**S.I.S.S.A./I.S.A.S.**

# MASTER THESIS

# *Emx2* antisense transcripts in cerebral cortex development


**Candidate**

**Dr. Chiara Gedressi**

**Supervisor**

**Prof. Antonio Mallamaci**

“The question of whether "you" would continue in multiple parallel brain vats raises issues that come perilously close to the theological notion of souls, but I see no simple way out of the conundrum. Perhaps we need to remain open to the Upanishadic doctrine that the ordinary rules of numerosity and arithmetic, of "one vs. many", or indeed of two-valued, binary yes/no logic, simply doesn't apply to minds — the very notion of a separate "you " or "I" is an illusion, like the passage of time itself.”

**V.S. Ramachandran**

“Fantasy is a black sky and the eyes to paint on it”

**24 Grana**

**INDEX** **PAG.**

# Chapter I

# INTRODUCTION

## 1.1 Cerebral cortex formation

### 1.1.1 Development of mammalian telencephalic vesicles

The mammalian brain arises from three vesicles forming at the anterior end of the neural tube: the prosencephalon (from Greek pre-brain), the mesencephalon (from Greek mid-brain) and the rhombencephalon (from greek rhomboid shaped-brain, also called hindbrain). These vesicles are generated at very early stage of the development; for example, in the mouse embryo the prosencephalon develops at around embryonic day 9 and in human embryos around week 6. Later, the prosencephalic vesicle gives rise to two laterally enlarged bulges, termed telencephalon (from Greek end-brain) and a medial secondary vesicle, the diencephalon (from Greek intermediate-brain) (fig. 1.1).

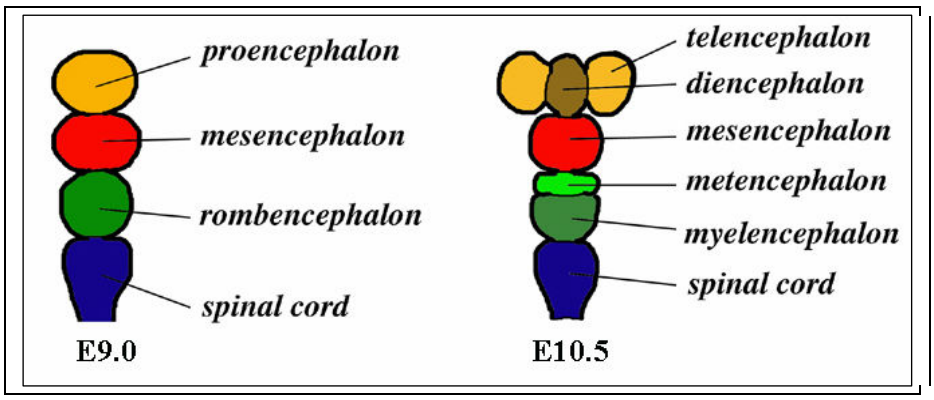


**Fig. 1.1 Mouse anterior neural tube from three-vesicle stage to six-vesicle stage**.

Anterior regions of the neural plate give rise to the ventral telencephalon (basal ganglia) and posterior regions generate the dorsal telencephalon (cerebral cortex) (fig. 1.2). The development of the telencephalic hemispheres does not proceed uniformly. Anterior-lateral regions are more advanced than posterior-medial regions. Regional specification becomes visible after the telencephalic hemispheres are formed.

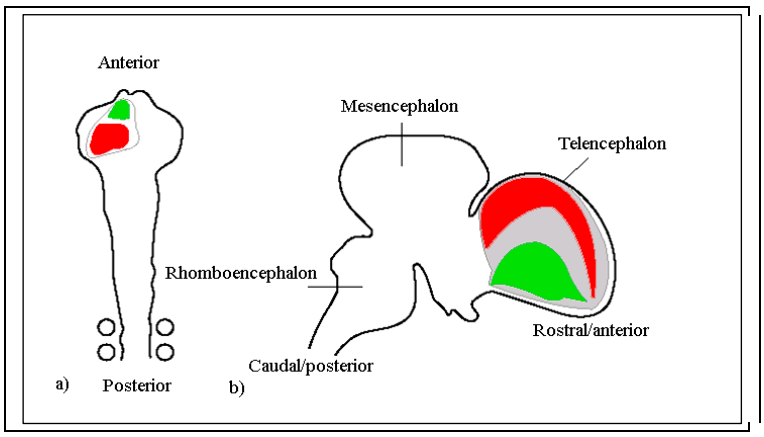


***Fig. 1.2 Developmental origin of vertebrate telencephalic regions****.(a) The neural plate viewed from the top, (b) The developing brain at a later stage viewed sideways. The dorsal telencephalon is depicted in red, the basal in green.*

The dorsal part of the telencephalon gives rise to a thin sheet, the pallium, which will generate the cortex.

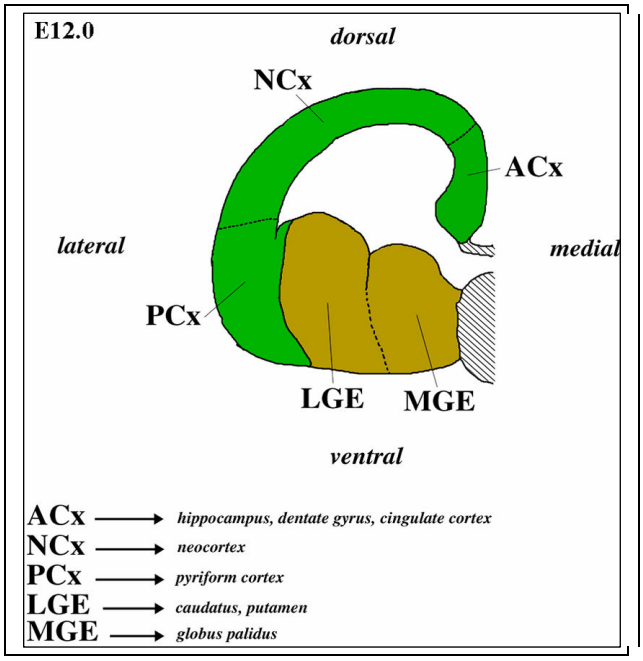


***Fig. 1.3 Schematic view of a section through the developing telencephalic vesicle at E12.0****. Acx, archicortex; Ncx, neocortex; Pcx, palaeocortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence.*

The ventral, subpallial region, develops two hill-like extensions, the lateral and medial ganglionic eminences, which will generate the basal ganglia (fig. 1.3).

The basal ganglia, placed in the bottom of the telencephalic vesicles, consist of four major group of neurons: the striatum, the globus pallidus, the subthalamic nucleus and the substantia nigra. The dorsal part of the striatum, involved in motor control, includes the caudate nucleus and the putamen.

Afferents reaching the basal ganglia come from two main sources: the cerebral cortex and the medial part of the thalamus. Efferents leave the basal ganglia from two points: the globus pallidum and the subtantia nigra.

Co-inactivation of *Pax6* and of another homeobox gene, *Emx2*, fully suppresses cortical specification and leads to homeotic conversion of the pallial precursor into striatum.

### 1.1.2 Development of mammalian cerebral cortex

The mammalian cerebral cortex has undergone an immense enlargement during evolution, characterized by an increase in cell number and a progressively more complex radial and horizontal organization. Two main different cortical regions can be distinguished: a phylogenetically older region, the allocortex, and a younger region, the isocortex (also called neocortex). The allocortex can be further subdivided into palaeocortex and archicortex. The former is thought to be the oldest part of the cerebral cortex, originating from olfactory regions, the latter, containing the hippocampal formation, is considered to be younger. These regions are distinguished on the basis of their lamination: neurons in the allocortex are organized in three horizontal layers, whereas those of the neocortex form six layers. Cortical layers located between the neocortex and the allocortex display three to six layers, reflecting their transitional nature. The six layered neocortex, which is the largest part of the mammalian brain, is divided into distinct areas according their functions and cytological organization (Brodmann.

1909). Each cortical layer contains two distinct neuronal types: pyramidal and non-pyramidal neurons (S. R. y Cajal. 1911). Pyramidal cells are the projection neurons of the cerebral cortex. They have a triangular soma and a characteristic dendritic arborisation pattern. These cells use glutamate as excitatory neurotransmitter (Parnavelas et al. *The central visual pathway*. In Handbook of Chemical Neuroanatomy (vol7) Integrated System of CNS(part2, A. Bjorklund at al. eds. pp 1-164, Elsevier). Non-pyramidal cells are the cortical interneurons, a heterogeneous group of cells that display a broad range of morphologies and molecular identities (Fairèn et al. Nonpyramidal neurons: general account. In *Cerebral cortex (vol1) Cellular components of the Cerbral Cortex* (A. Peters And E.G. Jones) pp 201-253, Plenum press). These neurons use the neurotransmitter GABA and also one or more neuropeptides.

Cerebral cortical neurons are mainly generated in the germinal ventricular zone which lies at the surface of the lateral ventricles. At the beginning of neurogenesis (around E10.5-E11 in the mouse embryo), newborn neurons leave the ventricular zone and migrate towards the margin of the cerebral wall to form a transient layer, called primordial plexiform layer or preplate. Later generated neurons infiltrate the preplate and split it into the superficial marginal zone (or layer I), located just under the pial surface, and the subplate, placed in the deep cortical wall (fig. 1.4), (Marin Padilla. 1971, Uylings et al. *The prenatal and postnatal development of rat cerbral cortex.;* B.Kolb and R.C. Tees. eds pp 35-76, MIT press). They accumulate in between marginal zone and subplate, where they give rise to the cortical plate, the forerunner of layers II to VI. These neurons take their final positions in an "inside-out" sequence: neurons generated early in the development settle in the deep cortical plate, while neurons generated later overcome the first ones and are finally placed above them (fig. 1.5), (Berry and Rogers. 1965, Rakic. 1974).

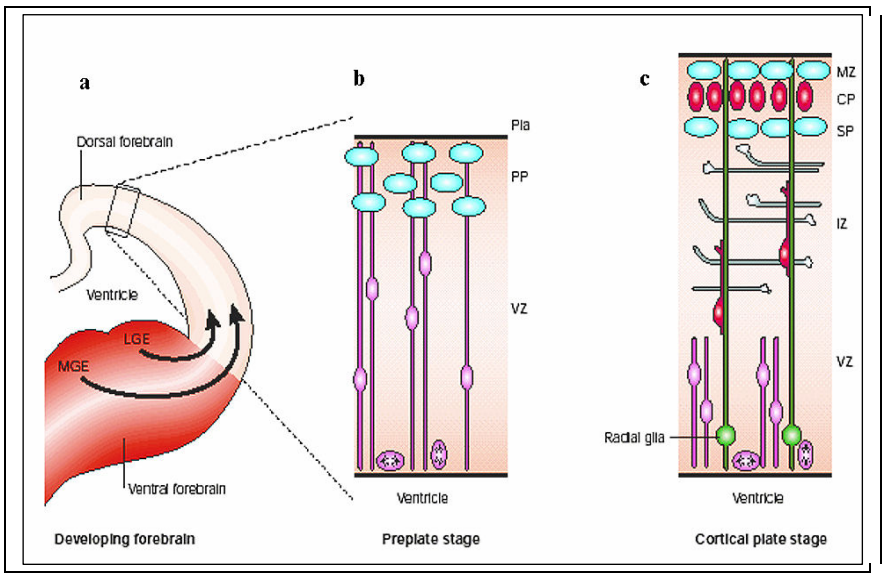


***Fig. 1.4 Schematic view of a section through the developing telencephalic vesicle****. The cerebral cortex arises from dorsal telencephalon. The lateral ganglionic eminence (LGE) and the medial ganglionic eminence (MGE) of the ventral forebrain generate neurons of the basal ganglia and the cortical interneurons. In the dorsal forebrain, the first cohort migrating neurons gives rise to the preplate (PP) (b). Subsequently generated neurons (pyramidal cells) migrate along radial glia and through the intermediate zone (IZ) to split the PP layer into the outer marginal zone (MZ) and inner subplate (SB)(c). These cells generate the cortical plate (CP).*

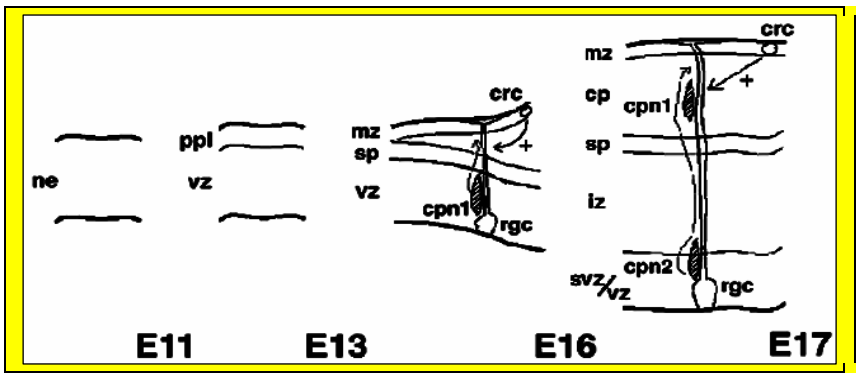


***Fig. 1.5. Schematic representation of the cortical wall during the cerebral cortex development****. Ventricular zone is at the bottom and marginal zone is at the top. Ne, neuroepithelium; ppl, preplate; vz, ventricular zone; mz, marginal zone; sp, subplate; crc, Cajal-Retzius cell; cpn, cortical late neuron; rgc, radial glia cell; cp, cortical plate; iz, intermediate zone; svz, sub-ventricular zone.*

As the cortical plate emerges, an another proliferative cell layer, placed above the main proliferative epithelium, appears. This is the so-called sub-ventricular zone, thought to be the main source of pyramidal neurons belonging to upper layers and to glial cells (II through IV), (Tarabikyn et al. 2001).

Among neurons located within the marginal zone (layer I), there are the Cajal-Retzius cells which provide diffusible signals, crucial for the migrations of cortical plate neurons (Frotscher. 1997), (fig. 1.5). Cajal-Retzius cells secrete the REELIN glycoprotein, essential for neuronal glia-mediated migration (De Rouvroit et al. 2001). REELIN induce neurons to migrate past their predecessors (D'arcangelo and Curran.1998), but it has also been thought to provide a stop signal to migrating cells for them to detach from the radial glia. Indeed, REELIN has been shown to inhibit neuronal migration via its interaction with *α3-β1* integrin, a downstream component of its signaling pathway (Dulabon et al. 2000). This migration-inhibiting activity of REELIN should be relevant at the end of radial migration, when the neuronal leading process contacts the marginal zone and pyramidal neurons detach from the radial glial scaffold (Nadarajan et al. 2001).

Radial migration of pyramidal neurons from proliferative layers to the forming cortical plate has been the subject of intense experimental investigations and two moving patterns have been recently demonstrated to take place. During early stages of the cerebral cortex development, when the cerebral wall is relatively thin, neurons move by "somal translocation" and later, during the cortical plate formation, they proceed by "glia-guided locomotion" (Nadarajan et al. 2001). The cells that undergo somal translocation typically have a long, radially oriented basal process that terminates at the pial surface, and a short, transient trailing process. The migratory behaviour is characterized by continuous advancement that results in a faster rate of migration. By contrast, cells that adopt glia-guided locomotion have a shorter radial process that is not attached to the pial surface. These cells show a characteristically slow saltatory pattern of locomotion. They perform short burst of forward movements that are interspersed with stationary phases, for this reason they display slow average speeds (Nadarajan et al. 2001). Interestingly, Nadarajan and colleagues have also revealed that neurons that show this saltatory pattern of movement switch to somal translocation in the terminal phase of their migration, once their leading process reach the marginal zone.

Cortical GABAergic neurons are generated in sub-pallial telencephalon, in the lateral and medial ganglionic eminences. They leave the proliferative epithelium and then initiate their long tangential migratory routes, moving parallel to the surface of the telencephalon. They cross the cortico-striatal boundary and enter the cortical wall, reaching their appropriate laminar-areal locations (Anderson et al. 1997, Tan et al. 1998, Tamamaki et al. 1997), (fig. 1.6).

