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FoxG1 promotes neuritogenesis and the formation of dendritic spines – a potential mechanism for West syndrome

THESIS SUBMITTED FOR THE DEGREE OF "DOCTOR PHILOSOPHIAE"

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To my beloved women: mom, wife and daughter.

I, Minh Duc Do, declare that the experimental data reported in this thesis are original and generated by me myself during my PhD work at SISSA.

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ABSTRACT

Foxg1 is an ancient transcription factor gene specifically expressed in the developing rostral brain. Here it is implicated in genetic control of multiple aspects of cerebral cortex morphogenesis, including early distinction between pallial and subpallial fields, dorsoventral patterning of the pallium, regulation of the balance between neural proliferation and differentiation, neocortical layering and tuning of astrogenesis rates. Proper Foxg1 allele dosage is crucial to normal brain morphogenesis and function. Rare microduplications of chromosome 14 fragments including Foxg1 are associated to a variant of the West Syndrome (WS), namely a devastating infantile pathological entity, characterized by seizures, abnormal interictal EEG activity, and a profound damage of cognitive abilities, persisting beyond the attenuation of EEG anomalies which often occurs around the third year of life. Aim of this thesis was to explore basic histological mechanisms possibly linking exaggerated Foxg1 expression levels by neocortical projection neurons to WS.

Three were the main findings of this work. First, I found that, upon transient overexpression of Foxg1 within the pallial neuronogenic lineage, neurons originating from the engineered proliferating pool - more numerous than in controls - retain the glutamatergic phenotype and, upon transplantation into neonatal neocortex, they survive at rates comparable with wild type controls. This suggests that an increased ratio between excitatory neocortical neurons and astrocytes occurring in WS patients may jeopardize the removal of ions and metabolites released in the extracellular space upon neuronal hyperactivity. Second, I discovered that neurite overgrowth triggered by Foxg1, previously documented in vitro, takes

also place in vivo, upon transplantation of engineered neurons into neonatal neocortex, regardless of neuron birthdate. Moreover, I found that the neuritic overgrowth triggered by Foxg1 was mainly restricted to dendrites. There was also an increase in axonal length and branching, however this did not reach statistical significance. Remarkably these cytoarchitectonic abnormalities may ultimately result into larger afferent basins impinging on excitatory neurons, which can ease neuronal synchronization over larger distances and contribute to EEG abnormalities of WS patients. Third, I discovered that neuronal overexpression of Foxg1 elicits a considerable increase of spines on proximal dendrites and that this effect is exacerbated upon stimulation of neuronal hyperactivity.

These findings will be the starting point of an ad hoc follow-up study, aimed at unveiling molecular mechanisms which connect Foxg1 overexpression with the development of such histological anomalies. Hopefully, they will be of help for rationale design of novel therapeutic approaches aimed at alleviating and limiting the neurological damages triggered by Foxg1 duplications.

CHAPTER I. INTRODUCTION

1. FoxG1 in the developing telencephalon.

The embryonic telencephalon gives rise to a diverse array of neuronal and glial cells that undergo a tightly precise migration to reach their final positions in the mature cerebral cortex and basal ganglia.

The complicated dynamical process that form such a complex structure consists of different morphogenic processes: (1) rostrocaudal (R-C) and dorso-ventral (D-V) specification of the telencephalic field, (2) cortical arealization, (3) control of cell cycle kinetics and (4) cortical histogenesis of the six layered neocortical structure. A lot of genes are involved in the molecular control of these processes. Among them, developmentally regulated transcription factors (TFs) have a particularly interesting role, because they act as molecular hubs to co-regulate many different aspects of cortical developments including lineage fate choices, cell cycle control, cell differentiation, cell migration, etc…

We are especially interested in the transcription factor FoxG1 which is crucial to several processes of telencephalic development. Here we will provide a synthesis of these processes, paying special attetntion to the role that FoxG1 plays in their regulation and coordination.

1.1 FoxG1 in Rostro-caudal (R-C) and Dorso-ventral (D-V) specification

During early embryogenesis, one certain part of the dorsal ectoderm is specified to form neural ectoderm. This ectoderm region later undergoes three stages of development: to

transform into the neural plate, then the neural groove and finally the neural tube. This process is called neurulation (fig.1). Rostral and caudal part of neural tube later will form the brain and the spinal cord, respectively.

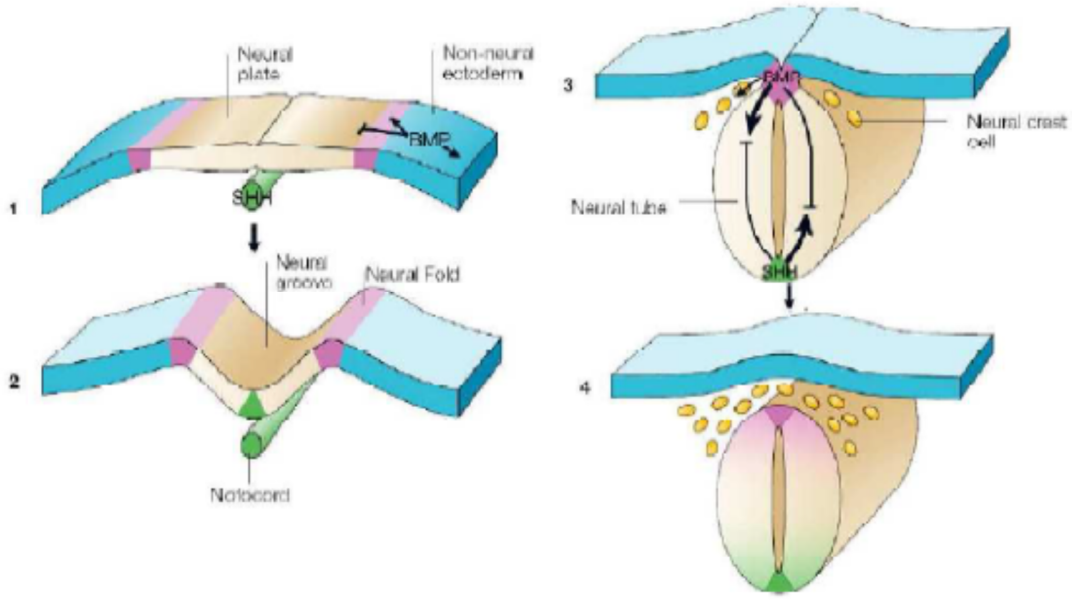

Figure 1. Schematic view of the neural tube formation and the signaling sources involved.

(1) opened neural plate (2) neural groove formation (3) closed the neural tube (4) delaminating neural crest

The very first molecular mechanism in the neurulation process is promoted by a large set of factors, released by the underlying mesoderm. For example, the Bone Morphogenetic Protein (BMP)-inhibiting signaling promoted by Cerberus, Chordin, Noggin, Follistatin changes the general ectoderm identity into its neural state; inhibiting signaling of Wnts and activating signaling of Fgf also contribute to the neural induction. This model, first described in Xenopuslaevis, now seems basically applicable for all vertebrates (fig.2)

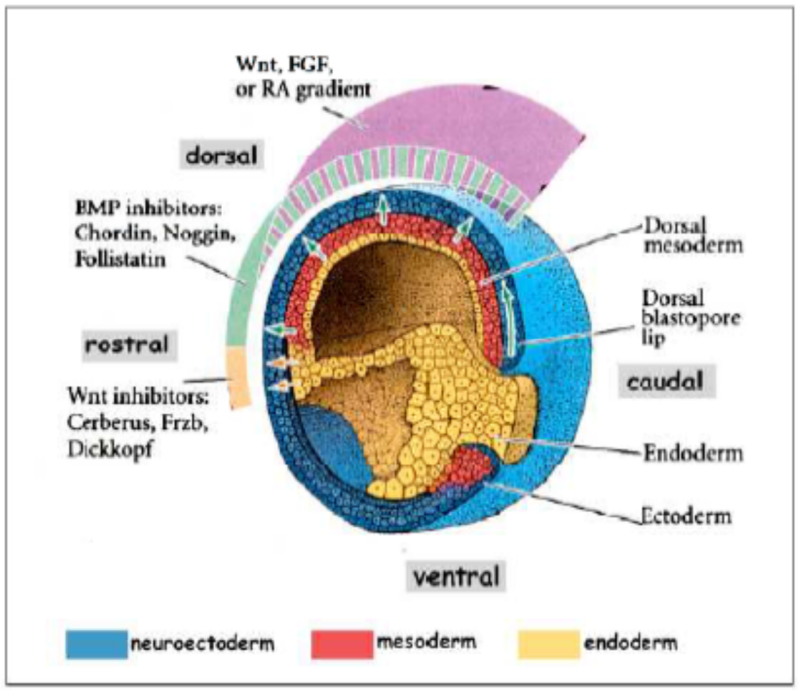

Figure 2. Model of determination of neural plate in Xenopusleavis

Adapted from Gilbert, Developmental Biology

After the neural tube is formed (E9.0 in mouse), its most anterior part undergoes dramatic changes and gives rise to a series of primary vesicles separated by ring-like constructions, including prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain) (fig.3)

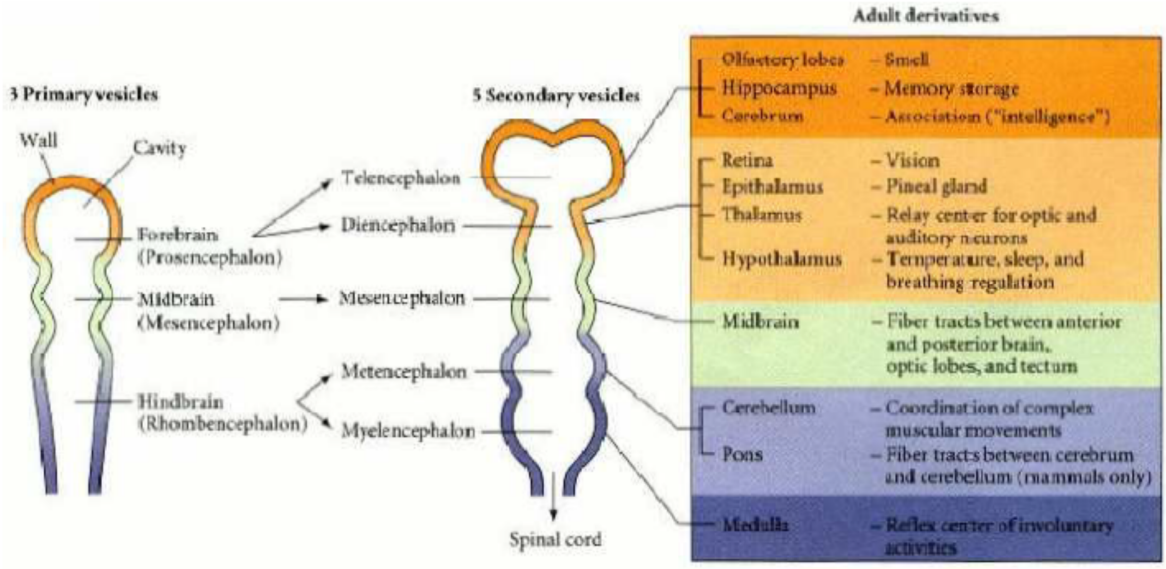

Figure 3.Early human brain development.Left : primary brain vesicles. Right: adult derivative formed by the walls and cavities of the brain

Adapted from Gilbert, Developmental Biology

The prosencephalon then divides into the telencephalon and diencephalon (fig.3). As the Rostral- Caudal and Dorsal-Ventral specification goes on, the dorsal telencephalon (pallium) gives rise to the archicortex (subiculum, hippocampus and dentate gyrus), the neocortex and the paleocortex (olfactory piriform cortex and enthorinal cortex) (fig.4). Conversealy, the ventral telencephalon (subpallium) is divided into the medial ganglionic eminence (MGE), which is more ventrally located and is the precursor of the globuspallidus, and lateral ganglionic eminence (LGE), which is more dorsal and generates the striatum. A third subpallial eminence, called caudal ganglionic eminence (CGE), supplies for the amigdala (fig.4).

According to classical neuroembryology, the diencephalon gives rise the thalamus and hypothalamus, the brain regions that received signals from many neural structures. The mesencephalon does not undergo further division, its lumen becomes the cerebral aqueduct.

The rhombencephalon divides into posterior myelencephalon and more anterior metencephalon (fig.3).

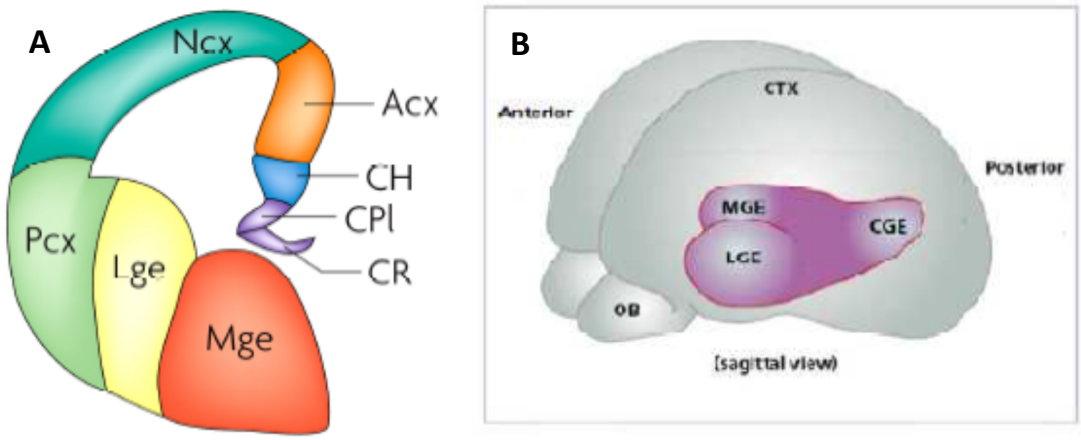

Figure 4. (A) Schematic view of a coronal section through the developing mouse telecephalic vesicles at E12. (B) Sagittal view of the embryonic vertebrae telencephalon as a transparent structure to reveal the ganglionic eminences.

CGE: caudal ganglionic eminence, CTX: cortex, LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence, OB: olfactory bulb.

Images modified from (A) Molyneaux, 2007 and (B) Corbin, 2001.

1.1.1 Rostro-caudal specification:

The R-C specification starts at E7 when signals that affect cell fate, histogenic organization and growth of adjacent tissues in a specific manner, are released by the so-called "primary organizers": the primitive node (Hensen's node in birds or Spemann organizer in amphibians) and the anterior visceral endoderm (AVE) in mammals(Thomas and Beddington, 1996). The primitive node secretes BMP antagonists such as Noggin, Chordin and Follistatin which are important for the neurulation as mentioned above. The visceral endoderm is an extra-embryonic tissue that underlines the neural plate and secretes antagonists of Wntfactors,

fibroblast growth factors (Fgfs) family members as well as retinoic acid. (fig.5) (Sasai and De Robertis, 1997; Altmann and Brivanlou, 2001). Among the molecules released by AVE, major roles are performed by Cerberus and Dickkopf(Ciani and Salinas, 2005).

At this stage, Wnt signaling, Fgfs and retinoic acids also start functioning to perform their character in the neural plate.

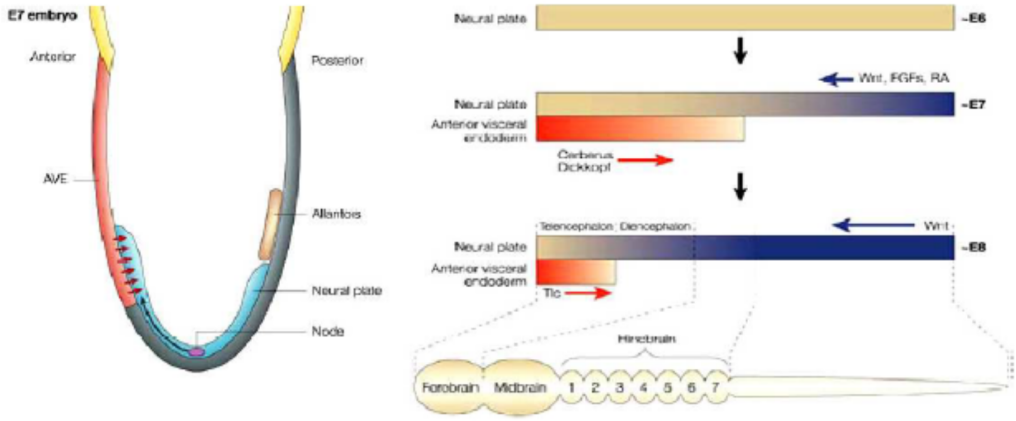

Figure 5.Anterior-posterior patterning of the telencephalon, neural induction.

Signals that come from the node establish gross anterior pattern (black arrow). The anterior visceral endoderm (AVE) together with the early node, acts to induce and/or maintain anterior neural character. The AVE is located beneath the future neural plate and expresses molecules such as ceberus and dickkopf (red arrow) that inhibit factors that would otherwise act to posteriorize the neural plate (Wnts, FGF, RA)

Adapted from Rallu, 2002

As the consequence of Wnt signalings and their antagonists action, two different domains are defined along the anterior-posterior axis of the tube: Otx2 and Gbx2 (fig.6). Wnt signaling promotes Gbx2 while inhibits Otx2 expression. The more rostral Otx2-expressing region will form forebrain and midbrain, in the other case, the more caudal Gbx2-expressing

region will become hindbrain and spinal cord. The anatomical border between these two regions is the isthmus, corresponding to the boundary between mesencephalon and metencephalon in the developing neural tube. Besides, Wntsignalingsalso affects the expression of the two genes Irx3 and Six3 in a very similar way to Gbx2 and Otx2 (fig.6). Wnt signaling confines Six3 expression to the most anterior part and promotes Irx3 expression in the posterior part of the neural tube. The border between these two domains is the zona limitanintrathalamica (ZLI), subsequently placed between thalamus and prethalamus.

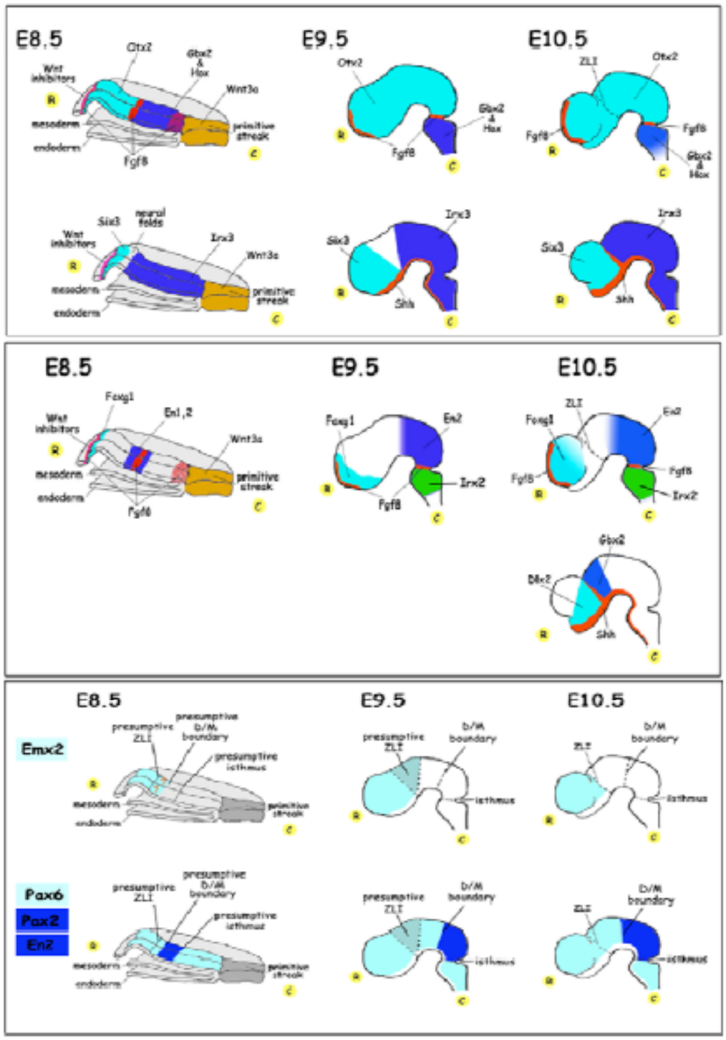

Figure 6. Schematic expression domains of the principal transcription factors involved in the anterior/posterior patterning of the mouse central nervous system at E 10.5.

