading processes of migratin neurons anchors them to the marginal zone containg Reelin. In reeler mouse, the apical dendrites of neurons are not fixed to the cortical surphace and as a consequence, they are dispersed in various directions. Image taken from Frotscher *et al.*, 2009.

2.3.2 Tangential migration

GABAergic interneurons (for a review see Marin and Rubistein, 2001) and oligodendrocytes are generated from progenitors in the subpallium and reach the cerebral cortex via tangential migration (**Fig.31**). Also glutamatergic subpopulation of neurons has been shown to tangentially migrate, as in the case of Cajal-Retzius cells and Satb2^{+} neurons, reaching the neocortex and the hippocampus, respectively (Bielle *et al.*, 2005; Britanova *et al.*, 2006).

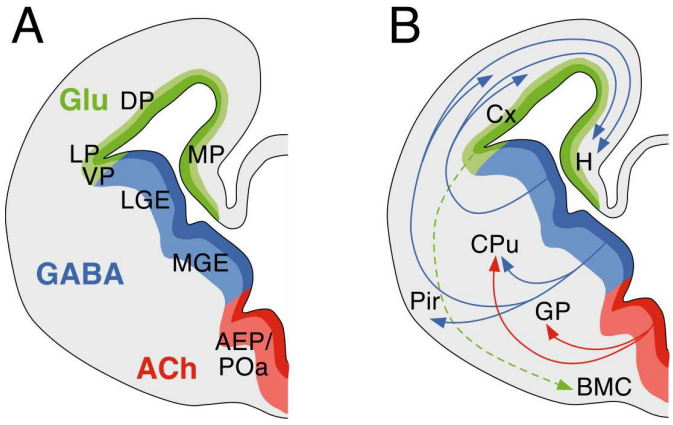


Figure 31 | Tangential migratory routes of GABAergic, cholinergic and glutamatergic cortical neurons. Abbreviations: AEP, anterior entopeduncular area; Cx, ocortex; DP, dorsal pallium; H, hippocampus; LGE, lateral ganglionic eminence; LP, lateral pallium; MGE, medial ganglionic eminence; MP, medial pallium; Pir, piriform cortex; POa, anterior preoptic area; VP, ventral pallium.

Several ventrally expressed transcriptor factors are important for either the differentiation or the migratory capacity of ventrally derived neurons. Mice lacking ventral homeobox genes allow studying the contributions of distinct parts of the ganglionic eminence to the generation of cortical interneurons. In mice knock-out for *Dlx1* and *Dlx2*, (both of them expressed throughout the early ventral telencephalon), tangential migration is dramatically impaired and only a quarter of neocortical GABAergic neurons can be found in the dorsal telencephalon (Anderson *et al.*, 1997). In *Nkx2.1*$^{-/-}$ mice, lacking a functional MGE but displaying a normal LGE, the number of neocortical GABAergic neurons is roughly half of that found in wild type brains (Sussel *et al.*, 1999). Conversely, in *Gsh2*$^{-/-}$ mice, the LGE is severely affected, but no change is detected in the neocortical interneuron complement (Corbin *et al.*, 2000). All this suggests that the MGE is the main source of GABAergic interneurons. In particular, the MGE produces somatostatin and parvalbumin subclasses of GABAergic interneurons (as well as a population of neuropeptide Y expressing interneurons) that migrate laterally and spread throughout the cortex. The CGE primarily produces calretinin and vasoactive intestinal (VIP)-expressing neurons, that migrate predominantly towards the caudal telencephalon. By contrast, LGE produces a substantial population of interneurons that migrate rostrally to the olfactory bulbs (**Fig.32**), as well as inhibitory projection neurons that populate ventral regions (Metin *et al.*, 2006).

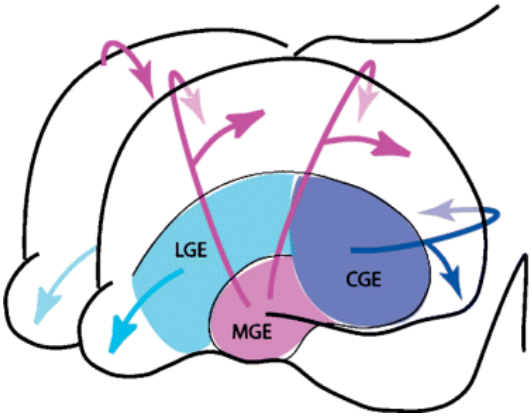


Figure 32 | Main migratory paths of interneurons derived from the three subdivisions of the ganglionic eminences. Abbreiviations: CX, cerebral cortex; HI, hippocampus; OB, olfactory bulb. Adapted from Metin *et al.*, 2006

In the mouse, three general and partially overlapping phases of migration can be distinguished (**Fig.33**). First, at E11.5, an early migration from MGE and anterior entopeduncular area (AEP) can be identified: neurons course superficially to the developing striatum and invade the cortical marginal zone and subplate. Second, at around mid-embryonic stages (E12.5-14.5), neurons from MGE migrate either deep or superficially to the developing striatum, and they populate both the SVZ, lower-intermediate zone (IZ) and the SP, from where they move into the cortical plate. Third, at late stages of the development (E14.5-16.5), migrating cells derive from both the LGE and the MGE (Reviewed in Metin *et al.*, 2006).

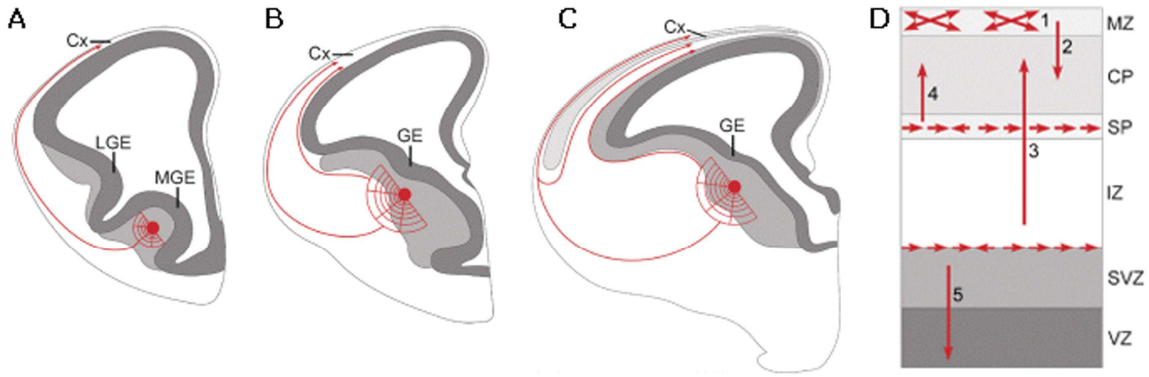


Figure 33 | Routes of tangential migration of interneurons from the basal telencephalon to the cortex. At early stages interneurons arise from the MGE and AEP and they follow a superficial route. At E13.5, interneurons are primarily generated from the MGE and follow a deep route to the developing striatum; some interneurons also migrate superficially. (c) At later stages, cortical interneurons also arise from the LGE and follow a deep route. H, hippocampus; GP, globus pallidum, LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, piriform cortex; VZ, ventricular zone; AEP, anterior entopeduncolar area; Str, striatum. (adapted from: Adapted from Metin *et al.*, 2006).

2.4. Development of thalamo-cortical afferents

Most sensory input to the cerebral cortex comes via the thalamus. During embryonic development, thalamic axons travel long distances to reach their target cortical areas. Thalamic input terminates principally in layer IV of the neocortex, although there are some terminations also in upper layers and layer VI. Only dorsal thalamic neurons send projections to the cerebral cortex; the epithalamus and the ventral thalamus do not. Layer VI neurons of primary cortical areas send corticofugal projections back to the thalamus, in correspondence of one of the four principal thalamic nuclei (Jones, 2007). To reach their targets, fibers coming from the thalamus or from the cortex have to cross several emerging boundary zones, including the diencephalic-telencephalic and the pallial-subpallial boundaries. Different cellular and molecular cues, distributed along the path followed by thalamocortical axons, guide thalamic growth cones to reach their cortical targets.

The early formation of the thalamocortical pathway is achieved by the growth cones of thalamic neurons from the dorsal thalamus; they proceed initially ventrally and turn dorsolaterally at the boundary between the telencephalon and the diencephalon, where they enter the internal capsule by E13 in the mouse embryo (**Fig.34**). Thalamocortical and corticofugal fibers interact in the internal capsule and after that, they resume their advance, in association with each other, and proceed towards their targets (Molnàr *et al.*, 1998).

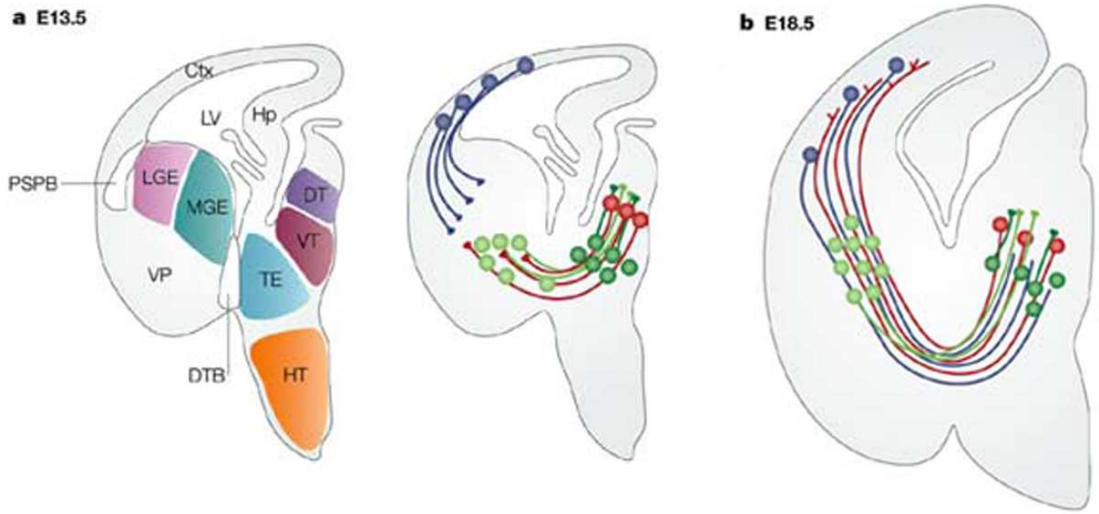


Figure 34 | Early growth of thalamocortical afferents from the dorsal thalamus to the cortex. At E13, thalamic axons (red lines) initially growth ventrally, then turn extending trough the medial and lateral ganglionic eminences. Contemporary, cortical fibers (blu lines) leave the cortex and after a pause at the pallial–subpallial boundary reach their final targets by E18, in a topographically organized manner. Abbreviations: DTB, diencepic-telencephalic border; PSPB, pallial-subpallial boundary. Image taken from Lopez-Bendito and Molnar, 2003.

First thalamic efferents reach the mouse cortex at E15 (**Fig.35**). The axons start to accumulate in subplate, although some axons and side branches penetrate the deep part of the cortical plate. During the early postnatal period (P0), most thalamic fibers invade the deep layers of the cortical plate, until reach layer IV (Reviewed in Lopez-Bendito and Molnar, 2003). Once the cortex is reached, activity dependent mechanisms become increasingly important in shaping topographic and feature maps (Katz and Shatz, 1996).

Several transcription factors are required for the proper development of thalamocortical connections. Among them, *Mash1*, *Pax6* and *Gbx2* (Tuttle *et al.*, 1999; Pratt *et al.*, 2002; Hevner, 2002), expressed at distinct, strategic points along the forming axonal pathway: *Gbx2* in the dorsal thalamus, *Mash1* in ventral thalamus and ventral telencephalon, *Pax6* not only in the thalamus, but also along the pathway taken by thalamocortical axons and in the cortex itself.

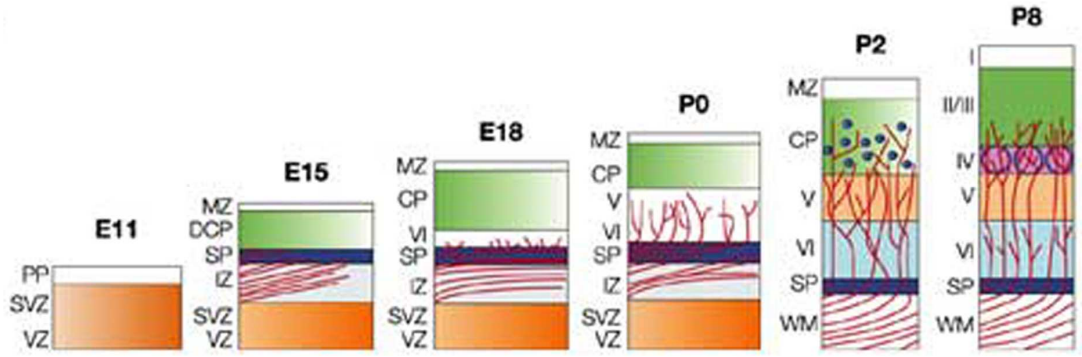


Figure 35 | Ingrowth of thalamic fibres on schematic coronal sections of mouse cortex during development. By E16, thalamocortical axons reach the cortex where they extend tangentially in the intermediate zone. Between E18 and P2, thalamocortical axons extend side branches into the more superficiall regions of the cortex, initially interacting with the subplate, then growing into layer 4. By P8, the mature arrangement of thalmocortical axons in established. Abbreviations: MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. From Lopez-Bendito and Molnar. 2003.

3. COMPARATIVE ASPECTS OF CORTICO-CEREBRAL DEVELOPMENT

3.1. Telencephalic organization in vertebrates

Gene expression and function studies in different animal models provide evidence for a common organization of the developing telencephalon in vertebrates. In particular most parts of the brain are remarkably conserved across amniotes, a group including reptiles, birds and mammals.

Amphibians have the simplest brain organization, with no large cell masses and little neuronal migration. Reptilian brains are slightly more complex, having a three-layered cortex, but are still relatively small. A relevant change during evolution has been the appearance of a six-layered structure, called isocortex or neocortex, that has come with mammals. The neocortex is formed by an inside-out gradient, whereas the reptilian dorsal cortex develops from an outside-in gradient.

Comparative studies in mammals, birds and reptiles (Smith-Fernandez *et al.*, 1998, and Puelles *et al.*, 2000) show a conserved pattern of gene expression, suggesting homologies between regions in distant species. The medial and dorsomedial regions of the reptilian brain are homologous to the mammalian hippocampus, the lateral cortex corresponds to the olfactory cortex, and the dorsal cortex is the homologue of the mammalian neocortex (**Fig.36**) (Aboitiz *et al.*, 2002). The avian hyperpallium is also comparable to the neocortex. In mammals, both hippocampus and dentate gyrus are three-layered structures, but only the dentate gyrus retains the ancestral outside-in gradient.

A second unique feature of the mammalian neocortex is the tremendous increase in surface area it has undergone in the mammalian lineage, from lissencephalic brains to highly convoluted brain in humans. The evolution and expansion of the neocortex is associated with the intelligence and social complexity of mammals. For example, the surface area of the human cortex is 1000 fold greater than that of a mouse, but is only 3-5 times as thick (Rakic, 1995). This pattern of morphological change indicates that brain size increases have resulted from a lateral, rather than radial, expansion of the neuroepithelium.

In conclusion, a general strong conservation in cortical development is present among mammals, bird and reptiles, but important changes have occurred during evolution contributing to the formation of the mammals' six-layered neocortex. Relevant differencies can be found at the level of the cortical proliferative zones, of the striatocortical junction (cortical hem in mammals) and in the amplification of the Reelin signals (Molnar *et al.*,

2006). Comparative and genetic aspects of cortical development are important to understand how evolution works and which mechanisms and rules it operates through.

