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DEFECTS IN NEURONAL DIFFERENTIATION AND AXONAL CONNECTIVITY IN MICE MUTANT IN THE SOX2 TRANSCRIPTION FACTOR GENE: IN VITRO AND IN VIVO STUDIES

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CHAPTER 1

GENERAL INTRODUCTION

1. The development of Central Nervous System in vertebrates

The vertebrate Central Nervous System (CNS) originates from the ectoderm, which is one of the three primordial embryonic layers together with the mesoderm and endoderm. These germinal layers are derived from the process of gastrulation that occurs at early stages during the embryogenesis, at about 6.5 days postcoitum (dpc).

In particular, at the end of gastrulation, the ectoderm differentiates in two different tissues: the epithelial ectoderm (or epiblast) that gives rise to the epidermis, and the neural ectoderm (or neuroblast) which gives rise to the nervous system.

The neural ectoderm extends along the dorso-medial embryonic region and differentiates, in the course of gestation, in the neural plate. The neural plate margins are subsequently raised to form the neural folds. The fusion of neural folds leads to the formation of the neural tube, the cavity of which is significantly larger in the more cephalic region. This process is called neurulation.

Since the early stages of CNS development, an antero-posterior and dorso-ventral regional identity is established. This is the first step for the subsequent development of the CNS. Before the fusion of neural folds is already possible to distinguish two different regions of the encephalon: the prosencephalic region (more rostrally) and the deuterencephalic region (more caudally). Through the expansion and the appearance of constrictions, primitive encephalon divides in three vesicles that give rise to the different portions of the brain: the prosencephalic vesicle, the midbrain vesicle and the romboencephalic

vesicle. Further development requires a subdivision of these vesicles: the prosencephalon divides in the telencephalon (rostrally) and in the diencephalon (caudally). The latter continues in the midbrain, followed by metencephalon and mielencephalon, which are derived from the romboencephalic vesicle and extend to form the spinal cord.

The telencephalic vesicle originates two lateral vesicles: the cerebral hemispheres. The ventral part of the telencephalon forms the corpus striatum. The dorsal telencephalon develops into archipallium, in mammalian called hippocampus, paleopallium and neopallium, which develop enormously to form cerebral cortex.

The diencephalon is anatomically divided in three areas: epithalamus, thalamus and hypothalamus along the dorso-ventral axis. Among these three structures, the thalamus is divided in a dorsal part, that processes sensory input, and a ventral part, that processes motor input.

2. Cortical and thalamic development

During the embryonic development, the forebrain is divided into two major regions, the telencephalon (rostral) and the diencephalon (caudal). The telencephalon will give the cerebral cortex; from the diencephalon develops the thalamic structure.

The cerebral cortex of mammalian brain is a complex, highly organized structure divided into discrete subdivisions (or areas) that process particular aspects of sensation, movement, and cognition. The cortex contains hundreds of different neuronal cell types and diverse range of glia (Peters and Jones, 1984).

The mechanism that control neocortical regionalization involves a rich array of signals, with interplay between intrinsic mechanisms, such as differential gene expression autonomous in neocortex, and extrinsic mechanisms, such as input from thalamocortical afferent.

The thalamus is a structure that contains multiple sensory nuclei and serves as relay station in which specific thalamic nuclei receive and project set of fibers to targeted cortical areas.

Sense organs and subcortical motor centers send input to one or more thalamic nuclei, and these nuclei have well defined reciprocal connections with the cortical regions where the sensory information are processed. The reciprocal connections have area and lamina specificity, highly conserved among species. Most of the thalamic input terminates in layer IV of the neocortex. Neurons of layer V, VI and subplate of each area send corticofugal projections to the corresponding thalamic nuclei.

2.1 Organization of cortex

At early stage of development, there is an expansion of neuroepithelium in the dorso-lateral wall of rostral neural tube. The layer adjacent the ventricle is named Ventricular Zone (VZ). The cortex, or pallium, develops from a morphological uniform VZ located in dorso-caudal part of the telencephalic vesicle.

The vertebrate CNS contains a great diversity of neurons and glial cells, which are generated in the embryonic neural tube at specific times and positions. Patterning centres, located at the perimeter of the dorsal telencephalon, produce morphogenetic diffusible molecules, which establish the differential expression of transcription factors that specify the area identity of cortical progenitors (Ragsdale and Grove,

2001). Signals of morphogenetic molecules are translated into transcription factor codes for regional specification, which leads to neurogenesis of the diversity of cell types in each brain region (Guillemot, 2007 a-b).

The first postmitotic neurons are accumulated below the pial surface forming a new layer called preplate. As the development proceed, between the VZ and the preplate forms an additional proliferative layer named Subventricular Zone (SVZ) (Bayer and Altman, 1991). Subsequently, at embryonic day 12 (E12), neurons generated in VZ/SVZ migrate using radial glia as scaffold, to form the Cortical Plate (CP) which splits the preplate into a superficial Marginal Zone (MZ) and a deep subplate. The later born neurons arrive at the cortical plate and migrate over the earliest born neuron forming the superior layers of cortex. So, the cortical plate differentiates in a deep to superficial (inside-out) pattern, forming layers from VI to II of the adult neocortex. (Bayer and Altman, 1991; Anderson et al., 2002; Xu et al., 2004). The subplate disappears after birth incorporated by layer VI (Allendoerfer and Shatz, 1994).The other five layers derived from the cortical plate. The cortex becomes also patterned along antero-posterior and medio-lateral axes (Bayer and Altman, 1991).

The cortex, also named pallium, is divided into Medial Pallium (MP), Dorsal Pallium (DP), Lateral Pallium (LP) and Ventral Pallium (VP), which give rise respectively to the hippocampus, neocortex, olfactory/piriform cortex and claustrum and part of amygdale (Puelles and Rubenstein, 2003). Each of these domains is subdivided into subdomains, such as the functional areas of the neocortex.

The neocortex is the largest region in the mammalian cerebral cortex. This is the part that shows the most extensive expansion and specialization during evolution. (Northcutt and Kaas, 1995; Krubitzer and Huffman, 2000).

Cortical areas differ by location, molecular property, histological organization, pattern of connectivity and function. Rostral region regulate motor and executive functions, caudal regions process somatosensory, auditory and visual input. These different cortical areas have a precise connectivity with nuclei in the dorsal thalamus.

In the mature cortex are distinguishable two broad classes of cortical neurons: the interneurons, that make local connections, and projection neurons, that extend axons to distant intracortical, subcortical and subcerebral targets.

2.2 Organization of thalamus

The thalamus develops from a progenitor region in the diencephalon: this region can be divided into three transverse domains: the Presumptive Pretectum (p1), the Presumptive Thalamus (p2) and the Presumptive Prethalamus (p3) (Rubenstein et al., 1994; Puelles and Rubenstein, 2003). In the alar plate of diencephalon resides the Zona Limitans Intrathalamica (ZLI) that divides p2 and p3 and functions as organizer (Vieira et al., 2005): indeed it expresses Shh, which is a key signal for patterning the thalamus. Additionally, Wnts expression is required for establishment of thalamic regional identities (Braun et al., 2003; Zhou et al., 2004) and Fgf8 controls the patterning of thalamic and prethalamic nuclei (Kataoka and Shimogori, 2008).

Thalamus and cortex develop synchronously. The majority of thalamic neurons are born between embryonic day E13 and E18,

(Altman and Bayer, 1979) which coincides with the period of neurogenesis in the cortex.

The thalamic nuclei are generated between E10.5 and E15.5 (Altman and Bayer, 1988), and are completely defined by gene expression at E15.5 (Nakagawa and O'Leary, 2001). They became morphologically distinguishable postnatally.

The Ventrobasal Nucleus (VB) is connected to Somatosensory (S1) and Motor (M1) Cortex, and the Dorsal Lateral Geniculate Nucleus (dLGN) is connected to the Visual Cortex (V1).

The Lateral Geniculate Nucleus (LGN) is generated between E12 and E14 (Lund and Mustari, 1977). Thalamus and hypothalamus can be distinguished after E12. On E13 begins to developing dorsal and ventral thalamus.

Only dorsal thalamic neurons are connected with the cortex. In addition to cerebral projection, thalamic regions send axons to striatum, amygdale, olfactory tuberculum, piriform cortex and hippocampus.

3. Neuronal differentiation and axon pathfinding

3.1 Neuronal differentiation and migration

The process that leads to the formation of the mature neurons is named neurogenesis, and consists of a progressive differentiation of the cells in the three main cell-types of the mature nervous tissue: astrocytes, neurons and oligodendrocytes.

All the cells that form the mature nervous tissue are derived from neural precursors, undifferentiated cells with high proliferative capacity. During differentiation, these cells give rise to neural

progenitors, a committed cell-type with a more restricted differentiation potential and with a limited regenerative capacity, that lead to the different cell-types of mature CNS through a process of maturation.

The differentiation reflects a qualitative change of the features (i.e. the acquisition of functional properties and the expression of specific genes by the cell), while the maturation leads to an increasing in the levels of specific genes expression.

During the differentiation process and the subsequent maturation process the cells migrate from the VZ of the neural tube to their final destination, giving rise to the specific functional areas of the CNS. Two types of migration are described: radial migration of excitatory neuron precursors and tangential migration of interneurons (see below).

The differentiation and patterning of the neural tube occurs by patterning centres that impart positional information. These neural centres produce signalling molecules which are able to impart regional identity to the various embryonic areas. These signalling molecules act according to a gradient, then, neural precursors respond differently to different concentrations of the signal undergoing to a region-specific specialization. The cells that will become part of the same defined area, will express the same specific genes that confer them characteristics closely related to the regional specificity.

Neuronal migration is the method by which neurons leave their birth place and reach their final position in the brain. In the neocortex are present two principal models of neuronal migration: the radial

migration of projection neurons ant the tangential migration of GABAergic neurons.

Radial migration. At the E12 the first postmitotic neurons begin to migrate radially outward from the VZ of the dorsal telencephalon along the ventricular-pial axis, and form the several structures that will give rise to the mature cortex in a well-described inside-out pattern. Radial glia fibers serve as scaffold for migrating cells. Most of the cells derived from dorsal proliferative zones will become pyramidal projection neurons.

Projection neurons are excitatory, glutamatergic neurons with a particular pyramidal morphology. There are three types of progenitors that give rise to this class of neurons residing in VZ/SVZ: neuroepithelial cells, radial glia and intermediate progenitors. Neuroepithelial cells are the earlier cells forming a single sheet of cells, which progressively transform in radial glia. The radial glia contributes to cortical neurogenesis (Malatesta et al., 2000; Noctor et al., 2001; Malatesta et al., 2003) generating pyramidal neurons at the apical surface of ventricular zone or producing intermediate progenitors (Noctor et al., 2004). The intermediate progenitors migrate to SVZ where they undergo a symmetric division producing two neurons (Noctor et al., 2004; Miyata et al., 2004), probably addressed to upper cortical layers. So, the production of mature neurons is tightly controlled in time from E11.5 to E17.5 (Rakic, 1974; Caviness and Takahashi, 1995), and postmitotic neurons position themselves in the developing neocortex through defined neurogenic gradients.

Tangential migration. At the beginning of neurogenesis, the proliferative zones of ventral telencephalon (the Ganglionic Eminences, GE) generate neurons which migrate tangentially into developing cortex to constitute most of GABAergic inhibitory interneurons (Anderson et al., 1997).

Interneurons are inhibitory neurons containing GABA (γ-amminobutirric acid). They comprise the 20-30% of cortical neurons. GABAergic interneurons derived from VZ/SVZ of the ventral (subcortical) telencephalon. The Medial Ganglionic Eminence (MGE) is the primary source of cortical interneurons (Anderson et al., 2001; Wichterle et al., 2001), but also Lateral Ganglionic Eminence (LGE) and Caudal Ganglion Eminence (CGE) give rise to cortical interneurons (Anderson et al., 2001; Jimenez et al., 2002; Nery et al., 2002). Within this group are recognizable subpopulations expressing distinct calcium-binding proteins (parvalbumin, calretinin, calbindin). Has been demonstrated that different interneurons subgroups have distinct spatial and temporal origin (Kubota et al, 1994; Gonchar and Burkhalter, 1997). Calretinin expressing interneurons originate within CGE, somatostatin and parvalbumin expressing interneurons derive from MGE.

Interneuron maturation in completed postnatally (Gao et al., 2000)

3.2 Axon pathfinding

In mice, between E13 and E18, neocortex and dorsal thalamus start to link with each other through reciprocal connections.

Corticothalamic and thalamocortical connections have area and lamina specificity. The thalamocortical fibers run from VB to layer IV

of S1, and from dLGN to layer IV of V1. Neurons in layer VI of the same areas project to respective thalamic nuclei.

Thalamocortical and corticothalamic projections have to cross several boundary zone to reach their final target, like Diencephalic-Telencephalic (DTB) and Pallial-Subpallial Boundaries (PSPB), which are demarcated by distinct molecular properties (Puelles et al., 2000).

The developing thalamocortical axons proceed ventrally from the dorsal thalamus and then turn dorsolaterally at the DTB to enter the Internal Capsula (IC) at E13. Then they advanced rapidly and pause before cross the PSPB at E15.

Projection originated from the preplate in the neocortex pause at PSPB at E14 (Molnár and Cordery, 1999). Projections from different cortical region arrive at this zone according the cortical developmental gradient, but the front of corticofugal projection lines up along PSPB (Molnár and Cordery, 1999). After crossing the PSPB corticofugal projections enter the IC, and in the region thalamocortical and corticofugal fibers interact and become dependent on each other, advancing intimately associated towards their targets (Molnár et al., 1998).

Certain macromolecules, diffusible or membrane bound, generated in specific region of the developing brain seem to participate in establishing the correct thalamocortical connectivity (Barbe and Levitt, 1992; Suzuki et al., 1997; Gao et al., 1998; Donoghue and Rakic, 1999). The release of attractive and repulsive factors, and axon guidance molecules, guide the growing axons though the forebrain

and help the projection to reach their specific target (O‘Leary and Nakagawa, 2002).

Some of these molecules have different function at different developmental stages, acting both attractive and repulsive guidance cues during the projection patterning.

Growing axons interact with cells resident on the future path of thalamocortical connectivity (like perirhinal cortex, thalamic reticular nucleus or ganglionic eminence); these cells contribute to guide projection along their trajectory (McConnell et al., 1989; De Carlos and O’Leary, 1992; Mitrofanis and Baker, 1993; Molnár et al., 1998).

Further, is necessary for thalamic axons to form an intimate relationship with the scaffold of preplate axons, and vice versa (Stoykova and Gruss, 1994; Hevner et al., 2002; Jones et al., 2002)

In mammals, the fibers arrive at the appropriate cortical region around E18.5, before their ultimate target neurons are born (Rakic, 1976; Shatz and Luskin, 1986; Molnár and Cordery, 1999), and they have to wait two or three days before they can establish their final connections.

4. The Sox transcription factors family

Sox genes encode a wide group of transcription factors (TFs) that play key roles in the regulation of embryonic development and in the determination of the cell fate (Kamachi et al., 2000). In fact, Sox proteins are expressed in various phases of embryonic development and cell differentiation.

All Sox proteins interact with DNA through the HMG domain (High-Mobility Group domain), allowing them to function as

transcription factors. The HMG domain encodes a 79-amino acid protein motif that binds the minor groove of DNA in a sequence-specific manner.

Initially, Sox genes were identified on the basis of their grade of similarity to the HMG domain of Sry (sex-determining region of Y chromosome) gene, which encodes for the mammalian testis-determining factor. Approximately, 26 vertebrate Sox (sry-related HMG box) genes have been identified and are classified into 7 subgroups (A-G) based on sequence identity of their HMG domain (Pevny and Placzek, 2005). The class comprising SOX1, SOX2 and SOX3, share greater than 90% amino acid residue identity in the HMG-DNA binding domain and are classified as subgroup B1. During the embryogenesis, the early onset of the expression of SoxB1 genes, directly correlates first, with ectodermal cells that are competent to acquire a neural fate, and second, with the commitment of cells to a neural fate. These data suggest a role for SoxB1 transcription factors in establishing neural fate during the embryogenesis (Pevny and Placzek, 2005).

4.1 The SoxB1 subgroup

The SoxB1 genes, Sox1, Sox2 and Sox3 are expressed throughout cells that are competent to form the neural primordium, and then become restricted to cells that are committed to a neural identity.

Sox1 is involved in neural determination, since the onset of its expression appears to coincide with the induction of neural ectoderm (Pevny et al., 1998).

In chick embryos, Sox3 is initially expressed throughout ectoderm that is competent to form nervous tissue before neural induction.

Sox2 expression marks neural primordial cells at various stages of development. Furthermore, its expression highly correlated with the multipotent neural stem cell state (see below). Because Sox2 is expressed uniformly in the early neural tube, it is regarded as a "pan-neural" marker in early embryonic stages. Another important aspect of Sox2 regulation is that its expression in the CNS is first activated upon neural induction elicited by signals from the organizer (Fernandez-Garre et al., 2002; Streit et al., 1997). Therefore, initiation of Sox2 expression must be an essential part of the mechanism of neural induction (Uchikawa et al., 2003).

After neural induction, Sox1, Sox2 and Sox3 are co-expressed in proliferating neural precursors along the entire antero-posterior axis of the developing embryo, and are detected in neurogenic regions in the postnatal and adult CNS (Pevny and Placzek, 2005). Their expression is modified by signalling molecules involved in neural induction.

Several evidences underline that SoxB1 factors are required for the maintenance of neural progenitor identity. First, two independent studies in chick embryos, have shown that SoxB1 proteins have a role in maintaining the undifferentiated state of neural progenitors (Bylund et al., 2003; Graham et al., 2003). Specifically, over-expression of SOX2 and/or SOX3 (by in ovo electroporation of chicken neural tube) inhibits neuronal differentiation of neural progenitors and causes them to retain their undifferentiated properties, including the ability to proliferate and express progenitor markers. Conversely, expression of a dominant negative form of SOX2 and/or SOX3 (interfering with the endogenous genes function) in neural progenitors results in their premature exit from the cell cycle and the onset of neuronal

differentiation, with the consequent exhaustion of neural progenitors pool. In a second study in rat embryos, investigating the molecular mechanisms regulating the conversion of Oligodendrocytes Precursors (OPCs) into multipotent Neural Stem-Like Cells (NSLCs), identified Sox2 as a key player in this process (Kondo and Raff, 2004). The conversion of OPCs into NSLCs directly depends on the reactivation of Sox2 expression, while inhibition of Sox2 expression results in premature exit from the cell cycle and neuronal differentiation of OPCs (Kondo and Raff, 2004).

SoxB1 factors must be key players in the timing of differentiation from a proliferating neural progenitor to a postmitotic neuron, regulating self-renewal, proliferation and crucial steps in several differentiation events.

4.2 The Sox2 gene

Sox2 is one of the earliest transcription factors expressed in the developing neural tube and is highly conserved among different species. This gene is composed by a single exon that encodes for a 2.4 Kb transcript. The encoded protein includes three main regions: an N-terminal hydrophobic region; a central region containing the HMG-DNA binding domain (by which the protein interacts with DNA and which is also the major interface for protein-protein interactions); an activation domain close to the C-terminus.

During mouse embryonic development, Sox2 expression is first detected in totipotent cells at the morula stage (2.5 dpc) and in the blastocyst inner cell mass (3.5 dpc). Later, Sox2 expression persists throughout the epiblast (the embryonic ectoderm, 6 dpc) and after gastrulation becomes restricted to the presumptive neuroectoderm, and

then in all the neural tube from the earliest stages of its development (neural plate, 7-7.5 dpc). In the following days of the embryonic development (by 9 dpc) Sox2 is expressed uniformly in the early neural tube (Avilion et al., 2003); it is regarded as an embryonic "pan-neural" marker. This pan-neural Sox2 expression results from the combined actions of many regulatory enhancers, each functioning in a specific area of the brain. These transcriptional enhancers correspond to extragenic sequence blocks widely conserved between different species (including chicken, mouse and human) and arranged colinearly in the different genomes (Uchikawa et al., 2003; 2004).

Mutant mice carrying Sox2-null mutation in homozygosis, failed to survive shortly after implantation (Avilion et al., 2003) because of the progressive loss of pluripotent stem cells of the epiblast. In vitro studies shown that Sox2, at early stages, is required to maintain cells of the epiblast in an undifferentiated state. In fact, in its absence pluripotent cells of the epiblast cease to proliferate and self-renew, and change their identity becoming trophoblast cells.

As the embryonic development proceeds, Sox2 expression is uniformly present in neurogenic regions: the neural plate and, thereafter, the entire neural tube. In the differentiating neural tube, Sox2 expression persist in the proliferating ventricular zone, and is diminished proceeding to the outer layers, where differentiation takes place (Ferri et al., 2004). In the adult brain, high-levels of Sox2 expression are seen in the two main adult neurogenic regions:

a) the subventricular zone (SVZ) of the lateral ventricle, from where expression extends along the entire rostral migratory

stream (RMS), along which dividing precursors migrate to the olfactory bulb;

b) the germinative layer of the hippocampus dentate gyrus.

In vitro cultures experiments, showed that, the ventricular zone cell population that expresses Sox2, in both embryos and adult mice, includes cells with functional properties of neural stem cells, i.e. self-renewal and multipotentiality (Zappone et al., 2000; Ferri et al., 2004). These results highlight that Sox2 function is related to important aspects of the biology of, at least, two types of stem cells: epiblast stem cells and neural stem cells.

In addition to neural proliferation/maintenance defects, adult Sox2 deficient mice, in which Sox2 expression is decreased by about 70%, (Sox2 "knockdown" mutants) exhibit important cerebral malformations (parenchymal and ventricle enlargement, circling behaviour and epilepsy) and neuronal abnormalities (degeneration and cytoplasmic protein aggregates) features common to different human diseases (Ferri et al., 2004). These observations suggest a role for Sox2 also in the maturation and survival of embryonic and adult neurons.

In vitro differentiation studies on neural stem cells cultured from embryonic and adult brains of Sox2 "knockdown" mutants, was observed that mutant cells produce reduced numbers of mature neurons (in particular GABAergic neurons), but generate normal glia. Most of the cells belonging to the neuronal lineage failed to progress to mature neurons showing morphological abnormalities. In vitro over-expression of Sox2 (by lentiviral infections) in neural cells at early, but not late, stages of differentiation, rescued the neuronal

maturation defects of mutant cells. Further, Sox2 over-expression suppresses the endogenous GFAP gene, a marker of glial differentiation. These results propose that Sox2 is required in early differentiating neuronal cells, for maturation and for suppression of alternative lineage markers (Cavallaro et al., 2008).

5. The Emx2 gene

The transcription factor Emx2, is one of the genes implicated in the process of "cortical arealization", which leads to the definition of the various areas composing the developing cerebral cortex (Mallamaci et al., 2000 a-b). Emx2 is a homeobox-containing TF. The homeobox sequence encodes a DNA-binding motif present in numerous proteins that regulate gene expression during development (Taylor, 1998). Functionally the homeobox proteins act as transcriptional regulators, targeting responsive genes via interaction between the homeodomain, regulatory sequences, and other cofactors.

Emx2 is expressed in dorsal telencephalon from early embryonic stages (8.5 dpc). Emx2 is expressed by progenitor cells in a low rostro-lateral to high caudo-medial gradient across the germinative ventricular zone of the cerebral cortex (Bishop et al., 2000; 2002). Its expression is maintained in adult brain neurogenic regions, the SVZ of the lateral ventricle and the hippocampus Dentate Gyrus (DG) (Gangemi et al., 2001; Galli et al., 2002). In $Emx2^{-/-}$ brains, there was a selective reduction of cortical areas with more caudo-medial identities, together with an expansion of rostro-lateral territories. $Emx2^{-/-}$ brains have a reduction in the size of the cerebral hemispheres and the olfactory bulbs. In particular, the hippocampus is greatly

reduced in size and the dentate gyrus is completely absent (Pellegrini et al., 1996; Yoshida et al., 1997). Emx2 mutant embryos also have an abnormally thick VZ in the medial embryonic cortex, and a thinner, less developed cortical plate, possibly due to a delay in cortical neurogenesis or a failure of cells to leave the cell cycle and migrate away from the VZ (Tole et al., 2000). These data suggest a dual role for the Emx2 gene: a more general effect on the patterning of forebrain regions and a more specific role in proliferation and/or specification of precursor cells of the medial cortex.

Emx2 expression is restricted to the proliferating precursors of the ventricular zone of the developing cerebral cortex and the adult brain, and is down-regulated in post-mitotic cortical neurons (Gulisano et al., 1996, Gangemi et al., 2001, Galli et al., 2002).

Emx2 regulates the proliferation of adult neural stem cells in a negative fashion, probably by diminishing their capacity for self-maintenance (Galli et al., 2002). Emx2 could be involved in pushing neural stem cells toward an asymmetric mode of cell division, increasing the proportion of more mature precursors in the cell population (Gangemi et al., 2001). Taken together these data suggest that Emx2 may be involved in the transition between neural stem cells and more mature precursors that migrate out of the ventricular zone (Gangemi et al., 2006). Again, the comparison of the expression profile of cultured neurospheres derived from wild-type and Emx2-null brain, confirmed a role for Emx2 in regulating the differentiation and migration properties of neural precursor cells.

The expression pattern of Emx2 and the defects observed in Emx2 mutant mice point to a complex regulatory role of this TF. The altered

lamination of the cortex indicates an impairment of neural migration, and the thickening of the ventricular zone suggests that a defective or delayed maturation of less mature precursor cells may be responsible for an intrinsic inability to respond to migratory cues. Under these circumstances, the higher proliferating Emx2 null cells remain in the VZ, leading to an expansion of this area, together with a reduction of the cortical areas (Gangemi et al., 2006).

The knowledge of target for Emx2 is limited to very few genes.

Different studies revealed that the spatially restricted expression of Wnt1 in the developing CNS requires Emx2 control (Iler et al., 1995; Ligon et al., 2003). The Wnt1 gene encodes signalling molecules that plays a crucial role in the establishment of the appropriate boundaries during CNS patterning (Iler et al., 1995). Emx2 is a direct repressor of Wnt1 in the developing mammalian telencephalon acting via direct binding to regulatory sequences located in the Wnt1 3' enhancer.

Emx2 could be a more general transcriptional repressor of its target genes, acting by different mechanisms. In fact, there are evidences that Emx2 represses also the activity of the FGF8 promoter induced by the transcription factor SP8, but without binding to the FGF8 promoter itself, whereas via protein to protein interaction with SP8 (Sahara et al., 2007; Zembrzycki et al., 2007).

SCOPE OF THE THESIS

The general aim of my PhD research was the study of the role of the Sox2 gene in neuronal differentiation and maturation and in the creation of axonal networking.

First I participated to work (Cavallaro et al., 2008, presented in Chapter 2) in which we performed in vitro differentiation studies on neural stem cells cultured from embryonic and adult Sox2 “knockdown” mutant brains, expressing reduced levels of Sox2. We demonstrated that Sox2 deficiency causes impaired neuronal final differentiation. In particular, I contributed to this work studying ex vivo cultures of neurons explanted from newborn mice cortex. By immunofluorescences I found that the neuronal population explanted from mutant brains revealed a reduction in number of cells positive for GABAergic markers. These results, together with the in vivo observation of a reduced number and abnormal arborization of GABAergic neurons in adult cortex, suggest a role for Sox2 in differentiation of at least one neuronal subpopulation: the GABAergic inhibitory neurons.

In the second part of this work (Mariani et al., submitted, presented in chapter 3) I contributed to the study of interactions between Sox2 and others transcription factors in vivo. The study on Sox2 “knockdown” mutants had revealed that in postnatal hippocampus the population of neural stem cells (NSC) is significantly reduced. Emx2 mutant mice show delayed hippocampal development, and in vitro, mutant $Emx2^{-/-}$ NSC show increased proliferation in long term neurosphere cultures. By the study of double mutant mice expressing

reduced levels of both Sox2 and Emx2 we found that Emx2 deficiency counteracts (at least in part) the effects of Sox2 deficiency on neural stem cell proliferation ability in the postnatal hippocampus, and also rescued other brain morphological abnormalities of Sox2-deficient mutants. The parallel study of double mutant mice expressing reduced levels of both Sox2 and Pax6 showed no differences as compared with the Sox2 “knockdown” alone. This work allowed to conclude that Emx2 may controls NSC decision, acting like Sox2 negative modulator, and a reduction of 50% in Emx2 expression can restore Sox2 controlled functions, at least with respect to NSC.

The goal of my main project (ongoing work, presented in chapter 4) is to study the ability of projection neurons to reach their specific target in Sox2 mutant brains. Previous work had demonstrated that loss of Sox2 causes defective maturation of cortical GABAergic interneurons. Projection neurons are another subset of cortical neurons, included in the glutamatergic neurons family. This work shows that a reduction or ablation of Sox2 expression leads to abnormalities in corticofugal axonal growth. Corticothalamic projection neurons are not able to reach their thalamic nuclei target, independently by the cortical area from which they start. Also, I demonstrated that the defect does not appear to reside in a cortical role of Sox2, as in a cortical specific involvement in differentiation of projection neurons. The role of Sox2 deficiency in thalamus (where Sox2 is expressed in neurons), in particular with respect to the possibility of altered patterning or altered expression of attracting/repulsive cues, remain to be investigate.

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CHAPTER 2

IMPAIRED GENERATION OF MATURE NEURONS BY NEURAL STEM CELLS FROM HYPOMORPHIC SOX2 MUTANTS

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Impaired generation of mature neurons by neural stem cells from hypomorphic Sox2 mutants

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Abstract

The transcription factor Sox2 is active in neural stem cells, and Sox2 "knockdown" mice show defects in neural stem/progenitor cells in the hippocampus and eye, and possibly some neurons. In humans, heterozygous Sox2 deficiency is associated with eye abnormalities, hippocampal malformation and epilepsy. To better understand the role of Sox2, we performed in vitro differentiation studies on neural stem cells cultured from embryonic and adult brains of "knockdown"

mutants. Sox2 expression is high in undifferentiated cells, and declines with differentiation, but remains visible in at least some of the mature neurons. In mutant cells, neuronal, but not astroglial differentiation, was profoundly affected. β-Tubulin-positive cells were abundant, but most failed to progress to more mature neurons, and showed morphological abnormalities. Overexpression of Sox2 in neural cells at early, but not late, stages of differentiation, rescued the neuronal maturation defect. In addition, it suppressed GFAP expression in glial cells. Our results show an in vitro requirement for Sox2 in early differentiating neuronal lineage cells, for maturation and for suppression of alternative lineage markers. Finally, we examined newly generated neurons from Sox2 "knockdown" newborn and adult mice. GABAergic neurons were greatly diminished in newborn mouse cortex and in the adult olfactory bulb, and some showed abnormal morphology and migration properties. GABA deficiency represents a plausible explanation for the epilepsy observed in some of the knockdown mice, as well as in SOX2-deficient individuals.

Introduction

Sox genes (Gubbay et al., 1990) encode transcription factors that regulate critical developmental decisions (Kamachi et al., 2000; Wilson and Koopman, 2002; Wegner and Stolt, 2005). In mouse, Sox2 is expressed in, and essential for, multipotent stem cells of the blastocyst inner cell mass, and its ablation causes early embryonic lethality (Avilion et al., 2003).

In the nervous system, Sox2 is expressed, and is functionally important, at the earliest developmental stages, in both chick and

Xenopus (Kamachi et al., 2000; Pevny and Placzek, 2005; Wegner and Stolt, 2005). In humans, Sox2 neural expression is conserved, and heterozygous *SOX2* mutations cause hippocampal defects, forebrain abnormalities and anophtalmia (Fantes et al., 2003; Sisodiya et al., 2006; Kelberman et al., 2006). In the mouse nervous system, Sox2 is expressed in stem cells and early precursors, and in few mature neurons (Zappone et al., 2000; Ferri et al., 2004). Adult Sox2-deficient mice, in which Sox2 expression is decreased by about 70%, exhibit neural stem/precursor cell proliferative defects in the hippocampus and periventricular zone (Ferri et al., 2004). Moreover, neurons containing neurofilament/ubiquitin-positive aggregates are observed, together with dead neurons, in thalamic and striatal parenchyma, which are already substantially reduced in size at early developmental stages. These observations point to a possible role for Sox2 in the maturation and/or survival of embryonic and adult neurons. In these mutant mice, abnormalities of ependyma and choroid plexi (the source of growth and trophic factors/signalling molecules) (Lim et al., 2000) were also observed (Ferri et al., 2004). This raises the issue of whether neuronal defects observed in vivo represent an intrinsic defect, or a response to abnormalities in the environment.

We performed in vitro differentiation studies on neurosphere-derived neural cells. Neural stem cells from Sox2-deficient mice produce reduced numbers of mature neurons, but generate normal glia. Normal Sox2 levels are required at early differentiation stages. In vivo, subsets of GABAergic neurons are affected.

Materials and Methods

Neural stem cell culture and differentiation

Neurosphere cultures were derived from adult or E14.5 mouse forebrain (Zappone et al., 2000; Ferri et al., 2004). For differentiation, neurospheres were dissociated to single cells, and plated onto MATRIGEL (Becton-Dickinson)-coated chambered slides (LabTec, Nunc) at 1-5 x 10^4 cells/cm^2 (Zappone et al., 2000; Gritti et al., 1996, Gritti et al. 2001), with bFGF only as mitogen. After 3 days, the medium was changed to neural stem cell medium without bFGF, supplemented with 1% foetal calf serum (FCS). After further six days (differentiation day 9), cells were analyzed by immunocytochemistry.

Immunocytochemistry and immunohistochemistry

Immunocytochemistry was as described by Zappone et al. (Zappone et al., 2000). For single-cell Sox2 immunofluorecence quantitation, see Fig. S2 in the supplementary material. Apoptosis was assayed by the DedEnd Fluorimetric TUNEL system (Promega). Immunohistochemistry and BrdU labeling were as in Ferri et al. (Ferri et al., 2004); in the latter, sacrifice was 3 days after the last injection. Five olfactory bulb sections (20 µm; 1 every 16) were counted per animal.

Antibodies

Primary antibodies were: mouse anti-β-tubulin III (Covance 1:500), rabbit anti-β-tubulin III (Covance 1:2000), rabbit anti-calretinin (Chemicon 1:1000; 1:500 for immunohistochemistry), rabbit anti-connexin 43 (Sigma 1:2000), rabbit anti-GABA (Sigma 1:2000),

mouse anti-GALC (Chemicon 1:200), mouse anti-GFAP (Sigma 1:400), rabbit anti-GFAP (Zymed 1:100), mouse anti-GFP (Molecular Probes 1:100), rabbit anti-GFP (Molecular Probes 1:300), mouse anti-MAP2 (Biomeda 1:100), mouse anti-MAP2 (Immunological Sciences 1:200), rabbit anti-MAP2 (Chemicon 1:1000), mouse anti-nestin (Chemicon 1:200), mouse anti-NeuN (Zymed 1:100 or Chemicon 1: 400, for immunohistochemistry), mouse anti PSA-NCAM (AbCys 1:800), rabbit anti-Sox2 (Chemicon 1:200 or 1:500 for immunohistochemistry), mouse anti-Sox2 (R&D 1:10 or 1:50 for immunohistochemistry), rabbit anti-S100 (DakoCytomation, 1:400) and mouse anti-RC2 [Developmental Hybridoma Bank (ascites fluid) 1:250]. Secondary antibodies were: anti rabbit or anti mouse Alexa 488 (green) or Alexa 594 (red) (Molecular Probes 1:1000-1:2000), anti rabbit or anti mouse FITC or TRITC (Jackson 1:200).

For immunofluorescence, 4% paraformaldehyde-fixed cells were pre-incubated with 10% FCS, 0.2% Triton X-100 in PBS for 30-60 minutes at room temperature, than the primary antibody was added (in 10% FCS in PBS) and left overnight at 4°C (or 1 hour at 37°C); cells were washed in PBS, the secondary antibody was added (in 10% FCS in PBS) for 1 hour at room temperature, followed by wash in PBS, DAPI nuclear counterstaining (4-8 minutes), and mounting in Fluorsave. Cells immunopositive for the various markers were counted under a fluorescence microscope; a minimum of 3000 total cells distributed on five fields was evaluated. Negative controls (equal cell samples treated the same way but omitting the primary antibody) were always performed in parallel for each reported experiment, and gave no signal.

RT-PCR

DNAse-treated RNA was reverse transcribed and assayed by PCR for *Sox2* as described by Zappone et al. (Zappone et al., 2000). Results were normalized using 18S RNA primers:

5'TTTCGGAACTGAGGCCATGATTAAG3'

and 5'AGTTTCAGCTTTGCAACCATACTCC3'.

Chromatin immunoprecipitation (ChiP), electrophoresis mobility shift (EMSA) and transfections

For ChIP, see Weinmann and Farnham (Weinmann and Farnham, 2002). Antibodies were anti-Sox2 (R&D) and rabbit anti-SV40 large-T (Santa Cruz). Primers for GFAP upstream region were 5'AAAGAATTCCCTGTGTTAGTCAGGGTTCTCTAG3' and 5'AAACTCGAGTACAGTGAAT- GGGTAATAAAAATA3'. For SRR2 and nestin primers, see Miyagi et al. (Miyagi et al., 2006). For EMSA, see Catena et al. (Catena et al., 2004). Oligonucleotides are shown in Fig. 9.

For P19 transfection, the 0.6 Gfap region (Fig. 9; amplified with above ChIP primers) was cloned upstream to the TK promoter in the TK-luciferase vector (Miyagi et al., 2006). P19 cells ($5x10^5$), plated the previous day in 3 cm dishes, were transfected with 0.5 μg luciferase reporter and 0.5 μg Sox2 expression vector (the CMV-Sox2-GFP lentiviral genome described below, or the same empty vector) using Lipofectamine 2000 (Invitrogen). Lysates were assayed for luciferase (Promega-E1980 kit) after 24 hours.