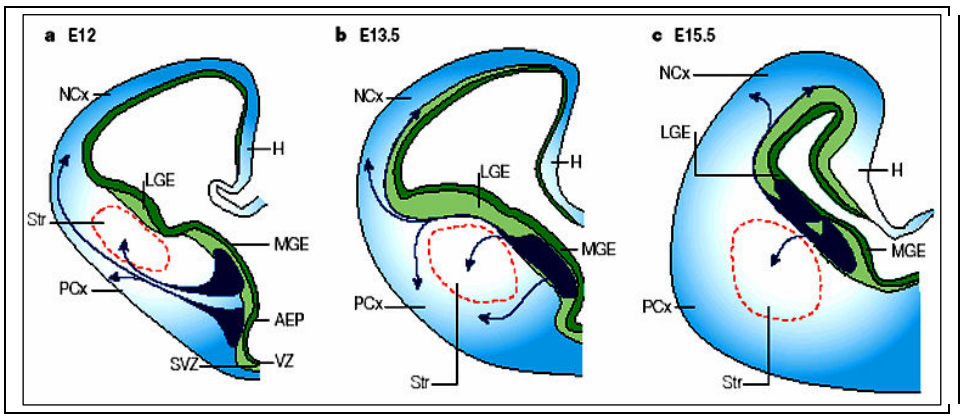


***Fig. 1.6 Tangential migration of immature interneurons from the basal telencephalon to the cortex****. Schematic transverse sections of the embryonic telencephalon,(a) at E12, interneurons arise from the MGE and AEP and they follow a superficial route.(b) At E13.5, interneurons are primarily generated from the MGE and follow a deep route to the developing striatum; some interneurons also migrate superficially. (c) At later stages, E15.5, cortical interneurons also arise from the LGE and follow a deep route. H, hippocampus; GP, globus pallidum, LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, piriform cortex; VZ, ventricular zone; AEP, anterior entopeduncolar area; Str, striatum.*

Their tangential migration seems to be largely independent of interactions with radial glia. Rather, an association of tangentially orientated cells in the developing cortex with bundles of the corticofugal fibre system has been reported (Metin and Godement. 1996) and the neural cell adhesion molecule TAG-1, located on the surface of cortico-fugal axons, has been shown to promote tangential migration of GABA-ergic neurons contacting them (Denaxa et al. 2001). The medial and not the lateral ganglionic eminence is the main source of the GABA-ergic interneurons.

Three general and partially overlapping phases of tangential migration of GABA-ergic neurons can be distinguished (fig. 1.6). First, an early migration from medial ganglionic eminence. These cells start to migrate around embryonic day 11.5 in the mouse. They

course primarily superficially to the developing striatum and invade the cortical marginal zone and subplate (Marin et al. 2001). Second, around mid-embryonic stages (E12.5-14.5), the medial ganglionic eminence seems to be the principal source of cells that migrate tangentially into the cortex. These neurons migrate either deep or superficially to the developing striatum, and they populate both the sub-ventricular zone/lower-intermediate zone and the subplate, from where they extend into the cortical plate (Anderson et al. 2001). Third, at late stages of the development (E14.5-16.5), cells that migrate tangentially into the cortex seem to derive from both the lateral and the medial ganglionic eminence (Marin and Rubenstein. 2001). Interestingly, some migrating cells are directed toward the proliferative regions of the cortex at this stage (Anderson et al. 2001), presently unknown is the biological significance of this phenomenon.

## 1.2 Cerebral cortex regionalization and aerealisation

### 1.2.1 Models for regionalization of the cortical primordium

Early during the development the mammalian cerebral cortex is a simple, continuous, sheet of proliferating neuroblasts, Later, it consists of several areas, each characterized by peculiar anatomical and functional properties. These areas form a map that is similar from one individual to another and displays large topological commonalities across mammalian species.

Two models have been proposed to explain how cerebral cortex gets organized into distinct areas. The first model, the protocortex model (O'Leary. 1989), suggests that the early cortical primordium would be like a "tabula rasa" and each region composing it would not display any areal bias at all. In such a model, cortical arealisation is primed by information born by afferents coming from different thalamic nuclei, each preferentially projecting to a different presumptive cortical area. Thalamo-cortical connections are highly conserved between species. Most of the thalamic afferents terminate in layer IV of the neocortex . Layer VI neurons of each area send projections back to the corresponding thalamic nucleus (Jones. 1985). Thalamic nuclei and cerebral cortex develop sinchronously: in the rat embryo, most of the thalamic neurons are co-generated with cortical plate neurons, between E13 and E19 (Altman and Bayer. 1979). Different projections coming from the dorsal thalamus find proper ways to their targets by recognizing sets of specific cues along their trajectories. Reciprocal connections between the neocortex and the thalamus start to appear during the second week of the mouse embryonic life, around E13-E18. Thalamic fibres arrive at the appropriate cortical regions before their ultimate target neurons are born (Shatz and Luskin. 1986), and they have to wait for two or three days before they can continue their growth and establish their final innervation pattern within the cortical plate.

The second model, the protomap model (Rakic. 1988), conversely postulates that cortical arealisation would take place on the basis of information intrinsic to the early cortical primordium. Positional values would be encoded by the graded expression of specific genes whitin the cortical proliferative epithelium. This positional information would be epigenetically transferred from neuroblasts to neurons in distinct cortical regions, eventually leading to the activation of different areal morphogenetic programs. Even if both models are based on large bodies of experimental data, strong evidence suggests that at least the early phases of cortical arealisation occur according the protomap model.

This model predicts that the cortex can show regional molecular differences early in corticogenesis, before the extrinsic afferents arrive to the cortex. In fact, many genes have recently been described to display patterned expression within the very early cortical primordium. Moreover, this model predicts that distinct parts of the early cortical primordium are differentially committed to express mature areal markers. Indeed a long list of early committed areal markers was been reported. Among these markers, there are the limbic system-associated membrane protein *Lamp* (Levitt. 1984), the lateral cortex marker *Latexin* (Arimatsu et al. 1999), the somatosensory areas-specific transgene H-2Z1 (Gitton et al. 1999) as well as a large set of hippocampal markers, including *KA1*, *SCIP*, *Steel* and *NK3* (Tole et al. 1997, Tole et al. 2001). Further support for the protomap model comes from molecular regionalisation of the cortical primordium taking place in *Gbx2*$^{-/-}$ and *Mash1*$^{-/-}$ mice despite the absence of any thalamo-cortical connections in these mutants. (Miyashita-lin et al. 1999, Tuttle et al. 1999). Finally, regions of the cortical primordium transplanted heterotopically into a host brain, have the capability to attract thalamic fibres appropriate for their place of origin (Frappe et al. 1999, Gaillard and Roger. 2000), suggesting that, rather than being patterned by thalamic fibres, a patterned cortex can orchestrate the area-specific sorting of thalamo-cortical axons. In this respect, a large set of areally patterned gene products, including *Lamp*, *Cdh6*, *Cdh8*,

*Cdh11*, *Coup-tf1*, *Ephrins* and *Eph* receptors, may participate in setting up this connectivity (Donoghue and Rakic. 1999, Barbe and Levitt. 1992, Zhou et al. 1999).

### 1.2.2 Signaling centres involved in cortical aerealisation

It was suggested that, like in the *Drosophila* wing morphogenetic field (Strigini and Cohen. 1999, Podos and Ferguson. 1999), diffusible ligands released by patterning edges at the borders of the cortical morphogenetic field could master regionalisation of it. This would be achieved by modulating the expression of cortical transcription factor genes in dose-dependent ways and by regulating, through them, the ultimate molecular machineries executing distinctive areal morphogenetic programs. According to these predictions, three complex signalling centres were described at the borders of the cortical field (Mallamaci A, Stoykova A. 2006) , (fig.1.7 panel A).

The first one, the cortical hem, lies along the medial edge of the cortex and expresses multiple *Wnt* and *Bmp* genes (Grove et al. 1998, Furuta et al. 1997), (fig.1.7 panel A). Abundant evidence supports the hypothesis that the hem is necessary for proper development of caudal-medial cortical areas. In mice deficient of *Wnt3a*, one of *Wnt* genes expressed at the hem, the hippocampus is nearly absent, whereas neighbouring neocortical areas appear grossly normal (Lee et al. 2000). Canonical Wnt signalling relies on a complex molecular machinery. Briefly, WNT ligands bind to plasmamembrane Frizzled receptors and low density lipoprotein receptor-related protein (LRP) co-receptors. This interaction triggers the signal inside the cell. In the cytoplasm, in the absence of extracellular stimulation, the soluble pool of is bound to a macromolecular complex, including Axin and the adematous-polyposis coli tumor suppressor gene (APC) protein, which targets *β-catenin* to proteosomal destruction. Upon binding of Wnts to their receptors, soluble *β-catenin* is no longer available for proteosomal degradation and, conversely, becomes detectable in the nucleus. Here, it forms a complex with TCF/LEF

cofactors, it further recruits other co-factors and the resulting complex activates the transcription of *Wnt* target genes.

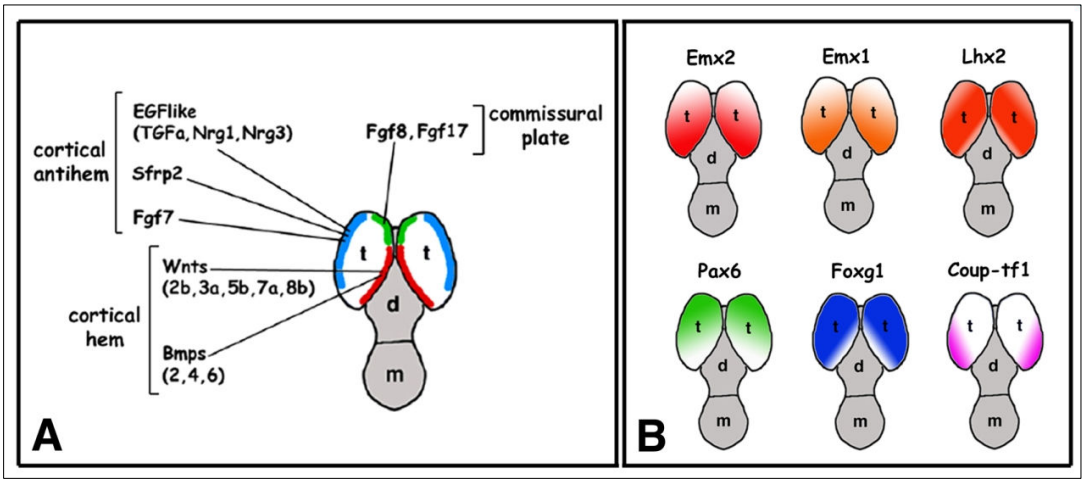


***Fig 1.7 Secreted ligands and transcription factors involved in cortical arealisation***. *The panel A) shows the signalling centres and their secreted products: in green the commissural plate, placed in the antero-medial edge of the cerebral cortex, in blue the cortical antihem, placed in the antero-lateral edge, in red the cortical hem, placed in the caudo-medial edge. The panel B) shows the graded expression of some trasciption factors involved in the cortical arealisation: Emx2, Emx1 and Lhx2 with a rostral/lateral$^{low}$-to-caudal/medial$^{high}$ expression gradient, Pax6 and Foxg1 with a caudal/medial$^{low}$-to-rostral/lateral$^{high}$ expression gradient and Coup-tf1 with a rostral$^{low}$-to-caudal$^{high}$ expression gradient.*

The second signalling border is the antero-medial edge of the telencephalon, the commissural plate, which is a source of FGF proteins (fig.1.7 panel A). Most FGFs are secreted proteins that affect target cells via high affinity receptors tyrosine kinase, RTKs, which act primarily through an evolutionary conserved *ras*-dependent intracellular signal transduction pathway (Crossley and Martin. 1995). Ectopic expression of FGF8 elicited either via bead implantation in chicken embryos or via electroporation in mouse explants,

perturbs expression patterns of transcription factors regionally expressed within the cortex (Okubo et al. 2002, Storm et al. 2003). In utero electroporation of *Fgf8* or a dominant negative form of FGF receptor 3 (*FgfR3*), causes molecular, histological and functional consequences on the developing cerebral cortex, which can be subsequently studied and characterized at appropriate post natal ages (Fukuchi-Shimogori and Grove. 2001). Overactivation of the FGF8 pathway in the anterior cortical primordium pushes somatosensory areas backward, like it happens in mutants lacking the transcription factor gene *Emx2* (Bishop et al. 2000, Mallamaci et al. 2000), whereas depression of this pathway elicits complementary consequences. Evidence suggests that the regulation of the cortical areal profile by *Fgf8* relies on mutual inhibitory interactions that this gene has with *Emx2*. For example, *Emx2* and *Fgf8* normally show complementary expression patterns, inactivation of *Emx2* leads to expansion of the *Fgf8* expression domain, up-regulation of *Fgf8* signalling depresses *Emx2* expression, down-regulation of *Fgf8* is followed by *Emx2* over-expression.

The third source of diffusible ligands is the lateral edge of the cortical field, or "antihem", identified by gene expression for three epidermal growth factor (EGF) family members, Tgf-α, Neuregulin1 and Neuregulin3, as well as for two other signaling molecules, Fgf7 and the secreted WNT antagonist Sfrp2. (Assimacopoulos et al. 2003), (fig.1.7 panel A). However, relevance of this structure to cortical arealisation has still to be proven. Except for *Sfrp2*, which could sequester Wnt molecules far from the hem, thus increasing the slope of the medial$^{high}$-to-lateral$^{low}$ gradient of these ligands, little is known about the supposed patterning activity exerted by other antihem ligands onto the adjacent cortical field.

### 1.2.3 Transcription factor genes regulating areas identity

Beyond secreted ligands released by the edges of the cortical field, a key role in controlling cortical arealisation has been suggested to be played by transcription factor genes, gradually expressed in this field, such as *Emx2, Pax6, Emx1, Lhx2*, *Foxg1, Couptf1* (Mallamaci A, Stoykova A. 2006), (fig.1.7 panel B). Their protein products would confer different area identities to cortical cells within distinct parts of the field, due to their differential affinity towards the promoters of areally restricted genes and according to complex combinatorial laws. In particular, these transcription factors would allow for proper expression of axon guidance molecules that control the area specific targeting of thalamo-cortical afferents. I'll briefly describe the activity of some of these genes.

Concerning *Foxg1* and *Lhx2*, they are expressed in the cortical primordium along rostral/lateral$^{high}$-to-caudal/medial$^{low}$ and rostral/lateral$^{low}$-to-caudal/medial$^{high}$ gradients, respectively (Mallamaci A, Stoykova A. 2006), (fig.1.7 panel B).

Both of them keep the cortical field free of any ectopic activation of cortical hem genes; the former is required for morphogenesis of the basal ganglia (Xuan et al. 1995). It has been reported that *Lhx2* is necessary for proper development of the archicortex (Monuki et al. 2001); conversely, relevance of *Foxg1* to cortical arealisation has still to be assessed.

*Emx2* is expressed in the primary proliferative layer of the cortex along a rostral/lateral$^{low}$-to-caudal/medial$^{high}$ gradient (Gulisano et al. 1996) and *Pax6* along a complementary rostral/lateral$^{high}$-to-caudal/medial$^{low}$ gradient (Stoykova et al. 1994), (fig.1.7 panel B). As assessed by molecular profiling, in the absence of either *Emx2* or *Pax6*, the full repertoire of areal identities is still encoded. However, in *Emx2*$^{-/-}$ mutants, occipital cortex and hippocampus are shrunken and frontal cortex is enlarged. Moreover, the areal distribution of the thalamo-cortical radiation is perturbed, coherently wih such

areal disproportions A complementary phenotype arises in *Pax6*$^{Sey/Sey}$ mutants, lacking functional PAX6, but the thalamic afferences do not reach the cortex. (Mallamaci et al. 2000, Bishop et al. 2000).

There is a complementarity in the expression pattern of *Emx2* and *Pax6*, in fact *Emx2* is normally expressed less intensely in regions of the cortical PVE where *Pax6* products are more abundant and vice versa.

In the cortical PVE, a direct mutual inhibition between *Emx2* and *Pax6* could normally occur. In the absence of functional EMX2 protein, the expression of *Emx2* mRNA is down-regulated near the medial-caudal edge of its domain, where it is normally more intensely expressed (L. Muzio et al. 2002). The fact that this down-regulation is not widespread, but can be detected just near this edge, provides clues about possible mechanisms by which *Emx2* normally sustains its own expression, involving WNT signalling (L. Muzio et al. 2005). In the absence of functional PAX6 protein, the expression of *Pax6* mRNA is up-regulated near the medial-caudal edge of its expression domain, where it is normally less intensely expressed. In other words, down-regulation of *Pax6* by EMX2 requires PAX6, suggesting that these two proteins could provide this function as heterodimers (L. Muzio et al. 2002).

The expression of *Emx2* and the activation of the beta-catenin pathway, even if mutually sustaining, do not normally spread all over the cortex, but remain mainly confined to its medial wall. Crucial for that could be the *Pax6*-dependent confinement of *Emx2* to dorso-medial cortex (Mallamaci et al. 2000) as well as the *Axin2*-dependent, beta-catenin self-limiting loop (Jho et al. 2002).

Finally, the Chick Ovalbumin Upstream Transcription Factor I *Coup-Tf1*, expressed in the ventricular zone, subplate and cortical plate along a high caudal to low rostral graded expression across the neocortex (Liu et al. 2000), (fig.1.7 panel B) was also suggested to play a crucial role in forebrain arealisation and this prediction has been tested in mice

knock-out for this gene. In these mice, all of the areal markers under examination, except for *Emx2* and *Pax6*, were expressed uniformly and lost their areal restrictions. This suggests that *Coup-tf1* is crucial for arealisation and, within this process, it acts downstream of *Emx2* and *Pax6*, being necessary to make the cortical field responsive to their patterning activity (Zhou et al. 2001).