Images adapted from Mallamaci, unpublished.

Following the “primary organizer”, the “secondary organizers” start to function. These “secondary organizers” are located at the border between the anterior neural and non-neural ectoderm (anterior neural ridge, ANR) and at the gap between midbrain and hindbrain.

The ANR is important for forming the forebrain. ANR ablation results in disruption of telencephalic markers FoxG1 and Emx1 (Shimamura and Rubenstein, 1997). FoxG1 is the chief transcription factor for telencephalic specification and it is promoted by ANR via Fgf8. Recently, ANR also has been found to secretes Fgfs directly, and when Fgf receptors are deleted the telencephalon does not form (Paek et al., 2009).

The ZLI, coming from the breaking down of the region between Six3 and Irx3 domains, secretes molecules from Sonic hedgehog (Shh) family. The ZLI splits the anterior, Otx2-expressing part of the neural tube into two main domains, able to respond to Fgfsignalling coming from ANR and isthmus, by activating FoxG1 and En2, respectively(Garcia-Lopez et al., 2004).

1.1.2 Dorsal-ventral specification:

The D-V specification of the neural tube is patterned by diffusible signals coming from extra-neuralsources: the epidermis and the notochord, which help inducing the dorsal and ventral identity of the neural tube, respectively. (fig.7)

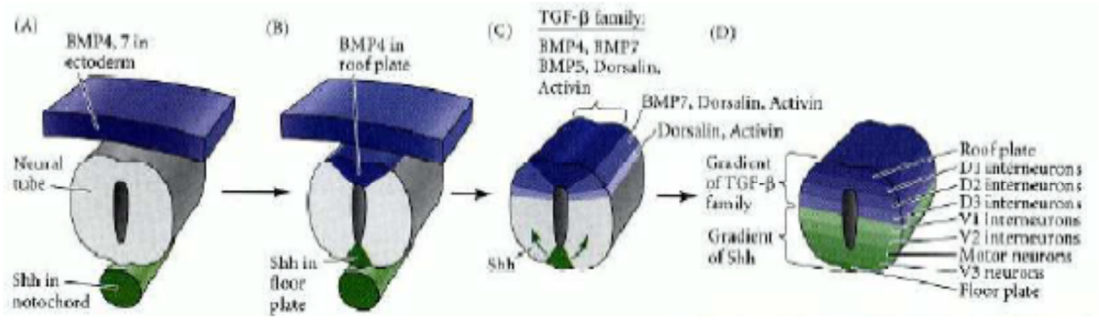

Figure 7.Dorsal-Ventral specification of the neural tube.

(A) Two signaling centers influence the newly formed neural tube: the roof of neural tube is exposed to BMP4 and BMP7 from the epidermis, while the floor is exposed to Shh protein from the notochord.

(B) Secondary signaling centers within the neural tube. The roof plate cells express and secrete BMP4, the floor plate is a source of Shh protein.

(C) BMP4 establishes a cascade of TGF factors, diffusing from the roof plate to the ventral neural tube. Shh proteins spread dorsally by gradient from the floor plate cells.

(D) The spinal cord identities are established by the exposure to BMP4/Shh gradients of paracrine factors.

Adapted from Gilbert, Developmental Biology

First, the epidermis secretes BMP4 and BMP7. These proteins together with dorsalin, activin and other proteins of the TGF-β super family promote the dorsal identity of the neural tube (Liem et al., 1995, 1997). Interestingly, a cell group, induced by these proteins above, becomes the secondary signaling center by expressing BMP4 in the dorsal part of neural tube (the roof plate). The BMP4 in roof plate cells promotes a cascade of TGF-β super family proteins in the adjacent cells (fig.9). These adjacent cells are exposed to these signaling proteins in a different concentrations and different time points (the nearer to the roof plate, the earlier and more concentrated the cells are exposed to the signaling). The temporal and concentration

gradients of the TGF-β super family proteins induce different types of transcription factors at different distances from the roof plate, so that the adjacent cells are given different identities.

The ventral specification takes place in a way similar to the dorsal one. The primary signaling for ventral specification is the Sonic hedgehog protein (Shh), originating from the notochord, and retinoic acid (RA), which may be produced by the adjacent somites(Pierani et al., 1999). These primary signalings convert the most ventral cells to become the floor plate, which also secretes Shh. Shh gradient is released highest at the most ventral part of the neural tube. The cells close to the floor plate receive different Shh concentration in different times, so that they are specified to have different identities. For example, the cells just beside the floor plate that are exposed to high concentration of Shh will become the ventral (v3) neurons, while the next cells, receiving slightly less amount of Shh will become motor neurons. The two next cell groups, receiving progressively less Shh will become V2 and V1 interneurons. (fig.8)

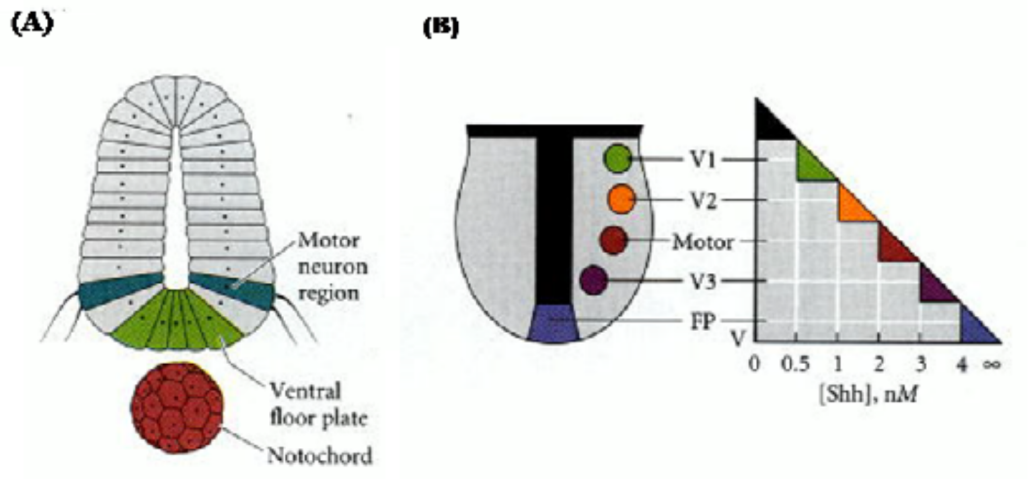

Figure 8. Induction of the ventral neural tube

(A) Cells closest to the notochord become floor plate neurons (green), motor neurons originates from the ventro-lateral sides.

(B) Relationship between Shh concentration and generation of different neuronal types in vitro

Adapted from Gilbert, Developmental Biology

Among the transcription factors that elicit the D-V specification within the rostral brain, the Zinc-finger transcription factor Gli3 and the Winged helix transcription factor FoxG1 perform major roles. (fig.9).

Gli3 counteracts Shh, preventing its ventralizing effect. In $Shh^{-/-}$ mice, the telencephalon size is reduced and ventral cells disappear but in double mutant $Shh^{-/-}$ and $Gli3^{-/-}$ mice, the phenotype is rescued. This phenomenon suggests that Shh simply inhibits Gli3 activities in this process (Rallu et al., 2002). Gli3 is expressed broadly throughout the telencephalic anlage and is induced by BMP signaling, and then it is downregulated in the ventral portion. Shh signaling neutralizes the repressive form of Gli3, blocking the conversion from the activator Gli3 to the repressor (Gli3R), thus promoting Fgfs expression. In the absence of Gli3, the whole dorsal telencephalon is disrupted (Hébert and Fishell, 2008).

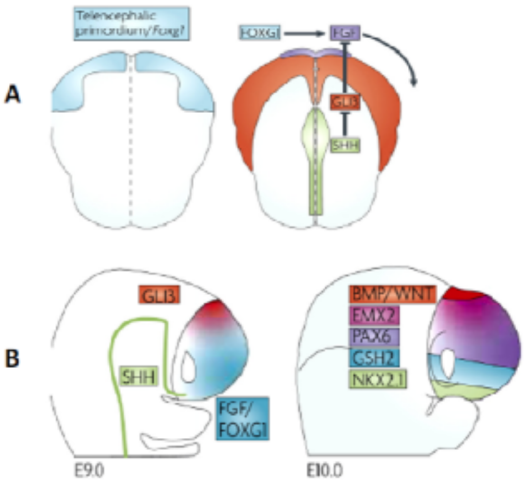

Figure 9.Dorsal-ventral patterning of the forebrain.

(A) The region that will become telecncephalon is defined by expression of FoxG1. FoxG1 (directly) and Shh (indirectly, via Gli 3) promotes Fgfs expression of the ANR. This patterns the nascent telencephalon. Dorsal view E8, E9.

(B) Dorsal telencephalon, expressing Gli3 at E9, is split, at E10, into BMP and Wnt expressing medial region and a more lateral cotical region expressing counter gradients of Emx2 and Pax6. The ventral telencephalon is subdivided into medial Nkx2.1-expressing domains and lateral Gsh2-expressing domains.Sagittal view E9, E10

Adapted from Hebert and Fishell, 2007

As stated above,Forkhead box G1 (FoxG1), formerly known as Bf-1, expressed in the anterior neural plate from E8.5, is the ealiest specific marker for telencephalic fate. Its expression defines the region that will become the telencephalon. FoxG1 promotes the expression of Fgfs from the ANR and in the contrary Fgf8 induces FoxG1 expression, forming a positive feedback loop. These Fgfsexpressionis necessary to form all the regions of the telencephalon so that when three Fgf receptors are deleted, the telencephalon does not form (Rallu et al., 2002). When FoxG1 is disrupted, the formation of the subpallium is abolished (Martynoga et al., 2005) (fig.10).

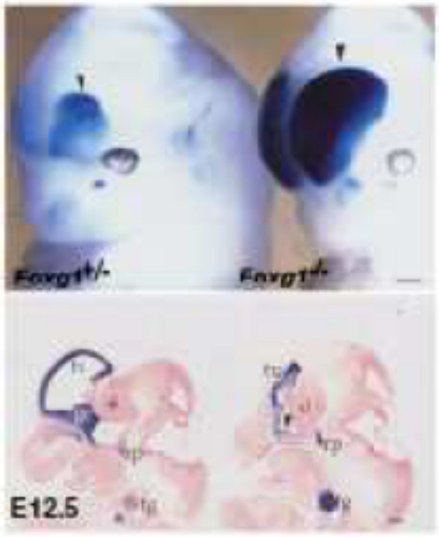

Figure 10. Forebrain development impairment in FoxG1$^{-/-}$.

FoxG1$^{-/-}$displays a smaller size of the telencephalic hemispheres compared to heterozygous mice. The ventral telencephalon formation is heavily impaired, the dorsal telencephalon size is also reduced.

X-gal histochemistry identifies structures that normally expressed FoxG1

Images adapted from Xuan, 1995.

Interestingly, it was shown that FoxG1 can intergrate signals from Shh, Wnt and Fgf8 pathway. This makes FoxG1 have a critical role in the forebrain specification.

In fact, FoxG1 can act as aWnt/β-catenin antagonist, as well as a downstream effector of Shh to specify the subpallial identities (Danesin et al., 2009). As a result of R-C and D-V patterning event, the prosencephalon divides into pallium and subpallium, characterized by the

expression of specific set of transcription factors. The subpallium then gives rise to the medial ganglionic eminence (MGE) and the lateral ganglionic eminence (LGE) which expresses Nkx2.1 and Gsh2, respectively.

No matter what the molecular mechanisms, FoxG1 usually functions as an transcriptional repressor in direct and indirect way (Li et al., 1995; Bourguignon et al., 1998; Dou et al., 2000; Yao et al., 2001; Seoane et al., 2004).

For example, FoxG1 can inhibit the TGF-β signaling by binding to Smad and FoxO transcription factor (Dou et al., 2000; Seoane et al., 2004) (fig.11). In other case, the proliferation-promoting effect of FoxG1 is based on protein-protein interaction and is not dependent on its DNA-binding ability (Dou et al., 2000; Hanashima et al., 2002); but in the neuronal differentiation-inhibiting effect, FoxG1 needs an intact DNA-binding domain to function (Hanashima et al., 2002).

Carrying out the critical role in ventral telencephalon formation, FoxG1 also physically interacts with a member of the Groucho/Transducin-like enhancer-of-split (TLE) family, TLE2. When FoxG1 or TLE is knocked down, the ventral telencephalon is disrupted. The interacting mechanism between FoxG1 and TLE2 is accomplished by a conserved FoxG1 N-terminal motif, whereas the C-terminal domain is not required although it has been previously proposed to be the biding site of TLE (Roth et al., 2010).

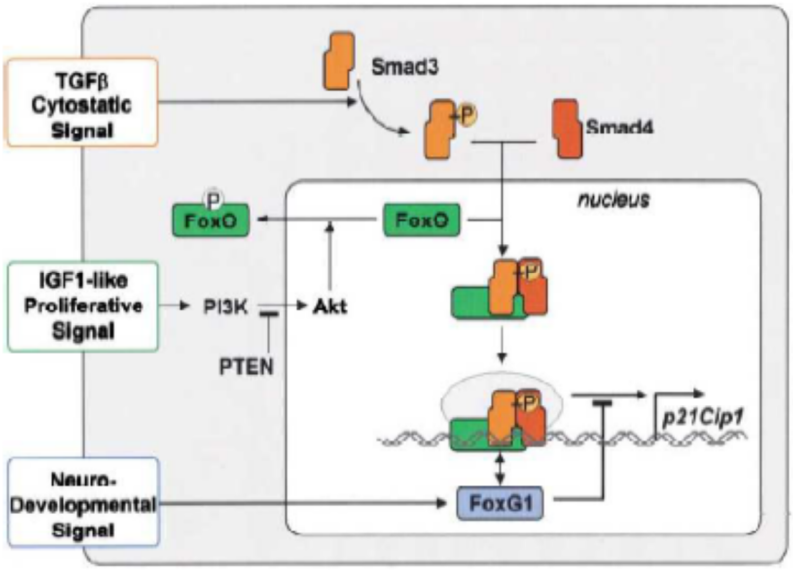

Figure 11.model of FoxO factors as a node for integration of TGF-/Smad, PI3K/Akt and FoxG1 pathways.

There are three pathways converging on FoxO to regulate the expression of p21Cip1 and cell proliferation. TGFβ receptors activation leads to Smad3 phosphorylation (P) and assembly of a Smad3-Smad4 complex in the nucleus. This complex associates with FoxO proteins and activate p21Cip1.

IGF1-like proliferative signals induce the PI3K/Akt pathway that in turn phosphorylates FoxO. This mechanism excludes FoxO from the nucleus and prevents Smad-FoxO dependent gene activation. FoxG1 binds to the FoxO-Smad complex, inhibiting its transcriptional activities

Adapted from Joan Seoane, 2004.

1.2 FoxG1 in cortical arealization.

The cortex is a very complex structure. It is divided into distinct areas with different specific properties. Even a slight change in size, position or connectivity of these areas may cause severe defects in brain function. The process in which the intrinsic and extrinsic factors of the cortical primodium interplay together to form specific areas is called cortical arealization.

Cortical arealization starts at early stage (E10.5 in mouse) and is mediated by a large set of molecules. There are two main categories of molecules which play critical roles in the regionalization of cortical primodium:

- Secreted ligand (SLs), released around the borders of cortical fields (fig.12)
- Transcription factors, gradually expressed within the proliferative layers of this filed (fig.13)

The secreted ligands come from three main sources around the cortical filed:

1. Rostrally, the commissural plate (derived from the anterior neural ridge) (between rostromedial cortex and the septum), secreting fibroblastic growth factors (Fgf 8,15, 17,18).
2. Dorsocaudally, the cortical hem (between the hippocampal field and the choroidal field), secreting BMP 2,4,6,7 (Furuta et al., 1997) and Wnt2b,3a,5a,7b,8a (Grove et al., 1998).
3. Lateroventrally, the anti hem (between paleocortex and the striatal anlage), secreting epidermal growth factors-like molecules (TGF-β, Nrg1 and Ngr3) Fgf7 together with the Wnt signaling inhibitor Sfrp2 (fig.12)(Assimacopoulos et al., 2003).

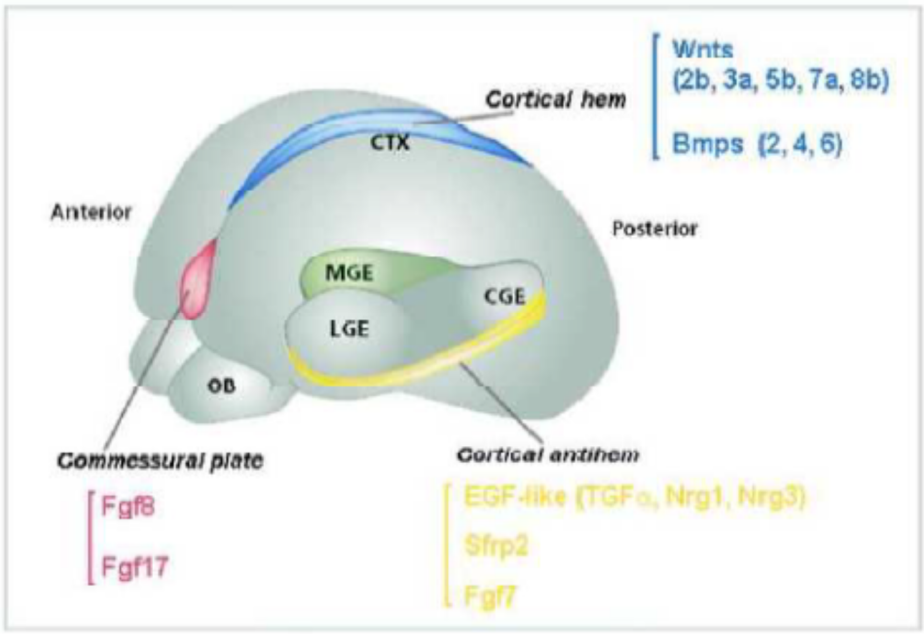

Figure 12. Localization of the signaling centers involved in cortical arealization

CGE: caudal ganglionic eminence, CTX: cortex, LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence, OB: olfactory bulb

Adapted from Corbin, 2001

The secretion of these molecules starts at very early stages and is tightly controlled, only a tiny change in dosages of these molecules may cause dramatic consequences.

Fgf8 secretion by the ANR starts at E8.5 in mouse. Fgf8 overexpression enlarges the rostral cortical area; when Fgf8 is inhibited by overexpressing the soluble form of Fgfr3 (one of its receptors), the rostral cortical area is shrinkage (Fukuchi-Shimogori and Grove, 2001).Wntsignalings are tranduced through a “canonical pathway”, strongly active in the early developing cerebral cortex. Wntsignalings apparently promotes caudal-medial fate of the

cortex, i.e. hippocampal and occipital neocortical identities.Within the rhombospinal domain, BMP signalings promote the dorsal fate and antagonize the ventral fate of the cortex.