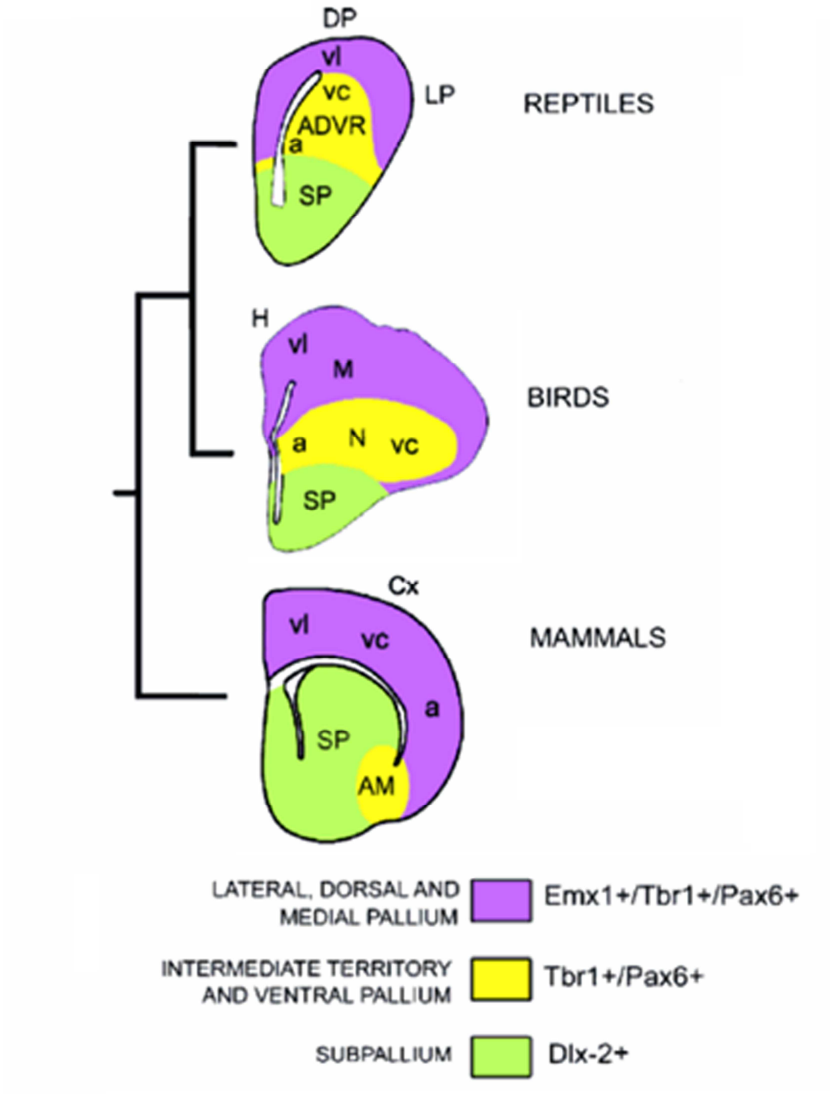


Figure 36 | Embryonic telencephalic territories based on regulatory gene expression data. Abbreviations: a, auditory pathway; AM, amygdala and ventral claustrum; ADVR, anterior dorsal ventricular ridge; Cx, cortex; DP, Dorsal pallium; DVR: Dorsal ventral ridge; H, Hippocampus; HV, hyperstriatum ventral; LP, Lateral pallium; MP, Medial pallium; N, neostriatum; SP, subpallium; vc, collicular and lemniscal pathways; vl, visual pathways. Image taken from Aboitiz *et al.*, 2002

3.2. Class-specific areal restrictions of neurons with distinct laminar identities

Even if homologous expression domains may be found at corresponding locations along the dorso-ventral axis of the mammalian, avian and reptilian telencephalon (*Emx1/Pax6/Tbr1*; *Pax6/Tbr1*; *Dlx2*), intriguingly, it has been reported that key molecular markers, specifically expressed by neurons belonging to different laminae of the mammalian neocortex, may harbor distinct regional restrictions in other amniotes.

For example, *Reln*, a hallmark of Cajal-Retzius cells spread at the surface of the entire mammalian pallium, is missing in a large sector of the sauropsid marginal zone, being confined to the medial-most part of it (turtle, lizard, chicken). Moreover, as it happens in mammals, where *Reln* is further expressed by layers V and IV, extramarginal sources of this glycoprotein have been widely described even in other Amniota (**Fig.37**). Reelin expression has been detected in cortical plate of turtle and lizard, in subplate of lizard and in periventricular proliferative layers of chicken and crocodile (Caviness, 1982; Bayer *et al.*, 1991; Bernier *et al.*, 1999; Goffinet *et al.*, 1999; Bernier *et al.*, 2000; Tissir *et al.*, 2003; Nomura *et al.*, 2008; Yoshida *et al.*, 2005).

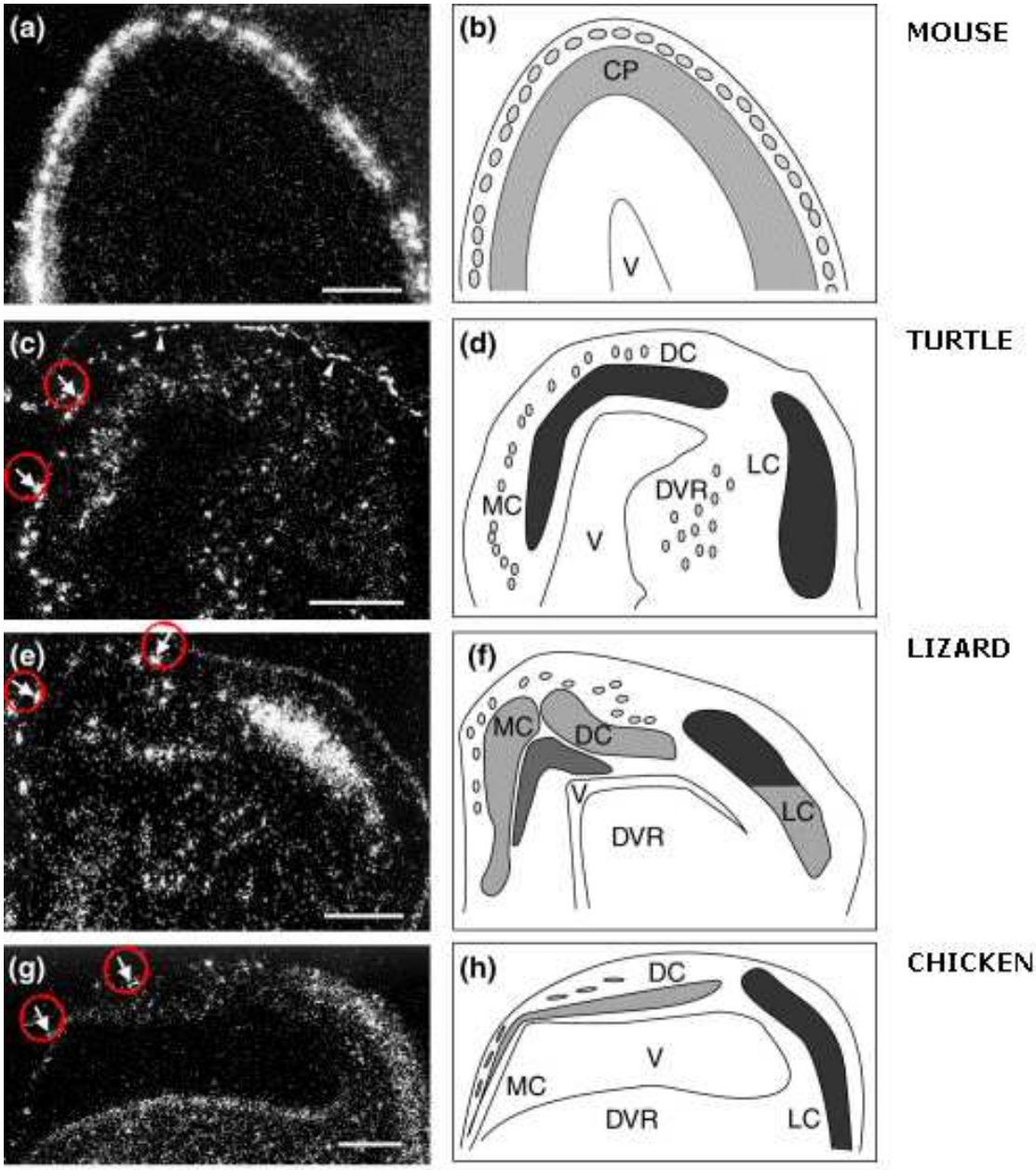


Figure 37 | Comparison of reelin mRNA expression. Frontal sections in the embryonic cortex of mouse (a-b), turtle (c-d), lizard (e-f) and chick (g-h). Expression patterns of reelin mRNA are shown in darkfield views in left panels. Right panles represent reelin-positive zones: ovals represent cells in marginal zone (MZ) and dark gray areas represent the more-diffuse expression in cortical plate or subcortex. In turtles (c-d), reelin-positive cells are dispersed in the MZ of the medial cortex and dorsal cortex and to a lesser extent in the lateral cortex and dorsal ventricular ridge (DVR). The CP in MC and DC is weakly. (e-f) show the lizard MC and DC in which reelin-positive neurons (arrows) are abundant in the MZ, and there is a second layer of reelin expression in the subplate [dark gray area in (f)], whereas the cortical plate is reelin-negative. The dorsal component of the lateral cortex expresses reelin (dark gray area in f). (g-h) show the chick in which subpial reelin-positive cells (arrows) are found only in the diminutive MC (hippocampus) and DC (parahippocampus), whereas the CP is negative. There is diffuse reelin expression in the LC. Abbreviations: V, ventricle; MC, medial cortex; DC, dorsal cortex; LC, lateral cortex. Image adapted from Bar *et al.*, 2000.

Another example is *Er81*. This gene, active in deep layers of the entire mammalian cortex as well as in the striatum, is not expressed by the avian hyperpallium (somehow corresponding to our neocortex), being confined to hippocampus/area parahippocampalis and arcopallium, on the medial and lateral sides of it, respectively (Nomura *et al.*, 2008) (**Fig.38**).

Finally, *Brn2*, expressed by a subset of layers II-III in the mammalian neo- and paleocortex, is absent in the avian hyperpallium, being specifically on in the mesopallium/nidopallium, on the latero-ventral side of it (Nomura *et al.*, 2008).

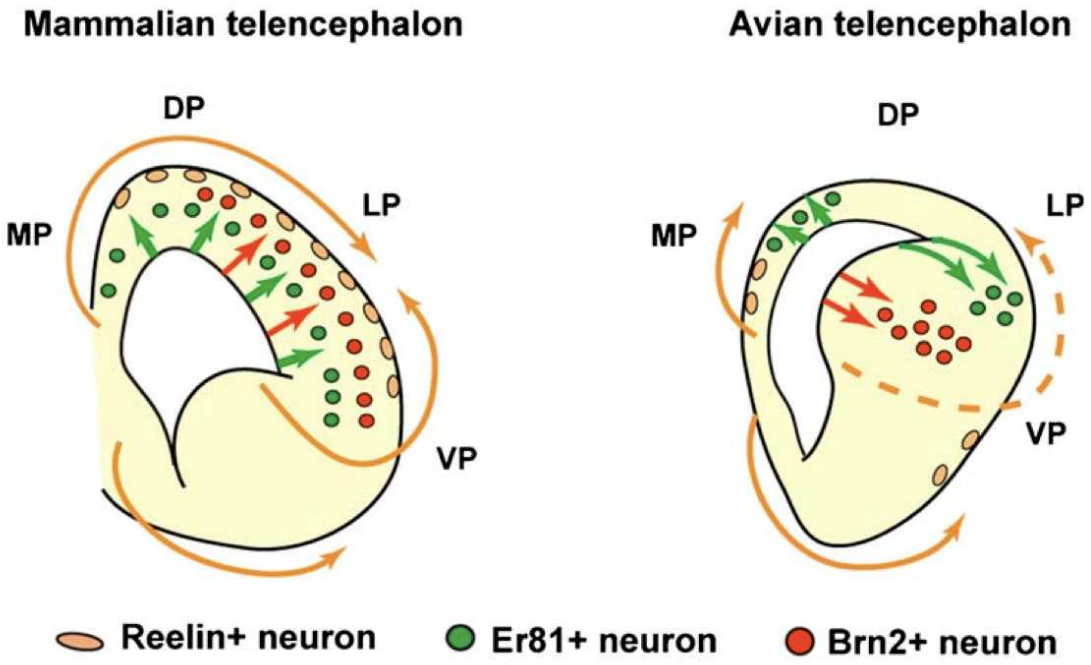


Figure 38 | Differences in neuronal specification and migration patterns between the mammalian and avian pallium. In the developing mammalian telencephalon, Reelin-positive neurons are derived from several origins including ventral pallium, and Er81 and Brn2- positive neurons are generated from entire pallial regions. In contrast, in the developing avian telencephalon, Reelin-positive neurons are not derived from the ventral pallium, and Er81 and Brn2-positive neurons are generated from distinct pallial regions (from Nomura *et al.*, 2008).

3.3. Evolutionary increase in the SVZ

In all amniotes, neurogenesis occurs in the so called ventricular zone (VZ), lying next to the lateral ventricle. In mammals, cortical neurons are also generated in the dorsal cortex by a mitotic compartment called the subventricular zone (SVZ), located above the VZ (Sturrock & Smart, 1980; Bayer & Altman, 1991). The progenitor cells of the mammalian SVZ and of abventricular regions differ from progenitors in the VZ.

The SVZ is non-existent in lizards (Goffinet 1983) and turtles (Martinez-Cerdeno *et al.*, 2006), but it can be identified in the dorsal ventricular ridge of chick (Molnar *et al.*, 2006). In mammals, the expanded size of the SVZ correlates with brain size, a trend that is particularly evident in primates, where two distinct SVZ regions can be identified: an inner SVZ (ISVZ) and a outer SVZ (OSVZ) (Smart *et al.*, 2002), (**Fig.39C)**. The OSVZ is histologically similar to the VZ and shows a compact radial organization. Contrary to what is observed in rodents, where the VZ is the main germinal compartment throughout corticogenesis, the primate VZ declines rapidly and is paralleled by the appearance of the SVZ followed by the OSVZ.

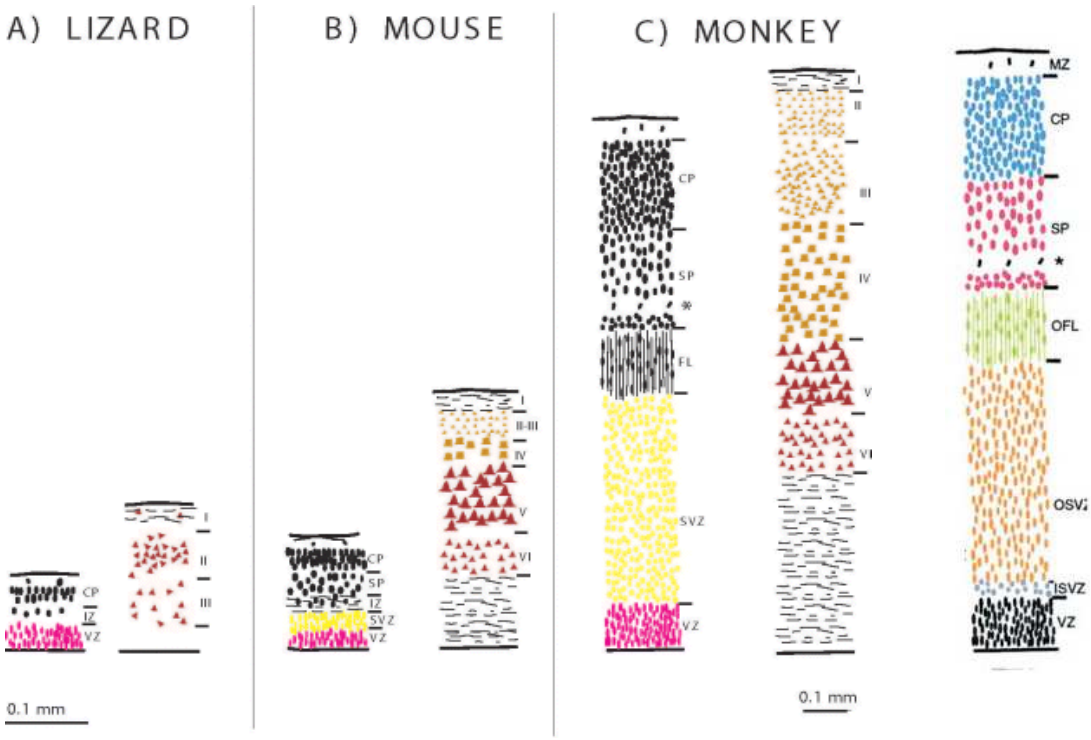


Figure 39 | Comparative relationship between SVZ density and cortical thickness and complexity of cerebral wall. The images represent the germinal layers during mid-neurogenesis (left panels) and the cortical layers of an adult brain (right panels). Image from Molnar *et al.*, 2006.

The laminar expansion from a three- to a six-layered cortex that occurred in the common ancestor of all mammals, and the tangential expansion of the cortex that has occurred within several mammalian orders, has to be correlated with the appearance of the cortical SVZ. In conclusion, neurogenesis in the SVZ could have increased neural production during embryonic development and may have been an important contributor to brain size evolution, leading to neocortical mammalian expansion.

3.4. Evolutionary increase in cortical interneurons

An increasing trend in the number of GABAergic neurons is evident during evolution. In Xenopus, GABAergic cells represent 3-11% of all pallial neurons, 10-12% in the pigeon, 15-20% in the mouse and 15-30% in humans.

Tangential migration to the pallium of GABAergic cells originating in the subpallium is widely conserved across species and has been observed also during development of lower vertebrates, such as chick and Xenopus, (Cobos*et al.,* 2001; Moreno *et al.,* 2008). However, tangential migration within pallial territories has been also described in turtles as well as in chick, (Tomioka *et al.,* 2000; Cobos *et al.,* 2001; Metin *et al.,* 2007) although the identity of the migrating neurons has not been fully characterized, but also in mouse, (about 5% of cortical interneurons) (Letinic *et al.*, 2002) and in humans, where 65% of GABAergic interneurons appear to be produced inside the pallium (Letinic *et al.*, 2001) (**Fig.40**), similarly to other primates (Petanjek *et al.*, 2009). GABAergic cells of the human neocortex born inside the pallium are produced from *Mash1* progenitor cells of the VZ and SVZ, a gene typically expressed in the subpallium and responsible for the differentiation of GABAergic neurons (Letinic *et al.*, 2002).