Even if with less thalamic fibres reach the cortex, the topography of thalamocortical connections of *Coup-tf1*$^{-/-}$ mutants was carefully studied. Authors find that the ventroposterior neurons in the thalamus could be retrogradely labelled from the caudal pole of the cortex, which is normally connected to the lateral geniculate nucleus. However, because *Coup-tf1* is not restricted to the cortex, but is also expressed by the thalamus, the significance of such mis-wiring for cortical arealisation is at the moment not clear at the present. (Zhou et al. 1999).

### 1.2.4 Role of *Emx2* in mouse cerebral cortex development

*Emx2* (orthologous to the gene *empty spiracles* of *Drosophila*) encodes for a homeobox TF gene, expressed within the PVE along a caudal-medial$^{high}$-to-rostral-lateral$^{low}$ and early$^{high}$-to-late$^{low}$ spatio-temporal gradient (A. Simeone et al. 1992), (fig.1.7 panel B). Its graded expression suggests that it can have a role in the process of arealisation and the phenotype of *Emx2*-/- mice confirms this hypothesis and indicates a role for *Emx2* in the lamination.

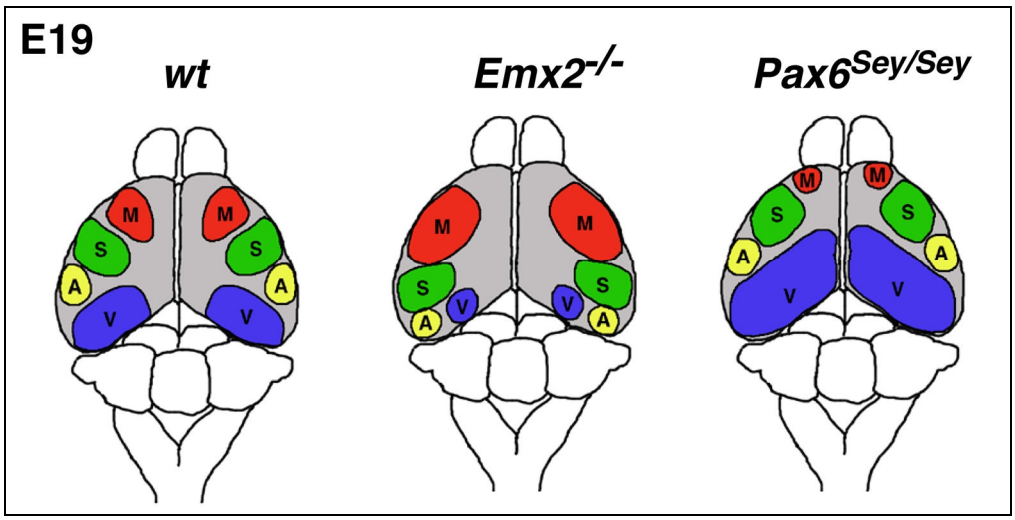


***Fig 1.8 The areal phenotype of Emx2 and Pax$^{sey/sey}$ null mutants at E19 with respect to the wild type mice (wt)***. *In Emx2-/- mice, the rostral-lateral areas are enlarged and caudally shifted and the caudal-medial areas are strongly reduced; the Pax$^{sey/sey}$ mice show a complementary phenotype, with the caudal-medial areas enlarged and rostrally shifted and the rastral-lateral areas strongly reduced.*

In Emx2-/- mice, rostral-lateral areas, where *Emx2* is poorly expressed (Mallamaci et al. 1998), were enlarged and caudally shifted (A. Mallamaci et al. 2000), while caudal-medial areas, where *Emx2* products are normally very abundant (Gullisano et al. 1998, Mallamaci et al. 1998) were strongly reduced (A. Mallamaci et al. 2000), indicating the involvement of *Emx2* in the caudal-medial area identity determination (fig.1.8).

Indeed, in the absence of *Emx2*, tangential expansion rates of the embryonic cerebral cortex PVE are reduced. The first reason is that cortical progenitors proliferate slower: in *Emx2*-/- mice, there is the elongation of TC (neuroblasts cycling time) due to lengthening of TS (DNA synthesis). Due to $T_C$ elongation, the proliferative pool of the mutant caudal-medial cortex "loses" one cell cycle out of four/five, with respect to its wild type counterpart; moreover, because of exaggerated neuronal differentiation, it is deprived of its components at even doubled rates. Moreover, the cortical progenitors divide more frequently along radial axes and exaggerately leave cell cycle.

Those kinetic changes are associated to increased proneural/antineural gene expression ratio, downregulation of lateral inhibition machinery and depression of canonical wnt signalling; by pharmacologically reactivating wnt signalling in *Emx2*$^{-/-}$ mutants, the neuronogenic rates are rescued. Wnt signalling and BMP signalling synergically promote *Emx2* transcription, through a beta-catenin/Smad1,4 binding module located within the *Emx2* telencephalic enhancer (Theil et al. 2002). In turn, *Emx2* up-regulates the final output of the canonical Wnt signalling machinery, thanks to concerted modulation of four distinct functional layers of it, ligands (*Wnt3a*, *Wnt8b*, *Wnt5a*, *Wnt2b*), surface receptors (*Fzd9*, *Fzd10*), intracellular beta-catenin agonists (*Lef1*) and intracellular beta-catenin antagonists (*Groucho*) (L. Muzio et al. 2005). In this way, near the cortical hem, a positive regulatory loop establishes between *Emx2* and Wnt signalling, crucial for proper sizing of occipital cortex and hippocampus.

All these phenomena are much more pronounced in caudal-medial than in rostral pallium, substantially contributing to selective hypoplasia of occipital cortex and hippocampus in the late gestation *Emx2* null embryos.

Moreover, radial migration of cortical plate neurons is specifically affected in *Emx2*-/- mice, similar to that observed in *reeler* mutant mice.

In *reeler* mice, early cortical plate neurons do not penetrate the preplate, which is not split in marginal zone and subplate and give rise to the so-called super-plate; moreover, late born cortical plate neurons do not overcome earlier ones, so that the classical inside-out rule is not followed. In *reeler* mutants, these migratory defects originate from constitutive functional ablation of the *Reln* gene, whose expression in the cortical marginal zone is necessary and sufficient to properly orchestrate neocortical neuron layering (D'Arcangelo et al. 1995).

In the *Emx2*-/- marginal zone, *Reln* mRNA expression is apparently normal at E11.5, it is reduced at E13.5 and completely absent since E15.5; in the same mutants, early phases of

cortical plate radial migration are poorly affected, late phases are impaired in a *reeler*-like way (A. Mallamaci et al. 2000). The *Emx2* mutant preplate is penetrated by cortical plate neurons, so that a subplate and a marginal zone are generated. However, late generated cortical plate neurons largely fail to overcome early born ones, so that the inside-out rule is hardly followed. In Emx2-/- mice, there is a striking temporal parallelism between *Reln* expression abnormalities and migratory abnormalities, which might be largely due to the specific, time-and-space-restricted, *Reln* knock-out occurring in these mutants, indicating that transient exposure to *Reln* signaling is sufficient to stable confer neurons fated to give rise to the subplate the property to be able to be subsequently "overcome" by cortical plate neurons, even at stages at which no more expression of *Reln* will be detectable. On the contrary, the reduction-absence of *Reln* products in the E13.5, E15.5 marginal zone would account for the inability of the majority of E13.5 born neurons to let E15 born ones to settle superficial to them. Late absence of *Reln* products in the marginal zone accounts for the abnormal, *reeler*-like neuronal packaging profile, as well as for late radial glia dysmorphologies, occurring to *Emx2-/-* mutants.

Takiguchi-Hayashy and collaborators (Takiguchi-Hayashy et al. 2004) demonstrated that the caudomedial telecenphalic wall and the cortical hem are the main source of neocortical Reelin-positive cells: these cells tangentially migrate beneath the pia mater, in an overall posterior-anterior direction, and finally distribute throughout the entire neocortex, along a caudomedial-high to rostrolateral-low cellular gradient. Embryos lacking *Emx2* display an impaired development of their cortical caudomedial region (A. Mallamaci et al. 2000). Thus, the Cajal-Retzius cells lacking in these mutants may be a subset of this regional phenotype, possibly arising from dramatic size-reduction of the caudal-medial proliferating pool that generated them.

Finally, *Emx2*, together with *Pax6*, plays a key role in telencephalic development as promoter of cortical versus non cortical morphogenetic programs. Indeed, if the two

homeobox genes *Emx2* and *Pax6*, normally expressed at high levels in the developing cortical primordium, are both inactivated, the telencephalic pallial neuroblasts are respecified as subpallial neuroblasts and this is followed by formation of an additional striatum-like structure in place of the cerebral cortex (L. Muzio et al. 2002).

## 1.3 Natural Antisense Transcripts (NATs)

### 1.3.1 Antisense transcription: structural and functional aspects

The role of antisense RNA in regulating gene expression is well established in prokaryotic systems. The first cellular antisense RNA, *micF*, was identified in E. coli in 1984 and was shown to repress translation of its target, *OmpF* mRNA, through base pairing with its 5'-end (Coleman et al., 1984). Since then other antisense RNAs have been described in bacteria and have been shown to repress (and in some cases activate) gene expression through RNA–RNA base pairing (Wagner and Flardh, 2002).

Recently, a large number of natural antisense transcripts (NATs) have been described in mice and humans. Antisense transcripts in eukaryotes are remarkably diverse. They can be distinguished by size, coding potential, and orientation with respect to complementary target sequences. The term "antisense regulation" generally refers to regulation of expression of an RNA target through direct base pairing with a complementary RNA. The most important distinction for purposes of classifying different antisense RNAs is that between *cis*-encoded and *trans*-encoded RNAs. *Trans*-antisense RNAs are those encoded at a locus separate from that of the target gene (for example the miRNA), while *cis*-antisense RNAs are those that are transcribed from two overlapping genes located on opposite strands of the DNA, in the same locus.

Although previous analysis of the mammalian transcriptome suggested that up to 20% of transcripts may contribute to sense-antisense (S/AS) pairs (Kiyosawa et al., 2003; Yelin et al., 2003; Chen et al., 2004), recent studies indicate that antisense transcription is more widespread (RIKEN Genome Exploration Research Group, Genome Science Group and the FANTOM Consortium 2005). Clustering the cDNAs into transcriptional units (TUs), in which members share sequence transcribed from the same strand, it was found that more than 72% of all genome-mapped TUs (43,553) overlap with some cDNA or ESTs (expressed sequence tag) mapped to the opposite strand.

Overlaps of cis S/AS pairs can target different portions of the corresponding TU, giving rise to three basic types of S/AS pairs: head-to-head or divergent, tail-to-tail or convergent, and fully overlapping. The divergent (head-to-head) classes are the most prevalent, the less numerous are the fully overlapping classes. As regards the coding potential, the S/AS pairs can be formed by two coding sequences, two noncoding sequences or one coding and one noncoding sequences; a very low number of the transcripts have exons overlapping with the antisense sequences.

The most frequent S/AS pairs are coding-non coding, while the less frequent ones are noncoding-noncoding. The relative expression of the two transcripts in the S/AS pair is concordant (co-expression) in the most of the cases, but it can be mutual or more complex. (RIKEN Genome Exploration Research Group, Genome Science Group and the FANTOM Consortium 2005).

A few epigenetic phenomena that involve noncoding antisense RNA were thoroughly studied and molecular mechanisms underlying them were disclosed to large extent. These phenomena are related to monoallelic expression and include X chromosome inactivation and genomic imprinting. X chromosome inactivation is a mechanism balancing expression of X chromosome-encoded genes in females. The silencing of one of the X chromosomes is mediated by a large noncoding RNA (*Xist*) that recruits a DNA- and histone-modifying protein complex (Clemson et al. 1996). An antisense transcript (*Tsix*) antagonizes the action of *Xist*; consequently, the X chromosome expressing *Tsix* remains active (Shibata and Lee, 2004). Imprinted genes are genes which are expressed or not, depending on their maternal or paternal origin. They are grouped in clusters and often display both DNA methylation and noncoding antisense transcripts. A mouse gene knockout study focusing on the paternally expressed *Air* antisense transcript has recently corroborated the importance of noncoding RNA expression in imprinting. *Air* overlaps the *Igf2r* gene in antisense direction and suppresses the paternal expression of *Igf2r*,

*Slc22a2*, and *Slc22a3*. Premature termination of the *Air* RNA resulted in both maternal and paternal expression of *Slc22a2*, *Slc22a3*, and *Igf2r* (Rougeulle and Heard, 2002; Sleutels et al., 2002). Further studies, however, indicated that parent-specific gene silencing did not require an RNA duplex formed between sense and antisense transcript (Sleutels et al., 2003).

However, little is still known about the function of natural antisense transcripts in the vast majority of cases. Several mechanisms can be taken into account, some hypothetical, some others corroborated by robust experimental evedences.

Antisense transcription may affect sense gene transcription, by altering the local state of chromatin. This may consist in a simple change of the DNA methylation profile. For example, the two *Sphk1*/*Khps1* RNAs overlap in the 5'-region in a head-to-head arrangement including the CpG island of the *Sphk1* promoter. Overexpression of the antisense transcript in the rat kidney-derived cell line NRK resulted in the demethylation of the CpG island (Imamura, 2004). Alternatively, the histone state may be changed, as supposed to happen in lymphocytes, at the level of immunoglobulins and T-cell receptor loci. Here extensive antisense transcription occurs before and during recombination and is believed to function by inducing an open chromatin structure that is accessible to recombination.

Antisense transcription might inhibit initiation of sense transcription because of competion between close, oppositely oriented promoters for the same trans-activators (divergent NATs).

Antisense transcription may also inhibit sense RNA elongation, because of collision among the two polymerase complexes or exaggerated supercoiling of the interposed DNA (convergent NATs). This was demonstrated in modified yeast strains, harboring the genes *GAL10* and *GAL7* made artificially convergent (Prescott and Proudfoot, 2002).

The antisense transcript may affect post-transcriptional maturation of the primary sense transcript. First, splicing may be affected. The best-characterized example is that of the α-thyroid receptor gene *TR-α*, encoding for two splice forms, TR-α1 and TR-α2, the latter lacking hormone-binding capacity and acting as a dominant repressor of the former. The antisense transcript *Rev-ErbAα* influences the processing of the *TR-α hnRNA* toward formation of the α1-isoform in a process that requires the overlap of *Rev-ErbAα* and *TR-α2* (Hastings et al., 2000). Alternatively, pairing between sense and antisense transcript may lead to post-transcriptional silencing, dependent on RNA editing. RNA editing includes the adenosine-to-inosine conversion by an enzyme family called "adenosine deaminase acting on RNA" (ADAR), acting on RNA duplexes. In some cases, hyper-editing of long, perfect RNA duplexes can result in their nuclear retention (Zhang and Carmichael, 2001) or cytoplasmic degradation (Scadden and Smith, 2001).

Finally, the antisense transcript may affect the half-life of the mature sense transcript. That may happen via activation of RNA interference (RNAi) by the resulting dsRNA. This process involves a protein complex that recognizes double-stranded RNA (Dicer) and cleaves the RNA duplex into small oligonucleotides of 21–23 base pairs (siRNAs). The strands are separated and become part of the RNA-induced silencing complex (RISC). RISC eventually degrades the cognate target mRNA in a sequence-specific fashion (Meister and Tuschl, 2004).

### 1.3.2 Transcripts in an antisense orientation with respect to transcription factors: *Emx2OS*

Several mouse natural antisense transcripts have been found to be associated with transcription factors involved in the nervous system development; in particular eight novel mouse NATs have been recently identified, associated with transcription factors (*Pax6, Pax2, Six3, Six6, Otx2, Crx, Rax* and *Vax2* ) that play an important role in eye

development and function (Alfano et al., 2005). In particular, the murine *CrxOS* harbors an ORF of 738 bp that is predicted to encode a protein of 246 amino acids, including two putative homeodomains. The overexpression of *CrxOS* in mouse adult retina using adeno-associated viral vectors caused a significant decrease in the expression levels of the corresponding sense gene, *Crx* (Alfano et al., 2005). *Pax6* has an associated antisense transcript too, *Pax6OS*, transcribed along a divergent orientation (head-to-head). *Pax6OS* is expressed in the adult mouse in the retina (with 6 alternative splicing forms) but not in the brain. However, *Pax6OS* is expressed in the early forebrain and spinal cord, at around E9.5, disappearing from the telencephalon since E10.5 (Anderson et al., 2002).

Recently a transcript encoded by the *Emx2* opposite strand, was found, in mouse and human, overlapping *Emx2* head-to-head. This transcript was called *Emx2OS* (Noonan et al., 2003) (fig.1.9).