Transcription factors implicated in arealization are expressed in proliferative layers of the developing neocortex along spatial gradients. These factors belong to different families and their gradients can be oriented in different ways. Major transcription factors are FoxG1, Emx2, Lhx2, Sp8, Pax6 and Coup-tr1 (fig.13)

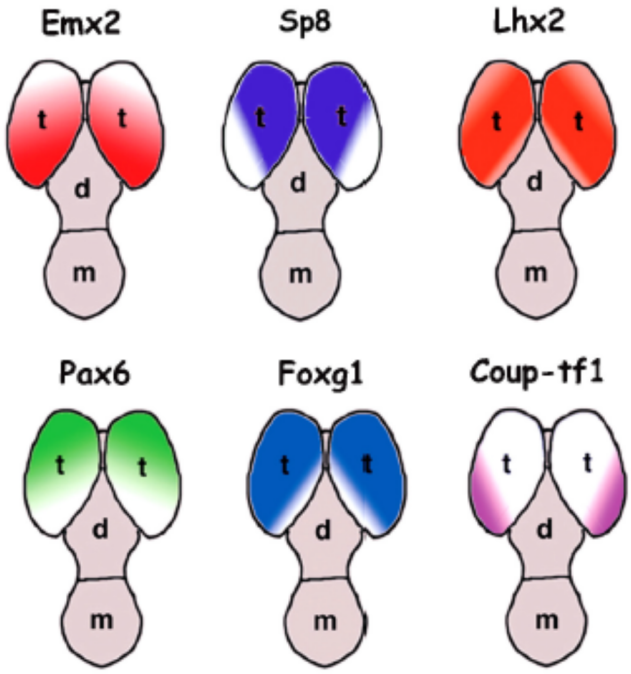

Figure 13.Some transcription factors involved in the cortical arealization.

E12.5 brains, dorsal view.

d: diencephalon, m: mesencephalon, t: telencephalon

Adapted from Mallamaci and Stojkova, 2006

The mechanism in which these TFs and ligands expressed at the borders of the cortical field interact with each other is very complex and includes both feed-forward and positive or negative feed-back processes. For example, rostral Fgf8 from the ANR/CoP is promoted by Shh from ventral telencephalon and the TFs Sp8 which is expressed in anterior medial area but this

signaling is limited in caudal areas by BMP from cortical hem together with the TFs Emx2 (Sahara et al., 2007).

Briefly, roles of the major TFs in the cortical arealization can be described below:

Emx2 is stimulated by BMPs and Wnts(Anderson et al., 1997; Wonders and Anderson, 2006) and inhibited by Fgf8. Meanwhile, Pax6 is strongly inhibited by Wnt signaling (Machon et al., 2007; Ivaniutsin et al., 2009) and promoted by Fgf8. These two TFs are expressed in the dorsal forebrain at opposite gradients. Emx2 knockout alone causes a remarkable enlargement of anterior cortical areas, at expenses of caudal medial ones. (fig.14). When Pax6 and Emx2 are both deleted, the choroidal roof and the subpallium are expanded and the cortex (including neocortex, paleocortex, archicortex, cortical hem, and the choroid plexus) does not form (Bishop et al., 2000; Muzio et al., 2002; Kimura et al., 2005) (fig.15).

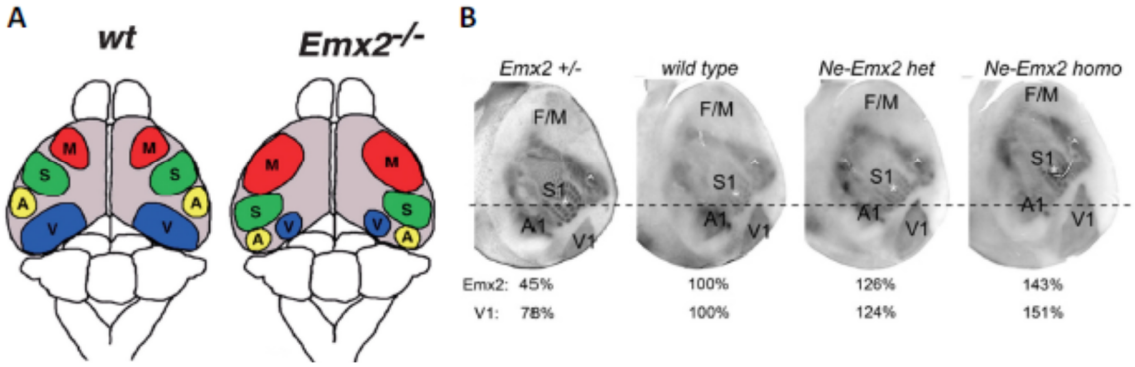

Figure 14. Area identity shifts in cerebral cortex of perinatal and adult Emx2$^{-/-}$ mouse.

(A) Emx2$^{-/-}$ mouse shows cortical area shift with enlargement of anterior areas and shrinkage of posterior and medial area. Adapted from Muzio and Mallamaci, 2003

(B) Direct involvement of Emx2 in cortical arealization has been confirmed by conditional gain and loss of function studies. Adapted from Hamasaki, 2004

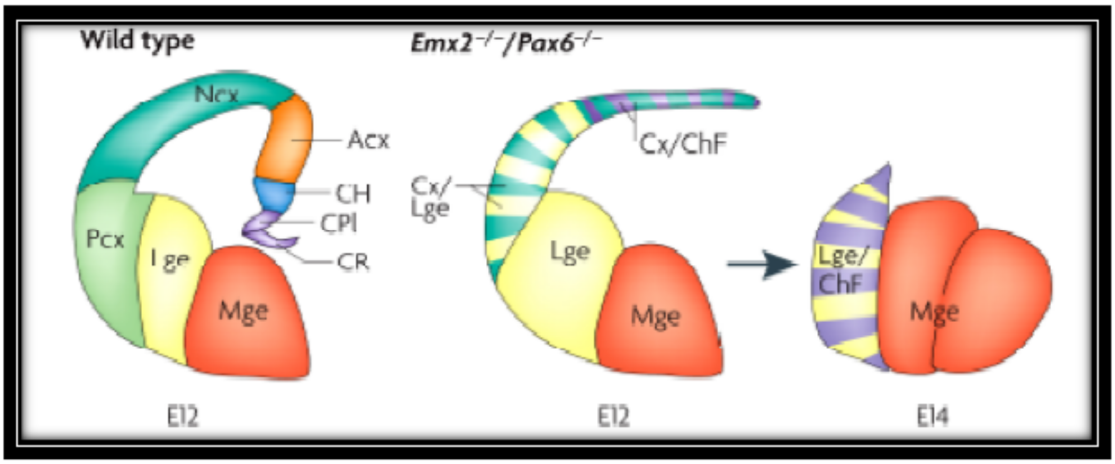

Figure 15.Emx2 involvement in dorsal forebrain specification.

Pax6 and Emx2 loss leads to the ventralization of cortical progenitors and neocortex (Ncx), Archicortex (Acx), cortical hem (CH), choroid plexus (CPI), choroid field (ChF) disappearances by embryonic day 14.

Adapted from Muzio and Mallamaci, 2003; Molyneaux, 2007

Lhx2 expression is stimulated by BMP (2 and 4) at low concentrations and inhibited by the same ligands at high concentrations. This may explain the expression profile of Lhx2: completelyabsent in the hem, high in archicortex and lower in neo/paleocortex(Monuki et al., 2001). In Lhx2 null mutant mouse embryos, the neocortex and the hippocampus are abolished while one or both structures is conserved in Pax6, FoxG1 and Emx2 null mutants. (fig.16,17).

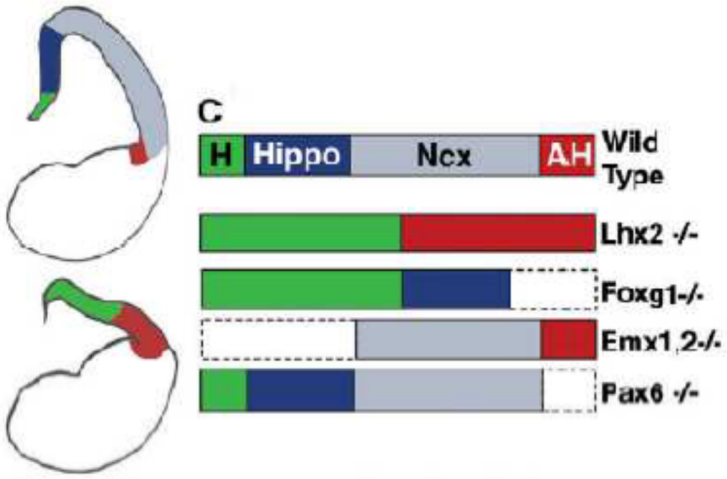

Figure 16.Schematics of mutant dorsal telencephalic phenotypes illustrating how the complete absence of hippocampus and neocortex is unique to Lhx2 mutant.

AH: anti hem, H: hem, hippo: hippocampus, Ncx: Neocortex.

Adapted from Vishaka, 2008

FoxG1 is inhibited by BMP (2, 4 but not 6, 7) (Furuta et al., 1997) and Wnt signaling (Ivaniutsin et al., 2009) and is strongly promoted by Fgf8 (Crossley et al., 2001; Garel et al., 2003; Storm et al., 2006). FoxG1 is highly expressed in the anterior-lateral telencephalic area. FoxG1 is not only crucial to establish the ventral identities of the telencephalon but also help Fgfs signaling to the partition of anterior-lateral and posterior-medial territories. In FoxG1 null mice, the ventral telencephalon does not form and the dorsal telencephalon, reduced in size, acquires molecular properties of archicortex(Muzio and Mallamaci, 2005) (fig.17).

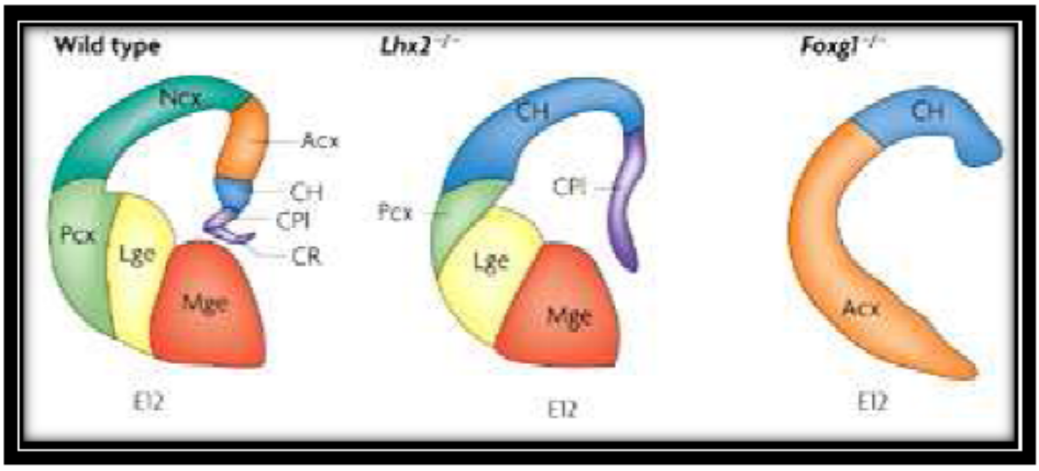

Figure 17.Mutant phenotypes of Lhx$^{-/-}$ and FoxG1$^{-/-}$ mice.

CH: cortical hem, CR: choroidal roof, ChP: choroid plexus, Cx: cortex, Lge: lateral ganglionic eminence, Mge: medial ganglionic eminence, Pcx: paleocortex.

Adapted from Molyneaux, 2007.

1.3 FoxG1 in cortical histogenesis.

Many cell types take part in the rodent cortical histogenesis. During this process, neurons are generated first, followed by astrocytes and finally oligodendrocytes in consecutive, however partially overlapping waves.

But not all the cells that populate the cortex are generated within the cortex itself. It has been shown that projection glutamatergic neurons of layers II-VI, astrocytes and a subset of oligodendrocytes (post-natal) come from the dorsal pallium (Emx1-expressing domain) (Gorski et al., 2002; Kessaris et al., 2006). On the contrary, glutamatergic Cajal-Retzius cells (CRs) (layer I), interneurons (all in rodents and a subset in primates) and a subset of oligodendrocytes (pre-

natal) are generated outside the pallium. They reach their final position in the cerebral cortex via tangential migration across the telencephalon (fig.18).

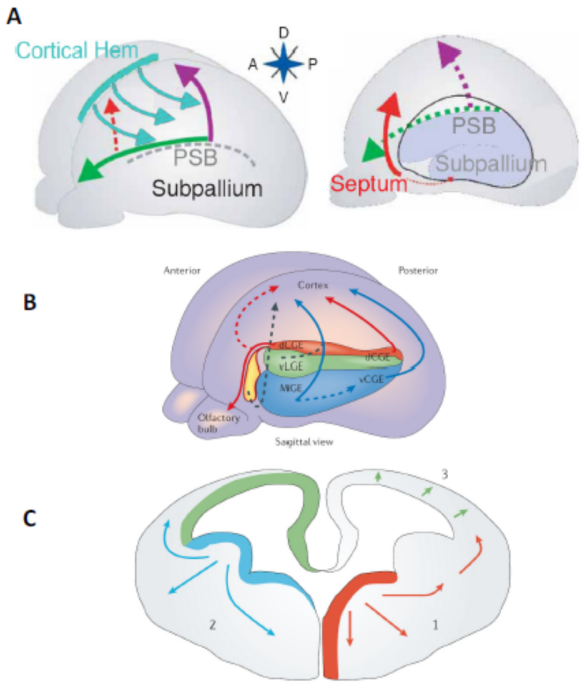

Figure 18.Extra cortical sources of cortical neural cell types.

(A) Cajal-retzius cells are generated in three main locations: the cortical hem, the pallial-subpallial boundary and the septum. From these points they will cover all the uppermost cortical layer. Modified from Bielle, 2005.

(B) The vast majority of interneurons are born in the subpallium in rodents from LGE, MGE and CGE. Modified from Wonders and Anderson, 2006

(C) Three waves of oligodendrocytes from different sources: MGE, LGE and cortex. However, only cortical derived oligodendrocytes retain after birth.

The CRs are transient population of glutamatergic neurons localized at the surface of the cerebral cortex during the cortical development (in layer I). In the post natal stage, CRs die massively (95% of them are eliminated in the first post natal week in rodents) (Derer and Derer,

1990). These cells are the main sources of reelin, a large extra cellular protein very important for the formation of the cortex. In fact, the newborn projection neurons (layer II-VI) need the Reelin signal to radially migrate from progenitor layers to the pia, overcoming previously born neurons (in agreement with the so-called inside-out rule) and ultimately detaching from the radial glial scaffold (Soriano and Del Río, 2005). There are three main extra cortical sources of CRs: the cortical hem (Meyer et al., 2002; Takiguchi-Hayashi et al., 2004; Yoshida et al., 2006), the ventral pallium and the septum (Bielle et al., 2005).

Interneurons comprise 35% of the total neurons in the cortex and use γ-amino butyric acid (GABA) as their neurotransmitter. Most of interneurons are born in the subpallium and migrate towards the cortex after their terminal mitoses, they can be distinguished based on their origin and molecular markers (somatostatin, parvalbumin and calretinin). Interestingly, some of the interneurons secrete Reelin, therefore contribute to the radial migrating mechanism of the projection neurons together with the CRs (Soriano and Del Río, 2005). In rodent, there is almost no evidence showed that interneurons are born in the cortex but in the other hand, in primates, the vast majority of GABA positive cells are born within the cortex itself (Letinic et al., 2002). This can be explained by the fact that some cells in the proliferative layers of human cortex strongly express Nkx2.1, a marker that is absent in the rodent cortex but particularly expressed in the MGE (Rakic and Zecevic, 2003).

In the case of oligodendrocytes, there are still controversies about their origin. Recently, it has been shown that the oligodendrocytes are not regional restricted, they are produced by three production waves respectively from the MGE, then the LGE-CGE and finally within the

pallium(Kessaris et al., 2006). Oligodendrocytes coming from the pallium possibly are the majority of those populating the mature cortex.

Cortical neural progenitors are initially generated from a proliferative layer that next to the lateral ventricle called ventricular zone (VZ). At E9.5 the neuroepithelial cells (NEs) in the VZ start to proliferate and differentiate, giving rise to first post mitotic neurons and other subsets of progenitors.

These first mitotic neurons form a transient structure called the preplate (PP). The PP remains until E13 and when the earliest migrating cortical neurons reach the PP, they form the cortical plate (CP) which divides the PP into a superficial marginal zone (MZ) and a deeply located subplate (SP). The cortical plate (CP), which will become the six-layer mature neocortex, is created by an “inside-out” neurogenetic gradient: later generated neurons go through early generated neurons to settle at the top of the CP, forming the upper layer of the cerebral cortex. (fig.19)

As the process goes on, an additional proliferative layer appears over the VZ, called the subventricular zone (SVZ). This zone later will gives rise to projection neurons and subsequently to glia.

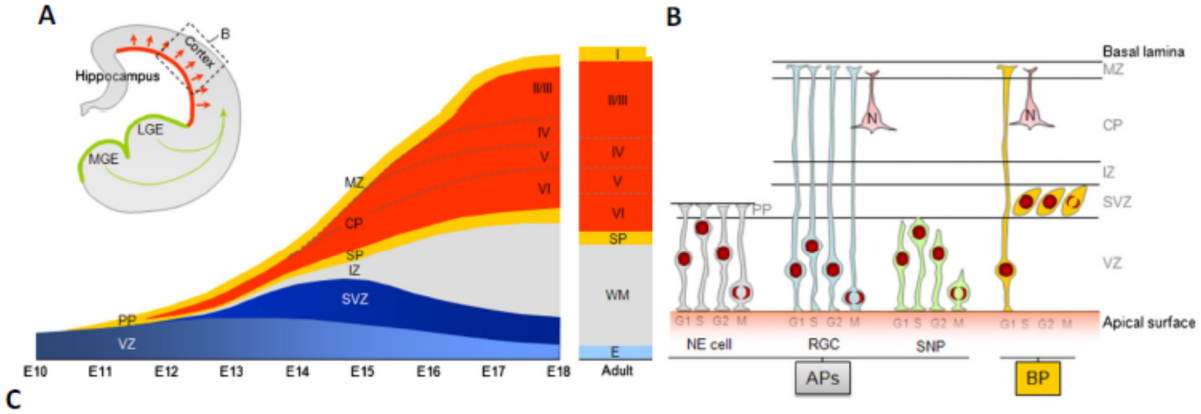

Table 1. Neurogenic progenitor cells in embryonic rodent cerebral cortex (mouse)

	NE	RG	SNP	IPC
Ages	E9.5–E13.5	E11.5–P0.5	E12.5–E16.5	E10.5–P0.5
Morphology	radial	radial	radial (VZ only)	multipolar (few radial)
Migration	IKNM	IKNM	IKNM	multipolar
Mitosis	apical	apical	apical	basal (rare apical)
Mitotic cycles	unlimited	many (≥10)	n.d.	1–3
Daughter cell fates	NE, RG, IPC, N	RG, IPC, N	SNP, N	IPC, N
Molecular expression	prominin 1 (CD133), nestin	Pax6, tenascin-C, BLBP, vimentin, nestin, GLAST	tubulin α_1 promoter	Tbr2 (some low-level Pax6)
Abundance	0–100% depending on age	approx. 50% of VZ progenitors (E13.5–E16.5)	approx. 50% of VZ progenitors (E13.5–E16.5)	10–50% of all progenitors (E10.5–P0.5)
References	1, 6, 7	6–8	9	1–3, 10–12

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

Figure 19. Cortical neurogenesis: the heterogeneity of proliferative layers.

