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TRANSCRIPTIONAL REGULATION, TARGET GENES AND FUNCTIONAL ROLES OF THE SOX2 TRANSCRIPTION FACTOR IN MOUSE NEURAL STEM CELLS MAINTENANCE AND NEURONAL DIFFERENTIATION

Jessica Mariani – No. 030667

Coordinator: Prof. Andrea Biondi
Tutor: Prof. Silvia K. Nicolis

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GENERAL INTRODUCTION

§ 1. Development of the central nervous system and telencephalon

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 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 estabilished. 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, the neural tube 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, that are derived from the romboencephalic vesicle and extend to form the spinal cord.

In the process that leads to the formation of the mature central nervous system or *neurogenesis*, can be distinguished three different phases closely related to each other:

1. Determination
2. Morphogenesis and patterning
3. Terminal differentiation

The first phase includes the neural induction, i.e. the formation of the neural plate, then the neural tube. The process of morphogenesis and patterning consists of a regionalization of the neural tube, in which the different regions of the future CNS express different genes and acquire different features, according to an antero-posterior and dorso-ventral patterning.

Finally, the third stage consists of a progressive differentiation of the cells in the three main cell-types of the mature nervous tissue: neurons, astrocytes 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 (or determined) 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.

During the differentiation process and the subsequent maturation process the cells migrate from the ventricular zone of the neural tube to their final destination, giving rise to the specific functional areas of the CNS. The former (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 latter (maturation) leads to an increasing in the levels of specific genes expression.

The differentiation and patterning of the neural tube occurs by patterning centres that impart positional information. These neural centers produce “signaling molecules” which are able to impart regional identity to the various embryonic areas. These signaling 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.

1.1. Genes implicated in the process of “cortical arealization”

Several genes are implicated in the definition of the various areas composing the developing cerebral cortex.

Among these genes, the homeodomain transcription factor Emx2 and the paired-box domain transcription factor Pax6 are genetic regulators of the area identities of cortical progenitors. These two transcription factors regulate the development of telencephalon and are expressed in opposing gradients at the level of neural progenitor cells. Emx2 is expressed in a high caudomedial to low rostrolateral gradient, whereas Pax6 is expressed in an opposing gradient, high rostrolateral to low caudomedial gradient. Their loss leads to several changes in the position and size of cortical areas (Bishop et al., 2000; 2002).

In $Emx2^{-/-}$mutant mice, the rostrolateral areas are expanded, whereas caudomedial areas are reduced. On the contrary, in $Pax6^{-/-}$ mutant mice, the rostrolateral areas are reduced and caudomedial areas are expanded. Therefore, gradients of signaling molecules are able to generate boundaries of gene expression that specify the territories corresponding to the future cortical areas and that impart positional identity to cortical neurons.

§ 2. Neural stem cells

Stem cells are defined, by functional properties, as cells that have the capacity to generate multiple types of differentiated cells (*multipotency*) and are able to undergo cell divisions in wich at least one of the daughter cells maintains stem cell potential (*self-renewal*) (Johansson et al., 1999 a-b). These functional properties distinguish stem cells from progenitor cells, that are cells with a more restricted potential (McKay, 1997).

In the nervous system, Neural Stem Cells (NSC) have the potential to differentiate into neurons, astrocytes and oligodendrocytes and to self-renew. NSC arise early in nervous system development (at 6.5 dpc) along the whole neural tube ectoderm. In the early embryonic phases, NSC undergo mainly symmetric divisions, which maintain stemness and lead to the expansion of the population. Later, in the neurogenic phase, stem cells divide asymmetrically to generate new stem cells and more committed proliferating precursors (Qian et al.,

1998, 2000; Temple, 2001 a-b). In the neurogenic phase, proliferating precursors produced mainly belong to the neuronal lineage. After the neurogenic phase, proliferating precursors develop predominantly into a glial progeny.

During the development, newborn neurons migrate, toward the radial glia, from the germinative ventricular zone to the external layers where they undergo terminal differentiation.

In the adult brain, small numbers of neural stem cells persist in selected regions: the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Sgubin et al., 2007). In these areas, stem cells are reppresented by mitotically quiescent cells (adiacent to the ventricle, or at the basis of the hippocampus dentate gyrus). From the SVZ, newly generated neuroblasts migrate along the rostral migratory stream (RMS) to reach their final destination in the olfactory bulb, where they turn radially and differentiate into granule and periglomerular neurons. In the hippocampus, SGZ astrocytes give rise to intermediate progenitors, which mature locally into granule neurons of the dentate gyrus (Doetsch and Hen, 2005; Galli et al., 2003).

Neural stem cells can be propagate in vitro under certain culture conditions. One of the reasons for studying their in vitro propagation and differentiation is their usefulness in cell-replacement therapy for the treatment of brain diseases (McKay, 2000).

In the presence of mitogens such as epidermal growth factor (EGF) and fibroblast growth factor (FGF), neural stem cells continue to proliferate and form a characteristic spheroid cell aggregate of tightly clustered cells, called “neurospheres”. All cells in these neurospheres are clonally derived from a single cell and can be propagated for several passages giving rise to secondary spheres. It should be noted that a neurosphere is a mixture of NSCs, differentiating progenitors and even differentiated neurons and glia, depending on the neurosphere size and time in culture. As in the primary culture, differentiating/differentiated cells rapidly die while the NSCs continue to proliferate, giving rise to many secondary spheres and exponential growth in vitro. In this way, stable NSCs lines can be obtained. On removal of the mitogens and the additin of serum to the culture medium, the progeny of NSC rapidly differentiate into the three main cell types of the CNS (astrocytes, oligodendrocytes and neurons) demonstrating that at least the initial cell was multipotent (Johansson et al., 1999 a-b; Galli et al., 2003).

Several genes play pivotal roles in controlling stem cells properties. Shh, Bmi-1 and Notch-1 are important for stem cell proliferation and maintenance. On the contrary, PTEN maintains quiescence, and its loss causes excessive stem cell proliferation.

In vivo, NSC reside in niches, that regulate their self-renewal, activation and differentiation (Spradling et al., 2001). Common components of stem cell niche are signaling from adiacent somatic cells, a basement membrane for cell anchoring and extracellular matrix (which modulate the adhesiveness and the activity of signaling molecules). Shh is also required for cell proliferation in the mouse SVZ stem cell niche and for the production of new olfactory interneurons in vivo (Palma et al., 2004).

§ 3. 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 first class, comprising SOX1, SOX2 and SOX3, share greater than 90% amino acid residue identity in the HMG-DNA binding domain and are classified as subgup 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).

3.1. Expression of the SoxB1 genes

The SoxB1 genes, Sox1, Sox2 and Sox3 are expressed throughout cells that are competent to form the neural primordium, and than 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, 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 signaling molecules involved in neural induction.

3.2. Functional roles of the SoxB1 genes

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 oligodendrocyte 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 depend on the re-activation of Sox2 expression, while inibition 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. The Sox2 gene: expression and function during brain development

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 form 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 pan-neurally (Avilion et al., 2003).

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 proceed, 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 enlargment, circling behaviour and epilepsy) and neuronal abnormalities (degeneration and cytoplasmic protein aggregates) features common to different human diseases (Ferri et al., 2004). These observation point to 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 suggests that Sox2 is required in early differentiating neuronal cells, for maturation and for suppression of alternative lineage markers (Cavallaro et al., 2008).

4.1. Sox2 transcriptional regulation in the central nervous system

In many different species, Sox2 is a marker of the nervous system from the beginning of its development (neural plate). As development proceeds, Sox2 is expressed in neural stem/progenitor cells residing in the ventricular zone of the developing brain and also in the neurogenic regions of the adult brain. Subsequently, Sox2 is down-regulated in the marginal zone where differentiating neurons reside.

Because Sox2 is expressed uniformly in the early neural tube, 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).

In mouse, at least two forebrain-specific enhancers are able to recapitulate Sox2 telencephalic expression throughout forebrain development (Zappone et la., 2000; Miyagi et al., 2004). These regulatory elements consist of to enhancer regions, one located 5’, the other 3’ to the Sox2 transcriptional unit. To examine the spatiotemporal activity of these two regulatory elements in developing brain, transgenic mice carrying a β-geo repoter gene uder the control of the 5’ or 3’ enhancer were generated, and analized for β-galactosidase activity at different developmental stages (Zappone et al., 2000; Catena et al., 2004; Miyagi et al., 2006). These studies shown that the expression of β- galactosidase by both these two regulatory elements is confined to the developing telencephalon, with the 5’ enhancer being more active in dorso-medial regions, and the 3’

enhancer in ventro-lateral regions of the telencephalon. The endogenous Sox2 expression in the telencephalon reflects the combined actions of these two regulatory regions. These two enhancers, are also active earlier in embryonic stem cells of the blastocyst inner cell mass (ICM).

As development proceeds, transgenes expression is maintained in the ventricular/proliferative zone (VZ), were undifferentiated neural stem/progenitor cells reside, but not in differentiating cells of the external layers. In order to evaluate whether the ventricular zone cells expressing the Sox2 5'/3' transgenes include *bona fide* neural stem cells, clonogenic analyses from the telencephalon of transgenic embryos were performed. These results confirm that the ventricular zone cell population that express the Sox2 transgenes include cells with functional properties of neural stem cells, i.e. self-renewal and multipotency (Zappone et al., 2000; Miyagi et al., 2006). Interestingly, the activity of the Sox2 5' and 3' regulatory sequences in telencephalic stem cells is not limited to the embryonic development, but persist also in the adult neurogenic regions of the brain, in the periventricular cells of the lateral ventricle (and along the rostral migratory stream reaching the olfactory bulb) and in the subgranular layer (SGL) of the hippocampal dentate girus (Zappone et al., 2000; Miyagi et al., 2006). The physiological significance of the presence of two distinct regulatory regions (5' and 3' Sox2 enhancers) which direct the expression of a single gene in the same cells is not known at present. In each case, it is possible to assume that two regulatory regions synergistically function to support the high level of gene expression in particular cell types.

The essential "core"elements of both Sox2 5' and 3'enhancers have been defined in vivo by transgenic assays and, in vitro, by transfection in Embryonic Stem (ES) Cells (Catena et al., 2004; Miyagi et al., 2004). Both core elements contain POU sites which have been shown to be functionally important in ES and brain cells (Catena et al., 2004; Miyagi et al., 2004, 2006), by binding specific transcription factors (Oct4 in ES cells, and Brn1/2 in neural cells). Thus, the 5' and 3' Sox2 enhancer are the first exemple of regulatory sequences that exert their activity in ES cells and neural stem/progenitor cells in a similar manner, by utilizing the common core sequence in which POU sites play a central role (Catena et al., 2004; Miyagi et al., 2004). Oct4 may regulates the transcriptional activity of both 5' and 3' enhancers in totipotent cells (ES cells), whereas Brn1/2 control their activity in neural stem/progenitor cells by binding the same sites (Catena et al.,

2004; Miyagi et al., 2004, 2006). Interstingly, like the regulatory elements of FGF-4 and UTF1 genes , the 3' Sox2 enhancer carries a Sox2-like binding sequence, adiacent to the funcitonal important POU site (Tomioka et al., 2002; Nishimoto et al., 1999, 2001; Yuan et al., 1995; Dailey et al., 2001). The combinatorial action of Sox2 and POU binding sites support the 3' enhancer activity. Importantly, studies in chick embryos showed that most of the regulatoty regions involved in Sox2 expression in the brain have Sox2 binding sites, suggesting the existence of an autoregulatory loop of Sox2 expression in the brain, at least in part conserved between chicken and mouse (Uchikawa et al., 2003; Tomioka et al., 2002; Miyagi et al., 2004, 2006).

The importance of studying the molecular mechanisms governing the transcription-regulatory activity of the Sox2 forebrain-specific enhancers, may lead to unravelling a broader aspect of a common regulatory network which defines the nature of the stem cell state of embryonic stem (ES) cells, neural stem cells (NSC) and possibly stem cells in general. The 5' and 3' elements, are the only Sox2 enhancers that function specifically in the telencephalon in mammals and are useful tools for marking and isolating neural stem/progenitor cells with region-specific properties. Pure telecephalic neural stem/progenitor cell population could be used for the treatment of major brain diseases. More in general, these regulatory regions will be useful for elucidating the molecular mechanisms of gene regulation, as well as the cell fate decisions made by telencephalic neural stem/progenitor cells during development.

4.2. Sox2 epigenetic regulation during neural differentiation

Many unique "epigenomes" can be created from a single genome by post-translational modification of both DNA and histones. DNA methylation creates permanent marks on the genome, which can be preserved through subsequent cell cycles. In this way, stable genomes, defining the function of different cell types are created. Then, these marks can be used to influence histone acetylation, resulting in localized changes in chromatin structure. For genes, this lead to either their activation in a cell specific manner, or their silencing. During brain development multiple cell types are generated, from a common

progenitor cell, each with their unique epigenomes responsible for their functional features.

The Sox2 gene, is a key neurodevelopmental transcription factor, expressed in neural stem/progenitor cells, in glial precursor and proliferating astocytes, but is silenced in neurons and quiescent astrocytes (Miyagi et al., 2004; Bani-Yaghoub et al., 2006). In an elegant study Sikorska at al (Sikorska at al., 2008) have shown that epigenetic mechanisms play a role in controlling cell-type-specific SOX2 expression. Interestingly, several CpGs located in the 5' and 3' enhancers of the Sox2 gene, highly conserved across different vertebrate species were identified. The Sox2 5' and 3' enhancers were differentially methylated (at these conserved CpGs) and acetylated, on a temporal basis, contributing to the generation of neuron- and asrtocyte-specific epigenomes from a common progenitor cell. Particularly, no methylated CpG in either of the two enhancer regions were found in cycling progenitor cells, correlating with the high levels of SOX2 expression. In contrast, the cells that differentiated into neurons and ceased to transcribe the gene, showed significant increases in CpG methylation at both Sox2 5' and 3' enhancers. In astrocytes, a progressive increase in the content of methylated CpGs in the Sox2 5' enhancer region was observed as cells moved from proliferation to quiescence and in which the SOX2 level drops down. On the other hand, the CpGs located in the Sox2 3' enhancer remained unmehylated regardless of proliferation vs. differentiation status of the astrocytes. In vitro, quiescent astrocytes could re-express SOX2 and be driven back into the cell cycle by the treatment with bFGF. In this process, again there was no change in the methylation status of the Sox2 3' enhancer. However, there was a transient DNA demethylation in the Sox2 5' enhancer, coincident with the increase in SOX2 expression. The region is subsequently remethylated as SOX2 expression levels declined.