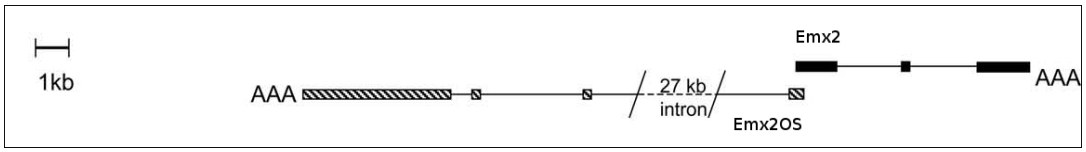


***Fig. 1.9 Genomic organization and relationship of sense and antisense genes at the EMX2 locus in mouse****. Emx2 and Emx2OS overlap head-to-head in a divergent orientation. Emx2OS has four exons and three introns and it is polyadenylated.*

The human *Emx2OS* is expressed in adult uterus, kidney and brain and its length is 8,5 Kb. The murine *Emx2OS* was identified from the adult uterus and kidney and its length is 5035 nucleotides, it has four exons and three introns and it is polyadenylated. Both of them show splicing variants. The nucleotide sequences of the human and murine *Emx2* opposite-strand sequences show no detectable homology other than those regions that overlap the *Emx2* transcripts. Several open reading frames of 450 nucleotides or less are present in the human and murine antisense transcripts. However, none of the predicted

peptides are conserved between the two species nor do they share similarity to sequences in GenBank.

In human the expression of *Emx2* and *Emx2OS* resulted concordant: they are both highly expressed in normal postmenopausal endometrium, both reduced in a subset of endometrial tumors and both not expressed in four of six endometrial cancer cell lines investigated. The murine *Emx2* and *Emx2OS* transcripts revealed endometrial cellular expression patterns identical to each other and with the human orthologs.

Main aim of my work was reconstructing the *Emx2OS* expression pattern in the developing murine cerebral cortex and casting light on the biological function of it.

# Chapter II

# MATERIALS & METHODS

## 2.1 Reagents and standard procedures

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

DH5α and XL1Blue strains. Restriction and modification enzymes were obtained from Roche and Promega, and used according to manufactures outlines. DNA fragments were purified from agarose gel by the Quiaquick DNA purification system (Quiagen). Small-scale plasmid preparations (mini-preps) from transformants were made by the alkaline lysis method as described by Maniatis et al. 1989. Large scale and midi-scale preparations (maxi and midi-preps) were done by purification on Quiagen columns (Quiagen).

## 2.2 *In situ* hybridization protocol

### 2.2.1 Preparation of OCT embed embryos

Embryos were dissected in cold PBS and fixed in 4% paraformaldehyde/PBS overnight at +4°C and then washed in PBS. They were equilibrated in PBS/Sucrose 30% solution and then included in Killik cryostat embedding medium and frozen on dry ice. Embryos were cut at 10µm using a Microm HM505N cryostat, and sections were mounted on Menzel-Glaser SuperFrost Plus slides. Slides were stored at –80 °C.

### 2.2.2 Preparation of single-stranded RNA probes

Single stranded RNA probes were synthetized by the in vitro transcription of sequences cloned into pGEM vectors which contains specific RNA polymerase promoter sites. By choosing the appropriate direction of transcription, either antisense or sense strand RNA

were synthetized. Plasmids for use in transcription reactions were prepared as described in 2.1. They were linearized, treated with phenol/chloroform and finally precipitated. Linearized plasmids were resuspended in distilled water at a concentration of 1µg/µl.

Preparation of $^{33}$P radiolabelled probe:

We dryed in SpeedVAc 12.5 µl of $\alpha^{33}$P UTP (Amersham) and we added: 1µg of linearized DNA; transcription buffer 5X: 4µl; DTT 0,1M: 2µl; rNTPs (rATP, rCTP, rGTP, 3,3 mM each): 3µl; RNA in 40U/µl: 1µl; RNA polymerase 20U/µl: 1µl; and H2O to 20µl. This reaction was incubated at room temperature overnight. The following day was added 1µl of RNA polymerase for 1 hour at 37°C. Finally we added 1µl of RNAse free DNAse 40U/µl at 37°C for 30 minutes. The reaction was stopped by adding 1µl of 0.5M EDTA and purificated on Mini Quick Spin RNA column (Roche) for 2 minutes at 1000g. We analyzed the transcription by counting at the beta counter the count per minute using 1µl of reaction in 3 ml of scintillation solution. Probes were stored at –80°C.

Preparation of Dig-labelled probe:

According to the manifacturers' instructions, we mixed the following reagents on ice in a eppendorf tube: linearized DNA: 1µg; transcription buffer 5X: 4µl; DTT 0,1M: 2µl; Dig labelling mix 10X (Roche): 2µl; RNAin 40U/µl: 1µl; RNA polymerase 20U/µl: 1µl; and H2O to 20µl. The reaction was incubated at room temperature overnight. The following day RNA transcripts were precipitated and resuspended in 30 µl of sterile water, 1 µl of RNA probe was run on a agarose gel for the quantification. Probes were stored at –80°C.

### 2.2.3 Pre-treatment of sections

In order to improve signal and reduce the background, sections were subjected to several pre-treatment steps before the hybridization probe was applied. The following protocol is adapted for the cryostat-cut sections.

Slides were left to dry for at leats 30 minutes. After this step slides were immersed in 4% paraformaldeyde in PBS for 10 minutes and then washed with PBS, twice for 5 minutes. Slides were immersed in HCl 0.2 M for 5 minutes. We washed with PBS, three times for 2 minutes, and incubated in 0.5 μg/ml of proteinase-K (Roche) in 50 mM Tris-Hcl pH 8, 5mM EDTA, at 30°C for 10 minutes. The proteinase K reaction was stopped by washing slides in Glycine 4 mg/ml in PBS for 5 minutes, twice. Slides were washed with PBS for 5 minutes, twice. Slides were immersed in 4% paraformaldeyde in PBS for 10 minutes. Slides were washed, for 4 minutes, twice. We quickly washed the slides in distilled water and finally slides were placed in container with 0.1 M Triethanolamine-HCl pH 8 set up with rotating stir bar for 5 minutes. We added 0.4 ml of acetic anhydride twice for 5 minutes. Slides were washed in sterile water twice for 2 minutes. We left the slides to dry and used them on the same day for hybridization.

### 2.2.4 Hybridization and washing of sections

The hybridization mix (Denhardts Salts 1X, DTT 50mM, Polyadenylic acid 500 μg/ml, Ribonucleic acid transfer 53,5 μg/ml, Dextran sulfate 10 %, Formammide 50%) including probe was heated at 80°C for 13 minutes and applied to the slides. Clean coverslips increased spread the hybridization mix over the sections. Slides were placed horizontally in a sealed plastic slide box, together with paper soaked in 50% formamide, 5X SSC and incubated overnight at 60°C. The following day, slides were removed and placed in a slide rack in a solution of 5X SSC, 0.15% β-mercaptoethanol at room temperature for 30 minutes, in order to remove the coverslips. Slides were incubated in stringent buffer (50%

formamide, 2X SSC, 0.15% β-mercaptoethanol) at 60°C for 30 minutes. They were then washed with NTE buffer (0.5M NaCl; 10 mM Tris-HCl pH 8; 5 mM EDTA) two times for 15 minutes each, and finally treated with ribonuclease-A (Roche) 20 μg/ml in NTE buffer for 30 minutes at 37°C. Slides were then washed with NTE for 15 minutes, and, after this step, we repeated the stringent buffer wash at 60°C for again 30 minutes. Slides were incubated with 2X SSC for 15 minutes, then 0.2X SSC for 15 minutes.

### 2.2.5 $^{33}$P radiolabelled probe *in situ* hybridization protocol

Dipping of sections:

Sections were dehydrated by quickly putting them through 30%; 60%; 80%; and 95% ethanol, all including 0.3M Ammonium Acetate, followed by 100% ethanol twice. Sections were left to dry and then for the autoraradiography. The autoradiography emulsion LM-1 (Amersham) was used for the detection of these radiolabelled slides. The emulsion was melted at 42°C and it was poured into a dipping chamber (Amersham), and the sections were dipped vertically into the emulsion and were stored in light-tight boxes at +4°C for 3-5 days.

Processing the emulsion:

Slides were removed from storage and left to equilibrate to room temperature in the light-tight boxes. Developer, stop and fix solutions were prepared fresh each time. Under darkroom conditions, slides were exposed in the following solutions: developer (D19 Sigma) for 5 minutes, stop solution (5% Acetic Acid) for 30 seconds, and finally fix solution (Sigma) for 25 minutes. After these treatments, slides were washed in water for 15 minutes, counter-stained with Hoecsht, and deidrated using the ethanol series. Coverslips was mounted using DPX (BDH) mounting solution.

### 2.2.6 Dig *in situ* hybridization protocol

Slides were pre-treated as described in 2.2.3 and washed as described in 2.2.4. From this point, slides were incubated in B1 solution (0.1 M Tris-HCl pH 7.4; NaCl 0.15 M) for 5 minutes. Sections were blocked in B1 solution containing 10% of Heat inactivated fetal bovin serum (FBS-Gibco) for 1 hour at room temperature. Slides were next incubated in B1 containing 0.5% FBS and the anti-Dig-AP (Roche) at the concentration of 1:2000, overnight at +4°C. The following day sections were washed three times in B1 solution before incubation into B3 buffer (0.1 M Tris-HCl pH8; 0.1 M NaCl; 50 mM MgCl2) containing 3.5µl of NBT and 3.5µl BCIP (Roche) for each ml. The development of these sections was followed using the microscope.

### 2.2.7 Probes used in *in situ* hybridization

The following probes were used: *Reln* (probe corresponding to nucleotides 5818-5973 of the Genbank file NM_011261), used in a Dig *in situ* hybridization protocol; *Emx2OS* (probe corresponding to nucleotides 3073-3866 of the Genbank file NR_002863), used in a Dig *in situ* hybridization protocol; *OS3'* (probe corresponding to nucleotides 124-444 of the Genbank file BF_470610), used in a 33P radiolabelled probe in situ hybridization protocol.

### 2.2.8. Microphotography and editing

Microphotographs of the *in situ* hybridized tissues were taken by a Nikon Eclipse 600 microscope equipped with an SV Micro CV3000 digital microscope camera (Taunton). Electronic files were processed on a MacIntosh G3 computer by Adobe Photoshop 6.0 software.

### 2.3 Quantification of *Emx2OS* and *BF470610* transcripts on dissected mouse embryos cerebral cortex

Cortices from 12 embryos of wt genotype were dissected at E12.5, E14.5, E18.5. RNA was extracted by RNAeasy kit (QIAGEN) and cDNA was generated by SuperScriptII (Gibco), in the presence of gene-specific oligos Emx2OS/RT1 (5' CTG CCA AGG ATG CTT GAG GTC C 3') or Emx2OS/RT2 (5' GAG AGA TTG ACT GCA TC 3') and H3A/RT (5' CAC TTG CCT CCT GCA AAG CAC C 3'). Real-time PCR was performed by Platinum SYBR Green qPCR UDG SuperMix (Invitrogen) on a Lightcycler (Roche), according to manifacturers' instructions. The following primers were used: Emx2OS/P1 (5' CCC GCG CCC GGG TCA CTG AGA TGG CTT CG 3' and Emx2OS/P3 (5' GAT GAG CAG GTG AGT GGT AGA TGG TTG TAA GCT GTAC 3') to amplify Emx2OS; Emx2OS/P4 (5' AAG TCT ATG GTC TTG GAA GAG AAA CTC AGT GCT TG 3') and Emx2OS/P5 (5' CTC ATC ATA ATG GCC ATT CAG AAC TTT TAA GTC TGT 3') to amplify BF470610; H3A/P1 (5' GTG AAG AAA CCT CAT CGT TAC AGG CCT GGT AC 3') and H3A/P2 (5' CTG CAA AGC ACC AAT AGC TGC ACT CTG GAA GC 3') to amplify Histone H3A for normalization.

### 2.4 Electroporation of E14.4 wt mouse embryos cerebral cortex

First, I prepared the glass micro-capillaries to inject the DNA. Then, I prepared the injection solution: for each capillary I used 0.25 µl of fast green 0,1%, 0.5 µl of *NLS-LacZ* 2 µg/µl, 0.25 µl of water (DNA solution). I anesthetize the pregnant mouse, 14 day after the fertilization, using a saline solution with ketamine and xylazine, by peritoneal injection. I cut the skin, the peritoneum and the endometrium. I chose an embryo and used the capillary to inject the DNA solution in the lateral ventricle. I positioned the electrodes near the lateral cortex of both the hemispheres, with the positive electrode next

to the injected hemisphere; the electroporation program was 3 pulses of 20V for 100ms with an interval of 450ms.

Then, I positioned the embryo in the abdominal cavity and I sew the peritoneum and the skin. The day after I sacrificed the pregnant mouse and I dissected the brain of the embryo, I fixed it in 4% paraformaldehyde/PBS 20 min. at +4°C, washed it in PBS and processed it for β-gal reaction according to standard methods.

If the reaction produced a bleu colored lateral cortex, the cortex was electroporated.

# Chapter III

# RESULTS

### 3.1 Search and quantification of transcripts in antisense orientation with respect to *Emx2* in mouse cerebral cortex

I used the software AntiHunter 2.0 to find ESTs in an antisense orientation with respect to *Emx2*. The input was the *Emx2* contig, the output was a group of ESTs. Most of them mapped on the *Emx2OS* gene, but one, *BF470610*: the former ones overlapped head-to-head (5'-5'), the latter tail-to-tail (3'-3').

I designed a set of primers for RT-PCR and real time RT-PCR and measured the levels of *Emx2*, *Emx2OS* and *BF470610* in the mouse embryo neocortex at different developmental stages (E12.5, E14.5, E18.5). In agreement with previous literature, I found that, during neocortical development, *Emx2* expression decreases from top level shown at E12.5 to 33% and 22% of this, at E14.5 and E18.5, respectively. *BF470610* expression was very low, less than *Emx2* one, and it progressively decreased during neocortical development, to the 27% (at E14.5) and to the 18% (at E18.5) of the *BF470610* level detected at E12.5. *Emx2OS* expression was on average very high, apparently higher than the *Emx2* one, and it varied during neocortical development, equalling 46% and 108% of the *Emx2OS* level detected at E12.5, at E14.5 and E18.5, respectively. So, during mouse neocortical development, the *Emx2OS* expression displays a complex temporal pattern, if compared with the *Emx2* one.

### 3.2 *Emx2OS* expression pattern in the E12.5, E14.5, E18.5 and adult mouse telencephalon

I designed and cloned two in-situ hybridization probes, one for *Emx2OS*, one for *BF470610.* I labeled the *Emx2OS* probe (794 bp) by digoxygenin (dig) and the *BF470610* probe (321 bp) by $^{33}P$, allowing a more sensitive signal detection, because of the low expression of *BF470610* and the shortness of its probe.

*BF470610* was undetectable at all in mouse embryo telencephalon, even by radioactive in-situ hybridization (data not shown).

*Emx2OS*, conversely, could be detected in the mouse embryonic brain, by dig in-situ hybridization (fig. 3.1 A, B, C), but was not detectable in the adult telencephalon (fig. 3.1 D). At E12.5 *Emx2OS* shows a wide expression in almost all the telencephalon (fig. 3.1 A), at E14.5 and at E18.5 it is prevalently expressed by pallial regions (fig.3.1 B, C). As for the E12.5 subpallium, *Emx2OS* is restricted to the deepest part of the LGE (lateral ganglionic eminence) and does not display any radial restriction within the MGE (medial ganglionic eminence) (fig. 3.2 A, B).