Sox2 lentiviral transduction

The Sox2 cDNA (*Xho*I-*Bsu*36I 1.3kb fragment) was cloned into the pRRLsin.PPT.CMV.NTRiresGFPpre lentiviral vector (Brunelli et al., 2007), between the CMV promoter and the IRES-GFP. The same vector, empty or carrying a Cre gene, was used as negative control (with comparable results). Lentiviruses were prepared as described by Brunelli et al. (Brunelli et al., 2007). Cells were transduced at MOI 100 at day 1 or 4 (Fig.1A) overnight. The following day the medium was changed to proliferation (day 1 transductions) or differentiation medium (day 4 transductions), and differentiation continued to day 9.

Primary cultures of cortical neurons

P0 Cortical neurons (Wagenaar et al., 2005, Li et al., 2005) were plated on polyethyleneimine-laminin-coated slides at 10^6 cells/ml. After 3hours, the plating medium was replaced with Neurobasal medium with B27, 1mM glutamine, 5ng/ml bFGF. The culture was maintained for 4-10 hours, prior to fixation with 4% paraformaldehyde.

Results

In vitro differentiation of normal and mutant neurospheres

Neurosphere cultures were derived from the subventricular zone (SVZ) of adult normal and Sox2-hypomorphic mice, carrying a null allele (Sox2$^{\beta\text{-geo}}$) together with a “knockdown” allele (Sox2$^{\Delta ENH}$) (Ferri et al., 2004). The null allele is a “knock-in”, where the β-geo gene replaces Sox2. In the “knockdown” allele an upstream Sox2 enhancer

is deleted. The level of Sox2 mRNA in Sox2$^{\beta\text{-geo}/\Delta ENH}$ neurosphere cultures is 25-30% of the wild type (Ferri et al., 2004).

In vitro, the growth (Zappone et al., 2000) of undifferentiated cultures (measured as numbers of total cells, or neurospheres) from mutant mice was not significantly different from that of normal controls (not shown).

Differentiation was carried out according to Gritti et al. (Gritti et al., 1996; Gritti et al., 2001) (Fig.1A). Undifferentiated neurospheres, dissociated to single cells, were made to adhere to slides, in the presence of bFGF. After 3 days, bFGF was removed, and 1% FCS was added, leading to differentiation within 9 days from initial plating. We studied differentiation of neurons and glia, as well as Sox2 expression, during this time window. For Sox2 evaluation, we used mouse monoclonal (R&D) and rabbit polyclonal (Chemicon) antibodies, of which we carefully confirmed the specificity (Fig.1B; see Fig. S1 in the supplementary material) by testing wild-type cells versus Sox2 conditionally deleted (null) cells.

Sox2 expression during in vitro NSC differentiation

In undifferentiated neurospheres, Sox2 is expressed, together with nestin (a marker of undifferentiated precursors) in virtually all cells (not shown). In differentiating cells, Sox2 is expressed at variable levels (dim to bright) in most cells until day 9, although the bright population was much reduced after differentiation day 1 (Fig.1C; see Fig. S2 in the supplementary material); nestin colocalized with Sox2 at day 1 (Fig.1C) but disappeared in most cells by day 3 (see Fig. S4 in the supplementary material). This result is mirrored by a 80%

reduction of Sox2 mRNA in differentiated cells (Fig.1D). In mutant cells, at the beginning of differentiation, Sox2 mRNA (Ferri et al., 2004) and protein (Fig.1E) are lower than in normal cells, as expected. By single-cell immunofluorescence, at day 1, the Sox2-bright population is much decreased in mutant cells; between days 5 and 9, the difference between normal and mutant cells is progressively reduced (see Fig. S2 in the supplementary material).

β-Tubulin-positive cells (neuronal lineage) appear towards day 5, and persist until day 9; MAP2, a more differentiated marker, is well visible at day 9. Neuronal lineage cells express relatively high levels of Sox2 (Fig. 2A,B); however, not all Sox2-bright cells expressed these markers. Similarly, the few GALC-expressing cells (oligodendrocytes) clearly retained Sox2 expression (Fig. 2C). However, the predominant population of (GFAP-positive) astroglia exhibited little Sox2-fluorescence (however, glial nuclei are more expanded than other nuclei, and thus may tend to be less Sox2 bright) (Fig. 2D). As in wild-type cultures, most mutant MAP2-positive (Fig. 2B) and β-tubulin- and GALC-positive cells (see Fig. S2 in the supplementary material and data not shown) retained significant, though slightly decreased (see Fig. S2C in the supplementary material), Sox2 expression.

Sox2 mutant neural stem cells generate morphologically immature β-tubulin III-positive neurons

In cultures from normal adults, most neuronal cells show mature morphology, with extensive arborization, at differentiation day 9 (Fig. 3A,B, left). However, in mutant cultures, β-tubulin-positive cells with

developed arborization were very rare (Fig. 3A,B, right) and most (undeveloped) β-tubulin-positive cells showed much weaker staining (Fig. 3A). Thus, although the total number of β-tubulin-positive cells is similar between normal and mutant cultures, the absolute number of morphologically "mature" mutant neurons is strikingly decreased (see Table S1 in the supplementary material; Fig. 3).

Sox2 is important for the in vitro generation of mature neurons, but not of glia

The immature morphology of mutant β-tubulin-positive cells correlates with impaired expression of mature neuronal markers (Fig. 4). In normal cells, most β-tubulin-positive cells were positive for NeuN (80%) or MAP2 (60%) (Fig. 4, see Table S1 in the supplementary material), whereas in the mutant, cells positive for β-tubulin/NeuN, β-tubulin/MAP2 and PSA-NCAM were strikingly decreased (Fig. 4). We obtained similar results using cultures from E14.5 forebrains (not shown).

Differentiated neuronal cells express the GABA neurotransmitter (Fig. 5) (Gritti et al., 1996; Gritti et al., 2001), and Ca^{2+}-binding proteins (calretinin and calbindin), which define inhibitory neurons and their different subpopulations (Wonders and Anderson, 2006; Levitt et al., 2004; Makram et al., 2004). We evaluated, at day 9, the number of cells expressing GABA or calretinin as a proportion of β-tubulin or MAP2-positive cells (Fig. 5; see Table S1 in the supplementary material). Only cells giving strong signals, covering cell body and processes, were scored positive. In both embryonic and adult cultures from normal mice, most of the strong β-tubulin- or MAP2-positive cells were also GABA positive (Fig. 5; see Table S1

in the supplementary material); a few GABA-positive cells (10-15% of the GABA-positive population) were MAP2 negative. In the mutant, most of the (rare, see Table S1 in the supplementary material) MAP2- and (well-developed) β-tubulin-positive cells were also GABA positive, as in the normal cells, but absolute numbers were reduced by more than ten times (Fig. 5); in addition, many GABA-positive cells were MAP2 negative (Fig. 5). Similarly, calretinin expression in the normal cells was frequent in MAP2-positive cells (30-40%), whereas in the mutant it was very rare (Fig. 5; see Table S1 in the supplementary material).

We further studied differentiation into GFAP-positive astroglia, and GALC-positive oligodendroglia. Contrary to results with neuronal differentiation, GFAP-positive cells with mature astroglia morphology were detected in similar proportions in cultures from normal and mutant cells (not shown and see Table S1 in the supplementary material).

Unexpectedly, in mutant cultures, some (~30%) of the β-tubulin-positive cells also showed clear, although quite low, GFAP expression (Fig. 6). These cells often showed some neuron-like arborization (Fig. 6, rows 2, 3), but it was not as developed as in wild type β-tubulin-positive cells; however, these cells were obviously distinguished from normal astrocytes, which were highly GFAP-positive (but β-tubulin-negative) and morphologically well developed (Fig. 6, row 4). In normal cultures, we never observed such cells, although a very low proportion of β-tubulin-positive cells (~3%) showed double staining (Fig. 6, top, arrowhead); these cells, however, were very poorly developed, and might represent an early maturation stage.

Interestingly, β-tubulin/GFAP double-positive cells were observed in differentiated cultures of glioblastoma multiforme neural stem cells (Galli et al., 2004; Lee et al., 2006a). Notably, these cells aberrantly express Sox2 (Hemmati et al., 2003; Lee et al., 2006a; Nicolis, 2007; Pomeroy et al., 2002). Finally, oligodendrocytes were slightly reduced (not shown; see Table S1 in the supplementary material).

The observed results are neither caused by differentiation delay nor by increased apoptosis of mutant cells, as indicated by normal kinetics of nestin and β-tubulin expression and by TUNEL assays (see Fig. S4 in the supplementary material). In conclusion, Sox2 is important mainly in neuronal, but not in astroglial differentiation.

High levels of Sox2 are required at early, but not late stages of neural differentiation

As shown above, Sox2-mutant cells show significantly lower levels of Sox2 than normal cells at the onset of differentiation (Fig. 1E, see Fig. S2 in the supplementary material); but not at later stages (see Fig. S2A-C in the supplementary material).

To evaluate if restoration of Sox2 levels might rescue the differentiation defect of mutant cells, we used a Sox2-IRES-GFP lentiviral construct. We transduced mutant cells at the end of day 1 after plating (Fig. 1A); after 16 hours, we washed the well to remove the virus, adding fresh medium to allow differentiation to proceed until day 9. Control cells were treated similarly, without virus or with control virus expressing only GFP. In an alternative experiment, cells were transduced at day 4, after the switch from mitogen-containing medium to mitogen-free, serum-containing medium. A high proportion (75-80%) of the cells were transduced, expressing GFP and

Sox2 (Fig. 7A). Transduction at day 1 did not change the overall number of β-tubulin-positive cells, but resulted in a dramatic increase in the proportion of well-arborized β-tubulin-positive cells (Fig. 7B,C,D), and of cells expressing the more mature MAP2 marker (Fig. 7C,D).

Importantly, well-arborized morphology in β-tubulin or MAP2-positive cells was observed almost exclusively in efficiently transduced (i.e. GFP-positive) cells (Fig. 7C; arrowheads). Most of the untransduced (GFP-negative) β-tubulin-positive cells showed poor arborization (Fig. 7C; arrow). This latter result represents an "internal" control, indicating that the rescue of the normal phenotype is due to viral-dependent expression, but not to any "environmental" change (caused by the transduction procedure) affecting the efficiency of differentiation. Moreover, control virus expressing GFP but not Sox2 had no effect (Fig. 7B,D). In contrast to the results obtained when the virus was transduced at day 1, no significant effect of Sox2 transduction was observed at day 4 (Fig. 7B,D). Thus, appropriate Sox2 levels are required at a crucial early stage of differentiation.

Ectopic Sox2 represses GFAP expression in differentiating cells

We further examined the astroglia population from cultures transduced with the Sox2-GFP-expressing lentivirus. Unexpectedly, cells expressing high levels of GFP (thus presumably of Sox2) showed reduced or no GFAP expression, while retaining astroglia morphology (Fig. 8A, left) and expression of astrocyte markers S100 and connexin 43 (Fig. 8B; see Fig. S3 in the supplementary material); by contrast, cells that had not been transduced showed the expected astroglia morphology with high GFAP expression (Fig. 8A, left). The loss of

GFAP expression is not due to toxicity from high levels of GFP, as cells transduced with a GFP-lentivirus without the Sox2 gene were not affected (Fig. 8A, right). Furthermore, the inhibitory effect of excess Sox2 levels on GFAP expression was observed both when the virus was added at day 1 and at day 4 (Fig. 8A).

This surprising result prompted an investigation of the possibility that Sox2 might directly affect GFAP expression. Upstream to the GFAP promoter (Morita et al., 1997; Kuzmanovic et al., 2003) lies a region containing three potential consensus Sox2-binding sites (conserved between mouse and man) (Fig. 8C). We cloned this region upstream to the thymidine kinase (TK) minimal promoter, linked to a luciferase reporter, and transfected this construct into P19 embryonic carcinoma cells, together with a Sox2 expression vector or, as control, the same vector without Sox2. The upstream promoter region stimulated luciferase activity by twofold in the absence of Sox2; however, the stimulation was abolished by Sox2 overexpression (Fig. 8D). This suggests that Sox2, expressed at high levels, is a repressor at this regulatory element.

In gel shift analysis (Fig. 8E), recombinant Sox2 (expressed in COS cells) or endogenous Sox2 from P19 cells (Fig. 8E left panels, lanes 1, 4) forms a retarded complex with a GFAP probe containing the two upstream putative Sox2 sites. This complex has mobility similar to that formed on a bona fide Sox2-binding site from an Oct4 gene enhancer (Chew et al., 2005) (Fig. 8E, left panels, Oct4 probe, lanes 2, 5). The complex was abolished by mutation of the Sox2 sites of the probe (MutGfap, lanes 3, 6) and by competition with excess unlabelled Oct4 (not shown) and wild-type, but not mutant, GFAP

oligonucleotide (Fig. 8E, right). Furthermore, in in vivo chromatin immunoprecipitation (ChIP) experiments, an anti-Sox2 antibody specifically precipitates the upstream GFAP regulatory region in chromatin from both P19 (which express Sox2) and embryonic (E12.5) neural tube cells (Fig. 8F). Control experiments with other Sox2-binding sequences (SRR2 and nestin) indicate that the anti-Sox2 antibody correctly precipitates these chromatin regions in P19 and spinal cord cells, respectively, although SRR2 is not precipitated in spinal cord cells, as expected (Miyagi et al., 2006). These experiments, which demonstrate binding of Sox2 to the GFAP upstream region in vivo and in vitro, and Sox2-dependent transcriptional inhibition (Fig. 8C-F), demonstrate that the repression of GFAP by Sox2 shown in differentiating neural cells (Fig. 8A) may be mediated, at least in part, by direct Sox2 regulation of transcription.

In vivo analysis of neurons in mutant mice

In vitro studies provided three main observations: (1) mutant cells show impaired neuronal maturation, with cells exhibiting abnormal morphologies; (2) GABAergic markers are significantly reduced; and (3) Sox2 levels are higher in early than in more differentiated neural cells, but significant Sox2 protein is retained in many neurons.

To analyze in vivo neuronal differentiation, we examined cortical neurons of newborn mice and newly generated rostral migratory stream (RMS) neurons. P0 cortical neurons derive from embryonic radial glia, and had only a few days to mature since their terminal cell division. Neurons, made to adhere to slides, were stained for neuronal markers. Most cells were positive for β-tubulin and MAP2 at variable intensities and had comparable levels of staining between normal and

mutant brains (see Fig. S5 in the supplementary material). However, GABA-positive and calretinin-positive cells were decreased by 50-60% in mutant cortical cells (Fig. 9A-C), confirming a defect, in mutant brain in vivo, of at least one class of mature neurons: the GABAergic neurons.

Cortical GABAergic neurons originate from precursors in the ganglionic eminences, which migrate after terminal division by tangential routes (Makram et al., 2004; Wonders and Anderson, 2006). In normal E17.5 embryos, we found several calretinin-positive (i.e. GABAergic) cells within the cortical plate (Fig. 10A-D), whereas in mutant embryos calretinin-positive cells were detected along subcortical fiber bundles but were very scarce or absent in the cortical plate (Fig. 10E-H). This migration abnormality might be part of the suggested differentiation defect. GABA staining at the same stage reveals a disorganized labeling pattern of GABAergic neurons in the mutant (Fig. 10I-N). GABAergic cells which reach their final destination in the cortex progressively develop postnatally into several more mature interneurons subtypes, which include calretinin-positive ones (Markram et al., 2004; Wonders and Anderson, 2006). In adult mutant cortex, calretinin-positive cells showed significant abnormalities, such as reduced dendritic and axonal arborizations (Fig. 11). In conclusion, a subpopulation of embryonically generated neurons (GABAergic neurons) is not only decreased in numbers in postnatal cortex, but also shows significant morphological abnormalities in embryo and adult.

In adult mouse, stem cells within the SVZ generate neurons (many of them GABAergic) that migrate to the olfactory bulb, where they

complete differentiation with the expression of mature markers (NeuN in all neurons, calretinin and calbindin in GABAergic neurons subclasses) (Doetsch, 2003; Lledo et al., 2006). We administered BrdU to adult mice, and measured the proportion of NeuN-positive cells within the BrdU-positive population in the olfactory bulb. The newly generated neurons (BrdU/NeuN-positive cells) are substantially (~40%) decreased in granule (GL) and in periglomerular (PGL) layers of mutant mice (Fig. 12A), indicating a significant maturation defect.

Does this maturation defect result in reduced steady-state levels of GABAergic neurons? Calretinin-positive cells are strongly decreased (40%) within the most external (periglomerular) layer, where mature calretinin-positive cells reside (Fig. 12B). This suggests that mutant cells destined to develop as calretinin-positive cells in the periglomerular layer may fail to reach it and/or complete their maturation. Additionally, calretinin-positive cells in the external layers of the olfactory bulb showed an important decrease in their degree of arborization (Fig. 12C).

Discussion

In mouse, Sox2 deficiency causes defects in adult hippocampal and subventricular zone stem/progenitor cells, decreased neurogenesis and neuronal defects (Ferri et al., 2004). Here, we show that normal Sox2 levels are essential for proper neuronal differentiation in vitro and, in vivo, for at least one class of neuron, the GABAergic neuron.

Sox2 is expressed in differentiating neural cells in vitro

In vitro, Sox2 expression is high in undifferentiated cells, significantly declines during differentiation, but is not completely extinguished in many cells (Figs 1, 2). The observed Sox2 expression is not due to antibody crossreactions, as shown by control experiments, using Sox2-null neural cells. (Fig. 1B; see Fig. S1 in the supplementary material), and by RT-PCR (Fig. 1D). This agrees with Bani-Yaghoub et al. (Bani-Yaghoub et al., 2006), who showed significant Sox2 expression in P3 cortex (glia and neurons), relative to high levels in embryonic cortex (mostly neural precursors).

Both in vitro and in vivo, Sox2 expression is decreased in the mutant, although much more in early than in more mature cells (Fig. 1E; see Figs S2, S5 in the supplementary material). It is possible that the enhancer that is deleted in the knockdown allele may be less relevant in mature cells, allowing some compensation. Notably, in vivo (Ferri et al., 2004) (see Fig. S5 in the supplementary material) Sox2 expression is maintained in subsets of differentiated neurons, within P0 cortical neurons, in adult SVZ-generated precursors/neurons in the olfactory bulb and in other cells. In the mutant, Sox2 is already decreased within early precursors, but much less significantly in neurons (see Fig. S5 in the supplementary material), in agreement with the in vitro observations.

Sox2 is important at early stages of neuronal differentiation in vitro

In vitro, Sox2-deficient cells exhibit a striking differentiation defect, characterized by abnormal morphology and decreased expression of mature differentiation markers. As the defect is apparent at

differentiation day 5 (Fig. 3C), Sox2 is already required at early stages. This is confirmed by the in vitro rescue experiment with a Sox2-expressing lentivirus (Fig. 7). Sox2 overexpression in mutant cells at the onset of differentiation is necessary to rescue the well-arborized β-tubulin-positive, MAP2-positive phenotype observed in normal, but not mutant cells. However, late expression does not rescue the phenotype (Fig. 7). Preliminary data (in preparation) indicate that neurons originate only from cells that are still dividing at early differentiation stages (day 2, but not day 4); moreover, progenitors at early, but not late stages, express transcription factors known to be involved in neuronal differentiation. Correct expression of Sox2 at early stages may be required to establish a downstream transcriptional program for differentiation, perhaps by generating a "poised" chromatin structure at loci crucial for subsequent neuronal development (as exemplified for Sox2 itself in ES cells) (Boyer et al., 2005; Boyer et al., 2006a; Boyer et al., 2006b; Szutoriz and Dillon, 2005; Azuara et al., 2006; Bernstein et al., 2006; Lee et al., 2006b). When such a program is compromised by insufficient Sox2 levels, as in the mutant, all successive maturation steps (from β-tubulin to MAP2/NeuN expression) would be altered. Indeed, clearly decreased levels of Sox2 are found, in the mutant, at early, but not at late, stages of neurogenesis. (Fig. 1E; see Figs S2, S5 in the supplementary material).

The rescue experiment, while highlighting an essential role of Sox2 in early cells, does not rule out additional, but not yet demonstrated, roles of Sox2 at later stages, as suggested by the presence of Sox2 in well-developed MAP2-positive cells in vitro (Fig. 2) and a few

neurons in vivo (see Fig. S5 in the supplementary material) (Ferri et al., 2004).

In the mutant, some cells with poorly developed neuronal morphology co-express a neuronal (β-tubulin) with a glial (GFAP) marker (Fig. 6). In neuronal committed cells, Sox2 might act to repress part of a gliogenic transcription program. Indeed, Sox2 binds to the GFAP promoter in vitro and in vivo (Fig. 8E,F); moreover, when overexpressed, it silences the endogenous GFAP activity in differentiating neural cells (Fig. 8A), and inhibits a co-transfected GFAP promoter-driven reporter transgene (Fig. 8D). Thus, at least part of the Sox2-dependent inhibition of GFAP is explained by a direct repressor activity of Sox2.

We hypothesize that Sox2 has a dual role in neural cell differentiation; in early precursors committing themselves to neurogenesis, it "programs" later neuronal differentiation events, while repressing some alternative (glial-specific) transcription programs. In cells undergoing gliogenesis, its decline would allow proper glial-specific gene expression. Similar models have been proposed for other differentiation systems (Enver and Greaves, 1998; Hu et al., 1997; Laslo et al., 2006; Mikkola et al., 2002; Nutt et al., 1999). In mutant neural precursors, Sox2 levels would be too low to upregulate the neuronal differentiation program efficiently and/or to switch-off the glial program.

Different roles for Sox2 in stem and in differentiating cells?

An important role of Sox2 in neural stem/precursor cells proliferation/maintenance was identified previously (Graham et al., 2003; Bylund et al., 2003; Ferri et al., 2004). This is consistent with

the high level of Sox2 detected in such cells (Fig. 1B-E; see Fig. S5 in the supplementary material). Our present results point to an additional role of Sox2 in differentiated cells. Sox2 might participate in different networks of transcription factors in stem versus differentiating cells. A precedent exists for Oct4, a factor co-expressed with Sox2 in ES cells, the levels of which affect both pluripotency and differentiation (Niwa et al., 2000).

Graham et al. (Graham et al., 2003) and Bylund et al. (Bylund et al., 2003) showed that increasing Sox2 levels in normal chick embryo neural tube prevents their initial (day 1) differentiation into β-tubulin-positive cells and maintains their self-renewal. Bani-Yaghoub et al. (Bani-Yaghoub et al., 2006) obtained similar results in embryonic neural precursors in vitro. These results are apparently at variance with our observation that Sox2 overexpression in Sox2-mutant cells increases their differentiation (Fig. 7).

Several important differences in species, cellular models, stages and differentiation techniques may explain these discrepancies. In particular, we transduced Sox2 in cells that had previously been induced to initiate differentiation by adherence to matrigel, whereas the above-mentioned authors overexpressed Sox2 in proliferating early precursors prior to their entry into differentiation. Furthermore, most importantly, we overexpressed Sox2 in mutant cells that already have an abnormally low Sox2 level, whereas the above authors overexpressed Sox2 in wild-type cells expressing the physiological level of Sox2. Thus, the rescue we observe may simply reflect the reestablishment of Sox2 levels appropriate for differentiation in cells that already entered the differentiation pathway; the fact that the

majority, but not all, of the transduced cells were rescued may indicate the need for a critical Sox2 level, that is neither too low (as in some transduced cells, Fig. 7A) nor too high. By contrast, their results may be due to Sox2 levels too high to allow entry of stem and early precursor cells into the differentiation pathway.

Sox2 overexpression in mutant cells did not change the balance between neuronal (as measured by β-tubulin expression) and glial cells. Rather, it modulated their differentiated characteristics (increased neuronal maturation, decreased glial GFAP expression). Thus, Sox2 does not control the choice between neuronal and glial differentiation.

In vivo defects in a subset of neuronal cells

In agreement with in vitro neural defects, we detect, in vivo, significant abnormalities of a subset of neurons, GABAergic neurons. These are decreased by 40-60% in P0 cortical cells and in the olfactory bulb, indicating that both embryonic and adult genesis of this neuronal type is compromised (Figs 9, 12). Additionally, we detect morphological abnormalities in embryonic GABAergic neurons, during their migration to the cortex from the ganglionic eminences, and in early postnatal cortex (Figs 10, 11), as well as, to a lower extent, in newly generated calretinin-positive cells in the adult olfactory bulb (Fig. 12C). These results confirm the in vitro results (Figs 3, 4 and 5) and extend preliminary in vivo evidence of loss of neural parenchyma and reduced maturation of postnatal neurons (Ferri et al., 2004).

From a quantitative point of view, the overall population in the P0 cortex and postnatal olfactory bulb is not as deeply affected as in the

in vitro experiments. We suggest several, not mutually exclusive, explanations for this discrepancy.

First, only selected neuronal populations may be vulnerable to low Sox2 dosage; these might be more represented in vitro than in vivo. Indeed, in vivo, among the neuron types tested, only the GABAergic subset is detectably compromised; significantly, in our in vitro system, the majority of differentiated neurons are of this type (Fig. 5) (see Gritti et al., 2001; Conti et al., 2005).

Second, in vitro stem cells may differ to some extent from in vivo stem cells. Indeed, most bona fide in vivo stem cells are in a low cycling state, and are a radial glia cell type (Doetsch, 2003), whereas in vitro stem cells are highly proliferating. Moreover, many in vitro stem cells actually arise from more differentiated in vivo precursors (transit-amplifying progenitors, astroglia and oligodendrocytes), which have been reprogrammed in vitro to a stem cell status by growth factor stimulation (Doetsch et al., 2002). Interestingly, reprogramming of oligodendrocyte precursors to stem cells requires Sox2 reactivation (Kondo and Raff, 2004); thus, Sox2 mutant neural stem cells might have been "reprogrammed" less efficiently than wild-type cells.

Third, in vitro culture conditions, while allowing efficient differentiation of normal neural stem cells, might be subtly deficient relative to the in vivo environment. This might exaggerate the proportion of mutant Sox2 cells that fail to undergo appropriate differentiation in vitro. Indeed, in vitro not all differentiated markers are developed, and very few cells express appropriate

electrophysiological properties, in contrast to ex vivo neurons (Gritti et al., 1996; Gritti et al., 2001).

Finally, cell selection effects normally operate in vivo, and only a minority of post-migratory cells survive (Ferrer et al., 1992; Muotri and Gage, 2006; Oppenheim, 1991). Abnormal neurons, that fail to properly develop and establish connections, will probably be selected against in vivo. The neuronal loss observed in vivo in specific brain areas (striatum, thalamus), and the reduced cortical extension (Ferri et al., 2004), might reflect these phenomena.

Conclusions

The in vitro culture system, by demonstrating a role for Sox2 in neuronal differentiation, will allow the identification of early Sox2 targets important for neuronal differentiation, by functional rescue experiments. Rare cases of Sox2 deficiency in man are characterized by hippocampal abnormalities, epilepsy, eye and pituitary defects (Fantes et al., 2003; Ragge et al., 2005; Sisodiya et al., 2006; Kelberman et al., 2006), also reported in mutant mice (Ferri et al., 2004; Taranova et al., 2006). Loss of GABAergic inhibitory neurons leads to epilepsy in mouse and man (Noebels, 2003; Cobos et al., 2005). Our observation of GABAergic neuron deficiency in mouse points to a plausible cellular basis for epilepsy in humans with *SOX2* mutations. Other neuronal subsets remain to be tested for their Sox2 requirement.

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Figures

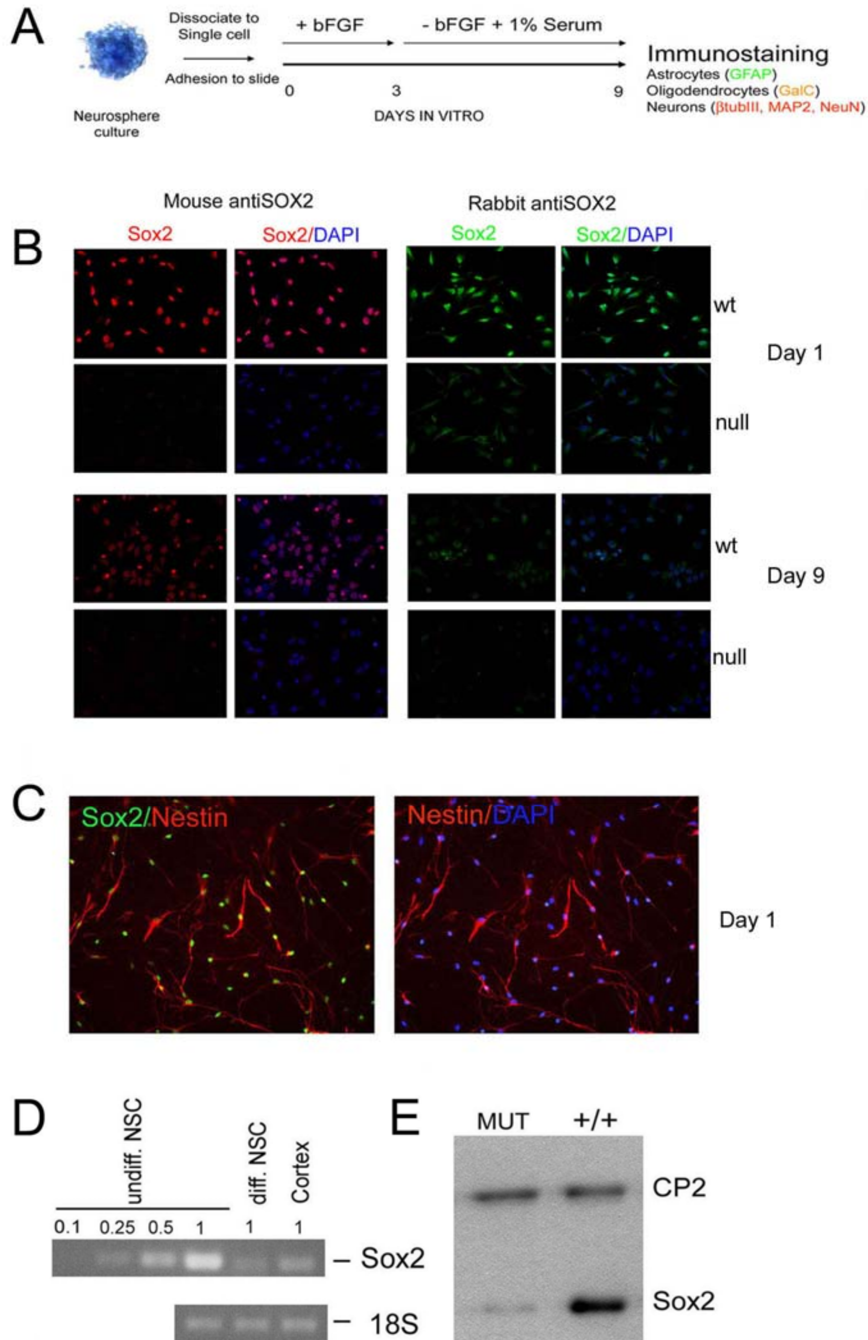


Figure 1 – Sox2 expression during in vitro neural stem cell differentiation. (**A**) In vitro neural stem cell differentiation scheme. (**B**) Specificity of the anti-Sox2 antibodies used in immunocytochemistry. Differentiation day 1 and 9 of wild-type

(wt) and Sox2 conditionally deleted (null) cells are shown. Left, R&D antibody; right, Chemicon antibody (see also Fig. S1 in the supplementary material). A clear nuclear signal is visible in wild-type, but not in Sox2-null, cells. A slight cytoplasmic staining can be seen with the rabbit antibody (Chemicon) in wild-type and null cells, thus likely representing a nonspecific background. (**C**) Sox2 and nestin immunofluorescence on differentiation day 1. We used Chemicon's anti-Sox2 antibody, confirming with R&D antibody. (**D**) RT-PCR of Sox2 expression in undifferentiated neurospheres (Undiff. NSC), day 9 differentiated cells (diff. NSC) and P0 cortical cells. Top: cDNA dilutions from undifferentiated NSC (0.1, 0.25, 0.5, 1) allow an estimate of Sox2 expression levels in differentiated (diff. NSC) and cortical cells. Bottom: 18S RNA PCR, for normalization. (**E**) Western blot of Sox2 (R&D antibody) in normal (+/+) and mutant (MUT) undifferentiated neurospheres. Upper band: ubiquitous CP2 transcription factor (loading control). Sox2 protein in the mutant is 15-25% of normal by densitometry.

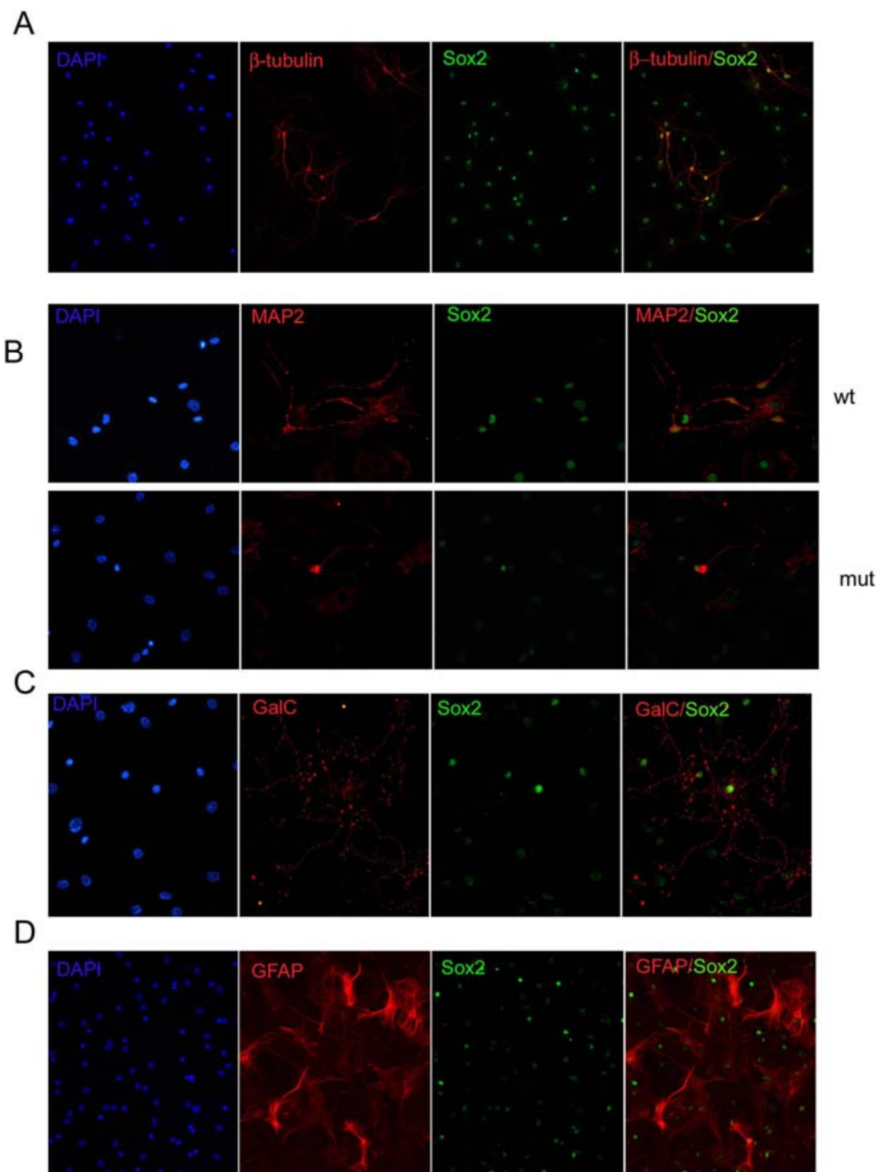


Figure 2 – Immunofluorescence for Sox2, neuronal and glial markers at differentiation day 9. (**A**) Sox2 and β-tubulin in normal cells. β-Tubulin-expressing cells show relatively high Sox2 positivity. (**B**) Sox2 and MAP2. Top: normal; bottom: mutant. MAP2-positive cells show significant Sox2 levels in both normal and mutant. (**C**) Sox2 and GALC, marking oligodendrocytes. (**D**) Sox2 and GFAP.

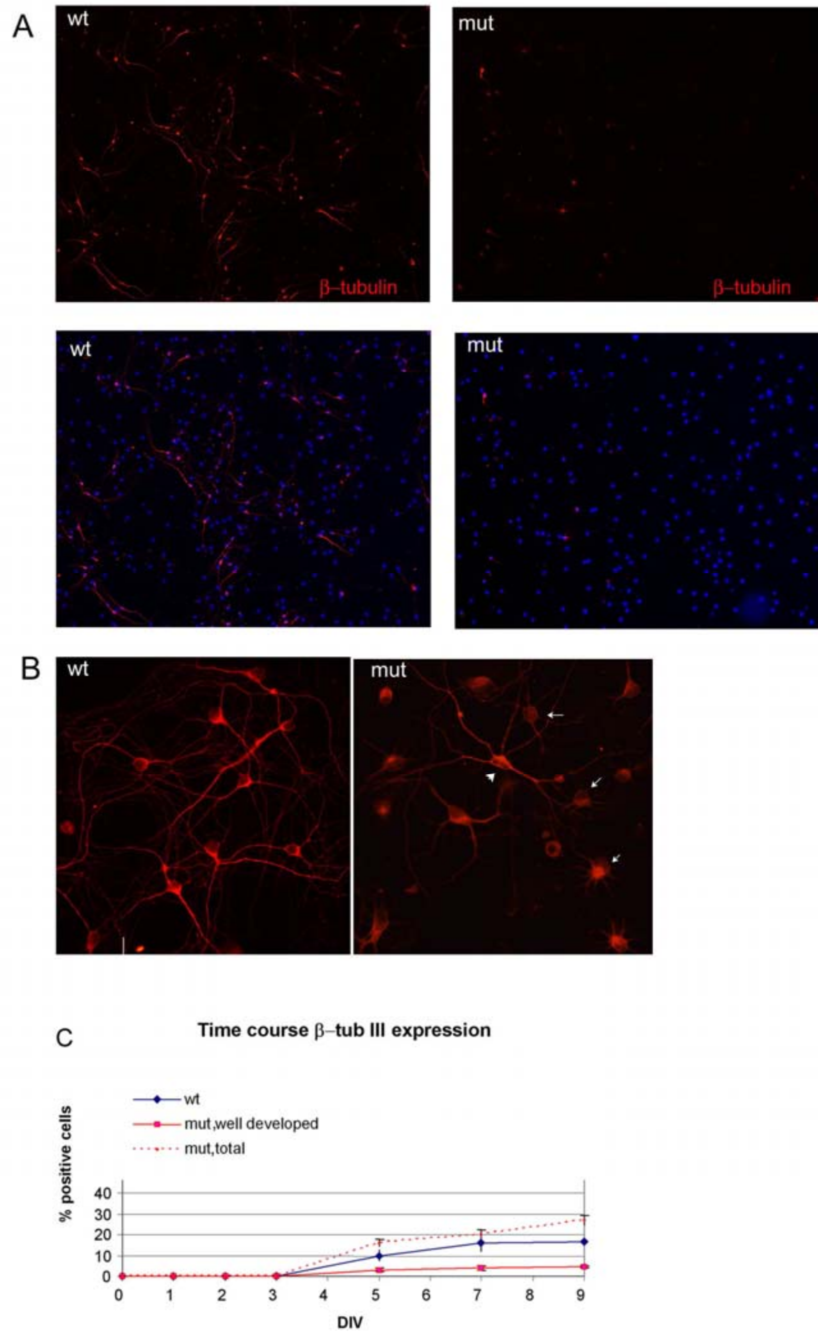


Figure 3 – β-Tubulin-positive cells are abnormal in differentiated Sox2 mutant cell cultures from adult mouse. (**A**) β-Tubulin immunofluorescence of normal (left) and

mutant (right) day 9-differentiated cells. Bottom: DAPI. Many of the mutant poorly arborized, less intensely stained cells are barely visible in this low-magnification image. (**B**) Higher magnification of normal and mutant β-tubulin staining. In mutant, the arrowhead indicates a cell with well-developed neuronal morphology and long arborizations; arrows indicate abnormal cells with short processes and often weak β-tubulin staining typical of the mutant. (**C**) Time course of β-tubulin expression during differentiation. "Mut, well developed" indicates cells with long arborizations (B, wt or arrowhead in mutant); "mut, total": total β-tubulin-positive cells (including those indicated by arrows in B, mut). The abnormal phenotype is already observed at day 5, the earliest stage when significant numbers of β-tubulin-positive cells appear.