The acetylation of histone H3 is a marker for chromatin remodeling, which also occurs at these Sox2 enhancers. High level of histone H3 acetylation was observed at both Sox2 5' and 3' enhancers as well as in the coding region in progenitor cells, where both enhancers were unmethylated. The degree of histone acetylation decreased as cells differentiated into neurons and quescent astrocytes, especially at the Sox2 3' enhancer and in parallel with the downregulation of SOX2 expression. By contrast, an increasing in H3 acetylation, at the Sox2 3' enhancer, was observed following bFGF treatment of quiescent astrocytes, as SOX2 was re-expressed.

Thus, both the Sox2 5' and 3' enhancers were unmethylated and associate with acetylated histone H3 in progentitor cells, in which the gene is expressed at high level. The same enhancers, were differentially methylated and acetylated in astrocytes vs. neurons. In this way, astrocytes retain the ability to re-express SOX2, whereas, in neurons, the gene is silenced. The methylation of the downstream Sox2 3' enhancer, was specific for neurons and coincided with gene silencing, whereas methylation of the pustream Sox2 5' enhancer occurred in both cell types and appeared to parallel cell cycle exit and down-regulation of SOX2. These data indicate that the signals affecting cell-cycle-related down-regulation of SOX2, act mainly at Sox2 5' enhancer and are different from those acting via the Sox2 3' enhancer that are responsible of SOX2 silencing.

Epigenetic mechanisms play a role, not only in programming genome end states (i.e. progenitors, neurons and astrocytes) but also in the reversible reprogramming of gene expression in differentiated cells (i.e. quiescent vs. proliferating astrocytes) and may participate in regulating expression of key genes (like Sox2) that are expressed only transiently in differentiating cells.

§ 5. The Emx2 gene

The vertebrate central nervous system (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 molecules, which establish within cortical progenitors the differential expression of transcription factors (TFs) that specify their area identity (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 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 an homeobox-containing TF. The homeobox sequence encodes a 62-amino acid homeodomain, that is 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 fated to give rise to the cerebral cortex, 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, postnatally, in adult brain neurogenic regions, the subventricular zone (SVZ) of the lateral ventricle and the hippocampus dentate gyrus (DG) (Gangemi et al., 2001; Galli et al., 2002). $Emx2^{-/-}$ mutant mice, die within few hours after birth, most likely due to kidney and other urogenital defects (Pellegrini et al., 1996). In $Emx2^{-/-}$ brains, there was a selective and relevant 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 ventricular zone (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 is down-regulated in post-mitotic cortical neurons, controlling some parameters of cortical neurons proliferation (Gulisano et al., 1996).

High levels of Emx2 expression are also present in the adult brain, specifically in neurogenic areas (the SVZ of the lateral ventricle and the DG of the hippocampus). Emx2 mRNA is specifically present in all undifferentiated neural stem cells of the adult SVZ and its expression is down-regulated upon their differentiation into neurons and glia (Gangemi et al., 2001; Galli et al., 2002). Abolishing or, increasing Emx2 expression in adult neural stem cells greatly enhances or reduces their rate of proliferation, respectively (Galli et al., 2002). In particular, when Emx2 expression is abolished, the frequency of symmetric cell divisions generating two stem cells

increases, whereas it decreases when Emx2 expression is enhanced. Thus, Emx2 regulates the proliferation of adult neural stem cells in a negative fashion, probably by diminishing their capacity for self-maintenance. 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 capable of migrating out of the ventricular zone, becoming post-mitotic and differentiating into the appropriate cell type (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. Emx2-null neurospheres have an alterated clonogenicity, together with an impaired migration capacity.

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).

5.1. Emx2 downstream transcriptional target genes

In spite of the importance of Emx2 in brain development, one of the major limitation in understanding how this homeodomain protein regulate gene expression during brain development is the knowledge of very few target genes.

One of the direct transcriptional targets of Emx2 during CNS development is the Wnt1 gene. The Wnt1 gene encodes a signaling molecules that plays a crucial role in the establishment of the appropriate boundaries during CNS patterning (Iler et al., 1995). Wnt1 is expressed in the presumptive midbrain, with a rostral limit in the diencephalon, and extends caudally to a region spanning the dorsal midline of the CNS. Its expression is excluded from the telencephalon and cortical hem. 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). In the entire 5.5 Kb Wnt1 3' enhancer, necessary and sufficient for the appropriate expression of Wnt1 (Danielian et al., 1997), a single DNA fragment containing two putative Homeodomain-Binding Sites (HBS1 and HBS2) was identified. Mutation or deletion of HBS1 resulted in the ectopic expression of a reporter transgene in the dorso-medial telencephalon and in cortical hem (Iler et al., 1995; Rowitch et al., 1998). Particularly, strong ectopic expression of Wnt1 (in the same regions) was observed in $Emx2^{-/-}$ embryos. Footprinting assays demonstrated that Emx2 is able to bind the HBS sites in the Wnt1 3' enhancer region (Iler et al., 1995). Taken together, these findings indicate that Emx2 is a direct repressor of Wnt1 in the developing mammalian telencephalon acting via direct binding to HBS 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).

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SCOPE OF THE THESIS

The aims of my PhD research were: to examine molecular mechanisms underlying the transcriptional regulation of the Sox2 gene during forebrain development; to examine the role of Sox2 for the proper neuronal differentiation of neural stem cells; and to examine the role of Sox2 in controlling the maintenance of neural stem cells (in vivo and in vitro).

The aim of the first work (presented in Chapter 1) was to investigate the transcription factors and the regulatory sequences that control transcription of the Sox2 gene in the developing brain and neural stem cells.

Our laboratory previously identified Sox2 regulatory sequences able to drive expression of a reporter β-geo transgene to neural stem cells of the brain in transgenic mice. I focused on two mouse forebrain-specific enhancers able to recapitulate Sox2 telencephalic expression throughout forebrain development, also active in neural stem cells of the adult and embryonic brain (Sox2 5' and 3' enhancers).

The main goal of this study was to identify:

a) regulatory sequences cis-regulating the transcription-regulatory activity of the Sox2 forebrain-specific enhancers
b) regulatory proteins/transcription factors able to trans-regulate Sox2 transcription in the developing brain and in neural stem cells
c) interactions between regulatory proteins/transciption factors with the forebrain-specific regulatory sequences during the development and differentiation of the CNS

This work showed that Emx2 acts as a direct transcriptional repressor of both Sox2 telencephalic enhancers, acting in two different ways to repress their transcriptional activity: by directly binding to a specific site within these regulatory elements, thus preventing the binding of activators, or possibly by protein to protein interaction sequestring the activators, thus antagonizing their activity.

By the study of double mutant mice (expressing reduced levels of Sox2 and Emx2) we further found that Emx2 deficiency counteracts (at least in part) the deleterious 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. It is likely possible that a simultaneous decrease of Emx2 levels (a Sox2 repressor) may antagonize these defects, by restoring Sox2 levels.

In the second line of my research (presented in Chapter 2) we performed in vitro differentiation studies on neural stem cells cultured from embryonic and adult brains of Sox2 "knockdown" mutants (expressing reduced levels of Sox2) where Sox2 deficiency impairs neuronal differentiation.

In particular, my contribution to this work was to evaluate the in vitro differentiation defects of Sox2 mutant neurospheres by immunofluorescence staining for different glial and neuronal markers. Strikingly, I 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.

To evaluate if restoration of Sox2 levels is able to rescue the differentiation defects of mutant cells, I engineered Sox2-expressing lentiviral vector, which I used to infect neural cells at early or late differentiation stages. I found that, Sox2 overexpression is able to rescue the neuronal maturation defects of mutant cells only if administered at early stages of differentiation. Further, I observed that Sox2 suppresses the endogenous GFAP gene, a marker of glial differentiation. These results suggests that Sox2 is required in early in vitro differentiating neuronal cells, for maturation and for suppression of alternative lineage markers.

The third research (presented in Chapter 3) investigated neurogenesis and neural stem cells properties in mice carrying a conditional mutation in the Sox2 gene ($Sox2^{flox}$). Here, Sox2 was deleted via a nestin-Cre transgene that leads to complete Sox2 loss in the central nervous system by 12.5 dpc. These studies showed that embryonic neurogenesis was not importantly defective, however shortly after birth, NSC and neurogenesis are completely lost in the hippocampus. The expression of cytokine-encoding genes, essential

for stem cell niche, is also strongly perturbed and leads to impaired stem cell maintenance (in vivo and in vitro).

In vitro, NSC cultures derived from Sox2-deleted forebrain become rapidly exhausted, losing their proliferation and self-renewal properties. In Sox2-deleted neurospheres, Shh is extremely downregulated. However, the conditioned medium from wild type NSC cultures or the administration of a Shh agonist efficiently rescue the proliferation defects. These results suggest that the effect of Sox2 on neural stem cells growth and maintenance is partially mediated by Shh secretion, and that the Shh gene must be a direct target of Sox2. To confirm this hypothesis, I infected Sox2-deleted NSC with a Sox2-IRES-GFP expressing lentivirus just prior to the beginning of the growh decline, and I observed that the re-expression of Sox2 induces the ability to re-express Shh and rescues the formation of neurosphere. These findings indicate that NSC control their status, at least in part, through non cell-autonomous mechanisms (such as activation of important cytochine-encoding genes) which depend on Sox2.

CHAPTER 1

(submitted to *Stem Cells*)

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

J. Mariani[1], C. Lancini[1], G.Vaccari[2], R. Favaro[1], A. Ferri[1], D. Tonoli[1], E. Latorre[1], S. Ottolenghi[1], S. Miyagi[3], G. Corte[4] , A. Okuda[3] , V. Zappavigna[2] and S.K. Nicolis[1]

[1] Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy
[2] Department of Animal Biology, University of Modena and Reggio Emilia, Via G. Campi 213/d, Modena 41100, Italy
[3] Division of Developmental Biology, Research Center for Genomic Medicine, Saitama Medical School, Saitama 350-1241, Japan
[4] Department of Biology, Biology and Genetics, University of Genova; IST – National Institute for Cancer Research, Genova, Italy

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) of the embryo and adult, 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]. This mutant expresses Sox2 at a level 25-30% that of wild type, and shows loss of hippocampal stem cells, corpus callosum interruption, parenchymal loss in striatum and thalamus, decreased numbers of GABAergic neurons, together with neurological defects, including epilepsy [5, 7]. More recently [10], we showed that Sox2 embryonic deletion leads to complete perinatal loss of hippocampal stem cells. NSC from the forebrain of such mutants become exhausted in in vitro neurosphere culture after few passages.

The Emx2 transcription factor is expressed in the developing dorsal telencephalon, including the prospective hippocampus and cerebral cortex, from early embryogenesis [11, 12]. Its expression is maintained postnatally in adult brain neurogenic regions, the subventricular zone (SVZ) and hippocampus dentate gyrus (DG)[13, 14].

Emx2 inactivation in mouse causes delayed hippocampal development, with reduced cerebral cortex and abnormal specification of cortical areas at birth [reviewed in 12,15-17]. In vitro, mutant

$Emx2^{-/-}$ NSC show increased proliferation in long term neurosphere cultures [14].

Following our initial description of the brain abnormalities of 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, 10, 12, 15]. Moreover, NSC from both $Sox2^{-/-}$ and $Emx2^{-/-}$ mutants exhibit important abnormalities in in vitro culture [10, 14]. 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 $Emx2^{+/-}$ heterozygosis. Loss of a single Emx2 allele significantly ameliorated the brain phenotype of Sox2 hypomorphic mice, suggesting that Emx2 may play antagonistic roles to Sox2.

To study the functional relationships between Emx2 and Sox2, we investigated the possibility that Sox2 might be negatively regulated by Emx2. We report that Emx2 is a direct transcriptional repressor of Sox2. Further, 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 initially 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. Contrary to our expectations, 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 (Supplementary 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 is expressed exclusively in the telencephalon. The SRR2 transgene [18, 19] is driven by the tk-promoter linked to an enhancer normally located immediately 3' to the Sox2 coding region (these mouse lines will be referred to as 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 E15.5 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 brain is rather abnormal in the latter ones, as expected [12]).

We confirmed these results in detail by staining brain sections for beta-galactosidase activity (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, we detected a similarly increased LacZ expression in the 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 "core"elements of the 5'- and 3'-enhancers were defined in vivo by transgenic assays and, in vitro, by transfection in Embryonic Stem (ES) Cells [18-20]. Both core elements contain POU sites, known to be functionally important in ES and brain cells [18-20], which bind specific transcription factors (Oct4 in ES , Brn1 and Brn2 in neural cells) [18-20]. The 5' enhancer was reduced by transgenic experiments to approximately 400 nucleotides , retaining most of the activity of the full enhancer [20]. This enhancer contains, in addition to the two POU sites, several ATTA sites (numbered 1 to 6 in Fig. 2A, and called hereafter ATTA-1 to ATTA-6 sites), 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 [18] (Fig. 2A).

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

Emx2 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 an empty vector gave no significant repression. Similarly, Emx2 strongly repressed the activity of the 3' Sox2 telencephalic enhancer [18, 19], when assayed with both a full size and a "core" enhancer [18] 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 activity of the mutants to the background level corresponding to the activity of the tk-promoter-

luciferase construct (the mutant in ATTA sites1/2/4/5/6 was essentially inactive already in the absence of Emx2, and thus Emx2 did not significantly inhibit it).

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

To understand the mechanisms of the effects described above, we characterized by electrophoretic mobility shift assays (EMSA) the binding of recombinant Emx2 to all of the ATTA sites in the core 5' enhancer. An oligonucleotide including the combined ATTA/POU site (ATTA-3) was previously shown to bind the ES cell factor OCT4 and its homologues Brn1 and Brn2, which are expressed in the nervous system [20, 22].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, Emx2 bound to ATTA-3, generating 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).