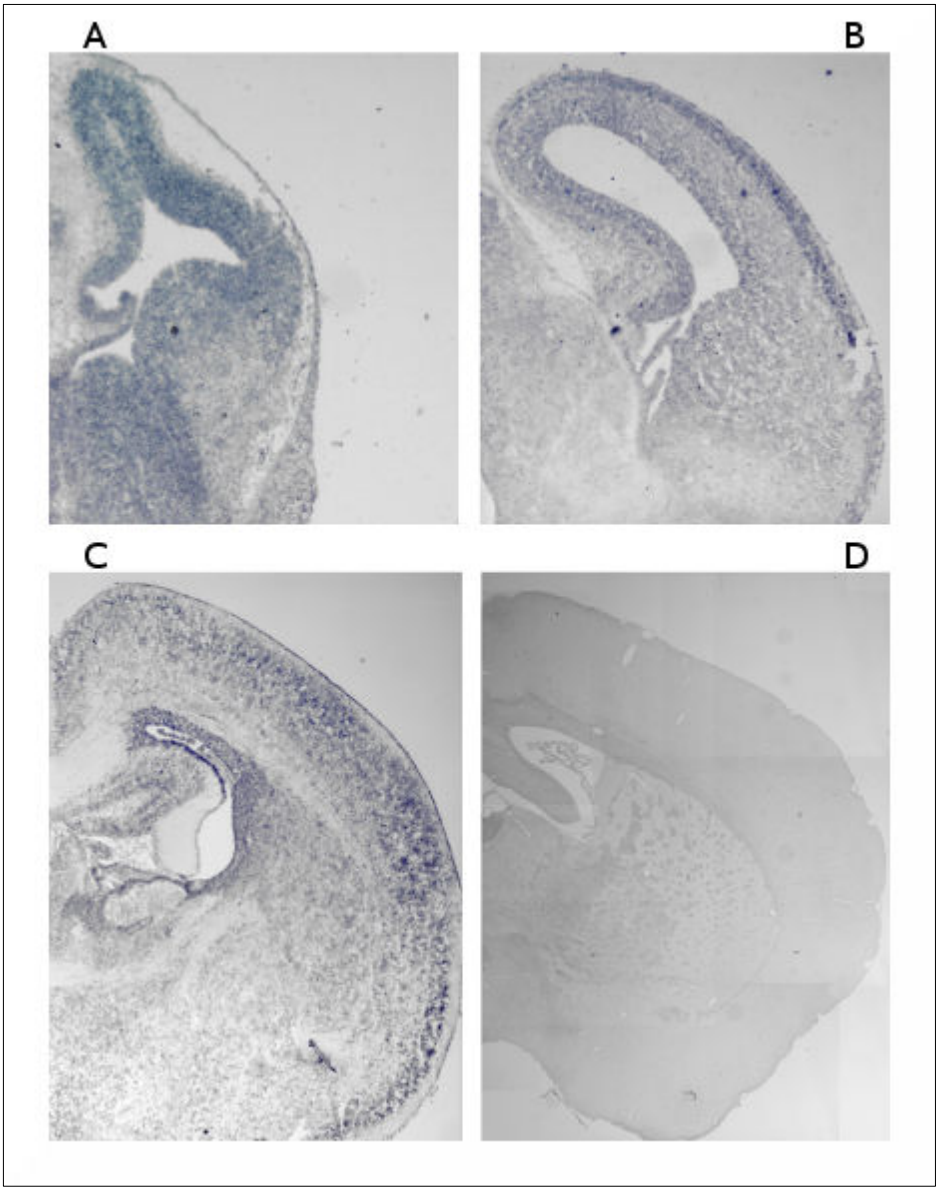


***Fig. 3.1 Emx2OS expression in the developing mouse telencephalon.*** *In-situ hybridization with a specific anti-Emx2OS Dig-labelled probe on mid- frontal sections of E12.5 (A), E14.5 (B), E18.5 (C) and adult (D) mouse telencephalons. Emx2OS is highly expressed in the cortical field at all the analyzed embrionic stages (A, B, C) but its expression is not detectable in the adult mouse cortex (D).*

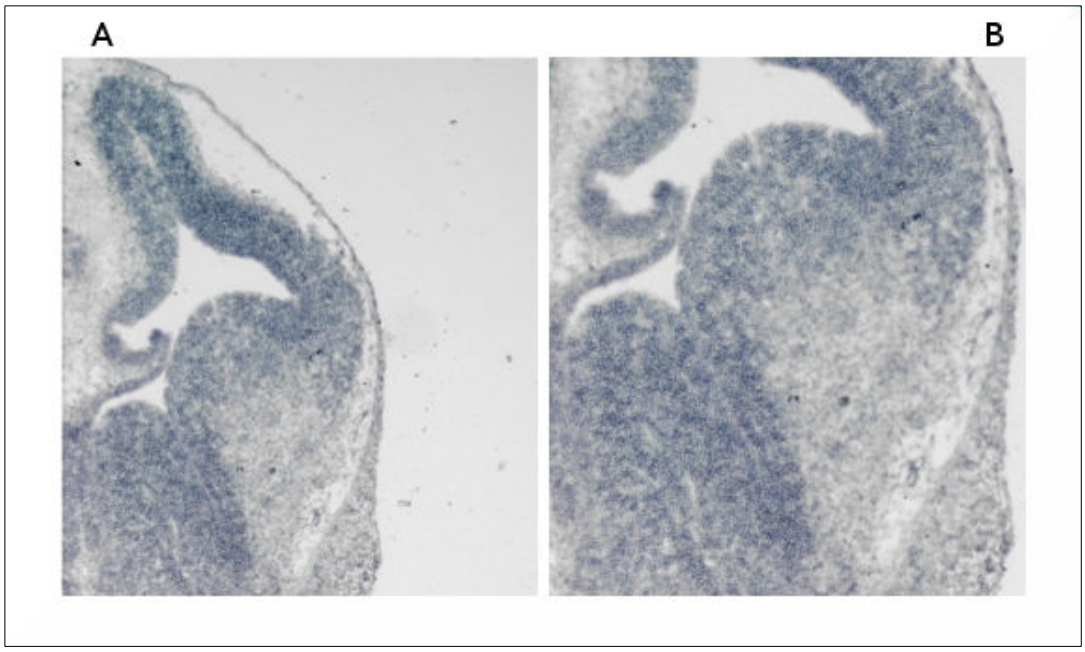


***Fig. 3.2 Radial restriction of Emx2OS RNA to ventricular layers of LGE.*** *In-situ hybridization with a specific anti-Emx2OS Dig-labelled probe on frontal sections of E12.5 (A) and, in particular, the magnification the ganglionic eminences (B) shows that the expression of Emx2OS is strong in the entire MGE (medial ganglionic eminence), but it is restricted in the LGE (lateral ganglionic eminence) to the deepest part of it.*

### 3.3 *Emx2OS* radial expression profile in the E12.5, E14.5, E18.5 and adult mouse neocortex

Within the embryonic cortical field, *Emx2OS* RNA shows a complex expression profile (fig. 3.3 A, B, C), while it is undetectable in the adult cerebral cortex (fig. 3.3 D). At E12.5 it is expressed in proliferating progenitors of the ventricular zone, where *Emx2* is expressed too (fig 3.3 A). At E14.5 it is strongly expressed in the ventricular zone and at a lower level in the subventricular zone. As for post-mitotic layers, it may be found in the subplate and in the cortical plate (where *Emx2* mRNA is absent) but is not detectable in the marginal zone (where *Emx2* mRNA is present) (fig 3.3 B). At E18.5, *Emx2OS* is still expressed in periventricular layers, in the subplate and in the cortical plate, and it is again not detectable in the marginal zone (fig 3.3 C). Interestingly, at both E14.5 and E18.5, *Emx2OS* shows the strongest expression in the most marginal part of the cortical plate, (fig 3.3 B and C). Now, because later born neurons leaving proliferative layers overcome earlier born ones and settle above them, cells populating this region at distinct embryonic ages are different neurons, caught at around the end of their radial migration. This means that strong *Emx2OS* expression is not peculiar to specific cortical laminae, but is rather a hallmark of a defined step of the general migration-differentiation process undergone by cortico-cerebral neurons.

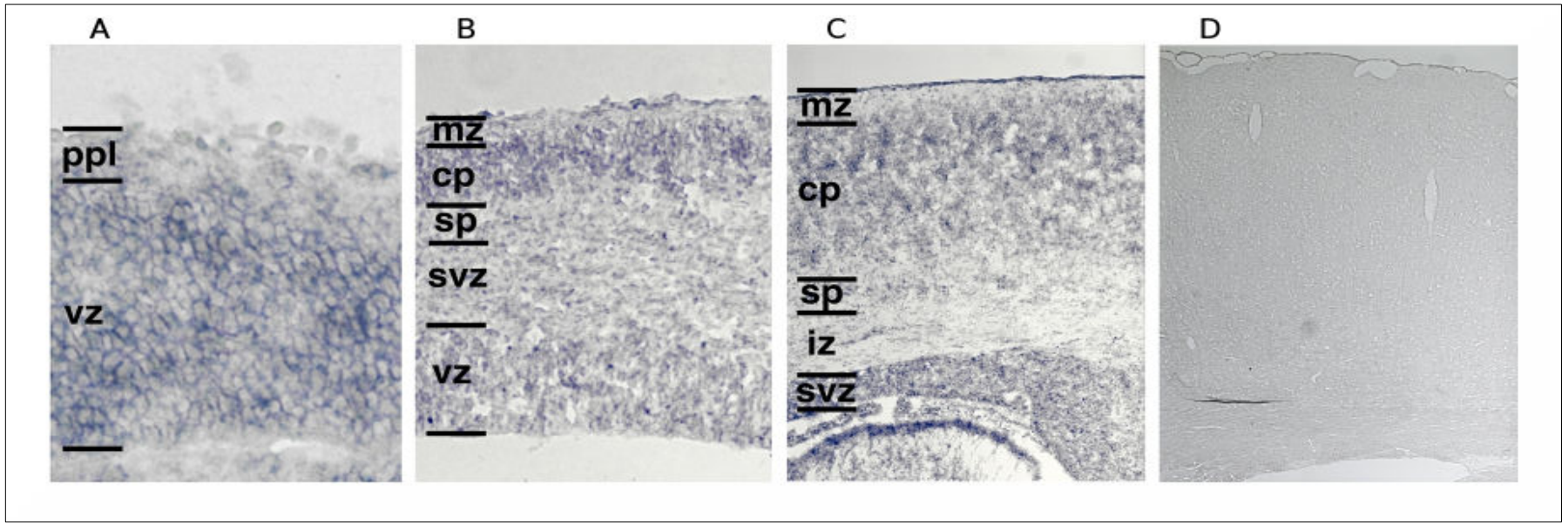


***Fig. 3.3 Emx2OS expression on frontal sections of mouse cerebral neocortex.*** *Emx2OS is highly expressed in the proliferating progenitors of the ventricular zone at E12.5 (A); it is expressed in the ventricular and subventricular zone, in the subplate and in the cortical plate – in particular in the youngest marginal neurons – at E14.5 (B) and E18.5 (C); it is not detectable in the adult neocortex.*

### 3.4 *Emx2OS* is not expressed in Cajal-Retzius cells

Cajal-Retzius cells are *reelin*-secreting neurons (Frotscher 1997), orchestrating neocortical and hippocampal lamination (D'Arcangelo et al., 1995; D'Arcangelo and Curran, 1998; Mallamaci et al., 2000; De Rouvroit et al., 2001) and guiding proper formation of cortico-hippocampal connections (Borrell et al., 1999). *Reelin*-secreting Cajal-Retzius cells of the developing mouse cerebral cortex are in marginal zones of neocortex (layer I) and archicortex (hippocampal stratum lacunosum-moleculare) (Alcantara et al., 1998), (fig 3.4 B, D and F). They express the EMX2 protein (Mallamaci et al., 1998) and require it for their proper development. In fact, late gestation *Emx2*$^{-/-}$ embryos lack *Reelin* mRNA in their MZ and display severe impairment of neuronal radial migration (A. Mallamaci et al. 2000).

Remarkably, Cajal-Retzius cells do not express *Emx2OS*, as shown by adjacent E18.5 telencephalic frontal sections hybridized with probes specific for *reelin* (fig 3.4 B, D, F) and *Emx2OS* (fig. 3.4 A, C, E), respectively. Here, despite of wide expression of *Emx2OS* in both neocortical and hippocampal fields (fig. 3.4 C, D E, F), no *Emx2OS* signal may be found in the reelin-expressing neo-cortical layer I and hippocampal stratum lacunosum-moleculare.

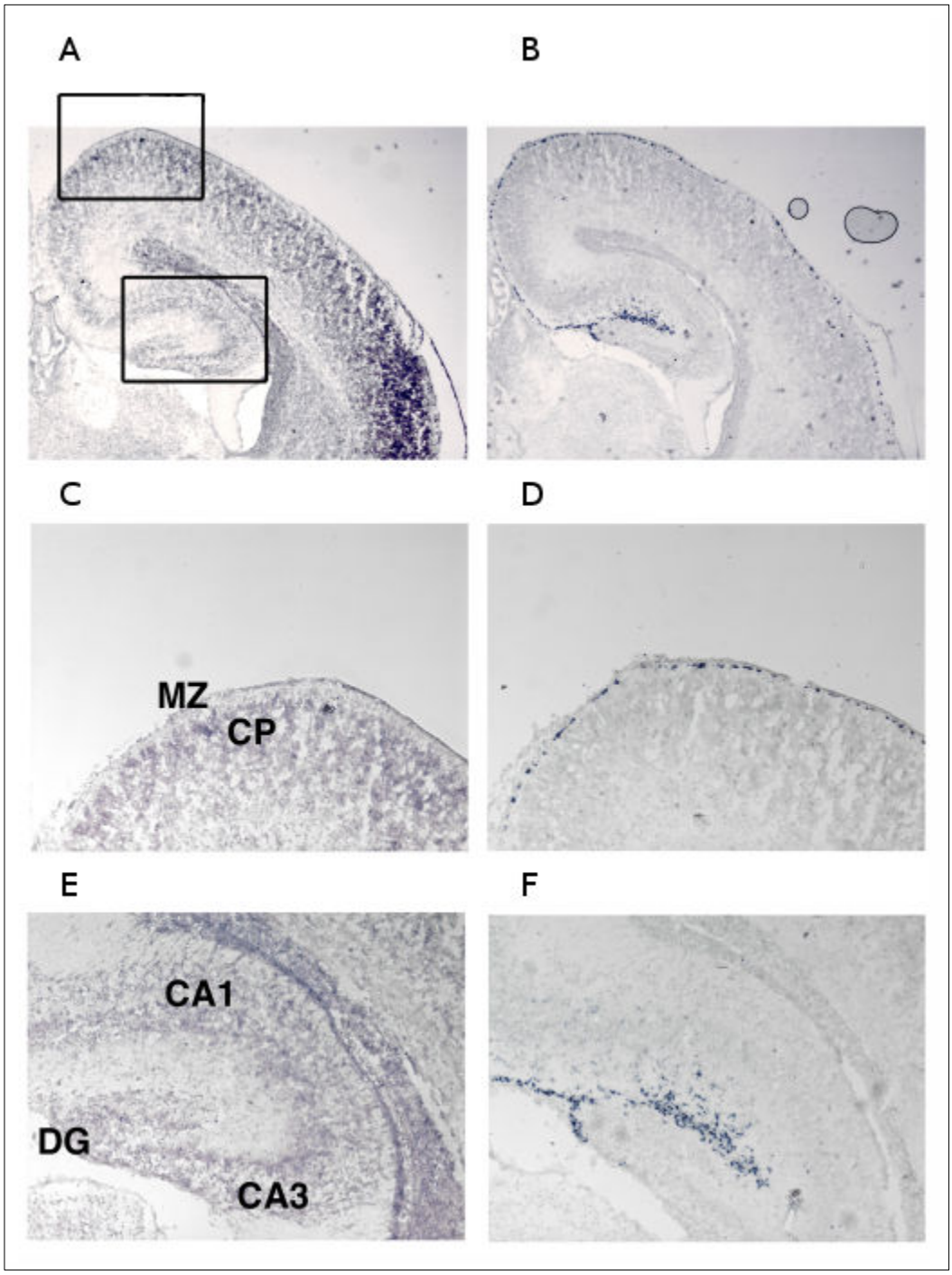


***Fig. 3.4 Undetectable expression of Emx2OS in the Cajal-Retzius cells of the marginal zone and hippocampus.*** *In-situ hybridization with a specific anti-Emx2OS Dig-labelled probe (A, C, E) or with a specific anti-Reelin Dig-labelled probe (B, D, F) on frontal sections of mouse telencephalon at E18.5 (A, B). The magnification of the cortical field (C, D) and of the ippocampal region (E, F) shows that the Cajal-Retzius cells, marked by Reelin mRNA, do not express Emx2OS at detectable levels.*

### 3.5 Investigating *Emx2OS* function

As for biological meaning of *EmxOS* RNA expression, three main classes of hypotheses may be conceived. First, *EmxOS* RNA might act as a classical mRNA, i.e. via bioactive peptides encoded by itself. Second, it might act as such, possibly influencing expression of other genes, lying within its locus or elsewhere in the genome. Third, it might play no role per se, being rather the transcription of its gene crucial to the proper working of the locus it belongs to.

Full empirical assay of these three hypotheses would obviously be a very tough task. Given my time constraints, I focussed my first efforts: (1) to assay the first hypothesis; (2) to run preliminary tests aimed at disclosing the occurrence of a possible mutual inhibition between *Emx2* and *Emx2OS*, as suggested by the mutual spatial exclusion occurring in vivo between these two transcripts.

To understand if *Emx2OS* may act as an mRNA, I analysed the human and the mouse *Emx2OS* sequences, looking for any conserved open reading frames (ORFs).

By using the Translate tool, on the ExPASy proteomic server, and selecting the ORFs longer than 240bp, I found four ORFs in the mouse *Emx2OS* sequence (240, 264, 269 and 354 nucleotides) and four ORFs in the human *Emx2OS* sequence (276, 326, 357 and 459 nucleotides). However none of the four mouse *Emx2OS* ORFs showed homology with the human *Emx2OS* ORFs (as assayed by Blast2seq software), neither at nucleotide sequence level (program: blastn, default parameters) nor at presumptive aminoacid sequence level (program: blastp, default parameters). Moreover, by using the the Prosite tool on the ExPASy proteomic server, I also searched for simpler conserved protein domains or functional sites in the human and murine *Emx2OS* ORFs, but I only found three low complexity regions in three of the eight analysed sequences.

In the absence of any biologically plausible and/or phylogenetically conserved *Emx2OS* RNA open reading frames, I looked for regulatory interactions possibly occurring

between the *Emx2OS* transcript and the *Emx2* mRNA. For this purpose, I performed two preliminary sets of experiments.