(A) Projection neurons arise from progenitors in the dorsal-lateral ventricular wall (red line) and migrate radially to populate the cortex (red arrows).

(B) and (C) Morphological, cellular and molecular properties of apical and basal progenitor subpopulations.

(A) and (B) are adapted from Merot, 2009; (C) is adapted from Pontious, 2008.

The cortical progenitors are complex population. These progenitors can be classified by their nature, position and expression of different markers. Two principal classes have been identified on the basis of their nucleus position during the M phase of the mitotic cycle:

(1) Apical progenitors which divide at the ventricular (apical) surface of the VZ and express Pax6 gene (Englund et al., 2005). They consist of NEs, radial glial cells (RGCs) and short neuronal precursors (SNPs).

(2) Basal progenitors or intermediate progenitors (IPCs), undergoing division away from the VZ and express Tbr2 (Götz and Huttner, 2005; Kriegstein et al., 2006)

NE cells are the founder population of all cortical progenitors, they appear in the telencephalon at the onset of cortical histogenesis. At early stage, they undergo symmetric division, leading to an increasing the surface area of the VZ. NE cells have peculiar radial morphology, with apical and basal processes which reach the ventricular lumen and the PP, respectively. When NEs divide, they undergo an interkinetic nuclear migration (IKNM), a process in which the NEs synthesize DNA when they reach the most basal position in the PP and they undergo division when their nuclei are close to the apical lumen.

Around E10.5 in mouse, the division of NEs progressively becomes more asymmetric. NEs begin to give rise to first neurons and radial glial cells (RGCs). RGCs share with NEs many histological, morphological and molecular properties, including full histogenetic potentials, radial morphology and IKNM. Both populations, NEs and RGCs, express Pax6 and Nestin(Hartfuss et al., 2001). Interestingly, the basic helix-loop-helix (bHLH) Hes transcription factors are shown to be important for the transition from NEs to RGCs. Mice without Hes1 and Hes5 have normal NEs at E8 but RGC differentiation is defective at E9.5 (Hatakeyama et al., 2004).

The short neuronal precursors (SNPs) are similar to RGCs, however, their basal process does not reach the MZ, so giving rise to a “pin like” morphology. They also undergo IKNM and can be distiguished from the other two apical progenitors by the activity of thealpha1 tubulin promoter (pTα1) (Sawamoto et al., 2001; Gal et al., 2006).

Basal progenitors originate from apical progenitors and undergo a few symmetric divisions (1-3 mitoses) away from the VZ. They show multipolar morphology, express the transcription factor Tbr2 and do not go through the IKNM. Notably, IPCs precursors are only a minority in the progenitor pool of the cortex, while they are the majority population in the ventral telencephalon (Martínez-Cerdeño et al., 2006).

Until now, these progenitors’ properties are well characterized but the relationship between them and their contribution to the cortical histogenesis are extensively controversial.

In the case of glial restricted progenitors (GRPs) and oligodendrocyte restricted progenitors (ORPs), the story is much more complicated. Recently, it has been shown that there is specific telencephalic precursor population which is able to generate both astrocytes and oligodendrocytes but not neurons (Strathmann et al., 2007). The existence of astrocyte restricted progenitors (ARPs) in the telencephalon is still not well demonstrated yet. Anyway, the proposal model in fig.20 somehow presents glial cell generation in the telencephalon.

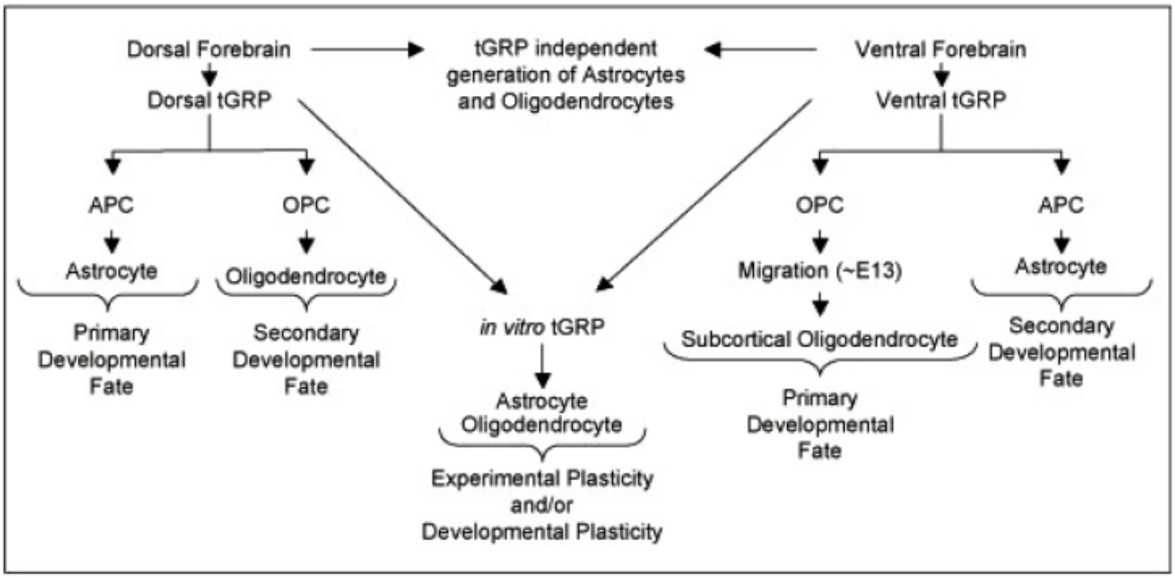

Figure20.A model for glial cell generation in the telencephalon.

The dorsal and ventral telencephalon would give rise to glial restricted progenitors (GRPs) with a primary developmental fate towards astrocytes and oligodendrocytes restricted progenitors (ORPs), respectively. As the dorsal and ventral telencephalon continues through development, each GRP population would have the potential to participate in a secondary developmental fate towards astrocytes ventrally or OPCs dorsally.

Adapted from Strathmann, 2007

Neural precursors proliferate and differentiate to assure the intact population of neurons and glial cells in the cortex at the end of the embryonic life. Cell cycle progression plays a major role in this complicated process which is responsible for the final outputs of all cell types.

It has been shown that both the length of cell cycle and the fraction of cells exiting from cell cycle (Q) increase during the neurogenesis process. Particularly, prolonged G1 phase is accountable for the lengthening of cell cycle (Takahashi et al., 1995) and the increase of Q is due to a shift of progenitors from self-renewing state to a more differentiated state (fig.21A).

As the G1 phase length increases, the cell division switch from the symmetrical self-renewal pattern to the asymmetrical neurogenic differentiated one and finally to asymmetrical, fully diffrerentiative one (fig.21B) (Calegari and Huttner, 2003; Götz and Huttner, 2005).

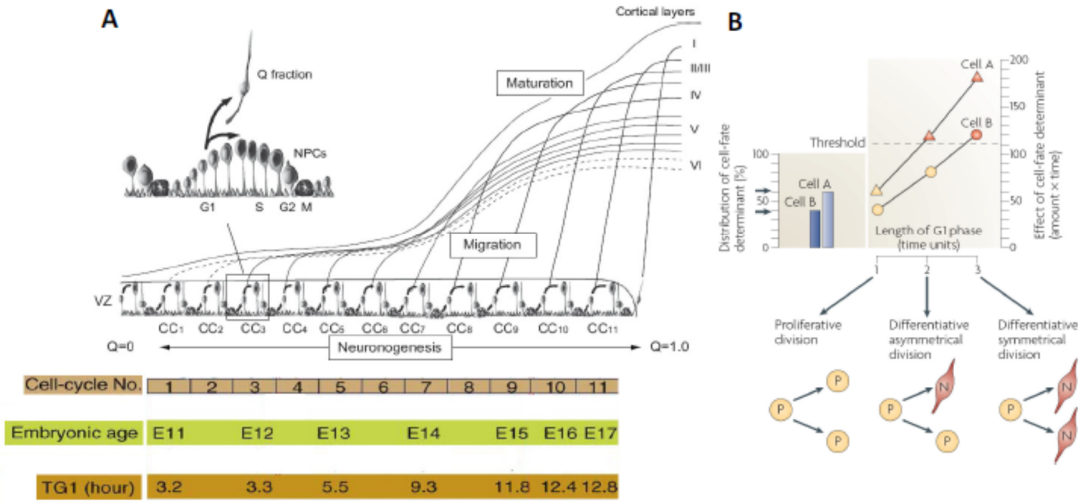

Figure 21. Cell cycle and division pattern control in cortical histogenesis.

(A) During the neurogenic period (E11-E17) the velocity of the cell cycle is not constant, there is an increase of time of G1 phase (TG1). At the same time, the fraction of cell leaving cell cycle (Q) increases from 0 to almost 1. Modified from Caviness, 2009; Mitsuhashi and Takahashi, 2009

(B) TG1 has been linked to the progression of a symmetric proliferative division pattern to differentiative one. Adapted from Deahay and Kennedy, 2007

Therefore, the G1 to S phase transition seems to be crucial for proliferative and differentiative fate choices. Cdk-cyclin complex inhibitors of the Kip/Cip family (p21Cip1, p27Kip1, p57Kip2) and Ink4 (p16Ink4, p15Ink4, p19Ink4 p18Ink4) could exert major roles in the balance between the two process. p21Cip1 knockout lead to exhaustion of proliferative pools in embryonic cortical progenitor in vitro culture (Kippin et al., 2005).

FoxG1 is highly expressed in proliferative layers during cortical development. In FoxG1 null mouse, the cortex is hypoplastic and previous study has demonstrated that the reason for this phenomenon is because of reduction of proliferative rate, increase of differentiative rate, cell cycle lengthening and increase of cell death. (Xuan et al., 1995; Hanashima et al., 2002; Martynoga et al., 2005). Moreover, FoxG1 has been shown to be necessary to mediate Bmi-1 promotion of cortical precursors self-renewal (Fasano et al., 2009) but the same author also claim that FoxG1 overexpression is not capable to promote NSCs self-renewal but only protect them from apoptisis. Studies from FoxG1-null heterozygous mice made the story more complicated, since they showed no appartent alteration in the apical progenitor compartment size but only a slight alteration of the SVZ. A plausible explaination for that can be the blockade of the neural precursor transition from the VZ to the SVZ, exerted by FoxG1 via suppression of $p21^{Cip1}$(Siegenthaler et al., 2008).

1.3.1 Neuronogenesis

Projection neurons are produced and formed different cortical layers from embryonic day E11.5 to E17.5 in mouse. These neurons migrate radially from the VZ and SVZ and detach from the RGC scaffolds to reach their final destination. They are arranged in an inside-out fashion: the later born neurons migrate over the early born one. Eventually, these neurons form the six layered cortex, in which the deeper layers (layer V, VI) contain neurons mostly project to subcortical structures and upper layers (layer II, III) contain neurons projecting to ipsilateral and controlateralcortical structures (Angevine and Sidman, 1961; Rakic, 1974) (fig.22).

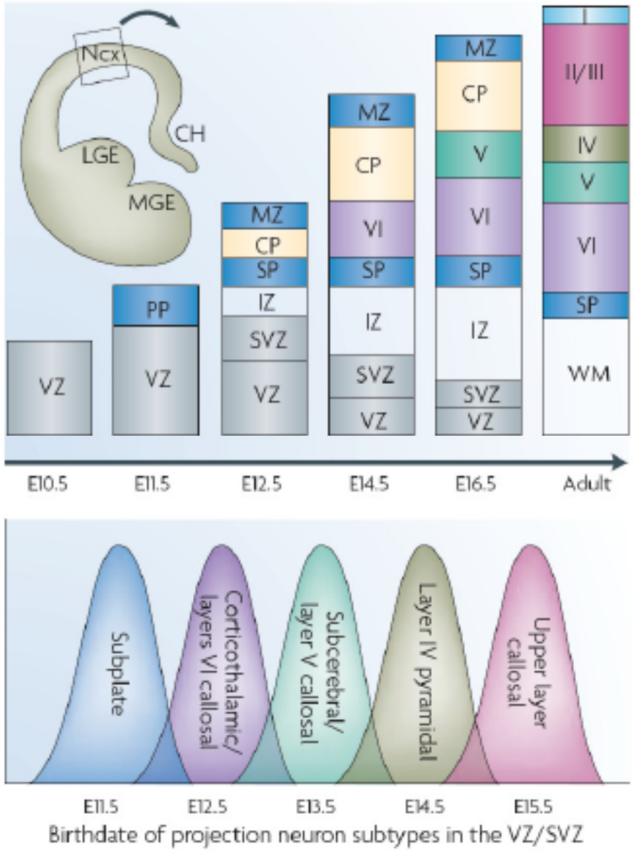

Figure22. Timing of cortical neurogenesis.

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

Adapted from Molyneaux, 2007

FoxG1 plays a complex role in differentiation of pallial glutamatergic neurons, contributing to specify their laminar identity as well as in orchestrating their radial migration from periventricular layers to the cortical plate.

Concerning laminar specification, first, in the absence of Foxg1, the majority (not the totality) of pallial neurons expresses the Cajal-Retzius cell marker Reelin and fails to express markers peculiar to neocortical plate (Hanashima et al., 2004; Muzio and Mallamaci, 2005). It is been suggested that this happens as Foxg1 is crucial for the proper transition of pallial precursors from early generation of Cajal-Retzius cells to later generation of neurons belonging to layers VI-to-II (Hanashima et al., 2004). However, this interpretation has been questioned. In

fact, normal confinement of Cajal-Retziusneuronogenesis to the borders of the cortical field (cortical hem, septum and antihem) and the capability of FoxG1 to normally confine hippocampal and cortical hem programs to the dorsomedial most telencephalicprimodium rather suggest that this phenomenon could be alternatively explained in terms of defective repression of caudal-medial areal programs (Muzio and Mallamaci, 2005). Later, it has been shown that conditional ablation of Foxg1 after the onset of cortical plate neuronogenesis forces pallial precursors to revert to generation of Cajal-Retzius cells. Moreover, it has been shown that a subset of these neurons displays a molecular signature of antihem-derived ones (Hanashima et al., 2007), suggesting that Foxg1 ablation may favor Cajal-Retziusneuronogenesis because of both areal mispatterning and impaired histogenetic progression of pallial precursors. Moreover, protracted Cajal-Retziusneuronogenesis has been demonstrated to occur - upon Foxg1 knock-down - also in clonal studies on isolated pallial precursors, so proving that Foxg1 acts on laminar/areal specification in a cell-autonomous way (Shen et al., 2006).

Second, in addition to promoting the transition from Cajal-Retziusneuronogenesis to cortical plate neuronogenesis, Foxg1 is specially needed to sustain the activation of layer V-differentiation programs. Specifically, Foxg1 robustly represses the transcription of the transcription factor gene Tbr1 (activated in the majority of neocortical glutamatergic neurons just upon their birth) in layer V neurons, so relieving Tbr1-dependent transcriptional repression of the main determinant of layer V identity, Fezf2 (Toma et al., 2014).

Concerning radial migration, it has been show that transient down-regulation of Foxg1 after exit of neural precursors form cell cycle is necessary in order to allow newborn neurons

withmultipolar morphology to activate Unc5D and progress from early to late phases of their multipolar stage. Moreover, it has been shown that subsequent reactivation of Foxg1 in postmitotic neurons is strictly needed to allow these neurons to get connected to radial glia and undertake their radial migration from the intermediate zone into the growing cortical plate (Miyoshi and Fishell, 2012).

1.3.2 Gliogenesis:

Mouse gliogenesis is a temporally restricted process which occurs mainly in the first month of post embryonic life. After the neurogenesis period, cortical precursors gradually generate glial cells, this shift is induced by both extrinsic and intrinsic factors. Differentiated cortical neurons feedback on precursors, via cardiotrophin 1-gp130-JAK-STAT pathway, to activate gliogenesis(Barnabé-Heider et al., 2005). Also long range signals BMP2/4, Smad signalling show neurogenenic character at the early stage and gliogenic at later stage of development (Gross et al., 1996; Li et al., 1998; Nakashima et al., 2001). Together with these extrinsic signals, cell intrinsic mechanisms also play an important role in this fate choice. Downregulation of proneural transcription factors of the bHLH (helix-loop-helix) family, like Neurogenin1 (Neurog1), attentuates neurogenesis and contribute to the activation of gliogenesis (Sun et al., 2001). Remarkably, double knockdown of Coup-tf1/2 prolongs neuronogenesis and impair gliogenesis both in vitro and in vivo (Naka et al., 2008). Foxg1 is apparently implicated in regulating astrogenesis rates. An in vitro study by Brancaccio et al (2010) showed that overexpression of Foxg1 in pallial stem cells halves their astroglial output,

possibly because of impaired commitment of these cells to panglial fates and defective progression of early glia committed progenitors to more advanced cell types. In this way, the progressive downregulation of Foxg1 which occurs within the apical proliferative compartment of the neuronogenicpallium might be instrumental to "prepare" the transition from neuronogenesis to astrogenesis, which normally takes place around E17 in the mouse.

2. FoxG1 in adult brain.

At the end of the developmental period, most of the cells in the telencephalon lose their proliferative properties and make their fate choice toward different cell types. However, it has been demonstrated that newborn neurons and glial cells are still generated both in neonatal and adult mammalian brain in specific regions. There are two major sites that still have the ability to produce neurons in physiological conditions: the subventricular zone of the lateral ventricle wall (aSVZ) (Luskin et al., 1993; Lois and Alvarez-Buylla, 1994) and the subgranular zone of the dentate gyrus in the hippocampus (SGZ) (Altman and Das, 1965; Gould and Cameron, 1996; Kempermann et al., 1997) (fig.23).

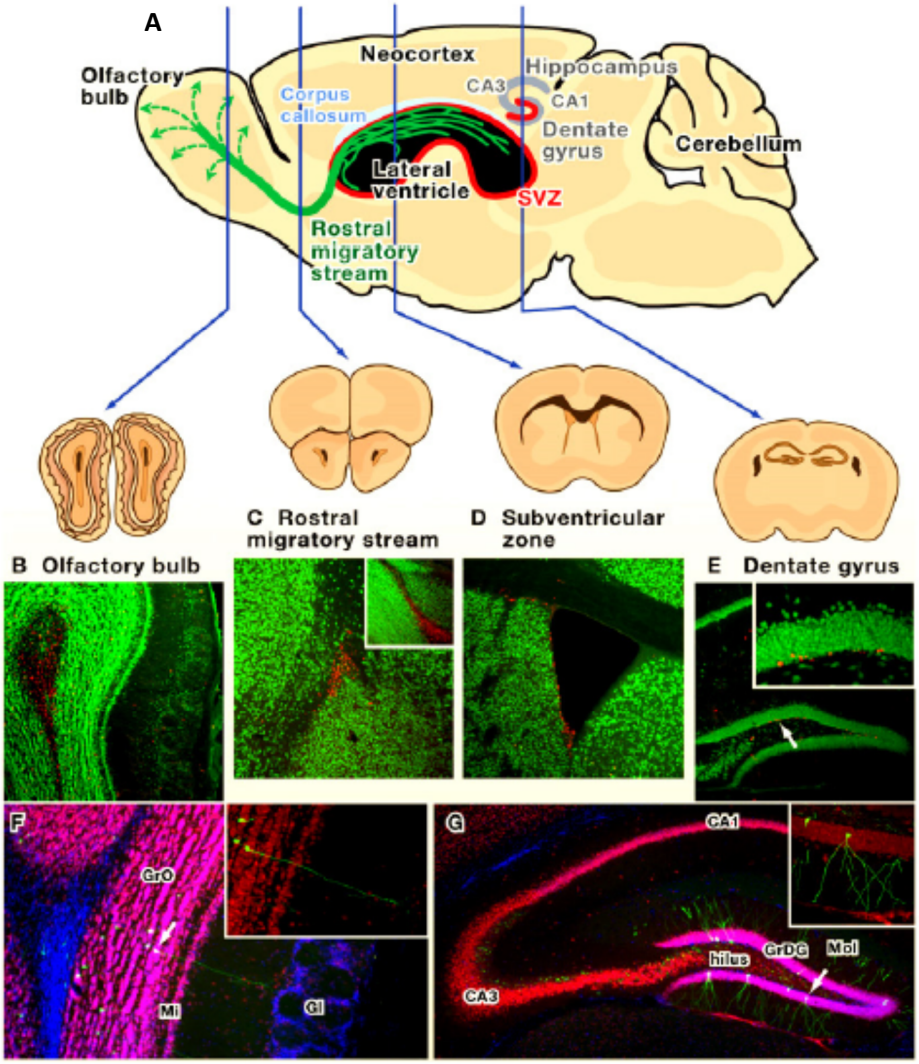

Figure 23.Adult neurogenic niches.