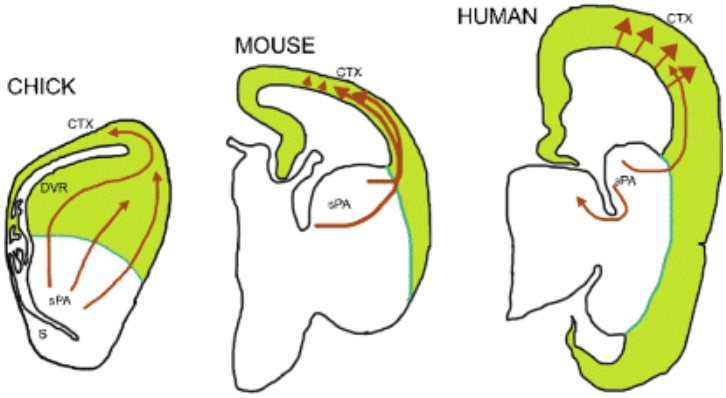


Figure 40 - Comparative tangential migration routes of GABAergic neurons from subpallium to the cortex. In humans and other primates, noumerous interneurons are produced also inside the dorsal telencephalon and migrate radially toi reach their final laminar position. Abbreviations: CTX, cortex; DVR, dorsal ventricular ridge; sPA, subpallium.

4. MARSUPIALS

Marsupials represent one of the three subclasses of mammals that include prototherians (monotremes), metatherians (marsupials), and eutherians (placentals). Like placental mammals, marsupials are a highly diverse group that have adapted to a number of different habitats and lifestyles. Extant marsupials have been subdivided into seven different orders and over 270 species have been identified (Karlen and Krubitzer, 2007) (**Fig.41**).

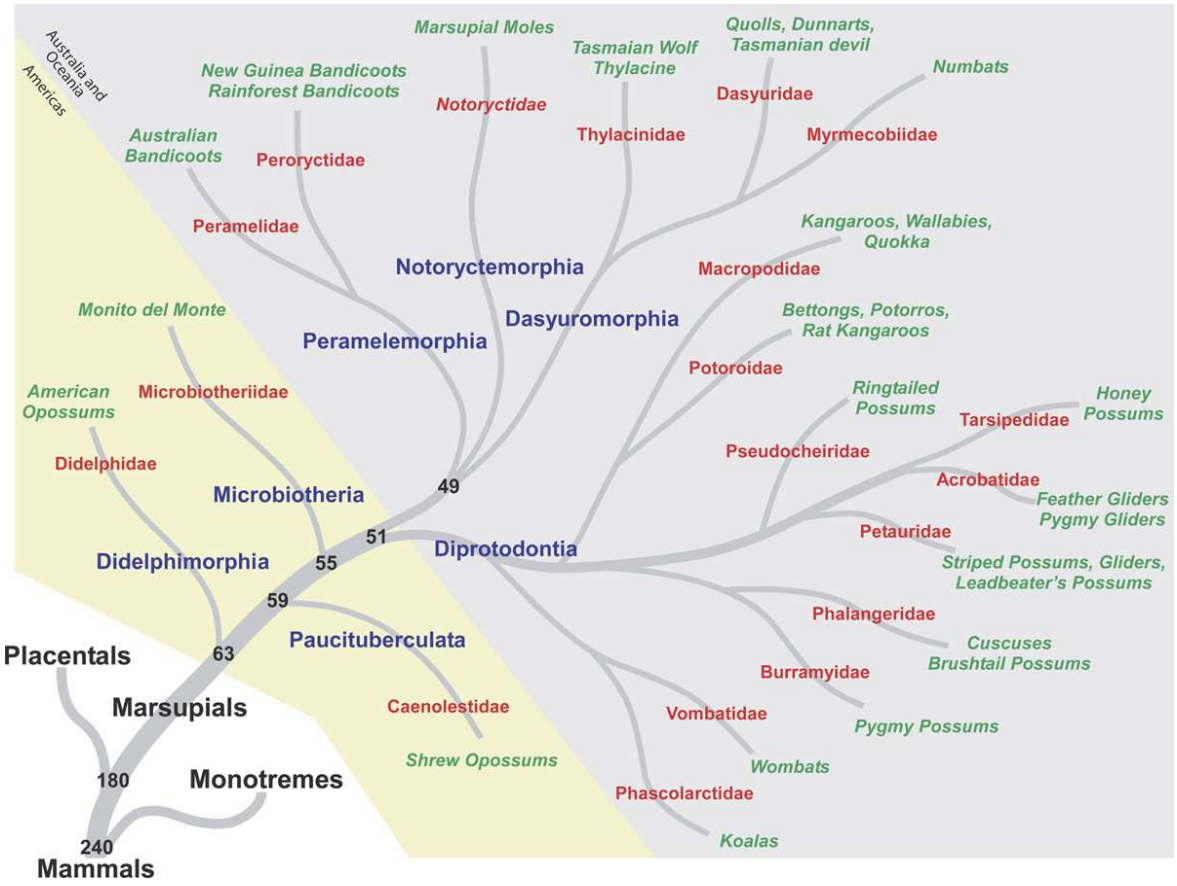


Figure 41 | A phylogenetic tree illustrating the branching among Monotremes, Marsupials and Placentals and major marsupial lineages. Image taken from Karlen and Krubitzer, 2007

Marsupials are important for understanding larger issues of cortical evolution and development. First, because marsupials occupy a wide range of habitats and have evolved a large array of unique adaptations, they make excellent models for studying animal ecology and the relationship between brain and behavior. Second, since they have retained a number of features of cortical organization from their ancient ancestor, radiated from

stem mammals over 180 million years ago (My), many of them are considered to reflect the ancestral mammalian state more than most other present day mammals. Moreover, because of the extremely immature state at birth, marsupials can be easily micro-surgically manipulated and serve as important models for studying neural development and regeneration. A large amount of regenerative studies have been carried out in the South-American short-tailed opossum, *Monodelphis domestica*, an animal model particularly suitable for laboratory studies since it is small, has a short gestational period (two weeks) and is highly prolific throughout the year (**Fig.42**) (Saunders *et al.*, 1989). Opossum nervous system can be dissected out in its entirety and maintained in culture for long period (weeks). Spinal cord damaged by experimental trauma in the cervical region, exhibits successful axon regeneration if the lesion is done before P9. By contrast, 12-day-old preparations show no regeneration (Reviewed in Mladinic *et al.*, 2009).

Another precious tool, to help on working with the opossum, is that its complete genome has been recently sequenced (Mikkelsen *et al.*, 2007), facilitating molecular studies on this model.

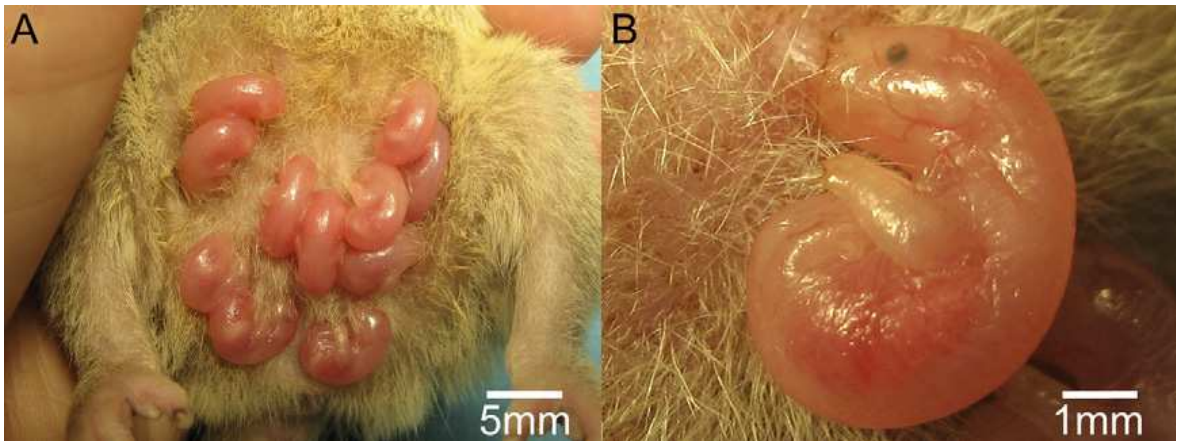


Figure 42 | Litter of short-tailed opossum on the day of birth (P0). Image taken from Karlen and Krubitzer, 2007.

5. AIMS OF THE THESIS

The aims of this thesis were to study in details key aspects of cortical histogenesis in marsupials trying to fill gaps in our knowledge of opossum corticogenesis. *Monodelphis domestica* does not only represent an interesting animal model for neurobiological aspects and regenerative studies, but also could give insight into evolutionary mechanisms that had occurred at the level of central nervous system from the common mammal ancestor of first placentals.

Taking advantage of the large body of molecular tools and methodologies nowadays used for developmental studies on placental's cortex and the availability of *M. domestica* genomic sequence data, we focused our attention on investigating in particular:

- the origin of cortical neurons (projection neurons and interneurons)
- laminar differentiation and conservation of cortical laminar markers
- neural migration profiles
- proliferative compartments

A more detailed knowledge of basic aspects of the system could provide a solid framework for more advanced developmental studies on marsupial corticogenesis.

MATHERIALS AND METHODS

1. ANIMALS

Opossums (*Monodelphis domestica*) at different postnatal ages were obtained from the colony maintained at the animal house facility of the University of Trieste, Italy. Mice (*Mus musculus*, strain CD1) were purchased from Harlan-Italy. Opossums were staged by systematic daily inspection of the colony for newborn litters, P0 corresponding to the day of birth. Mice were staged by timed breeding and vaginal plug inspection. Animals handling and subsequent procedures were in accordance with European laws [European Communities Council Directive of November 24, 1986 (86/609/EEC)] and with National Institutes of Health guidelines. In particular, to harvest opossum brains (from P1-P40), young animals were killed by decapitation, after hypothermia. When the entire CNS had to be recovered, the animals were alternatively killed by aorta resection, again after hypothermia. In the case of P60 animals, they were terminally anesthetized by urethane and transcardially perfused with 4% paformaldehyde. As for mouse embryos (E10.5-E18.5), they were harvested from pregnant dames killed by cervical dislocation.

2. BROMODEOXYURIDINE ADMINISTRATION

Bromodeoxyuridine (BrdU) was administered to P1-P18 opossum pups at the dose of 200 μg/g of body weight in 0,9% NaCl, by subcutaneous injections. During administration, pups were left attached to the mother, previously anesthetized with isofluorane. All injected animals were sacrificed at the age of P30 and their brains used for kinetic studies.

3. ORGANOTYPIC CULTURES

Floating organotypic brain cultures were used for this study. Opossum CNSs were dissected from anaesthetized P10 young placed a small, Sylgard-filled Petri dish with tiny pins (Minuten Nadelen) placed through the paws and repeatedly washed in DMEM-F12-Glutamax/

0.6%Glucose/ Fungizone/ Pen/ Strept, paying particular attention to avoid any damages. After further manipulations (DNA injection and electroporation), CNSs were transferred to 50 mL Falcon tubes (one per tube), each containing 15 mL of DMEM-F12-Glutamax/ 0.6%Glucose/ N2/1% FBS/ Fungizone/ Pen/ Strept. Cultures were maintained at 32°C for two days, in the presence of bubbling 95%O_2–5%CO_2. To verify brain vitality, a final pulse of BrdU (5μg per ml of medium) was given 90 min before explants' fixation.

4. BRAINS ELECTROPORATION

Electroporation was performed on acutely dissected P10 opossum CNSs, put in a petri dish filled with 0,6% Glucose/1X PBS. 1 μl of an aqueous 1 μg/μl solution of plasmid DNA (containing 0,01% of fast green dye) was injected into a lateral ventricle, using glass capillaries (Biological Instruments, 1B100-3) prepared by the micropipette puller P-97 (Sutter Instrument Co.). Two parallel, 5cm-spaced, rectangular electrode plates (4cm x 6cm) were placed on both sides of the telencephalon and three 100 V pulses (each 100 ms long, interval between consecutive pulses 450 ms) were delivered, using an electro-square-porator (BTX 830). Plasmids pTα1-EGFP (kindly provided by E. Ruthazer) and pDsRed2-N1 (Clontech) were used for electroporation.

5. HISTOLOGY

CNS specimens (both mouse and opossum, both whole brains and explants) were fixed in 4% paraformaldehyde-PBS overnight at +4°C, cryoprotected in 30% Sucrose/PBS and cut coronally at 10 μm. Cryosections were mounted on Fischer SuperFrost Plus slides, and subsequently processed for *in situ* hybridization or immunohistochemistry.

To accurately determine the fraction of $Tbr2^+$ cells also expressing β-tubulin, 6 freshly dissected P10 opossum cortices were pooled and dissociated to single cells by gentle trituration. Cells were resuspended in DMEM-F12/1% Serum, plated onto slides previously covered with 20 μg/ml poly-D-lysine, and left to attach for 1 hour at RT. Slides were processed for immunofluorescence, as elsewhere described.

6. IMMUNOFLUORESCENCE

6.1. Immunofluorescence protocol

Immunofluorescence was performed after drying sections for at least one hour. They were post-fixed 5 minutes in 4% praformaldeyde, followed by three washes in PBS. Generally, sections were then boiled for 5' in 10 mM pH=6 citrate buffer. This step was omitted in the case of β-tubulin, β-tubulin/GFP and GABA stainings. In case of BrdU detection, sections were also treated for DNA depurination (2 M HCl, for 15' min a RT) and then neutralized (in 0.1 M borate buffer, pH 8.5, for 15 min at RT). In case of other combined immunofluorescences (BrdU/Tle4, BrdU/Cux1, BrdU/Tbr2 and IdU/GABA), HCl concentration was reduced to 0,2 M. Sections were incubated for 1 h at RT under blocking mix (1X PBS;10% FBS; 1mg/ml BSA; 0.1% Triton X100) and then incubated at 4°C overnight with primary antibody in blocking mix. The following day sections were washed in PBS for 5 minutes, three times, and incubated for two hours with secondary antibodies diluted in blocking mix. Slides were washed three times in PBS for 5 minutes, stained with DAPI and mounted in VECTASHIELD Mounting Medium (Vector).

6.2. Antibodies used

Primary antibodies used were as follows: mouse monoclonal anti-BrdU (clone B44, Becton Dickinson), 1:50; rat anti-BrdU (clone ICR1, Abcam), 1:500; goat anti-Brn1 (Santa Cruz), 1:30; mouse monoclonal anti-Calretinin (clone M7245, Dako), 1:50; rabbit anti-Cux1 (Santa Cruz), 1:30; rabbit anti-Foxp2 (Abcam), 1:1500; rabbit anti-Gaba (Sigma), 1:10000; rabbit anti-GAD (Sigma), 1:1000; rabbit anti-Gfap (Dako), 1:500; chicken anti-GFP (Abcam), 1:800; mouse monoclonal anti-O4 (clone O4 R&D systems), 1:600; rabbit anti-Pax6 (Abcam); 1:300; rabbit anti-pH3 (Upstate), 1:600; rabbit anti-S100β (Dako), 1:200; rabbit anti-Tbr1 (kindly provided by Robert Hevner), rabbit anti-Tbr2 (Abcam), 1:500; Rabbit anti-Tle4 (Santa Cruz), 1:30; mouse anti-neuron-specific class III β-tubulin (clone Tuj1, Covance), 1:500. Finally, immunoreactivity was revealed after 2h incubation with secondary Alexa antibodies, 488 and 594 (Molecular Probes), 1:400. A table with Placental-vs-Marsupial conservation of epitopes recognized by a selection of these antibodies is provided in the Supplementary Material section (Table 1).

7. *IN SITU* HYBRIDIZATION PROTOCOL

7.1. Preparation of Dig-labelled RNA probe

Non-radioactive *in situ* hybridization was performed to study *Reln* mRNA expression. The probe used corresponded to exons 1-12 of *M. domestica Reln* coding region (*Ensembl* mdo-chr.8: nt 155064879-154744337).

The following PCR primers, containing appropriate restriction sites, 5'-GAGAAGTGCTCATTTCCCTGCACATTG-3' and 5'-CCACATTCATTGCCAATGACAGCTCC-3', were designed to amplify 1,2kb of the *Reln* transcript that was cloned into pBluescript KS(-) (Stratagene) *E. coli* expression vector, which contains specific RNA polymerase promoter sites.

All basic DNA standard methods (extraction, purification, ligation) as well as bacterial cultures, transformation, media and buffer preparations and agarose gel electrophoresis were performed according to Maniatis *et al.*,1989. DNAs were transformed in *E.coli* TOP10 strain (Invitrogen).

Ligation reactions were performed with LigaFast Rapid DNA Ligation System (Promega). DNA fragments were purified from agarose gel with the Qiaex II DNA purification system (Qiagen). Small-scale plasmid preparations (mini-preps) from transformants were made by purification on Sigma columns (Mini prep Kit, Sigma). Large scale preparations (maxi-preps) were done by purification on Qiagen columns (Plasmid Maxi Kit, Qiagen).

Plasmids for use in transcription reactions were linearized with appropriately placed restriction enzymes, purified by phenol/chloroform precipitation and finally resuspended in deionized water.

According to the manufacturers' instructions, the following reagents were assembled on ice in an eppendorf tube: 1 μg linearized DNA; 4μl Transcription 5x Buffer (Promega); 2μl 0,1M DTT (Invitrogen); 2μl 10x Dig labelling mix (Roche); 2μl 20U/μl RNAseOUT (Invitrogen); 1μl 20U/μl RNA polymerase (either T3 or T7, Promega); deionized water to 20μl. The reaction was incubated at room temperature overnight. The following day RNA transcript was precipitated and resuspended in 20 μl of sterile deionized water, 2 μl of RNA probe were run on denaturing agarose gel against known weight markers for quantification. Probes were diluted and then stored at –80°C.