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 other neural transcription factors. We [18, 19, 20] previously demonstrated the binding of the POU factor Brn2 to POU sites in the 5' and 3' Sox2 enhancers.

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 mechanisms (possibly protein to protein interaction, 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 [20], and other sequences containing an ATTA motif bind Brn1 and Brn2 [25, 26, 27] (see Fig. 3A), we tested all ATTA sites in the 5' enhancer for binding to these transcription factors, and for interactions with Emx2. 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 an anti-Brn2 antibody (lanes 3,4), which confirms its identity. Finally, the addition of excess unlabeled ATTA-1/2 oligonucleotide competed the binding of the previously validated Brn2-binding site, ATTA-3 in the 5' enhancer ([20] and present paper) as efficiently as the 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, and data not shown).

In additional control 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 in the Nestin gene neural enhancers [25, 27].

These experiments demonstrate that Emx2 is able to prevent the binding of transcription factors (in this case Brn2) to their cognate motifs via mechanisms independent of Emx2 binding to DNA.

A possible mechanism for Emx2-dependent repression of Brn2 binding to the DNA 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. 4D). 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 [20] or of the POU site in the 3' enhancer [18,19] 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 test for 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 and data not shown). The Brn2-dependent stimulation of the ATTA-1/2 construct was efficiently repressed to basal levels (i.e. just above the level of the tk-luc reporter, compare lane 9 to 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, [20]) 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 $Sox2^{\beta\text{-geo}/\Delta Enh}$ mutant [5, 7], that expresses Sox2 (from the single residual knock-down allele) in brain at about 25-30% of wild type 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 a 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 [28]).

To interpret this result, we examined Sox2 expression in wild type mice in the prospective hippocampal area during development. 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. 7C). On the other hand, the Emx2 level was higher [12, 16] in the medial as compared to the lateral wall, pointing to an inverse relation between Sox2 and Emx2 expression. Consistent with this interpretation, we noted an abrupt drop of Emx2 expression in the lower part of the medial wall, where Sox2 is concomitantly upregulated (Fig. 7C, asterisk).

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. 7C). 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 the growth of the hippocampus 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 [16, 17, 23, 24, 29-31]. We report in vivo and in vitro data showing 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 expression along the neural tube is regulated by a multiplicity of different enhancers, active at specific locations [2, 5, 18-20, 32]. In mouse, the best characterized enhancers are the 5' and 3' Sox2 enhancers employed in these studies [2, 18-20, 33]. Both enhancers direct transgenic reporter gene expression exclusively 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 [17, 20, 28, 29].

At the cellular level, there is considerable overlap of Sox2 and Emx2 expression domains within neuroepitelial cells of the ventricular zone [5, 28]. 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. 7C). Coexpression of Sox2 and Emx2 is also observed in a small number of adult hippocampal cells (Supplementary Fig. 2).

Here, we showed that loss of either one or both copies of the Emx2 gene 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 [18, 19]), within the full Sox2 locus. We propose that this effect of Emx2 deficiency depends on direct effects of Emx2 on Sox2 regulatory regions.

Based on EMSA and on transfection data, Emx2 appears to act in two different ways to repress Sox2 enhancer activity (Figs. 3-5).

First, it can directly bind to characterised 5' (ATTA-3) and 3' enhancer sites (ATTA-4) (Fig. 3); the nucleotide sequence of these sites bears similarity to the sequence in the Wnt1 gene through which Emx2 directly represses Wnt1 expression in the developing telencephalon (Fig. 3A; see [24]). The sites in the Sox2 enhancers are bound by the POU factors Brn1 and Brn2 ([20] and Fig. 3), two factors that were previously implicated in Sox2 regulation on the basis of transfection, transgenic and ChIP experiments [18-20]. 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 gel shift 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.

The second mechanism whereby Emx2 may repress the Sox2 enhancers is by antagonizing the binding to DNA of activator transcription factors, likely through protein to protein interaction, without directly binding to the DNA. In fact, the in vitro binding of Brn2 to ATTA-sites in Sox2 enhancers and to other previously described and validated Brn2 sites [20, 25, 27] is prevented by Emx2 addition, in the absence of any binding of Emx2 itself to the same sequences (Fig. 4). This suggests that Emx2 might be able to antagonize Brn2 by sequestering it, thus 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 [30]; moreover, Emx2 and SP8 proteins physically interact [31]. 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 [21]. 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 [20].

In conclusion, we propose that Emx2 contributes to the regulation of Sox2 expression by antagonizing activators, such as Brn2 (and possibly other factors able to bind the ATTA core sequence, [21]). 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 both Sox2 and Emx2 genes have important functions in this brain region [5, 10, 12]. This indicates that homozygous mutation of Sox2 or Emx2 may cause the loss of separate, and essential, functions in hippocampal development. In addition to its essential role in hippocampal development, Emx2 has an antagonistc function 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 [10]. 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, 34] in the hippocampus is importantly decreased ([5] and Fig. 7, present paper).

Our present experiments show that loss of a single Emx2 allele (that, by itself, has little phenotypic effects [12, 16, 29]) 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 [28]) This result 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 [14] 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 [14]. Thus, the decision between self-renewal (which requires maintenance of adequate Sox2 levels, [10] 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 development of the hippocampus, together with the significant decrease in cortex growth and patterning defects in Emx2 homozygous mutants [16, 29] are the result of complex mechanisms. Although a direct patterning activity of Emx2 was demonstrated by transgenic Emx2 overexpression [35], 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 [16, 28, 36].

The identification of Sox2 as a potential target of Emx2 repressive action, together with strong evidence that Sox2 controls NSC maintenance, suggests the speculation that Emx2 gradients might affect Sox2 levels in different developing cortical regions, thus helping control the balance between self-renewal and commitment to differentiation of stem cells. In this work, 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 deficiency of Emx2 in relation to Sox2-dependent phenotypes.

Materials and Methods

Mouse lines and immmunohistochemistry

For the 5' and 3' enhancer-reporter mice see refs. 2, 18-20. The Sox2-hypomorphic (Sox2 $^{\Delta Enh}$) and null (Sox2 $^{\beta\text{-geo}}$) mutant alleles were as in [5]. The Emx2 null mutant mouse was described in [12].

X-gal staining, GFAP/nestin and BrdU immunohistochemistry (IHC) and histological analyses were as reported [5]. IHC with anti-Emx2 antibodies was as described [13].

Luciferase reporter constructs and transfection assays

The 400 bp Sox2 5' telencephalic enhancer core region was PCR amplified from the 0.4a-Sox2 promoter-βgeo vector [20] and cloned into the pGL3-based luciferase reporter plasmid, upstream to a 215 bp minimal tk promoter (5' enh-tk-luc). Mutated enhancer versions were

obtained by PCR-mutagenesis. Luciferase reporters for 3' enhancer activity were described [18, 19]; their core sequence was as in [38], 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 (in pSG5), Brn2, GATA1 and GATA2 (in pBluescript) were produced using in vitro transcription-translation reticulocyte lysate system (TNT, Promega). For GST-pull-down experiments, Emx2 (or CP2 control, [39]) cDNAs, cloned in pGEX2T, were expressed in *Escherichia coli* BL21 strain cells, purified and quantitated. 1 μg of total protein (GST-Emx2, GST-CP2 and GST-only resins) was used for GST-pulldown of ^{35}S Brn2-containing TNT reaction as in [39].

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

EMSA was essentially as in ref. 40 using in vitro transcribed/translated proteins; ChIP was as described in [6].

Online Methods

Mouse lines, X-gal staining and immmunohistochemistry

The 5' and 3' enhancer-βgeo transgenic mice lines, and X-gal staining protocols, were described in [2, 18-20]. The Sox2-hypomorphic (Sox2 $^{\Delta Enh}$) and null (Sox2 $^{\beta\text{-geo}}$) mutant alleles were as in [5]. The Emx2 null mutant mice (kindly provided by A. Mallamaci) were described in [12].

GFAP/nestin and BrdU immunohistochemistry (IHC) on hippocampus and all histological analyses were carried out as previously reported [5]. IHC with anti-Emx2 antibodies was as described [13].

Experimental procedures involving animals were approved by the Italian Ministry of Health.

Luciferase reporter constructs

The Sox2 5' telencephalic enhancer core region of 400 bp was PCR amplified from the 0.4a-Sox2 promoter-βgeo vector [20] using the following primers:

Fw: 5' CGAGGTACCGTCAAATAGGGCCCTTTTCAG 3'

Rv: 5' TATCTCGAGAAGCCAACTGACAATGTTGTGG 3'

containing a KpnI and XhoI restriction sites (underlined), for further cloning into the pGL3-based plasmid containing a 215 bp minimal tk promoter (a gift of Prof. Hitoshi Niwa) (5' enh-tk-luc).

The reporter plasmid carrying mutations in the ATTA-3 site (ATTA-3 site mut) was obtained as above, starting from 0.4a-MUT Sox2 promoter-βgeo vector [20]. All other ATTA-site mut reporter constructs were similarly obtained by PCR-based site-directed mutagenesis.

Primers for the ATTA-1/2 site mut plasmid:

Fw:

5'CGAGGTACCGTCAAATAGGGCCCTTTTCAGATTTTA**AGGA** CAAAATAAA**AGGA**G TCTGCTC 3'

Rv:

5'TATCTCGAGAAGCCAACTGACAATGTTGTGG 3' containing the desidered mutations (in bold) and KpnI/XhoI restriction sites (underlined).

The ATTA-4 site mut plasmid was generated by replacing a PstI cassette of the 5' enh-tk-luc with a corresponding cassette, containing the desidered mutation (in bold), obtained after amplification with the following primers:

Fw:

5'ACTCTGCAGGTCCCCTGCCGTTCGCCTTCATTTCCATA**AG GA**G AGGAGGAGAGG AGG 3'

Rv:

5' CGGGTCGCTGCAGGGTCGCTCGGTGTTCG 3'

PstI restriction site (underlined) in both primers.

The ATTA-5/6 sites mut plasmid was generated using two overlapping primers containing the desidered mutations (in bold) to separately amplify the 5'- and 3'-portions of the 5' enhancer, in conjunction with external primers flanking the KpnI and XhoI sites of the 5' enh-tk-luc vector. The full mutated enhancer was obtained by reamplification of the obtained fragments with the same external 5' enh-tk-luc primers. The sequences of the primers used are:

Fw1:
5’GCATCAACCTAGTAAGATGCTTGGCTAGTTCTCGCTA**AGG**
TCTGCAAC 3’
Rv1: (XhoI-external primer):
5’TATCTCGAGAAGCCAACTGACAATGTTGTGG 3’
Fw2: (KpnI-external primer):
5’CGAGGTACCGTCAAATAGGGCCCTTTTCAG 3’
Rv2:
5’GTTGCAGACCTTAGCGAGAACTAGCCAAGCATCTTACT**AG**
GTTGATGC 3’

The reporter plasmid carrying mutations in five ATTA-sites was obtained by combining the mutations via PCR.

For constructing the 3X POU/ATTA site 3 plasmid, the combined POU/ATTA site (in bold type caracters) was multimerized to tree copies, and subcloned into the KpnI/XhoI site of the pGl3-tk luciferase vector, using the following primers:
Fw:
5’CACTGC**TAATTAGCAAT**GCTAGGGTGC**TAATTAGCAAT**G
CTAGGGTGC**TAATTAGCAAT**GCTAGC 3’
Rv:
5’TCGAGCTAGC**ATTGCTAATTA**GCACCCTAGC**ATTGCTAA**
TTAGCACCCTAGC**ATTGCTAATTA**GCAGTGGTAC 3’
For constructing the 2X ATTA site 1,2 plasmid, the ATTA site 1,2 core sequence, 5’TTA**ATTA**CAAAATAAA**ATTA**GTCTGCTCTTC 3’, was dimerized (as a synthetic oligonucleotide) and subcloned into the KpnI/XhoI site of the pGL3-tk luciferase vector.

The Luciferase reporter vectors bearing BamHI/SalI genomic DNA fragments of the 3’ enhancer were described [18, 19]; their core sequence was essentially as in [38], Fig 3:
5’GGATCCCTA**ATTA**ATGCAGAGACTCTAAAAGAATTTCCCG
GGCTCGGGCAGCCATTGTGATGCATATAGG**ATTA**TTCACGT
GG**TAAT**GAGCACAGTCGAC 3’

These fragments were subcloned into the BamHI/SalI site located 3’ to the Luciferase gene.

P19 transfection assays

For transfection experiments, P19 cells were grown in MEM-ALPHA medium supplemented with PenStrep, L-glutamine and 10%

fetal bovine serum. $2x10^5$ /well exponentially growing P19 cells were plated in 6-well-plates, and transfected the following day with Lipofectamine 2000 (Invitrogen) according to supplier's instructions. Briefly, medium in each well was replaced with 1ml of Opti-MEM medium (Invitrogen) with 10μl of Lipofectamine 2000 (Invitrogen), with DNA. For experiments in Fig. 2, we used 1μg of the luciferase reporter plasmid, and increasing amounts (from 10 ng to 750 ng) of the Emx2 expression vector Emx2 (pCAGGS-Emx2, a gift from V. Broccoli) per well, as indicated (Fig. 2B,C,E: 500 ng Emx2 vector; Fig. 2D: as indicated). In control experiments, equimolar amounts of the Emx2 "empty" vector (pCAGGs) or the Otx2 expression vector (pCAGGS-Otx2, a gift from V. Broccoli), were used. The pBluescript vector was added to each transfection to equalize the total amount of transfected DNA to 2 μg total in each reaction. After 24h, total cellular extracts were prepared and Luciferase activity was measured according to the Promega Luciferase reporter system protocol.