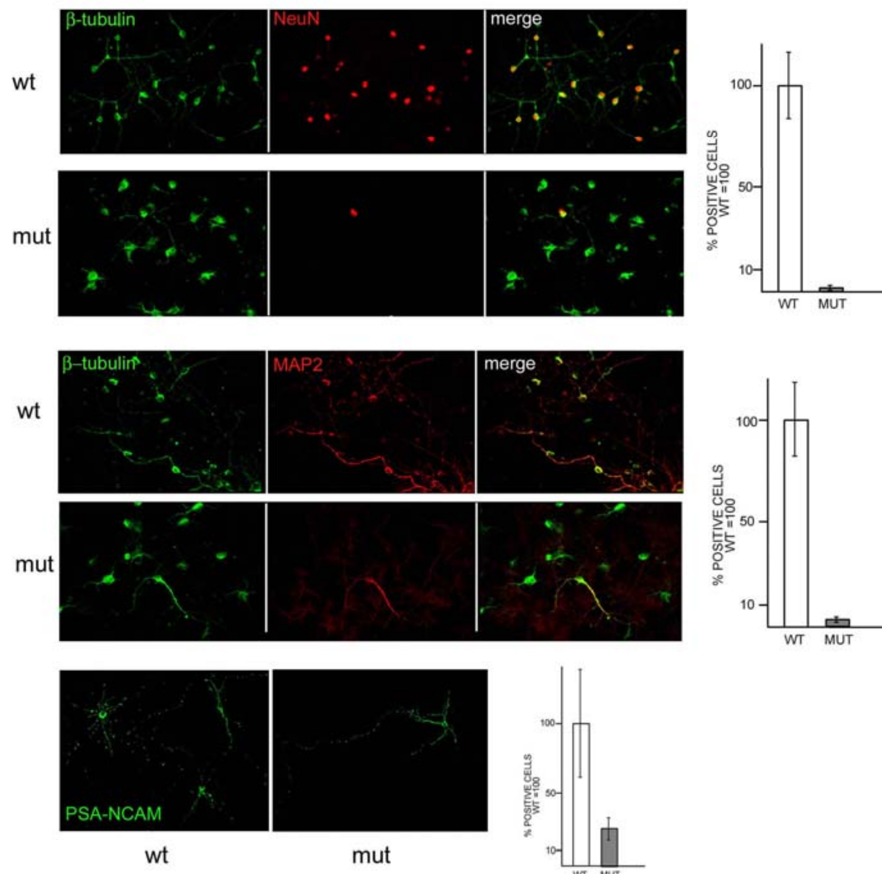


Figure 4 – Cells expressing mature neuronal markers are very reduced in differentiated Sox2 mutant cultures. Neuronal markers in normal and mutant cells at differentiation day 9 (NeuN/β-tubulin, rows 1, 2; MAP2/β-tubulin, rows 3, 4; PSA-NCAM, row 5). Most β-tubulin-positive cells in normal are positive for mature markers NeuN or MAP2; by contrast, very few mutant cells are positive for these markers. Histograms show percentage of cells positive for NeuN/β-tubulin, rows 1, 2; MAP2/β-tubulin, rows 3, 4; PSA-NCAM, row 5, with wild-type average of 100%. Results from *n*=4 normal and *n*=4 mutant mice (see Table S1 in the supplementary material).

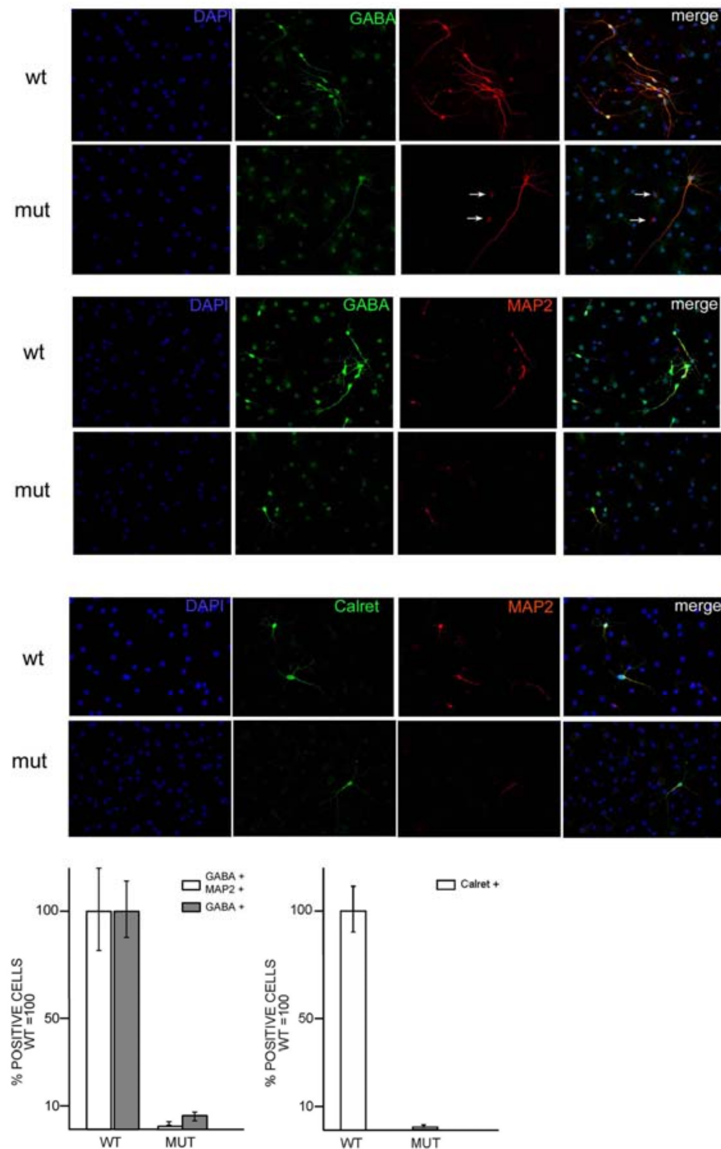


Figure 5 – Cells expressing GABAergic markers are very reduced in differentiated Sox2 mutant cultures. Double-immunofluorescence with general neuronal markers (β-tubulin, rows 1, 2; MAP2, rows 3, 6; red), GABA (rows 1-4) and calretinin (5-6), in normal and mutant day 9-differentiated cultures. Histograms: percentage of positive cells, with wild-type average of 100%. Most β-tubulin-positive cells in normal (top) are GABA positive. In mutant (second row), two immature-looking β-tubulin-positive cells are very weakly GABA positive (or negative) (arrows), in contrast to the adjacent well-arborized GABA-positive cell. In normal cultures, most GABA- and virtually all calretinin-positive cells (rows 3, 5) express the mature

neuronal marker MAP2; these cells are extremely reduced in mutant cultures (rows 4, 6 and histogram). Results from *n*=4 normal and *n*=4 mutant mice (see Table S1 in the supplementary material).

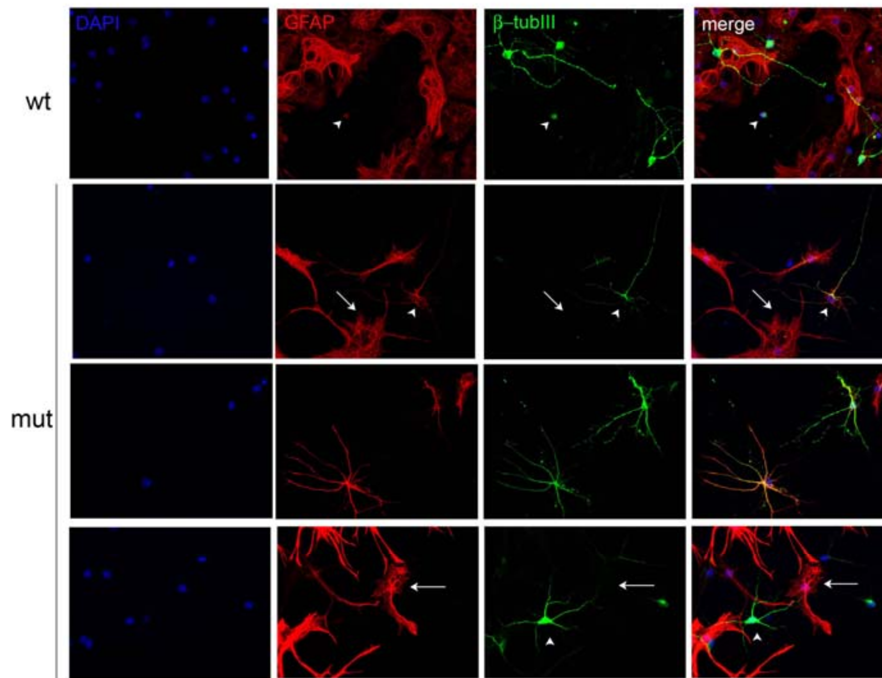


Figure 6 Co-expression of neuronal and glial markers in individual cells in Sox2 mutant cultures. Double-immunofluorescence (β-tubulin and GFAP) of normal (wt) and mutant (mut) day 9-differentiated cells. Typical wild-type neurons (β-tubulin positive) show extensive arborization, are closely associated with glia (which are GFAP positive), and are GFAP negative (top row). Rare cells with a very undifferentiated morphology are weakly positive for both markers (top, arrowhead). In mutant, various arborized cells are positive for both β-tubulin and GFAP (second row, arrowhead; third row, two arborized cells). Well-developed astrocytes are GFAP positive, but β-tubulin negative (arrows, rows 2, 4). In mutant, some intensely β-tubulin stained cells with neuronal morphology are also present (fourth row, arrowhead); these cells are GFAP-negative, as in wild type.

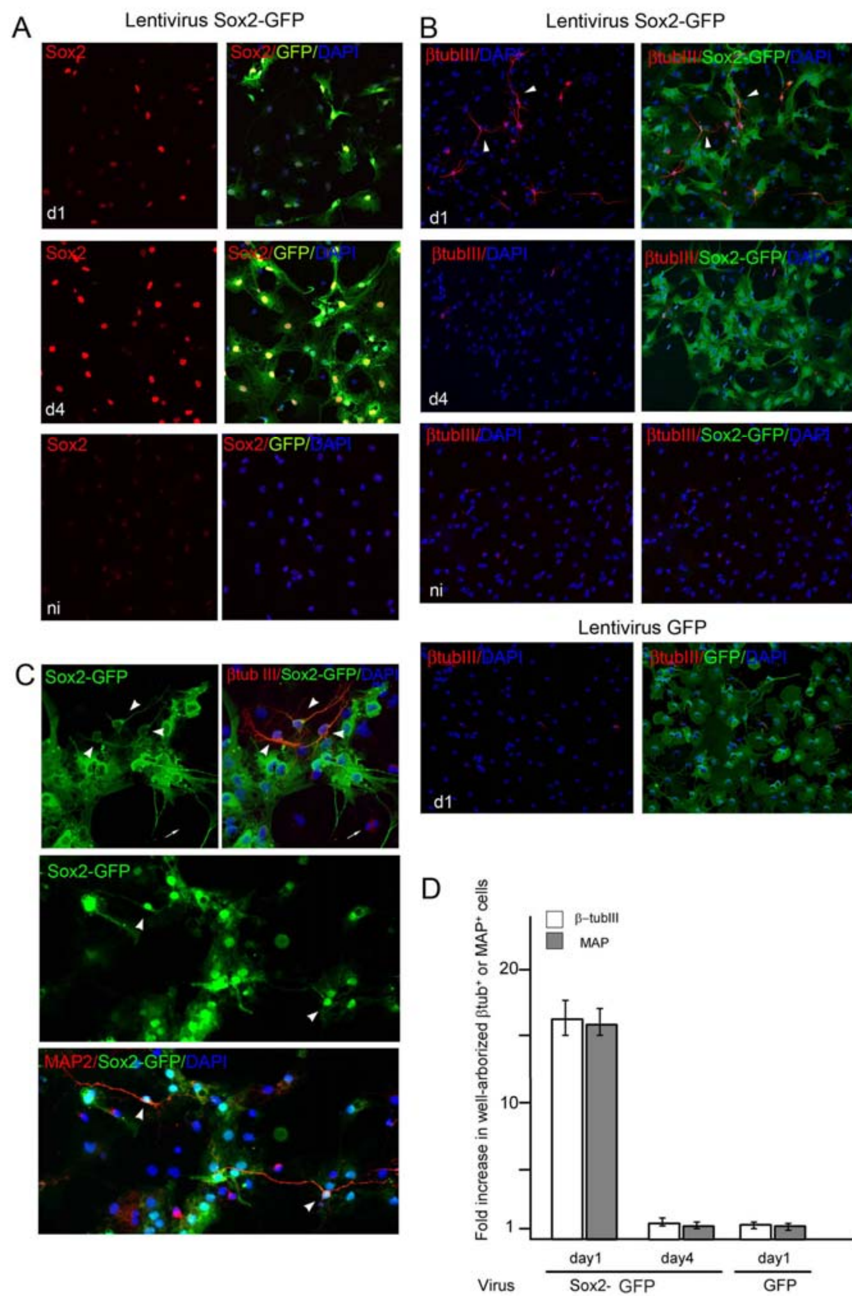


Figure 7 – Rescue of neuronal maturation in mutant cells by lentiviral Sox2 expression at early stages of in vitro differentiation. (**A**) Immunofluorescence for Sox2 (red) (R&D) and GFP (green), encoded by Sox2-IRES-GFP lentivirus, in cells infected at day 1 (d1) or day 4 (d4), compared with non-infected (ni) control. Immunofluorescences were performed the day after infection. Efficient infection

(high proportion of GFP-positive cells) is coupled to clear Sox2 overexpression, which is observed at variable levels in transduced cells. (**B**) β-tubulin- and GFP immunofluorescence, at differentiation day 9, of mutant cells transduced with Sox2-GFP lentivirus at day 1 (d1), or day 4 (d4), compared with non-infected (ni) control, or the control infected with GFP-only transducing virus. Abundant well-arborized β-tubulin-positive cells (arrowheads indicate two of them) are observed in cultures transduced at day 1 with the Sox2-expressing virus, but not in cells transduced at day 4, or in controls. (**C**) GFP (green) and β-tubulin (red, top) or MAP2 (red, bottom) immunofluorescence shows that well-arborized neuronal cells (arrowheads) are always double-positive for the neuronal marker and for GFP, indicating that they derive from a Sox2-transduced cell. By contrast, some poorly developed neuronal cells (arrow) are not green, thus presumably originating from non-transduced cells. (**D**) Fold-increase in numbers of MAP2-positive and well-arborized β-tubulin-positive cells in mutant cells infected with Sox2-lentivirus at differentiation day 1, when compared with infection at day 4, or with control virus (day 1) expressing GFP but not Sox2. Values represent fold increase in numbers of MAP2-positive or well-arborized β-tubulin-positive cells (arrowheads in B,C for examples) relative to non-infected control. In day 1 transduced cells, numbers of well-arborized β-tubulin-positive and of MAP2-positive cells were 3.7% and 4.3%, respectively. In a parallel experiment using wild-type control cells mock-treated in the same way with a non-Sox2-expressing virus, the corresponding values were 5.7 and 6.2%. Data from two experiments in duplicate.

Figure 8 – Sox2 regulates GFAP expression and directly interacts with upstream regulatory DNA sequences of the GFAP gene in vitro and in neural cells chromatin. (**A**) Sox2 overexpression in differentiating cells represses endogenous GFAP expression. Double immunofluorescence (confocal microscopy) of day 9-differentiated cells transduced with Sox2-expressing lentivirus (Sox2-GFP; left) or

control lentivirus (GFP; right) at day 1 (d1) or 4 (d4), with antibodies against GFP (green, revealing Sox2-IRES-GFP, or GFP for control virus), and the astroglial marker GFAP (red). Sox2-lentivirus-transduced cells show no, or very little, GFAP expression, whereas strongly GFAP-positive cells in the same field are Sox2-GFP-negative (left). By contrast, in cells transduced with control virus, GFP and GFAP colocalize within most cells. (**B**) Double immunofluorescence for GFAP and astrocytic markers S-100 (left) or connexin 43 (CX43; right) (Nagy and Rash, 2000) in differentiation day 9 cells; not transduced (nt) or day 1 transduced with Sox2-GFP-expressing lentivirus (d1). Virtually all cells positive for GFAP co-express S-100 or CX43 in non-transduced cells. In Sox2-transduced cells, numerous cells can be seen which have low or absent GFAP expression; and are positive for S-100 (left) or for CX43 (right), confirming their astroglial identity. (**C**) Putative Sox2-binding sites within a 0.6 kb region (0.6GFAP) just upstream to a previously investigated 2.5 kb GFAP promoter/enhancer. The sequence highlights the Sox2 consensus sequences investigated (red). Gfap is the oligonucleotide used in EMSA experiments in E; MutGfap is its mutated version (nucleotide substitutions in green). CDS: coding sequence. (**D**) Co-transfection experiments in P19 cells. Activity of a luciferase reporter gene driven by the 0.6 GFAP region linked to a TK minimal promoter (0.6GfapTK), or by the TK promoter only (TK), when co-transfected with Sox2 expression vector, or control "empty" vector (as indicated). Asterisk indicates a statistically significant difference (paired *t*-test, $P<0.005$). Results are average of n=4 transfections in duplicate. (**E**) EMSA with probes (indicated below the panels) encompassing the Sox2 consensus binding sites in the 0.6 GFAP region (Gfap), or the same probe mutated as in 8B (MutGfap), or a control probe carrying a Sox2-binding site from an Oct4 gene enhancer (Oct4). Nuclear extracts (P19; SOX2/COS, COS cells transfected with Sox2 expression vector; COS, untransfected COS cells), and competitor oligonucleotides with the molar excesses used for the competition experiments in the right panel, are indicated above the figure. (**F**) ChIP with anti-SOX2 antibodies of the 0.6 Gfap region in P19 and E12.5 spinal cord cell chromatin, compared with control SRR2 (which is bound by Sox2 in P19, but not in E12.5 spinal cord cell chromatin) (Miyagi et al., 2006) or nestin (bound by Sox2 in P19 and E12.5 spinal cord cell chromatin) (Tanaka et al., 2004; Miyagi et al., 2006) regulatory regions. The anti-Sox2 antibody precipitates both GFAP and SRR2 chromatin in P19 cells, but only GFAP chromatin in spinal cord cells, as expected. Antibodies are indicated above the panels; cell types and amplified DNA regions are indicated below the panels. Arrowheads indicate the positions of PCR bands corresponding to amplified target regions. Low-intensity diffused bands at the bottom are non-reacted primers. Results are representative of three experiments. unrel, unrelated control antibody against SV40 large-T antigen; Input chrom, input chromatin (not immunoprecipitated) - a positive control for the PCR reaction.

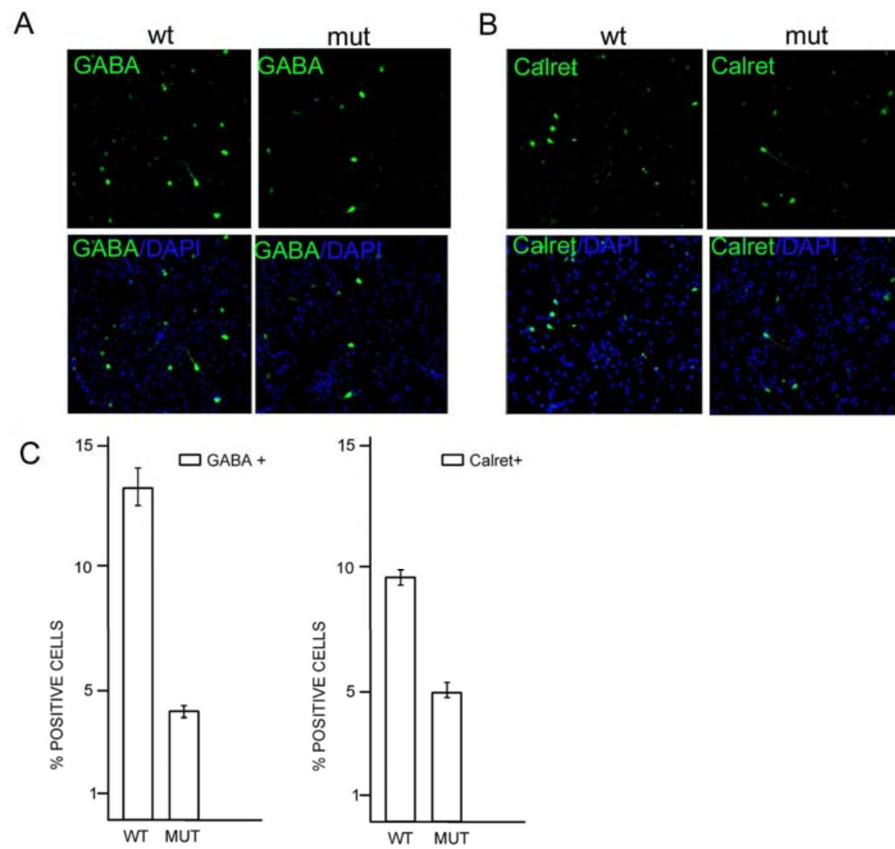


Figure 9 – Neurons expressing GABAergic markers are reduced in Sox2 mutant neonatal brains. (**A**,**B**) GABA (A) and calretinin (B) immunofluorescence of P0 cortical neurons (normal, left; mutant, right). Lower panels are counterstained with DAPI. (**C**) Percentage of GABA- or calretinin-positive cells in normal or mutant P0 cortical neurons. Results from *n*=3 normal and *n*=3 mutant mice.

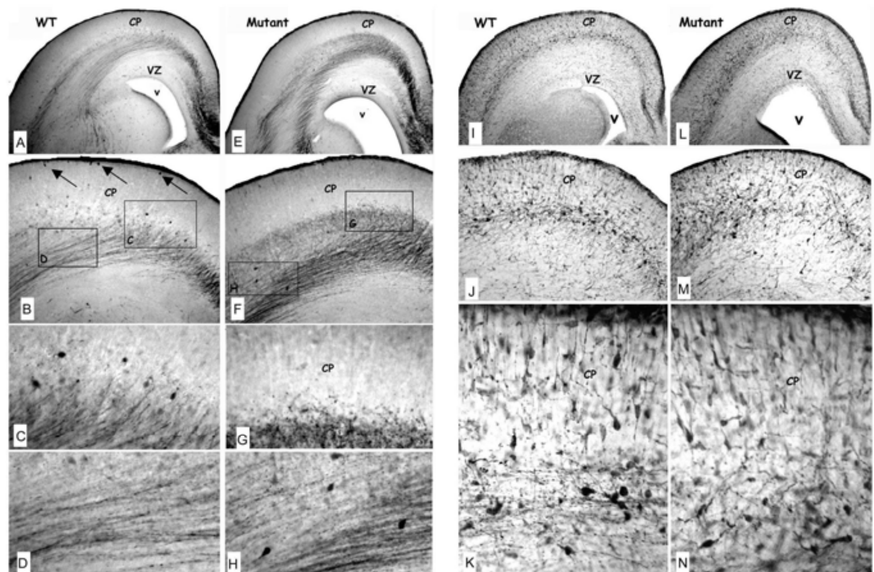


Figure 10 – Abnormal calretinin- and GABA-positive neurons in E17.5 mutant brain. Calretinin (**A-H**) or GABA (**I-N**) immunohistochemistry in sections from normal (A-D,I-K) and mutant (E-H,L-N) forebrains. (A,E,I,L) General views of normal and mutant forebrain sections (dorsal region). Lower panels show progressively more enlarged details. (B,F,J,M) Details of the cortical region. The boxed regions in B and F are shown in C,D and G,H, respectively. Arrows in B indicate calretinin-positive neurons that reached the more external cortical layers following migration. Neurons in these positions are much rarer in the corresponding mutant section (F). C shows neurons that reached deep layers of the cortical plate; in the corresponding region of the mutant (G), no cells are seen. (D) Subcortical fiber bundles (along which calretinin-positive cells migrate from ganglionic eminences to cortex at earlier stages); no cells are seen here in the wild type. In the corresponding region of the mutant (H), calretinin-positive cells are still seen along this migratory route. (K,N) Enlarged details of J and M. In mutant (N), general disorganization of the GABA-positive neurons and of their arborizations is seen. V, ventricle; VZ, ventricular zone; CP, cortical plate.

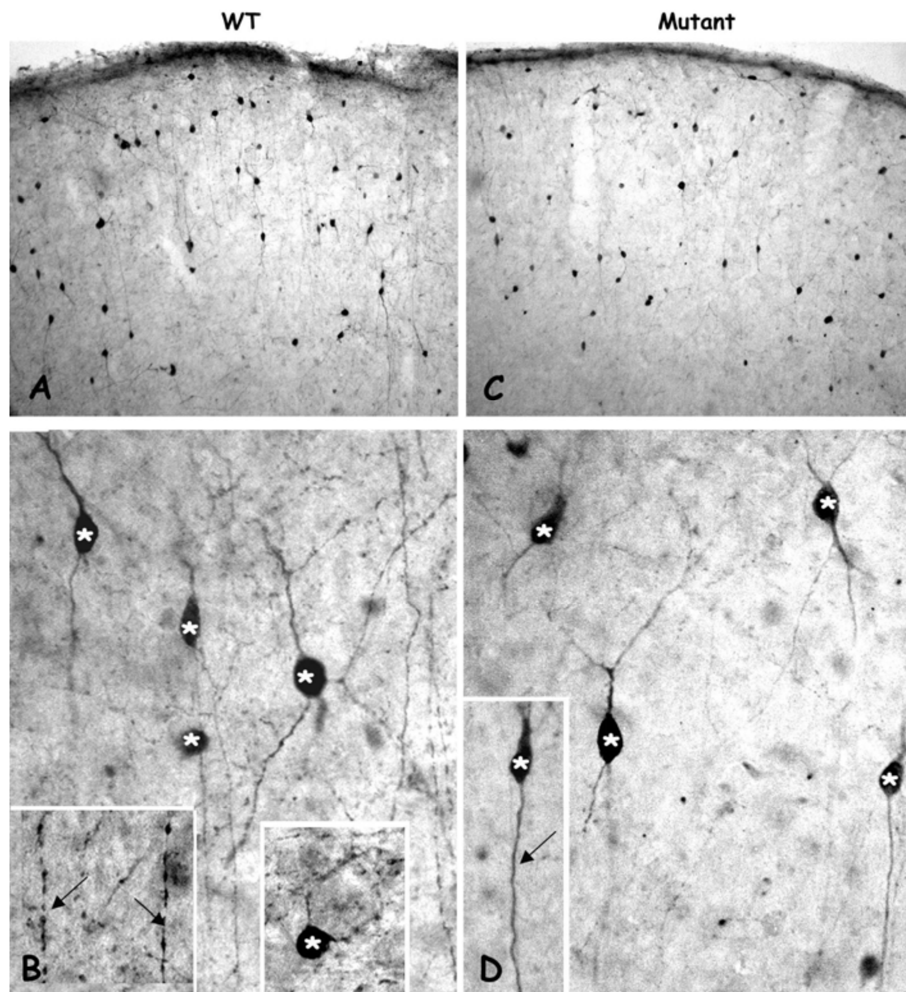


Figure 11 – Decreased frequency and arborization of calretinin-positive neurons in adult mutant somatosensory cortex. (**A**,**C**) Calretinin immunohistochemistry reveals lower frequency of calretinin-positive neurons in mutant (C) versus wild-type (A) mice. (**B**,**D**) Higher magnification shows reduction of dendritic arborizations and of axonal varicosities (the swellings where transmitter-containing vesicles accumulate) in calretinin-positive neurons (asterisks) of mutant (D) versus wild-type (B) brains. Insets in B show, on the left, two vertically oriented varicose processes (arrows) and on the right a highly ramified calretinin-positive neuron (asterisk). Inset in D shows a poorly ramified calretinin-positive neuron (asterisk) with a vertically oriented smooth process (arrow). Original magnifications: A,C 940x ; B,D 2400x; insets 3200x.

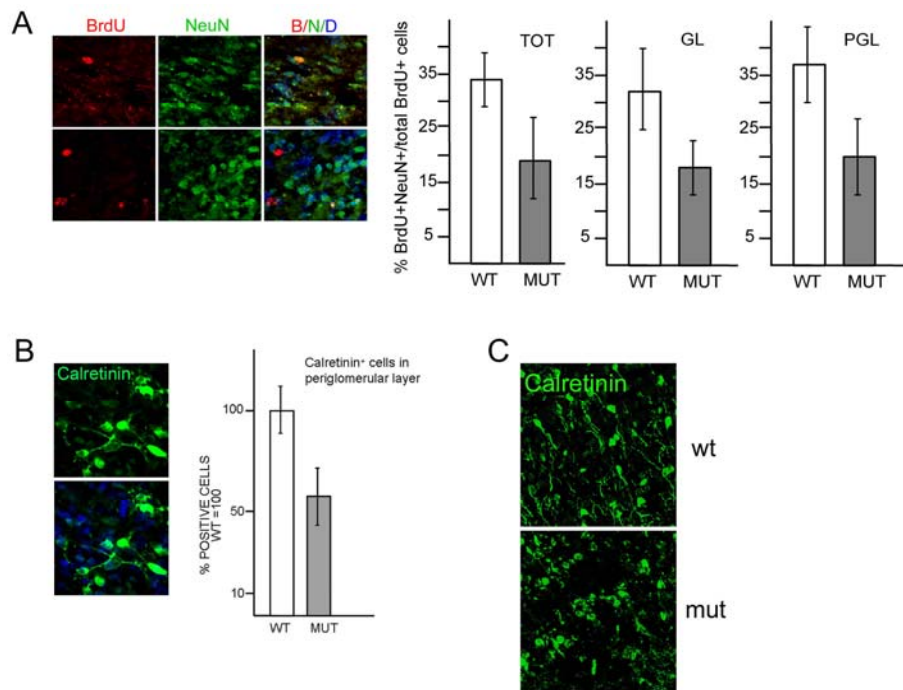
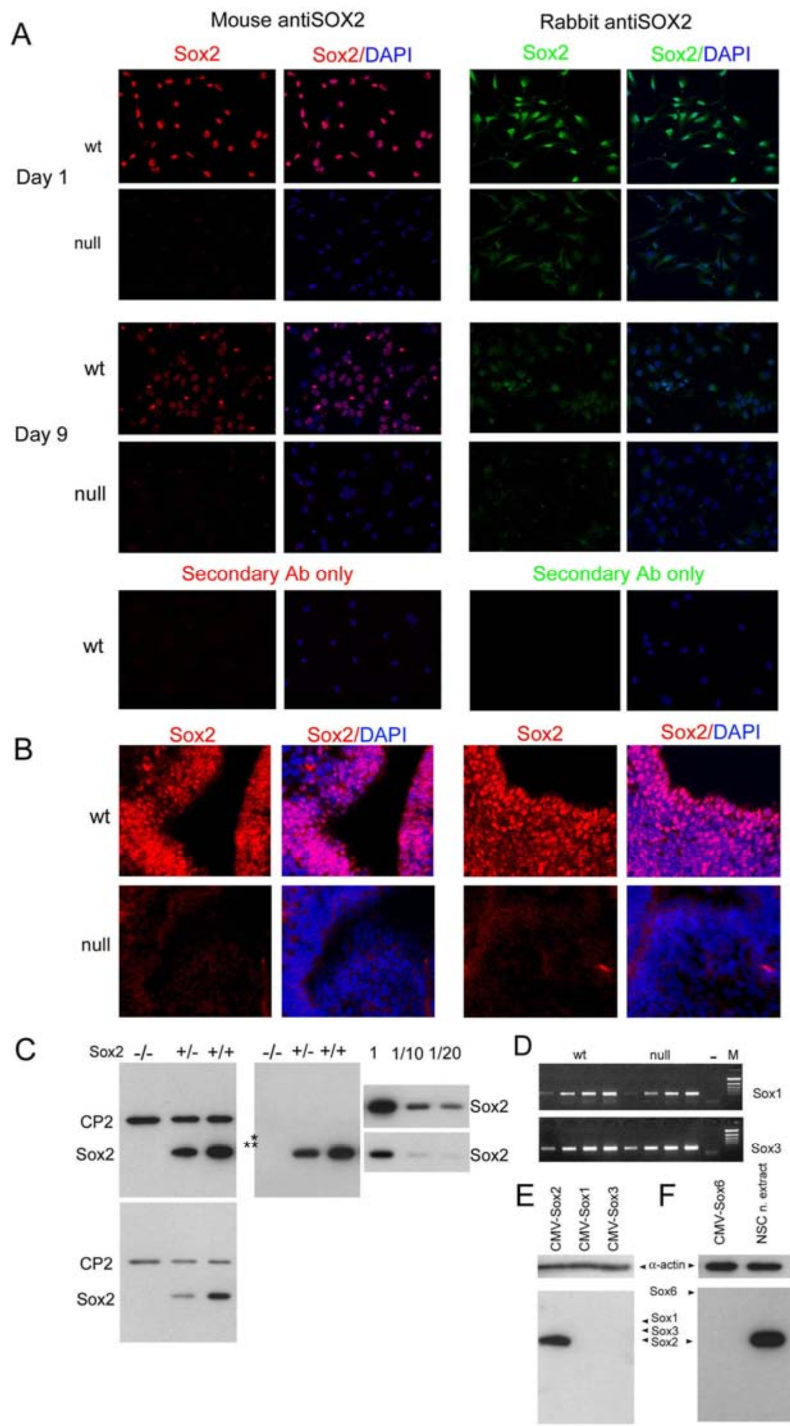


Figure 12 – Impaired neuronal maturation in adult olfactory bulb of Sox2 mutant mice. (**A**) Immunofluorescence of BrdU/NeuN-double positive (red and green, yellow in overlay; first row) and BrdU-single-positive (red only; second row) cells in olfactory bulb sections. Histograms: percentage of BrdU/NeuN double-positive cells within the total BrdU-positive population in normal (WT) and mutant (MUT) olfactory bulb, in the entire bulb (TOT) or specifically in the granule layer (GL) and periglomerular layer (PGL) neuronal populations. Results from wild-type (n=4) and mutant mice (n=6). (**B**) Calretinin-positive cells (green) in olfactory bulb. Histograms: quantitation of calretinin-positive cells in normal (WT) and mutant (MUT) olfactory bulb within the periglomerular layer (four wild type, six mutants). (**C**) Confocal microscopy of calretinin-positive cells in the olfactory bulb reveals very limited arborization of mutant (mut) cells compared with wild type (wt). This morphology was clearly detected in two out of the four mutant mice analyzed.