7.2. Pretreatment of sections

In order to improve signal and reduce the background, sections were subjected to several pre-treatment steps. The following protocol, adapted for cryostat-cut sections, was applied. Slides were left to dry for at not less than 1 hour, then immersed in 4% paraformaldeyde in PBS for 10 minutes and then washed with PBS, twice for 5 minutes. Slides were immersed in HCl 0.2 M for 5 minutes, then washed in PBS, three times for 2 minutes, and incubated in 0,5 μg/ml of proteinase-K (Roche) in 50 mM Tris-HCl pH 8,5mM EDTA, at 30°C for 15 minutes. The proteinase K reaction was stopped by washing slides in Glycine 4 mg/ml in PBS for 5 minutes, twice. Slides were washed in PBS for 5 minutes, twice, then immersed in 4% paraformaldeyde in PBS for 10 minutes and subsequently washed in PBS, for 4 minutes, twice. The slides were rinsed in distilled water and finally placed in a container with 0,1 M Triethanolamine-HCl pH 8 set up with a rotating stir bar for 5 minutes. 0,4 ml of acetic anhydride were added twice for 5 minutes each, then slides were washed in deionized water twice for 2 minutes. Finally, the slides were left to dry for not less than 1 hour and immediately used for hybridization.

7.3. Hybridization and washing of sections

The hybridization mix (Denhardts Salts 1X, DTT 50mM, Polyadenylic acid 500 μg/ml, Ribonucleic acid transfer 53,5 μg/ml, Dextran sulphate 10 %, Formamide 50%) was additioned with either 30 μl probe/slide, then heated to 80°C for 10 minutes and applied to the slides. Clean glass coverslips were applied to increase the uniform spreading of the hybridization mix over the sections. Slides were placed horizontally in a sealed plastic slide box, together with paper soaked in 50% formamide, 5X SSC and incubated overnight at 60°C. The following day, slides were removed and placed in a slide rack in a solution of 5X SSC, 0.15% β-mercaptoethanol at room temperature for 30 minutes, in order to remove the coverslips. Slides were incubated in stringent buffer (50% formamide, 2X SSC, 0.15% β-mercaptoethanol) at 60°C for 30 minutes. Then, they were washed with NTE buffer (0.5M NaCl; 10 mM Tris-HCl pH 8; 5 mM EDTA) two times for 15 minutes each. Finally, slides were sequentially incubated with 2X SSC, and 0.2X SSC, for 15 minutes each.

7.4. Digoxigenin revelation

After pre-treatement and washing, slides were incubated in B1 solution (0.1 M Tris-HCl pH 7.4; NaCl 0.15 M) for 5 minutes, and then blocked in B1 solution containing 10% of heat inactivated fetal bovin serum (FBS, Gibco) for 1 hour at room temperature. Slides were next incubated in B1 containing 0.5% FBS and the anti-Digoxigenin antibody conjugated with alkaline phosphatase enzyme (Dig-AP, Roche) at the concentration of 1:2000, overnight at +4°C. The following day sections were washed three times in B1 solution before incubation in B2 buffer (0.1 M Tris-HCl pH 9.5; 0.1 M NaCl; 50 mM $MgCl_2$) containing the chromogenic substrates: 3.5 µl of NBT (Nitro blue tetrazolium chloride, Roche) and 3.5 µl BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt, Roche) for each ml. The ongoing development of these sections was followed using a bright field Olympus CHT microscope.

8. IMAGING AND CONFOCAL MICROSCOPY

Fluorescent labeled sections were imaged and analyzed using a fluorescent Nikon (Tokyo, Japan) Eclipse 80i microscope and a DS-2MBWC digital microscope camera. Confocal photos were taken by a TCS SP2 Leica confocal microscope; they were generally collected as 1.0 µm-thick Z-stacks and as 3.0 µm-thick Z-stacks, in the case of pTα1-EGFP electroporated cells. All images were processed by Adobe Photoshop CS3 software.

9. SAMPLE SIZING

Unless otherwise stated, each experiment was performed at least in triplicate. In cases of laminar birthdating, per each BrdU pulsing time, 3 mid-frontal 800 µm-wide neocortical sectors, from 4 cortices were profiled. $pH3^+$ cells countings were performed in similar ways, but throughout the neocortical field. The same applies to $Tbr2^+$, $Tbr2^+/BrdU^+$ and $Pax6^+$ cells countings, conversely restricted to 200 µm-wide parietal sectors. Error bars represent standard deviations.

RESULTS

1. MOLECULAR DIVERSIFICATION OF NEURONS BELONGING TO DIFFERENT NEOCORTICAL LAMINAE AND THEIR RADIAL MIGRATION

The placental neocortex is formed of six layers, each expressing a well defined set of molecular markers (reviewed in Molyneaux *et al.*, 2007). To assay possible conservation of the neocortical laminar profile between Placentals and Marsupials, we looked at the distribution of a selection of these markers in the opossum neocortex at postnatal day 30 (P30), a developmental age at which radial neuronal migration seems to be largely completed (**Fig. 1A**). Tbr1, expressed by mouse subplate, marginal zone and layer VI (as well as, to a lesser extent, by layers III and II) (Hevner *et al.*, 2001), was detectable in the opossum in two stripes of cells. The deeper, corresponding to layer VI, included stronger labeled neurons; the more superficial, corresponding to layers II and III, displayed less intense immunoreactivity. Only a few weakly labelled $Tbr1^+$ cells were found in layer I, if any. Foxp2 and Tle4, markers of deep layers in Placentals, were both confined to the deep grey matter of the opossum. As expected, Tle4, expressed by mouse layers VI and V, displayed a wider radial domain as compared to Foxp2, restricted to murine layer VI only (reviewed in Molyneaux *et al.*, 2007). Conversely, Cux1 and Brn1, markers of upper layers in Placentals, were both confined to the superficial grey matter. Again as expected, Brn1, also labeling a subset of layer V neurons in the mouse (McEvilly *et al.*, 2002), displayed a wider radial domain as compared to Cux1, a marker of layers II-IV only (Nieto *et al.*, 2004).

Next, to reconstruct the temporal order of layers generation, we followed two complementary approaches. First, we assayed the time-course of expression of selected laminar markers, Tle4, Foxp2, and Cux1 for cortical plate (CP) as well as Calretinin and *Reelin* (*Reln*) mRNA for preplate (PPL). Second, we performed systematic bromodeoxyuridine (BrdU) pulse-chase birthdating analysis.

Time-course analysis of laminar markers gave results similar to the mouse. There were, however some differences. As for Foxp2, no neocortical signal was detectable at P0, when a few strongly labeled cells were conversely present in basal ganglia (data not shown). A Foxp2 signal appeared in lateral neocortex by P4. At P8, this signal spread to the entire cortical plate, getting progressively confined to the deepest part of it at later developmental ages **(Fig. 1B)**.

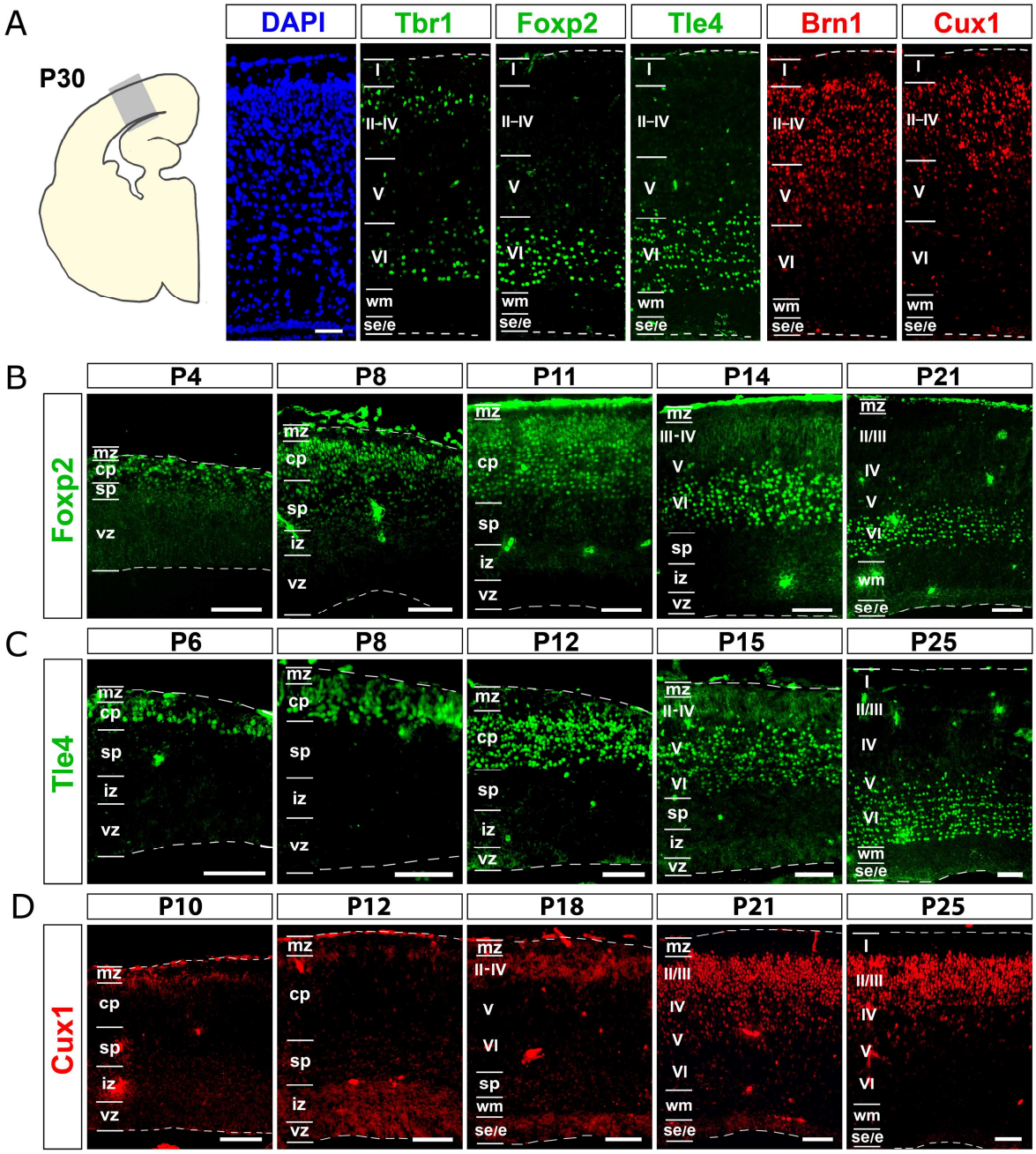


Figure 1 | Expression profiles of layer-specific markers in the opossum neocortex. (*A*) DAPI staining and Tbr1, Foxp2, Tle4, Brn1 and Cux1 immunofluorescence, on adjacent P30 mid-frontal neocortical sections. *(B-D)* Time course immunoprofiling of Foxp2, Tle4 and Cux1 on mid-frontal neocortical sections from P4 to P25 ages. Abbreviations: cp, cortical plate; e, ependyma; mz, marginal zone; ppl, preplate; se, subependymal zone; sp, subplate; iz, intermediate zone; vz, ventricular zone; wz, white matter; I, II, III, IV, V, VI are cortical layers. Scale bars: 100µm.

A similar profile was displayed by the deep cortical plate marker Tle4 (data not shown and **Fig. 1C**). As for Cux1 (**Fig. 1D**), two weak and hardly detectable signals were found at P10, in periventricular layers and in a few cells in the upper cortical plate. These signals were stronger at P12, and, by P18, the abventricular expression domain became wider than

the periventricular one. By P25 Cux1^{+} cells were tightly clustered in the most superficial cortical plate and no more Cux1 was detectable near the ventricle. Calretinin (Calb2), expressed by mouse subplate and Cajal Retzius cells, was detectable in the opossum telencephalon throughout neuronogenesis (**Fig. 2A**). At P1, Calretinin^{+} cells were mainly localized in the ventral telencephalon and, within the cortex, restricted to the most marginal-lateral part of it (**Fig. 2Aa,a',** arrowheads). At P4, positive cells were throughout the cortical plexiform layer (PPL), including the hippocampus; within the lateral cortex, the Calretinin^{+} domain was split in two stripes, the more superficial including the marginal zone (MZ), the deeper corresponding to the subplate (SP). (**Fig. 2Ab,b'**). This SP domain, relatively wider as compared to the mouse one (**Fig. 2Ac,c',d,d'**), persisted up to P18 (**Fig. S1A**), disappearing around P20 (**Fig. S1B,C**).

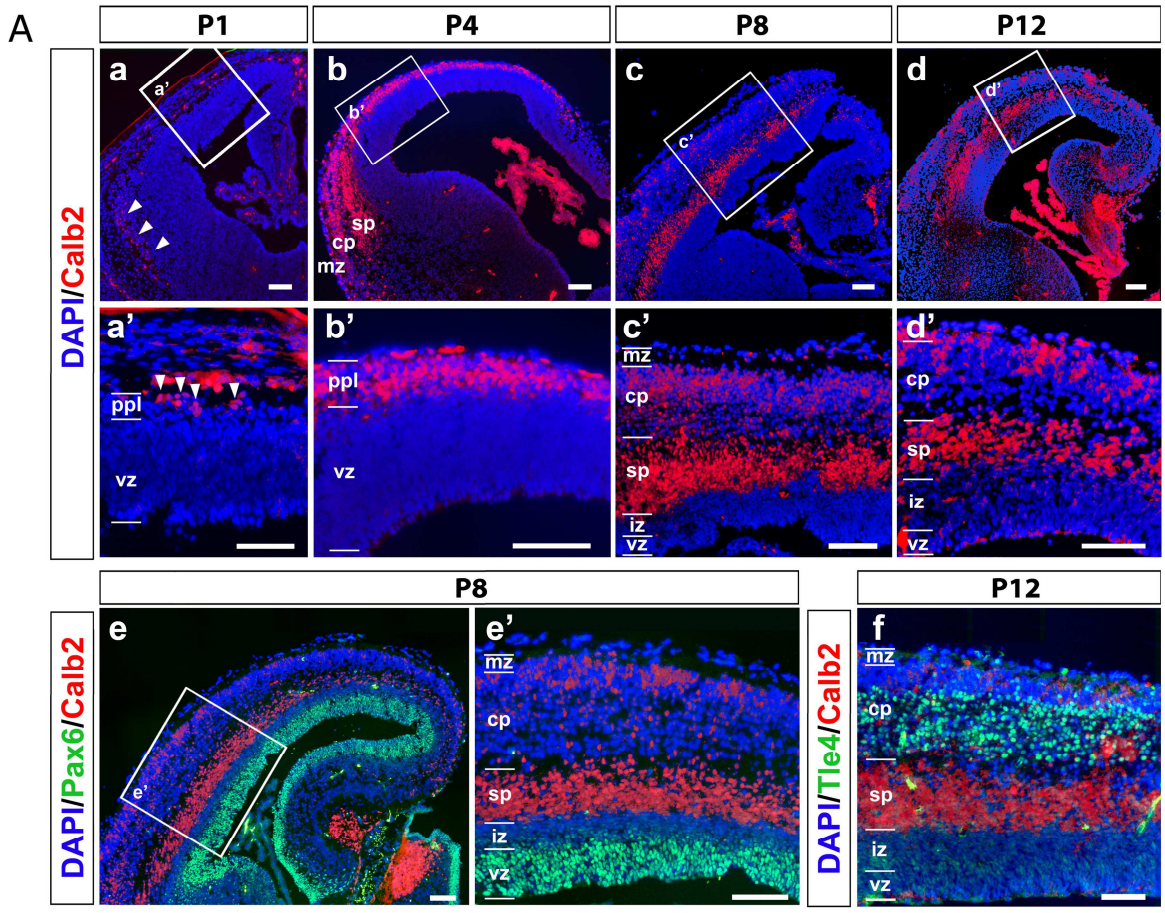


Figure 2A | Dynamics of Calretinin expression in the developing opossum telencephalon. (*Aa-d*) Time course immunoprofiling of Calretinin (Calb2), from P1 to P12. (*Aa'-d'*) Magnifications of boxed areas in (*Aa-d*). (*Ae-f*) Comparisons among distributions of Calretinin, Pax6 (*Ae,e'*) and Tle4 *(Af)*. Abbreviations: cp, cortical plate; mz, marginal zone; ppl, preplate; sp, subplate; iz, intermediate zone; vz, ventricular zone. Scale bars: 100µm.

Such SP domain was separated from the ventricular Pax6 domain by the interposed intermediate zone (IZ) (**Fig. 2Ae,e'**) and abutted the layer VI-V Tle4 domain on its marginal side (**Fig. 2Af**). However, Calretinin was not restricted to PPL and its derivatives. From P4 until P18, weaker labelled Calretinin$^+$ cells were detectable within the outmost cortical plate, where the last generated neurons settle (**Fig. 2Ab,c',d'**; **Fig. S1A**). Starting from P20 and, better, at P30, a distinct, areally restricted, strong Calretinin expression domain was evident a few cell rows deeper to the MZ (**Fig. S1B-F**).