For cotransfection experiments with Brn2 and Emx2 expression vectors, Brn2 expression vector (gift from D. Mejiers) (or the "empty" control) was transfected in increasing amounts in the experiment in Fig. 6A (+, ++, +++: 125, 500, 1000 ng); in Fig. 6B and 6C, Brn2 was added at the fixed amount of 500ng/transfection, and increasing amounts of pCAGGS-Emx2 expression vector were added (+, ++,+++,++++: 100, 200, 500,1000 ng). The "empty" vector was added to each transfection at the proper concentration to equalize the total amount of DNA transfected in each reaction to 2 μg.

Recombinant protein expression and purification

Recombinant Emx2 (in pSG5), Brn2, GATA1 and GATA2 (in pBluescript) were produced using in vitro transcription-translation reticulocyte lysate system (TNT, Promega), according to the manufacturer's indication, in a total volume of 50 μl for 1.5 hours at 30°C, using 2 μg plasmid template, and then frozen at -80°C. Amounts of TNT reaction used are indicated in Figure legends. In Fig. 4A, to use equivalent amounts of in vitro-synthethized proteins, TNT reactions were performed in the presence of ^{35}S methionine, the amounts of protein produced (Brn2, Emx2, GATA1 or GATA2) were estimated by autoradiography of western blot, normalized for the numbers of methonines in each protein, and equivalent amounts of each recombinant protein were used.

The Emx2 (or CP2 control, [39]) cDNAs were cloned in frame into the pGEX2T vector. The *Escherichia coli* BL21 strain cells were trasformed with the above plasmid and cultures were grown at midlogaritmic phase (0.6 A_{600}). Protein expression was induced with 0.1mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 37°C. The GST-EMX2 protein present in the soluble fraction was bound to GST-Sepharose 4B (Amersham Bioscience) and purified according to the manufacturer's instructions.

Protein was eluted from sepharose, quantitated by Coomassie blue staining in comparison to BSA standards, and 1 μg of total protein (for GST-Emx2, GST-CP2 and GST-only resins) was used for GST-pulldown of ^{35}S Brn2-containing TNT reaction as in [39].

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

EMSA was performed [40] by preincubating in vitro transcribed/translated proteins for 30 min on ice in 20μl of binding buffer (75 mM NaCl, 20% Ficoll, 10mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10 mM DTT, 1μg of poly(dI-dC), together with 2μl ($5x10^4$ cpm) of ^{32}P-end-labeled oligonucleotide probes. The incubation mixture was resolved by electrophoresis on a 5 or 6% polyacrylamide gel (29:1, acrylamide/bisacrylamide ratio) in 50mM Tris borate, 1mM EDTA, pH 8.2 (0.5XTBE) buffer run at 4°C at 150 V for 3h. Gel were dried and exposed to a Kodak X-AR film at -80°C. For "supershift" reactions, 1μl of the 1:10 diluted mouse α-Emx2 antibody (mouse ascites, kindly provided by F. Mavilio) or 8μl of the goat α-Brn2 antibody (undiluted) (Santa Cruz Biotechnology) were added to the complete binding reaction just prior to the addition of the labeled probe. Unlabeled competitor oligonucleotides in Fig. 4B were added in a 25-fold molar excess.

The following double-stranded oligonucleotides were used as probes for EMSA (only the top strand is shown) (compare to sequences in Fig. 3A) (underlined sequences correspond to mutated nucleotides):
ATTA-site 3:
5'-TCGTCAAACTCTGCTAA**ATTA**GCAATGCTGAGAAA-3';
ATTA-site 3 mut1:
5'-TCGTCAAACTCTGC<u>ATCC</u>T<u>T</u>GCA<u>GA</u>GCTGAGAAA-3';
ATTA-site 3 mut2:

5’-TCGTCAAACTCTGCTACGGCGCAATGCTGAGAAA-3’;
3’-Enh:
5’-GGCAGGTTCCCCTCTAATTAATGCAGAGACTC-3’;
ATTA-1/2 sites:
5’-GGGCCCTTTTCAGATTTTAATTACAAAATAAAATTA
GTCTGCTCTTCCTCGG-3’;
ATTA-1/2 sites mut:
5’-GGGCCCTTTTCAGATTTTAAGGACAAAATAAAAGGA
GTCTGCTCTTCCTCGG-3’;
Delta1-Enh:
5’-AGAGAGCAGGTGCTGTCTGCATTACCATACAGCTGA
GCGC-3’;
Nestin-Enh:
5’-GTGTGGACAAAAGGCAATAATTAGCATGAGAATCGG
CCTC-3’.
ChIP was as described in [6].

Figures

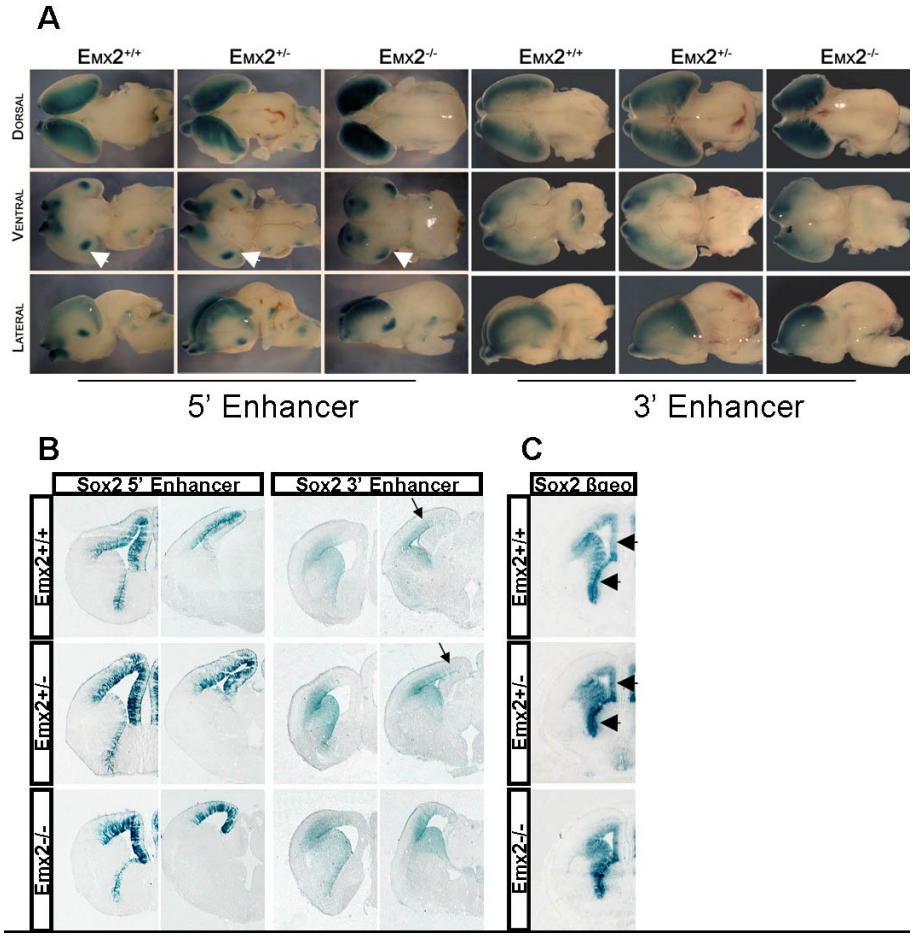


Figure 1 – Emx2 deficiency increases activity of Sox2 telencephalic enhancers-driven lacZ transgenes. (**A**) X-gal stained E15.5 brains carrying beta-geo transgenes driven by the 5' Sox2 telencephalic enhancer (left) or by the 3' enhancer (right), of $Emx2^{+/+}$, $Emx2^{+/-}$, or $Emx2^{-/-}$ genotype, 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 $Emx2^{+/+}$.

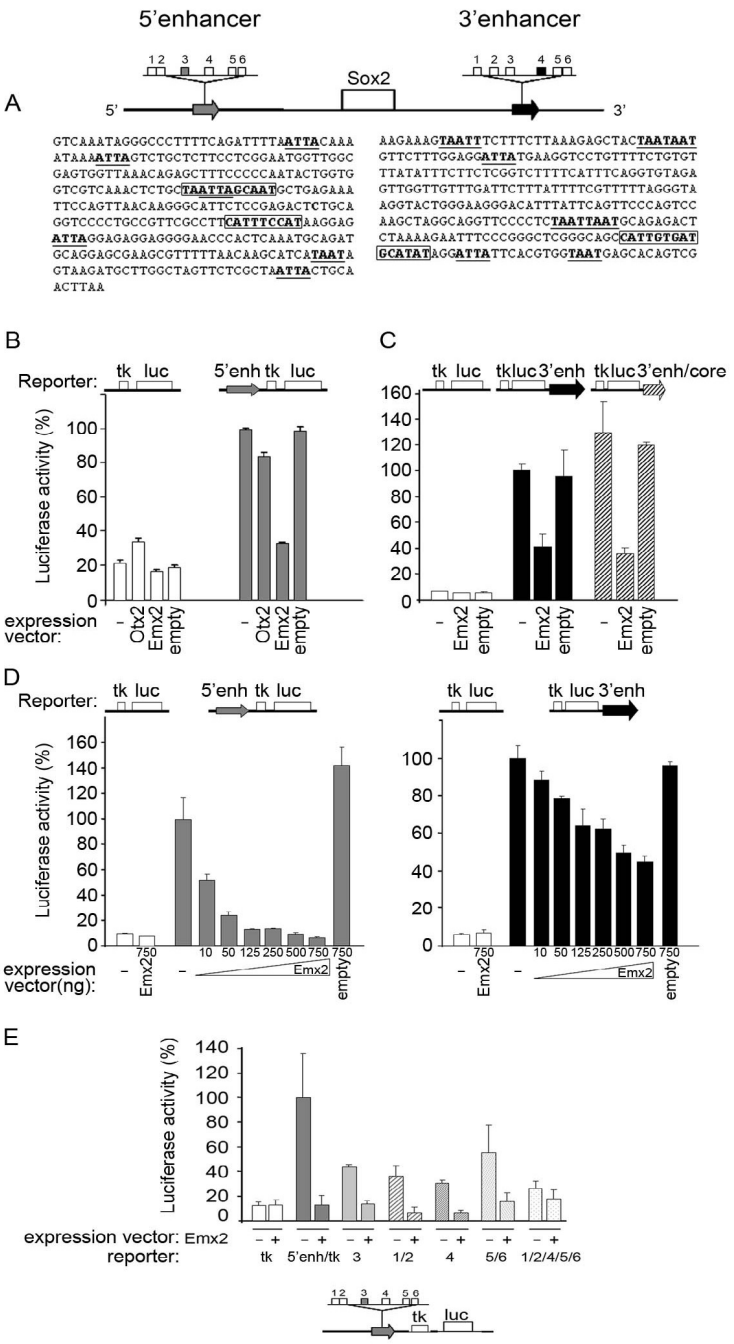


Figure 2 – Emx2 represses the activity of the 5' and 3' Sox2 telencephalic enhancers in transfection assays. (**A**) 5' and 3' Sox2 telencephalic enhancers. Numbered squares: ATTA sites, underlined and bold in the sequences below. Boxed

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).

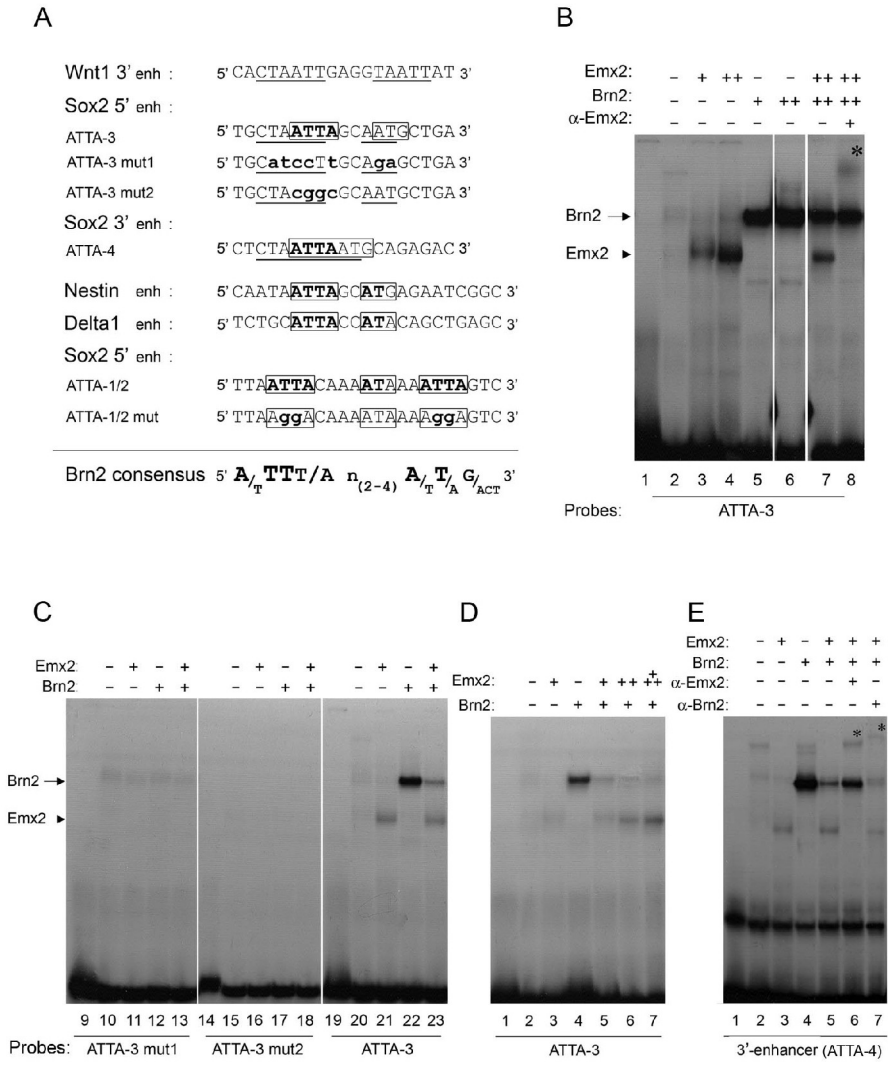


Figure 3 – Emx2 binds to ATTA sites within the Sox2 5' and 3' enhancers, and antagonizes binding of the activator Brn2. (**A**) ATTA sequences binding Emx2 and/or Brn2. Lowermost line: Brn2/POU consensus based on TFBS cluster and our data. Letter size is proportional to nucleotide frequency. The spacer (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).