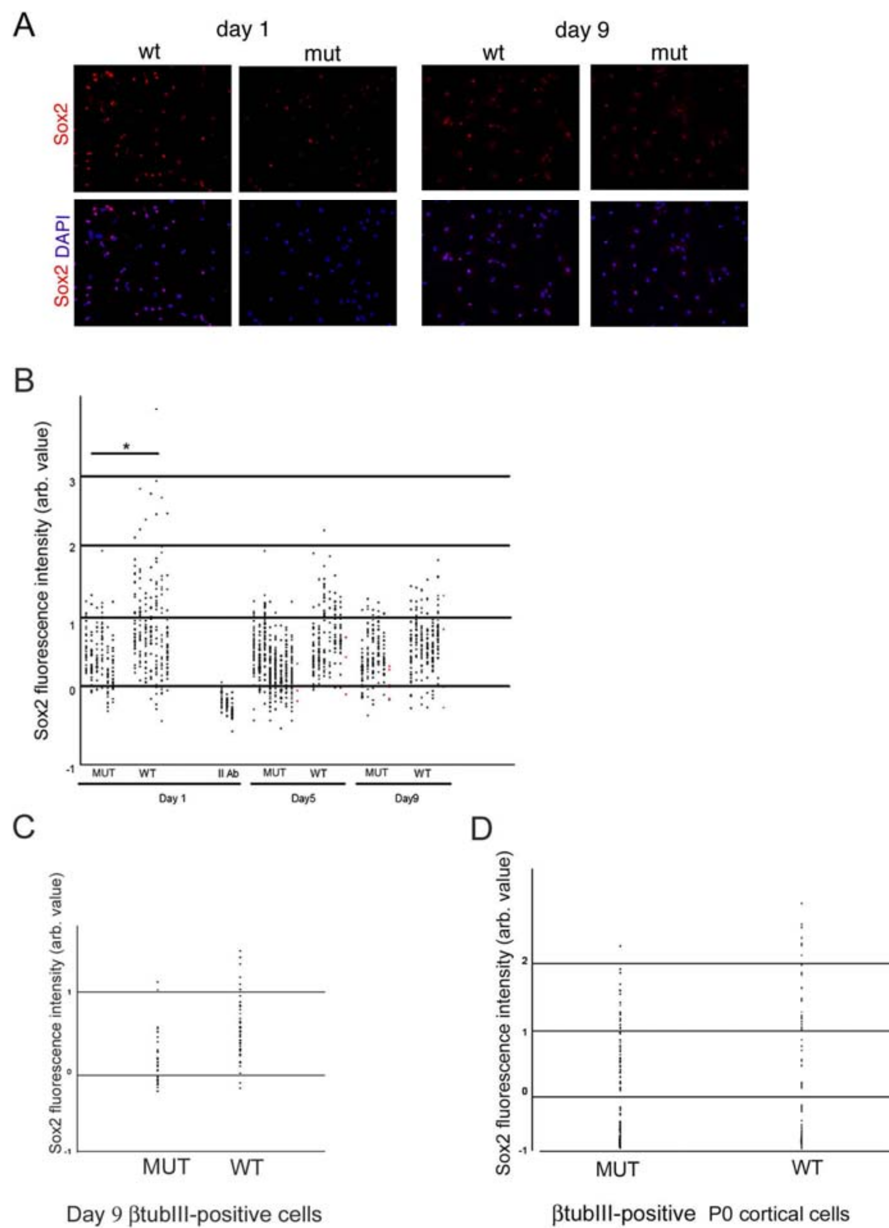
Supplementary figures



A
Mouse antiSOX2
Rabbit antiSOX2
Sox2
Sox2/DAPI
Sox2
Sox2/DAPI
Day 1
wt
null
Day 9
wt
null
Secondary Ab only
Secondary Ab only
wt
B
Sox2
Sox2/DAPI
Sox2
Sox2/DAPI
wt
null
C
Sox2 -/- +/- +/+
-/- +/- +/+
1 1/10 1/20
CP2
Sox2
Sox2
Sox2
CP2
Sox2
D
wt
null
M
Sox1
Sox3
E
CMV-Sox2
CMV-Sox1
CMV-Sox3
F
CMV-Sox6
NSC n. extract
α-actin
Sox6
Sox1
Sox3
Sox2

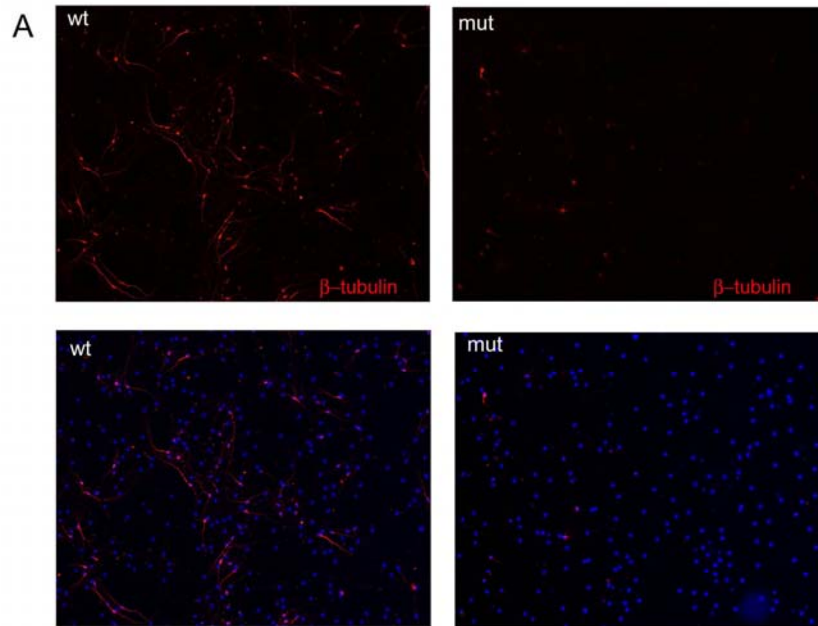
Supplementary Figure 1 – Evaluation of anti-Sox2 antibodies by immunocytochemistry, immunohistochemistry and western blot analysis of wild-type and Sox2-null neural cells, and of recombinant Sox proteins by western blot. We evaluated the Sox2 specificity of two commercial antibodies (R&D, mouse monoclonal; Chemicon, rabbit polyclonal). Sox2-null neural cells, obtained by in vivo nestin-driven Cre-mediated deletion (R.F. et al., unpublished), were compared with wild-type cells. Both antibodies gave clear nuclear staining in most of the wild-type cells, but failed to show any reactivity with nuclei of Sox2-null cells. (A) Dissociated neurospheres allowed to attach to a slide were probed with the indicated antibodies at the beginning (day 1) or at the end (day 9) of the differentiation protocol described in Fig. 1. With both antibodies, a clear nuclear signal is visible in wild-type, but not in Sox2-null cells. Expression decreases with differentiation, but is still clearly detected in day 9 differentiated cells. A slight cytoplasmic staining can be seen with the rabbit antibody (Chemicon) at both day 1 and day 9, in wild type and null cells, thus likely representing a nonspecific background. Secondary antibodies only (bottom panels) yield no signal. (B) In vivo, neither antibody stains nuclei in brain sections of mutant null newborn mice. Immunohistochemistry with both mouse (left panels) and rabbit (right panels) anti-Sox2 antibodies detects abundant nuclear Sox2 expression in wild-type (wt), but not in Sox2-deleted (null) ventricular zone at P0. Some background staining seen in the null mouse sections does not localize to nuclei. (C) Western blot studies with the R&D antibody, confirming that it does not crossreact with any proteins in undifferentiated neurosphere lysates of Sox2-null cells, even in the presence of a large excess of protein and with long exposures. Proteins from neurosphere cultures of wild-type (+/+), Sox2 heterozygous (+/−) and Sox2-deleted (−/−) mice were probed with anti-Sox2 antibody. Positions of Sox2 and CP2 (ubiquitous nuclear protein, as loading control) are indicated. Left panels: two different exposures of a filter probed with anti-Sox2 and anti-CP2 antibodies. Genotypes are indicated above the lanes. The longer (top) exposure shows failure of the antibody to detect any non-specific signal in the −/− sample; the lower (shorter) exposure allows better comparison of the CP2 signal, demonstrating that equal amounts of extracts were loaded in all lanes. Middle panel: the same filter probed with the Sox2 antibody, prior to re-probing with the CP2 antibody. No signal is seen in the Sox2-null (−/−) extract, even with this long (1 minute) exposure. Asterisks indicate the expected position of the Sox1 (*) and Sox3 (**) transcription factors, which are expressed in the same cells at normal levels (see D). Right panels: progressive dilutions (1/10, 1/20) of the amount of extract (1 corresponds to the amount loaded in the +/+ lane of the upper left and middle panels) still yield a clearly visible Sox2 signal, even when the same filters exposed for only 6 seconds (lower panel), instead of 1 minute (top panel). Thus, a 10-fold overexposure of an amount of extract 20-fold in excess to that required for Sox2 detection, still does not yield any non-specific signal. (D) RT-PCR analysis of expression of SoxB family members Sox1 and Sox3 (co-expressed with Sox2 in neural precursors), in wild-type and Sox2-null neurosphere cultures. Samples shown were taken from the PCR reactions at 25, 30, 35 and 40 cycles for both wild-type and null. Expression levels of Sox1 and Sox3 are similar between wild-type and Sox2-null cells. −, control reaction with reverse transcriptase-negative null control (40 cycles); M, marker. (E,F) Lack of cross-reaction of the anti-Sox2 antibodies with recombinant Sox1, Sox3 and Sox6. NIH3T3 (E) or HeLa (F) cells were transfected with CMV promoter-driven expression vectors (pCDNA3) for Sox2, or

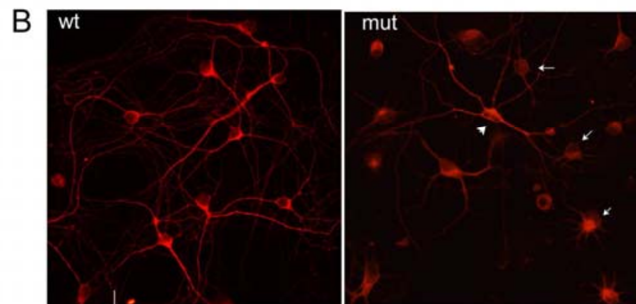
Sox1, Sox3 and Sox6. Cell extracts were probed with R&D anti-Sox2 antibody. The Sox1, Sox3 (E) and Sox6 (F) positions are indicated beside the panels. Although Sox2 was easily detected, no reactivity was obtained with extracts from cells transfected with the other Sox expression vectors. In conclusion, anti-Sox2 antibodies do not significantly crossreact with protein present in neural cells at various differentiation stages. The staining experiments reported in the paper were always performed with both antibodies (as indicated in figures), with essentially identical results. When quantitation of the staining was required, the R&D antibody was used.

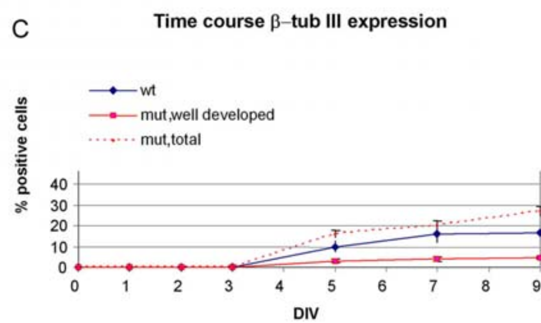


Supplementary Figure 2 – Evaluation of Sox2 immunofluorescence at the single-cell level. To evaluate Sox2 immunofluorescence at the single-cell level, digital images of Sox2 immunofluorescence-labeled nuclei were acquired, and individual nuclei were delimited and evaluated (on the monochromatic image taken on the appropriate fluorescence channel) with the image-processing algorithm of the Region Of Interest (ROI) program provided with the Leica TCS2 Confocal

Microscope (Leica Microsystems), or the ImageJ.exe processing and analysis program (http://rsb.info.nih.gov/ij/), and expressed in arbitrary units as the sum of the background-subtracted pixel values within each ROI (nucleus). Background levels were established measuring nuclei of Sox2-null cells (see Fig. S1) or of cells treated with secondary antibody only (B), giving comparable values. The ratios between positive signals and internal background (measured on five different positions within each field) were plotted and statistical significances were assessed by nonparametric tests (heteroskedastic ANOVA, T-test; **P*<0,05). (A) Examples of Sox2 immunofluorescence of normal and mutant cells at day 1 (left) or day 9 (right) of in vitro differentiation. In day 1 cells, a Sox2-bright cell population is seen in the normal, which is very reduced in the mutant. At day 9, fluorescence levels are very similar between wild type and mutant. (B) Evaluation of Sox2 immunofluorescence (R&D antibody) at the single-cell level in wild type (WT) and mutant (MUT) cells, on the overall population at days 1, 5 and 9 of in vitro differentiation (as indicated). Each dot represents the Sox2 fluorescence level of a single cell nucleus; each vertical dot series represents the values within an individual microscope field evaluated (see Materials and methods below). "II Ab" indicates nuclear fluorescence values obtained with the secondary antibody only; the "0" level was set just above the highest values obtained with this negative control, as shown in B (the same applies to C and D). Red dots identify the β-tubulin-positive cells within the samples shown (see also C). At least 500 nuclei per differentiation day per genotype were quantitated, within at least six different fields. The asterisk indicates a significant difference at day 1, but not at days 5 and 9, between wild-type and mutant Sox2 fluorescence distributions (one-way ANOVA, *P*<0.03; two-tailed *t*-test, *P*<0.001). (C,D) Evaluation of Sox2 immunofluorescence within the β-tubulin-positive cell population at day 9 of in vitro differentiation (C) or in in vivo differentiated P0 cortical cells (D), in normal (WT) and mutant (MUT). Fluorescence levels are indicated as explained in B. Examples of Sox2/β-tubulin-double-positive cells in differentiation day 9 cells and P0 cortical neurons are shown in Fig. 2A, Fig. S5B, respectively. In the in vitro-differentiated β-tubulin positive cells (C), the Sox2 level was slightly, but significantly, decreased in mutants (two-tailed *t*-test, *P*<0.01). This is at variance with the analysis reported in Fig. S2B for the overall population, where most cells are glia. A comparison between normal and mutant MAP2-positive cells for Sox2 expression was not performed, owing to the rarity of MAP2-positive cells in the mutant (see text). In D, the data document a slight (statistically non-significant) difference between the wild- type and the mutant (two-tailed *t*-test, *P*<0.34). At least 200 nuclei from β-tubulin-positive cells were analyzed in C and D, for n=2 wild type and n=2 mutants.

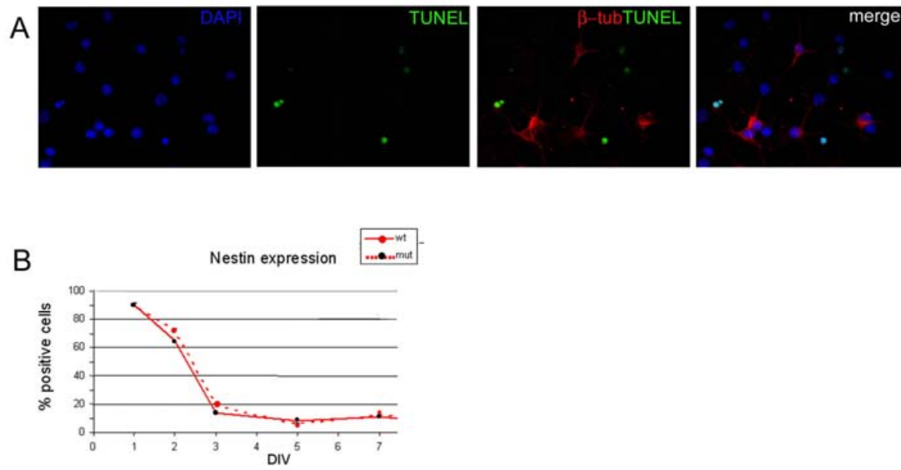




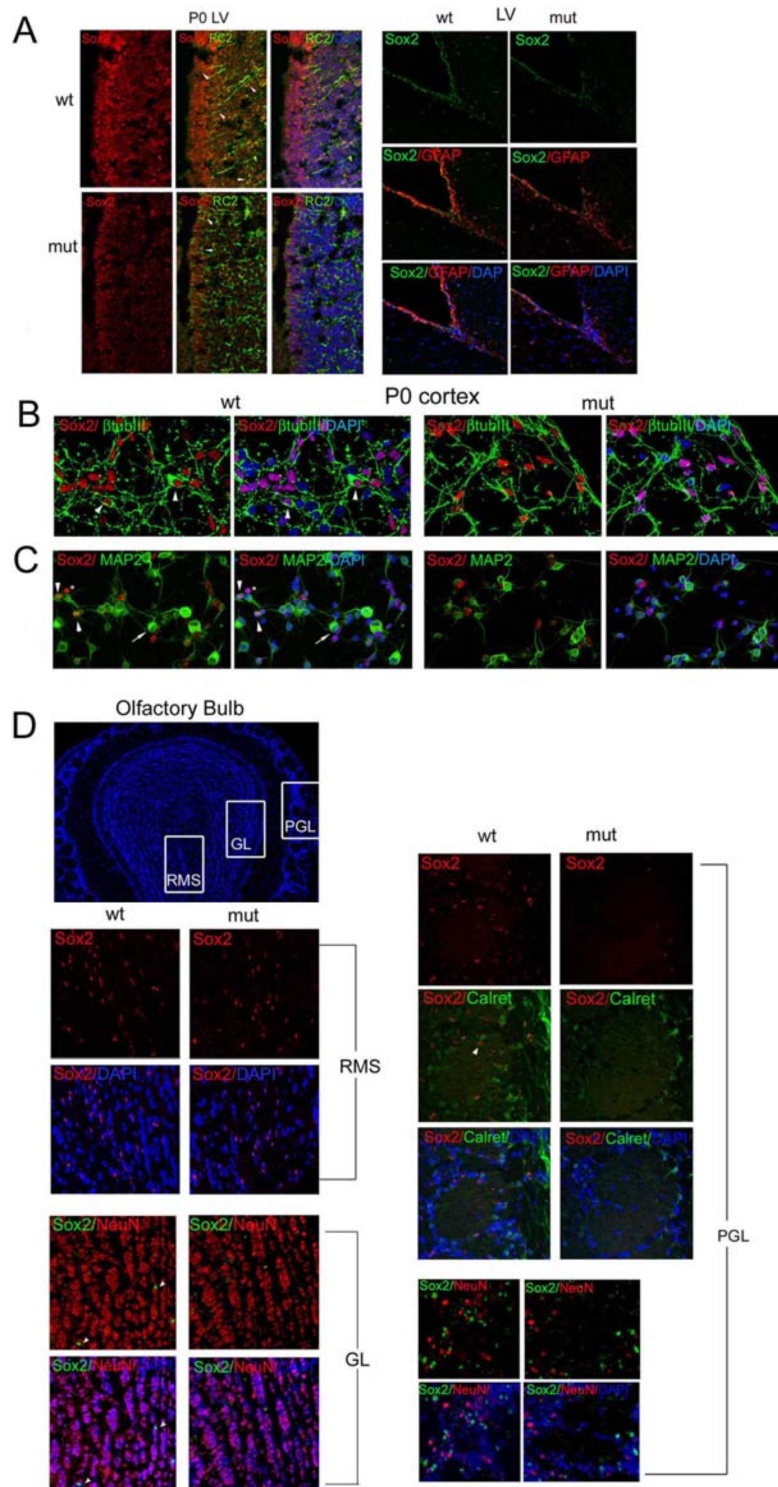


Supplementary Figure 3 – Expression of astrocytic markers S-100 and connexin 43 (CX43) (Nagy and Rash, 2000) in GFAP-positive in vitro differentiated

astrocytes (untransduced, or day 1 transduced with Sox2-expressing lentivirus). (A) Double immunofluorescence for GFAP and S-100 (top panels) or CX43 (bottom panels) in differentiation day 9 cells, untransduced (left) or transduced with Sox2-GFP-expressing lentivirus (right). Virtually all cells positive for GFAP co-express S-100 (top panels) or CX43 (bottom panels) in untransduced cells. In Sox2-transduced cells, numerous cells can be seen which have low or absent GFAP expression (see Fig. 9) and are positive for S-100 (top) or for CX43 (bottom), confirming their astroglial identity (arrows indicate examples). (B) Double immunofluorescence for GFP (marking cells transduced with the Sox2-GFP-expressing lentivirus) and for S-100 (top) or CX43 (bottom). The vast majority of Sox2-transduced cells (where downregulation of endogenous GFAP is observed, see Fig. 8) express S-100 (top panels) and CX43 (bottom panels), consistent with an astrocytic identity. S-100 may be somewhat reduced in occasional Sox2-transduced cells. No fluorescence signal is observed in Sox2-GFP virus-transduced cells prior to antibody staining (lower right image, indicating that GFP endogenous green fluorescence is not detected in cells after fixation), nor with secondary antibodies only (not shown). Images are by non-confocal microscopy; see also Fig. 8 for confocal images of GFAP/S-100 and GFAP/CX43 immunofluorescence.



Supplementary Figure 4 – The block in neuronal maturation in Sox2 mutant cultures is not associated with apoptosis, nor with persistence of undifferentiated cells characteristics (nestin positivity). (A) Apoptosis between initial β-tubulin expression and MAP2/NeuN activation can be ruled out. In fact, between day 5 and 9, ~15% of the cells show TUNEL positivity (green), both in normal and mutant; however, >98% of β-tubulin-positive cells (red) do not show TUNEL positivity. Shown are differentiation day 7 mutant cells. Furthermore, the total number of cells in mutant cultures at day 9, and the number of β-tubulin-positive cells were comparable between normal and mutant cells (see Table S1 in the supplementary material; data not shown), indicating that the maturation block is not associated with, or dependent on, apoptotic cell death. Numbers of Ki67-positive (dividing) cells were also similar (not shown). (B) Time course of nestin expression. The kinetics of decrease of the number of cells positive to nestin (a marker of the undifferentiated state) is very similar between wild-type and mutant cultures. Note that β-tubulin appeared at day 5 in mutant, as in normal cells (see Fig. 3C). Thus, initial differentiation steps are not significantly delayed in mutant cells.


A
P0 LV
wt
mut
Sox2
RC2
LV
Sox2
Sox2/GFAP
Sox2/GFAP/DAPI
B
P0 cortex
Sox2/ βtubIII
Sox2/βtubIII/DAPI
C
Sox2/ MAP2
Sox2/ MAP2/DAPI
D
Olfactory Bulb
RMS
GL
PGL
Sox2/DAPI
Sox2/NeuN
Sox2/Calret
Sox2/Calret/DAPI

Supplementary Figure 5 – Sox2 expression in the lateral ventricle (A), and in regions of neuronal differentiation (within the neonatal cortex, B,C, and in adult olfactory bulb, D), in normal and mutant mice. (A) Left: Sox2 (red) (Chemicon) and RC2 (green, a radial glia marker) (Merkle et al., 2004) immunofluorescence on sections of P0 lateral ventricle (P0 LV) of normal (wt) and mutant (mut) mice (confocal microscopy). Arrowheads: examples of Sox2/RC2 double-positive cells. Right: Sox2 (green) (Chemicon) and GFAP (red) immunofluorescence in adult lateral ventricle (LV) of wild type (wt) and mutant (mut). (B,C) Immunofluorescence of isolated P0 cortical neurons from normal (wt) and mutant (mut) brains with Sox2 (R&D) and β-tubulin (B) or MAP2 (C) antibodies (confocal microscopy). A large proportion of β-tubulin or MAP2-stained neurons are clearly Sox2-positive.Within the MAP2-positive population, the intensity of Sox2 staining inversely correlates with that of differentiated marker, and the most strongly MAP2-labeled cells are completely devoid of Sox2. Arrowheads: examples of Sox2/β-tubulin or Sox2/MAP2 double-positive cells. Sox2/MAP2 double-positive cells are generally weakly positive for both markers. Arrows indicate strongly MAP2-positive cells (generally Sox2-negative). Asterisks indicate strongly Sox2-positive cells (generally MAP2-weakly positive or negative). (D) Immunofluorescence analysis of Sox2 expression in the olfactory bulb. Top: Low-magnification image of an olfactory bulb section (DAPI nuclear staining); white boxes highlight the regions of the rostral migratory stream (RMS) and, more externally, sections of the peripheral layers where terminal neuronal differentiation is completed: the granule layer (GL) and periglomerular layer (PGL). Lower panels show higher magnifications of these regions (as indicated) analyzed in wild-type (wt) and mutant (mut), with the indicated antibodies In the RMS, Sox2 is expressed in numerous cells, many of which are positive for PSA-NCAM (Ferri et al., 2004), a marker of transit-amplifying progenitors (Doetsch, 2003; Lledo et al., 2006). In the differentiated peripheral layers, some weakly Sox2-positive cells are still visible; they are rare in the GL, but more numerous in the PGL, where calretinin-positive neurons differentiate 14-20 days after their birth (Lledo et al., 2006). Here, however, few if any calretinin or NeuN-positive cells show Sox2. In the mutant, the number of Sox2-positive cells is diminished, as expected on the basis of the observations on the SVZ. Arrowheads in GL indicate Sox2-positive NeuN-negative cells. Arrowhead in PGL indicates cell appearing weakly positive for Sox2 and calretinin.

Supplementary Table 1: expression of lineage-specific markers in differentiated neural stem cells from Sox2-deficient mice

	WT	**MUT**
ß-tubulin[a] with well-developed neuronal morphology, extensive arborization	13,2% ± 1,5%	1,3% ± 0,9%
Poorly developed, limited arborization, generally less intenslely stained	<0,5%	18,9% ± 1,9%
NeuN[b]	11,4% ± 1,9%	0,25% ± 0,12%
MAP2[b]	7,9% ± 1,4%	0,26% ± 0,1%
PSA-NCAM	3,8% ± 1,5%	1% ± 0,4%
GABA[c]	8,9% ± 1,9%	0,8% ± 0,4%
CALRETININ[d]	3,1 % ± 0.7%	<0,1%
GFAP	60% ± 1,3%	58% ± 2,3%
GALC	3% ± 0,8%	2,5% ± 1%

These data were obtained from differentiation of neural stem cells from adult brain (similar data were obtained with E14.5 embryonic cells, not shown). In one set of experiments ß-tubulin, NeuN, MAP2, PSA-NCAM, GFAP and GAL-C were evaluated in slides from differentiated cultures obtained from n=4 wt and n=4 mutant mice; MAP2 and NeuN were counted in double immunofluorescence labellings with ß-tubulin. GABA and calretinin were evaluated in a separate experiment, in which n=2 wt and n=2 mutants (already assayed for the markers above) were differentiated, and assayed by double labelling with ß-tubulin or MAP2 (similar percentages of ß-tubulin and MAP2-positive cells were obtained in all these experiments). The total number of cells at the end of differentiation was always very similar between wild type and mutant. **a**: see Fig. 2 for the different appearance of ß-tubulin-positive cells in the mutant; **b**: NeuN and MAP2-positive cells are also ß-tubulin-positive in double immunofluorescence labellings; **c**: GABA-bright cells are indicated. GABA-bright cells were nearly always MAP-2 positive in double immunofluorescence labellings in the wild type (see Fig. 4). A dimmer GABA positivity was observed in most ß-tubulin-positive cells in the wild type, though not (or much less) in the mutant (see Fig.4); **d**: CALRETININ-positive cells were essentially always MAP2-positive in double immunofluorescence labellings; they constituted about 38% of the total MAP2-positive cells.

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CHAPTER 3

EMX2 IS A DOSE-DEPENDENT NEGATIVE REGULATOR OF SOX2 TELENCEPHALIC ENHANCERS

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Emx2 is a dose-dependent negative regulator of Sox2 telencephalic enhancers

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Abstract

The transcription factor Sox2 is essential for neural stem cells (NSC) maintenance in the hippocampus and in vitro. The transcription factor Emx2 is also critical for proper hippocampal development, and its loss causes an unbalance between NSC self renewal and commitment to differentiation in vitro. In a search for "modifier" genes affecting the Sox2 deficiency phenotype in mouse, we observed that loss of a single

Emx2 allele substantially increased the telencephalic LacZ transgenic expression driven by the 5’ or 3’ enhancer of Sox2. In vitro electrophoresis mobility shift assays, protein to protein interaction and transfection studies indicated that Emx2 represses 5’ and 3’ Sox2 enhancer activities. Emx2 bound to overlapping Emx2/POU binding sites, preventing binding of the POU transcriptional activator Brn2 to its target sequence. In addition, Emx2 directly interacted with Brn2 without binding to DNA, sequestering it. Loss of a single Emx2 allele increased Sox2 levels in the medial telencephalic wall, including the hippocampal primordium.

In hypomorphic Sox2 mutants, retaining a single copy of a “weak” Sox2 allele, loss of a single Emx2 allele resulted in a substantial rescue of hippocampal radial glia stem cells and of neurogenesis, indicating that Emx2 functionally interacts with Sox2 at the stem cell level. These data show that Emx2 negatively modulates Sox2 expression, and may thus control important aspects of NSC function in development.

Introduction

The transcription factor Sox2 is essential in pluripotent stem cells of the blastocyst inner cell mass [1]. Sox-2 is also highly expressed in neural stem cells (NSC) and in their early progeny, and repressed upon differentiation [2-6]. The decreased expression of Sox2 in a mouse hypomorphic Sox2 mutant causes important brain and neurologic defects [5, 7], which mimic significant aspects of the pathology of Sox2-deficient patients [8, 9]. In this hypomorphic mutant, we combined the deletion of one Sox2 allele ($Sox2^{\beta\text{-geo}}$ knock-

in) with the deletion, on the other allele, of an upstream enhancer of Sox2 (Sox2$^{\Delta Enh}$), important for its expression in telencephalic NSC [2, 5, 10]. In the hypomorphic mutant, Sox2 expression is 25-30% as that of the wild type; this mutant shows hippocampal stem cells loss, corpus callosum interruption, parenchymal loss in striatum and thalamus, decreased numbers of GABAergic neurons and neurological defects, including epilepsy [5, 7]. Recently [11], we showed that Sox2 embryonic deletion leads to complete perinatal loss of hippocampal stem cells. NSC from the forebrain of such mutants become rapidly exhausted in in vitro neurosphere culture.

The Emx2 transcription factor is expressed in the developing dorsal telencephalon, including prospective hippocampus and cerebral cortex, from early embryogenesis [12, 13]. Its expression is maintained postnatally in adult brain neurogenic regions, the subventricular zone (SVZ) and hippocampus dentate gyrus (DG)[14, 15].

Emx2 inactivation in mouse causes delayed hippocampal development, with reduced cerebral cortex and abnormal specification of cortical areas at birth [reviewed in 13,16-18]. In vitro, mutant Emx2-/- NSC show increased proliferation in long term neurosphere cultures [15].

Following our description of brain abnormalities in hypomorphic Sox2 mutants, we wished to investigate possible effects of “modifier genes” on the Sox2 hypomorphic phenotype.

A common aspect of the defects in Sox2 and Emx2 mutants is the abnormal hippocampal development [5, 11, 13, 16]. Moreover, NSC

from both $Sox2^{-/-}$ and $Emx2^{-/-}$ mutants exhibit important (opposite) abnormalities in in vitro culture [11, 15]. Therefore, we looked for genetic interactions between Sox2 and Emx2 in double mutants in which the Sox2 hypomorphic genotype ($Sox2^{\beta\text{-geo}/\Delta Enh}$)[5] was combined with loss of a single Emx2 allele. This significantly ameliorated the brain phenotype of Sox2 hypomorphic mice (Suppl. Fig.1) This suggested that Emx2 may play antagonistic roles to Sox2, possibly by negatively modulating its activity.

We report that Emx2 is a direct transcriptional repressor of Sox2. Loss of a single Emx2 allele substantially rescues the number of hippocampal NSC in the dentate gyrus of hypomorphic Sox2 mutants. Thus, Emx2 functionally interacts with Sox2 at the stem cell level.

Results

Emx2 represses transgenic and knock-in Sox2-LacZ reporters

We crossed $Sox2^{\beta\text{-geo}/+}$, $Emx2^{+/-}$ double heterozygotes with homozygous Sox2 knock-down ($Sox2^{\Delta Enh/\Delta Enh}$) mice, obtaining double mutants in which the Sox2 hypomorphic genotype, $Sox2^{\beta\text{-geo}/\Delta Enh}$ [5], was combined with the loss of a single Emx2 allele. The brain phenotype of these double mutants was significantly ameliorated relative to Sox2 hypomorphic mice from the same litter, in which both Emx2 alleles were still present (Suppl. Fig. 1). This suggested that Emx2 might transcriptionally repress Sox2, or somehow antagonize it.

To evaluate the effect of Emx2 in Sox2 regulation, we crossed mice carrying Sox2-lacZ transgenic or knock-in reporters to $Emx2^{+/-}$ mice. The Sox2-β-geo transgene [2] is driven by 5.7 kb of the Sox2

promoter/enhancer, and its neural expression is progressively confined to the telencephalon, after E11.5. The SRR2 transgene [19, 20]) is driven by the tk-promoter linked to an enhancer normally located immediately 3’ to the Sox2 coding region (these mouse lines are denominated 5’ and 3’ enhancer lines, respectively). In the knock-in line, a $Sox2^{\beta\text{-geo}}$ construct [2], was inserted by homologous recombination into the Sox2 locus, allowing regulation of a properly integrated construct; note, however, that this knock-in lacks the 3’ enhancer, that is part of the region replaced with β-geo.

Breeding with Emx2-mutant mice, we obtained E14.5 progeny consisting of embryos carrying the transgene in the heterozygous state, together with the three possible Emx2 genotypes (wild type,+/+; heterozygote, +/-; homozygote, -/-).

For each construct, loss of one Emx2 allele is associated to significantly increased LacZ expression both dorsally and ventrally (Fig. 1A); a further strong increase is observed in $Emx2^{-/-}$ mice (note, however, that the $Emx2^{-/-}$ brain is abnormal, as expected [13]).

We confirmed these results by beta-galactosidase staining of brain sections (Fig. 1B). The 5’enhancer construct is expressed in dorsal and medial areas of the telencephalic ventricular zone and, ventrally, along the medial ganglionic eminence , whereas the 3’ enhancer construct is more active in ventrolateral areas. In $Emx2^{+/-}$ heterozygotes, the respective domains of expression were more intensely stained, both anteriorly and posteriorly; additionally, the extension of the LacZ-positive region was somewhat increased towards the midline, in mice carrying the 3’enhancer construct (arrows). In $Sox2^{\beta\text{-geo}}$ knock-in ; $Emx2^{+/-}$; heterozygotes, LacZ expression was similarly increased

LacZ in medial and ventral regions (arrows), where the residual 5'enhancer is active. As expected, homozygous $Emx2^{-/-}$ mutants showed increased LacZ expression, although matching the different areas is problematic due to morphological abnormalities.

These results indicate that Emx2 represses, in vivo, the activities of both the 5' and 3' enhancers of Sox2.

Emx2 transfection in Sox2-positive P19 teratocarcinoma cells represses reporter genes driven by the 5' or 3' Sox2 enhancer

The 5'- and 3'-enhancers "core"elements were defined in vivo by transgenic assays and, in vitro, by transfection in Embryonic Stem (ES) Cells [19-21]. Both elements contain POU sites, known to be functionally important in ES and brain cells [19-21], which bind specific transcription factors (Oct4 in ES , Brn1 and Brn2 in neural cells) [19-21]. In transgenic mice, approximately 400 nucleotides of the 5' enhancer are sufficient for full activity [2]. This enhancer contains, in addition to the two POU sites, several ATTA sites (referred to as ATTA-1 to ATTA-6, Fig.2A), which represent the core of potential homeobox transcription factor-binding motifs [21], including Emx2. The more 5' POU site is combined with ATTA-3 site within a single overlapping sequence. The 3' enhancer similarly contains several ATTA sites, together with a previously characterized POU-binding element [19](Fig. 2A).

To evaluate the role of Emx2 in the control of Sox2 expression, we transfected into P19 teratocarcinoma cells a luciferase reporter gene, driven by the minimal tk promoter linked to the core 5'Sox2 enhancer, in the absence or presence of an Emx2-expression vector. P19 cells

express Sox2 at high levels, but are negative for both Emx2 and the putative Sox2 activators [21] Brn1 and Brn2.

Emx2 cotransfection strongly repressed the activity of the enhancer, to a level just above that of the control enhancer-less tk-luciferase vector (Fig. 2B). Cotransfection with a vector expressing Otx2, a related homeobox gene, or with empty vector gave no significant repression. Similarly, Emx2 strongly repressed the activity of the 3'Sox2 telencephalic enhancer [19, 20], when assayed with both a full size and a "core" enhancer [19] construct (Fig. 2C). The repression caused by Emx2 was dose-dependent for both the 5 and 3' enhancers (Fig. 2D).

To identify the site where Emx2 binds to repress transcription, we mutated, in different combinations, each of six sites characterized by the ATTA sequence in the 5'enhancer. Unexpectedly, all the mutations strongly decreased the activity (in the absence of cotransfected Emx2)(Fig. 2E); the simultaneous mutation of five out of six sites (1/2/4/5/6, leaving only ATTA-3), essentially abolished the activity of the core enhancer (Fig. 2E).In these experiments, Emx2 cotransfection further reduced the residual activity of the mutants to the background level corresponding to the activity of the tk-promoter-luciferase construct.

These experiments suggest that the mutation of the ATTA sites destroys the binding of some (yet unidentified) activator protein. In contrast, as the repressive Emx2 activity is not abolished by any of the mutations, Emx2 either binds to other unidentified sites, or somehow antagonizes the activator at each of the defined sites.

Emx2 binds a composite POU/Emx2 binding-site (ATTA-3), and inhibits the binding of Brn2 to the same site

We characterized by electrophoretic mobility shift assays (EMSA) the binding of recombinant Emx2 to all of the ATTA sites in the core 5' enhancer. ATTA-3 resembles (Fig. 3A) one of the few characterised Emx2-binding sites, that of the Wnt1 gene [23, 24]; furthermore, a similar site is located in the 3' enhancer (ATTA-4) just upstream to the already studied [18, 19], functionally important, POU site. In EMSA, recombinant Emx2 (Suppl.Fig.2, panel A) bound to the Wnt-1 oligonucleotide (originally characterized only by foot-printing) generating a complex, that was supershifted by an anti-Emx2 antibody (Suppl.Fig.2, panel B). Similarly, ATTA-3 generated with Emx2 a strong retarded band (Fig. 3B, lanes 3-4; Fig. 2C, lane 21); two different mutations of ATTA-3 abolished Emx2 binding (Fig. 3C, lanes 11 and 16, versus lane 21). Further, the ATTA-3/Emx2 binding was efficiently competed by excess unlabelled Wnt-1 or ATTA-3 oligonucleotides, with similar kinetics. (Suppl. Fig. 2B).

An oligonucleotide including the combined ATTA/POU site (ATTA-3) binds [21, 23] the ES cell factor OCT4 and its brain homologues Brn1 and Brn2.As Emx2 inhibits the activity of Sox2 telencephalic enhancers in brain (Fig. 1), we asked if Emx2 binding to the POU sites in brain cells might interfere with the binding of Brn factors. Brn2 bound, as expected, the composite POU/ATTA-site 3 (ATTA-3) of the 5'enhancer, that was shown to bind Emx2 (Fig. 3B, lanes 5,6). When Brn2 and Emx2 were added together, no ternary Emx2-Brn2-probe complex was detected, suggesting that the binding was mutually exclusive. Addition of anti-Emx2 antibody caused the

loss of the Emx2 band and its supershift, but did not affect the Brn2 band (Fig. 3B, lanes 7,8). Importantly, Brn2 binding was abolished (Fig. 3C, lanes 12 and 17 as compared to lane 22) by the same mutations that cause loss of Emx2 binding.