As for Reelin, this glycoprotein is a hallmark of Cajal-Retzius cells in the mouse, where additional *Reln*$^+$ cells can also be found in layers IV-V of the late cortical plate (D'Arcangelo *et al.*, 1995; Yoshida *et al.*, 2005). We studied *Reln* expression in the opossum by a riboprobe corresponding to exons 1-12 and found a spatio-temporal profile similar to the mouse one (**Fig. 2B**). No *Reln* signal was detectable in the pallium at P3, i.e. just before the appearance of the CP (**Fig. 2Ba,a')**. Three days later, however, at P6, numerous *Reln*$^+$ cells were found in the neocortical MZ (**Fig. 2Bc,c'**), as well as in the marginal cingulate cortex (**Fig. 2Bb,b'**) and in the stratum lacunosum-moleculare of the hippocampus (**Fig. 2Bc,c''**), i.e. near two of the main birthplaces of Cajal-Retzius cells described in Placentals (Takiguchi-Hayashi *et al.*, 2004; Bielle *et al.*, 2005).

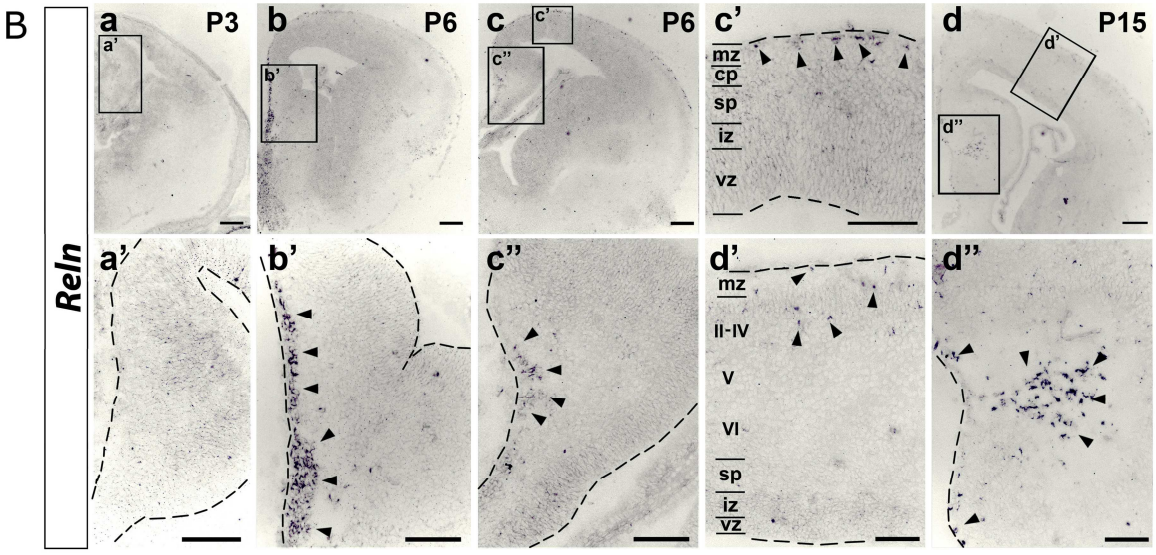


Figure 2B | Dynamics of *Reln* expression in the developing opossum telencephalon. *In situ* hybridization of *Reln* mRNA on coronal sections of P3, P6 and P15 opossum telencephalons. (*Ba'-d''*) are magnifications of boxed areas in (*Ba-d*). Abbreviations: cp, cortical plate; mz, marginal zone; sp, subplate; iz, intermediate zone; vz, ventricular zone; II-IV, V, VI are cortical layers. Scale bars: 100µm.

This expression pattern was retained at least up to P15. At this age *Reln*$^{+}$ cells within the neocortical MZ were much more sparse and additional *Reln*$^{+}$ elements were detectable within the developing neocortical CP (**Fig. 2Bd-d''**).

To reconstruct the temporal order of layer generation, we also performed systematic BrdU pulse-chase birthdating analysis. By this approach, cells which were in S-phase at the time of BrdU injection and exited the cell cycle immediately afterwards, remained heavily labeled and as such, easily traceable upon completion of their radial migration. We injected opossum pups at different developmental ages (P1; P4; P6; P8; P10; P12; P14; P16; P18) with a single pulse of saturating BrdU and left them to develop until the age of P30, when all neurons have reached their final laminar position (as shown in **Fig. 1** e **Fig. 2**). We recovered their brains and analyzed the cortices by BrdU immunofluorescence (**Fig. 3A**).

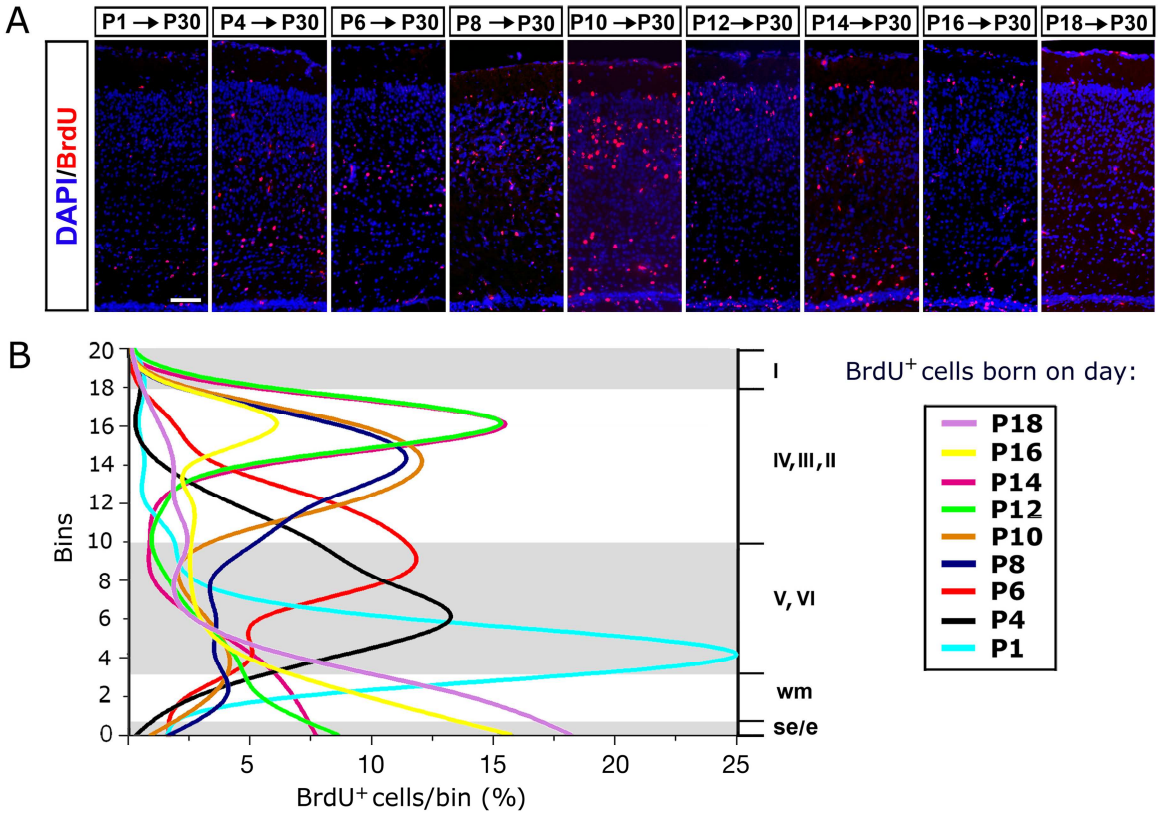


Figure 3(A/B) | BrdU-birthdating of opossum neocortical neurons. (*A*) BrdU immunoprofiling of mid-frontal neocortical sections, from opossums injected with a single pulse of BrdU at the ages of P1, P4, P6, P8, P10, P12, P14, P16 and P18, and fixed at P30. (*B*) Diagramatic representation of BrdU^{+} cells sampled in (*A*): the cortical wall is divided into 20 equally spaced bins, numbered from ventricular to marginal; radial extension of cortical laminae is indicated by white/grey shading; plots representing percentages of BrdU^{+} cells falling into each bin, for each injection time, are superimposed. Abbreviations: e, ependyma; se, subependymal zone; wz, white matter; I, II, III, IV, V, VI are cortical layers. Scale bar: 100µm in (*A*).

To compare radial distribution and laminar identities of $BrdU^+$ cells in distinct brains, we divided the cortical wall into 20 equally spaced bins, numbered from ventricular to marginal, and on this framework reported the approximative radial extension of distinct cortical laminae: layer I (evaluated by loose DAPI staining), layers II-IV (by Cux1 immunofluorescence), layers V-VI (by Tle4 immunofluorescence). Then, for each injection time, we counted $BrdU^+$ cells, calculated the percentage of them falling into each bin and plotted the data. Finally, we superimposed the resulting curves, so obtaining a synopsis of the whole radial migration process (**Fig. 3B**). We found that neocortical neurons were generated in a wide temporal window, mainly from P1 to P14. Cells born at P16 reached superficial layers only to a limited extent, suggesting that at that age neurogenesis was over, and P18 cells prevalently remained beneath the CP. Deep cortical plate neurons were prevalently born between P1 and P6, upper cortical plate neurons between P8 and P14. Colocalization of Tle4 and Cux1 with BrdU in P30 animals injected at P4 and P12, respectively, confirmed this conclusion (**Fig. 3C,D**).

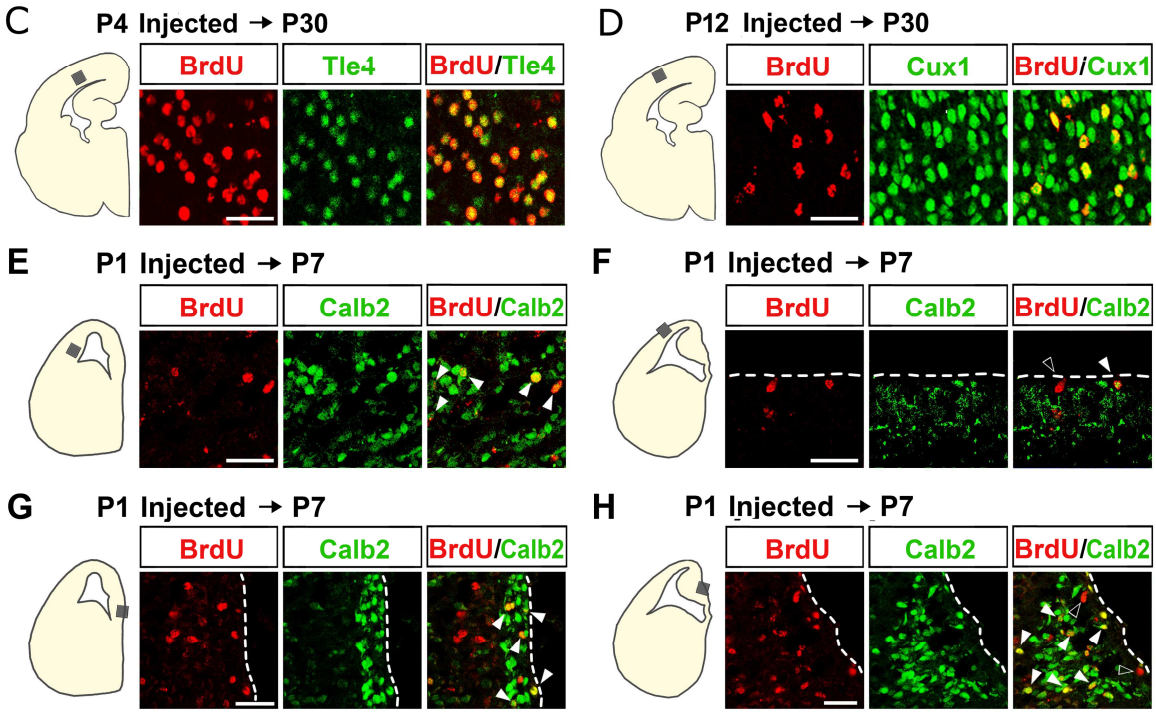


Figure 3(C/H) | BrdU-birthdating of opossum neocortical neurons. *C-H*) Colocalization of layer-specific markers, Tle4 (*C*), Cux1 (*D*), Calb2 (*E-H*), with BrdU injected at P4 (*C*), P12 (*D*) and P7 (*E-H*), respectively. Solid arrowheads in (*E-H*) point to $Calb2^+/BrdU^+$ cells; empty arrowheads in (*F,H*) point to $Calb2^-/BrdU^+$ cells. Abbreviations: e, ependyma; se, subependymal zone; wz, white matter; I, II, III, IV, V, VI are cortical layers. Scale bar: 40µm in (*C-H*).

Concerning the subplate (SP), as this structure is not anymore distinguishable at P30, we assayed the date of birth of its neurons in distinct, dedicated experiments. By administering P1 pups with BrdU and recovering their brains at P7 and P12, $BrdU^+$/$Calretinin^+$ cells were detectable beneath the CP, especially in lateral cortex (**Fig. 3E** and data not shown), suggesting that the SP is mainly generated around birth. Finally, consistently with *Reln* data, P1-BrdU-pulsed/$Calretinin^+$ cells, corresponding to presumptive Cajal-Retzius cells (Alcántara *et al.*, 1998), were also detectable at P7 in the neocortical MZ (**Fig. 3F**), the marginal cingulate cortex (**Fig. 3G**) and the hippocampal stratum lacunosum-moleculare (**Fig. 3H**).

In conclusion: (1) in the opossum, neocortical neuronogenesis begins at the time of birth and ends up two weeks later, at P14 – P16, and radial migration is completed by P25; (2) the molecular laminar profile is very similar in Marsupials and Placentals; (3) after preplate splitting, cortical plate neurons are laid down in both mammalian subclasses according to the same “inside-out” rule.

2. DOES A BASAL PROGENITORS COMPARTMENT EXIST IN THE OPOSSUM?

In Placentals, neocortical projection neurons are prevalently generated by basal progenitors (BPs) or intermediate progenitor cells (IPCs), which lie around the pallial subventricular zone/ventricular zone (SVZ/VZ) border and divide far from the ventricular surface (Sessa *et al.*, 2009, Kowalczyk *et al.*, 2009). In the developing opossum cortex, a subventricular zone is not morphologically distinguishable (Saunders *et al.*, 1989) and, based on the distribution of mitotic elements, a true basal progenitor compartment seems to be absent as well (Abdel-Mannan *et al.*, 2008). The mitoses survey by Abdel-Mannan *et al.*, (2008) - however - was restricted to the P8-P16 window; moreover, no basal molecular marker was assayed. So, we readdressed this issue, extending the mitosis survey to a wider developmental window and scoring molecular markers peculiar to neuronogenic progenitors, including kinetics of their expression.