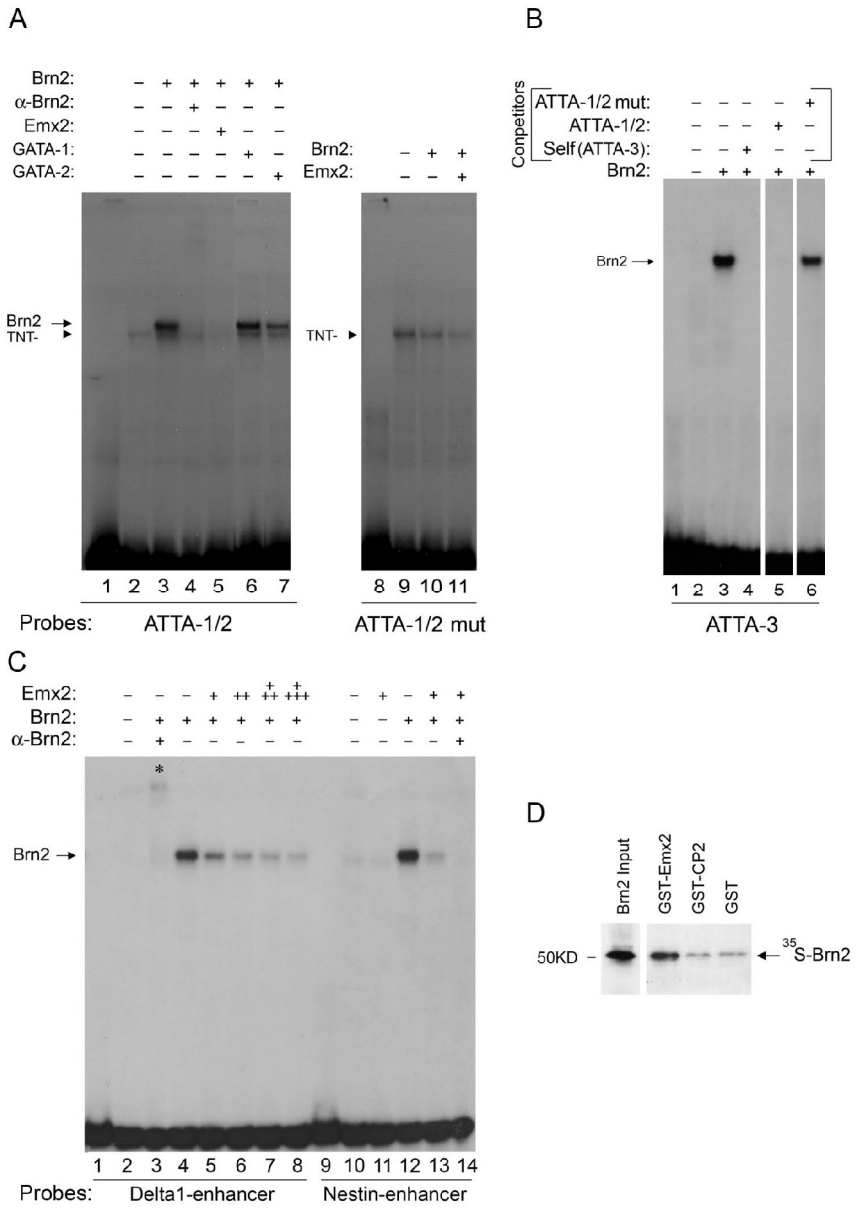


Figure 4 - Emx2 antagonizes the binding of Brn2 to ATTA-1/2 sites in the 5' enhancer, and to previously characterized Brn2 binding sites in other neural enhancers. (**A**) EMSA with a probe containing ATTA sites 1 and 2 (5' enhancer); added recombinant proteins, and Brn2 antibody, are indicated above 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 [18-20]; 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)** 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).

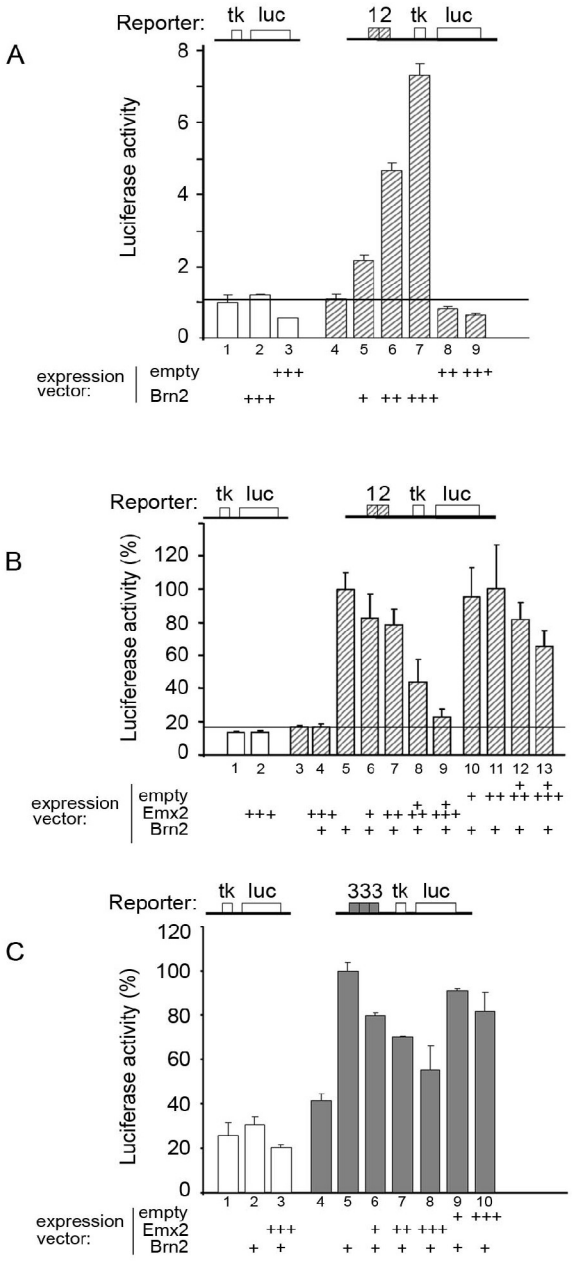


Figure 5 – Emx2 represses Brn2-transactivated ATTA-1/2 and ATTA-3 sites – tk luciferase reporter constructs in a dose-dependent way. (**A**) Brn2 dose-dependent transactivation of ATTA-1/2 sites (5' enhancer). (**B,C**) Emx2 dose-dependent

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.

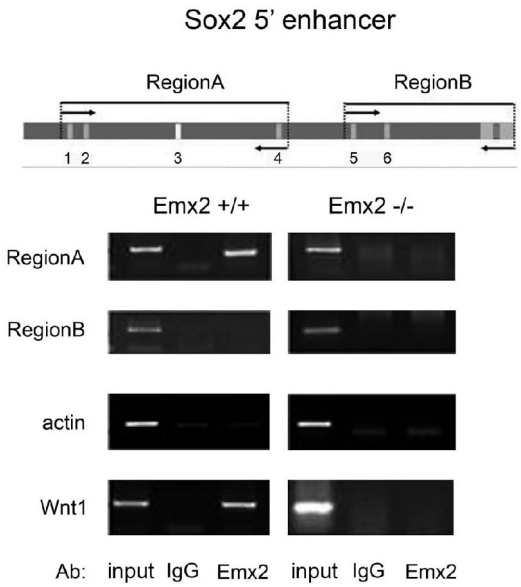


Figure 6 – Emx2 is bound to the Sox2 enhancer in vivo. ChIP with anti-Emx2 antibodies of E14.5 embryonic brain chromatin from wild type and $Emx2^{-/-}$ control embryos. Region A, containing ATTA-3 site is immunoprecipitated from wild type, but not Emx2-null chromatin. The previously described Wnt1 enhancer 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.

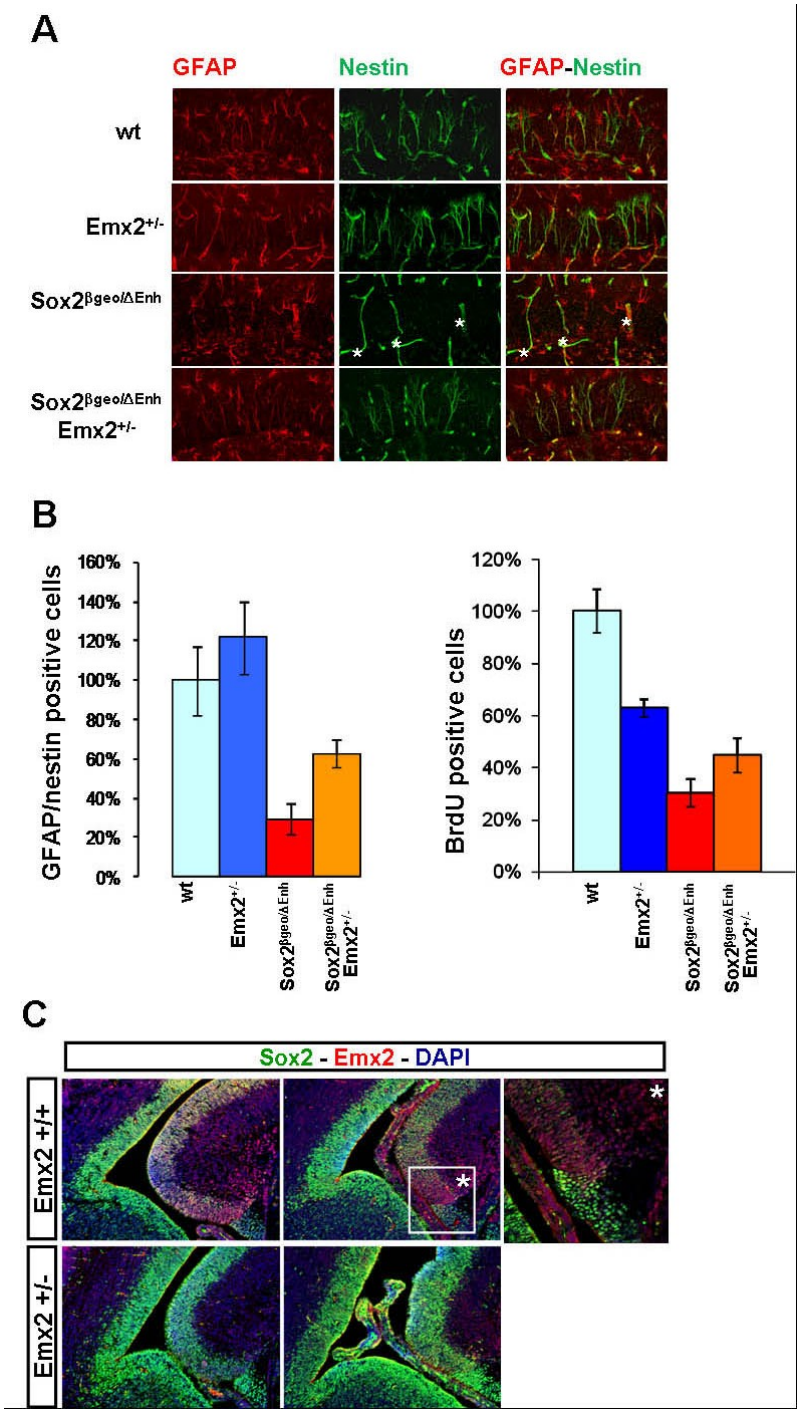
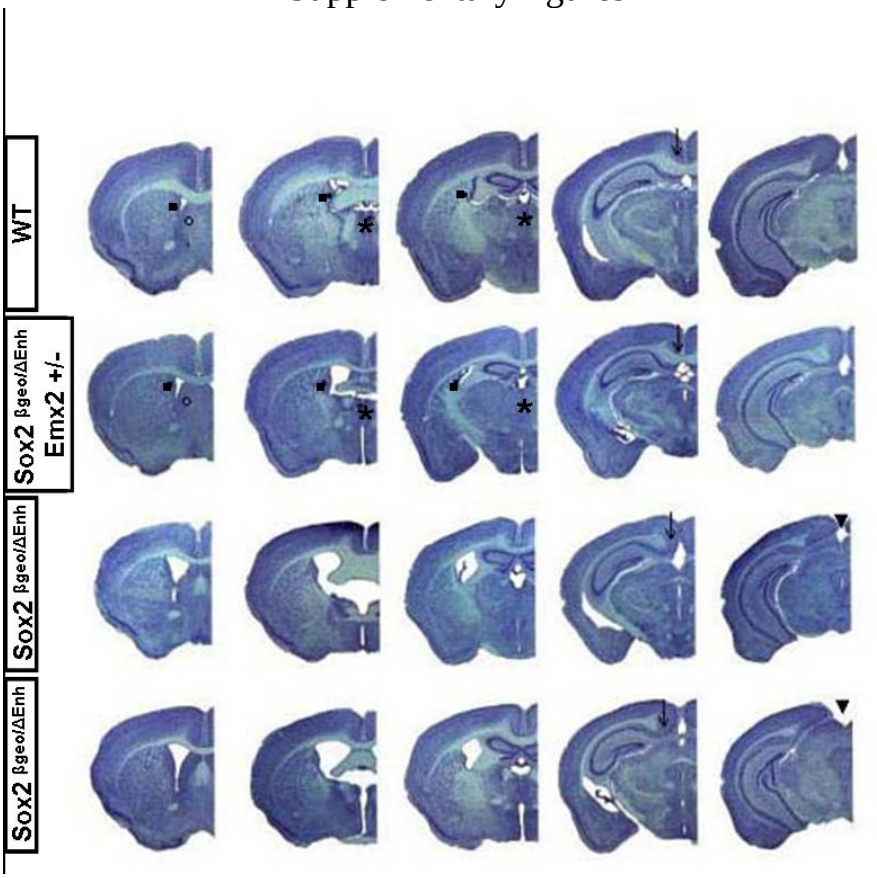