Adding increasing amounts of Emx2, in the presence of a fixed amount of Brn2, proportionally increased Emx2 binding, whereas Brn2 binding was strongly decreased. (Fig. 3D lanes 5-7). The repression of Brn2 binding was observed already at relatively low levels of added Emx2 (and Emx2 binding), and under conditions of a large excess of labelled oligonucleotide; this suggests that the repression of Brn2 binding is not simply the result of a direct competition on the same DNA molecule, but rather entails other indirect mechanisms (see below).

We performed similar experiments using the 3' enhancer. Again, 3'enhancer ATTA-4 site (Fig. 3A) bound both Brn2 and Emx2 (Fig. 3E), and addition of Emx2 greatly decreased the binding of Brn2 (Fig. 3E, lanes 4,5). Similarly to the 5' site, mutation of this site abolished the binding of both Emx2 and Brn2 (not shown).

Emx2 inhibits Brn2 binding to ATTA sites 1,2 without directly binding to DNA

The ATTA motif is part of a large number of core sequences of distinct transcription factor-binding motifs, which are difficult to identify purely on the basis of the DNA sequence. As the POU/ATTA sequence (ATTA-3) binds both Oct4 and Brn1/Brn2 [21], and other sequences containing an ATTA motif bind Brn1 and Brn2 ([26- 28]; see Fig. 3A), we tested all ATTA sites in the 5' enhancer for binding to these factors. Brn2 bound (Fig. 4A) an oligonucleotide containing

both sites 1 and 2 (ATTA-1/2), whereas Emx2 did not bind (the weak band migrating slightly faster than Brn2 in lane 3, arrowhead, is due to a protein contained in the TNT extract used for Brn2 synthesis, see lane 2). Mutation of the conserved TT doublet in the ATTA motif abolished Brn2 binding, leaving only the fast TNT-derived band (lanes 10-11). The Brn2 band was almost completely ablated by addition of anti-Brn2 antibody (lanes 3,4). Finally, excess unlabeled ATTA-1/2 oligonucleotide competed the binding of the previously validated Brn2-binding site, ATTA-3 in the 5' enhancer ([21] and present paper) as efficiently as unlabelled ATTA-3 site oligonucleotide did (Fig.4B, lanes 4,5, versus lane 3). In contrast, a mutated ATTA-1/2 site oligonucleotide failed to compete (lane 6). We conclude that ATTA-1/2 site is a genuine Brn2-binding site.

As shown in Fig. 3D, Emx2 might inhibit the binding of Brn2 to the POU/ATTA site (ATTA-3) oligonucleotide both by direct DNA binding and by other indirect mechanisms. We tested the effects of Emx2 addition to the ATTA-1/2 site oligonucleotide, in the presence of Brn2. Emx2 addition (Fig. 4A, lane 5) almost completely abolished Brn2 binding, already at low Emx2 concentrations. Similar or higher amounts of the hematopoietic transcription factors GATA-1 and GATA-2 did not interfere with Brn2 binding (Fig. 4A, lanes 6,7).

In additional experiments (Fig. 4C) Emx2 prevented Brn2 binding, in a dose-dependent fashion, to two independently characterized Brn2-binding sites (Fig. 3A), those in the Delta and Nestin genes neural enhancers [26, 28].

Finally, we asked if "endogenous" Brn2 from neural stem/progenitor cells behaves as recombinant Brn2. We used nuclear extracts from a

murine adult hippocampal stem/progenitor cell line (AHP)[29], which coexpresses Sox2, Brn2 and Emx2 in a substantial proportion of cells (Suppl. Fig. 3), and from neurosphere cells. The ATTA 3 site is known to bind endogenous Brn2 [21]. The ATTA1/2 site generated, with AHP nuclear extracts, strong retarded bands (arrows) of mobility similar to that observed with the ATTA-3 site oligonucleotide (Fig. 4D). Both bands were supershifted by anti-Brn antibodies, but not anti-GATA-1 antibody; excess unlabeled ATTA1/2 oligonucleotide (but not its mutated version) efficiently competed, at low concentration, the binding to ATTA-3 labelled oligonucletide. Endogenous Emx2 is low in AHP and neurosphere nuclear extracts (Suppl. Fig. 2A and not shown), and did not generate a retarded band with either the canonical Wnt1 Emx2-binding sequence, or the ATTA-3 oligonucleotide. However, when recombinant Emx2 was added to nuclear extracts, the binding of Brn2 to ATTA-3 site was strongly inhibited already at low concentrations (similar results, not shown, with the ATTA1/2 site); upon addition of larger Emx2 amounts, the expected Emx2 band appeared (Fig. 4E). Addition of (control) GATA-1 protein had no effect. Thus, Emx2 antagonizes the binding of endogenous Brn2 to the ATTA-3 site.

Overall, the experiments reported above (Figs. 3,4) demonstrate that Emx2 prevents the binding of transcription factors (in this case Brn2) to their cognate motifs via mechanisms independent of its binding to DNA; one possible mechanism might be protein to protein interaction between Emx2 and Brn2. In a GST-pull down assay, a GST-Emx2 fusion protein retained in vitro synthesized Brn2 (Fig. 4F). We conclude that Emx2 and Brn2 proteins are able to physically interact.

Emx2 functionally antagonizes Brn2

POU factors, including Oct4 and neural transcription factors Brn1 and Brn2, were characterized as activators of the Sox2 3' enhancer in co-transfection experiments, and the mutation of the POU/ATTA site (ATTA-3 site) in the 5'enhancer [21] or of the POU site in the 3'enhancer [19,20] substantially decreased the activity of Sox2 transgenic constructs, suggesting that Brn1 and Brn2 factors may be positive regulators of Sox2 transcription in the brain.

To evaluate the respective roles of Brn2 and Emx2 in transfection experiments we linked to the minimal tk-promoter the ATTA-1/2 or the POU/ATTA (ATTA-3) site (the latter as a trimer) from the 5'enhancer. We transfected the construct into P19 cells in the presence of different amounts of Brn2-and/or Emx2 expression vectors (Fig. 5). In the absence of Emx2, Brn2 strongly stimulated the activity of the ATTA-1/2 construct in a dose-dependent way and, to a lesser extent, that of the ATTA-3 construct (Fig. 5A,C). The Brn2-dependent stimulation of the ATTA-1/2 construct was repressed to basal levels (just above the level of the tk-luc reporter, lane 9 versus lanes 1 and 2), by cotransfection of progressively increasing amounts of the Emx2-expression vector (Fig. 5B). Cotransfection of control " empty" vector, instead of Emx2- expression vector, yielded a slight repression only at the highest tested levels, ensuring specificity of the Emx2 repression observed (Fig. 5B, lanes 10-13). Similarly, on the ATTA-3 construct, Brn2-dependent stimulation was repressed by Emx2 (Fig. 5C). Thus, Brn2 is an activator at both the ATTA-3 (as previously shown in vivo and in vitro, [21]) and the ATTA-1/2 sites, and Emx2 represses the transcriptional activity at the same sites,

antagonizing Brn2-dependent stimulation. As Emx2 does not bind to ATTA-1/2 site sequences (Fig. 4A), this repression is caused by mechanisms that do not strictly require Emx2 binding to the DNA. The somewhat lower effect of Emx2 in the Brn2-dependent system, as compared to the drastic effect observed with the full “core” element (in the absence of cotransfected Brn2)(Fig. 2), probably reflects the modest enhancer activity of the individual ATTA sites in isolation, as compared with the cooperative activity of the multiple sites active in the full enhancer (Fig. 2).

Emx2 binds to the 5’enhancer in vivo

To ascertain if Emx2 interacts in brain cells with the Sox2 regulatory elements, we performed in vitro Chromatin Immunoprecipitation (ChIP) with anti-Emx2 antibodies, using chromatin from embryonic telencephalon (E14.5), from wild type and Emx2-null (negative control) embryos. A fragment comprising the ATTA-3 and the adjacent ATTA-1/2 sites was bound by Emx2 in wild type chromatin, but not in Emx2-null chromatin (Fig. 6). No binding was detected in an adjacent region B, comprising ATTA-5 and 6 sites, and lying 3’ to the bound DNA region. We conclude that Emx2 likely functionally interacts with the Sox2 regulatory region in vivo.

Loss of a single Emx2 allele significantly rescues the hippocampal NSC deficiency of hypomorphic Sox2 mutant mice

To ascertain if the Emx2-dependent inhibition of Sox2 expression, demonstrated in vitro, has any in vivo effects on Sox2-dependent brain phenotypes, we selected for further studies the hippocampus neural stem/progenitor cells of the hypomorphic Sox2$^{\beta\text{-geo}/\Delta\text{Enh}}$ mutant

[5, 7], that expresses Sox2 (from the single residual knock-down allele) at low levels. In these mice, postnatal neurogenesis is strongly diminished, particularly in the hippocampus. In particular, the number of nestin/GFAP double-positive radial glia cells (a stem/progenitor cell expressing Sox2 [5, 6]) is drastically decreased [5].

In Sox2 hypomorphic mutants, heterozygosis for a mutated Emx2 allele was sufficient to substantially rescue the number of GFAP/nestin stem/progenitor cells from about 20% to 60% of wild type levels (Fig. 7A,B); additionally, the radial glia was converted from a thin, poorly-developed appearance typical of cells of the hypomorphic mutant, to quasi-normal morphology (Fig. 7A). In agreement, BrdU incorporation (Fig. 7B) was substantially increased to 45% of wild type levels in $Sox2^{\beta\text{-geo}/\Delta Enh}$; $Emx2^{+/-}$, versus about 30% in $Sox2^{\beta\text{-geo}/\Delta Enh}$; $Emx2^{+/+}$ controls (even if loss of a single Emx2 allele, per se, causes some decrease of BrdU incorporation, Fig. 6B, Discussion, and [30]).

To interpret this result, we examined Sox2 expression in wild type mice in the prospective hippocampal area during development. In this area, Sox2, Brn2 and Emx2 are coexpressed in a large proportion of cells (Fig. 7C). At E 15.5, both the medial and lateral walls of the hippocampus expressed Sox2; however the medial wall of the lateral ventricle, from which the hippocampus will originate, expressed Sox2 at comparatively lower levels than the lateral wall (Fig. 7D). On the other hand, the Emx2 level was higher in the medial as compared to the lateral wall (Fig.7D, see also refs.13, 17]), pointing to an inverse relation between Sox2 and Emx2 expression.

In Emx2$^{+/-}$ heterozygotes we noted a significant upregulation of Sox2 expression in the medial telencephalic, relative to the lateral wall, when compared to wild type mice (Fig. 7D). This suggests that, within the area from which the hippocampus will arise, Emx2 negatively modulates Sox2 levels. This result is consistent with the possibility that the loss of a single Emx2 allele in Sox2 hypomorphic /Emx2$^{+/-}$double mutants contributes, by upregulating the deficient Sox2 expression, to the observed radial glia rescue.

Discussion

We studied the effect of Emx2, a transcription factor involved in hippocampal growth and in cortex patterning, on the expression of Sox2, a transcription factor critical for NSC maintenance. In spite of the importance of Emx2 in brain development, very few direct target genes (Wnt1 and possibly FGF8) are known [17, 18, 24, 25, 31-33]. In vivo and in vitro experiments show that Emx2 negatively regulates Sox2 at defined enhancer sites. Our results, together with data of the literature, suggest that Emx2 may control NSC decisions, at least in part by regulating Sox2 levels.

Emx2 negatively modulates Sox2 expression in the telencephalon by a direct action on Sox2 telencephalic enhancers

Sox2 neural expression is regulated by multiple enhancers, active at specific locations [2, 5, 19-21, 34]. In mouse, the best characterized enhancers are the 5' and 3' Sox2 enhancers studied here [2, 19-21, 35]. Both enhancers direct transgenic reporter gene expression to the

telencephalon, the 5’ enhancer being more active in dorso-medial regions, and the 3’ enhancer in ventro-lateral regions.

Emx2 is expressed in the dorsal telencephalon according to a posterior medial to anterior lateral concentration gradient, that intercepts the Sox2 expression domain [18, 21, 30, 31].

At the cellular level, Sox2 and Emx2 expression domains substantially overlap within the ventricular zone [5, 30]. In particular, in the late embryo, both genes are active in the prospective hippocampal domain; at this stage, in the lateral ventricle, regions of high Sox2 expression show relatively lower Emx2, and regions of high Emx2 expression have lower Sox2 levels (Fig. 7D). Coexpression of Sox2 and Emx2 is also observed in adult hippocampal cells in vivo (Supplementary Fig. 4), and in adult hippocampal AHP cells (Supplementary Fig. 3).

Loss of either one or both copies of Emx2 greatly increases the expression of transgenes driven by the 5’ or the 3’ Sox2 enhancers (Fig. 1); we observed a similar result with the $Sox2^{\beta\text{-geo}}$ knock-in allele, that retains the 5’ enhancer (but has lost the 3’ enhancer [19, 20]), within the full Sox2 locus. We propose that this effect of Emx2 deficiency depends on direct effects of Emx2 on Sox2 regulatory regions.

To interpret at the molecular level these in vivo data, we performed EMSA and transfection experiments, mainly with a cell line (P19) that, although non-neural, expresses Sox2 and can be manipulated by transfection to express Brn2 and/or Emx2 (absent in the basal state). Based on these data, we propose two different mechanisms whereby Emx2 might downregulate Sox2 enhancer activity (Figs. 3-5, Suppl.

Fig. 5). First, it can directly bind to 5' (ATTA-3) and 3'enhancer (ATTA-4) sites (Fig. 3); these sites resemble the Emx2-binding site which represses Wnt1 in the developing telencephalon (Fig. 3A; see [25]). ATTA-3 and ATTA-4 are also bound by the POU factors Brn1 and Brn2 ([21] and Fig. 3), that were previously implicated in Sox2 regulation on the basis of transfection, transgenic and ChIP experiments [19-21]. As mutations at the ATTA-3 site abolish the binding of both Emx2 and Brn2, it is likely that their binding is mutually exclusive; indeed, we did not detect in EMSA experiments (even at high concentration of protein relative to probe, not shown) any band of mobility slower than that of Brn2, that might suggest the formation of a ternary complex of DNA with both factors. Therefore, Emx2 might directly prevent Brn2 activity at these sites by binding to the overlapping Emx2-Brn2 DNA motifs.

Additionally, Emx2 may repress the Sox2 enhancers by antagonizing the binding to DNA of transcription factors, likely through protein to protein interaction, without direct DNA binding. In fact, the binding of Brn2 to ATTA-sites in Sox2 enhancers and to other previously described and validated Brn2 sites [21, 26, 28] is prevented by Emx2 addition, in the absence of any binding of Emx2 itself to the same sequences (Fig. 4). Thus, Emx2 might antagonize Brn2 by sequestering it, preventing its binding. Evidence in favour of this mechanisms is provided by GST pull-down experiments showing that Brn2 and Emx2 may physically interact (Fig. 4D). Emx2 represses SP8 trancription factor-dependent activity of the FGF8 promoter without binding to the promoter itself [32]; moreover, Emx2 and SP8 proteins physically interact [33]. Our data extend these

observations, pointing to Emx2-dependent modulation of Brn2 activity via protein to protein interaction. It is worth noting that the binding sequence recognized by Brn2 in our experiments is a rather degenerate one, centred on an ATTA motif that is potentially recognized by many transcription factors [22]. Presently, we cannot rule out that, in addition to Brn2, other transcription factors, particularly the Brn1 homolog or Oct6, might bind to this sequence, and could thus be antagonized by Emx2.

Additional data suggest that these mechanisms do operate in vivo. In fact, Emx2 binds to a fragment comprising the POU/ATTA-site (ATTA-3) in nuclei from normal telencephalon, in ChIP experiments (Fig. 6). This fragment lies within a 120 bp DNA region that mediates POU site-dependent reporter gene expression in the telencephalon of transgenic embryos [21].

In conclusion, we propose that Emx2 contributes to the regulation of Sox2 expression by antagonizing Brn2 (and possibly other activators able to bind the ATTA core sequence, [22]). The mechanism provides a wide scope for modulation, depending on the affinities of Emx2 for its DNA target and or protein interactors, and on the relative ratios between Emx2 and brain transcription factors at different locations.

Loss of a single Emx2 allele significantly antagonizes the hippocampal NSC loss in Sox2 hypomorphic mutants

Sox2 hypomorphic, Sox2 conditional-null and Emx2 homozygous mutants all show severe hippocampal defects, indicating that separate Sox2 and Emx2 activities are required for hippocampal development [5, 11, 13]. In addition to its essential role in hippocampal development, Emx2 has antagonistc functions towards Sox2, as

demonstrated by the increased Sox2 expression observed in the medial lateral ventricle wall, including the prospective hippocampus, upon the loss of a single Emx2 allele (Figs. 1 and 7). An important question is whether the loss of a single Emx2 allele (and the resulting moderate Sox2 overexpression) has any phenotypic consequences on Sox2-dependent functions.

Sox2 is critically required for NSC in the hippocampus. Embryonic deletion of Sox2 (by E12.5) does not immediately result in NSC loss, but this becomes evident at later stages, starting by P2 and resulting in complete ablation of hippocampal neurogenesis and dentate gyrus severe hypoplasia by P7 [11]. In adult Sox2 hypomorphic ($Sox2^{\beta\text{-}geo/\Delta Enh}$) mutants, the number of nestin/GFAP radial glia cells (a neural stem/progenitor cell type expressing Sox2 [5, 6, 36] in the hippocampus is importantly decreased ([5] and Fig. 7, present paper).

Our experiments show that loss of a single Emx2 allele (that, by itself, has little phenotypic effects [13, 17, 31]) slightly raises the number of nestin/GFAP radial glia cells in Sox2 wild type mice (Fig. 7); importantly, however, in Sox2 hypomorphic mutants, the loss of a single Emx2 allele strongly increases the number of nestin/GFAP radial glia cells, as well as, to a lesser extent, BrdU incorporation (note that heterozygous Emx2 deficiency, per se, decreases BrdU incorporation (Fig. 7)(see also [30]). This demonstrates that Emx2 deficiency critically affects at least one well characterized Sox2-dependent phenotype. There may be several mechanisms for this effect. One possibility, suggested by the effect of the deletion of a single Emx2 allele on Sox2 expression (Figs. 1 and 7) is that Emx2 deficiency ($Emx2^{+/-}$), by raising the activity of the single

“knockdown” Sox2 allele in the hypomorphic mutant, may contribute to a better embryonic/perinatal development of hippocampal NSC and thus to the rescue of the nestin/GFAP hippocampal stem cells (Fig. 7A). Note that the gap in Sox2 expression level between the severely affected hypomorphic mutant (25-30% of normal) and the essentially normal Sox2 heterozygote (about 65% of normal Sox2 activity, [5,7]) is relatively small, suggesting that limited derepression of the Sox2 knockdown allele due to Emx2 deficiency might be sufficient to reach a threshold level adequate to improve stem cell maintenance.

Although it remains possible that other activities of Emx2 besides that on Sox2 regulation contribute to the observed results, our interpretation is in keeping with suggestions [15] that Emx2 functions at the level of the decision of the NSC between self renewal (symmetrical division) and commitment to differentiation (asymmetrical division). In fact, in neurosphere long term cultures of Emx2-/- mutants, the growth rate and the proportion of symmetrical stem cell divisions were increased relative to wild type cells [15]. Thus, the decision between self-renewal (which requires adequate Sox2 levels, [11] and commitment to differentiation (linked to Sox2 downregulation [7]) might be influenced by the level of Emx2 expression at least in part through Sox2 regulation.

Perspectives

The defective hippocampal development, together with the significant decrease in cortex growth and patterning defects in Emx2 homozygous mutants [17, 31] are the result of complex mechanisms.

Although a direct patterning activity of Emx2 was demonstrated by transgenic Emx2 overexpression [37], the cortex growth deficiency, failure of hippocampal development and, to a lesser extent, patterning activity, are explained, in part, by indirect mechanisms, such as changes in gradients of diffusible factors [17, 30, 38].

The identification of Sox2 as a potential target of Emx2 repressive action, together with strong evidence that Sox2 controls NSC maintenance, suggests that Emx2 gradients might affect Sox2 levels in different developing cortical regions, thus helping control the balance between NSC self-renewal and commitment to differentiation. Here, we limited our study of Sox2-dependent functions (Fig. 7) to heterozygous Emx2 mutants, which retain normal brain morphology. Future studies may address the role of complete Emx2 deficiency in relation to Sox2-dependent phenotypes.

Materials and Methods (see also Online Methods)

Mouse lines and immmunohistochemistry

Mouse lines were described: 5’ and 3’ enhancer-reporter, refs. 2, 19-21; Sox2-hypomorphic (Sox2 $^{\Delta Enh}$) and null (Sox2 $^{\beta\text{-geo}}$) mutant alleles, ref.5; Emx2 null mutant, ref.13.

X-gal staining, immunohistochemistry (IHC) and histology were as reported [5]. For anti-Emx2 IHC, see ref.14; for anti-Brn2 IHC, a SantaCruz goat antibody [21] was used (1:100).

Reporter constructs and transfection

The 400 bp Sox2 5’ telencephalic enhancer [21] and its PCR-mutated versions were cloned into the pGL3-based luciferase reporter,

upstream to a 215bp minimal tk promoter (5'enh-tk-luc). Luciferase reporters for 3'enhancer activity were described [18, 19]; their core sequence was as in [40], Fig 3. Exponentially growing P19 cells were transfected with Lipofectamine 2000 (Invitrogen) and luciferase activity assayed after 24 hrs.

Recombinant protein expression and purification

Recombinant Emx2, Brn2, GATA1 and GATA2 were produced in the reticulocyte lysate system (TNT, Promega). For GST-pull-down experiments, Emx2 (or CP2 control, [41]) cDNAs, cloned in pGEX2T, were expressed in *Escherichia coli* BL21ce. Purified proteins (1 μg of total protein, as GST-Emx2, GST-CP2 and GST-only resins) were used for GST-pulldown of ^{35}S Brn2-containing TNT reaction as in [39].

Electrophoretic mobility shift assay (EMSA) and Chromatin Immunoprecipitation (ChIP)

EMSA (ref.42) utilized TNT-produced proteins or nuclear extracts; ChIP was as in [6].

Acknowledgements

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Figures

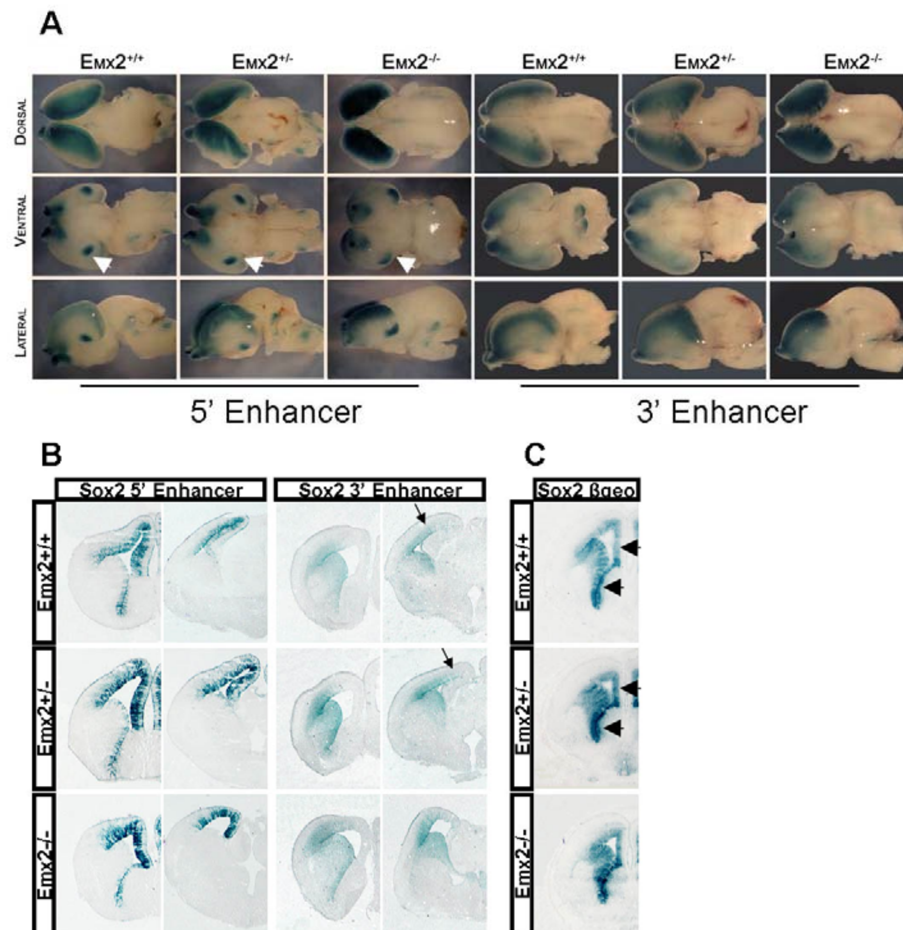
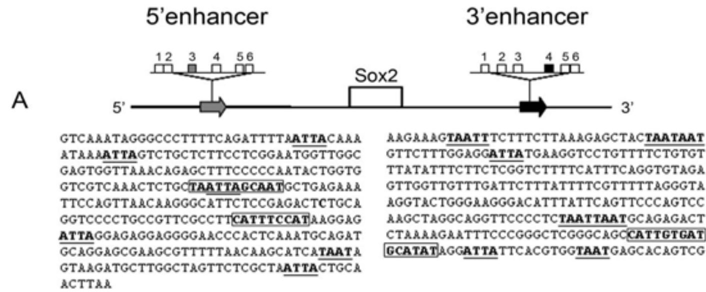


Figure 1 – Emx2 deficiency increases activity of Sox2 telencephalic enhancers-driven lacZ transgenes. (**A**) X-gal stained E15.5 brains carrying beta-geo transgenes driven by the 5' Sox2 telencephalic enhancer (left) or by the 3' enhancer (right), of Emx2$^{+/+}$, Emx2$^{+/-}$, or Emx2$^{-/-}$ genotype, as indicated. Dorsal (top row), ventral (middle row) and lateral (bottom row) views are shown. Increased X-gal staining is seen, most clearly in dorsal views, in Emx2$^{+/-}$ as compared to Emx2$^{+/+}$ brains, and in Emx2$^{-/-}$ as compared to Emx2$^{+/-}$ brains. In the 5' enhancer-transgenic brains, an X-gal-positive spot on the ventral telencephalic vesicles, visibile in the ventral (arrow) and lateral views, has comparable intensity in Emx2$^{+/+}$ and Emx2$^{+/-}$ brains, acting as an internal control for staining. Overall, 7/7 Emx2$^{+/-}$ transgenic embryos (5' construct, E15.5) showed increased lacZ expression relative to Emx2$^{+/+}$ from the same litter (4 embryos). Similarly, 7/8 Emx2$^{+/-}$ embryos carrying the 3' transgene

showed increased lacZ activity relative to Emx2$^{+/+}$ controls (4 embryos). Homozygous Emx2$^{-/-}$ 5' transgenic embryos were always (7/7) more intensely stained than their control heterozygotes (Emx2$^{+/-}$) littermates (11 embryos); 7/7 of the Emx2$^{-/-}$ 3' transgenics were more stained than their Emx2$^{+/-}$ heterozygous controls (10 embryos). (**B, C**) X-gal stained brain coronal sections of 5' or 3' enhancer-lacZ transgenic forebrains (**B**), and of Sox2$^{\beta\text{-geo}}$ knock-in heterozygous brains (**C**), of Emx2$^{+/+}$ (top row). Emx2$^{+/-}$ (middle) and Emx2$^{-/-}$ (bottom) genotype. Arrow in B (3' enhancer) points to some dorsal expansion of X-gal staining signal in Emx2$^{+/-}$, as compared to Emx2$^{+/+}$ brain. Arrows in **C** point to the medial telencephalic wall (including the prospective hippocampus) and the medial ganglionic eminence, where increased X-gal staining is clearly visibile in Emx2$^{+/-}$ brains as compared to Emx2$^{+/+}$.


5'enhancer
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GTTGGTTGTTTGATTCTTTATTTTCGTTTTTAGGGTA
AGGTACTGGGAAGGGACATTTATTCAGTTCCCAGTCC
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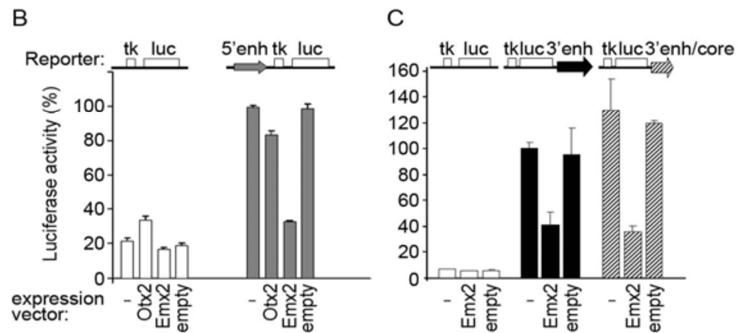
B
Reporter:
tk luc
5'enh tk luc
Luciferase activity (%)
0
20
40
60
80
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expression vector:
–
Otx2
Emx2
empty
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Otx2
Emx2
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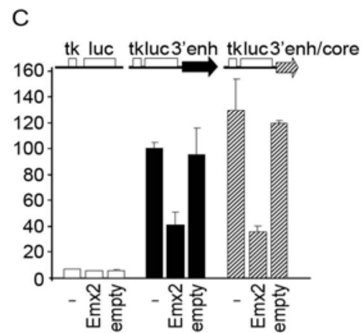
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tk luc
tkluc 3'enh
tkluc 3'enh/core
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Emx2
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Emx2
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Emx2
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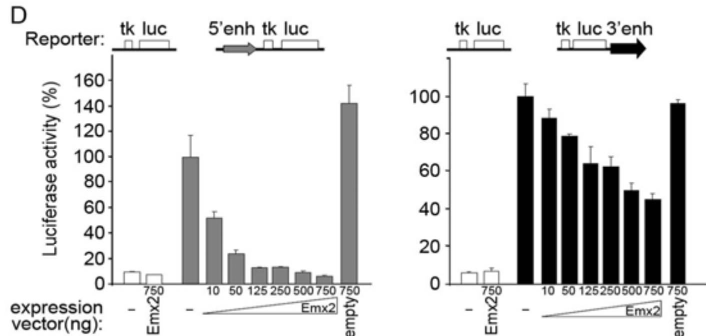
D
Reporter:
tk luc
5'enh tk luc
tk luc
tk luc 3'enh
Luciferase activity (%)
0
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40
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expression vector(ng):
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750
Emx2
–
10 50 125 250 500 750
Emx2
750
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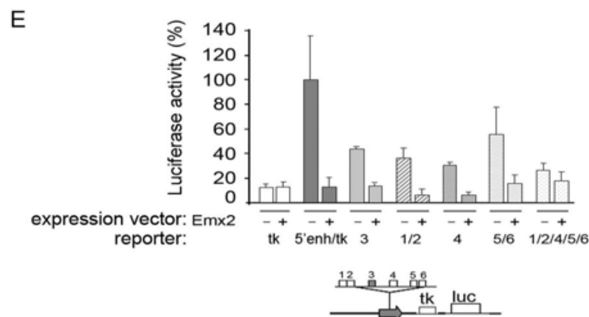
E
Luciferase activity (%)
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20
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expression vector: Emx2
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reporter:
tk
5'enh/tk
3
1/2
4
5/6
1/2/4/5/6
1 2 3 4 5 6
tk
luc

Figure 2 – Emx2 represses the activity of the 5' and 3' Sox2 telencephalic enhancers in transfection assays. (**A**) 5' and 3' Sox2 telencephalic enhancers. Numbered squares: ATTA sites, underlined and bold in the sequences below. Boxed bold sequences: POU sites [18-20] in 5' and 3' enhancers (**B,C**) Cotransfection of 5' or 3' enhancer-driven (black bars, full enhancer; striped bars, "core" enhancer) tk-luciferase vectors, or "empty" tk-luciferase vector (white bars), with Emx2 or Otx2 expression vectors, or with "empty" vector. The mean activity of the enhancer-driven constructs (with no cotransfected expression vector) is set = 100% luciferase activity. (**D**) Co-transfection of 5' and 3'-enh. luciferase constructs with increasing amounts of Emx2-expression vector. (**E**) Luciferase activity of 5' enhancer constructs carrying mutations in the indicated ATTA sites, and their response to co-transfection of the Emx2 expression vector (500 ng).

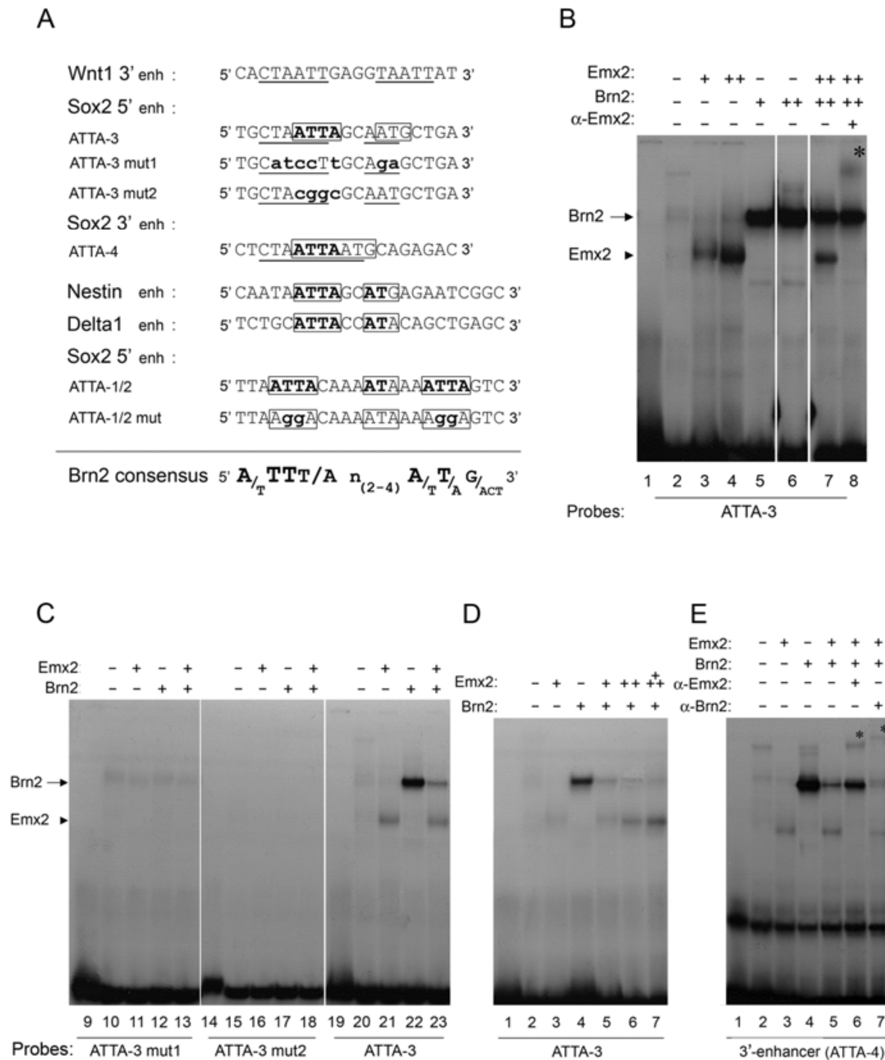


Figure 3 – Emx2 binds to ATTA sites within the Sox2 5' and 3' enhancers, and antagonizes binding of the activator Brn2. (**A**) ATTA sequences binding Emx2 and/or Brn2. Lowermost line: Brn2/POU consensus based on TFBS cluster and our data. Letter size is proportional to nucleotide frequency. The spacer (n) is 2-3 nucleotides in previously validated sites [25, 27]. For the interaction of a POU factor with its binding site, and spacer length, see [37]. Boxed sequences are homologies to the Brn2 consensus. Underlined sequences correspond to the previously reported Emx2 binding sequence (footprint) in the Wnt1 enhancer [23, 24], and to

homologous sequences within the 5’ and 3’ Sox2 enhancers. (**B**) EMSA with an ATTA-3 site probe (5’ enhancer) and recombinant Emx2 and Brn2 proteins (as indicated above the lanes). Anti-Emx2 antibody was added in lane 8. Asterisk: supershifted band. (**C**) EMSA with wild type (lanes 19-23) and two different mutated (lanes 9-13; 14-18) ATTA-3 site probes (5’ enhancer). (**D**)Addition of increasing amounts of Emx2 (lanes 5-7) to ATTA-3 site probe (5’ enhancer) together with a fixed amount of Brn2 (as in lane 4). An Emx2 retarded band appears, while the Brn2 band progressively disappears. (**E**) EMSA with a probe from the 3’ enhancer ATTA-4 site, showing ability to bind Emx2 or Brn2. Addition of Emx2 together with Brn2 (lane 5) antagonizes Brn2 binding. Asterisks indicate bands supershifted by antibodies (lanes 6,7).