First, we systematically studied the distribution of progenitors undergoing mitosis at distinct radial positions, starting from P1 up to P25, by scoring the mitotic marker phospo-Histone 3 (pH3) (**Fig. 4A**). For each developmental age, we divided the cortical wall into four unequally spaced bins, *l* (*luminal*, including the two ventricular most cell rows), *p* (*periventricular*, corresponding to the densely packed zone over the ventricle minus the *l* belt), *i* (*intermediate*, corresponding to the region between *p* and MZ), *m* (*marginal*, corresponding to the MZ), and plotted the percentages of $pH3^+$ cells falling in each of them (**Fig. 4B**). We observed that the vast majority of $pH3^+$ cells were aligned along the ventricular surface, as proper apical progenitors, whereas only a few of them were scattered elsewhere. Specifically at P4-P6 a few mitoses could be found in bin *p* (prevalently in its rostro-lateral part), corresponding to the main zone where basal progenitors divide in Placentals (**Fig. 4Ab, Fig. 4B** and data not shown). Abventricular mitoses could also be found in bins *i* and *m*, mainly after neuronogenesis completion (**Fig. 4Af, Fig. 4B**), as described for placental MZ glial progenitors (Costa *et al.*, 2007). As a complementary approach, we looked for cortical expression of the T-box transcription factor Tbr2, a hallmark of basal progenitors in Placentals (Englund *et al.*, 2005). We found numerous $Tbr2^+$ cells at all stages under examination, from P1 to P18 and later (**Fig. 4C** and data not shown). On 10µm-thick sections, their linear frequency gradually arose from 37±2 cells/100 µm, at P1, to 97±14 cells/100 µm, at P8 (P1-P8 is the time window when PPL, deep CP and part of superficial CP are generated), subsequently declining to 45±3 cells/100 µm at P15 (that is just after the end of neuronogenesis) and even less at P18 (**Fig.**

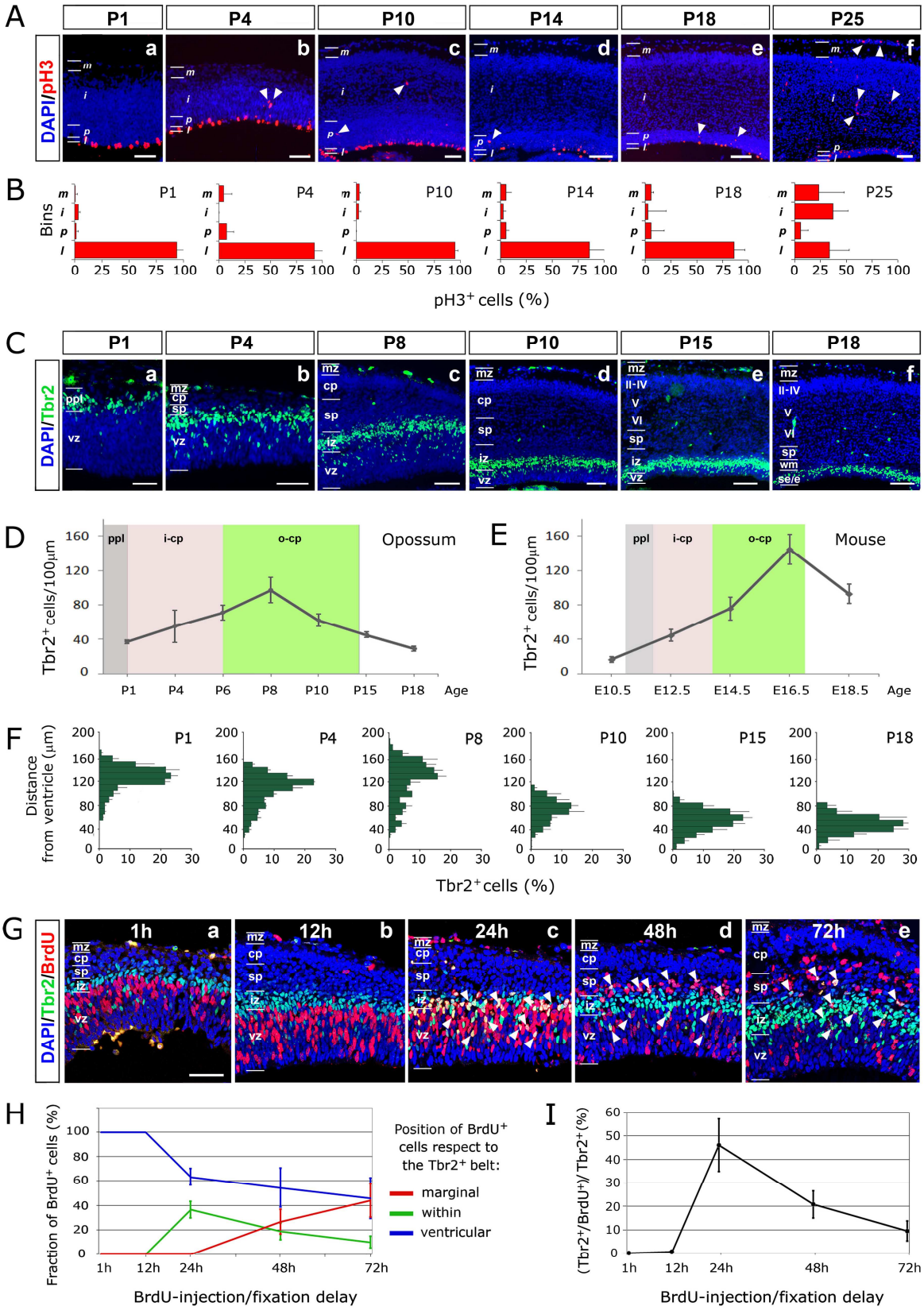
4D). Remarkably, this time course mimed that of murine $Tbr2^+$ cells (**Fig. 4E**), which - however - were more numerous and reached their peak linear density slightly later, close to the end of neuronogenesis. Finally, as for radial distribution, modal distance between $Tbr2^+$ cells and ventricular surface varied in the opossum from about 120-140 µm at P1-P8, to 60 µm at P15. In synthesis, $Tbr2^+$ cells were detectable throughout the neuronogenetic window, prevalently clustered at around the border between VZ and IZ, but also scattered within the VZ, exactly like Placentals basal progenitors (Englund *et al.*, 2005) (**Fig. 4F**).

So, based on pH3 data, a basal proliferative compartment does not seem to exist throughout the cortical field and at all developmental stages (except – maybe, in the early rostral-lateral pallium). Based on Tbr2 expression, a compartment molecularly similar to the basal one of Placentals can be distinguished, interposed between ventricular precursors and abventricular neurons. How to solve this conundrum? Rather than being due to absence of proliferative activity, paucity of abventricular $pH3^+$ cells might reflect a very slow cell cycle progression in front of a short duration of M-phase (Takahashi *et al.*, 1993 and 1995; our unpublished observations), possibly leading to an underestimation of proliferative population. To rule out this possibility, we looked for presumptive basal cells in S-phase, reasonably several times more frequent than the corresponding M-phase ones (Takahashi *et al.*, 1993 and 1995). So, we pulsed P4 - P10 opossum pups with BrdU, fixed their brains and looked for cortical $Tbr2^+/BrdU^+$ cells. Such cells were extremely rare, if any, and the vast majority of $Tbr2^+$ cells lay above $BrdU^+$ ones (data not shown and **Fig. 4Ga**). This suggests that, in Marsupials, $Tbr2^+$ cells are not proper basal progenitors, but represent a *subventricular postmitotic* compartment, interposed between intermitotic apical progenitors and postmitotic abventricular neurons.

To corroborate this interpretation, we further investigated the origin and fate of $Tbr2^+$ cells. In rodents, $Tbr2^+$ cells are generated by a subset of neuronally committed apical progenitors, called “pin-like” cells or short neural precursors (SNPs), which, facing the ventricular cavity and not contacting the pial surface, can be distinguished from radial glial cells (RGCs) for specific firing of the α1-tubulin promoter ($pT\alpha1^+$ cells) (Gal *et al.*, 2006; Ochiai *et al.*, 2009). We electroporated a pTα1-EGFP construct into the P10 opossum cortex and fixed electroporated brains 2 days later, after a final pulse (60 min) of BrdU. Once verified their vitality and cytoarchitectonic integrity (by scoring radial distribution of $BrdU^+$ and $Tbr2^+$ cells, **Fig. S2**), we finally looked for $GFP^+/Tbr2^+$ cells (**Fig. 4J**). Many of them were found (**Fig. 4Jb-b’’**), confirming that opossum $Tbr2^+$ cells

derive from apical progenitors, as in rodents. We then compared the distribution of Tbr2 and the neuronal marker β-tubulin on coronal slices of P10 opossum brains (**Fig. 4K**). $Tbr2^+$ cells lay below the main β-tubulin domain, but a substantial overlap between the two antigens was detectable as well (**Fig. 4Ka,b**): in particular, around 30% of acutely dissociated neocortical cells expressing Tbr2 were also positive for β-tubulin (**Fig. 4Kc-h**), consistently with the hypothesis that Tbr2 is transiently expressed before the activation of neuron-specific markers.

Finally, to get a better temporal resolution of the developmental process under examination, we pulsed P6 pups with BrdU, fixed their brains after different times, (from 6h up to 72h), and immunoprofiled $BrdU^+$ cells, performing time-course analysis of their radial distribution (**Fig. 4G** and **Fig. 4H**). Comparing profiles got at different times, $BrdU^+$ cells seemed to move along a wave, from the ventricular side towards the marginal aspect of the cortical wall. In particular, up to 12h, all $BrdU^+$ cells lay deep to the $Tbr2^+$ belt (**Fig. 4Gb**); starting from 24h, some of them were detectable within this belt (**Fig. 4Gc**); at 48h, about 1/3 of them were over it (**Fig. 4Gd**); finally, at 72h, the majority had overcome the $Tbr2^+$ belt, so that only a few remained near the ventricle (**Fig. 4Ge**). Remarkably, the percentage of Tbr2 cells also immunoreactive for BrdU displayed a biphasic trend, arising from about 0% at 12h up 46% at 24h and subsequently declining to less than 10% at 3 days (**Fig. 4I**). In synthesis, neural progenitors exiting the cell cycle leave the VZ and activate Tbr2 18±6 hours after the last DNA synthesis; one day later, the same cells massively move to more marginal positions, where they downregulate Tbr2, while activating neuron-specific markers (**Fig. 4Kc-h**).


A
P1
P4
P10
P14
P18
P25
DAPI/pH3
B
Bins
pH3+ cells (%)
C
P1
P4
P8
P10
P15
P18
DAPI/Tbr2
D
Opossum
Tbr2+ cells/100µm
Age
E
Mouse
F
Distance from ventricle (µm)
Tbr2+ cells (%)
G
1h
12h
24h
48h
72h
DAPI/Tbr2/BrdU
H
Fraction of BrdU+ cells (%)
Position of BrdU+ cells respect to the Tbr2+ belt:
marginal
within
ventricular
BrdU-injection/fixation delay
I
(Tbr2+/BrdU+)/ Tbr2+(%)
BrdU-injection/fixation delay

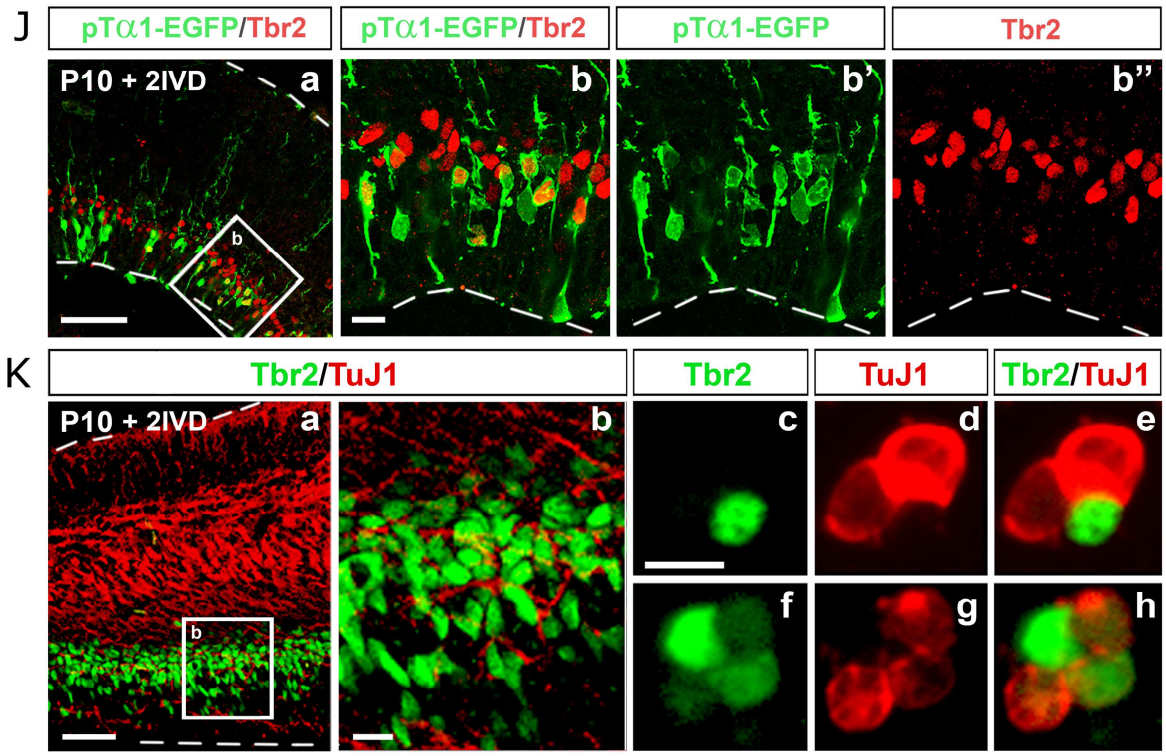


Figure 4 | Dynamics of phospho-histone3 (pH3) and Tbr2 expression in the developing opossum cortex. (*A*) pH3 immunoprofiling of mid-frontal neocortical sections of P1-P25 opossums. Immunopositive cells are prevalently aligned near the ventricle, solid arrowheads point to rare abventricular $pH3^+$ mitotic cells. (*B*) Diagramatic representation of $pH3^+$ cells sampled in (*A*), classified in: *l* (*luminal*, including the two ventricular most cells rows), *p* (*periventricular*, corresponding to the densely packed zone over the ventricle minus the *l* belt), *i* (*intermediate*, corresponding to the region between *p* and marginal zone), *m* (*marginal*, corresponding to the marginal zone). (*C*) Tbr2 immunoprofiling of mid-frontal neocortical sections of P1-P18 opossums. (*D,F).* Linear densities *(D)* and radial distributions *(F)* of $Tbr2^+$ cells sampled in *(C). (E)* Linear densities of $Tbr2^+$ cells in the mouse. Grey, pink and green shading in (*D*) and (*E*) demarkate peak neuronogenesis windows for primordial plexiform layer (ppl), inner cortical plate (i-cp) and outer cortical plate (o-cp), respectively. (*G*) Confocal Tbr2/BrdU immunoprofiling of neocortical coronal sections from opossum pups, pulsed with BrdU at P6 and sacrificed after different times: 1h, 12h, 24h, 48h and 72h. Arrowheads in (*Gc-e*) point to cells immunoreactive for both BrdU and Tbr2. (*H*) Relative radial distribution of $BrdU^+$ cells sampled in (*G*) compared to the $Tbr2^+$ belt. (*I*) Time course of percentages of $Tbr2^+$cells sampled in (*G*) also immunoreactive for BrdU. (*J,K*) Confocal Tbr2/EGFP (*Ja-b''*) and Tbr2/β-tubulin (*Ka,b*) immunoprofiling of opossum cerebral cortex, dissected out at P10, acutely electroporated with a pTα1-EGFP plasmid and kept in vitro culture for 48 hours. Magnifications of boxed areas in (*Ja*) and (*Ka*) are shown in (*Jb-b''*) and (*Kb*), respectively. (*Kc-h*) Colocalization of Tbr2 and β-tubulin on acutely dissociated cells from P10 opossum cortex. Abbreviations: cp, cortical plate; e, ependyma; iz, intermediate zone; mz, marginal zone; ppl, preplate; sp, subplate; se, subependymal zone; vz, ventricular zone; wm, white matter; II-IV, V, VI are cortical layers. Scale bars: 100µm in (*A,C,G,J,K*), 20µm in (*Jb, Kb,c*).

3. THE APICAL PROLIFERATIVE COMPARTMENT AND ITS DYNAMICS

It stems from previous results that the apical progenitor compartment is the place where pallial projection neurons are generated. To reconstruct its dynamics, we performed time-course analysis of its hallmark Pax6 (Englund *et al.*, 2005). As in Placentals, this homeoprotein was specifically expressed in the pallial ventricular zone, throughout neuronogenesis (**Fig. 5A**). On 10µm-thick sections, linear frequencies of Pax6$^+$ cells were 160±16 cells/100 µm at P4, 132±4 cells/100 µm at P8, 92±8 cells/100 µm at P10, 66±11 cells/100 µm at P15 (**Fig. 5**B). These values were slightly lower than the corresponding mouse ones (185±16 cells/100 µm at E12.5, 156±13 at E14.5 and 96±13 cells/100 µm at E16.5; data not shown), however the two temporal progressions were basically very similar. All that suggests that decreased neuronal density and reduced thickness of the opossum cortex (Saunders *et al.*, 1989), rather than reflecting a change of the apical proliferative compartment size, mainly originates from the absence of a basal, transient amplifying population.

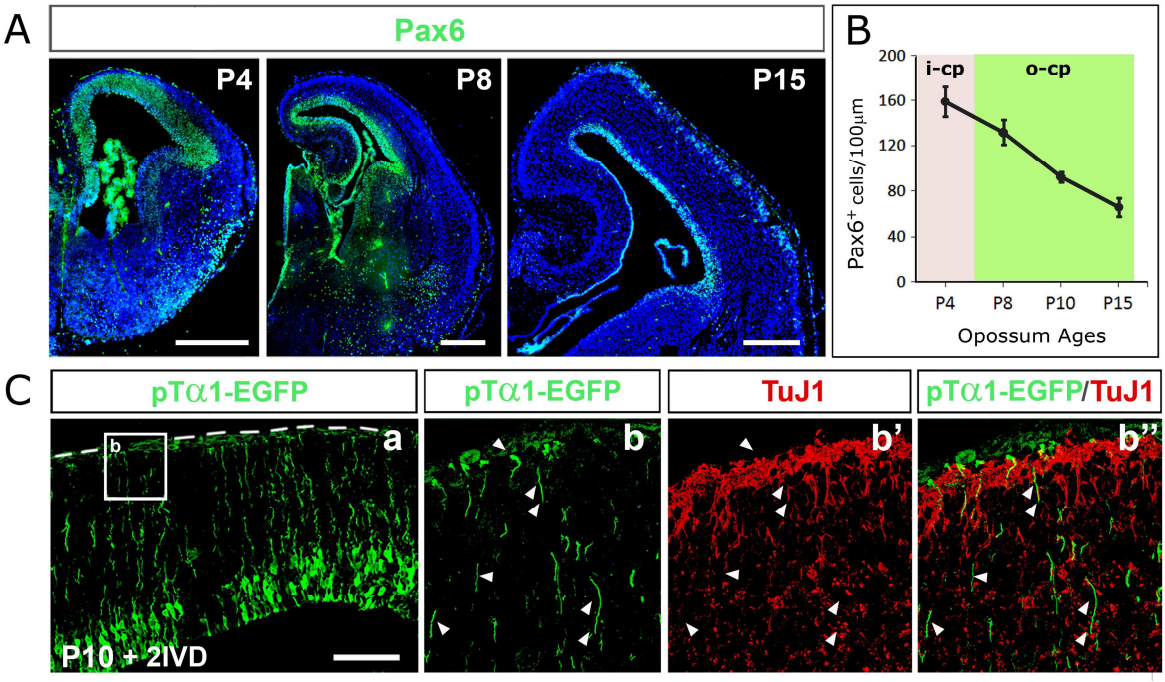


Figure 5 | Analysis of the apical progenitors compartment. (*A*) Pax6 immunoprofiling of a selection of coronal sections from P4-P15 opossum telencephalons. (*B*) Linear densities of Pax6$^+$ cells sampled in (*A*). (*C*) EGFP/β-tubulin immunoprofiling of opossum cerebral cortex, dissected out at P10, acutely electroporated with a pTα1-EGFP plasmid and kept in vitro culture for 48 hours. Magnifications of the boxed area in (*Ca*) are shown in (*Cb-b''*). Arrowheads in (*Cb-b''*) point to pial processes of electroporated cells, immunoreactive for EGFP, but not for β-tubulin. Scale bars, 400µm in (*A*), 100µm in (*C*).