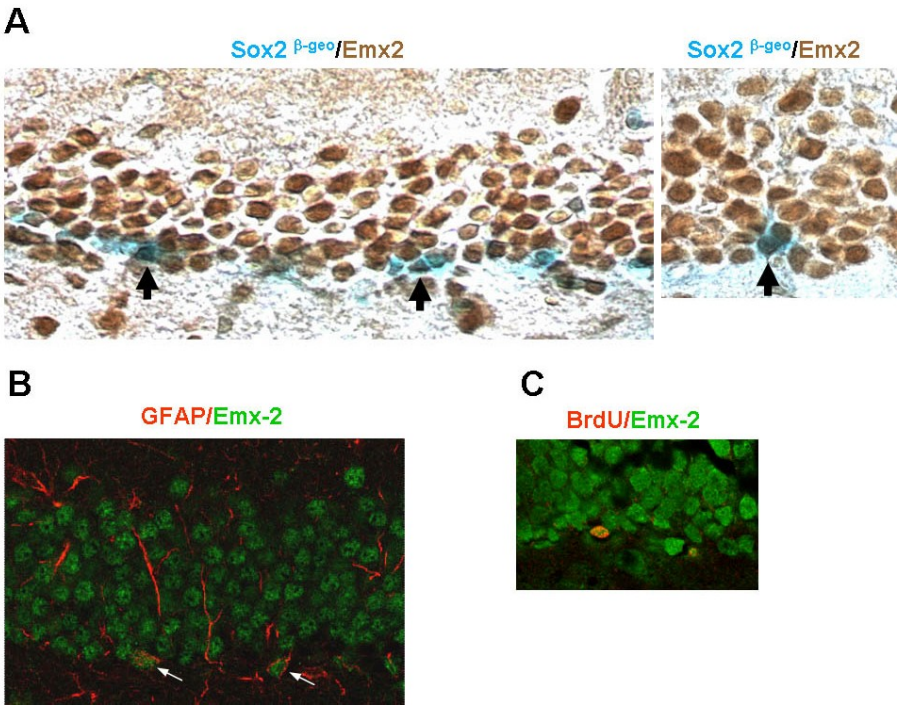
Figure 7 – Emx2 deficiency ($Emx2^{+/-}$) rescues GFAP/nestin stem cells impairment in the hippocampus of Sox2-deficient ($Sox2^{\beta\text{-geo}/\Delta Enh}$) mutant mice. (**A**) GFAP/nestin

double immunofluorescence of hippocampus dentate gyrus in the indicated genotypes. GFAP/nestin-positive cells, strongly depleted in Sox2-hypomorphic ($Sox2^{\beta\text{-}geo/\Delta Enh}$) mutants, recover to a significant extent in $Sox2^{\beta\text{-}geo/\Delta Enh}$;$Emx2^{+/-}$ 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**) 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. The panel marked by asterisk, a higher magnification of the wild type region of the medial telencephalic wall (boxed), highlights a region of Sox2 and Emx2 expression in cell nuclei of the ventricular zone showing the abrupt transition between a Sox2 high-Emx2 low and a Emx2 low-Sox2 high region.

Supplementary figures



Supplementary Figure 1 – Emx2 deficiency significantly rescues the brain morphological defects seen in $Sox2^{\beta\text{-geo}/\Delta Enh}$ 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 – (**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].

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CHAPTER 2

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

Maurizio Cavallaro[1#], Jessica Mariani[1#], Cesare Lancini[1#], Elisa Latorre[1], Roberta Caccia[1], Francesca Gullo[1], Menella Valotta[1], Silvia DeBiasi[2], Laura Spinardi[1,3], Antonella Ronchi[1], Enzo Wanke[1], Silvia Brunelli[4,5], Rebecca Favaro[1], Sergio Ottolenghi[1] and Silvia K. Nicolis[1]

[1]Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, piazza della Scienza 2, 20126 Milano, Italy
[2]Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, via Celoria 26, 20133 Milano, Italy
[3]Direzione Scientifica Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Via Francesco Sforza 28, 20122 Milano, Italy
[4]Dip. di Medicina Sperimentale, Facoltà di Medicina, Università degli Studi di Milano-Bicocca, Via Cadore, 48 - 20052 Monza, Italy
[5]Stem Cell Research Institute, DIBIT H San Raffaele, Via Olgettina 58, 20132 Milano,
Italy
[#]These authors contributed equally to this work

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.

Acknowledgements

We thank Akihiko Okuda for the TK-luciferase vector, Anna Ferri and Valentina Tosetti for help with experiments, and Daniela Santoni for animal care. This work was supported by grants from Telethon (GGP05122), Fondazione Cariplo (2004-1503 and NOBEL) and MIUR (Cofin 2005; FAR 2004-6) to S.K.N.

Figures

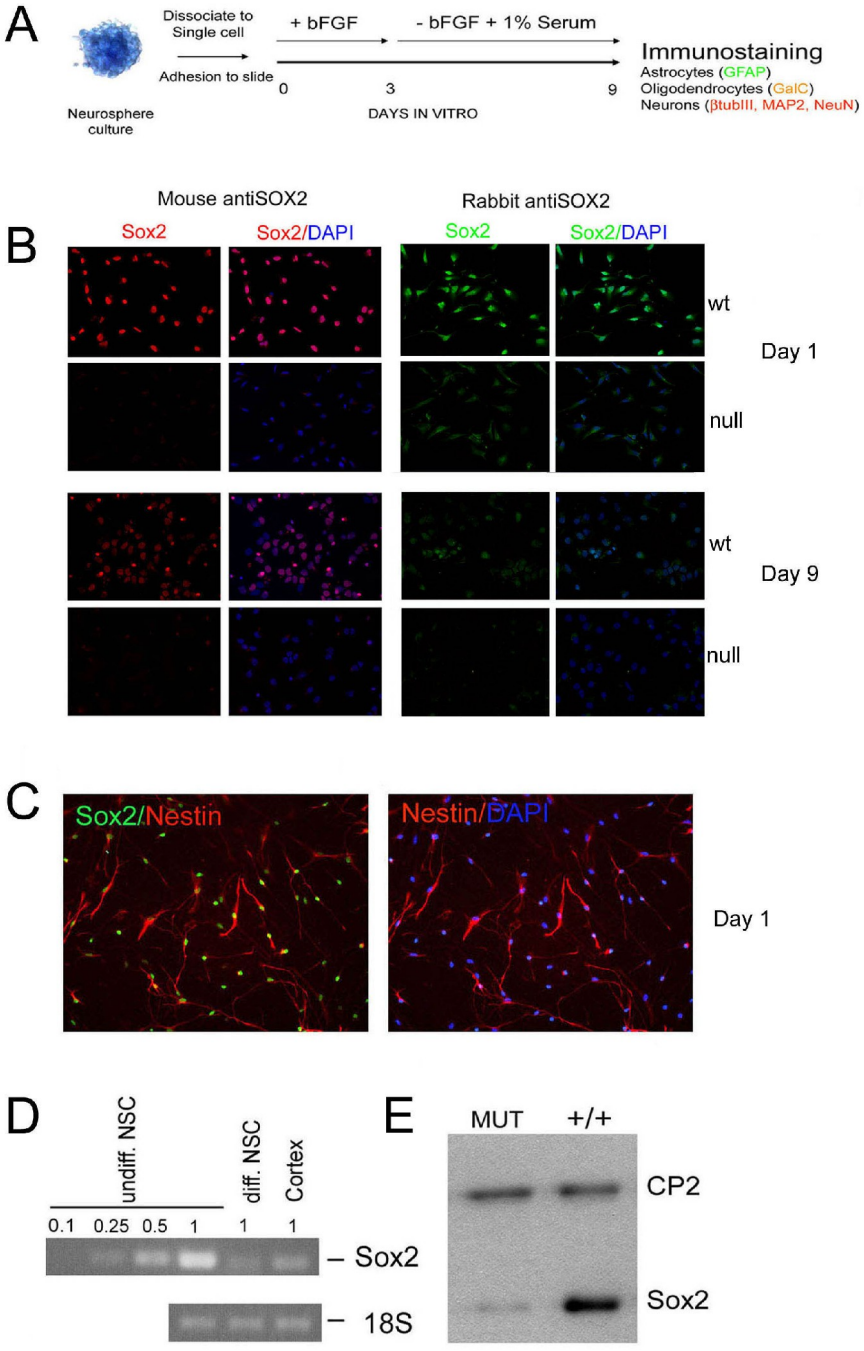


Figure 1 – Sox2 expression during in vitro neural stem cell differentiation. (**A**) In vitro neural stem cell differentiation scheme. (**B**) Specificity of the anti-Sox2

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.

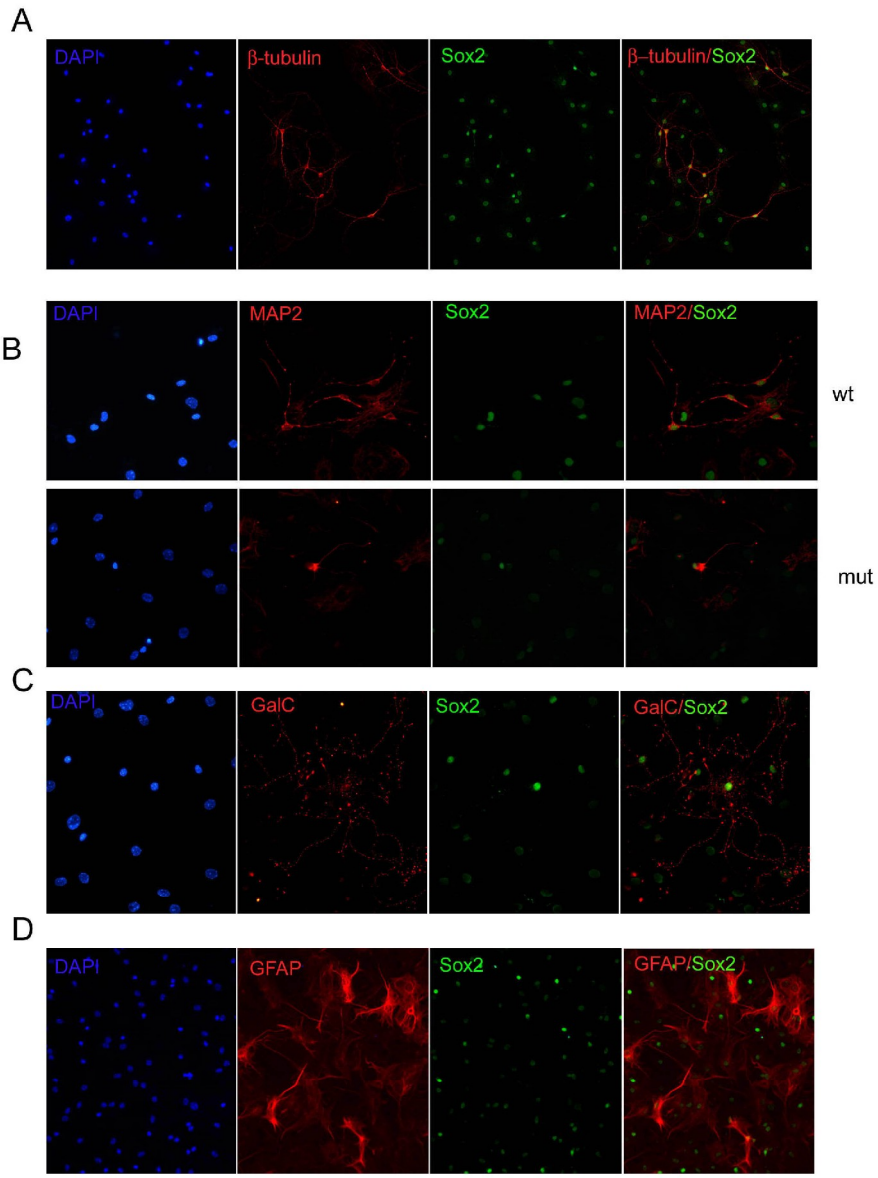


Figure 2 – Immunofluorescence for Sox2, neuronal and glial markers at differentiation day 9. (**A**) Sox2 and β-tubulin in normal cells. β-Tubulin-expressing cells show relatively high Sox2 positivity. (**B**) Sox2 and MAP2. Top: normal; bottom: mutant. MAP2-positive cells show significant Sox2 levels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

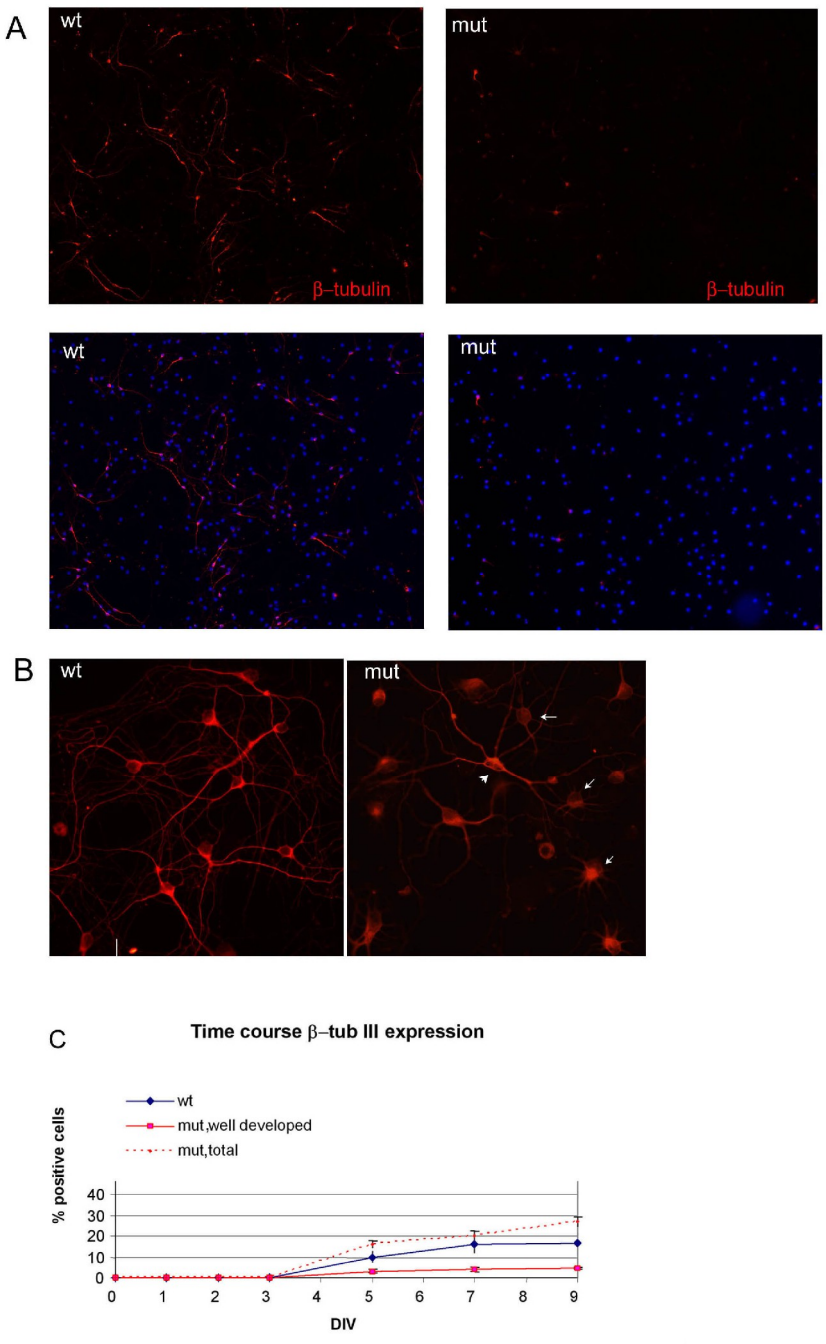


Figure 3 – β-Tubulin-positive cells are abnormal in differentiated Sox2 mutant cell cultures from adult mouse. (**A**) β-Tubulin immunofluorescence of normal (left) and mutant (right) day 9-differentiated cells. Bottom: DAPI. Many of the mutant poorly

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.