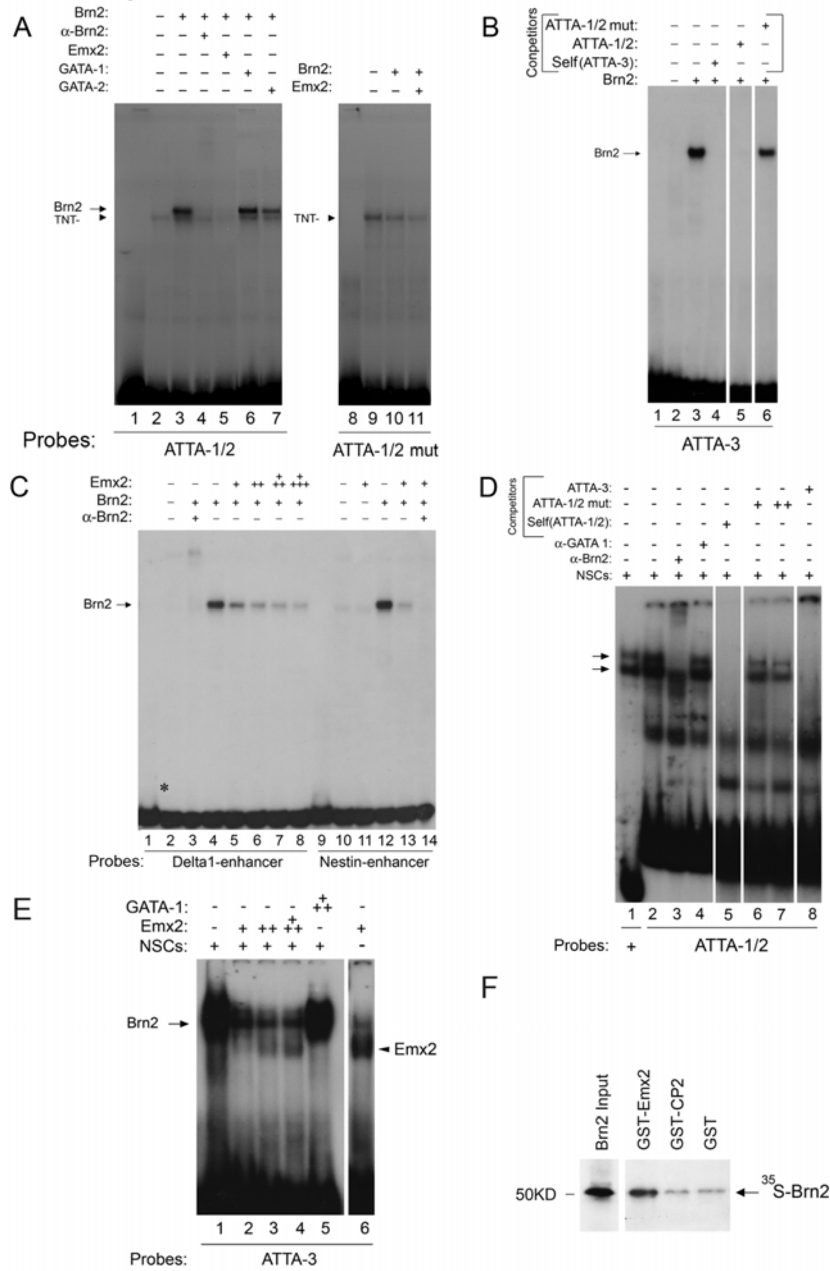


Figure 4 – Emx2 antagonizes the binding of Brn2 to ATTA-1/2 sites in the 5' enhancer, and to previously characterized Brn2 binding sites in other neural enhancers. (A) EMSA with a probe containing ATTA sites 1 and 2 (5' enhancer); added recombinant proteins, and Brn2 antibody, are indicated above the lanes. The

probe binds recombinant Brn2 (arrow), but not Emx2 (TNT- arrowhead indicates a non-specific band seen also with TNT extract only). Addition of Emx2 antagonizes Brn2 binding (lane 5). No antagonism is seen upon addition of GATA1 or GATA2 (lanes 6,7). (B) EMSA with an ATTA-3 site probe (a previously validated Brn2 binding site in the 5' enhancer [19-21]; binding of Brn2 is efficiently competed by wild type non-labelled ATTA-1/2 sites oligonucleotide (lane 5), but not by its mutated version (lane 6). Competition is as efficient as with the "self" oligonucleotide (lane 4). (C) EMSA with probes containing previously validated Brn2 binding sites in the nestin and Delta-1 enhancers. Brn2 binding (arrow) is antagonized by simultaneous Emx2 addition in a dose-dependent way. Asterisk: Brn2 antibody-supershifted band. (D) EMSA with ATTA-1/2 site probe and nuclear extracts from AHP neural cells. Two complexes are generated (arrows) with both ATTA-3 (lane 1, "+" as in [21]) and ATTA-1/2 (lane 2), which are supershifted by anti-Brn2 (lane 3), but not anti-GATA1 antibodies (lane 4). Binding of Brn2 to ATTA-1/2 is efficiently competed by unlabelled ATTA-3 (lane 8), by "self" ATTA-1/2 (lane 5), but not by mutated ATTA-1/2 (lanes 6,7) oligonucletides. (E) EMSA with ATTA-3 probe and nuclear extracts from AHP cells. Added recombinant proteins (Emx2, GATA-1) are indicated above the lanes.The Brn2 retarded complex (lane 1, arrow) (see also [21] and panel D) is sharply decreased following addition of Emx2 (lanes 2-4), but not of control GATA-1 (lane 5).The lower, Emx2-containing complex, is progressively increased in parallel with the addition of Emx2. This complex has the same mobility of that generated by direct binding of recombinant Emx2 to the ATTA-3 probe (lane 6). (F) Emx2 and Brn2 directly interact in a GST pulldown assay. Brn2 is retained by GST-Emx2, but not by GST-CP2 control resin (which gives a weak signal equivalent to that seen with the "empty" resin (GST).

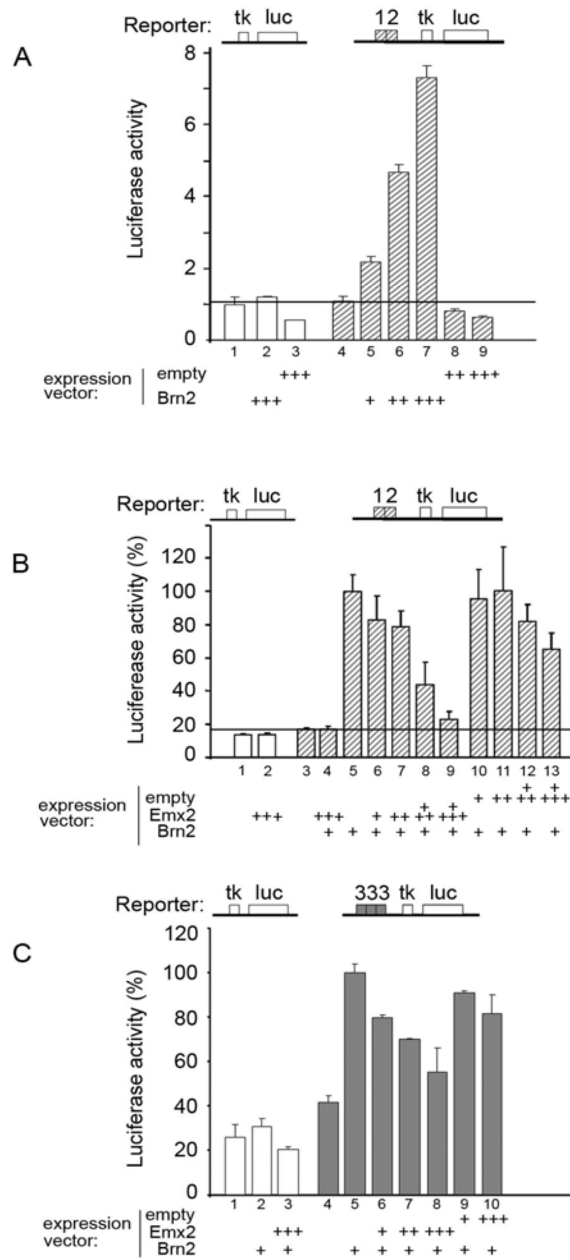


Figure 5 – Emx2 represses Brn2-transactivated ATTA-1/2 and ATTA-3 sites – tk luciferase reporter constructs in a dose-dependent way. (**A**) Brn2 dose-dependent

transactivation of ATTA-1/2 sites (5' enhancer). (**B,C**) Emx2 dose-dependent repression of Brn2-dependent transactivation of ATTA-1/2 sites construct **(B)** and of ATTA site 3 construct (**C**). In **A**, luciferase activity is expressed in arbitrary units, where 1 is the activity of the tk luc reporter; in **B** and **C**, 100% luciferase activity is set to the maximum observed activity. The horizontal line in A and B represents the background activity of the ATTA-1/2 site construct in the absence of cotransfected Brn2.

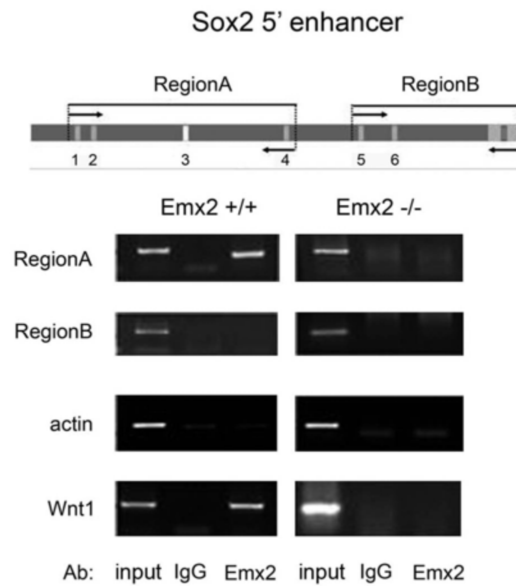


Figure 6 – Emx2 is bound to the Sox2 enhancer in vivo. ChIP with anti-Emx2 antibodies of E14.5 embryonic brain chromatin from wild type and $Emx2^{-/-}$ control embryos. Region A, containing ATTA-3 site is immunoprecipitated from wild type, but not Emx2-null chromatin. The previously described Wnt1 enhancer containing an Emx2 binding site [24] is used as a control (Wnt1), and is similarly precipitated from wild type, but not mutant, chromatin. Antibodies used are indicated below the lanes. Input: input chromatin. IgG: anti-IgG control antibodies. Emx2: anti-Emx2 antibodies.

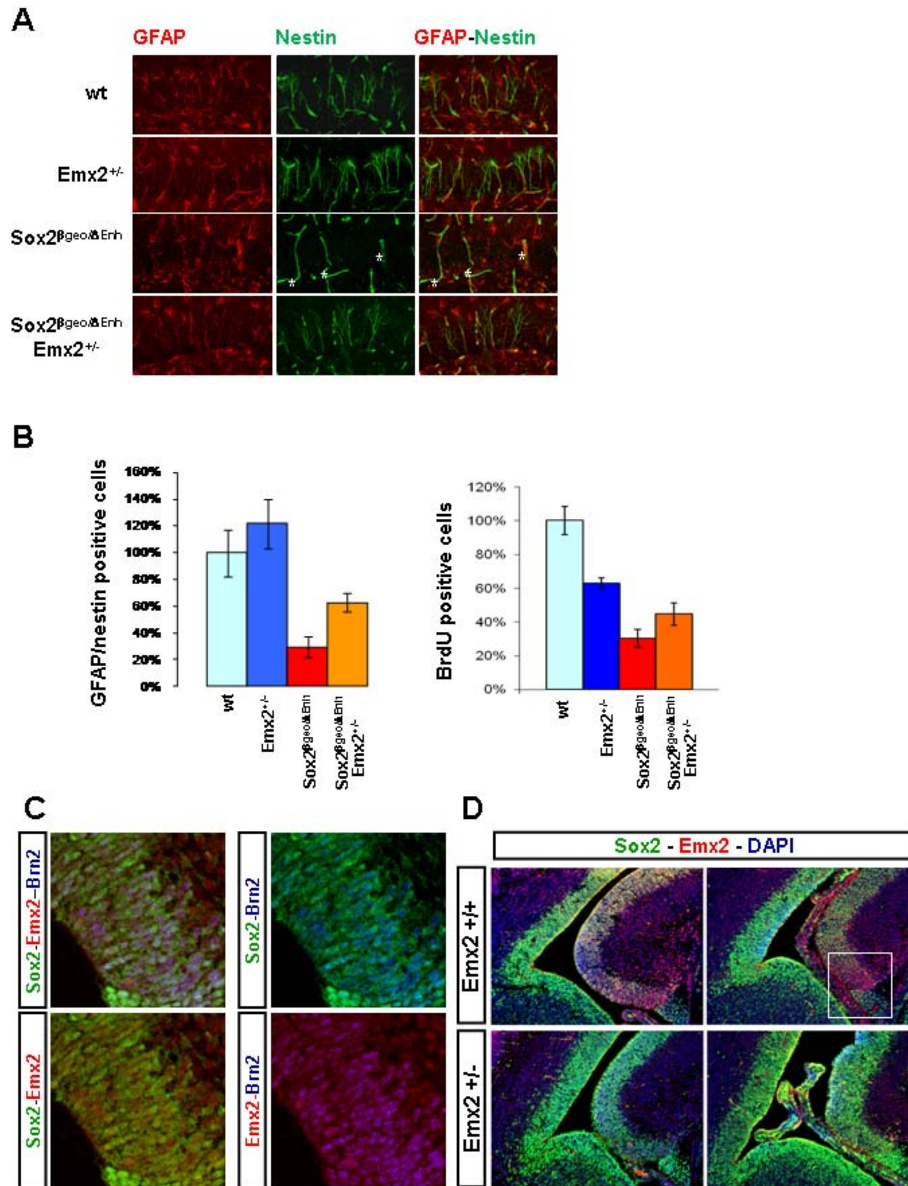
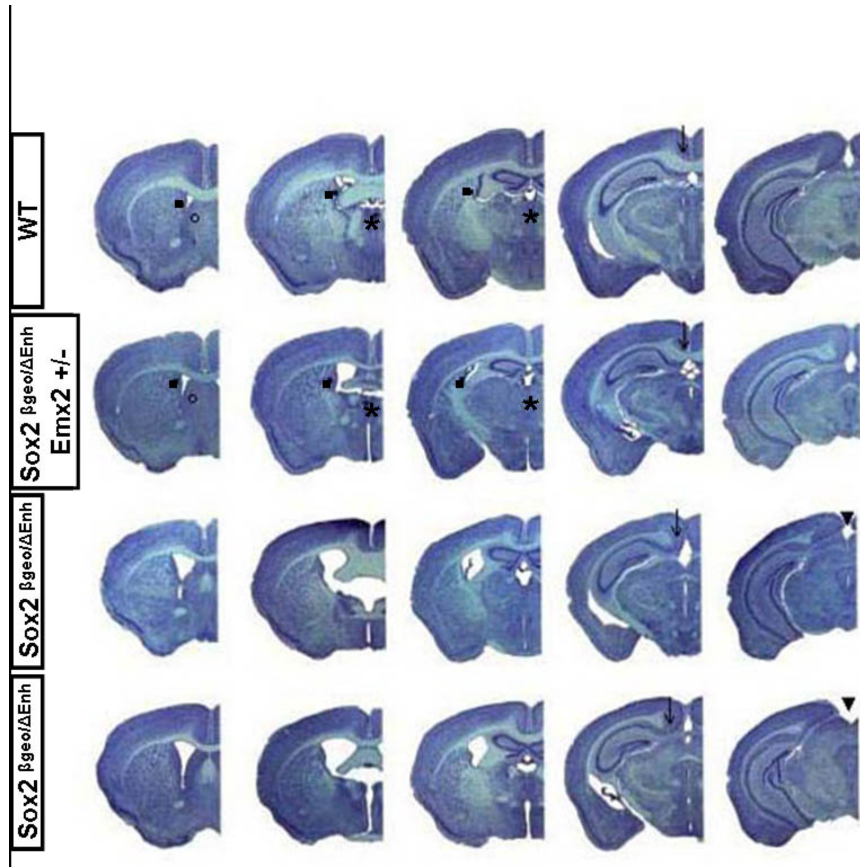


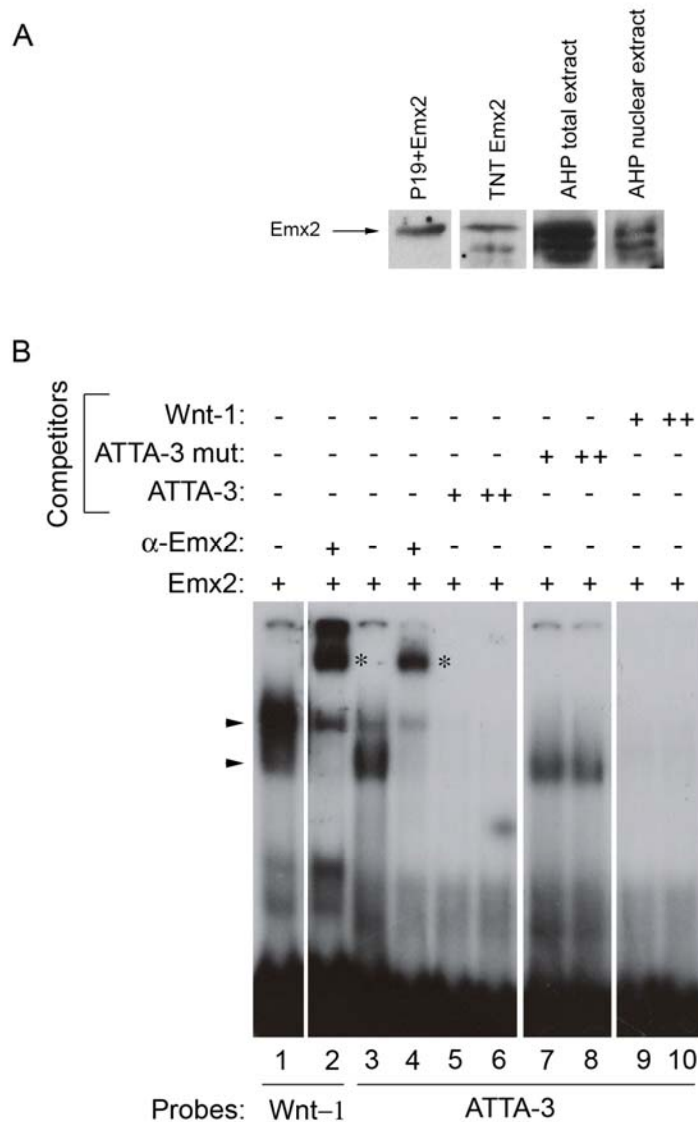
Figure 7 – Emx2 deficiency (Emx2$^{+/-}$) rescues GFAP/nestin stem cells impairment in the hippocampus of Sox2-deficient (Sox2$^{\beta\text{-geo}/\Delta\text{Enh}}$) mutant mice. (**A**) GFAP/nestin double immunofluorescence of hippocampus dentate gyrus in the indicated genotypes. GFAP/nestin-positive cells, strongly depleted in Sox2-hypomorphic (Sox2$^{\beta\text{-geo}/\Delta\text{Enh}}$) mutants, recover to a significant extent in Sox2$^{\beta\text{-geo}/\Delta\text{Enh}}$;Emx2$^{+/-}$ double mutants (asterisks mark vessels, showing non-specific fluorescence). (**B**) GFAP/nestin-positive cells and BrdU-positive cells (n=8 mice per genotype). Wild

type is set = 100%. (**C**) triple immunofluorescence (confocal microscopy) with anti Sox2 (green), anti Emx2 (red) and anti Brn2 (blue) on E15.5 telencephalic sections detects extensive coexpression of Sox2, Emx2 and Brn2 in the ventricular zone. The image shows an area within the medial telencephalic wall, that approximately corresponds to the region boxed in D. (**D**) double immunofluorescence with anti Emx2 (red) and anti Sox2 (green) antibodies on E15.5 telencephalic sections (confocal microscopy), in wild type ($Emx2^{+/+}$, top) and $Emx2^{+/-}$ heterozygotes (two different mice/genotype). In $Emx2^{+/-}$ brains, compared to $Emx2^{+/+}$ controls, an increase in the intensity of Sox2 staining is seen in the medial telencephalic wall (comprising the prospective hippocampus), as compared with the outer/lateral wall within the same section.

Supplementary figures

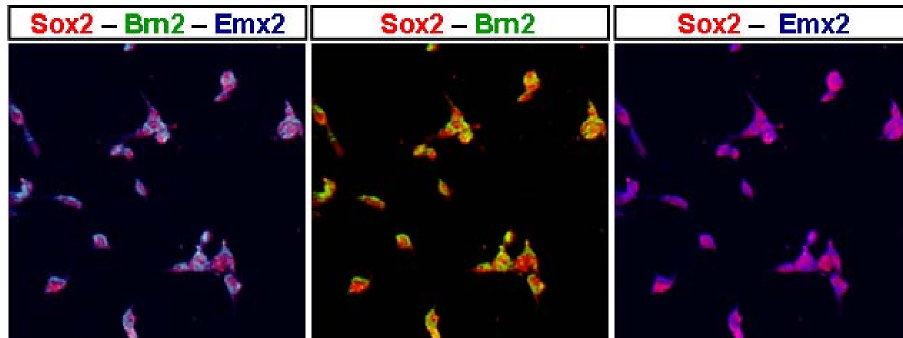


Supplementary Figure 1 – Emx2 deficiency significantly rescues the brain morphological defects seen in Sox2$^{\beta\text{-geo}/\Delta\text{Enh}}$ hypomorphic mutant adult brain (parenchymal loss in thalamus/striatum; reduced corpus callosum; reduced cortex). Sections through adult brains of the indicated genotypes are shown (anterior, left, to posterior, right). In particular, the ventricle enlargement and parenchymal loss in the striatum (filled squares), septum (empty circles) and thalamus (asterisks), tipical of the hypomorphic Sox2 mutant, were greatly diminished; further, the corpus callosum (arrows) was not interrupted and the extension of the cortex (arrrowheads), particularly the posterior and medial parts, was close to normal, in contrast with the usual findings in the hypomorphic mutants (n=5 mice/genotype assayed).

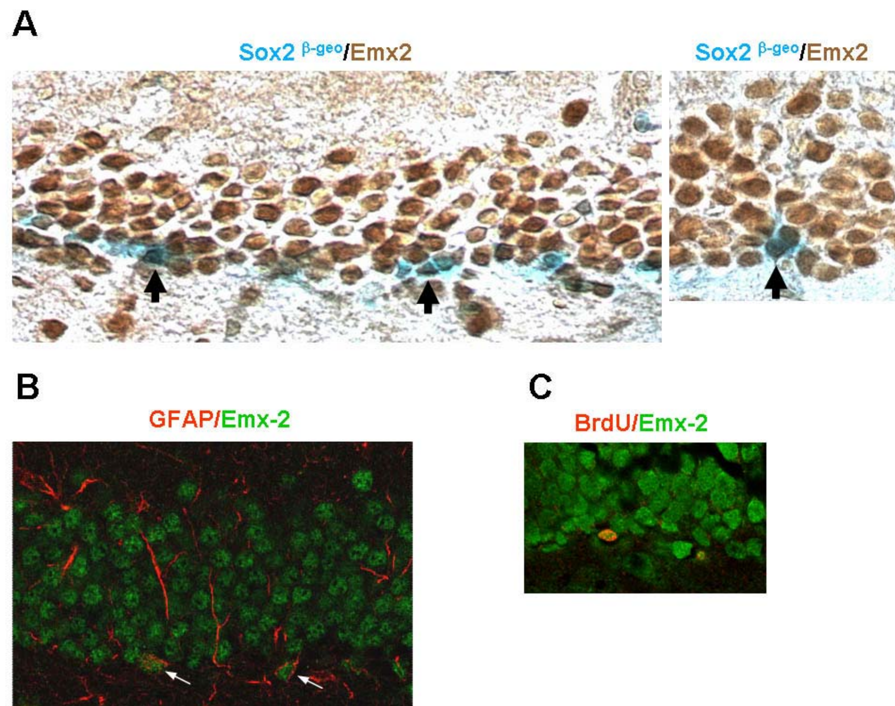


Supplementary Figure 2 – Emx2 binds to the Wnt1 and Sox2 (ATTA-3) enhancers. (**A**) Western blot showing Emx2 recombinant protein produced in P19 cells transfected with Emx2 expression vector (lane 1), in the TNT in vitro system (lane 2), compared to extracts from the hippocampal AHP cell line (lanes 3,4). Note that Emx2 levels are much lower in nuclear (lane 4) than in total extracts (lane 3)(same cell numbers used). (**B**) EMSA with recombinant Emx2 and probes containing the sites in the Wnt1 3' enhancer (lanes 1,2), or the ATTA-3 site in the

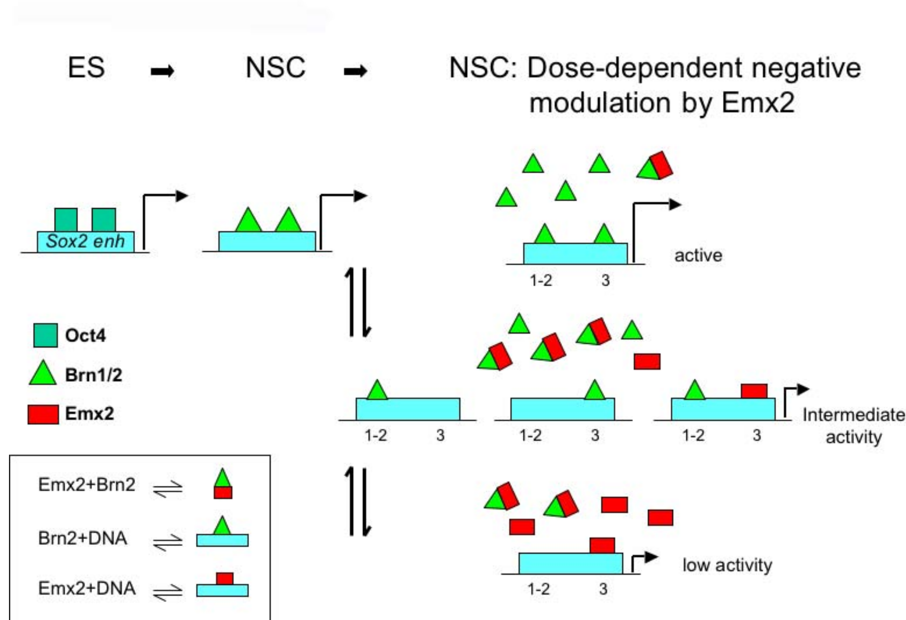
Sox2 5' enhancer (lanes 3-10)(see Fig. 3A for sequences). Added competitor oligonucleotides and antibodies are indicated above the lanes. Two bands are generated by Emx2 binding to the Wnt1 probe (lane 1, arrowheads), consistent with the Wnt1 site being a double site (Fig. 3A); both bands are supershifted by anti-Emx2 antibody (lane 2, asterisk indicates the supershifted band). The ATTA-3 probe generates with Emx2 a complex (arrow), which is supershifted by anti-Emx2 antibodies (lane 4, asterisk), and is competed by unlabelled ATTA-3 oligonucleotide (50-100 molar excess)(lanes 5,6), but not by mutant ATTA-3 (lanes 7,8). Unlabelled Wnt1 oligonucleotide competes binding to the ATTA-3 probe as efficiently as ATTA-3 itself (lanes 9,10; compare to lanes 5,6). Equivalent data were obtained with neurosphere extracts (not shown).



Supplementary Figure 3 – AHP cells coexpress Sox2, Emx2 and Brn2. Triple immunofluorescence with anti-Sox2, Emx2 and Brn2 antibodies of the AHP line detects coexpression of all three proteins in numerous cells. Note the presence of cytoplasmic as well as nuclear Emx2.



Supplementary Figure 4 – (**A**) Emx2 (brown, antibody staining) is coexpressed with Sox2 (Sox2$^{\beta\text{-geo}}$, blue, X-gal staining) in cells of the DG SGZ (arrows point to examples of double-positive cells). (**B**) Emx2 (green, immunofluorescence, confocal microscopy) is expressed in GFAP-positive (red) radial glia cells in the DG (arrows), as seen for Sox2 [5]. (**C**) Emx2 (green, immunofluorescence, confocal microscopy) is expressed in BrdU-positive cells (red, antiBrdU antibody) at the basis of the DG, as previously seen for Sox2 [5].



Supplementary Figure 5 – A hypothesis for the dose-dependent negative modulation of the Sox2 neural enhancer by Emx2. The Sox2 5' enhancer ("1-2" and "3" are tthe ATTA-1/2 and ATTA-3 transcription factor binding sites) is bound by POU activators Oct4 (in ES cells) and Brn1/2 (in neural stem/progenitor cells, NSC)([19-21] and present work). Emx2 antagonizes Brn2 function in two ways: preventing Brn2 binding to DNA (to both ATTA-1/2 and ATTA-3 sites) by protein-to-protein interaction, and by direct binding to DNA (to ATTA-3 site), to a sequence overlapping that recognized by Brn2. This mechanism operates on multiple Brn activator binding sites (the two sites ATTA-1/2 and 3, represented here; possibly to all six ATTA sites in the enhancer, see Fig. 2). Hence, at high Brn2/Emx2 ratios (top), Brn2 is bound to DNA at all sites (1-2 and 3), and the enhancer is fully active; at higher Emx2 concentrations relative to Brn2, some sites (1-2, or 3) are no longer bound by Brn2, and Emx2 is bound to some of them (site 3), giving rise to intermediate levels of activity (middle); at higher Emx2 concentrations, the Brn activator is no longer bound, leading to low activity (bottom).

Disclosure of potential conflicts of interest

The authors indicate no potential conflicts of interest.

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CHAPTER 4

ABNORMAL DEVELOPMENT OF CORTICOTHALAMIC CONNECTIONS IN SOX2 HYPOMORPHIC AND CONDITIONAL KNOCK OUT MICE

Caccia R., Broccoli V. and Nicolis S.K.

On going work

Abnormal development of corticothalamic connections in Sox2 hypomorphic and conditional knock out mice

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Introduction

The neocortex, the largest region of the cerebral cortex, is divided in different functionally area. Besides, the cortex presents also a laminar morphology, with six recognisable layers.

Sense organs, excluded olfactory organs, send the sensory input to one or more thalamic nuclei. These nuclei have well defined and reciprocal connections with specific cortical regions that process the information. The connections have area and lamina specificity.

The corticothalamic projection neurons are a part of cortical neurons. They are generated in the ventricular/subventricular zone of the lateral ventricle and migrate to form the cerebral cortex in an “inside out” pattern to form layers from VI to II. The populations of neurons located in the subplate and in the layer VI send long distance projections to link the cortex and the rest of central nervous system.

Thalamic nuclei are generated between E10.5 and E15.5.

Cortex and thalamus develop synchronously, and start to form connections around E13.5. Corticothalamic and thalamocortical

connections have to cross several zone to reach their ultimate target. These comprise pallial-subpallial boundary (PSPB) and diencephalic-thelencephalic boundary (DTB). These zones act as barrier zone and corridor for elongating neurons.

Thalamocortical projections proceed ventrally towards the ventral thalamus, then turn dorsolaterally at DTB and arrive to the internal capsula (IC) at E13.5: and then pause. Projections from the neocortex arrive at PSPB at E14.5 (projections from different regions reach the boundary asynchronously, according to the cortical developmental gradient) and pause. After entering in the IC, at E15.5 corticothalamic and thalmocortical axons interact then proceed associated with each other towards their targets.

Previous work performed in our laboratory demonstrated reduced cortical size and parenchymal loss with cell death in the thalamus of $Sox2^{\beta geo/\Delta enh}$ mutants (Ferri et al. 2004). We hypothesized that thalamocortical and/or corticothalamic connections may be affected in mice deficient for the Sox2 transcription factor. Here, we investigate corticothalamic connections in four different mouse strains mutant in Sox2: a *knockdown* mouse strain, expressing reduced amount of Sox2 an three different strains with *complete* deletion of Sox2 in different regions of brain (the whole brain, the cortex only and the dorsal thalamus only).

We find that Sox2 is required for the development of corticothalamic axons after E12.5. we demonstrated that the defect does not reside in the developing neurons; more probably, the environment surrounding growing axons have a defect in program of expression of molecules involved in axon guidance.

Corticothalamic projection abnormalities exist in $Sox2^{\beta geo/\Delta enh}$ "knockdown" mutant mouse

$Sox2^{\beta geo/\Delta enh}$ mice carry a regulatory ($Sox2^{\Delta enh}$) mutation together with a null ($Sox2^{\beta geo}$) mutation; these "knockdown" mice express 20-30% of the normal amount of Sox2 in the developing brain. (Ferri et al., 2004)

To obtain our experimental model, we crossed mice carrying the null mutation with mice homozygous or heterozygous for the Sox2 regulatory mutation (Fig. 1). Mice carrying only the regulatory mutation ($Sox2^{\Delta enh/+}$), which do not show any phenotypical abnormality (Ferri et al., 2004), were considered as controls.

To study if cortical neurons are able to develop and form correct interactions in $Sox2^{\beta geo/\Delta enh}$ mice, we analyzed embryonic brains at E18.5. At this stage of embryonic development, the axonal projections have finished to grow and are establishing final synaptic contacts with their specific target.

To visualize the pattern of axon elongation we used DiI crystals (1, 1-dioctadecyl –3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate). DiI is a fluorescent dye able to bind the plasma membrane by hydrophobic interaction with its lypophilic portion. DiI crystals are placed by manual insertion in specific points of brain. The molecules of dye diffuse along the biological membranes localized near the site of implant, including the membrane of the long axonal projections.

DiI crystals were implanted, in separate experiments, in the three major functional areas in the neocortex. The Primary Somatosensory

Cortex (S1) is located in the medial region of brain, and sends projections that will synapse onto the Ventrobasal Nucelus (VB) of the thalamus. The Primary Motor Cortex (M1), the more rostral region of the neocortex, also sends its axons towards the VB nucleus, but its final target is more rostral than that of S1. The more caudal region of cortex is the Primary Visual Cortex (V1), which sends its projections to the dorsal Lateral Geniculate Nucleus (dLGN).

In the wild type, as expected, fibers start to grow from their specific area in the neocortex (Fig. 2). The outgrowth of axons begins around E13.5. They elongate first ventrally to arrive at PSPB, then turn towards the midline of brain and arrive to the IC. After exiting the IC and crossing the DTB (that happens at E15.5) they turn their trajectory towards the dorsal thalamus, where dorsal thalamic nuclei reside (Fig. 2).

In all mutant brains studied, the initial tract of corticofugal projections is normal: the axons start to grow towards the ventral part of the telencephalon and turn towards the midline to reach in the IC. Subsequently, however, abnormalities become apparent in mutant brains.

In three out of four mutant brains examined for the connections between M1 and medial VB nucleus, the axons exiting the IC and reaching their target are reduced in number (Fig. 3; Table 1). In the fourth brain analyzed, as well in all controls, the progression and the number of corticofugal axonal projections reaching the VB is comparable and normal (Fig. 3).

Similarly, five out of eight mutant brains implanted in S1 show reduced numbers of axons exiting the IC and arriving to the VB (Fig.

4). The other three mutant brains do not show significant anomalies (Fig. 4).

Similar observations are made about the implants in V1. In two out of seven mutant brains, the initial tract of projections is normal, but the fibers that cross the boundary between telencephalon and diencephalon and reach the dLGN are very reduced in number compared to wild type brains. In the other five brains, no significant differences were observed between wild type and mutant in the number of axons reaching dLGN (Fig. 5).

In summary, in $Sox2^{\beta geo/\Delta enh}$ mutant brains important abnormalities are present in the development of corticothalamic axons. These abnormalities do not affect the correct routing of projection neurons along the initial tract of their trajectory, but rather affect the number of axons that arrive to their final target, which is severely reduced in some mutants, more mildly in others. Out of nineteen mutant brains implanted, about 50% (10/19) show a substantial depletion in the numbers of axons crossing the DTB and reaching their specific nucleus (Table 1; Figs. 3-5). In particular, we observe this depletion in 75% (3/4) of the brains implanted in M1, in 65% (5/8) of brains implanted in S1 and in 28% (2/7) of brains implanted in V1.

The severity of abnormalities in these brains is variable, from almost total absence of axons reaching the thalamus, to a milder phenotype,. (Fig. 6)

Abnormal corticothalamic connections in the $Sox2^{\beta geo/flox}$;Nestin*cre* mutant mouse

We then investigated a mouse model in which *complete* ablation of Sox2 could be obtained by the action of a Cre recombinase.

This model carries a Sox2 null allele ($Sox2^{\beta geo}$) together with a Sox2 allele flanked by two loxP sites ($Sox2^{flox}$; Favaro et al., 2009), the substrate of Cre recombinase; a transgene specifically expressed in the developing central nervous system is also present, in which the expression of the Cre recombinase gene is driven by the regulatory regions of the Nestin gene, (Medina et al., 2004) The deletion of the $Sox2^{flox}$ gene is complete by E12.5 in the whole brain, including cortex and thalamus (Favaro et al., 2009). The mating plan is presented in Figure 7.

The analysis of projection neurons in these mutants was also performed on E18.5 brains, using the DiI tracer. The implants of DiI crystals was made always in the three major functional areas of the neocortex (M1, S1 and V1). We implanted four mutant brains in M1, five mutant brains in S1 and seven mutant brains in V1, with their respective controls.

In all wild type brains, the projection axons show the expected pattern (Fig. 8; Table 2). In the mutant brains($Sox2^{\beta geo/flox}$;Nestin*cre*), the initial tract of axonal development is normal, with the correct turn of trajectory towards the ventral area first, and the midline later, as previously seen in the hypomorphic ($Sox2^{\beta geo/\Delta enh}$) brains (not shown). However, all sixteen mutant brains analyzed show that the axons exiting the IC and crossing the telencephalon-diencephalon boundary are extremely reduced in number (Fig. 8; Table 2).

In conclusion, Sox2 is required for correct axon pathfinding after day E12.5. Complete ablation of Sox2 by E12.5 leads to important abnormalities in all the mice studied; Sox2 reduction ("knockdown" model) causes similar abnormalities, but with reduced penetrance (50%) and greater variability.

Cortical specific deletion of Sox2 does not lead to abnormalities in axonal pathfinding

There are two possible explanations for the abnormal growth of axons in mice lacking Sox2 protein:

- Sox2 expression is required in the cortex for the birth and maturation of cortical projection neurons
- Sox2 expression is required in the thalamus, to produce molecular signals required for the correct elongation and pathfinding of corticothalamic axons

To investigate if the problem resides in the cortex or in the thalamus we deleted $Sox2^{flox}$ by specific cortical, or thalamic, Cre-expressing mice.

To obtain the ablation of Sox2 gene expression in neocortex only, we crossed (Fig. 9) mice carrying the $Sox2^{flox}$ allele with mice carrying a Cre recombinase gene inserted into the Emx1 locus by homologous recombination, downstream to an IRES (Internal Ribosome Entry Site) element in the gene 3'-UTR. (Gorski et al., 2002). In this "knock-in" construct, Cre is inserted into the Emx1 locus in a way that does not affect the normal expression of the Emx1 gene, and is expressed according to the Emx1 expression pattern (the cortex only from E9.5)(Gorski .et al., 2002).