Moreover, in placental neocortex, the $Pax6^+$ compartment is not homogeneous. It includes neural stem cells with morphology of radial glial cells (RGCs) and $pT\alpha1^+$ neuronally committed progenitors, having lost their contact with pial surface. It is noteworthy that, upon electroporation of the pTα1-EGFP transgene into the opossum cortex, fluorescent cells displayed morphology of proper RGCs, extending from the ventricular edge to the pial surface (**Fig. 5Ca**). Moreover, despite of the time elapsed after electroporation, pial processes and end-feet of many of these cells were still not immunoreactive for neuron-specific β-tubulin, ruling out that they were neurons (**Fig. 5Cb-b''**). All that means that in Marsupials, either pTα1 fires in RGCs, or retraction of the pial process by neuronally committed progenitors is delayed as compared to rodents.

4. DISTRIBUTION AND GENERATION OF CORTICAL GABAERGIC CELLS

In the mouse, GABAergic neurons are generated within ventral forebrain and reach the cortex by tangential migration, according to a well characterized spatio-temporal pattern (reviewed in Marin and Rubenstein, 2001; López-Bendito *et al.*, 2004). First interneurons enter the cortex at around E12.5, the migratory wave reaches the mid-neocortex at E13.5, the same gets into the hippocampus by E15.5. Early migration is mainly superficial, by E13.5 a robust periventricular migratory route appears, becoming predominant at later stages. In order to establish developmental correspondences between mouse and opossum, we systematically scored the distribution of GABA immunoreactive cells in the developing post-natal telencephalon of the marsupial. Since P1 and up to P14, a huge number of $GABA^+$ cells were detected within the ventral telencephalon. Here, they were early confined to abventricular layers, and later also detectable near the ventricle (**Fig. 6Aa-d**).

Cortical distribution was more complex. At P1 only a diffuse and light immunoreactivity was detectable throughout the marginal pallium (**Fig. 6Aa')**, and rare $GABA^+$ somata were localized in presumptive paleocortex (**Fig. 6Aa** and data not shown). At P4 a substantial number of $GABA^+$ cells were present in the abventricular half of both paleo- and neocortex, but not in the archicortex (**Fig. 6Ab,b'**). At P8, abventricular $GABA^+$ cells were present throughout the cortex, including the marginal hippocampus, ventricular $GABA^+$ cells were conversely limited to paleo- and neocortex (**Fig. 6Ac,c'**). Finally, at P14, $GABA^+$ cells were detectable throughout the cortex, clustered in a narrow periventricular belt and more loosely distributed elsewhere (**Fig. 6Ad,d'**). Remarkably, spatio-temporal distribution of immunoreactivity against glutamate-decarboxylases 65 and 67 (GAD65 and GAD67) was consistent with the GABA pattern (**Fig. 6B**).

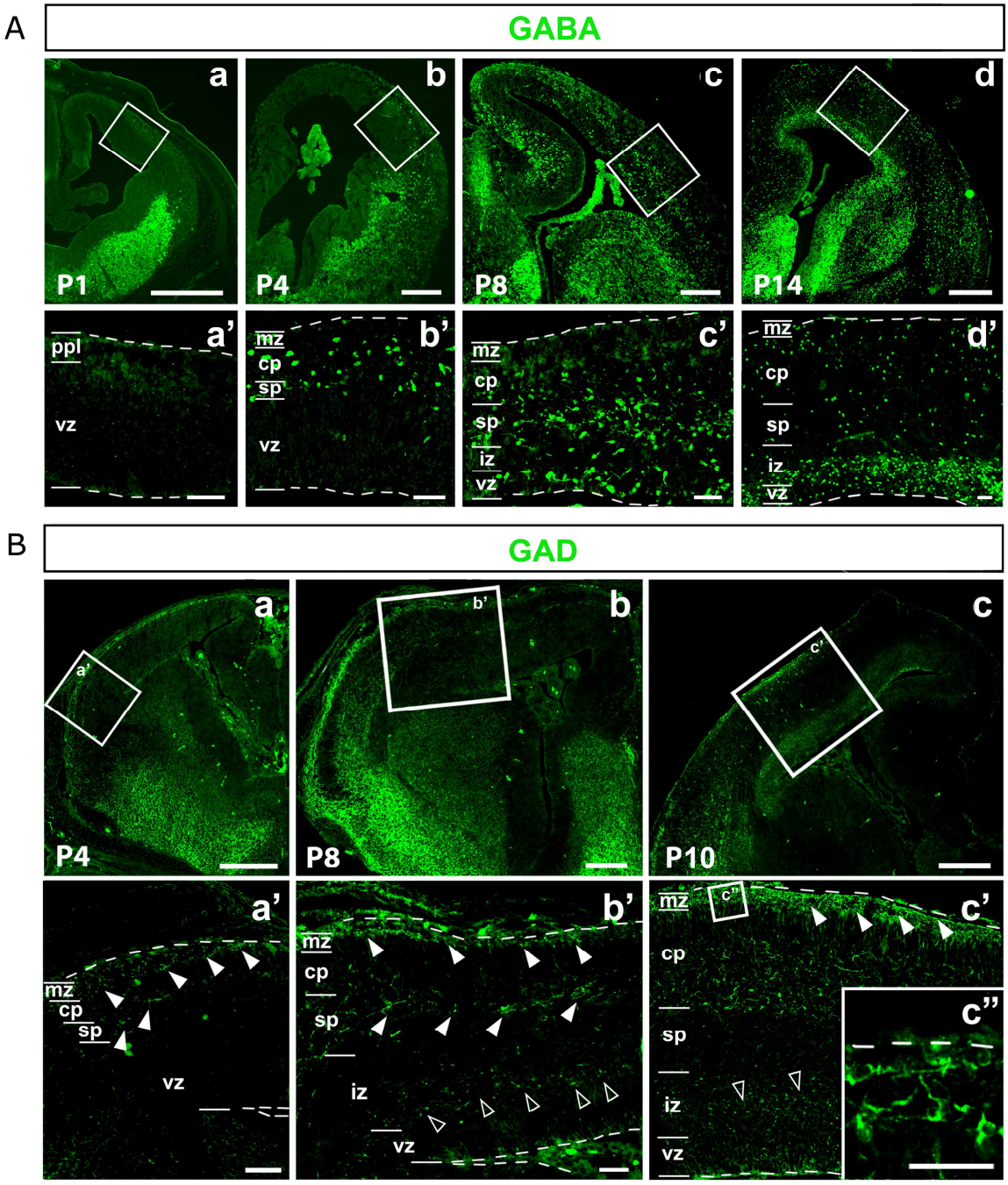


Figure 6 | Distribution of GABA and GAD immunoreactivity in developing opossum cortices. (*Aa-d*) Distribution of GABA$^+$ cells on frontal sections of P1-P14 cortices. (*Aa'-d'*) Magnifications of boxed regions in (*Aa-d*). (*Ba-c*) Distribution of GAD$^+$ cells on frontal sections of P4, P8 and P10 cortices. (*Ba'-c'*) Magnifications of boxed regions in (*Ba-c*). GAD$^+$ cells are evident in the MZ and the SP since P4 (*Ba'-b'*, solid arrowheads). At P8, an additional reactivity is detectable in proliferative layers (*Bb'*, empty arrowheads). Finally, at P10, both MZ and periventricular GAD signals are strengthened (*Bc',c''*) and additional GAD$^+$ cells are present throughout the cortical plate (*Bc'*). Abbreviations: cp, cortical plate; iz, intermediate zone; mz, marginal zone; sp, subplate; vz, ventricular zone. Scale bars: 400µm in *(Aa-d; Ba-c)*, 50µm in (*Aa'-d'*; *Ba'-c''*).

5. POST-NEURONAL HISTOGENESIS: GENERATION OF ASTROCYTES AND OLIGODENDROCYTES

In rodents gliogenesis follows neuronogenesis and that happens in the developing opossum cortex as well. To assess astrocytogenesis progression, we looked at the distribution of the Glial Fibrillary Acid Protein antigen (Gfap), from P8 (i.e. the middle of the neuronogenic window) onward. A periventricular signal was detected from the beginning, restricted to hippocampus at P8 (**Fig. 7Aa**), spread throughout the cortical field at P12 (**Fig. 7Ab**). This signal presumptively corresponds to the soma of radial glial cells, which - in primates - share this marker with true astrocytes (Levitt and Rakic, 1980; Sancho-Tello *et al.*, 1995). An abventricular Gfap signal associated to cells with morphology of astrocytes, could be found only later, starting from P18-P20 (data not shown and **Fig. 7Ac-f**). These cells were detectable in the MZ, the grey matter and white matter. A subset of them coexpressed the mature astrocyte marker S100β. (**Fig. 7Bg-i''**). The differentiation of oligodendrocytes was studied by monitoring immunoreactivity for the marker O4 (Sommer and Schachner, 1981). A strong signal was found at both analyzed ages, P40 and P60, restricted to white matter (wm), internal capsule (ic) and hippocampal commissure (hc) (**Fig. 7Ca,b**). [*Monodelphis* has no corpus callosum (Abbie *et al.*, 1939)].

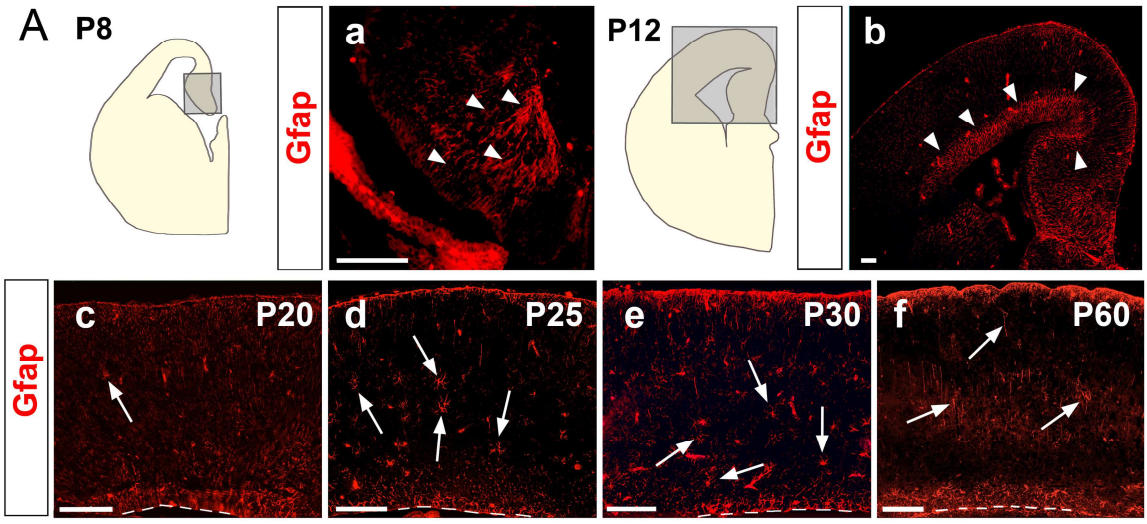


Figure 7 (A) | Gliogenesis in the opossum cortex. (*Aa-f*) Gfap immunoprofiling of coronal sections from P8-P60 cortices. Arrowheads in (*a*) and in (*b*) point to Gfap$^+$ presumptive radial glial cells, within P8 hippocampus and P12 cortical periventricular layers, respectively. Arrows in (*c-f*) indicate Gfap$^+$ cells with astrocyte morphology. Scale bar: 100 µm.

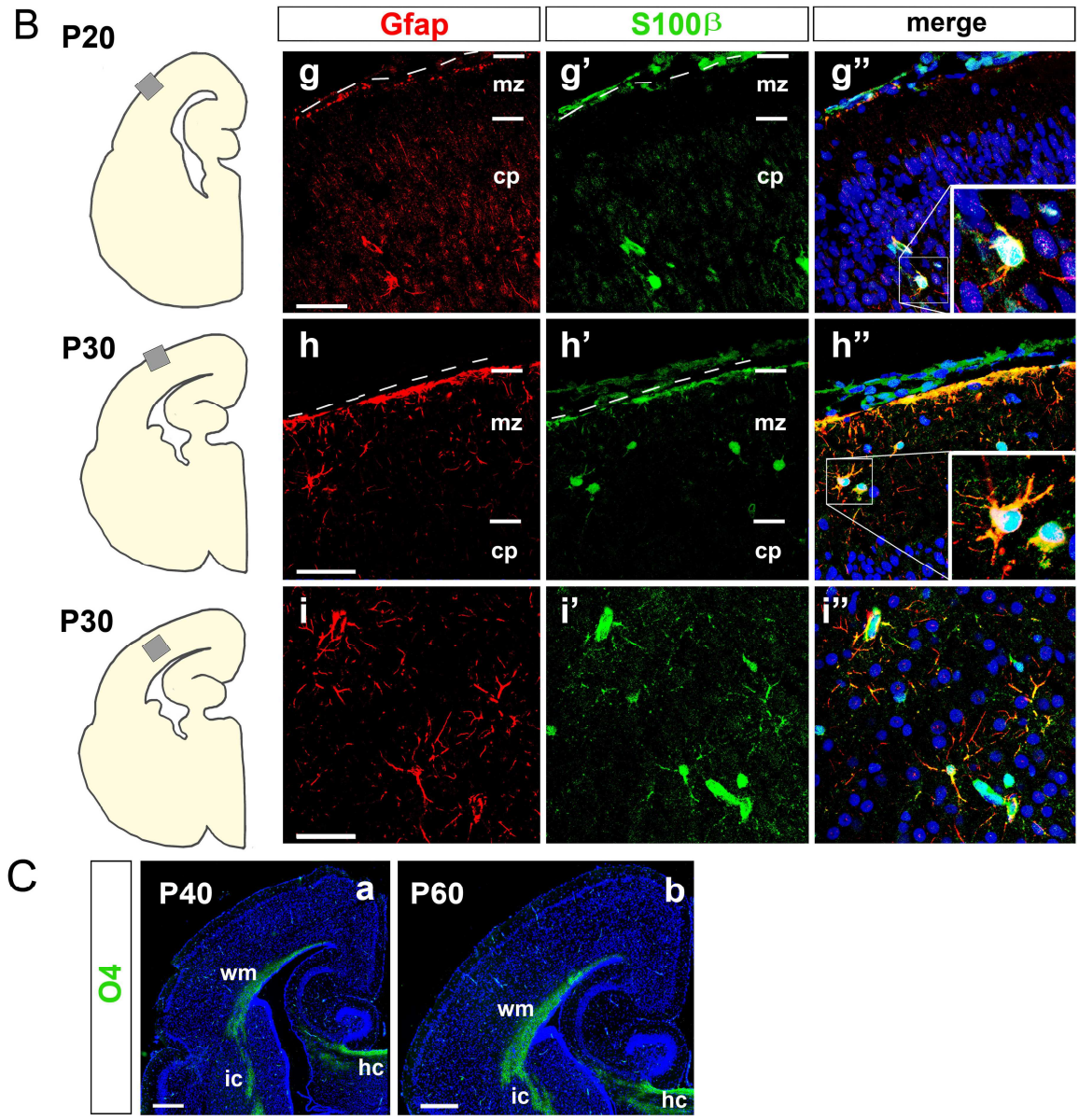


Figure 7 (B/C) | Gliogenesis in the opossum cortex. (*Bg-i''*) Combined Gfap/S100β immunoprofiling of P20-P30 cortices, showing specific colocalisation of these two antigens within more mature astrocytes, in cortical plate (CP) and marginal zone (MZ). (*Ca,b*) Immunoprofiling of P40-P60 mid-frontal cortical sections for the oligodendrocyte-specific marker O4: an intense staining may be found in white matter (WM), internal capsule (ic) and hippocampal commissure (hc). Scale bars: 100 µm in (*Aa-f*), 50 µm in (*Bg-i*), 200 µm in (*B*).

DISCUSSION

In the present study we found that the cortico-cerebral neuronal complement is generated in the opossum pup between P1 and P16, molecular diversification of neurons belonging to distinct laminae largely resembles that of Placentals, migration of cortical plate neurons follows the "inside-out" rule. We demonstrated that opossum cortico-cerebral neurons are almost entirely generated by apical progenitors, and that Tbr2, the hallmark of placental basal progenitors, is only transiently expressed by opossum post-mitotic elements, prior to the activation of neuron-specific genes. Moreover, we showed that such absence of a basal transient amplifying population is the main reason for reduced thickness and decreased neuronal density, peculiar to the Marsupials cortex (Saunders *et al.*, 1989; see also http://brainmaps.org/). As for GABAergic neurons, we found that they are mainly generated in the subpallium, like in rodents (reviewed in Marin and Rubenstein, 2001), and invade the cortex by P4. Finally, like in Placentals, cortical histogenesis continues with astrocytogenesis (from P18 onward) and ends up with oligodentrocytogenesis (around P40 and later).