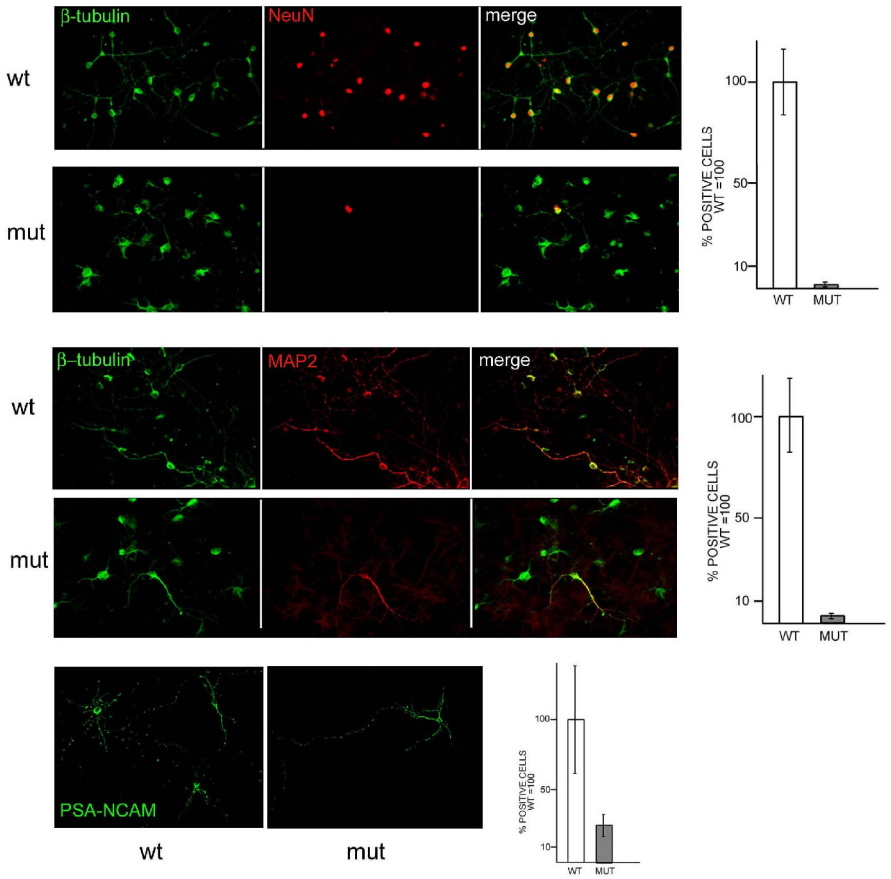


Figure 4 – Cells expressing mature neuronal markers are very reduced in differentiated Sox2 mutant cultures. Neuronal markers in normal and mutant cells at differentiation day 9 (NeuN/β-tubulin, rows 1, 2; MAP2/β-tubulin, rows 3, 4; PSA-NCAM, row 5). Most β-tubulin-positive cells in normal are positive 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).

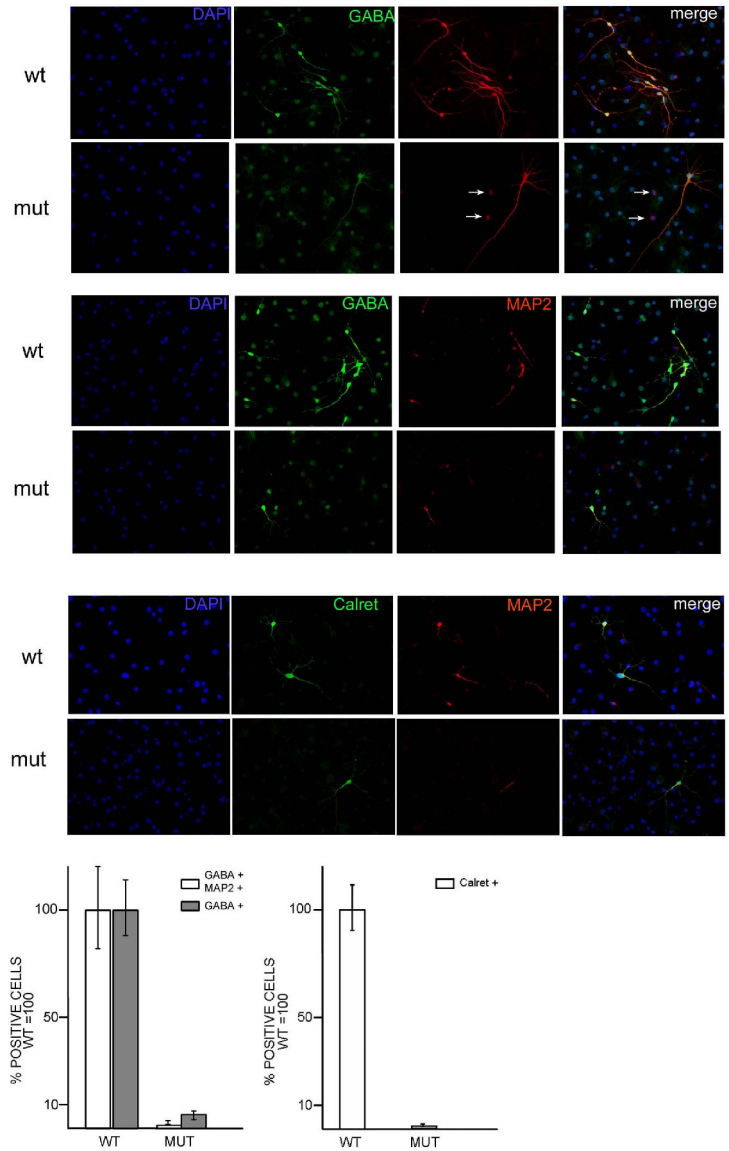


Figure 5 – Cells expressing GABAergic markers are very reduced in differentiated Sox2 mutant cultures. Double-immunofluorescence with general neuronal markers (β-tubulin, rows 1, 2; MAP2, rows 3, 6; red), GABA (rows 1-4) and calretinin (5-6), in normal and mutant day 9-differentiated cultures. Histograms: 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).

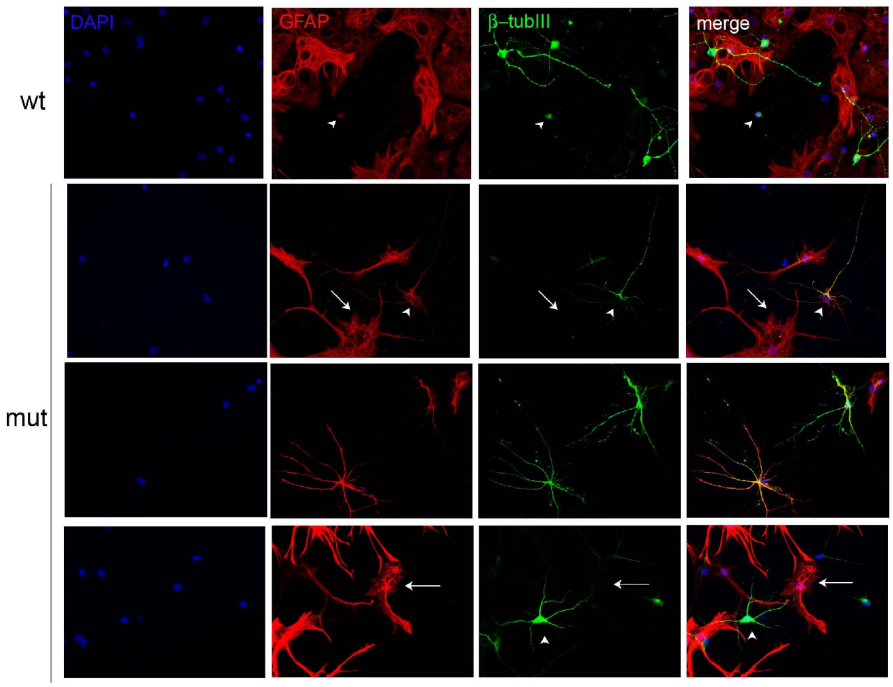


Figure 6 Co-expression of neuronal and glial markers in individual cells in Sox2 mutant cultures. Double-immunofluorescence (β-tubulin and GFAP) of normal (wt) and mutant (mut) day 9-differentiated cells. Typical wild-type neurons (β-tubulin positive) show extensive arborization, are closely associated 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.

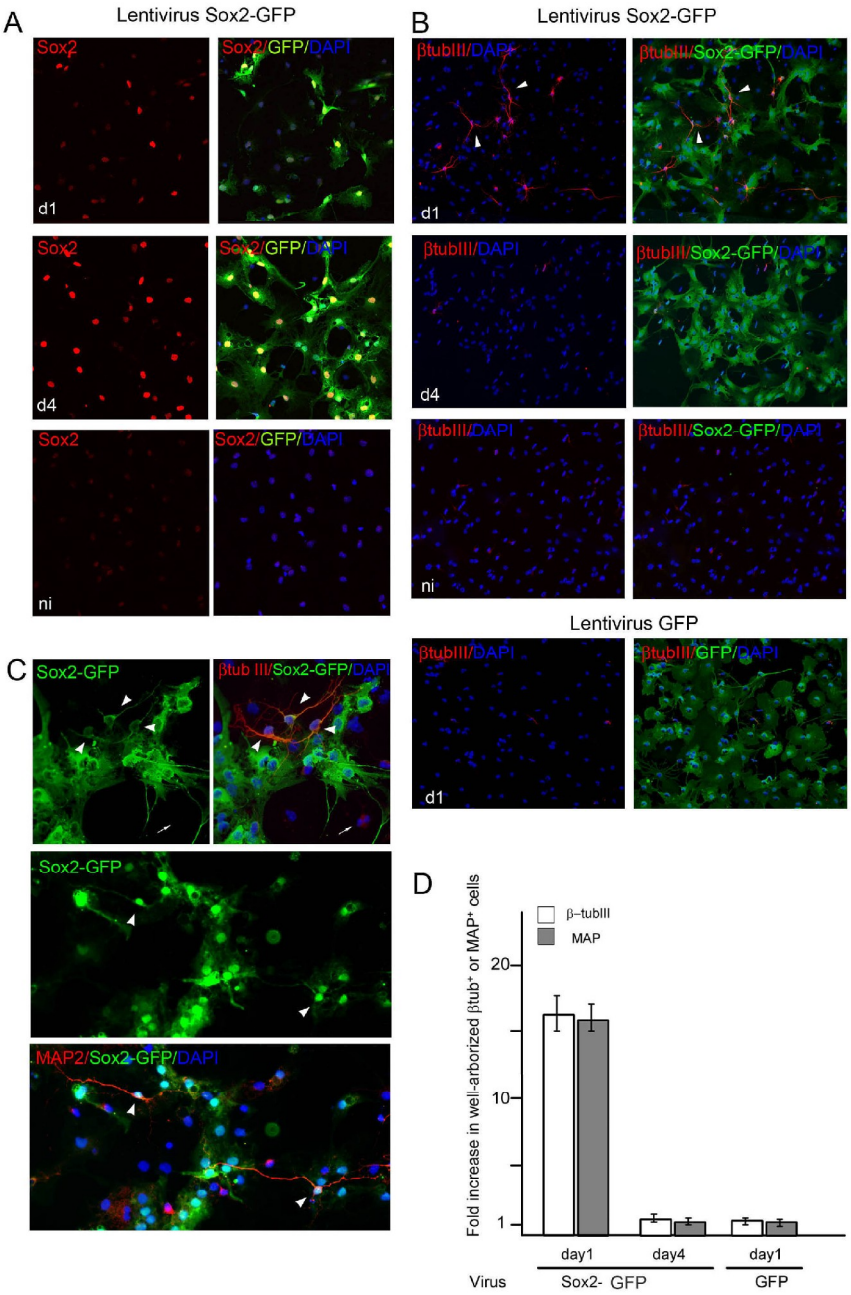


Figure 7 – Rescue of neuronal maturation in mutant cells by lentiviral Sox2 expression at early stages of in vitro differentiation. (**A**) Immunofluorescence for Sox2 (red) (R&D) and GFP (green), encoded by Sox2-IRES-GFP lentivirus, in cells infected at day 1 (d1) or day 4 (d4), compared with non-infected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