By E12.5 (with timing similar to Necstin*cre*), Sox2 expression is completely ablated specifically in the neocortex, whereas it is not affected in other regions of the brain, including the thalamus (Fig. 10).

Also in this case DiI crystals were placed in the three major functional cortical areas, M1, S1 and V1, of E18.5 brains. The implants were made in two mutant brains in M1, six mutant brains in S1 and six mutant brains in V1, and an equal number of wild type control brains (Table 3).

No one of the implanted mutant (Sox2$^{\beta geo/flox}$;Emx1IRES*cre*) brains shows significant differences in the numbers of axons reaching their final target as compared to wild type (Sox2$^{flox/+}$) control brains. In both mutant and control brains the fasciculation of fibers starts from neocortex and proceeds to the ventral region, turns towards the midline, passes the IC crossing the DTB, so reaching the final target (Fig. 11).

Hence, deletion of Sox2 restricted to the neocortex does not seem to affect the development and routing of corticothalamic projection neurons.

Finally, we attempted a thalamic specific deletion. We used mice carrying the Cre recombinase inserted (“knock in”) in the locus of the RORα gene. RORα is expressed as early as E12.5 in dorsal thalamus by presumptive ventroposterior neurons (Nakagawa and O’Leary, 2003). We expected to see the deletion of Sox2 around E14.5. However, in Sox2$^{\beta geo/flox}$;RORα*cre* mice Sox2 expression is still present at E15.5 (Fig. 12) in amount comparable to controls. Zhou and colleagues (Zhou et al., 2008) have seen that in RORα*cre* mice, cre expression was restricted to a subset of thalamic dorsal cells. It is

possible that this partial expression of cre recombinase does not permit a ablation of Sox2.

Indeed we did not observe abnormalities in the mutant brains carrying the RorαIRES*cre* (data non shown).

Discussion

In this study we show that Sox2 is required for correct elongation of corticothalamic axonal connections. In brains in which Sox2 expression is decreased ("knockdown" mutants), or conditionally ablated from day E12.5 ($Sox2^{flox/flox}$;Nestin*cre* mutants) the initial tract of axonal projections navigating through the ventral telencephalon is unaffected, and the fibers are able to reach the internal capsula. The second tract of growth is abnormal: axons able to exit the internal capsula and make the correct turning towards the dorsal thalamus are very reduced in number.

Sox2 is an important transcription factor expressed in the central nervous system from the beginning of its development, and the complete knock out mouse is early embryonic lethal (Avilion et al. 2003). Previous studies performed in our laboratory have utilized as model the $Sox2^{\beta geo/\Delta enh}$ hypomorphyc mice, which expresses 20-30% of the normal amount of Sox2; in these mutants brains show several abnormalities, including decreased cortical size, defects in neurogenesis and parenchymal reduction and cell death in thalamus (Ferri et al. 2004). To better understand the physiological role of Sox2 we also generated conditional mutant mice, in which Sox2 is flanked by loxP sites ($Sox2^{flox}$) and can be ablated by driven expression of Cre recombinases. Using a Cre recombinase driven by a regulatory region

specific to the developing central nervous system (Nestin*cre*), also these mice show brain abnormalities including reduced hippocampus, a moderate lateral ventricle enlargement and slight size reduction of the posterior ventrolateral cortex (Favaro et al. 2009). On the basis of the defects found in neurons, we started to study the networking of long range development of corticothalamic axons in mouse brain.

Sox2 is required for correct development of thalamic tract of corticothalamic projection neurons

DiI labeling experiments in E18.5 brains indicate that the lack of Sox2 results in failure of corticofugal projections to grow into the thalamus, but is not accompanied by a misrouting of the fibers; rather, few fibers exit the internal capsula and cross the diencephalic-telencephalic boundary. Most of the projections seem to stall into the internal capsula without exiting. Preliminary data suggest that thalamocortical connections are not affected in Sox2 mutant brains (data not shown).

The aberrant development of the terminal thalamic projection tract, present as a constant character in conditional mutant mice, shows greater variability in hypomorphic mice. Probably the incomplete ablation of Sox2 gene products in “knockdown” mutants can explain the wide spectrum of phenotypes observed, suggesting that small differences in amounts of Sox2 can be sufficient to elicit great variability in the development of corticofugal projections.

In contrast, the phenotype of Nestin*cre* conditional knock out mice is more similar in all mutant brains analyzed, with a consistent reduction in number of axons reaching their target. This is an evidence that Sox2 is important for the correct development of corticofugal axons after

E12.5. Corticofugal axons begin their growth towards thalamic targets around E13.5, and complete the process at E18.5. So, the total loss of SOX2 protein occurs before the beginning of axonal development.

Axon guidance is a complex process involving many molecules. Different genes encoding transcription factors, nuclear receptors, cell adhesion molecules, axon guidance receptors and ligands were described (reviewed in Lopez-Bendito and Molnar 2003).

Several hypothesis can be made to explain the abnormalities in corticofugal projections development.

A first possibility is a cell autonomous defect due to an abnormal differentiation program of projection neurons. The growth cone is programmed to respond to specific cues and the environment is specified to produce them. Axons might lack ability to respond to normal cues along the elongation pathway. Notably, in vivo Sox2 expression is maintained in a subset of differentiated neurons, including cortical pyramidal neurons (Ferri et al. 2004, Cavallaro et al. 2008) (it remains to be elucidated if this Sox2 positive population comprises the corticofugal projection neurons). In Sox2 hypomorphic cells, neuronal differentiation is impaired, with cells exhibiting a not developed arborization; this immature morphology correlates with impaired expression of some mature neuronal markers (Cavallaro et al. 2008). Lentiviral Sox2 transduction experiment in Sox2-deficient mutant cells differentiating in vitro showed that Sox2 is required at early stages of differentiation, not at later stages. Probably, at early stages Sox2 establish a downstream transcriptional program for a correct differentiation. Normal axons are able to growth towards the right synaptic partner because they express several specific molecular

receptors on their growth cone. It is possible that the mutant projection neurons are able to initially grow towards ventral telencephalon, but are unable to respond to later stimuli, because the lack of Sox2 causes a defective transcriptional program leading to the non expression of some particular receptors involved in guidance in the thalamic tract.

The growing axons also express several cell adhesion molecules on their surface. These molecules bind to similar proteins on nearby cells. It has been demonstrated that corticofugal and thalamocortical fibers interact physically and proceed dependent on each other (Molnar et al. 1995, 1998). This interaction happens in the internal capsula. Errors in pathfinding of both corticofugal and thalamocortical connections were described in mice with mutations in transcription factors Tbr1, Gbx2 and Pax6 (Stoykova and Gruss, 1994; Hevner et al., 2002; Jones et al., 2002). Because we have seen that the defects appears only after axons growing into the subpallium, and entering the internal capsula, another possibility is that there is misexpression of one or more of these cell adhesion molecules.

Alternatively, pathfinding defects at the level of the internal capsula could be caused by abnormal development of sourrounding subpallium cells. Sox2 expression was found in sparse mature neurons in the striatum (Ferri et al. 2004). The striatum is a major forebrain nucleus that integrates cortical and thalamic afferents. Spiny projection neurons, a subset of striatal neurons, reside in dorsal striatum, and receive glutamatergic projection from cerebral cortex, which form well defined synapses (Wolf, 1998). We do not know if these are the neurons expressing Sox2, but it is possible that the region

of residence, overlapping the region of internal capsula, can contribute to regulate the growth of projection neurons.

Another possible explanation is a defective growth of axons due to anomal expression of thalamic attractive/repulsive cues. Sox2 is an important transcription factor expressed in developing and postmitotic thalamus, including dorsal thalamic nuclei (Vue et al. 2007). The area of Sox2 expression in dorsal thalamus overlaps the region of residence of thalamic nuclei. It is possible that Sox2 could be involved in regulating the production of one or more terminal guidance cues or, more in general, in the patterning of the thalamus.

Several studies have demonstrated that the diffusible molecule Sonic Hedgehog is involved in the guidance of commissural axons (Charron et al. 2003, Okada et al. 2006) acting by regulating the attractive Netrin1 signal. Recent work (Parra and Zou 2010) demonstrates that Shh is also involved in the repulsive response to semaphorine of commissural axons. Shh expression is present along the axial midline not only in the spinal cord, but also in the forebrain. Rostrally, Shh is expressed in ventral forebrain. In previous work we demonstrated that Shh is a direct target of Sox2 and the complete ablation of Sox2 gene expression from E12.5 causes a progressive reduction of Shh expression in telencephalon and diencephalon, but not in midbrain (Favaro et al. 2009). Shh expression along the midline of diencephalon, reduced in Sox2 conditional knock out mice, could be involved in the response of growing axons that normally leads axonal projections to turn towards the dorsal thalamus.

Shh is also a well known signaling center in the developing diencephalon, that patterns the thalamus in mice (Ishibashi and

McMahon 2002). Additionally, other signaling molecules involved in thalamic pattern are Wnts, required for establishing regional thalamic identitites (Braun et al 2003, Zhou et al. 2004) and Fgf8 that controls the pattern of thalamic and prethalamic nuclei along the anteroposterios axis (Kataoka and Shimogori, 2008); Sox2 can be involved in regulating directly or indirectly the development of dorsal thalamus by acting on the expression of these genes .

Sox2 deficient projection neurons do not show growth defects

To elucidate if the defect resides in the neurons resident in the cortex or in signalling molecules of the thalamus, we generated mice in which the expression of Sox2 is ablated specifically in the cortex.

To obtain cortical specific deletion we used mice in which the Cre recombinase is driven by the Emx1 regulatory regions (Gorski et al. 2002). Emx1 is expressed in the cerebral cortex from E9.5. Immunohistochemistry shows that by E12.5 the ablation of Sox2 protein is complete in the cerebral cortex, but is not altered in other regions of brain, including prospective dorsal thalamus (Fig.10). Because the deletion in the neural tube in conditional knock out mice previously studied (Nestin*cre*, Favaro et al. 2009) is also complete at E12.5, the timing of gene ablation is correct to perform this analysis.

DiI labeling experiments in E18.5 brains deleted specifically in the cortex reveals absence of abnormalities in corticofugal projection. The connections are normal, both in routing and abundance (Fig.11; Table 3).

The deletion of Sox2 gene restricted to the cortical region does not lead to an abnormal phenotype comparable to that seen with Nestin*cre*, despite the fact that the timing of deletion is at least as

early (see above). This suggests that neurons born in the ventricular zone and resident in the cortex are able to elongate their projections towards the final target also in the absence of Sox2 in the region of origin. So, after E12.5, Sox2 is not necessary for the creation of a functional growth cone and a correctly elongating axon.

An approach to delete Sox2 in thalamus

To obtain thalamic specific deletion I begun to work with mice carryng the Cre recombinase driven by regulatory element of RORα. RORα gene is expressed as early as E12.5 in the presumptive thalamus and cerebellum (Nakagawa and O'Leary 2003). Immunohistochemistry on E15.5 brains (the time of exiting of axons from the internal capsula) revealed that Sox2 protein is still present in the dorsal thalamus at levels undistinguishable from wild type. Moreover, the thalamic nuclei develop between E10.5 and E15.5 in mice (Altman and Bayer 1988). So, this deletion, also if happened later than the time points we analyzed, is not useful for this analysis: the nuclei are generated and are presumably already "programmed"; the projections are already routed towards their target and have almost finished to grow.

Conclusions

In summary, we found that the thalamic tract of corticofugal axonal growth is dependent on the expression of Sox2 at midgestation (after E12.5). Sox2 expression is not necessary in the cortex after E12.5 for normal development of projections. We hypothesize that Sox2 expression is needed in the thalamus where it would be involved in the mechanism of correct elongation of axons.

In the future, we would like to better investigate the problem of elongation of cortical projection neurons in the thalamic tract. The projection neurons elongate, after crossing the DTB, towards the midline, then turn towards dorsal thalamic nuclei. Since Shh is expressed in the ventral midline, and is greatly reduced in NestinCre-deleted Sox2 mutant mice (Favaro et al., 2009), we may try to delete Sox2 using a Cre transgene under the control of SBE2 (Shh brain enhancer-2). The SBE2 element is active in the hypothalamus, and partially in the dorsal thalamus, of transgenic mouse embryos (Jeong et al., 2006); hence, we could drive Sox2 deletion along the midline of diencephalon, the region of expression of Shh. This experiments can give us a first answer about the potential role of Sox2 in axon guidance via Shh regulation.

It will be very important to perform in situ hybridization analysis to study the expression of specific axon guidance molecules (like netrin1 and semaphorins) in different regions and at different stages of the developing thalamus.

Materials and Methods

Animals

The generation of $Sox2^{\beta geo}$ allele and $Sox2^{\Delta enh}$ allele has been described (Zappone et al., 2000; Avilion et al., 2003; Ferri et al., 2004). Hypomorphic experimental mice embryos were derived from intercrosses of heterozygous mice carrying a null Sox2 allele ($Sox2^{\beta geo/+}$) with mice carrying regulatory mutant allele in heterozygosis or homozygosis ($Sox2^{\Delta enh/+}$ or $Sox2^{\Delta enh/\Delta enh}$). Generation of $Sox2^{flox}$ allele has been described (Favaro et al., 2009). Conditional

knock out mutants were obtained through two generation of crossing. First, mice carrying $Sox2^{\beta geo}$ allele were crossed with mice carrying Nestin*cre* transgene (Medina et al, 2004) to obtain double heterozygotes. Experimental mice were obtained crossing $Sox2^{flox/flox}$ mice with $Sox2^{\beta geo/+}$;Nestin*cre* mice. Regional specific knock out mice were derived in two generations: mice carrying $Sox2^{\beta geo}$ allele were crossed with mice carrying Emx1IRE*cre* (Gorski et al., 2002) to obtain double heterozygotes. $Sox2^{\beta geo/+}$;Emx1IRES*cre* mice were then intercrossed with $Sox2^{flox/flox}$ mice to obtain experimental animals. The day of vaginal plug is consider E0.5. Foetuses were removed and anaesthetized by hypothermia before decapitation. Brains were dissected at stages E18.5 for tracing with carbocyanine dyes, and E15.5 for immunohistochemistry. Whole embryos was collected at E12.5 for immunohistochemistry. All brain and embryos were fixed at 4°C in 4% (wt/vol) paraformaldehyde in phosphate buffered saline (PBS). Experimental procedures involving mice were approved by the Italian Ministry of Health.

Genotyping

Screening of embryos was carried out by allele specific PCR.

Tracing with carbocyanine dye

Brains at E18.5 were fixed 48h at 4°C in 4% paraformaldehyde. To label corticofugal fibers, small holes were made into the cerebral cortex in three different sites: rostral, medial and caudal. Single crystals of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate, Molecular Probes) were placed into them by using a tungsten wire under a binocular dissecting

microscope. Brains were incubated in 4% paraformaldehyde at room temperature at dark for 24h and in 2% paraformaldehyde at room temperature at dark for 5-6 weeks. The brains were then washed with PBS and embedded in 4% agarose and were sectioned in 200 μm coronal slices by a vibratome (LEICA). Tissue was counterstained with DAPI (4',6-diamidino-2-phenylindole) 5μg/ml, washed in PBS and coverslipped in FluorSave reagent (345789, Calbiochem). Slices were analysed by a fluorescent microscope: all images were collected on a Zeiss Axioplan 2 microscope and processed with Adobe Photoshop 7.0 software (Adobe Systems).

Immunohistochemistry

E12.5 embryos and E15.5 dissected brains were fixed overnight at 4°C in 4% (wt/vol) paraformaldehyde in PBS, cryoprotected with sucrose 30% in PBS and cryostat sectioned onto slides (SuperFrost Plus). For Sox2 immunohistochemistry antigen unmasking was carried out by boiling sections in 0.01 M citric acid and 0.01 M sodium citrate for 3 min in a microwave, before blocking. Sections were then washed in PBS and blocked with FBS 1% in PBS 1h at room temperature. After extensively washing in PBS sections were incubated overnight at 4°C with primary antibody (mouse antibody to SOX2, 1:50, R&D MAB2018) diluted in 1% FBS in PBS, extensively washed in PBS and then incubated for 1h at room temperature with a secondary antibody conjugated with a fluorochrome (goat antimouse IgG Alexa 546, 1:500, Molecular Probes). Slides are counterstained with DAPI and mounted in PBS. Section were analysed with fluorescent microscope. All images were collected on a Zeiss Axioplan 2

microscope and processed with Adobe Photoshop 7.0 software (Adobe Systems).

Acknowledgements

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Figures

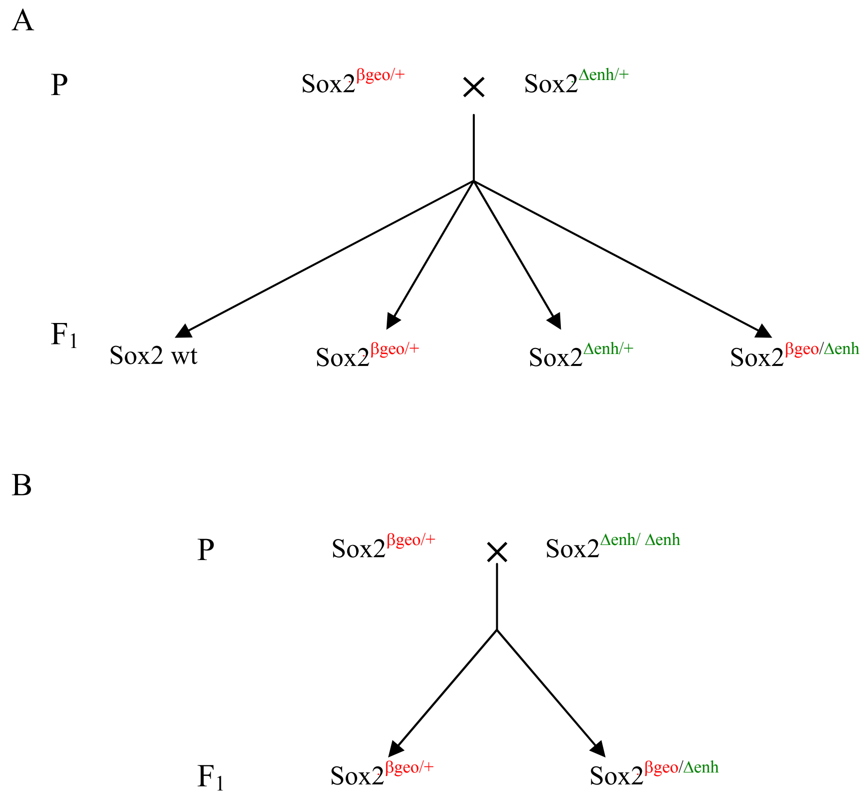
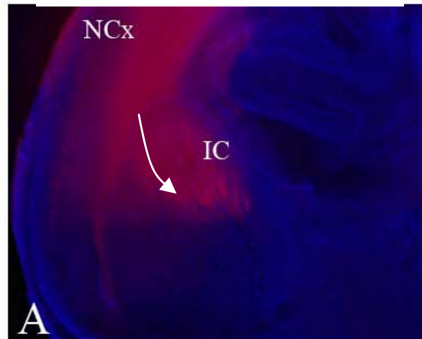
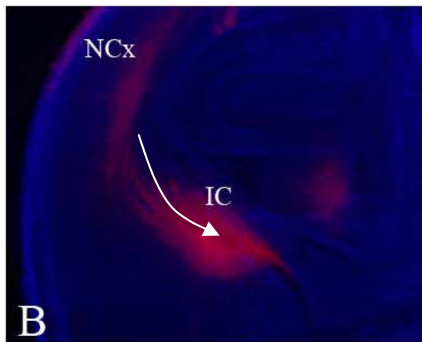
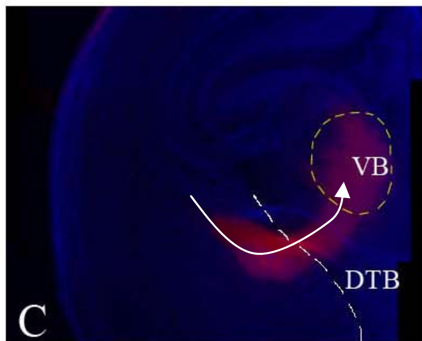


Fig. 1 Breeding scheme to obtain Sox2 "knockdown" mutant mice
To obtain $Sox2^{\beta geo/\Delta enh}$ "knockdown" mice, $Sox2^{\beta geo/+}$ mice were crossed to mice heterozygous (A) or homozygous (B) for the regulatory mutation ($Sox2^{\Delta enh/+}$). The mating between mice carrying a null mutation and mice carrying the regulatory mutation in heterozygosis produces 25% of $Sox2^{\beta geo/\Delta enh}$ mutant in offspring (A). The mating between mice carrying the same null mutation and mice carrying the regulatory mutation in homozygosis produces 50% of $Sox2^{\beta geo/\Delta enh}$ mutant in offspring (B).

E18.5 $Sox2^{+/+}$







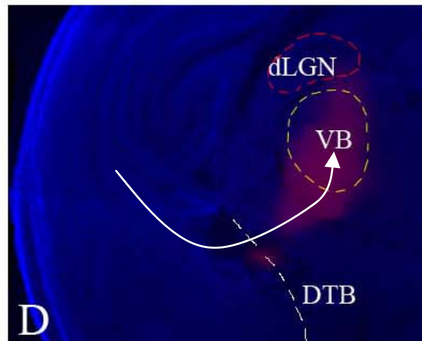


Fig. 2 normal outgrowth pattern of fibers labeled with DiI in the forebrain

E 18.5 coronal sections (A more rostral to D more caudal), implanted with DiI crystals and counterstained with DAPI. In E18.5 $Sox2^{+/+}$ brains axons projecting from cortex show a normal pattern of elongation. (A) Axons leave the neocortex (Ncx) and elongate towards the ventral telencephalon, then (B) turn towards the midline and enter the internal capsula (IC); axons exiting the IC cross the diencephalic-telencephalic boundary (DTB, white outline) turning towards the dorsal thalamus to reach the appropriate thalamic nucleus (C,D), highlighted with yellow outline(ventrobasal nucleus, VB) and red outline (dorsal Lateral geniculate nucleus, dLGN). Arrows indicate normal routing of axonal growth.

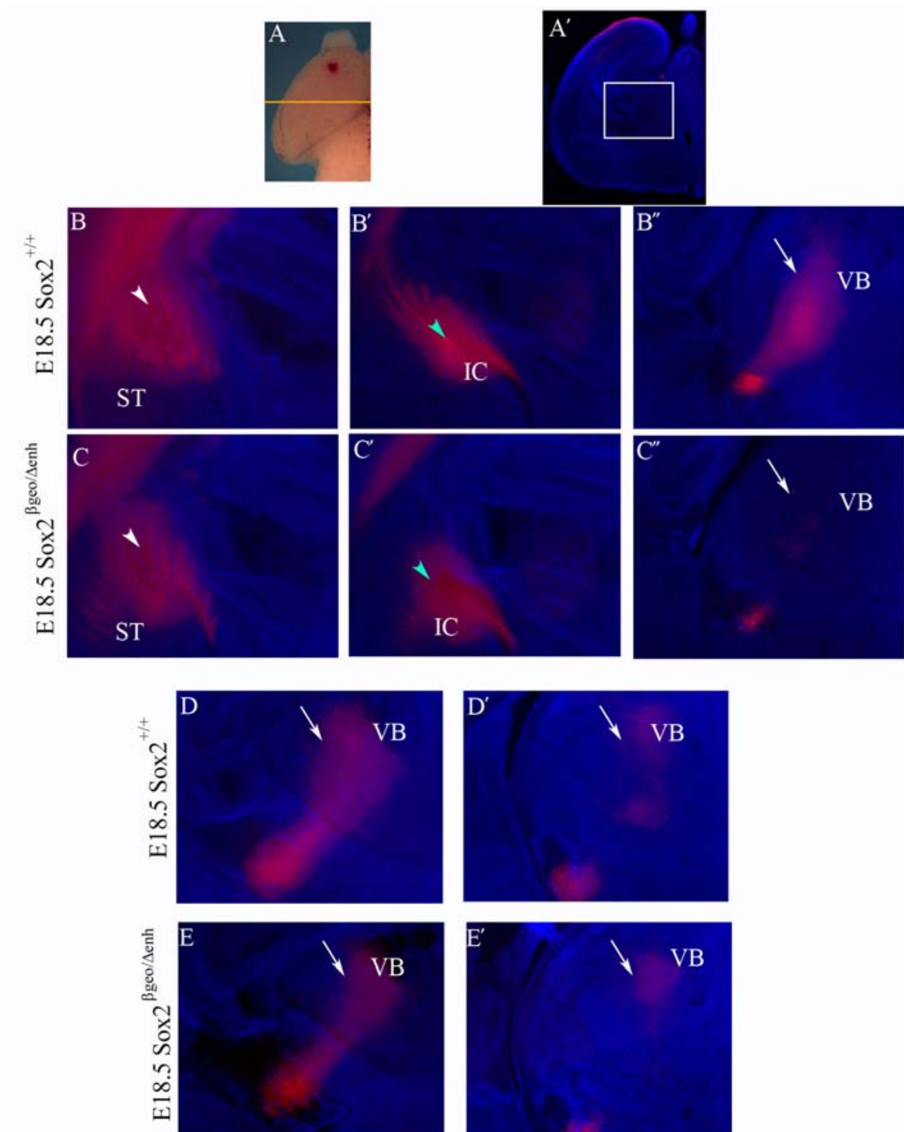


Fig. 3 Labeling of projections starting from primary cortical motor area (M1) reveals a reduction in number of axons reaching the VB in most Sox2$^{\beta geo/\Delta enh}$ brains

Corticofugal fibers are labeled with DiI crystals placed in the more rostral region of cortex at E18.5 (A). Yellow line indicates the levels of sections shown. Details of 200 μm coronal sections (A', white square) of implanted brains counterstained with DAPI (B-E'). The position of VB is indicated by arrows. Adjacent rostral sections of wild type (B, B') and Sox2$^{\beta geo/\Delta enh}$ (C, C') brain show that the initial tract of projection is identical: the fibers enter in the striatum (ST) and form the internal capsula (IC) (B, B', C, C'). More posterior sections show that in wild type (B", D, D') the corticofigal connections reach the ventrobasal nucleus (VB), as expected. In

Sox2$^{\beta geo/\Delta enh}$ (C”) brain the number of fibers able to exit the IC and turn towards the VB is extremely reduced. In one single case (E, E’), Sox2$^{\beta geo/\Delta enh}$ brain shows that fibers reaching the VB in numbers comparable as the wild type.

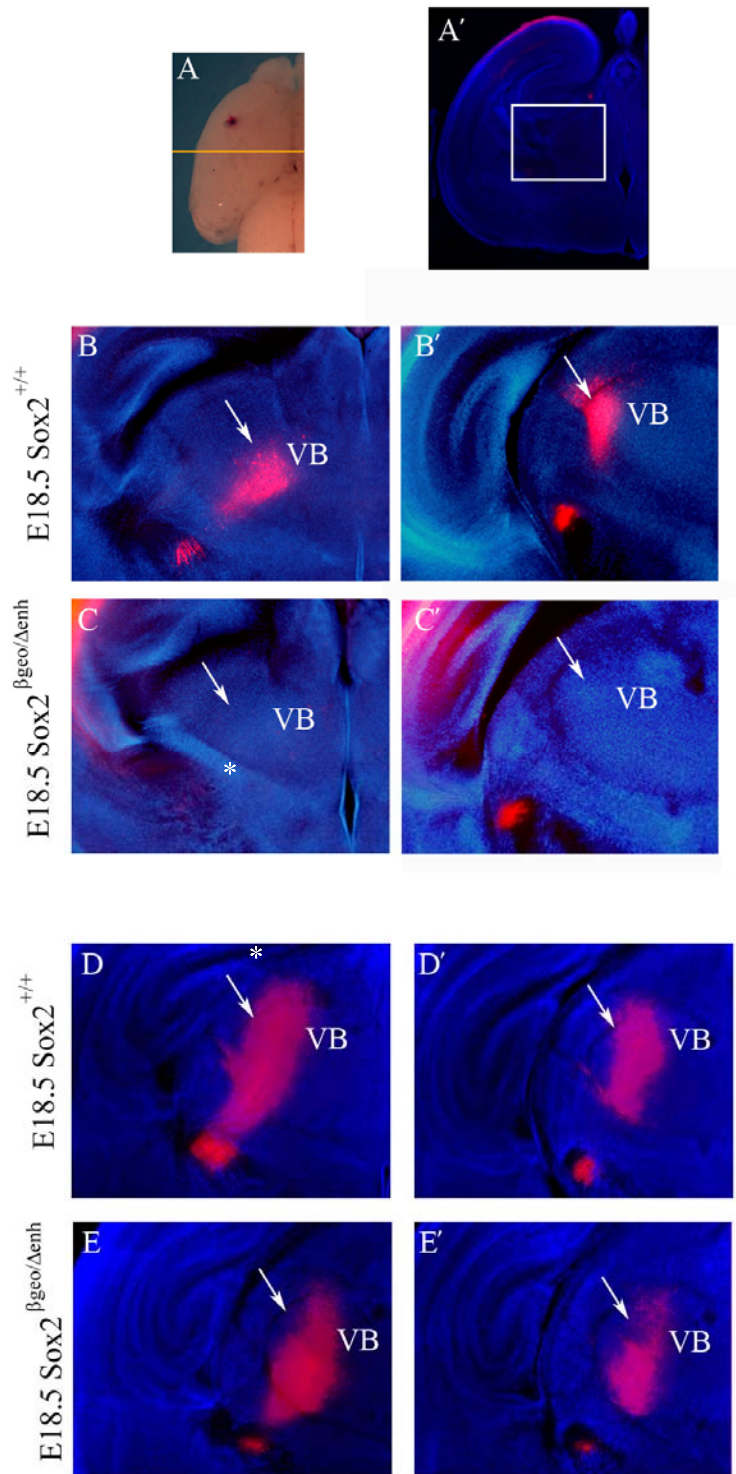

A
A′
B
B′
VB
VB
E18.5 Sox2+/+
C
C′
VB
VB
*
E18.5 Sox2βgeo/Δenh
D
D′
VB
VB
*
E18.5 Sox2+/+
E
E′
VB
VB
E18.5 Sox2βgeo/Δenh

Fig. 4 Projection neurons starting from primary cortical somatosensory area (S1) show a decrease of axons reaching the VB in most Sox2$^{\beta geo/\Delta enh}$ brains

In E18.5 brain, axons leaving the somatosensory area are labeled with DiI crystals placed in the medial region of the brain (A). Yellow line indicates level of sections shown. Details (white square in A') of adjacent 200 µm coronal sections of wild type (B, B', D, D') brain show the normal routing of the projections. In similar sections of Sox2$^{\beta geo/\Delta enh}$ brain (C, C') the corticofugal connections exiting the IC are greatly reduced in number, an few axon can arrive to the VB, indicates by arrows. Also in this case there are some (3/8) Sox2$^{\beta geo/\Delta enh}$ brains in which the abnormal phenotype is not present, and most axons reach the VB as seen with the wild type (E, E'). Asterisks indicate the pedunculum, that seems reduced; this characteristics does not correlate with normal or abnormal phenotype.

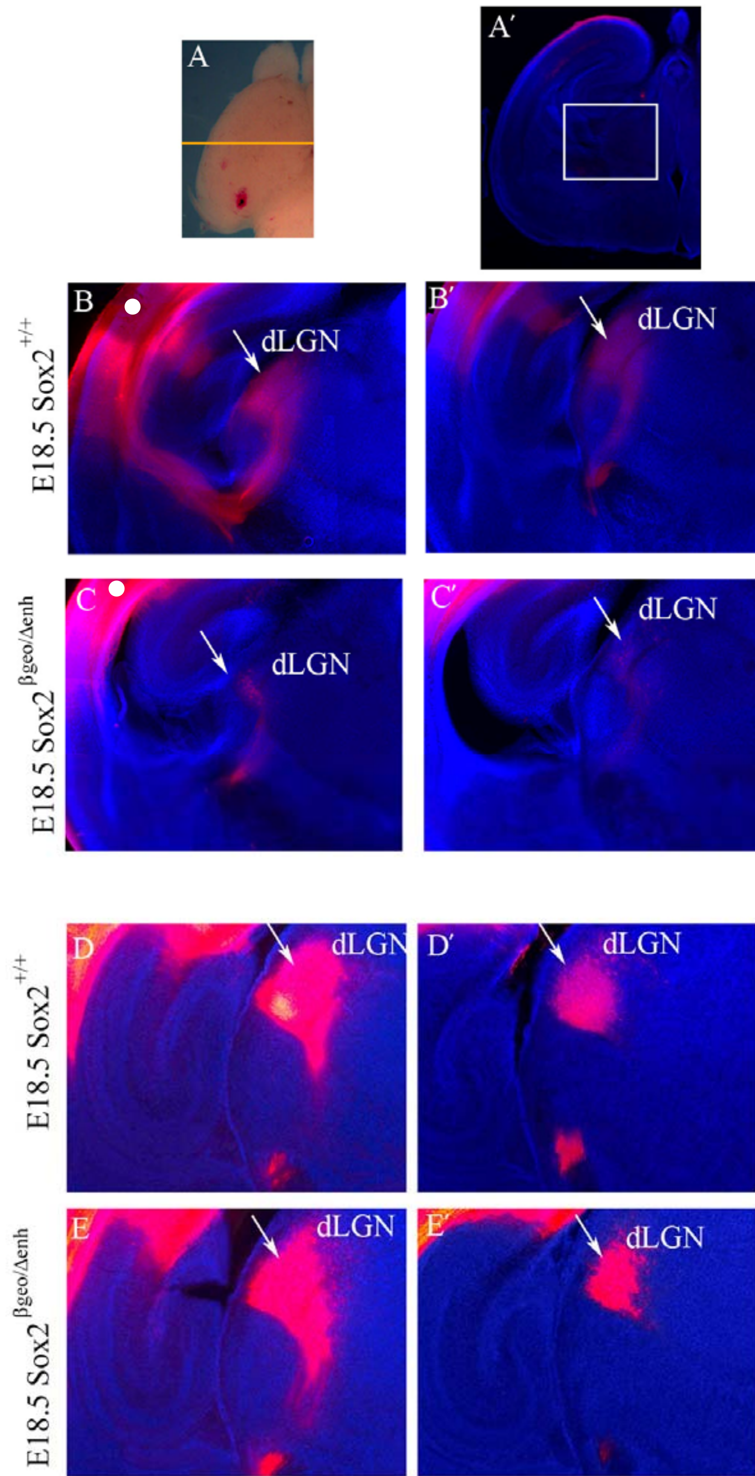
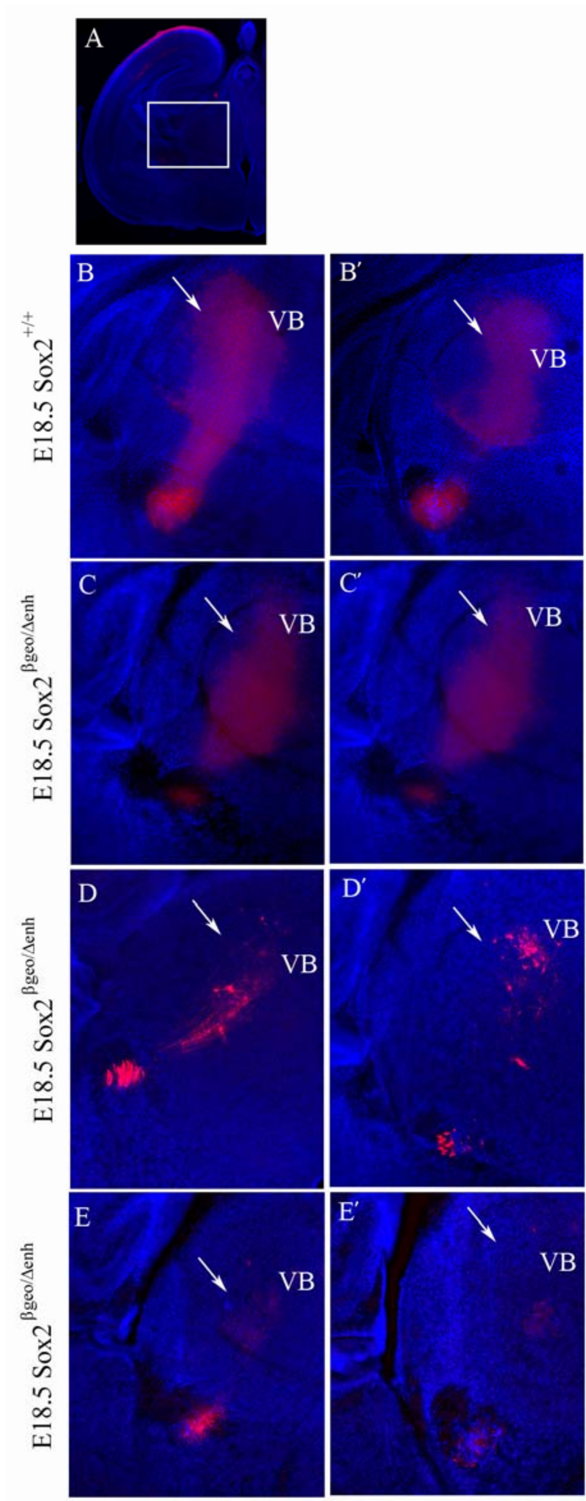

A
A′
B
B′
dLGN
dLGN
E18.5 Sox2+/+
C
C′
dLGN
dLGN
E18.5 Sox2βgeo/Δenh
D
D′
dLGN
dLGN
E18.5 Sox2+/+
E
E′
dLGN
dLGN
E18.5 Sox2βgeo/Δenh

Fig. 5 DiI placement in the primary cortical visual area (V1) reveals a depletion in the numbers of axons reaching the dLGN in Sox2$^{\beta geo/\Delta enh}$ brains

Placement of DiI crystals in the more caudal region of the neocortex at E18.5 (A) labels the corticofugal projections that reach the dorsal lateral geniculate nucleus (dLGN). Yellow line indicates the level of sections shown. Details (white square in A') of 200 μm section evidentiate the normal pattern of axonal fasciculation in wild type brain (B, B', D, D'), counterstained with DAPI. In Sox2$^{\beta geo/\Delta enh}$ brain a clear reduction of axon numbers exiting the IC and reaching the dLGN is observed (arrows in C, C'). In several Sox2$^{\beta geo/\Delta enh}$ brains the abnormal phenotype is not present and the number of axons reaching the dorsal thalamus is comparable to that in wild type brain (E, E'). White circles indicate the site of DiI crystals placement ; the diffused red colour is the fluorescent dye that labels all the membranes next to the implant site.