(A) In physiological conditions, there are only two sites of neurogenesis in the adulthood: the aSVZ and the SGZ (red).

(B), (C), (D), (E) Frontal view at different rostrocaudal levels, showing BrdU incorporation by proliferating cells. Red: BrdU, green: NeuN.

(F), (G) Newborn neurons in the olfactory bulb and dentate gyrus labeled by retrovirus mediated expression of green fluorescent protein. Red: NeuN, green: GFP, blue: DAPI.

Adapted from Zhao, 2008

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

Together with the findings above, many researchers agree that the aSVZ and the SGZ are neurogenic niches, retaining some embryonic environmental and cellular cues which are responsible for the maintenance of NSCs.

For example, Shh signaling is still essential for neurogenic niche functions through the activation of transcription factor Gli1, systemic administration of cyclopamin, a Shh inhibitor, strongly impairs aSVZprolireation in vivo and in vitro (Palma et al., 2005). Other morphogenes that take part in adult stem cell renewal are Bmp, Fgf, Wnt, Egf families (Basak and Taylor, 2009).

Among transcription factors, Pax6 and Emx2 are still expressed in some progenitors of the aSVZ and the SGZ. While Pax6 is necessary for the specification of periglomerular cells and granule neurons (Hack et al., 2005; Kohwi et al., 2005), overexpression of Emx2 leads to increasing of differrentiative asymmetrical divisions in these regions (Galli et al., 2002).

In adult brain, FoxG1 is expressed at high level in the neurogenic niches (Shen et al., 2006; Fasano et al., 2009) and in the olfactory bulbs (fig.24). FoxG1 heterozygous mice show a 60% reduction in total number of hippocampal dentate gyrus cells, this is because of proliferative impairment and cell death increasing. This reduction is also associated with behavioral abnormalities (hyperlocomotion, deficit in contextual fear conditioning) and resistance to anti-depressants (Shen et al., 2006; Kinsler et al., 2010).

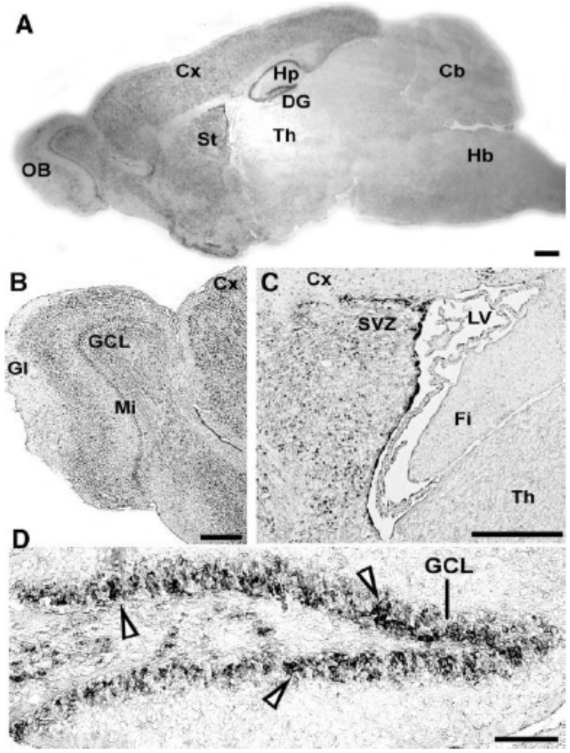

Figure 24.FoxG1 expression in the adult forebrain.

In situ hybridization reveals FoxG1 expression in:

(A) a sagittal section.

(B) rostral migratory stream (RMS)

(C) subventricular zone (SVZ)

(D) dentate gyrus (DG). Within the DG, cells with the highest level of FoxG1 mRNA are present in small clusters within or near the SGZ (arrow head)

GCL: granular cell layer, GI: glomerular layer, H: hilus, Hb: hindbrain, Hp: hippocampus, LV: lateral ventricle, Mi: mitral cell layer, OB: olfactory bulb.

Adapted from Shen, 2004

It has been demonstrated that Bmi1, a transcriptional repressor of the polycomb family is required for postnatal self-renewal of neural stem cells (Molofsky et al., 2003). Recently Fasano et al. have shown that Bmi1 stimulates specifically adult NSC self-renewal from telencephalon and this effect requires Foxg1 activity. However, according to these authors,

Foxg1 would be necessary, but not sufficient to stimulate NSCs self-renewal (Fasano et al., 2009).

3. West syndrome.

3.1 History

West syndrome, named after the English physician William James West, is a rare epileptic disorder in infants and children. The original case which was first published in The Lancet in 1841 described his own son James Edwin West. In 1952, Gibbs and Gibbs described a unique EEG pattern recorded in a large number of infantile spasm patients: hypsarrhythmia (from Greek, “hyp” means high, “arrhythmia” means lack of rhythm) which is characterized by random, high voltage, non synchronous spikes and slow wave activities. Until 1964, the triad of infantile spasms, mental retardation and hypsarrhythmia EEG pattern has been collectively called West syndrome. Afterwards, there are other names for this syndrome: "Generalized Flexion Epilepsy", "Infantile Epileptic Encephalopathy", "Infantile Myoclonic Encephalopathy", and "Salaam spasms".

3.2 Epidemiology

The incidence of West syndrome is about 1 per 2000 to 4000 live births (Hrachovy and Frost, 1989). Boys are more often affected than girls. The onset occurs between 3 and 7 months of age in 50-70% and before 12 months in 90% of cases (Kellaway et al., 1979). However, there are cases with later occurrence, up to 4 years old, so that it may cause delay in treatments.

3.3 Clinical manifestations

As mentioned above, West syndrome consists of symptomatic triad: infantile spasms, diffuse paroxysmal EEG abnormalities and mental retardation.

3.3.1 Infantile spams:

The spasm is usually sudden, symmetrical, bilateral, and affects the axial muscle group. Flexor and extensor muscle group may involve so that spasm may vary from extensive contraction of all flexor or extensor muscles to contraction of only neck muscles or abdominal recti (Hrachovy and Frost, 1989). Spasm may be flexor, extensor or mixed flexor-extensor. The most common type is mixed spasm, followed by flexor, finally extensor spasm and most infants have more than one of these contraction type.

The flexor spasm, though not the most common, is the most characteristic of West syndrome. When the abdominal flexor muscles are involved, the body may bend at the waist like a jackknife (jackknife seizure). The jackknife seizure plus the adduction of the upper extremities (when upper extremities are involved) is reminiscent of the ritual of salaam, thus this spasm is called with the term "salaam attacks." When only the neck flexor muscles are involved, the spasm may be a head nod. The involvement of the shoulder girdle may manifest as a shrug-like movement (Kellaway et al., 1979). A behavioral arrest may also occur as a seizure without associated spasm. Alteration in respiration is also a common associated phenomenon, whereas change in heart rate is rare (Kellaway et al., 1979).

The etiology and prognosis of West syndrome seem not to be affected by the type of spasms (whether it is flexion, extension, or mixed). In contrast, whether the spasms are symmetrical or not is important because asymmetrical spasm contributes to indicate some kind of cortical brain damage (Fusco and Vigevano, 1993).

Spasms tend to occur soon after awakening or on falling asleep. Most of the spasms occur in clusters (the interval between successive spasms is less than 60 seconds). Usually the intensity of spasms in a given cluster will peak gradually and then decline (Hrachovy and Frost, 1989). The frequency of spasms varies from only a few times a day to several hundred a day (Kellaway et al., 1979). They do not show a prediction for either day or night, although they appear to be temporally related to sleep. These spasms may be triggered by sudden loud noises or tactile stimulation, but not photic stimulation.

Following a spasm there may be periods of attenuated responsiveness. Crying may frequently follow a spasm, but this is not an ictal phenomenon.

3.3.2 EEG abnormalities:

The usual EEG abnormalities consist of diffuse, high amplitude, non-synchronous paroxysmal and slow wave theta and delta activity with loss of background features that is continuous when awake and fragmented in sleep (fig. 25). This hypsarrhythmic pattern may be symmetrical or asymmetrical because of additional foci, or unilateral. In other conditions, it consists of one or several spike foci when awake with secondary generalization in sleep.

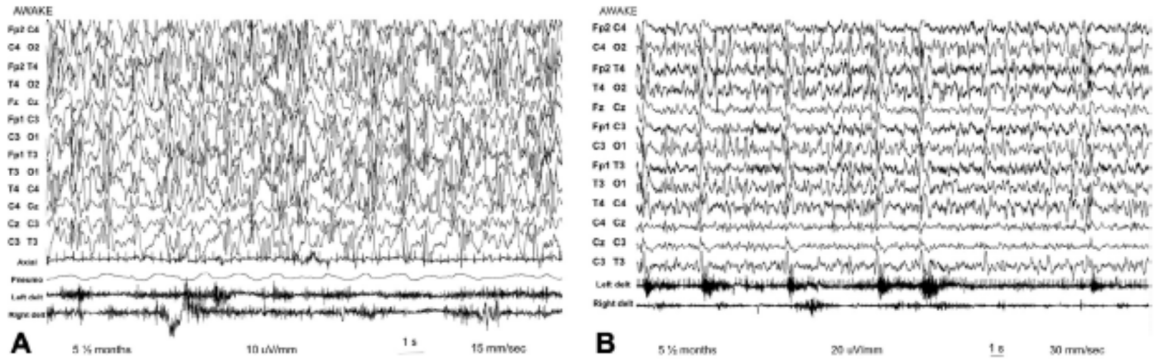

Figure 25. EEG recorded from a West symdrome patient.

(A) EEG and poly graphic recordings during wakefulness: slow waves of large amplitude mixed with almost continuous, independent and multifocal, high amplitude spikes, with a slight tendency to become synchronous, configuring a modified hypsarrhythmia.

(B) clusters of asymmetrical epileptic spasms.

Adapted from Bertossi, 2014.

The interictal EEG of infantile spasms is usually characterized by hypsarrhythmia with a continuous, irregular, random, ever-changing, disorganized, high-voltage spike and slow wave activity. This is sufficiently characteristic to be easily identified, and the term “chaos” may be inappropriate. It may be present during wakefulness and non-REM sleep or may be present only during sleep (Watanabe et al., 1993). During deep sleep, it may be discontinuous (Blume and Dreyfus-Brisac, 1982). Hypsarrhythmia, however, is usually seen in the early stages of infantile spasms, most often in younger infants, and is present in approximately 66% of the cases (Blume and Dreyfus-Brisac, 1982). The “chaotic” pattern becomes more organized with time (Hrachovy et al., 1984; Watanabe et al., 1993) and, between 2 years and 4 years of age, may evolve into the generalized slow sharp and slow-wave pattern of Lennox-Gastaut syndrome.

Infantile spasms are associated with several different ictal EEG patterns (Kellaway et al., 1979). The most common feature is a high-voltage, generalized, slow-wave transient followed by an attenuation of background activity that lasts more than 1 second, referred to as an electrodecremental response. In other instances, there is electrodecremental fast activity (Vigevano et al., 1993). The duration of the ictal EEG ranges from 0.5 seconds to 106 seconds. The longer episodes are associated with behavioral arrest. In some instances, the ictal discharge combines focal discharge with the cluster of spasms (Carrazana et al., 1993), the focal discharge either preceding, following, or being in the middle of the cluster of spasms. This combination strongly indicates either brain malformation or focal brain lesion.

3.3.3 Mental retardation:

Because the onset of West syndrome is early (3-7 months old), the psychomotor impairment and mental retardation signs and symptoms are quite poor and elusive, including:

- loss of hand grasping and simple muscular movements
- axial hypotonia and dsyphonia
- no visual attention and abnormal ocular movement
- no social response

Among these symptoms, loss of eye contact has a negative prognostic significance.

3.4 Etiology

Infantile spasms are either due to a variety of known etiological factors (symptomatic), or are without apparent causes (idiopathic/cryptogenic). There is a distinction between cryptogenic and idiopathic suggested by several authors: idiopathic referring to patients with a possible hereditary predisposition, such as a family history of epilepsy or febrile seizures or EEG genetic patterns, and cryptogenic referring to patients with a presumed underlying etiology, that cannot be demonstrated (Van der Berg and Yerushalmy, 1969; Dulac et al., 1993a; Vigevano et al., 1993). 9% to 15% of the cases are cryptogenic, the rest are symptomatic (Matsumoto et al., 1981; Riikonen, 1982). Unless the etiology is a specific genetic disorder, such as tuberous sclerosis or twin pregnancy, familial recurrence is rare (Kurokawa et al., 1980; Dulac et al., 1993b).

The symptomatic cases are associated with several prenatal, perinatal, and postnatal factors. Prenantal (CMV fetopathy) or perinatal (herpes virus or bacterial meninigitis) infection, neonatal ischemia following term (focal or diffuse) or premature delivery, or post-natal ischemia (near miss), various brain dysgenesis (lissencephaly, hemimegalencephaly, focal cortical dysplasia, septal dysplasia or callosal agenesis), chromosomal (including Down syndrome, del1p36) or single gene (ARX mutation) involvement, neurocutaneous syndrome (tuberous sclerosis, incontinentiapigmenti or Ito syndrome, neurofibromatosis…)

During the past several decades, immunization with various vaccines, especially the diphtheria-pertussis-tetanus vaccine, has been frequently considered as a causative agent in infantile spasms. The relationship is speculative, because the diphtheria-pertussis-tetanus immunization is given at a time when infantile spasms have their peak occurrence (ie, less than

6 months of age). Current available evidence indicates that the association between infantile spasms and diphtheria-pertussis-tetanus immunization is coincidental and that the two are not causally related (Fukuyama et al., 1977; Cody et al., 1981; Bellman et al., 1983).

3.5 Prognosis

The spasms and hypsarrhythmic EEG tend to disappear spontaneously before 3 years of age. However, up to 55% to 60% of children with infantile spasms will develop other types of seizures and epileptic syndromes, for example, Lennox-Gastaut syndrome (Jeavons et al., 1973; Matsumoto et al., 1981; Riikonen, 1982).

The prognosis for West syndrome in terms of normal development is poor in spite of treatment. Overall, only about 5% to 12% of patients have normal mental and motor development. Approximately one-half are left with motor impairment and 70% to 78% are mentally retarded (Jeavons et al., 1973; Matsumoto et al., 1981; Riikonen, 1982; Glaze et al., 1988). The prognosis is better in the idiopathic or cryptogenic cases that have no known associated etiologic factor, no abnormality on neurologic examination, normal development before the onset of the spasm, and normal neuroimaging prior to therapy. Among this group of infants, 37% to 44% are neurologically and cognitively normal at long-term follow-up (Jeavons et al., 1973; Matsumoto et al., 1981; Riikonen, 1982; Glaze et al., 1988). In this subgroup of cryptogenic patients, a delay in initiation of treatment may be associated with worse outcome (Matsumoto et al., 1981; Riikonen, 1982).

Symptomatic cases with degenerative brain diseases have the worst prognosis (Glaze et al., 1988), with the mortality rate is around 25%. Recently, the mortality rate was reported to be reduced to 5%, which may be attributed to improved general medical care(Glaze et al., 1988).

3.6 Pathogenesis and pathophysiology

In the cryptogenic cases, nonspecific degenerative changes have been reported in the cerebral cortex and white matter (Satoh et al., 1986).

The pathogenesis of West syndrome is unknown. However, the following hypotheses have been advanced:

(1) Brainstem dysfunction of serotonergic neurons may cause infantile spasms (Hrachovy et al., 1981; Silverstein and Johnston, 1984). This hypothesis is based on the observation that patients with infantile spasms have decreased REM sleep duration. Brainstem serotonergic neurons are involved in sleep cycles and depletion of serotonin may decrease REM sleep. Langlais and colleagues provided data supporting a serotonin dysfunction hypothesis by demonstrating reduced levels of 5-HIAA, a metabolite of serotonin, in patients with infantile spasms, but it is yet undetermined whether this is primary or secondary to West syndrome (Langlais et al., 1991). In children who responded to adrenocorticotropic hormone treatment, there was a large increase in 5-HIAA following therapy, whereas in nonresponders, 5-HIAA levels decreased.

(2) An immunologic abnormality has also been proposed. Patients with infantile spasms have been reported to have an increased frequency of HLA-DRw52 and an increased number of activated B cells (Glaze et al., 1988). However, the relationship remains to be verified.

(3) Alteration in the brain-adrenal axis in patients with infantile spasms has recently been suggested. It has been shown that there is a lower adrenocorticotropic hormone and increased corticotrophine releasing hormone concentration in cerebrospinal fluid, although the differences in cerebrospinal fluid of cortisol or corticotropin-releasing hormone levels between infantile spasm patients and normal children are not significant (Baram et al., 1992). According to this hypothesis, excessive corticotrophine releasing factor due to stress or other precipitating factors could trigger the occurrence of spasms.

(4) Overexpression of axonal collaterals and excitatory synapses that play a major role in the development of cortical functions determine major hyperexcitability of the developing brain cortex and could be responsible of continuous spiking activity, particularly in combination with some brain damage. Lack of myelin at that age would account for the absence of interhemispheric synchrony, thus producing the hypsarrhythmic pattern (Dulac et al., 1994). Continuous paroxysmal activity would account for the cognitive decline. It would also determine subcortical disinhibition, with paroxysmal discharges in the basal ganglia (Chugani et al., 1990). Thus, a loop including the cortex and basal ganglia would be involved in the genesis of West syndrome (Desguerre et al., 2003).

4. Relationship between West syndrome and FoxG1

Recently, it has been reported that a number of microduplications of chromosome 14q12 sharing the FoxG1 locus are associated with developmental delay, delayed/absent speech, and infantile epilepsies (Bertossi et al., 2014; Pontrelli et al., 2014). In particular, in 14 dup(14) patients, the size of duplication varied from 88Kb to 84Mb and 9/14 patients developed seizures in the first month of life. Moreover, most of them (8/9) presented infantile spasms and hypsarrhythmia/modified hypsarrhythmia EEG patterns (Bertossi et al., 2014). This observation, together with the notion of the essential role of FoxG1 in neurogenesis and cortical neural differentiation, has led to the hypothesis that duplication of FoxG1 may be the main cause of West syndrome phenotype. There were a very few reports of single individuals with 14q12 duplication, including FoxG1, with normal phenotype, normal intellect and no epilepsy (Shaikh et al., 2009; Amor et al., 2012). However, it is commonly accepted that these phenotypic variabilities might be explained by an incomplete penetrance of FoxG1 duplication, the variable involvement of its regulatory elements, other genes in the duplicated region and genetic mosaicism (Brunetti-Pierri et al., 2011; Tohyama et al., 2011; Falace et al., 2013).