First, I used the *Pax6*$^{Sey/Sey}$ mutants, lacking *Pax6*-dependent inhibition of *Emx2* in the rostro-lateral pallium and already available to me, as a chip (but imperfect) model of *Emx2* overexpression. I measured *Emx2* and *Emx2OS* transcript levels in total RNA extracted from their cerebral cortices at E14.5, via real-time RT-PCR. As expected (Muzio et al. 2000a), *Emx2* resulted upregulated in Pax6$^{Sey/Sey}$ mutants by 25% as compared to wild type controls. Interestingly, *Emx2OS* was upregulated in Pax6$^{Sey/Sey}$ mutants too, by about 35%. All that suggested that no relevant inhibition of *Emx2OS* expression by *Emx2* takes normally place.

Second, I analysed (by GeneBee software) the whole *Emx2OS* gene sequence, to find modules encoding for putative RNA stem & loops, possibly displaying sequence similarity with known miRNAs (listed in the miRBase Sequence Database, at the Sanger Institute website) and able to target in silico the *Emx2* transcript. I found a piece of *Emx2OS* intronic sequence (uacagggaaauuguaacggggu), embedded within a pre-miR-like palindromic module, similar to miR-130a (uacgggaaaauuguaacgugac) and differing from miR-130a by 5 mismatches outside the seed region. By using miRBase Targets (at the Sanger Institute website), I found that miR-130a has a presumptive target within the 3'UTR region of the murine *Emx2* gene. Remarkably, *Emx2OS* miR-130a-like anneals in silico on the same target as well, even if more loosely (a U-A pair lost and two G-T imperfect pairs in place of canonical Watson & Crick A-T pairs) (Fig. 3.5).

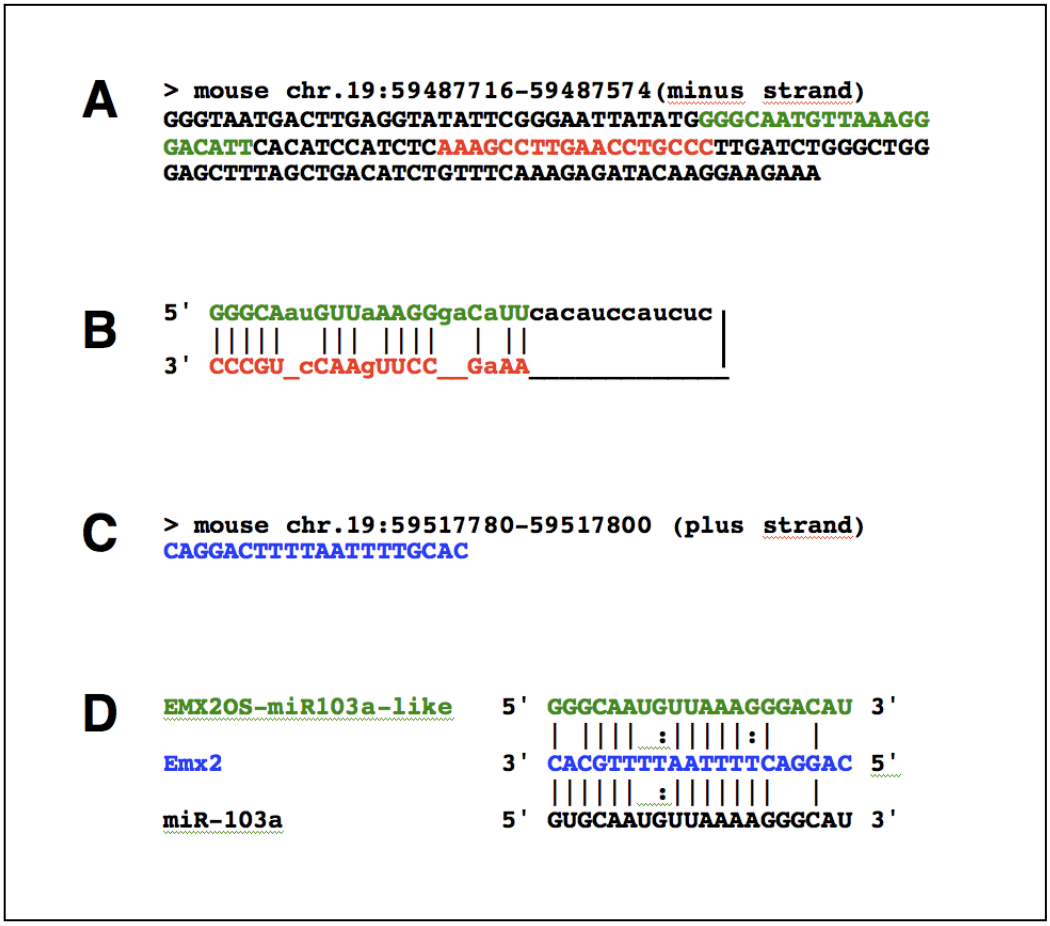


***Fig. 3.5. A presumptive miR directed against Emx2 mRNA is encoded by the first intron of Emx2OS.*** *(A) Region of mouse chromosome 19 within the first intron of Emx2OS, conserved among Homo sapiens, Mus musculus, Rattus norvegicus, Danio rerio and Xenopus tropicalis; (B) module presumptively encoding for a pre-miR-like stem-and-loop structure, embedded within the former; (C) putative target of the resulting Emx2OS miR-130a-like product, lying within Emx2 mRNA 3'UTR; (D) in silico pairing of canonical miR-130a and Emx2OS miR-130a-like product with this target.*

To assess if such *miR-130a*-like product is really generated in vivo, I designed a LNA probe against the mir130a-like *Emx2OS* sequence and I performed an in-situ hybridisation on brain slices of mouse embryos at different stages. However, I did not find any signals (data not shown). This means that no *Emx2OS*-derived mir130a-like

short RNA is present in the embryonic mouse brain or that this product falls under the detectability threeshold of our technique.

Upon these preliminary functional investigations, I decided to systematically assay for the occurrence of any regulation of *Emx2* by the *Emx2OS* transcript as such (in trans), via in utero somatic electroporation of dedicated gain- and loss-of-funtion constructs.

For this purpose, I trained myself in electroporation, using a a basic reporter construct, able to drive strong constitutive expression of the *NLS-LacZ* reporter gene. I used pregnant females, 14 days after fertilization. After anaesthesizing them, I cut the peritoneum and a part of the uterus, corresponding to the chosen embryo, I injected a colored solution of DNA into the embryo ventricular cavity and applied a potential difference with a pair of electrodes next to the parietal lobe, to make the DNA go in the periventricular cells. I put the embryo back into the abdomen and sewed the peritoneum. The day after the electroporation, I sacrificed the mouse, dissected out the brain and left it in the presence of X-gal, a substrate converted by the *LacZ* gene product into a blue precipitate (fig 3.6).

In the meantime, I began to reconstruct the entire *Emx2OS* cDNA. For this purpose I collected, cloned and sequenced a set of partial cDNAs fragments, obtained via RT-PCR from total RNA from E18.5 mouse brain. These fragments will be subsequently spliced in vitro and transferred into the pcDNA expression vector as full *Emx2OS* cDNA, for in vivo overexpression.

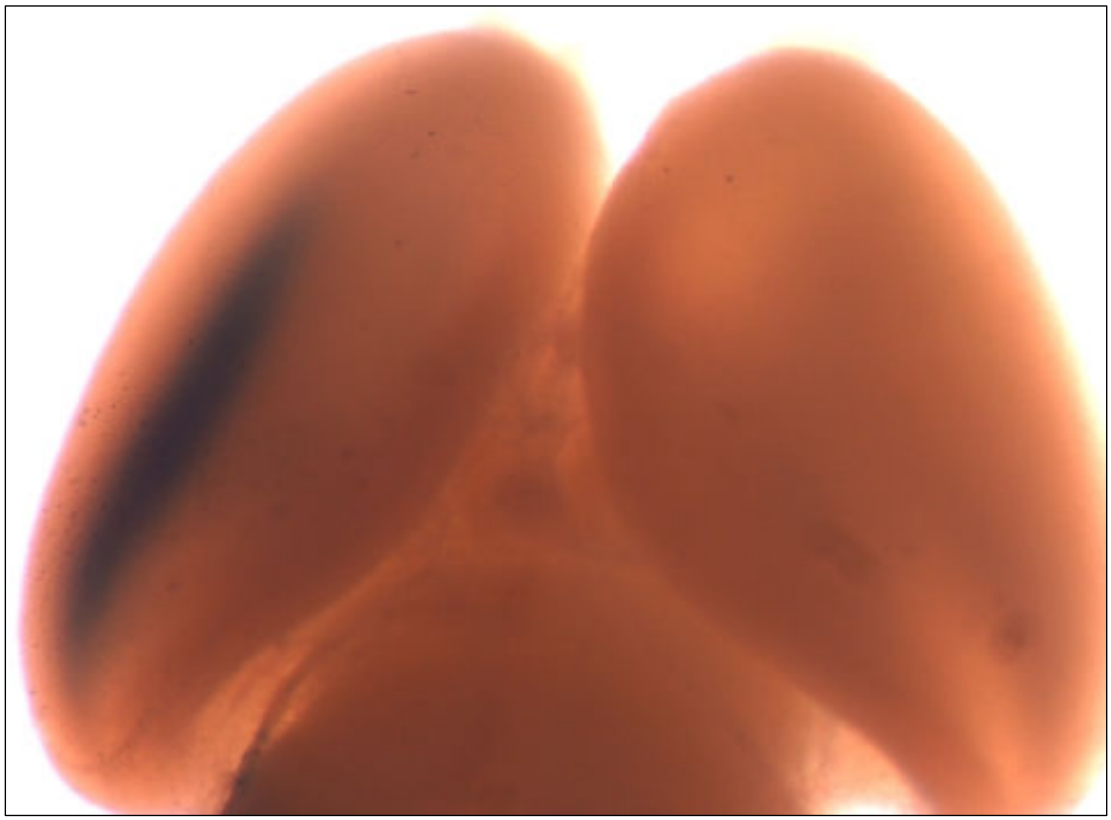

***Fig. 3.6 NLS-LacZ exo-uterus electroporation of the lateral cerebral cortex of an E14.5 mouse embryo****. On day after the electroporation the mouse embryo was sacrifized and its brain was fixed and let react with X-GAL, the substrate of the LacZ gene product: it was metabolized to a blue-colored compound, proving the expression of LacZ.*

# Chapter IV

# DISCUSSION

The *Emx2* homeobox gene is crucial to proper development of the mouse embryo, being necessary for morphogenesis of the urogenital tract (Pellegrini et al., 1997) as well as for determination, regionalisation and arealisation of the cerebral cortex (reviewed in Mallamaci and Stoykova, 2006). Its spatio-temporal expression pattern is very complex and molecular mechanisms underlying its establishment and mainteinance were poorly addressed and only partially disentangled. Analysis of conventional, polypeptide-dependent transcriptional regulation of *Emx2* led to the discovery of two cis-active elements, *EMXB* and *EMXC*, mediating HOXA10-dependent repression of *Emx2* within the female urogenital tract during the post-proliferative phase of the oestral cycle (Troy et al., 2003), as well as of two elements, *DT-1* and *DT-2*, mediating Smad1,4/Tcf-Lef dependent activation of *Emx2* in the developing cerebral cortex (Theil et al., 2002). Recently, more and more impressive experimental evidence accumulated, pointing to genome-wide existence of additional functional layers controlling transcription, distinct from classical, transcription factors-dependent regulation, and conversely relying on synthesis and activity of plenty of ncRNAs (not coding RNAs) (Mattick, 2007). Remarkably, *Emx2* antisense transcripts were described in both the urogenital system and the adult brain (Noonan et al., 2003) and multiple *Emx2*-associated ncRNAs are currently reported in web-accessible databases, such as NCBI and UCSC ones, as well. A systematic study of temporal and spatial distribution of these *Emx2*-associated antisense RNAs in the developing cerebral cortex as well as a preliminary analysis of their possible involvement in the control of developmental processes (including transcriptional regulation of *Emx2*) were subject of my work.

By combining quantitative RT-PCR and not-radioactive in situ hybridisation, I found that the main antisense transcript associated to *Emx2*, *Emx2OS*, overlapping the former in a head-to-head (5'-5') fashion, is expressed within the developing embryo brain at high levels and according to a complex spatio-temporal pattern (fig 3.1). *Emx2OS* expression

is not confined to cerebral cortex. Among its extra-cortical domains, there are the MGE (medial ganglionic eminence), expressing it in a generalized way, and the LGE (lateral ganglionic eminence), where its expression is restricted to the most ventricular layers (fig 3.2). Within the cortical field, *Emx2OS* shows a highly dynamic expression profile (fig. 3.3). It is expressed by periventricular proliferative layers, throughout the embryonic neuronogenetic window, here overlapping its sense correlate, *Emx2* mRNA. Outside periventricular layers, *Emx2OS* and *Emx2* expressions segregate. Pioneer Cajal-Retzius neurons belonging to the marginal zone (the forerunner of neocortical layer I), express *Emx2* sense at very high levels (Mallamaci et al., 1998), but do not express *Emx2OS* at all. Almost no trace of *Emx2* products can be found in subplate and cortical plate (the forerunner of neocortical layers II-to VI) (Simeone et al., 1993), conversely giving rise to sustained expression of *Emx2OS*. Remarkably, *Emx2OS* expression within the developing cortical plate is not uniform, but consistently more pronounced in the marginal-most part of it, at all of embryonic developmental stages subject of investigation. This marginal cortical plate is occupied by young neurons, fated to form distinctive laminae of the mature cortex, which have just completed their radial journey from periventricular proliferative layer to their final radial location and are going to be overcome by later born siblings, directed to more superficial locations (Bayer and Altmann, 1991). So, *Emx2OS* cannot be properly considered a "layer-specific" gene, but seems rather to peak in all neocortical pyramidal neurons, during a restricted, well-defined phase of their maturation, slightly after their exit from mitotic cycle. In agreement with this interpretation, *Emx2OS* is completely off in the adult neocortex, where bulk neuronogenesis already ended up.

The peculiar spatio-temporal relationship occurring between *Emx2* and *Emx2OS* transcripts suggests that they might reciprocally influence their expression, possibly in a negative fashion. On the other side, the absence of appreciable *Emx2OS* down-regulation

in *Pax6*$^{Sey/Sey}$ mutants, which overexpress *Emx2*, further suggests that the *Emx2-Emx2OS* interaction, if any, might be unidirectional, i.e. from *Emx2OS* to *Emx2*, and not viceversa. Moreover, the absence of relevant and phylogenetically conserved polypeptide-encoding sequences within *Emx2OS* allows us to reasonably rule out it may act as a conventional mRNA. So, *Emx2OS* possibly regulates *Emx2*, as such (in *trans*) or via the effects that its own transcription may exert on the locus encoding for both of them (in *cis*).

Actually, assaying *cis*-effects of *Emx2OS* would require very complex and time-consuming genomic manipulations, which, because of time constraints, cannot fall within the workprogram of this thesis. Conversely, addressing activities of *Emx2OS* RNA in *trans*, i.e. activities mediated by the molecule (and by by-products of its synthesis) as such, looks like a more tractable task. In this respect, I found a phylogenetically conserved miR, putatively encoded by the first intron of *Emx2OS*, highly similar to miR-130a and able in silico to specifically bind to a target located within the *Emx2* mRNA 3'UTR. Unfortunately, in vivo levels of this putative miR, if any, fell under the detectability threeshold of in situ hybridisation, so it is hard at the moment to draw an acceptable conclusion about its real existence and function. Well-designed gain-of-function experiments might help circumventing the problem and casting light on this point. On the other side, main bio-active product of *Emx2* antisense transcription might be just the mature *Emx2OS* RNA. So I initiated to reconstruct its cDNA in vitro. Hopefully, it will be later transferred into a standard expression vector and over-expressed in vivo, via somatic electroporation into the embryonic developing cortex, so allowing to clarify this point.