We found that molecular diversification of the cortical plate is highly conserved between Marsupials and Placentals, with Foxp2 and Tle4 restricted to deeper layers and Brn1 and Cux1 mainly confined to upper layers. This suggests that the neocortical hexalaminar profile arose before the branching between these two subclasses, about 180 My ago (Murphy *et al.*, 2004). Minor differences were observed in derivatives of preplate, namely the phylogenetically most ancient component of our cortex (Marin Padilla, 1998). Based on Calretinin immunostaining, the opossum subplate appeared quite prominent, as previously assessed by simple histological inspection (Saunders *et al.*, 1989), far thicker than in rodents (Mallamaci *et al.*, 2000). Cells expressing *Reln,* a hallmark of Cajal Retzius (CR) neurons, were detectable beneath the pia mater, in both neo-and archicortex, like in Placentals. However their appearance did not predate the splitting of the preplate, as it happens in rodents (Mallamaci *et al.*, 2000). We reconstructed rules governing marsupial neocortical lamination, by following two complementary experimental approaches. First, we compared the mature distribution of neocortical laminar markers with the radial settling profile of neurons generated at different developmental times, as assessed by BrdU pulse-chase analysis. Second, we performed systematic time-course expression analysis of a selection of these markers, from their appearance to the end of the lamination process.

Both approaches indicated that, in *M. domestica,* radial neuronal migration takes place in a way similar to Placentals. Like in rodents (D'Arcangelo *et al.*, 1995; Yoshida *et al.*, 2005), such process is reasonably promoted by the glycoprotein Reelin, released by Cajal-Retzius cells and, later, by some CP neurons. This protein seems - however - dispensable for preplate splitting, which apparently occurs in the absence of detectable expression of its mRNA.

Beyond the study of laminar differentiation and radial migration, we paid special attention to the origin of cortical neurons, both glutamatergic and gabaergic. In Placentals glutamatergic neurons are generated within the dorsal telencephalon by two periventricular proliferative compartments, the apical and the basal, the former confined to the ventricular zone, the latter mainly lying in the subventricular zone (reviewed in Guillemot, 2005). It was previously reported that, whereas an apical compartment is present in the dorsal telencephalon of all vertebrates, the basal one seems to specifically emerge in Placentals (Saunders *et al.*, 1989, Abdel-Mannan *et al.*, 2008). We readdressed this issue by a variety of approaches, including scoring of $pH3^+$ mitoses and $Tbr2^+$ cells, immunoprofiling of acutely administrated BrdU and pTα1-driven EGFP, as well as BrdU pulse-chase analysis. It resulted that, in the opossum, cells lying at the VZ/IZ border and expressing Tbr2 do not form a transient amplifying population, but represent a postmitotic transitional compartment, passed through by neuroblasts in the process of switching Pax6 off and activating neuron-specific β-tubulin. Remarkably, such absence of a tight linkage between Tbr2 expression and basal progenitor identity is not surprising, as this transcription factor is also expressed in the pallium of other vertebrates missing a basal proliferative compartment, such as Anamnia and birds (Brox *et al.*, 2004; Mueller *et al.*, 2008, Bulfone *et al.*, 1999). Anyway, it has to be recalled that a few abventricular $pH3^+$ mitoses may be actually found in the lateral most opossum pallium, mainly at P4 – P6. It is tempting to speculate that such basal proliferative activity may selectively increase the final neuronal output of the small ventricular sector inbetween neopallial and striatal fields, in charge of generating paleocortex and other latero-ventral derivatives of the amniote telencephalon (Fernandez *et al.*, 1998).

CONCLUSIONS

Our analysis shows that three key aspects of cortico-cerebral histogenesis are conserved among mouse and opossum:

- in both system models, glutamatergic and gabaergic neurons are generated by progenitors within dorsal and ventral telencephalic periventricular layers, respectively;
- such neurons reach the cortical plate following shared patterns of radial and tangential migration, respectively;
- molecular differentiation of neurons belonging to different laminae takes place according to similar profiles;

So, based on temporal progression of morphogenetic subroutines subject of our analysis, we can sketch a scheme with presumptive correspondences among cortico-cerebral developmental ages of mouse and opossum (**Fig. 8**).

One main difference between mouse and opossum emerged from our study: this is the structure of proliferative compartments of the cortical primordium. In the opossum, only an apical proliferative compartment exists. A $Tbr2^{+}$ compartment, reminiscent of the murine basal compartment, can be found in between apical intermitotic progenitors and mature neurons, however it includes only postmitotic elements.

These findings suggest that, whereas the general blueprint of neocortex emerged before the branching between Eutherians and Methaterians, the development of a basal proliferative compartment is a subsequent acquisition of the Eutherians lineage, paving the way to the thickening of their cortical grey matter.

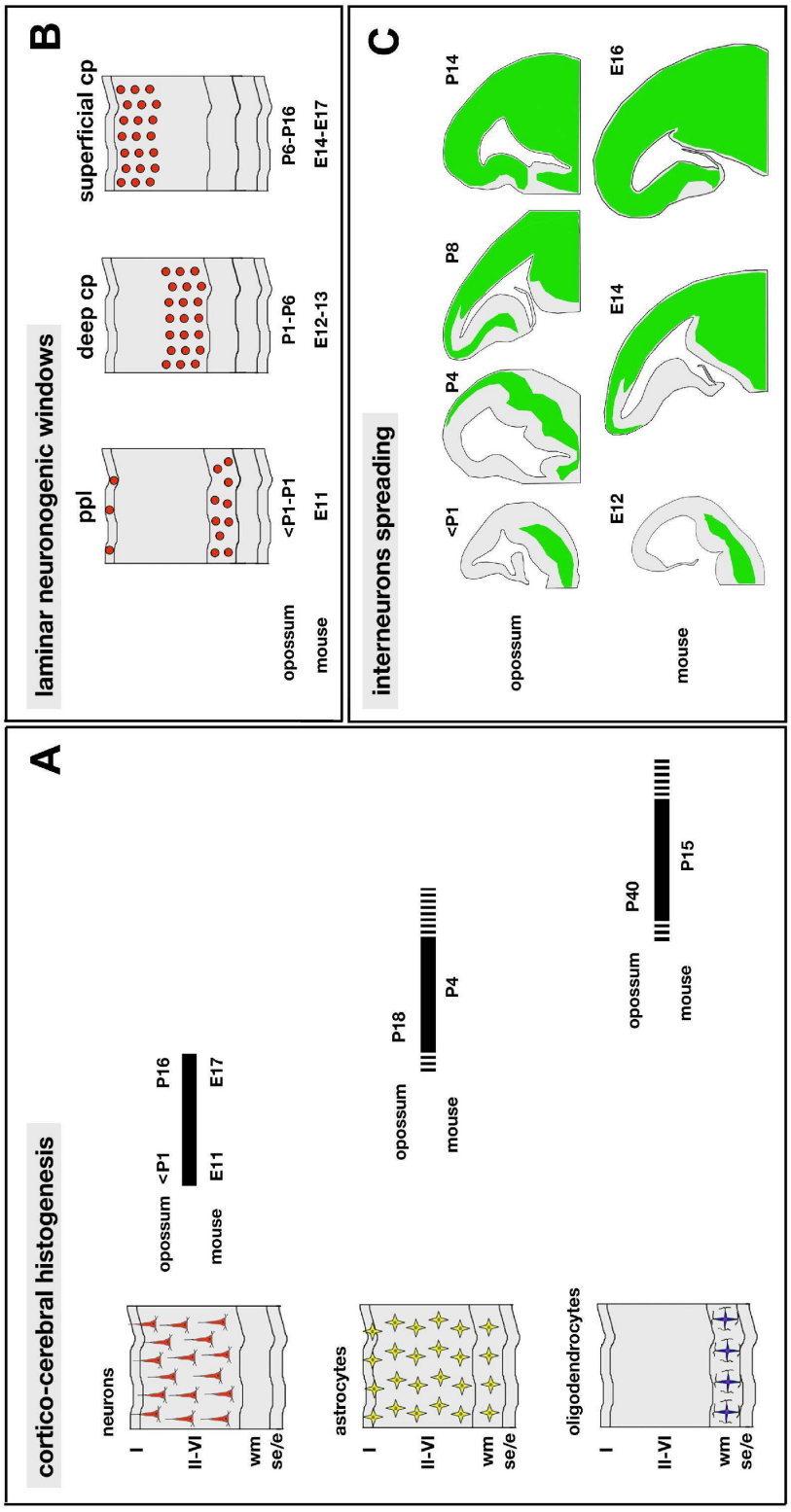


Figure 8 | Opossum and mouse cortico-cerebral histogenesis: a comparison. (*A*) Radial distribution and approximate temporal generation windows of neurons, astrocytes and oligodendrocytes in the opossum and mouse cerebral cortex. (*B*) Peak generation times for primordial plexiform layer, deep cortical plate and superficial cortical plate in opossum and mouse. (*C*) Temporal profile of interneurons spreading in the developing cerebral cortex of opossum and mouse. Abbreviations: e, ependyma; cp, cortical plate; ppl, preplate; se, subependymal zone; wm, white matter; I, II-VI are cortical layers.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE 1

Table 1 | Placental-vs-Marsupial immunogens conservation.

antibody	immunogen used to raise the antibody	opossum immunogen ortholog	aa % identity
anti-Brn1, goat polyclonal	hsa-Brn1: C-terminal aa 421-500 (ENSP00000355001)	mdo-Brn1 (ENSMODP00000022595) aa 402 -462	97.5% identity in 80 residues overlap
anti-Calb2 (Calretinin) mouse monoclonal	hsa-Calb2: all ENSP00000307508), 271 aa	mdo-Calb2-1 (ENSMODP00000017822) 264 aa	85.2% in 271 aa overlap
		mdo-Calb2-2 (ENSMODP00000028132) 273 aa	85.0% in 274 aa overlap
anti-Cux1	mmu-Cux1: aa 1111-1332 (ENSMODP00000016992)	mdo-Cux1 (ENSMUSP00000106740) aa 1035-1254	97.2% in 214 aa overlap
anti-Foxp2, rabbit polyclonal	has-Foxp2: aa 700-715 [EDDREIEEEPLSEDLE]	mdo-Foxp2 (ENSMODP00000019790) aa 678-694	100% in 16 aa overlap
anti-GAD65/67, rabbit polyclonal	hsa-Gad65: aa 570-585; hsa-gad67: aa 579-594 [DIDFLIEEIERLGQDL]	mdo-Gad65 (ENSMODP00000010945) aa 568-583; mdo-Gad67 (ENSMODP00000010606) aa 577-592	100% in 16 aa overlap
anti-Pax6, rabbit polyclonal	mmu-Pax6: aa 267-285 (ENSMUSP00000106716)	mdo-Pax6 (ENSMODP00000012008) aa 251 – 269	100% in 18 aa overlap
anti-Tbr2, rabbit polyclonal	mmu-Tbr2: aa 650-688 [TPSNGNSPPIKCEDINTEEYSKDTSKGMGAYYAFYTSP]	not available (seq available only for the first 402 aa: Ensembl ENSMODP00000018828)	not applicable (72.9% mouse-opossum identity, in 410 aa, limited to the available opossum 402 first aa)
anti-Tle4, rabbit polyclonal	mmu-Tle4: aa 273-473 (ENSMUSP00000057527)	mdo-Tle4 (ENSMODP00000011926) aa 132-332	98.5% in 200 aa overlap

FIGURE S1

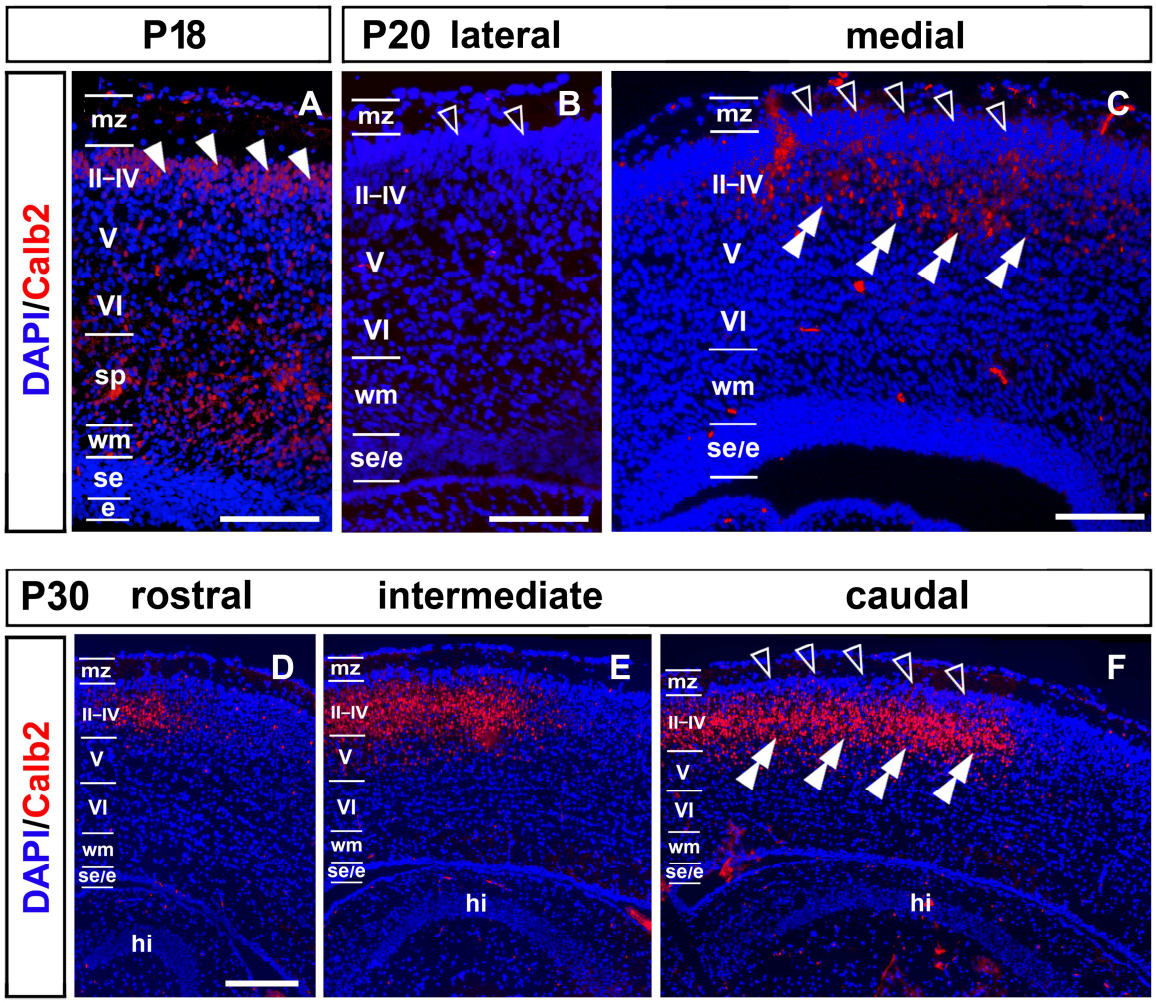


Figure S1 | Late expression of Calretinin in the opossum cortex. (*A-F*) Calretinin (Calb2) immunoprofiling of coronal sections from P18-P30 opossum cortex. Solid arrowheads point to immuno-positive cells in the outer cortical plate at P18 (*A*), double arrowheads demarcate the deeper, areally restricted expression domain visible starting from P20 onward (*C-F*). Empty arrowheads highlight the absence of Calb2^{+} cells in more superficial rows of the CP at P20 and later (*B,C,F*). Abbreviations: e, ependyma; hi, hippocampus; mz, marginal zone; se, subependymal zone; wm, white matter; II, III, IV, V, VI are cortical layers. Scale bar: 100µm.

FIGURE S2

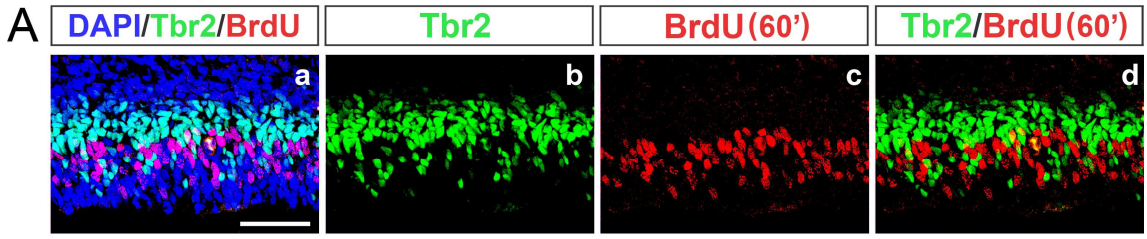


Figure S2 | Distribution of BrdU and Tbr2 immunoreactivity in the opossum cortex explanted at P10, electroporated by pTα1-EGFP, kept in floating culture for 48h and terminally administered with a 60' pulse of BrdU. Scale bar: 100µm.

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