Interstingly, while FoxG1 was overexpressed in neuronal cultures, effects of increasing neurite growth and imbalanced ratio between neurons and glial cells (increasing neurogenesis and reducing gliogenesis) have been found (Brancaccio et al., 2010). Increasing neurite growth (including axons and dendrites) may determine the hyperexcitabilities of the cortex, besides, lowering the astrocytes/neurons ratio would jeopardize neurotransmitter and potassium clearance upon neuronal firing. These phenomena may substantially contribute to abnormal

electrical activities within the brain (hypsarrhythmia) and therefore strengthen the putative role of FoxG1 in West syndrome.

CHAPTER II. AIM OF THE WORK:

As reported above, Foxg1 is implicated in genetic control of multiple aspects of cerebral cortex morphogenesis, including early distinction between pallial and subpallial fields, dorsoventral patterning of the pallium, regulation of the balance between neural proliferation and differentiation, neocortical layering and tuning of astrogenesis rates. Remarkably, rare microduplications of chromosome 14 fragments including Foxg1 are associated to a variant of the West Syndrome (WS), namely a devastating infantile pathological entity, characterized by seizures, abnormal interictal EEG activity, and a profound damage of cognitive abilities, persisting beyond the attenuation of EEG anomalies which often occurs around the third year of life.

Aim of this thesis was to explore basic histological mechanisms possibly linking exaggerated Foxg1 expression levels by neocortical projection neurons to WS, paying special attention to the size of the glutamatergic neuronal pool and its cytoarchitecture in vivo.

CHAPTER III. MATERIALS AND METHODS

1. Animals

Wild type mice (strains CD1 purchased from Harlan-Italy) and Tau-EGFP$^{+/-}$(Wernig et al., 2002), purchased from Jackson Laboratories, USA), used in this study were maintained at the SISSA animal facility. Embryos werestaged by timed breeding and vaginal plug inspection. Tau-EGFP$^{+/-}$ E12.5 embryos were distinguished from their wild type litter mates by inspection under fluorescence microscope.

2. Setting up in vitro cultures of CNS precusors and neurons

2.1. Proliferating cortical-cerebral precursor (cPC) cultures

cPC cultures were set up and carried on as follows. On day 1, neural precursors were isolated from E12.5 embryonic cortices and plated onto uncoated 24 multiwell (BD Falcon) after gentle mechanical dissociation to single cells. 3*10^{5}cPCs were plated for each well in 350µl of serum free pro-proliferative medium [1:1 DMEM-F12, 1X Glutamax (Gibco), 1X N2 supplement (Invitrogen), 1mg/ml BSA, 0.6% w/v glucose, 2µg/ml heparin (Stemcell technologies), 20ng/ml bFGF (Invitrogen), 20ng/ml EGF (Invitrogen), 1X Pen/Strept (Gibco), 10pg/ml fungizone (Gibco)]. 2µg/µl doxycycline (Clontech) was added to the medium, when required.

On day 3, 100 µl of medium [1:1 DMEM-F12, 1X Glutamax (Gibco), 1X N2 supplement (Invitrogen), 1mg/ml BSA, 0.6% w/v glucose, 2µg/ml heparin (Stemcell technologies), 80ng/ml

bFGF (Invitrogen), 80ng/ml EGF (Invitrogen), 1X Pen/Strept (Gibco), 10pg/ml fungizone (Gibco)] was added to each well.

On day 5, the neurospheres were harvested by centrifugation (300g for 3'30'' at RT) and dissociated by trypsin 1X (Sigma) at RT for 2'. Trypsin was neutralized by adding 1 volume of DMEM/F12 medium containing Soy bean Trypsin Inhibitor140 µg/ml and DNAseI 10 µg/ml. Afterwards, the cells were centrifugated (300g for 7' at RT) and resuspended in pro-proliferative medium at 600 cells/µl for further expansion.

Finally, on day 7 and day 8, cells were added with fresh medium and trypsinized, as on day 3 and 5, respectively.

2.2 Differentiating cPC cultures

For differentiation experiments, day 8 cPCs prepared and dissociated to single cells as described above, were plated onto 200µg/ml poly-L-lysine-coated 24-wells, at $3*10^5$ cells/well in 350µl of doxycycline-free differentiative medium [1:1 Neurobasal A, 1X Glutamax (Gibco), 1X B27 supplement (Invitrogen), 0,5mM glutamine, 25µM β-Mercaptoethanol, 1X Pen/Strept (Gibco), 10 pg/ml fungizone (Gibco)]. Differentiative medium was half-replaced every 3.5 days with fresh medium and neurons were allowed to differentiate for 7 and 14 days.

2.3 Cortico-cerebral neuronal cultures

Cortical tissue from E16.5 mice was chopped to small pieces for 5-8 minutes, in thesmallest volume of ice-cold 1X PBS-0,6% glucose-0,1% DNaseI. After digestion with 1mg/mltrypsin for 5 minutes, cortices were spinned down and transferred todifferentiative medium. The

suspension was pipetted 5-8 times with a P1000 gilsonpipetteandundissociated tissue was left to sediment for 2 minutes at 1g, in ice. The supernatant was harvested and the living cells counted. Cells were plated on poly-L-Lysine coated 1cm∅ glass coverslips in 24 multiwell-plates, at $3*10^5$ cells/well in 350µl medium. Medium was half-replaced with fresh one every 3.5 days.

In case of spine morphometry experiment, 50 mM of KCl was added to half the culture 12 hours before analysis.

3. Basic cloning

Basic DNA manipulations (extraction, purification, ligation) as well as bacterial culturesand transformation, media and buffer preparations were performed according to standardmethods. Plasmids were grown in E.Coli, Xl1-blue or Stbl4 strains.

Lentiviral vectors employed in this study include:

- LV_pCAGGS-LacZ, aka LV-lacZ, purchased from Addgene (#12108)

- LV_pPgk1-EGFP, aka pCCL-SIN-18PPT.Pgk.EGFP-Wpre (Follenzi and Naldini, 2002), a kind gift by L.Naldini.

- LV_pPgk1-Luc, constructed by transferring the NotI-BamHI 1.57kb fragment from LV_pPgk1-EGFP to NotI-BamHI digested LV_TREt-Luciferase (Raciti et al., 2013).

- LV_Pgk1-mCherry, constructed by transferring the mCherry module form LV_pTa1-mCherry(Brancaccio et al., 2010)to LV_pPgk1-EGFP (see above), in place of EGFP

- LV_TREt-Foxg1 (Raciti et al., 2013).

- LV_TREt-PLAP, constructed by replacing the XhoI/SalI fragment of pPgk1-EGFP by an XhoI-compatible/SalI-compatible element, including the XbaI-AgeI 0.35kb TREt fragment of P199 (Stegmeier et al., 2005) and the EcoRI/SalI 2.2kb IRES-PLAP fragment from pCLE (a kind gift by GordFishell).

- LV_pTα1-rtTA (Brancaccio et al., 2010).

4. Producing and titrating LVs

4.1. Lentiviral vectors packaging and titration

Third generation self-inactivating (SIN) lentiviral vectors were produced as previously described (Follenzi and Naldini, 2002) with some modifications. Briefly, 293T cells were co-transfected by LipoD (SignaGen) with the transfer vector plasmid together with the three auxilary plasmids (pMD2 VSV.G, pMDLg/pRRE, pRSV-REV), according to manufacturer's instructions. The conditioned medium was collected after 24 and 48hrs, filtered and ultracentrifuged at 50000 RCF on a fixed angle rotor (JA 25.50 Beckmann Coulter) for 165 min at 4°C. Viral pellets were resuspended in PBS without BSA (Gibco).

Lentiviral vectors encoding for fluorescent proteins were titrated on HELA TET-off cells (Clontech; fluoroproteincds driven by TREt) or on HEK293T cells (Invitrogen; fluoroproteincds driven by constitutive promoters), by endpoint fluorescence titration, as previously described (Follenzi and Naldini, 2002).

Other LVs were generally titrated by Real Time quantitative PCR after infection of HEK293T cells, as previously reported (Sastry et al., 2002). One end point fluorescence-titrated LV was

included in each PCR titration session and PCR-titers were converted into fluorescence-equivalent titers throughout the study.

4.2 Lentiviral infection of cPCs

After plating onto 24 multiwell plates, cPCs were immediately infected with LV combinations shown in Figures. Each LV was used at MOI=8. Lentiviral conditioned medium was washed away after 2 days in experiment described in Figure 4 and after 4 days in all other experiments.

5. In vivo transplantation

For transplatation experiments, neurospheres were harvested after 2 days or 7 days of propagation in serum-free, proliferative medium and dissociated by trypsin to single cells at $5*10^4$cells/µl in proliferative medium. Just prior to transplantation, engineered cPC populations were mixed as shown (referring to Figures 2-4, a-b1-c1 together with a-b2-c2 and a-b1-c2 together with a-b2-c1), at 1:1 ratio. 150,000 mixed cells (in 3µl) were injected by free-hands, with a pulled borosilicate pipette, into the fronto-parietal parenchyma of P0 CD1 wild-type mouse pups, preanasthetized by hypothermia. 0.1% Fast green FCF (Sigma) was used to trace the transplanted cells. Operated recipients were returned to mothers and allowed to develop up to P7 or P14. Their brains were fixed, cryoprotected, and sliced at 10 (Figure 2) or 28µm (Figures 3,4), according to standard procedures.

6. Immunoflorescence

For immunofluorescence on cortico-cerebral neurons, cells were fixed directly on poly-L-lysine coated 1 cm∅ glass coverslips in 24 multiwell plates with 4% paraformaldehyde (PFA) for 20 min at RT. Subsequently, cells were washed 3 times in 1X PBS containing 0.1% Triton X-100 (Sigma) (PBS-Triton).

As for immunofluorescence on brain sections, slices were allowed to dry for at least one hour at RT, they were post-fixed 5 minutes in 4% paraformaldeyde at RT, followed by three washes in PBS-Triton. In both cases, samples were subsequently treated with blocking mix (1X PBS; 10% FBS; 1mg/ml BSA; 0.1% Triton X100) for at least 1 hour at RT. After that, incubation with primary antibody was performed in blocking mix, overnight at 4°C. The day after, samples were washed in PBS-Triton 4 times and incubated with a secondary antibody in blocking mix, for 2 hours at RT. Samples were finally washed in PBS-Triton for 5 minutes, 3 times, and counterstained with DAPI (4', 6'-diamidino-2-phenylindole). When appropriate, they were mounted in Vectashield Mounting Medium (Vector).

The following primary antibodies were used: anti-Tubb3, mouse monoclonal, (clone Tuj1, Covance, MMS-435P, 1:1000); anti-GFP, chicken polyclonal (Abcam, ab13970, 1:500); anti-MAP2, rabbit polyclonal (Abcam, ab32454, 1:500); anti-mCherry, rabbit poloclonal, (MBL, PM 005, 1:500); anti-vGLUT1, rabbit polyclonal (Synaptic Systems, 135303, 1:1000); anti-GAD65, rabbit polyclonal (Synaptic Systems, 198103, 1:1000), anti-PSD95, mouse monoclonal (Abcam, ab2723, 1:1000), anti-Smi312, mouse monoclonal (Abcam, ab24574, 1:1000).

Secondary antibodies were conjugates of Alexa Fluor 488, and Alexa Fluor 594 (Invitrogen,1:500).

7. Images acquisitions

Immunoprofiled cultured neurons were photographed on Leica DM 6000 microscope equipped with 20X or 40X objectives and a Hamamtsu C4742-95 camera.

Immunoprofiled slices of transplanted brains were photographed on Leica confocal microscope equipped with 40X and 63X objectives. Samples were collected as 8µm Z-stacks (Figure 2) or 22µm Z-stacks (Figures 3-4) of 1024*1024 pixel images.

Immunoprofiled dendritic spines (Figure 6) were photographed on Leica confocal microscope equipped with 40X and 63X objectives. An additional 8X electronic zoom was added for monitoring spines. Dendrites were randomly chosen among those best representing the corresponding biological replicate and pictures along these dendrites were taken from approximately 10µm to 90µm far from the soma. Pictures were collected as 5µm Z-stacks of 1024*1024 pixel images.

All images were processed using Adobe Photoshop CS2 software.

8. Neurite morphometry

After images acquisition, neuronal silhouettes, including somas and neurites, were genereted by free hand, with GIMP2.6 software. These silhouettes were analyzed by the Neurphology(Ho et al., 2011) interactive plug-in, in ImageJ software. Four parameters were measured: number of somas, total neurite length, number of attachment points, number of endpoints. Numerical results were finally processed by Excel software.

9. Spines morphometry

After acquisition, images were analyzed for 2 parameters: number of spines and dendrite surface area. Spines was counted manually in each picture, while the dendrite surface was calculated by ImageJ software.

10. Statistical analysis

Data analysis (both manual and automated) was performed rigorously in double blind. Results were processed by Excel software. Average and standard error of mean (sem) were determined and normalized agains control average. Statistical significance of results was evaluated by t-test (one-tail; paired in cotransplantation studies, unpaired in all other experiments)

CHAPTER IV. RESULTS

1. Foxg1 overexpression leads to an enlargement of the pallial glutamatergic neuronal pool

It was previously shown that *Foxg1* overexpression in proliferating cortical precursor cells (cPCs) inhibits astrocyte generation and forces neuronogenic precursors to keep proliferating, which causes a pronounced enlargement of the neuronogenic pool (Martynoga et al., 2005; Brancaccio et al., 2010). All that ultimately results into an exaggerated neuron-to-astrocyte generation ratio, namely a potential key mechanism underlying etiopathology of the West syndrome. Actually, all these studies were performed in vitro. Moreover, the neurotransmitter identity of neurons originating from engineered Foxg1-gain-of-function (GOF) precursors was not investigated.

To assess if high Foxg1 levels affect the neurotransmitter identity, we performed gain-of-function assays on pallial tissue dissected out at E12.5, a developmental age when gabaergic interneurons originating in ventral telencephalon have not yet migrated into the cortico-cerebral primordium. cPCs originating from mechanical dissociation of this tissue were coninfected with two lentiviruses allowing conditional, doxycyclin-dependent overexpression of *Foxg1*, under the control of the neuronal lineage-specific Tubulin alpha 1 promoter (Brancaccio et al., 2010). Engineered, proliferating cPCs were grown for one week as neurophere cultures, under Fgf2/Egf and saturating doxycyclin. Then, to allow their neuronal differentiation, they were transferred to polylysin-coated coverslips, under Neurobasal A medium, in the absence of doxycyclin. Seven days later, neurons were fixed (Fig.26A,B). They were co-immunoprofiled for the pan-neuronal marker Tubulin beta 3 (Tubβ3), the glutamatergic marker vesicular glutamate

transplorter 1 (vGlut1) and the gabaergic marker glutamate decarboxylase 65 (Gad65). Naive, not-engineered age-matched neurons, from pallium and subpallium, were used as controls. Interestingly, upon *Foxg1* overexpression in pallial precursors, almost all neurons originating from these precursors were positive for vGlut1 and none of them expressed the gabaergic marker Gad65, like in wild type controls (Fig.26C,D).

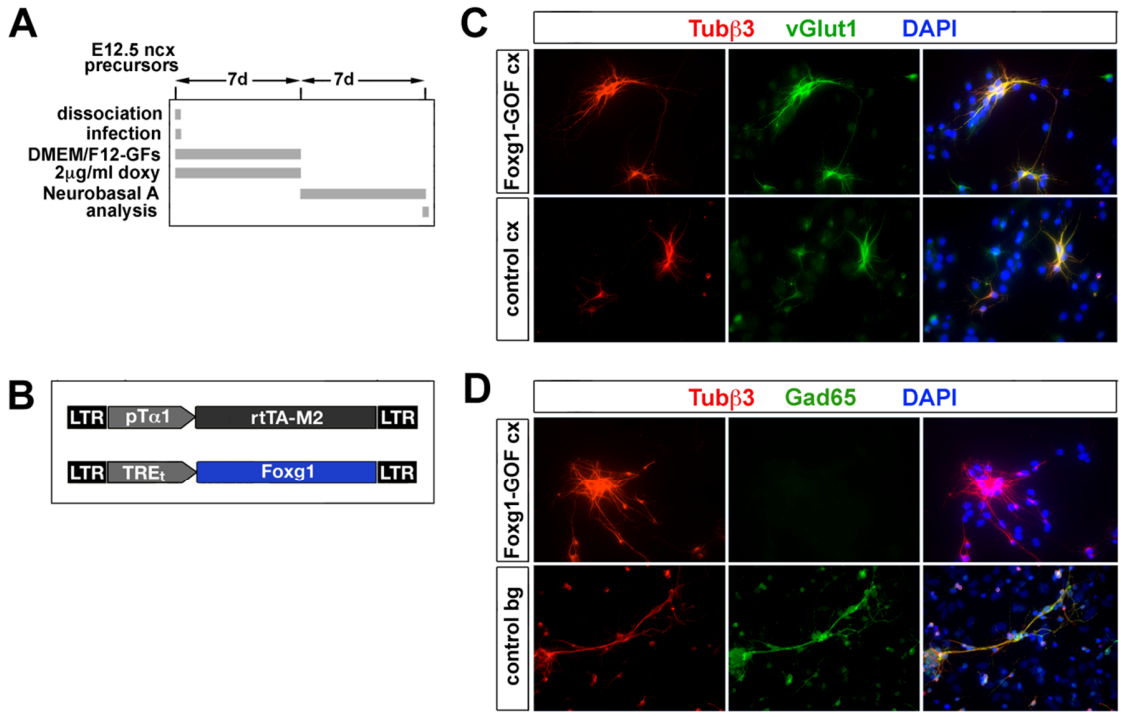

Figure 26. Neurotransmitter phenotype of *Foxg1* gain-of-function neurons.

(**A**) Temporal articulation of the tests. Dissociated neocortical precursors from E12.5 wild-type donors were acutely infected by LV_p(Ta1)-rtTA-M2 and LV_TREt-Foxg1, driving doxycyclin-dependent expression of *Foxg1*, they were kept in pro-proliferative medium under doxycyclin for 7 days as neurospheres, and transferred to polylysin-coated coverslips under Neurobasal A medium for differentiation. 7 days later, they were fixed and analyzed.

(**B**) Recombinant viruses employed for this test.

(**C, D**) Immunoprofiling of engineered Tubb3^{+} neurons for the glutamatergic marker vGlut1 and the gabaergic marker Gad65. Not-infected neurons from cortex (cx) and basal ganglia (bg) are shown as controls.