# Chapter V

# REFERENCES

-K. Ajiro et al. *Alteration of the cell cycle-depend histone phosphorylations by okadaic acid. Induction of mitosis-specific H3 phosphorylation and chromatin condensation in mammalian interphase cells* J. Biol. Chem. 271, 13197-13201 (1996)

-S. Alcantara et al. *Regional and Cellular Patterns of reelin mRNA Expression in the Forebrain of the Developing and Adult Mouse* The Journal of Neuroscience, 18, 7779–7799 (1998)

-G. Alfano et al. *Natural antisense transcripts associated with genes involved in eye development* Human Molecular Genetics, 14, 913–923 (2005)

-K. Allendorfer et al *Nerve growth factor receptor immunoreactivity is transiently associated with the subplate neurons of the mammalian cerebral cortex* Proc. Natl. Acad. Sci. USA 87, 187 (1990)

-K. Allendoerfer and C. Shatz. *The subplate, a transient neocortical structure: its role in the development of connections between thalamus and cortex.* Annu. Rev. Neurosci. 17, 185-218 (1994)

-L. Altman and S. Bayer. *Development of the diencephalon in the rat. V. Thymidine-radiographic observations on internuclear and intranuclear gradients in the thalamus.* J. Comp. Neurol. 188, 473-499 (1979)

-T. R. Anderson et al. *Differential Pax6 promoter activity and transcript expression during forebrain development* Mechanisms of Development 114, 171–175 (2002)

-T. Theil Alvarez-Bolado et al *Gli3 is required for Emx gene expression during dorsal telencehalon development* Development 126 3561-3571 (1999)

-M. E. Anderson and A. M. Graybiel Encyclopedia of life science Nature(2001)

-S. A. Anderson et al. *Interneuron migration from basal forbrain to neocortex: dependence on Dlx genes*. Science 278, 474-476 (1997)

-S. Anderson et al. *Distinct cortical migrations from the medial and lateral ganglionic eminence.* Development 128, 353-363 (2001)

-Y. Arimatsu et al. *Area- and lamina-specific organization of a neuronal subpopulation defined by expression ol latexin in the rat cerebral cortex* Neuroscience 88(1), 93-105 (1999)

-S. Assimacopoulos et al. *Identification of a Pax6-dependent EGF family signaling source at the lateral edge of the embryonic cerebral cortex* J. Neurosci 23 (16), 6399-6403 (2003)

-S. Bayer and L. Altmann *Neocortical Development* Raven Press (1991)

-M. Barbe and P. Levit *The early commitment of foetal neurons to the limbic cortex* J. Neurosci 11(2), 519 (1991)

-M. F. Barbe and P. Levitt *Atraction of specific thalamic input by cerebral graft depends on the molecular identity of the implat* Proc.Natl.Acad.Sci USA 89, 3707-3710 (1992)

-B. L. Bass RNA *editing by adenosine deaminases that act on RNA* Annu
Rev Biochem 71, 817–846 (2002)

-A. Bauer et al. *Pontin52 and Reptin52 function as antagonistic regulators of beta-catenin signaling activity* EMBO J. 19(22), 6121-6130 (2000)

-M. Berry and A.W. Rogers. *The migrations of neuroblasts in the developing cerebral cortex.* J. Anat. 99, 691-709 (1965)

-M. Bienz and H. Clevers *Armadillo/β-catenin signals in the nucleus-proof beyond a resonable doubt?* Nat cell Biology 5, 179-182 (2003)

-K. Bishop et al *Regulation of area identity in the mammalian neocortex by Emx2 and Pax6* Science 288, 344-349 (2000)

-K. Bishop et al. *Emx1 and Emx2 cooperate to regulate cortical size, lamination, neuronal differentiation, development of cortical afferents, and thalamocortical pathfinding* J. Comp Neurol. 17, 345-360 (2003)

-A. Bjorklund at al. eds. pp 1-164, Elsevier

-V. Borrell et al. *Reelin Regulates the Development and Synaptogenesis of the*

*Layer-Specific Entorhino-Hippocampal Connections* The Journal of Neuroscience, 19, 1345–1358 (1999)

-P. Briata et al *EMX1 homeoproteins is expressed in the cell nuclei of the developing cerebral cortex and in the axons of the olactory sensory neurons* Mech Dev 57, 169-180 (1996)

-K. Brodmann. *Vergleichende Lokalisationslehre der Grosshirnrinde in ihren Prinzipien dargestellt auf Grund des Zellenbaues*, J.A. Barth (1909)

-A. Bulfone et al. *Expression pattern of the Tbr2(eomesodermin) gene during mouse and chick brain development* Mech Dev 84, 133-138 (1999)

-S. Bulchand et al. *LIM-homeodomain gene Lhx2 regulates the formation of the cortical hem* Mech. Dev 100 165-175 (2001)

-S. R. y Cajal. *Histologie du Système Nerveux de l'Homme et des Vertébrés,* vol 2 Maloine (1911)

-G. Castelo-Branco et al. *Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a*. Proc Natl Acad Sci U S A. 100(22), 12747-12752 (2003)

-S. M. Catalano and C. J. Shatz *Activity-dependent cortical target selection by thalamic axons.* Science 281, 559-562 (1998)

-R. A. Cavallo et al. *Drosophila Tcf and Groucho interact to repress Wingless signaling activity* Nature 395, 604-608, (1998)

-V. S. Caviness et al. *The reeler malformation.* In Cerebral cortex,vol VII Development and maturation of the Cerebral cortex A. Peters and E.G. Jones eds 59-89 Plenum Press (1988)

-V. S. Caviness et al *Cell output, cell cycle duration and neuronal specification: a model of integrated mechanisms of the neocortical proliferative process.* Cereb. Cortex 13(6), 592-598 (2003)

-V. S. Caviness Jr. et al. *Cell output, cell cycle duration and neuronal specification: a model of integrate mechanisms of the neocortical proliferative process.* Integrated mechanisms of proliferation (2004)

-C. B. Chambers et al. *Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors* Development 128 689-702 (2001)

-P. Chapouton et al. *The role of Pax6 in restricting cell migration between developing cortex and basal ganglia* Development 126, 5569-5579 (1999)

- J. Chen et al. *Over 20% of human transcripts might form sense-antisense pairs* Nucleic Acids Res. 32, 4812 (2004)

-A. Chenn and C.A. Walsh. *Regulation of Cerebral Cortical Size by Control of Cell Cycle exit in Neural precursor* Science 297, 365-369 (2002)

-C. M. Clemson et al. *XIST RNA Paints the Inactive X Chromosome at Interphase: Evidence for a Novel RNA Involved in Nuclear/Chromosome Structure* The Journal of Cell Biology 132, 259-275 ( 1996)

-M. Cohen-Tannoudji et al. *Unexpected position dependent expression of H-2 and beta2-microglobulin/LacZ transgenes* Mol. Reprod. Dev. 33, 149-159 (1992)

-J. G. Corbin et al. *The Gsh2 homeodomain gene controls multiple aspects of telencephalic development*. Development 127, 5007-5020 (2000)

-J. Coleman et al. *The use of RNAs complementary to specific mRNAs to regulate the expression of individual bacterial genes* Cell. 37, 429-36 (1984)

-V. Coskun and M. B. Luskin *The Expression Pattern of the Cell Cycle Inhibitor p19 INK4d by Progenitor Cells of the Rat Embryonic Telencephalon and Neonatal Anterior Subventricular zone* J. Neurosci. 21, 3092-3103 (2001)

-P. H. Crossley and G.R. Martin. *The mouse Fgf8 encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo* Development 121, 439-451 (1995)

-G. D'Arcangelo et al. *A protein related to extracellular matrix proteins deleted in the mouse mutant reeler* Nature 374, 719-723 (1995)

-G. D'arcangelo and T. Curran. *Reeler: new tales on an old mutant mouse.* Bioessay 20, 235-244 (1998)

-M. Denaxa et al. *The adhesion molecule TAG-1 mediates the migration of cortical interneurons from the ganglionic eminence along the corticofugal fibre system.* Development 128, 4635-4644 (2001)

-L. De Rouvroit et al. *Neuronal migration.* Mech. Dev.105, 47-56 (2001)

-M. J. Donoghue and P. Rakic. *Molecular evidence for the early specification of presuntive functional domains in the embryonic primate cerebral cortex* J. Neurosci. 19, 5967-5979 (1999)

-C. L. Dou et al. *Dual role of Brain-factor-1 in regulating grwyh and patterning of the cerebral hemispheres* Cereb. Cortex 9, 43-55 (1999)

-L. Dulabon et al. *Reelin binds □3□1 integrins and inhibits neuronal migration.* Neuron 27, 33-44 (2000)

-K. L. Eagleson et al *Mechanisms specyfing are fate in cortex include cell-cycle-dependent decision and the capability of progenitors to express phenotype memory* Development 124, 1623 (1997)

-G. Estivill-Torrus et al. *Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors* Development 129, 455-466 (2002)

-D. S. Falconer. *Two new mutants "trembler" and "reeler" with neurological actions in the mouse house* J. Genet 50, 192-201 (1951)

-A. Fairèn et al. Nonpyramidal neurons: general account. In *Cerebral cortex (vol1)Cellular components of the Cerbral Cortex* (A. Peters And E.G. Jones) pp 201-253, Plenum press)

-R. T. Ferri and P. Levitt *Cerebral cortical progenitors are fated to produce region-specific neuronal populations* Cereb. Cortex 3, 187 (1993)

-M. Filali et al. *Wnt-3A/beta-Catenin Signaling Induces Transcription from the LEF-1 Promoter*. J. Biol.Chem. 277(36), 33398–33410 (2002)

-C. Fode et al. *A role for neural determination genes in specifying dorso-ventral identity of telencephalic neurons* Genes Dev. 14 67-80 (2000)

-Y. Foruta et al. *Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development* Development 124, 2203-2212 (1997)

-G. D. Frantz et al. *Regulation of the POU domain gene SCIP during the cerebral cortical dvelopment* J. Neurosci 14(2), 472-485 (1994) a.

-G. D. Frantz et al. *Otx1 and Otx2 define layers and regions in the developing cerebral cortex and cerebellum* J. Neurosci 14, 5725-5740 (1994) b.

-I. Frappe et al. *Transplants of fetal frontal cortex grafted into the occipital cortex of newborn rats receive a substantial thalamic input from nuclei normally projecting to the frontal cortex* Neuroscience 89, 409-421 (1999)

-M. Frotscher. *Dual role of Cajal-Retzius cells and reelin in cortical development*. Cell Tissue Res. 290, 315-322 (1997)

-T. Fukuchi-Shimogori and E. A. Grove. *Neocortex patterning by the secreted signaling molecule FGF8* Science 294, 1071-1074 (2001)

-T. Fukuda et al. *Immunohistochemical localization of neuron and L1 in the formation of thalomocortical pathway of developing rats* J. Comp. Neurol. 382, 141 (1997)

-J. Gadisseaux and P. Evrard. *Glia-neuronal relationship in the developing central nervous system. A histochemical-electron microscopy study of radial glial cell particulate glicogen in normal and reeler mice and human foetus* Dev Neurosci. 7, 12-31 (1985)

-A. Gaillard and M. Roger. *Early commitment of embryonic neocortical cells to develop area-specific thalamic connections* Cereb. Cortex 10, 443-453 (2000)

-N. Gaiano and G. Fishell. *The role of Notch in promoting glial and neural stem cell fate* Annu. Rev. Neurosci 25 471-490 (2002)

-I. Galceran et al *Hippocampus development and generation of dentate gyrus granule cells is regulated by* LEF1 Development 127, 469-482 (2000)

-A. Gallet et al. *Trunk specific modulation of wingless signaling in Drosophila by teashirt binding to armadillo* Curr. Biol. 8(16), 893-902 (1998)

-C. Ghiang et al. *Cyclopia and defective axial patterning in mice lacking Sonic Hedgehog gene function* Nature 383, 407-413 (1996)

-Y. Gitton et al. *Specification of somatosensory ares identity in cortical explants.* J. Neurosci. 19, 4889-4898 (1999)

-E. A. Grove et al *The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and it is compromised in Gli3 deficient mice* Development 125, 2315-2325 (1998)

-M. Gulisano et al. *Emx1 and Emx2 show different patterns of expression during proliferation and differentiation of the developing cerebral cortex in the mouse* Eur. J. Neurosci 8, 1037-1050 (1996)

-L. Gunhaga et al. *Sonic hedgehog signaling at gastrula stages specifies ventral telencephalic cells in the chick embryo*. Development 127(15), 3283-3293 (2000)

-L. Gunhaga et al. *Specification of dorsal telencephalic character by sequential Wnt and FGF signaling.* Nat. Neurosci. 6(7), 701-707 (2003)

-P. A. Hall and A .L. Woods. *Immunohistochemical markers of cellular proliferation: achievement, problems and prospects* Cell Tissue Kinet. 23, 505-522(1990)

-M. Hallonet et al. *Vax1 is a novel homeobox-containing gen expressed in the developing anterior ventral forbrain* Development 125, 2599-2610 (1998)

-C. Hanashima et al. *Controls the Proliferation and Differentiation of Neocortical Progenitor Cells through Independent Mechanisms* J. Neurosci. 22, 6526-6536 (2002)

-M.L. Hastings et al. *Post-transcriptional regulation of thyroid hormone receptor expression by cis-acting sequences and a naturally occurring antisense RNA* J Biol Chem 275, 11507–11513 (2000)

23.

-N. Heins et al. *Glial cells generate neurons: the role of the transcription factor Pax6* Nat Neurosci. 4, 308-315 (2002)

-X. He et al. *LDL receptor related protein 5 and 6 in Wnt/b-Catenin signaling: Arrows point the way* Development 131, 1663-1677 (2004)

-A. Ho and S. F. Dowdy. *Regulation of G 1 cell-cycle progression by oncogenes and tumor suppressor genes*. Current Opinion in Genetics & Development 12, 47–52 (2002)

-R. F. Hevner et al. *Cortical and thalamic axon pathfinding defects in Tbr1, Gbx2, and Pax6 mutant mice: evidence that cortical and thalamic axons interact and guide each other.* J. Comp. Neurol 447, 8-17 (2002)

-R. E. Hill et al. *Mouse small eye results from mutations in a paired-like homeobox-containing gene* Nature 354, 522-525 (1991)

-Y. Hirabayashi et al. *The Wnt/b-catenin pathway directs neuronal differentiation of cortical neural precursor cells* Development 131, 2791-2801 (2004)

-T. Imamura et al. *Non-coding RNA directed DNA demethylation of Sphk1 CpG island* Biochem Biophys Res Commun 322, 593–600 (2004)

-E. G. Jones. *The thalamus* Plenum New York (1985)

-E. H. Jho et al. *Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of a signaling patway* Mol. Cell Biol. 22, 1172-1183 (2002)

-H. Kiyosawa et al. *Antisense transcripts with FANTOM2 clone set and their implications for gene regulation* Genome Res. 13, 1324 (2003).

-A.S. Kim et al.*Wnt receptors and Wnt inhibitors are expressed in the developing telencephalon* Mech. Dev. 103(1-2), 167-172 (2001)

-J.D Kohtz et al. *Regionalization within the mammalian telencephalon is mediated by changes in responsiveness to Sonic Hedgehog* Development 125, 5079-5089 (1998)

-J. Y. Lai and S. Stifani. *The Winged-Helix Protein Brain Factor 1 Interacts with Groucho and Hes Proteins To Repress Transcription* Mol. Cell Biol. 21, 1962-1972 (2001)

-A. Lavdas et al *The medial ganglionic eminence gives rise to a population of early neurons in the developing cortex* J. Neurosci 99, 7881-7888 (1999)

-D.D.M. O'Leary. *Do cortical areas emerge from a protocortex?* Trends Neurosci. 12, 400-406 (1989)

-S. M. Lee et al. *The expression and post-translational modification of a neuron-specific b-tubulin isotype during chick embryogenesis* Cell Motil Cytoskel 17, 118-132 (1990)

-S. M. Lee et al. *A local Wnt3a signal is required for development of the mammalan hippocampus* Development 127, 457-467 (2000)

-P. Levitt *A monoclonal antibody to limbic system neurons* Science 223, 229 (1984)

-H. Van der Loos and T. Woolsley *Somatosensory cortex: structural alterations following early injury to sense organ* Science 179, 395 (1973)

-Q. Liu et al. *Differential expression of Coup-Tf1, Chl1 and two novel genes in developing neocortex identified by differential display PCR* J. Neurosci. 20, 7682-7690 (2000)

-D. Lyden et al. *Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts* Nature 401, 670-677 (1999)

-A. Mallamaci et al. *EMX2 protein in the developing brain and olfactory area* Mech of Dev. 77(2), 165-172 (1998))

-A. Mallamaci et al. *The lack of Emx2 causes impairment of Reelin signaling and defects in neuronal migration in the developing cerebral cortex* J. Neurosci. 20, (2000)

-A. Mallamaci et al. *Area identity shifts in the early cerebral cortex of Emx2-/- mutant mice* Nat. Neurosci. 3, 679-686 (2000)

-A. Mallamaci, A. Stoykova. *Gene networks controlling early cerebral cortex arealization* Eur J Neurosci. 23, 847-56 (2006)

-T. Maniatis et al. *Molecular cloning* Cold Spring harbour lab press 1989

-S. Maretto et al *Mapping Wnt/□catenin signaling during mouse development and in colorectal tumors* Proc.Natl.Acad.Sci USA 100, 3299-3304 (2003)

-O. Marin et al *Origin and molecular specification of striatal interneurons* J. Neurosci. 20, 6063-6076 (2000)

-O. Marin et al. *Sorting of striatal and cortical interneurons regulated by semaphorn/neuropilin interactions.* Science 293, 872-875 (2001)

-O. Marin and J. Rubenstein. *A long, remarkable journey: tangential migration in the telencephalon.* Nat. Rev. Neurosci. 2, 780-790 (2001)

-M. Marin Padilla 1971 *Early prenatal ontogenesis of the cerbral cortex(neocortex) of the cat (Felix domestica). A Golgi study.* Z.Anat.Entwickl-Gesh. 134, 117-145

-M. Marin-Padilla *Cajal-Retzius cells and the development of the neocortex* Trends in Neurosci. 21, 64-71 (1998))