A
B
VB
B′
VB
C
VB
C′
VB
D
VB
D′
VB
E
VB
E′
VB
E18.5 Sox2+/+
E18.5 Sox2βgeo/Δenh
E18.5 Sox2βgeo/Δenh
E18.5 Sox2βgeo/Δenh

Fig. 6 DiI labeling of projection neurons in Sox2$^{\beta geo/\Delta enh}$ brain demonstrates abnormalities in axonal projections of variable severity.

Details (white square in A) of 200 μm section of implanted brains. DiI crystals placed in the medial region of Sox2$^{+/+}$ brains (B, B')show that axons leaving this cortical region follow the expected route of growth and do not show abnormalities. Implant of DiI crystals in the same region of Sox2$^{\beta geo/\Delta enh}$ brain (B-D') leads to a variable axonal phenotype; this mutant genotype shows variability ranging from a wild type-like phenotype(B, B'), to a partial reduction in the number of axons entering the dorsal thalamus (C, C'), to a quite complete absence of axons reaching the appropriate nucleus (D, D'). Arrows indicate the ventrobasal nucleus (VB).

Table 1 Summary of total Sox2$^{\beta geo/\Delta enh}$ versus Sox2$^{+/+}$ brains analyzed by DiI implantation

Site of implant	DiI brain implants			
	wild type[a]		Sox2$^{\beta geo/\Delta enh}$	
	Axonal projection pattern			
	Normal	**Abnormal[b]**	**Normal**	**Abnormal[b]**
Anterior (M1)	4	0	1	3[c]
Medial (S1)	8	0	3	5[d]
Posterior (V1)	7	0	5	2[e]
TOTAL	**19**	**0**	**9**	**10**

a: Sox2$^{+/+}$ and Sox2$^{\Delta enh/+}$ are both identified as wild type

b: brains showing a visible reduction or absence of axonal projections reaching the appropriate target in thalamus are defined as abnormal

c: two out of three brains show severe phenotype(fibres are absent, as in fig. 6, E, E'), one shows a milder phenotype (as in figure 6, D, D')

d: two out of five brains show severe phenotype; three show a milder mile phenotype

e: all show severe phenotype

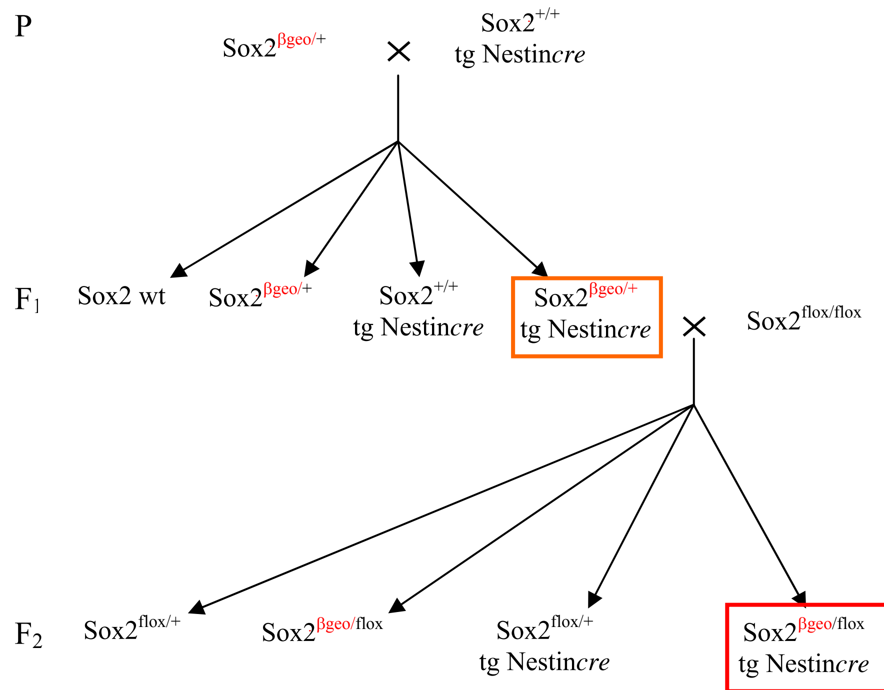
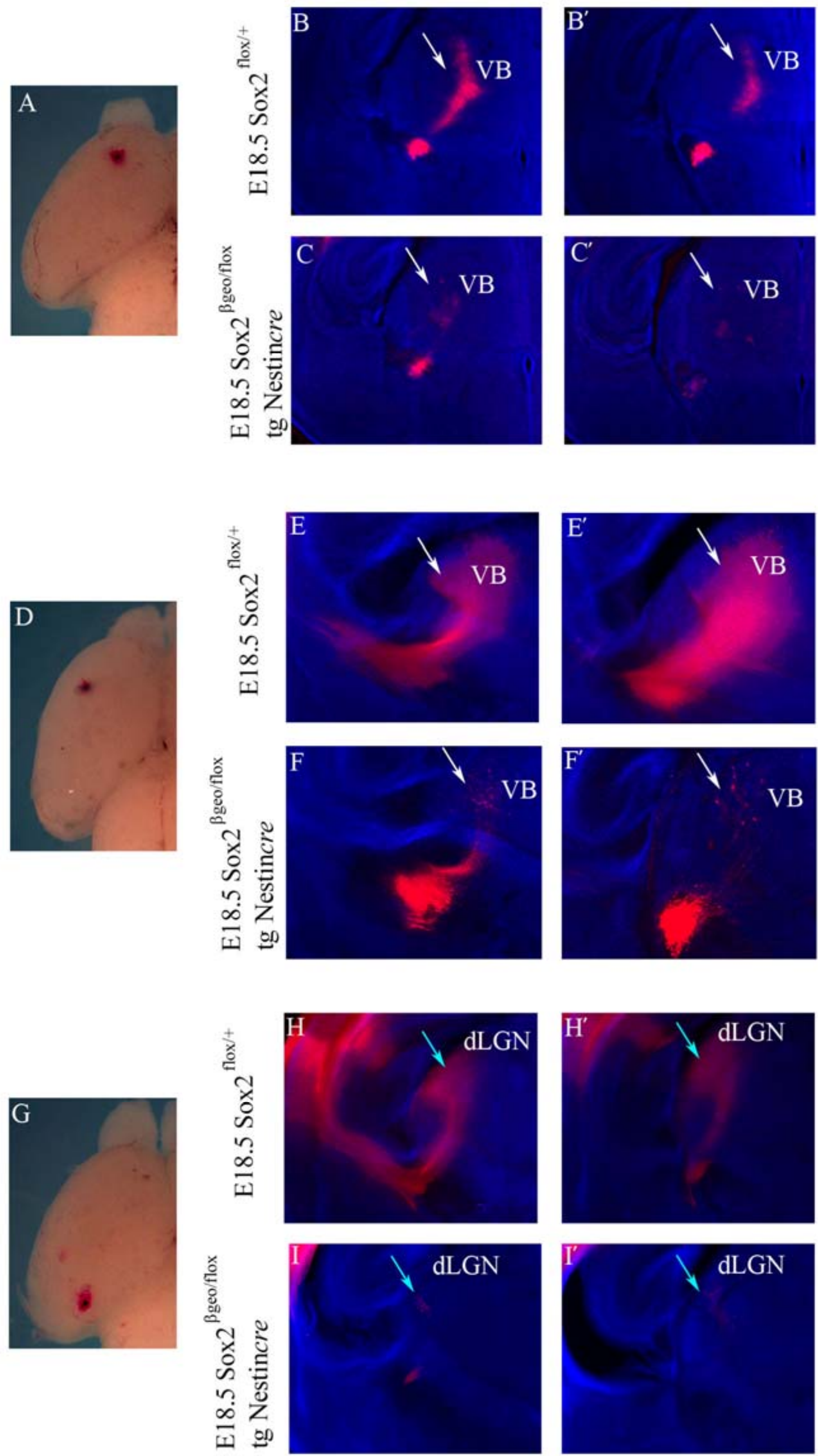


Fig. 7 Breeding scheme to obtain Sox2 conditional knock out mutant mice
To obtain Sox2$^{\beta geo/flox}$; tgNestin*cre* conditional mutant mice two generations of breeding were required. First, a Sox2$^{\beta geo/+}$ mouse was crossed to a mouse carrying the Nestin*cre* transgene. This mating allows to obtain 25% of offspring with both Sox2$^{\beta geo/+}$ genotype and Nestin*cre* transgene (orange box). Crossing the Sox2$^{\beta geo/+}$; tgNestin*cre* mouse to a mouse carrying the Sox2 allele flanked by the loxP sites in homozygosis produces 25% of Sox2$^{\beta geo/flox}$; tgNestin*cre* mouse (red box), the conditional knock out mouse in which Sox2 is completely absent in the entire neural tube from E12.5


A
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VB
B′
VB
E18.5 Sox2flox/+
C
VB
C′
VB
E18.5 Sox2βgeo/flox
tg Nestincre
D
E
VB
E′
VB
E18.5 Sox2flox/+
F
VB
F′
VB
E18.5 Sox2βgeo/flox
tg Nestincre
G
H
dLGN
H′
dLGN
E18.5 Sox2flox/+
I
dLGN
I′
dLGN
E18.5 Sox2βgeo/flox
tg Nestincre

Fig. 8 DiI labeling of corticofugal fibers in Nestin*cre* Sox2 deleted mice demonstrates a depletion in projections reaching the appropriate thalamic nucleus

Three sites of placement of DiI crystals in E18.5 brains label the corticofugal projections directed to the ventrobasal nucleus (VB, A, D) and dorsal lateral geniculate nucleus (dLGN, G). Details of 200 µm adiacent coronal sections counterstained with DAPI of $Sox2^{flox/+}$ brains (B, B', E. E', H, H') show the axons arriving to the appropriate nucleus as expected. DiI labeled axons in 200 µm adiacent coronal section counterstained with DAPI of $Sox2^{\beta geo/flox}$; tgNestin*cre* brain (C, C', F, F', I, I'), show a severe reduction in number of fibers reaching their appropriate thalamic nucleus in all brains analyzed. Crystals of DiI placed in the rostral (C, C') or medial (F, F') region, like the implants in caudal region (I, I'), show that the axons entering the thalamus and turning towards the dorsal thalamus are fewer in comparison with the $Sox2^{flox/+}$brains. White arrows indicate the ventrobasal nucleus (VB) and light blue arrows indicate the dorsal lateral genciulate nucleus (dLGN).

Table 2 Summary of Sox2$^{\beta geo/flox}$Nestin*cre* brains analyzed versus Sox2$^{flox/+}$ brains

Site of implant	DiI brain implants			
	Sox2$^{flox/+}$		Sox2$^{\beta geo/flox}$; Nestin*cre*	
	Axonal projection pattern			
	Normal	Abnormal[a]	Normal	Abnormal[a]
Anterior (M1)	4	0	0	4
Medial (S1)	5	0	0	5
Posterior (V1)	7	0	0	7
TOTAL	**16[b]**	**0**	**0**	**16[c]**

a: brains showing a visible reduction or absence of axonal projections reaching the appropriate target in thalamus are defined as abnormal

b: all the control brains analyzed show that axons leaving the cortical region follow the expected route of growth

c: all the mutant brains analyzed show severe reduction of axons reaching the appropriate thalamic nucleus.

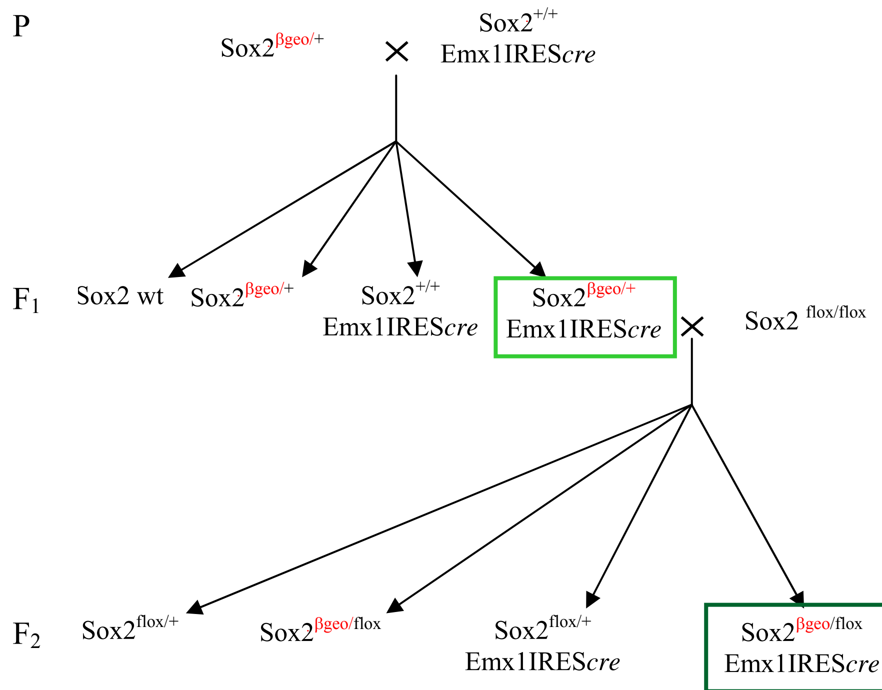

Fig. 9 Breeding scheme to obtain Emx1*cre* deleted Sox2 mice
To obtain $Sox2^{\beta geo/flox}$; Emx1IRES*cre* mutant mice two generation of breeding were been. First, a $Sox2^{\beta geo/+}$ mouse was crossed to a Emx1IRES*cre* mouse to obtain $Sox2^{\beta geo/+}$; Emx1IRES*cre* mice (the expected percentage is 25%, light green box). The second generation was obtained by crossing the $Sox2^{\beta geo/+}$; Emx1IRES*cre* mouse to a $Sox2^{flox/flox}$ mouse. 25% of the total offspring produced by this mating is represented by the cortical specific knock out mutant mice ,$Sox2^{\beta geo/flox}$; Emx1IRES*cre* (dark green box).

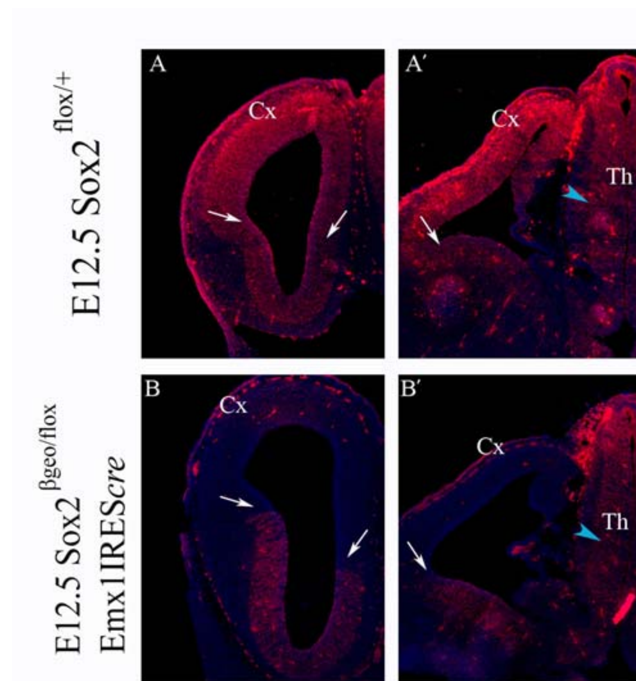

Fig. 10 Emx1IRES*cre* deletes Sox2 gene in developing cortex, but not in other cerebral regions, by E12.5.

Immunofluorescence with anti-Sox2 antoibodies (red) of 20 μm coronal slices of E12.5 brains, counterstained with DAPI (blue). In $Sox2^{flox/+}$ brain the expression of Sox2 protein is detectable in ventricular/subventricular zone of lateral ventricle and in the presumptive thalamus (A, A'). In $Sox2^{\beta geo/flox}$;Emx1IRES*cre* brain the expression of Sox2 protein is no longer detectable specifically in the dorsal telencephalon (B, B'), but is not affected in the ventral telencephalon (arrows indicate the boundary of expression between dorsal and ventral telencephalon). The espression of Sox2 in the thalamus is not compromised by the action of Cre recombinase (light blue arrowheads in A' and B' indicate the thalamic eminence expressing Sox2).

Table 3 Summary of Sox2$^{\beta geo/flox}$Emx1IRES*cre* brains versus Sox2$^{flox/+}$ brains

Site of implant	Implanted brains			
	Sox2$^{flox/+}$		Sox2$^{\beta geo/flox}$; Emx1IREScre	
	Axonal projection pattern			
	Normal	Abnormal[a]	Normal	Abnormal[a]
Anterior (M1)	2	0	2	0
Medial (S1)	6	0	6	0
Posterior (V1)	6	0	6	0
TOTAL	**14**[b]	**0**	**14**[c]	**0**

a: brains showing a visible reduction or absence of axonal projections reaching the appropriate target in thalamus are defined as abnormal

b: all the control brains analyzed show that axons leaving the cortical region follow the expected route of growth

c: all the mutant brains analyzed show that corticothalamic axons reach their appropriate nucleus.

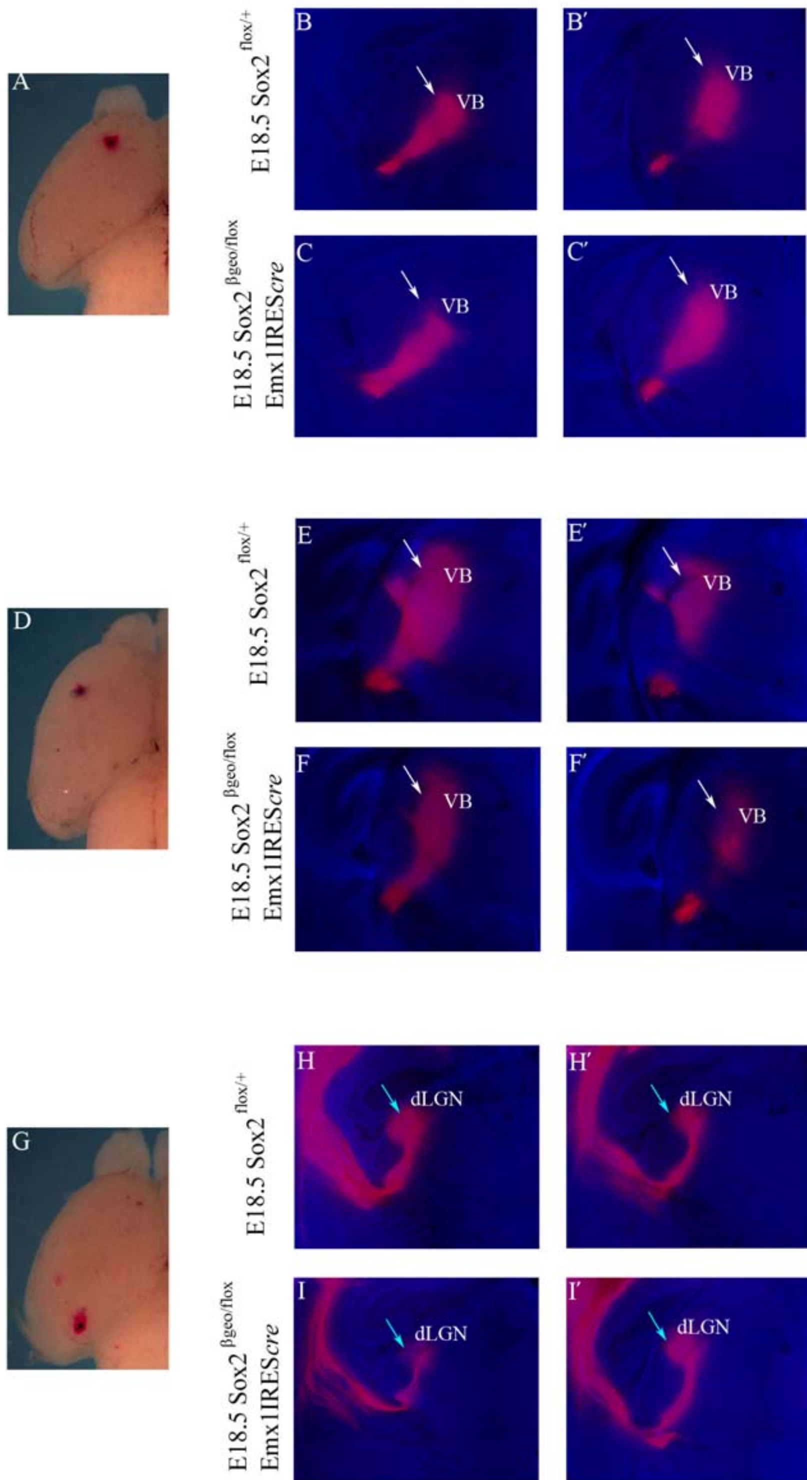
A
B
VB
B′
VB
E18.5 Sox2 flox/+
C
VB
C′
VB
E18.5 Sox2 βgeo/flox
Emx1IREScre
D
E
VB
E′
VB
E18.5 Sox2 flox/+
F
VB
F′
VB
E18.5 Sox2 βgeo/flox
Emx1IREScre
G
H
dLGN
H′
dLGN
E18.5 Sox2 flox/+
I
dLGN
I′
dLGN
E18.5 Sox2 βgeo/flox
Emx1IREScre

Fig. 11 Corticofugal axons labeled with DiI are not abnormal in Sox2$^{\beta geo/flox}$;Emx1IRES*cre* brain

DiI crystals were placed in the three major functional regions of cortex (motor cortex, A, somatosensory corte, D, visual cortex, G) in E18.5 brains to label the corticofugal prejections. (C, C'F, F', I, I') 200 µm adjacent coronal sections of braina lacking Sox2 specifically in the cortex show that the DiI stained fibers do not show abnormalities if compared with their Sox2$^{flox/+}$ controls (B. B', E, E', H, H'). The axons arriving from rostral (B, B', C, C') and medial (E, E', F, F') region project correctly towards the VB both in Sox2$^{flox/+}$ and in Sox2 mutant brain. Also the projections from the caudal region (H, H', I, I') show the same pattern of elongation in Sox2$^{flox/+}$ and in Sox2 cortical null brain. White arrows indicate ventrobasal nucleus (VB), light blue arrows indicate dorsal lateral geniculate nucleus (dLGN).

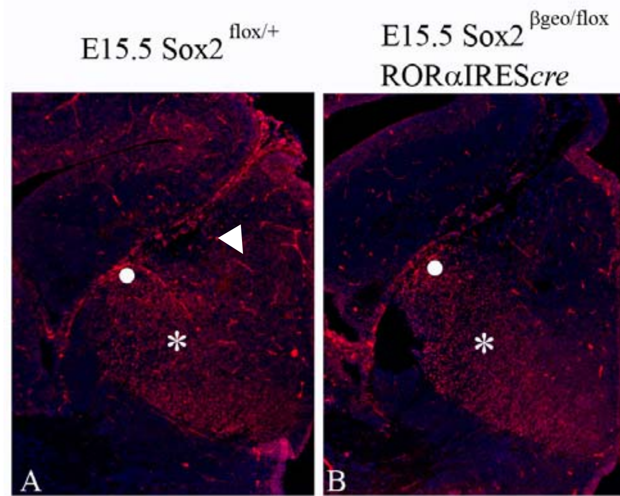

Fig.12 RORαIRES*cre* does not delete Sox2^{flox} in dorsal thalamus up to E15.5
Immunofluorescence with anti-Sox2 antoibodies (red) of 20 μm coronal slices of E15.5 brains, counterstained with DAPI (blue). Staining of Sox2$^{flox/+}$ (A) and Sox2$^{\beta geo/flox}$; RORα*cre* (B) show that expression of Sox2 is in dorsal thalamus is not affected by the expression of Cre recombinase (B, asterisks indicate the ventrobasal nucleus, the circles indicate the dorsal lateral geniculate nucleus; the triangle indicates aspecific red staining)

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CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

1. Sox2 is required for NSC maintenance

Sox2 is a transcription factor expressed in, and essential for, the multipotent stem cells of blastocyst inner cell mass. Its ablation causes early embryonic lethality (Avilion et al., 2003). Later Sox2 is a marker of nervous system from the beginning of its development. As development proceeds, Sox2 expression is restricted to neural stem cells and progenitors in the ventricular/subventricular zone (VZ/SVZ) of developing brain and in neurogenic regions in adult brain, SVZ and hippocampus dentate gyrus (Zappone et al., 2000; Ferri et al., 2004). It is known that Sox2 is functionally essential for maintenance of undifferentiated state of NSC (Graham et al., 2003; Ferri et al, 2004; Cavallaro et al., 2008), and its expression is progressively downregulated during the neuronal differentiation (Cavallaro et al., 2008). A residual expression of Sox2 is retained in some populations of differentiated neurons. Strikingly, it has been demonstrated that Sox2 can reprogram terminally differentiated cells to induced pluripotent stem (iPS) cells, acting together with three other transcription factors (Takahashi and Yamanaka, 2006).In our laboratory we investigated the role of Sox2 in developing brain and in neural stem cells by in vivo and in vitro studies on animal models whit reduced (hypomorphic mice, Ferri et al., 2004, Cavallaro et al., 2008) or absent (conditional knock out mice, Favaro et al., 2009) Sox2 expression.

Reduced level of Sox2 expression causes depletion of stem and progenitor cells and cerebral defects, including reduced neocortex size

and parenchymal loss. Moreover, this mice show some neurological problems, like epilepsy and motor defects (Ferri et al., 2004).

2. Sox2 is required for the differentiation of GABAergic neurons

Our study on neural stem cells derived from mice with reduced expression of Sox2 showed that neuronal terminal differentiation is specifically affected (Cavallaro et al., 2008). Sox2 deficient stem cells cultures originate a normal number of cells expressing markers of young neurons, even though with a poor morphology. This population, however, is not immunoreactive for neuronal terminal differentiation markers, such as MAP2 and NeuN. In particular, by study ex vivo and in vivo, we have seen that in newborn mouse cortex and in adult olfactory bulb GABAergic mature populations are greatly diminished in number (40-60%). Additionally, we detect defective migration of GABAergic neurons originated from precursors in ganglionic eminence: these cells are detected along the subcortical fibre bundles but are rare in cortical plate (Cavallaro et al., 2008).

So, we demonstrated that a normal level of Sox2 is required for correct neuronal differentiation; mutant cells generate a reduced number of mature neurons, in particular GABAergic neurons, but the production of glia is not affected (Cavallaro et al., 2008). Sox2 overexpression at early, but not later, stages of differentiation in cultured mutant cells is able to rescue the mutant phenotype. Neuron progenitors express at early stages transcription factors known to be involved in neuronal differentiation. We hypothesize that Sox2, commits early precursor to neurogenesis establishing a downstream

transcriptional program for later neuronal differentiation events and repressing alternative (glial) transcription programs. (Cavallaro et al., 2008).

These data demonstrate a role of Sox2 in neuronal differentiation at least for a subset of mature neurons, the GABAergic neurons.

3. Sox2 is required for the correct development of corticothalamic axons

Another subset of mature neurons that are affected by the reduction/absence of Sox2 is the population of cortical excitatory projection neurons. Many different genes encoding transcription factors, nuclear receptors, cell adhesion molecules, axon guidance receptors and ligands are involved in the mechanism of axon pathfinding (reviewed in Lopez-Bendito and Molnar, 2003).

By studies on Sox2 mutant mice, we have seen that Sox2 is required for the correct growth of corticothalamic axons after E12.5 (time of complete Sox2 deletion driven by Nestin*cre* transgene, Favaro et al., 2009). The Sox2 absence leads to an aberrant growth of axons, without misrouting of fibers: the corticofigsl projections arrive in the striatum without evident problems, then, they seem stall into the internal capsula and axonal growth in thalamus is absent,.

Normal axons are able to grow because express specific molecular receptors on their surface and growth cone. Besides, the growing of axons involved also several cell adhesion molecules that bind to similar proteins on nearby cells. Corticothalamic and thalamocortical projection interact physically in the internal capsula, then proceed dependent on each other (the "handshake hypothesis", Molnar and

Blakemore, 1995; Molnar et al., 1998; Hevner et al., 2002). We have seen that in Sox2 mutant mice the defects appears only after corticothalamic axons growing into the subpallium and entering the internal capsula, whereas seem that thalamocortical fibres are not affected, as seen in preliminary experiments. A first hypothesis to explane the abnormal corticothalamic growth is a defective expression of one or more adhesion molecules on corticothalamic axons surface. This can lead the axons to lack the ability to interact with thalamocortical fibres. This defect can be attributed to a cell specific altered differentiation program which does not allow progenitors differentiate and express correct molecule on their surface.

On the basis of previous evidences for the role of Sox2 in correct neuronal differentiation, we first investigated the possibilitiy of a cell autonomous defect, dues to a misregulation of a "differentiation program" established by Sox2, that leads to the lack of capacity to response to environmental stimuli.

Cortical projection neurons originate from progenitors expressing Emx1 (Britanova et al., 2006). We have ablated Sox2 specifically in the compartment of cells expressing Emx1, using an Emx1IRES*cre* deleter mouse. The timing of deletion is very similar to that one of the Nestin*cre*, with a complete dorsal telencephalic ablation by E12.5

This deletion does not affect the correct development of corticothalamic projections, ruling out the hypothesis of a cell autonomous differentiating defect of projection neurons.

Several other explanations are possible to clarify this defect.

The internal capsula resides in the dorsal striatum. The striatum is the region where cortical and thalamic afferents are integrated. Spiny

projection neurons reside in dorsal striatum, and receive and contact glutamatergic projection from cerebral cortex, which form well defined synapses (Wolf, 1998). In this region Sox2 is expressed in sparse neurons (Ferri et al., 2004).

Errors in pathfinding of both corticofugal and thalamocortical connections were described in mice with mutations in transcription factors Tbr1, Gbx2 and Pax6 (Stoykova and Gruss 1994; Hevner et al. 2002; Jones et al., 2002).

Mechanisms of guidance in IC are still poorly defined, but is known that this region expresses some guidance molecules, like Netrin1 (Métin et al., 1997; Richards et al., 1997), Ephrin-A (Dufour et al., 2003), Semaphorin 3A (Bagnard et al., 2001) and Semaphorin 6A (Garel et al., 2002). Additional positional cues are found at the DTB. Genetic defects affecting this region can stop axons traveling in either direction, or lead to misrouting (Garel and Rubenstein, 2004; Hevner et al. 2002).

It is possible that Sox2 controls the expression of signaling molecules in IC, or, more in general, in the striatal region, along the path of projections growth. In Situ Hybridization studies can elucidate if there is a variability in expression of striatal guidance cues between normal and Sox2 mutant mice.

Sox2 is expressed also in the dorsal thalamus, in territory including the region of thalamic nuclei (Vue et al., 2007).

Dorsal thalamusis the final target of corticothalamic projections. It is possible that the lack of Sox2 affects the expression of diffusible molecules involved in guidance events.

Sonic Hedgehog (Shh) is described acting in the pathway of guidance of commissural axons. It is involved both in attractive Netrin1 signaling (Charron et al., 2003; Okada et al., 2006) and in the repulsive Semaphorins signaling (Parra and Zou, 2010) in spinal cord. Besides, both Netrin1 and Sempahorins are involved as guidance cues for corticofugal axons (Metin et al., 1997; Bagnard et al., 1998). We demonstrated that Shh is a direct target of Sox2 (Favaro et al., 2009). Shh expression is detectable in the midline of ventral forebrain. In mice lacking Sox2, the expression of Shh is reduced in ventral foirebrain, but not in midbrain (Favaro et al., 2009). Shh in ventral forebrain could be involved in the pathway of expression of some guidance cue, like Netrin1 and Semaphorins. Sox2 would be involved in the same pathway, regulating the expression of Shh.

Zona limitans intrathalamica (ZLI) is a neuroepithelial domain that separates preumptive prethalamus from presumptive thalamus during thalamic development (Larsen et al., 2001) and functions as secondary organizer (Vieira et al., 2005). ZLI is a source of Shh, known to be an important signaling molecule in the patterning of thalamus in mice (Ishibashi and McMahon, 2002). Other diffusible factors involved in normal development of thalamus are Wnts , that contributes to establishment of regional thalamic identities (Braun et al., 2003; Zhou et al., 2004). Additionaly, Fgf8, which is expressed in ZLI, controls the patterning of thalamic and prethalamic nuclei (Kataoka and Shimogori, 2008). By In Situ Hybridization or Immunohistochemistry analysis is possible to investigate if Sox2 deletion causes defective expression of gene involved in thalamic patterning.

The thalamic nuclei are generated between E10.5 and E15.5 (Altman and Bayer, 1988). E15.5 is the earliest stage in which individual thalamic nuclei are defined by gene expression pattern (Nakagawa and O'Leary, 2001; Kataoka and Shimogori, 2008). In mutant brains, thalamic nuclei, at E18.5, show normal morphology, but remains the possibility that Sox2 deficiency causes alterations in their molecular identity by defective differentiation of dorsal thalamic neurons. It is interesting to perform the molecular chatacterization of thalamic nuclei in Sox2 deficient mice, by analysis of gene expression.

4. Emx2 acts as a regulator of Sox2

The identification of Emx2 as direct transcriptional repressor of Sox2 expression during brain development, together with strong evidences that Sox2 controls stem cell maintenance, suggest that Emx2 gradients might affect Sox2 levels in different cortical regions, controlling the balance between self-renewal and commitment to differentiation of stem cells. Thus, Emx2 may control NSC decisions, at least in part, by regulating Sox2 levels.

Emx2 seems to antagonize Sox2 expression by direct transcriptional repression of the two Sox2 telencephalic enhancers (Sox2 5' and 3' regulatory elements) both in vivo and in vitro.

The "core" elements of both the Sox2 5' and 3' enhancers contain POU sites, known to bind different positive regulators of their transcriptional activity in different cell types. Probably at later stages of development, Emx2 might repress transcription at these sites by negatively affecting the activators (by directly binding to the same sites or via protein to protein interaction) to regulate differentiation of

neural stem/progenitor cells and cortical patterning, thus allowing the downregulation of Sox2 expression in differentiating cells.

The ablation of Emx2 expression in neural stem cells enhances their rate of proliferation, and it is possible that Emx2 deficiency counteracts the effects of Sox2 deficiency on neural stem cells proliferation ability and neuronal differentiation, probably antagonizing the defect by rescuing Sox2 levels.

5. Sox2 and human diseases

In human, Sox2 deficiency is a rare condition found in patients with microphtalmia (small eyes) or anophtalmia (no eyes) (Fantes et al., 2003). Moreover, these patients show others important neural defects, including abnormalities in hippocampus and corpus callosum, epilepsy, pituitary defects and motor problems (Ragge et al., 2005; Sisodiya et al., 2006; Kelberman et al., 2006).

5.1 Sox2 deficiency and epilepsy in humans and mice

Genetic distruption of homeobox genes related to specification, regionalization and terminal differentiation results in epileptic phenotype. Sox2 have a role in neuronal terminal differentiation (Cavallaro et al., 2008) The Sox2 mutant mice reproduce several different characteristics of neurological diseases present in Sox2 deficient patients. In particular epilepsy and hippocampal defects are mirrored in both mutant mice generated in our laboratory (Ferri et al., 2004; Favaro et al., 2009).

Loss of GABAergic inhibitory neurons leads to epilepsy in mouse and man (Noebels, 2003; Cobos et al., 2005). The finding that

GABAergic inhibitory neurons show defective migration and are reduced in Sox2 mutant cortex, represents a plausible cellular basis for epilepsy in humans with Sox2 mutation (Cavallaro et al., 2008).

The in vitro culture system allowed to identify that Sox2 is important at early, not at later stages, of neuronal differentiation; moreover, this system will allow the identification of Sox2 target important for neuronal differentiation, by rescue experiments.

5.2 Are abnormalities in axon guidance involved in motor coordination defects present in Sox2 mutant patients?

Another characteristic present in Sox2 deficient patients is motor coordination problems (Sisodiya et al., 2006). Similar behavioural defects are present also in Sox2 mutant mice (Ferri et al., 2004).

Loss of projection neurons, in Otx1 mutant mice, leads to a rearrangement of local circuitry characterized by excess of excitation (Sancini et al., 2001). The sense organs send to cortex several complex informations. Corticothalamic axons projecting in the thalamus act as feedback system, that plays a crucial role in modulating the thalamic responses required to perform the complex information processing and integration that underlie mammalian behaviors (Jones, 2002; Alitto and Usrey, 2003; Temereanca and Simons, 2004). The reduction/absence of these connections, present also in Sox2 mutants, can leads to a lack of negative feedback, resulting in excess of motor response to environmental stimuli.

5.3 Sox2 and cell therapy

As Sox2 plays pivotal roles in controlling neural stem cells self-renewal/proliferation and differentiation (Ferri et al., 2004; Cavallaro

et al., 2008; Favaro et al., 2009), its study will be useful for elucidating such mechanisms that are of particular relevance for the improvement of stem-cell-based approaches.

Elucidating the molecular mechanisms which govern proliferation and differentiation of NSC give great hope for the treatment of neurological disorders. Different subtypes of differentiated neurones can be generated in vitro from stem cells of various sources including reprogrammed somatic cells (iPS). The transplantation of in vitro generated neurones, instead of undifferentiated NSC, have shown a major, long-lasting improvement in some patients (Rossi and Cattaneo, 2002; Lindvall and Kokaia, 2006). However, effective strategies must be developed to isolate, enrich and propagate homogeneous populations of NSCs, and to identify the molecules and mechanisms that are required for their proper integration and differentiation into the injured brain.

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autrice della tesi di DOTTORATO dal titolo:

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AND AXONAL CONNECTIVITY IN MICE MUTANT IN THE
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AUTORIZZA

La consultazione della tesi stessa, fatto divieto di riprodurre, in tutto o in parte, quanto in essa contenuto.

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