To investigate whether Foxg1-GOF precursors may generate an excess of neurons in vivo, we transplanted genetically modified cPCs into recipient brains and compared their engrafted neuronal progeny with controls. To distinguish transplanted elements from autochtonous ones, we employed cPCs originating from the E12.5 pallium of *Tau*$^{EGFP/+}$donors, which activate the EGFP reporter specifically upon neuronal differentiation. We engineered them by lentivectors driving overexpression of *Foxg1* (or the control, *human placental alkaline phosphatase* gene, *PLAP*, (Gaiano et al., 2000)) under the control of the pTα1 promoter, in a doxycyclin-dependent fashion. We grew engineered cPCs for seven days as neurosphere cultures, under Fgf2, Egf and doxycyclin, and we finally transplanted them by free-hand into the fronto-parietal cortical parenchyma of P0 wild type recipients (Fig.27A). To counteract the impredictable experimental variability originating from free-hand manipulation and other contingencies, we decided to introduce an appropriate internal control. Specifically, just at the end of the in vitro culture, we mixed two equally sized pools of Foxg1-GOF and PLAP-GOF cPCs and acutely employed the resulting mixed suspension for in vivo injections. To distinguish Foxg1-GOF cells from PLAP controls, we labelled the latter ones just after tissue dissociation by LV_pPgk1-mCherry, driving constitutive and strong expression of the mCherry fluoroprotein (Fig.27B). Seven days after transplantation, we fixed the brains and we monitored their cortices for the distribution of engrafted *Tau*$^{EGFP/+}$ neurons, mCherry^{-}/Foxg1-GOF and mCherry^{+}/PLAP-GOF. The frequency of mCherry^{-}/Foxg1-GOF neurons (as identified by Tau-promoter driven Egfp immuno reactivity (Wernig et al., 2002)) was almost doubled as compared to mCherry^{+}/PLAP-GOF controls (Fig.27C), consistently with old in vitro data by Brancaccio et al (2010). Interestingly, similar results were obtained by scoring transplanted brains seven more days

later, at P14 (Fig.27C,D). To rule out possible biases due to incomplete labelling of the PLAP-GOF population by LV_pPgk1-mCherry, we reversed the labelling scheme, associating mCherry to Foxg1, and repeated the full experiment set. Remarkably, despite a slight decrease of PLAP-normalized frequency of Foxg1-GOF neurons, again Foxg1 overexpression elicited a robust and statistically significant increase of the neuronal output in vivo, as evaluated at both P7 and P14 (Fig.27C,E).

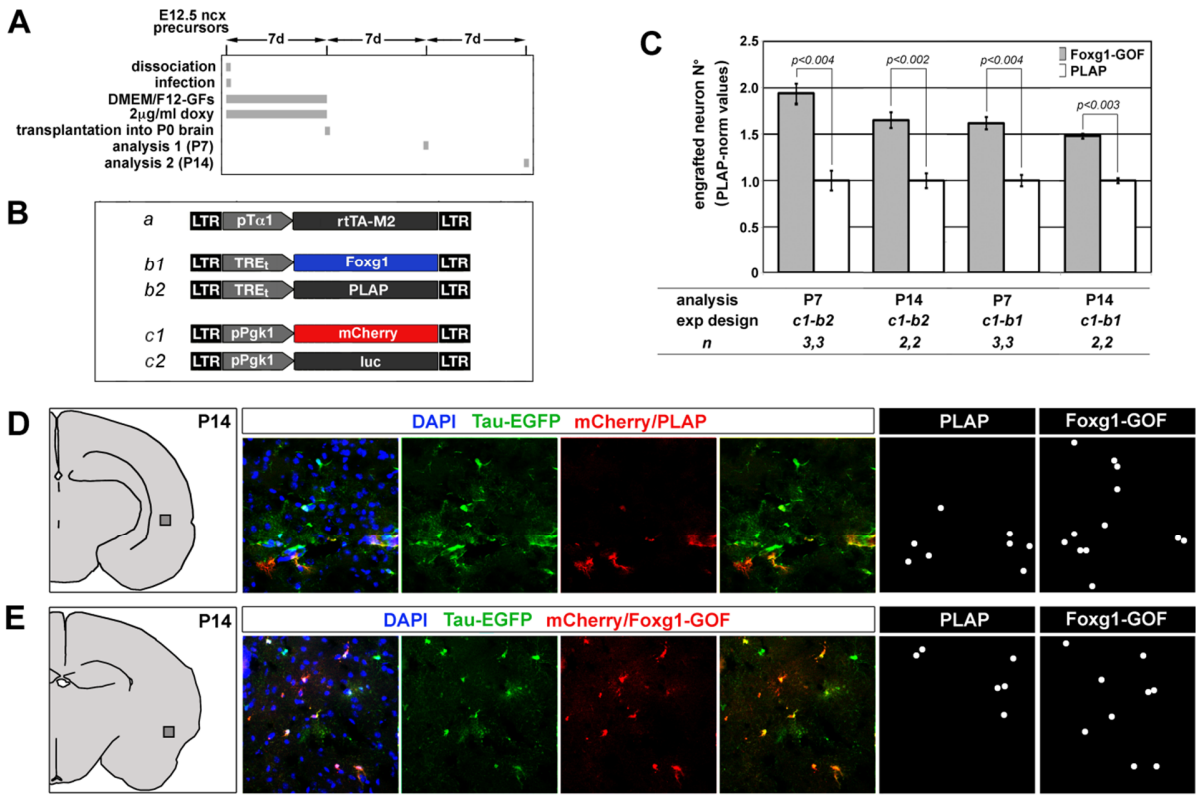

Figure 27.Engraftment and survival of neurons originating from *Foxg1* gain-of-function pallial precursors.

(**A**) Temporal articulation of the tests. Dissociated neocortical precursors from E12.5 *Tau-EGFP*$^{+/-}$donors were acutely infected by specific combinations of recombinant lentiviruses, driving doxycyclin-dependent expression of *Foxg1* or control (*PLAP*), they were kept in pro-proliferative medium under doxycyclin for 7 days as neuropheres, and finally transplanted into lateral neocortex of P0 wild-type recipients by intraparenchymal injection. Analysis was performed 7 or 14 days after transplantation. Transplanted neurons were identified thanks to their Tau-promoter-driven Egfp immunoreactivity. On average, for each brain, 312±38 cells were counted.

(**B**) The panel of recombinant viruses employed for these tests Precursors were alternatively infected by the four LV combinations: a-b1-c1; a-b1-c2; a-b2-c1 and a-b2-c2. Immediately before transplantation, co-infected precursors were pooled 1:1, as follows: a-b1-c1/a-b2-c2; a-b1-c2/a-b2-c1.

(**C**) Representation of numbers of engrafted neurons in the cortex of P7 and P14 recipients. Data normalized against *PLAP* controls; scalebars = s.e.m; *n* = number of brains analyzed. (**D,E**) Examples of engrafted neurons detected by anti-EGFP/anti-mCherry immunofluorescence. Confocal pictures in the middle refer to the small boxed area in the left panel. An idealized representation of transplanted neurons with different identities is shown in the

2. Foxg1 overexpression leads to exaggerated neuritic outgrowth in vivo

It was previously found that Foxg1 overexpression promotes neuritic outgrowth, whereas heterozygosity for a Foxg1 null allele leads to poorer neuronal architecture. However these experiments were run in vitro. Moreover, the selective impact of Foxg1 on dendritic or axonal morphology was not assessed (Brancaccio et al., 2010).

To investigate the effects of Foxg1 on neuronal architecture in vivo, we engineered E12.5 cortico-cerebral precursors, making them gain-of-function for Foxg1, and co-transplanted them with controls into recipient P0 brains, according to the design described above in Fig.27. Similarly to what shown in Fig.27, two alternative staining patterns were adopted, c1-b2 and c1-b1 (Fig.27B and 28B). As we previously found that both patterns gave consistent results, here we pooled primary Foxg1-GOF data originating from c1-b2 and c1-b1 tests and compared them with corresponding controls. Silhouettes of Foxg1-GOF engrafted neurons and PLAP controls were drawn by free hand and their morphology was analyzed by Neurphology software (Fig.28E,F). Four primary parameters were scored: number of somas, total neurite length, number of neurite attachment points, number of neurite endpoints. Based on them, three secondary morphometric indices were calculated: average neurite length/soma, number of attachment points per soma and ratio between numbers of endpoints and attachment points (Fig.27C). Interestingly all three indices were robustly upregulated upon Foxg1 overexpression, by 2.35 ± 0.20 ($p<0.001$), 1.52 ± 0.08 ($p<0.003$) and 1.21 ± 0.05 fold ($p<0.005$), respectively, compared to controls (Fig. 3D). This means that Foxg1 promotes emergence of neurites, their growth and arborization.

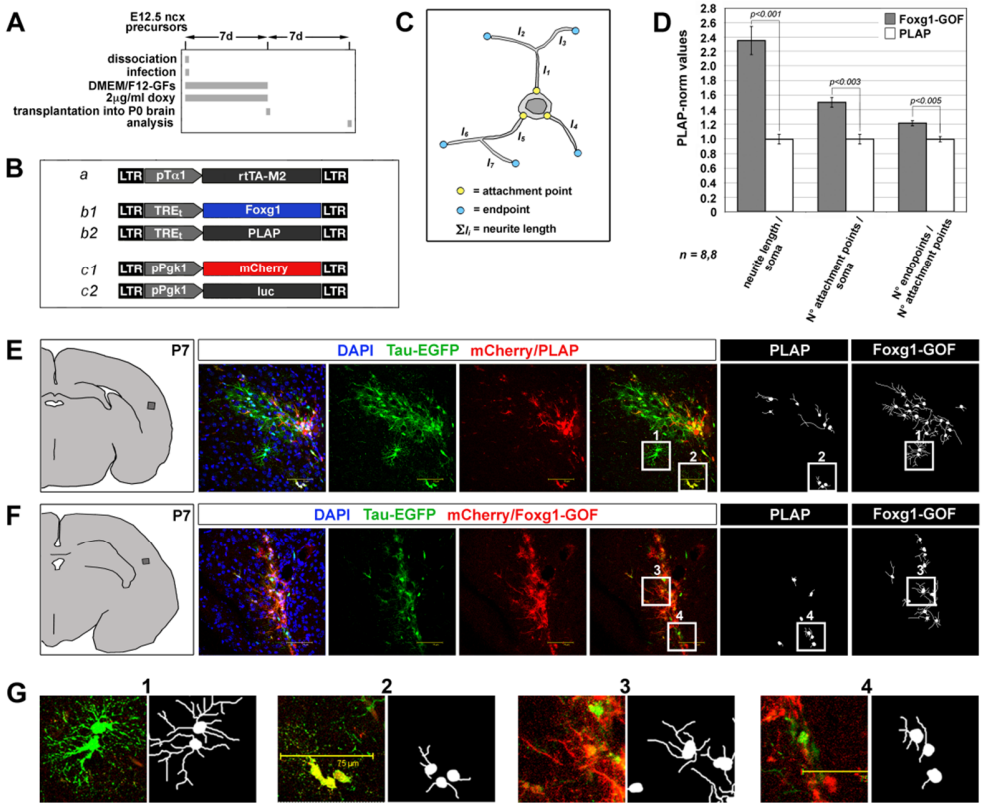

Figure 28.Morphometric profiling of *Foxg1* gain-of-function neurons: neurites, in vivo_I.

(**A**) Temporal articulation of the tests. It was as shown in Figure 27A. Analysis was performed 7 days after transplantation.

(**B**) Recombinant viruses employed for these tests. They were as shown in Figure 27B. (**C**) Representation of an idealized neuron with morphometric parameters used for this analysis. (**D**) Representation of three morphometric indexes, total neurite length/soma, number of attachment points/soma, and number of end points/ number of attachment points, in transplanted engineered neurons and controls. Here, data referring to *Foxg1*-GOF neurons labelled according to "a-b1-c1" and "a-b1-c2" designs were pooled (the same applies to controls). Analysis referring to all the neurites; data normalized against *PLAP* controls; scalebars = s.e.m; *n* = number of brains analyzed. Per each brain, 27±4 *PLAP* cells and 54±13 *FoxG1*-GOF cells were scored. Absolute average values of the three morphometric indexex in *PLAP* controls were as follows: total neurite length/soma = 37.7±2.7mm; number of attachment points/soma = 2.31±0.16; number of end points/number of attachment points = 1.33±0.06. (**E,F**) Examples of engrafted neurons detected by anti-EGFP/anti-mCherry immunofluorescence. Confocal pictures in the middle refer to the small boxed area in the left panel. Silhouettes of transplanted neurons with different identities are shown in the right panels. **(G)** High magnification of the analyzed neurons

Actually, neural precursors employed in Fig.28 test were kept under growth factors inhibiting neuronal differentiation for 7 days prior to transplantation (Fig. 28A), suggesting that engrafted neurons were prevalently born from aged precursors, at a stage roughly corresponding to E19 in vivo. To assess if pro-neuritogenic Foxg1 activity also applies to earlier born neurons, we repeated the entire experiment, shortening the in vitro expansion of precursors to two days only (Fig. 29A). Remarkably, also in this case all three morphometric indices resulted to be robustly upregulated upon Foxg1 overexpression. Specifically their values were increased by 3.00 ± 0.51 ($p<0.02$), 1.44 ± 0.09 ($p<0.03$) and 1.46 ± 0.07 fold ($p<0.0001$), (Fig. 3D-F), as for average neurite length/soma, number of attachment points per soma and ratio between numbers of endpoints and attachment points, respectively. This suggests that Foxg1 may enrich neuronal morphology regardless of neuron birthdate.

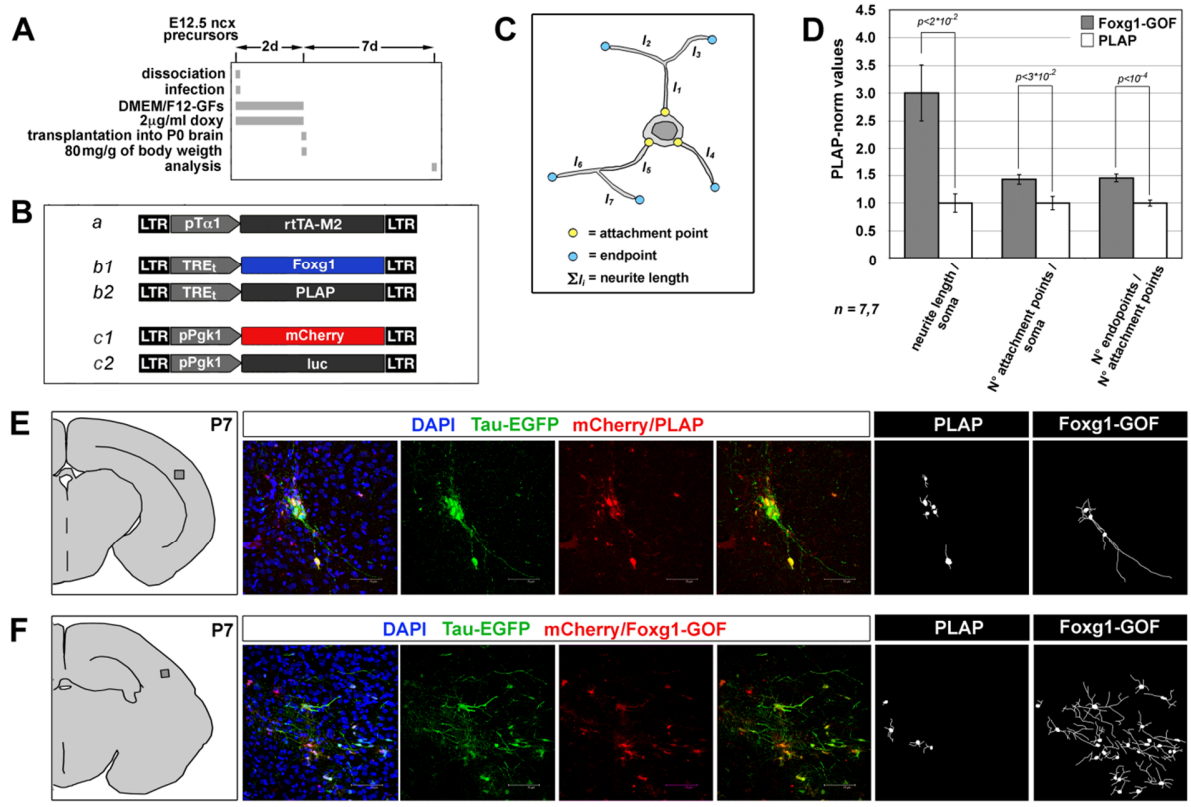

Figure 29.Morphometric profiling of *Foxg1* gain-of-function neurons: neurites, in vivo_II.

(**A**) Temporal articulation of the tests. Dissociated neocortical precursors from E12.5 *Tau-EGFP*$^{+/-}$donors were acutely infected by specific combinations of recombinant lentiviruses, driving doxycyclin-dependent expression of *Foxg1* or control (*PLAP*), they were kept in pro-proliferative medium under doxycyclin for 2 days as neuropheres, and finally transplanted into lateral neocortex of P0 wild-type recipients by intraparenchymal injection. Recipient mice were injected intraperitoneally with a single dose of 80 mg doxycyclin per gram of body weigth. Analysis was performed 7 days after transplantation.

(**B-F**) were as in Figure 3B-F. Per each brain 12.4±2.3 *PLAP* cells and 31±6 *FoxG1*-GOF cells cells were scored. Absolute average values of the three morphometric indexex in *PLAP* controls were as follows: total neurite length/soma = 24.2±4.1mm; number of attachment points/soma = 1.98±0.24; number of end points/number of attachment points = 1.20±0.06.

Then, to selectively assess the impact of Foxg1 on dendritic and axonal morphology, we engineered E12.5 wild type cPCs making them conditional GOF for Foxg1, under the control of the neuronal lineage-specific pTα1 promoter and doxycyclin. These cells were kept under proliferative medium and doxycyclin for 7 days and they were subsequently trasferred onto polylysin-coated coverslips under neurobasal A, for differentiation. 14 days later, these cells were immunoprofiled for the soma-dendritic marker Map2 and the soma-axonal marker Smi312 (Fig.30A,B). Neuronal silhouettes were generated and analyzed similarly to Fig. 28 and 29 (Fig. 30F). However, here separated analyses were run on the $Map2^{+}$ and the $Smi312^{+}$, dendritic and axonal parts, respectively, of the neuritic trees. Interestingly, Foxg1 overexpression increased dendrite length/soma, number of dendritic attachment points per soma and ratio between numbers of dendritic endpoints and attachment points, by 1.80 ± 0.12 ($p<0.01$), 1.30 ± 0.03 ($p<0.02$) and 1.15 ± 0.05 fold as compared to controls, respectively (Fig.30D). The corresponding axonal morphometric indices were upregulated as well (by 1.54 ± 0.39 ($p<0.21$), 1.96 ± 0.57 ($p<0.07$), and 1.14 ± 0.06 fold ($p<0.16$), respectively), however these changes were not statistically significant (Fig.30E).