-M. Marklund et al. *Retinoic acid signalling specifies intermediate character in the developing telencephalon*. Development 131(17), 4323-4332 (2004)

-J. S. Mattick *A new paradigm for developmental biology* J Exp Biol. 210, 26-47 (2007)

-D.A McLaughlin et al. *Wnt8b regulates cellular proliferation in the adult dentate gyrus* Soc. Neurosci 228, 228 (2000)

-H. Meinhard. *Cell determination boundaries as organizing regions for secondary embryonic fields* Dev. Biol. 96(2), 375-385 (1983)

-G. Meister and T. Tuschl *Mechanisms of gene silencing by double stranded RNA* Nature 431, 343–349 (2004)

-C. Metin and P. Godement. *The ganglionic eminence may be an intermediate target for corticofugal and thalamocortical axons.* J. Neurosci. 16, 3219-3235 (1996)

-I. Mikkola et al. *Superactivation of Pax6-mediated transactivation from paired-binding sites by DNA-independent recruitment of different homeodomain proteins J. Biol. Chem 276, 4109-4118* (2001)

-J. Misson et al *Identification of radial glial cells within the developing murine central nervous system: studies based upon a new histochemical marker* Dev. Brain Res. 44 95-108 (1988)

-Z. Molnàr and C. Blakemore. *How do thalamic axons find their way to the cortex?* Trends Neurosci. 18, 389-397 (1995)

-Z. Molnàr et al. *Mechamisms underlying the early establishment of thalamocortical connections in the rat.* J. Neurosci 18, 5723-5745 (1998)

-Z. Molnàr and P. Cordery. *Connections between cells of the internal capsule, thalamus, and cerebral cortex in embryonic rat.* J. Comp. Neurol. 413, 1-25 (1999)

-E. Monuki et al. *Patterning of the dorsal telencephalon and cerebral cortex by a roof plate-Lhx2 patway* Neuron 32, 591-604 (2001)

-T. Mitsuhashi et al. *Overexpression of p27 Kip1 lengthens the G1 phase in a mouse model that targets inducible gene expression to central nervous system progenitor cells* Proc. Natl. Acc. Sci 98 6435-6440 (2001)

-S. Miyama et al. *A gradient in the duration of the G1 phase in the murine neocortical proliferative epithelium.* Cereb Cortex 7, 678-689 (1997)

-E. M. Miyashita-lin et al. *Early neocortical regionalization in absence of thalamic innervation.* Science 285, 906-909 (1999)

-E. S. Monuki et al. *Patterning of the dorsal telencephalon and cerebral cortex by a roof plate-Lhx2 patways* Neuron 20, 591-604 (2001)

-L. Muzio et al. *Conversion of cerebral cortex into basal ganglia in Emx2$^{-/-}$Pax6$^{Sey/Sey}$ double mutant mice*. Nature Neuroscience 5(8), 737-745.(2002)

-L. Muzio et al. *Emx2 and Pax6 control regionalization of the pre-neuronogenic cortical primordium* Cerebr. Cortex 12, 129-139 (2002)

-L. Muzio and A. Mallamaci. *Emx1, Emx2 and Pax6 in specification, regionalization and arealisation of the cerebral cortex* Cereb. Cortex 13(6), 641-647 (2003)

-L. Muzio et al. *A mutually stimulating loop involving , Emx2 and cnonical Wnt signalling specifically promotes expansion of occipital cortex and hippocampus.* Cereb. Cortex 15, 2021-2028 (2005)

-B. Nadarajan et al. *Two models of radial migration in early development of the cerebral cortex.* Nat. Neurosci. 4, 143-150 (2001)

-J. R. Naegele et al. *Sharpening of topographical projections and maturation of geniculocortical axon arbors in the hamster.* J. Comp. Neurol. 277, 593-607 (1988)

-Y. Nakagawa et al *Graded and areal expression patterns of regulatory genes and caherins in embryonic neocortex independent of thalamocortical imput* J. Neurosci 19(24) 10877- (1999)

-F.C. Noonan et al. *Antisense transcripts at the EMX2 locus in human and mouse* Genomics 81, 58–66 (2003)

-S. I. Ohnuma and W. A. Harris. *Neurogenesis and the Cell Cycle* Neuron 40, 199-208 (2003)

-T. Ohtsuka et al. *Roles of the Basic Helix-Loop-Helix Genes Hes1 and Hes5 in Expansion of Neural Stem Cells of the Developing Brain* J. Biol. Chem 276 30467-30474 (2001)

-Y. Okubo et al *Coordinate regulation and synergic actions of Bmp4, Shh and Fgf8 in the rostral prosencephalon regulate morphogenesis of the telencephalic and optic vesicles.* Neuroscience 111, 1-17 (2002)

-D.M. Panchison et al. *Sequential actions of BMP receptors control neural precursor cell production and fate* Gene Dev. 15, 2094-2110 (2001)

-J.G.Parnavelas et al. *The central visual pathway*. In Handbook of Chemical Neuroanatomy (vol7) Integrated System of CNS(part2)

-T. Pratt et al. *A role of Pax6 in the normal development of dorsal thalamus and its cortical connections.* Development 127, 5167-5178 (2000)

-M. Pellegrini et al. *Dentate gyrus formation requires Emx2* Development 122, 3893-3898 (1996)

-M. Pellegrini et al. *Emx2 developmental expression in the primordia of the reproductive and excretory systems* Anat Embryol (Berl) 196(6), 427-33 (1997)

-S. D. Podos and E.L. Ferguson. *Morphogen gradients: new insights from DPP.* Trends Genet. 15, 396-402 (1999)

- Prescott and Proudfoot *Transcriptional collision between convergent genes in budding yeast* Proc Natl Acad Sci U S A. 99, 8796-801 (2002)

-L. Puelles et al. *Pallial and subpallial derivates in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx2, Emx1, Nkx2.1, Pax6 and Tbr1* J Comp Neurol 424, 409-438 (2000)

-C. Ragsdale et al. *Early patterning of the cerebral cortex may be shaped by gradients of receptors and binding proteins of the Fgf, Bmp and Wnt signaling patways* Soc Neurosci 18, 116 (2000)

-P. Rakic. *Neurons in Rhesus monkey visual cortex: systematic relation between time of origin and eventual disposition.* Science 183, 425-427 (1974)

-P. Rakic et al. *Computer-aided three dimensional reconstruction and quantitative analysis of cells from serial electron microscopy montages of foetal monkey brain* Nature 250, 31-34 (1974)

-P. Rakic. *Specification of cerebral cortical areas.* Science 241, 170-176 (1988)

-Rattner et al. *A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of the frizzled receptors* Proc. Natl. Acad. Sci. USA 94(7), 2859-2863 (1997)

-D. S Rice et al. *Disabled-1 acts downstream of Reelin in a signaling pathway that controls laminar organization of the mammalian brain* Development 125, 3719-3729 (1998)

-V. Riechmann et al. *Mutually exclusive expression of two dominant negative helix-loop-helix(dnHLH) genes, Id4 and Id3, in the developing brain of the mouse suggests distinct regulatory roles of these dnHLH proteins during cellular proliferation and differentiation of the nervous system* Cell Growth Differ. 6, 837 (1995)

- RIKEN Genome Exploration Research Group and Genome Science Group (Genome Network Project Core Group) and the FANTOM Consortium *Antisense Transcription in the Mammalian Transcriptome* Science 309, 1564 (2005)

-E. J. Robertson. *Embryo-derived stem cell line. In teratocarcinomas and embryonic stem cell: a practical approach.* Washington IRL press 71-112 (1987)

-H. Roelink and R. Nusse. *Expression of two members of the Wnt family during mouse development* Genes Dev 5, 381-388 (1991)

-K. Roovers and R. K. Assoian. *Integrating the Map kinase signal into the G1 phase cell cycle machinery* Bioassay 22, 818-831 (2000)

-C. Rougeulle and E. Heard *Antisense RNA in imprinting: spreading silence through Air* Trends Genet 18, 434–437 (2002)

-J. Rubenstein et al. *Genetic control of cortical regionalization and connectivity* Cereb. Cortex 9, 524 (1999)

- Scadden and Smith *Specific cleavage of hyper-edited dsRNAs* EMBO J. 20(15), 4243-52 (2001)

-R. Scardigli et al. *Direct and concentration-dependent regulation of the proneural gene Neurogenin2 by Pax6* Development 130, 3269-3281 (2003)

-A. Schlessinger et al. *The time of origin of neurons in Ammon's horn and the associated retrohippocampal fields* Anat. Embryol 154, 153 (1978)

-J. Seoane et al. *Integration of Smad and Forkhead Pathways in the Control of Neuroepithelial and Glioblastoma Cell Proliferation* Cell 117 211-223 (2004)

-A. J. Shatz and B. M. Luskin. *The relationship between the geniculocortical afferents and their cortical target cells during development of the cat's primary visual cortex.* J. Neurosci. 6, 3655-3668 (1986)

-S. Shibata and Jeannie T. Lee. *Tsix Transcription- versus RNA-Based Mechanisms in Xist Repression and Epigenetic Choice* Current Biology 14, 1747–1754 (2004)

-I. Shimamuta and J. L. Rubenstein. *Inductive interactions direct early regionalization of the mouse forebrain* Development 124, 2709-2718 (1997)

-A. Simeone, et al. *Two vertebrate homeobox genes related to the Drosophila empty spiracles gene are expressed in the embryonic cerebral cortex*. EMBO J.11(7), 2541-2550 (1992)

-A. Simeone et al. *Nested expression domains of four homeobox genes in the developing rostral brain* Nature 358, 687-690 (1992)

-A. Simeone et al. *A vertebrate gene related to Orthodenticle contains a homeodomain of the Bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo* EMBO J. 12, 2735-2747 (1993)

-F. Sleutels et al. *Imprinted silencing of Slc22a2 and Slc22a3 does not need transcriptional overlap between Igf2r and Air* EMBO J 22, 3696–3704 (2003)

-F. Sleutels et al. *The non-coding Air RNA is required for silencing autosomal imprinted genes* Nature 415, 810–813 (2002)

-P. Soriano et al. *Targeted distruption of the c-src proto-oncogene leads to osteoporosis in mice* Cell 64, 693-702 (1991)

-E. Storm et al. *Negative regulation between two Fgf signaling pathways a mechamism for generating distinct phenotypes in response to different levels of Fgf8* Proc. Natl. Acad. Sci. USA 100, 1757-1762 (2003)

-A. Stoykova and P. Gruss. *Roles of Pax genes in developing and adult brains as suggested by expression patterns.* J. Neurosci. 14, 1395-1412 (1994)

-A. Stoykova et al. *Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in the developing forebrain* Development 124, 3765-3777 (1997)

-A. Stoykova et al. *Pax6 modulates the dorsoventral patterning of the mammalian telencephalon* J. Neurosci. 20(21), 8042-8050 (2000)

-M. Strigini and S.M. Cohen. *Formation of morphogen gradients in the Drosophila wing.* Semin. Cell Dev. Biol. 10, 335-344 (1999)

-L. Sussel et al. *Loss of Nkx2.1 homeobox gene function result in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum* Development 126(15) 3359-3370 (1999)

-N. Tamamaki et al. *Origin and route of tangentially migrating neurons in the developing neocortical intermediate zone.* J. Neurosci. 17, 8313-8323 (1997)

-S. Tan et al. *Separate progenitors for radial and tangential cell dispersion during development of the cerebral cortex.* Neuron 21, 295-304 (1998)

-T. Theil et al. *Gli3 is required for Emx gene expression during dorsal telencephalon development* Development 126, 3561-3571 (1999)

-T. Theil et al. *Wnt and Bmp signalling cooperatively regulate graded Emx2 expression in the dorsal telencephalon* Development 129, 3045-3054 (2002)

-T. Takahashi et al. *Cell cycle parameters and patterns of nuclear movement in the neocortical proliferative zone of the fetal mouse* J. Neurosci. 13, 820-833 (1993)

-T. Takahashi et al. *Interkinetic and migratory behaviour of a cohort of neocortical neurons arising in the early embryonic murine cerebral wall* J. Neurosci 16, 5762-5776 (1996)

-T. Takahashi et al. *Sequence of neuron origin and neocortical lamibar fate: relation to cellcycle of origin in the developing murine cerebral wall* J. Neurosci 19 10357-10371 (1999)

-K. Takiguchi-Hayashy et al. *Generation of Reelin-positive marginal zone cells from the caudomedial wall of telencephalic vesicles* J. Neurosci. 24(9), 2286-2295 (2004)

-V. Tarabikyn et al. *Cortical upper layer neurons derive from the subventricular zone as indicated by Svet1 gene expression* Development 128 (11), 1983-1993(2001)

-T. Theil et al. *Wnt and Bmp signalling cooperatively regulate graded Emx2 expression in the dorsal telencephalon* Development 129(13), 3045-54 (2002)

-S. Tole et al. *Early specification and autonomous development of cortical fields in mouse hippocampus.* Development 124, 4959-4970 (1997)

-S. Tole et al. *Dorsoventral patterning of the telencephalon is disrupted in the mause extra-toes.* Dev. Biol. 217, 254-265 (2000)

-S. Tole et al. *Detailed field pattern is intrinsic to the embryonic mouse hippocampus early in neurogenesis.* J. Neurosci. 21(5),883-894 (2001)

-H. Toresson et al. *Genetic control of dorsal-ventral identity in the telencephalon: opposing role for Pax6 and Gsh2* Development 127 4361-4371 2000)

-P.J. Troy et al. *Transcriptional repression of peri-implantation EMX2 expression in mammalian reproduction by HOXA10* Mol Cell Biol. 23(1), 1-13 (2003)

-R. Tuttle et al. *Defects in thalamocortical axon pathfinding correlates with altered domains in Mash1 deficient mice.* Development 126, 1903-1916 (1999)

-H. B. M. Uylings et al. *The prenatal and postnatal development of rat cerbral cortex.* (B.Kolb and R.C. Tees. eds pp 35-76, MIT press)

-E. G. H. Wagner, K. Flardh *Antisense RNAs everywhere?* Trends Genet 18, 223–226 (2002)

-C. Walter and P. Gruss. *Pax6 a murine paired box gene, is expressed in the developing CNS* Development 113, 1435-1449 (1991)

-H. Wichterle et al. *Young neurons from medial ganglionic eminence disperse in adult and embryonic brain* Nat. Neurosci. 2, 461-466 (1999))

-H. Wichterle et al. *In utero fate mapping reveals distinct migratory patways and fates in neurons born in the mammalian forebrain* Development 128, 3759-3771 (2001)

-J. Willert et al. *A transcriptional response to Wnt protein in human embryonic carcinoma cells.* BMC Dev. Biol. 2(1),8 (2002)

-Y. Xiong et al. *p21 is a universal inhibitor of cyclin kinases* Nature 366, 701 (1993)

-S. Xuan et al. *Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres* Neuron 14, 1141-1152 (1995)

-T. Yagi et al. *A novel negative selection for homologous recombinants using diphteria toxin A fragment gene* Analyt. Biochem. 214, 77-86 (1993)

-R. Yelin et al. *Widespread occurrence of antisense transcription in the human genome* Nat. Biotechnol. 21, 379 (2003)

-M. Yoshida et al. *Emx1 and Emx2 functions in development of dorsal telencephalon* Dvelopment 124, 101-111 (1997)

-K. Yun et al. *Gsh2 and Pax6 play complementary roles in dorsoventral patterning of the mammalian telencephalon* Development 126, 193-205 (2000)

-Yun et al. (2001)

-D. Zechner et al. *beta-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system*. Dev Biol. 258(2), 406-418 (2003)

-Z. Zebedee and E. Hara. *Id proteins in cell cycle control and cellular senescence* Oncogene 20, 8317-8325 (2001)

- Zhang and Carmichael *The fate of dsRNA in the nucleus: a p54(nrb)-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs* Cell. 106(4), 465-75 (2001)

-A. Zhou et al. *The nuclear orphan receptor Coup-Tf1 is required for differentiation of subplate neurons and guidance of thalamocortical axon* Neuron 24, 847-859 (1999)

-A. Zhou et al. *An intrinsic factor for early regionalization of the neocortex* Genes Dev. 15, 2054-2059 (2001))

-F. Zindy et al. *Expression of INK4 inhibitors of cyclin D-dependent kinases during mouse brain development* Cell Growth Differentiation 8, 1139-1150 (1997)

**Acknowledgments**

First, I want to thank my supervisor, Prof. A. Mallamaci, who followed me in my research project in a very complete way, teaching me a lot of things in both scientific and technical fields and allowing me to work autonomously.

Then, I really thank Prof. Vincent Torre, who believed in me, and all the colleagues and friends, who supported me, both in Milan and in Trieste, in particular: Paola Gava, Alisia Carnemolla, Danila Baldessari, Maria Pannese, Nicola Maiorano and all my Ph.D. colleagues, for their help and for sharing both the achievements and the trouble.

Finally, the deepest thanks go to my family and to my boyfriend Carlo for their love and trust and for still making me believe that everything is possible.