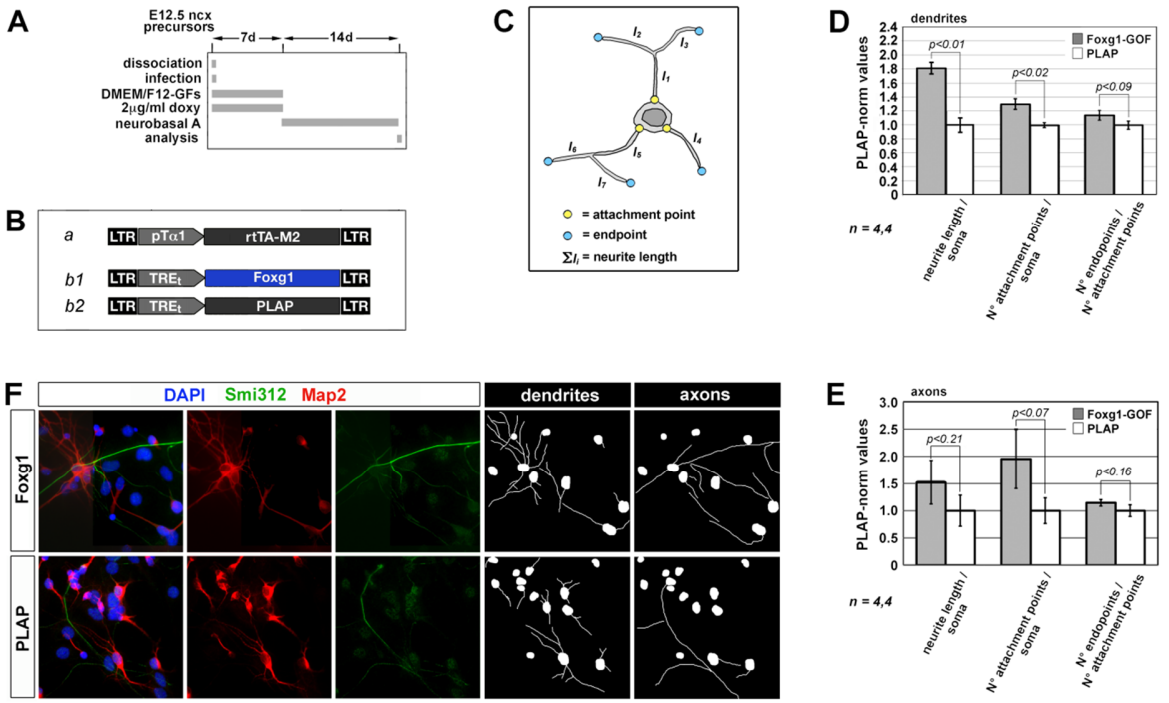

Figure 30.Morphometric profiling of *Foxg1* gain-of-function neurons, in vitro.

(**A**) Temporal articulation of the tests. Dissociated neocortical precursors from E12.5 wild-type donors were acutely infected by LV_p(Ta1)-rtTA-M2 and LV_TREt-Foxg1 (or LV_TREt-PLAP), driving doxycyclin-dependent expression of *Foxg1*(or *PLAP*), they were kept in pro-proliferative medium under doxycyclin for 7 days as neurospheres, and transferred to polylysin-coated coverslips under Neurobasal A medium for differentiation. 14 days later, they were fixed and analyzed.

(**B**) Recombinant viruses employed for this test.

(**C**) Representation of an idealized neuron with morphometric parameters used for this analysis.

(**D,E**) Representation of three morphometric indexes, total neurite length/soma, number of attachment points/soma, and number of end points/ number of attachment points, in engineered neurons and controls. Analysis restricted to dendrites (D) and axons (E); data normalized against *PLAP* controls; scalebars = s.e.m; *n* = number of brains analyzed. On average, 133±42 *PLAP* cells and 141±15 *FoxG1*-GOF cells were analyzed. Absolute average values of the three morphometric indexex in *PLAP* controls were as follows. Dendrites: total dendrite length/soma = 38.6±5.3mm; number of attachment points/soma = 1.62±0.05; number of end points/number of attachment points = 1.35±0.07. Axons: total axon length/soma = 24.4±7.6mm; number of attachment points/soma = 0.41±0.10; number of end points/number of attachment points = 2.27±0.24. (**F**) Immunoprofiling of Foxg1-GOF and PLAP control neurons for the axonal marker Smi312 and the somato-dendritic marker Map2. Primary immunofluorescences, to left, are flanked

3. Foxg1 overexpression stimulates the formation of dendritic spines.

Spatial frequency of excitatory synapses on glutamatergic cortical neurons is finely tuned in an activity-dependent fashion. It is among key determinants of neuronal excitability.

To assess whether Foxg1 stimulates the formation of dendritic spines, we plated E16.5 neocortical neurons and infected them acutely with lenti viral vectors driving doxycyclin-dependent expression of FoxG1 and PLAP, each at MOI = 8. Under these conditions, the almost totality of neural cells is transduced (Brancaccio et al., 2010 and data not shown). We allowed them to differentiate for 5 days in vitro, and we finally challenged them by Foxg1 overexpression and/or 50 mMKCl addition to the culture medium, as shown in Fig.31A,B. Eventually, we immunoprofiled them for the excitatory post-synaptic marker PSD95 and the dendritic marker Map2. We focussed on dendrite segments proximal to neuronal soma and there we evaluated the number of postsynaptic spines standing on a dendritic surface of defined area. As expected, 50mM K^+ increased superficial spine density, by 1.66$\pm$0.02 fold ($p<0.01$). Remarkably, Foxg1 overexpression upregulated such density as well, by 2.63$\pm$0.13 fold ($p<0.001$). Moreover, when combined with K^+, Foxg1 elicited an even more pronounced effect (3.34$\pm$0.22 fold, with $p<0.001$).

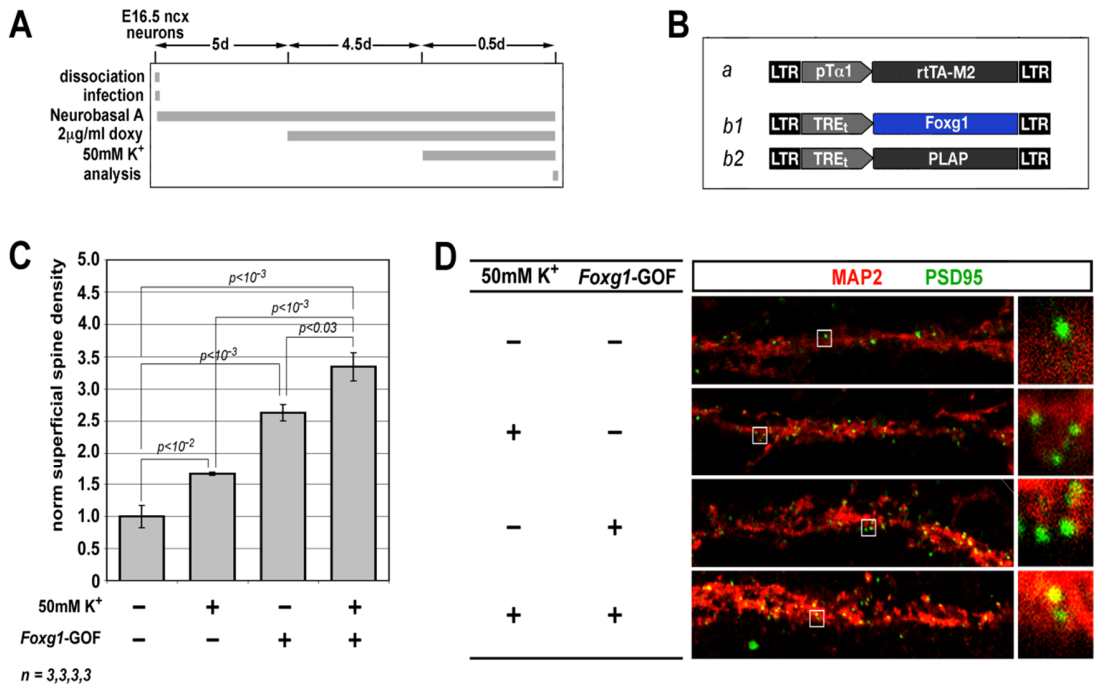

Figure 31.Morphometric profiling of *Foxg1* gain-of-function neurons: dendritic spines, in vitro.

(**A**). Temporal articulation of the tests. Dissociated neocortical precursors from E16.5 wild-type donors were acutely infected by LV_p(Ta1)-rtTA-M2 and LV_TREt-Foxg1, driving doxycyclin-dependent expression of *Foxg1*. They were cultured on polylysin-coated coverslips under Neurobasal A medium for 10 days. Doxycyclin was added starting from day 6. 50mM KCl was further added 12 hours before analysis

(**B**). Recombinant viruses employed for this test. They were as shown in Figure 25B.

(**C**). Representation of superficial spine density in *Foxg1*-GOF and control neurons, challenged or not by 50mM K^+. Density was as evaluated by anti-PSD95/anti-Map2 immunoprofiling of randomly picked, 80 mm-long dendritic segments, lying 10mm apart from soma. At least three dendrite segments were scored per each biological replicate. Data normalized against *PLAP* controls; scalebars = s.e.m; *n* = number of brains analyzed. Absolute superficial spine density of "*PLAP*-noK^+" controls was 0.17± 0.03 spines/mm^2.

(**D**). Examples of spines immunodetection.

CHAPTER V. DISCUSSION

In synthesis, four were the main findings of this work. First, we showed that, upon transient overexpression of Foxg1 within the pallialneuronogenic lineage, neurons eventually resulting from differentiation of the engineered proliferation pool - more numerous than in controls - retain the glutamatergic phenotype and, upon transplantation into neonatal neocortex, they survive at rates comparable with wild type controls. Second, we found that neurite overgrowth triggered by Foxg1, previously documented in vitro (Brancaccio et al., 2010), takes also place in vivo, upon transplantation of engineered neurons into neonatal neocortex, regardless of neuron birthdate. Third, we found that the neuritic overgrowth triggered by Foxg1 was mainly restricted to dendrites. We detected also an increase in axonal length and branching, however this did not reach statistical significance. Fourth, we discovered that neuronal overexpression of Foxg1 elicits a considerable increase (>2.5x) of spines on proximal dendrites and that this effect is exacerbated upon stimulation of neuronal hyperactivity.

FoxG1 is normally expressed in both proliferating precursors and – after a transient downregulation – in postmitotic neurons (Miyoshi and Fishell, 2012). To mimic and model what may happen in the brain of patients affected by the FoxG1-linked West syndrome, we overexpressed this gene under the control of the Tubulin-a1 promoter, which specifically fires

within the neurogenic lineage, both in proliferating precursors and postmitotic neurons (Sawamoto et al., 2001).

Actually, the overproduction of neurons we observed upon Foxg1 overexpression in pallial precursors was expected. A reduction of the proliferating neural pool upon Foxg1-LOF manipulations, due to precocious neuronal differentiation was previously described (Xuan et al., 1995; Hanashima et al., 2002). It is largely due to upregulation of the key cell cycle regulator Cip1p21, as well as to exaggerated Bmp (Hanashima et al., 2002) and Tgfb signalling (Dou et al., 2000). Moreover, our group previously showed that overexpression of Foxg1 in dissociated neural stem cells and/or neuronal progenitors may increase their neuronal output up to 2.4-fold, because of delayed exit from cell cycle and increase survival of postmitotic neurons (Brancaccio et al., 2010). What is remarkable is that these neurons retain their glutamatergic profile and survive in vivo, similarly to control neurons. Intriguingly, the same eccess of Foxg1 in cortico-cerebral stem cells also reduces their astroglial output up to 2-fold (Brancaccio et al., 2010). All that suggests that patients affected by Foxg1-duplication linked West syndrome could be affected by an increased ratio among excitatory neurons and astrocytes. This could lead to defective removal of metabolites and ions released into the extracellular space upon neuronal firing - glutamate and K+ among them - (Devinsky et al., 2013; Pannasch and Rouach, 2013), so contributing to neuron hyperexcitability and hyperactivity.

Concerning the cytoarchitectonic abnormalities we found in Foxg1-GOF neurons, at the moment we ignore the molecular mechanisms which link Foxg1 overexpression to their genesis.

Control of dendritic and axonal growth relies on a plethora of intertwined, dedicated devices (Yang et al., 2010; de la Torre-Ubieta and Bonni, 2011; Arikkath, 2012) and reconstructing these mechanisms is not a trivial issue.

Paradoxically, some primary molecular perturbations evoked by *Foxg1*-GOF manipulations should *antagonize* rather than *promote* dendritic growth. For example, the key effector of canonical Notch signalling *Hes1* is upregulated upon *Foxg1* overexpression by >10 times (Brancaccio et al., 2010). However, Notch1 activation in cortico-cerebral neurons *inhibits* neurite outgrowth (Berezovska et al., 1999; Sestan et al., 1999), whereas its consequences on dendrite branching may vary depending on the experimental context (Redmond et al., 2000; Bonini et al., 2011). On the other side, *Reelin*, encoding for a glycoprotein expressed by Cajal-Retzius cells and a subset of pyramidal cortical neurons (Deguchi et al., 2003), is dramatically down-regulated by Foxg1 (Miyoshi and Fishell, 2012). Puzzingly again, in the absence of canonical Reelin signalling, dendrites of cortical pyramids are *sensiblyshortened* and *poorly ramified* (reviewed by (D'Arcangelo, 2006)). All that suggests that molecular machineries mediating proneuritogenic activity of Foxg1 should be very effective, so overwhelming the concurrent antineuritogenic effects listed above.

Based on our present knowledge, we suspect that at least three specific molecular cascades might connect Foxg1 overexpression to dendritic overgrowth. The first of them includes the centrosome-anchored HLH protein Id1, known to provide cardinal inhibition of dendritic growth (Kim et al., 2009). *Id1* is transcriptionally stimulated by Bmps, via pSmad1,5,8 nuclear effectors, (Hollnagel et al., 1999), whereas its protein product is post-translationally

destabilized by the E3 ubiquitine ligase complex Cdc20-APC (Kim et al., 2009). Foxg1, inhibiting Bmp4 expression (Dou et al., 1999) and counteracting transmission of Bmp signals (Rodriguez et al., 2001), should ultimately downregulate Id1, so relieving its tonic inhibition of dendrite growth. The second cascade could involve the transcription factor gene NeuroD1. As shown in cerebellar (Gaudillière et al., 2004) and hippocampal granules (Gao et al., 2009), NeuroD1 is a powerful inducer of initiation, growth and eleboration of dendrites. Moreover, it requires CaMKIIa-mediated, activity-dependent phosphorylation of its Ser336 for this purpose (Gaudillière et al., 2004). Actually, NeuroD1 was described to be *upregulated* in *Foxg1-null* mutants (Miyoshi and Fishell, 2012). However, as its activation normally takes place just upon the exit of cortical precursors from celle cycle, this phenomenon could reflect the massive exit of neural precursors from cell cycle occurring in Foxg1-null mutants, rather than a genuine NeuroD1 repression by Foxg1. Consistently with this hypothesis, NeuroD1 expression, normally transient, is *dramatically prolonged* upon Foxg1 electroporation (Miyoshi and Fishell, 2012), suggesting a latent capability of the latter to sustain the former, masked in Foxg1-LOF approaches. Therefore, Foxg1 overexpression might sustain dendrite growth, just upregulating NeuroD1. (In this scenario, neuronal hyperactivity originating from increased excitatory inputs impinging on Foxg1-GOF neurons might further amplify the effects elicited by NeuroD1 upregulation on the dendritic tree). Finally, Foxg1 might impact dendrite morphology by inhibiting Tbr1 transcription (Toma et al., 2014) and consequently relieving Tbr1-dependent inhibition of Fezf2 (Han et al., 2011; McKenna et al., 2011), namely a primary corticofugal neuron determinant promoting dendritic growth (Chen et al., 2005; Zuccotti et al., 2014).

As for axons, their moderate elongation observable in Foxg1-GOF mutants might be due to perturbation of pathways ending on the cardinal promoter of axonal growth Ccd1 (Ikeuchi et al., 2009). Ccd1 is normally transactivated by SnoN(Ikeuchi et al., 2009), which is in turn destabilized by the Cdh1-APC/pSmad2,3 complex (Stegmüller et al., 2006, 2008). Foxg1 might interfere with this mechanism, chelating pSmad2 (Rodriguez et al., 2001) and consequently relieving the indirect down-regulation of Ccd1 by Cdh1-APC. These hypotheses will be tested in a follow-up study. Moreover, such approach will include an unbiased approach, based on systematic comparison of the transcriptome (and translatome) of neurons harboring different Foxg1 gene dosages.

Regardless of molecular mechanisms underlying the dendritic overgrowth elicited by Foxg1, such overgrowth can have a profound impact on cortical circuitries. Within the developing human embryo, neocortical dendritogenesis is finely regulated in time and space. It initiates immediately after pyramidal neurons have completed radial migration layer (Rakic, 2002), then it bursts upon the arrival of corticopetalfibres, until the third postnatal month, and it continues postnatally, peaking by the 16th and the 30th month, in infragranular and supragranular layers, respectively. Finally, a pronounced dendrite pruning occurs, particularly vigorous in adolescence (Petanjek et al., 2008). Accurate regulation of dendrite size and shape seems crucial to mental function, as suggested by the association of intellectual ability to robust cortical thickening in childhood followed by pronounced cortical thinning in adolescence (Shaw et al., 2006). Interestingly, the dendritic overgrowth we found upon Foxg1 overexpression may result into larger afferent basins impinging on excitatory neurons, which can ease neuronal synchronization over larger distances and contribute to EEG abnormalities of West patients.

Finally, as suggested by misreguation of spine densities, this scenario might be worsened by increased excitatory inputs, which could be conveyed to glutamatergic neurons upon Foxg1-GOF manipulations. Dendritic spines are small dendrite protrusions, on which the majority of excitatory synapses of neocortex and hippocampus form (inhibitory synapses are predominantly established on neuronal somata and dendritic shafts) (Spruston, 2008). Their geometry is supposed to profoundly impact electric properties of pyramidal neurons, enhancing perisynaptic potential variations elicited by postsynaptic excitatory currents, supporting locally-restricted electrical computation, and allowing *equalized* and *low-noise*, linear integration of information neuron-wide (Yuste, 2013). Spines are highly dynamic and neuronal activity promotes their formation (Papa and Segal, 1996; Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Kwon and Sabatini, 2011).

Here we induced neuronal hyperactivity by acute treatment of neurons with 50mM KCl(Kim et al., 2010) and, as expected, we detected an appreciable increase of spine density on proximal dendrite segments. Interestingly, Foxg1 overexpression produced an even more pronounced effect and, strikingly, it synergyzed with potassium. [Unfortunately, we did not categorize spines based on their morphology, namely a key parameter influencing their physiology (Yuste, 2013)]. Of course, all that is of particular interest for West syndrome etiopathogenesis, as it suggests a possible Foxg1-dependent mechanism increasing neuron excitability. But, how is this achieved?

Molecular control of spine morphogenesis is extremely complex. Spine dynamics largely relies on controlled translation (Holt and Schuman, 2013) and ubiquitylation-degradation (Yamada et al., 2013) of spine proteins. Moreover, activity-induced spine outgrowth requires opening of NMDARs (N-methyl-d-aspartate-type glutamate receptors), activation of protein kinase A (PKA) (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Kwon and Sabatini, 2011), and it depends on proteasome (Hamilton et al., 2012).Stimuli conveyed by these key molecular players (and others) result into specific alterations of dendritic cytoskeleton, local clustering of receptors, voltage-gated channels and adaptor proteins as well as into local plasma membrane distorsions, ultimately resulting in spine formation [reviewed by (Dillon and Goda, 2005; Ethell and Pasquale, 2005; Okabe, 2007; Sheng and Hoogenraad, 2007; Arikkath, 2012; Rochefort and Konnerth, 2012)]. Foxg1 could modulate transcription of key genes involved in this processes. Alternatively, it could act post-transcriptionally, regulating translation and/or protein stability. Intriguingly, in fact, Foxg1 translocates to the cytoplasm of postmitotic neurons upon Akt-dependent phosphorylation of its T236 (Regad et al., 2007) and physically interacts with the translational elongation factor Eef1g (Stelzl et al., 2005). On the other side, Foxg1, chelating pSmad2 and 3 proteins (Rodriguez et al., 2001), should prevent Tgf-beta-dependent transcriptional stimulation of the E3 ubiquitin ligase Mdm2 (Araki et al., 2010), leading to upregulation of the Mdm2 protein target PSD95 (Colledge et al., 2003) and ultimately limiting the internalization of the AMPA-kainate receptor AMPAR (Colledge et al., 2003). Like in case of mechanisms undelying neuritic outgrowth, even this working model will be addressed in a dedicated follow-up study, also including an unbiased transcriptome and translatome profiling of Foxg1-GOF neurons.

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