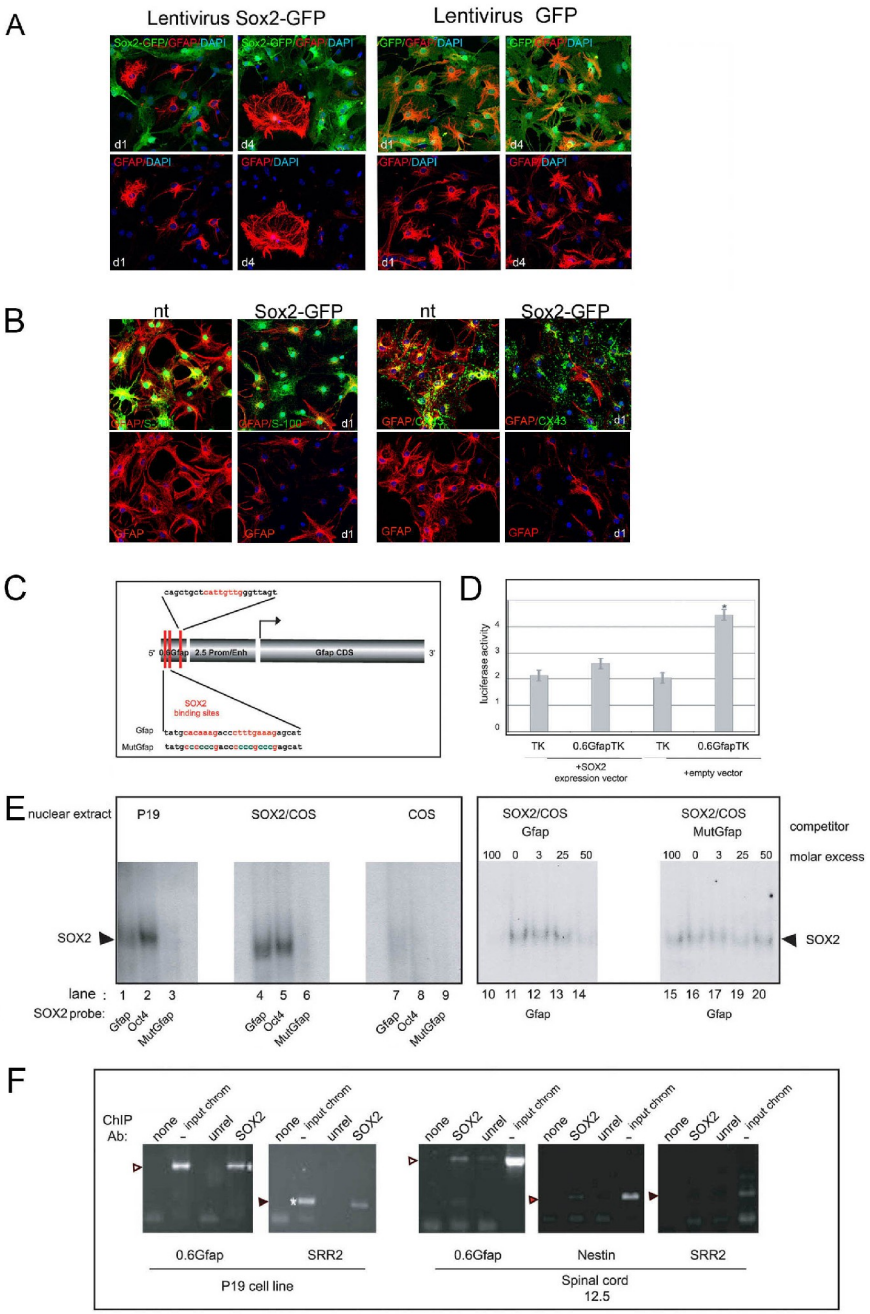


Figure 8 – Sox2 regulates GFAP expression and directly interacts with upstream regulatory DNA sequences of the GFAP gene in vitro and in neural cells chromatin. (**A**) Sox2 overexpression in differentiating cells represses endogenous GFAP expression. Double immunofluorescence (confocal microscopy) of 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.

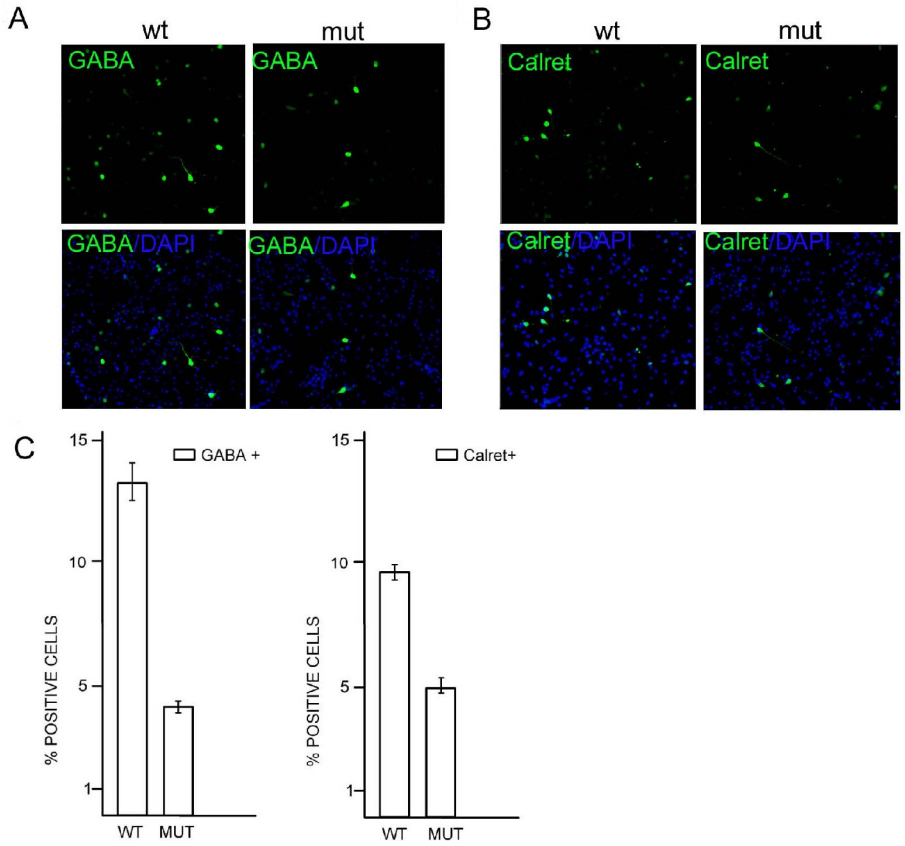


Figure 9 – Neurons expressing GABAergic markers are reduced in Sox2 mutant neonatal brains. (**A**,**B**) GABA (A) and calretinin (B) immunofluorescence of P0 cortical neurons (normal, left; mutant, right). Lower panels are counterstained with DAPI. (**C**) Percentage of GABA- or calretinin-positive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

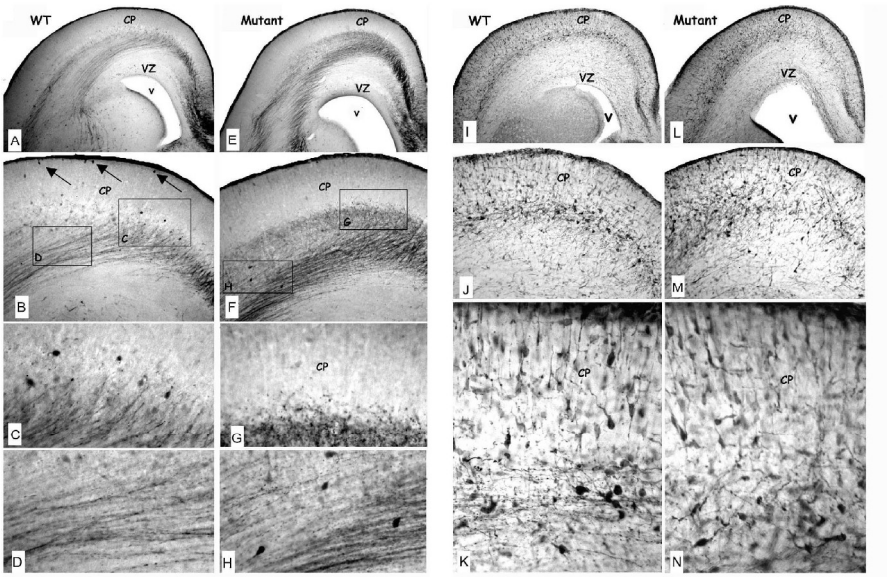


Figure 10 – Abnormal calretinin- and GABA-positive neurons in E17.5 mutant brain. Calretinin (**A-H**) or GABA (**I-N**) immunohistochemistry in sections from normal (A-D,I-K) and mutant (E-H,L-N) forebrains. (A,E,I,L) General views of normal and mutant forebrain sections (dorsal region). Lower panels 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.

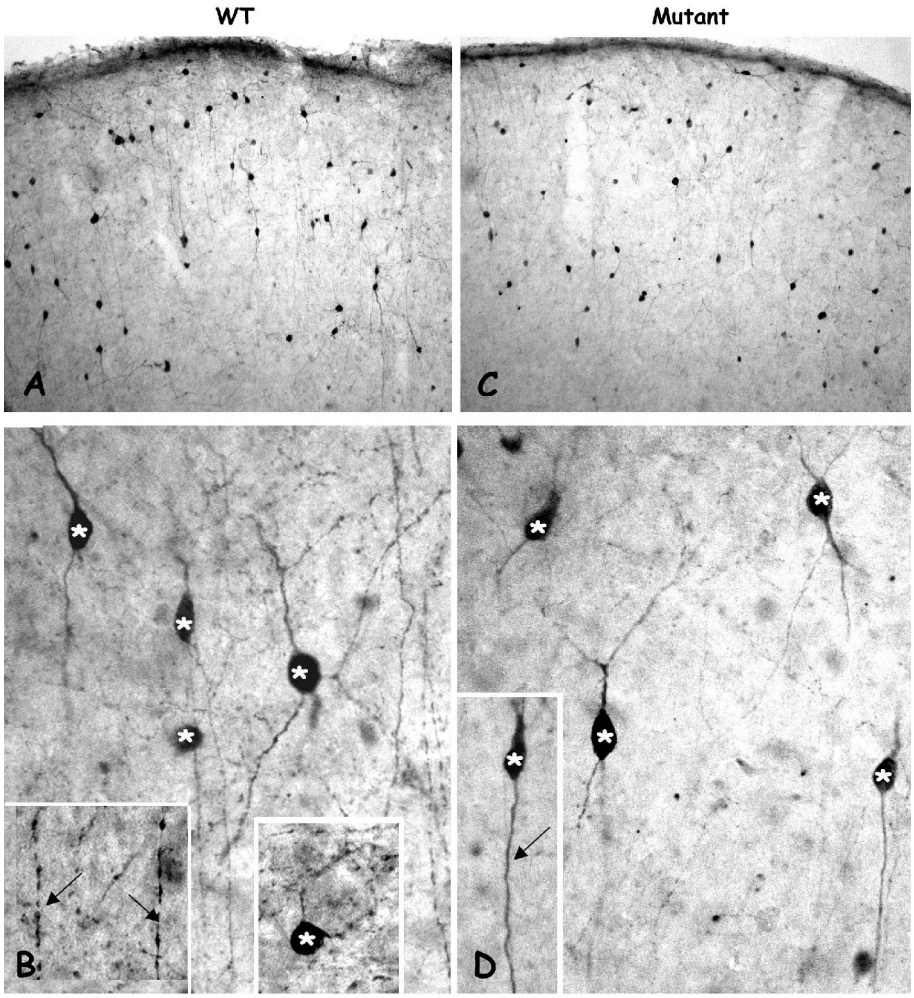


Figure 11 – Decreased frequency and arborization of calretinin-positive neurons in adult mutant somatosensory cortex. (**A**,**C**) Calretinin immunohistochemistry reveals lower frequency of calretinin-positive neurons in mutant (C) versus wild-type (A) mice. (**B**,**D**) Higher magnification shows 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.

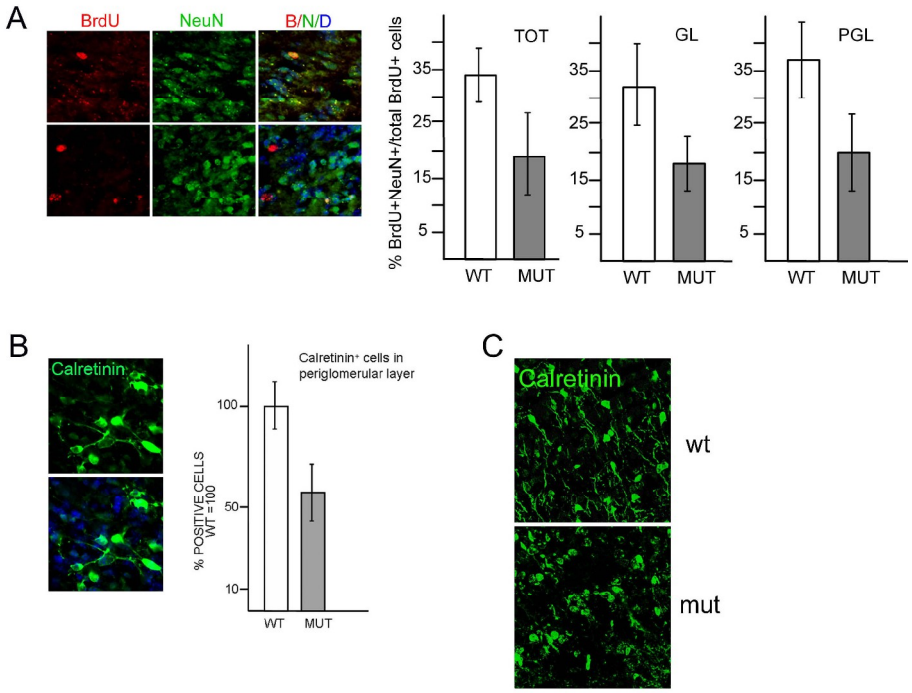
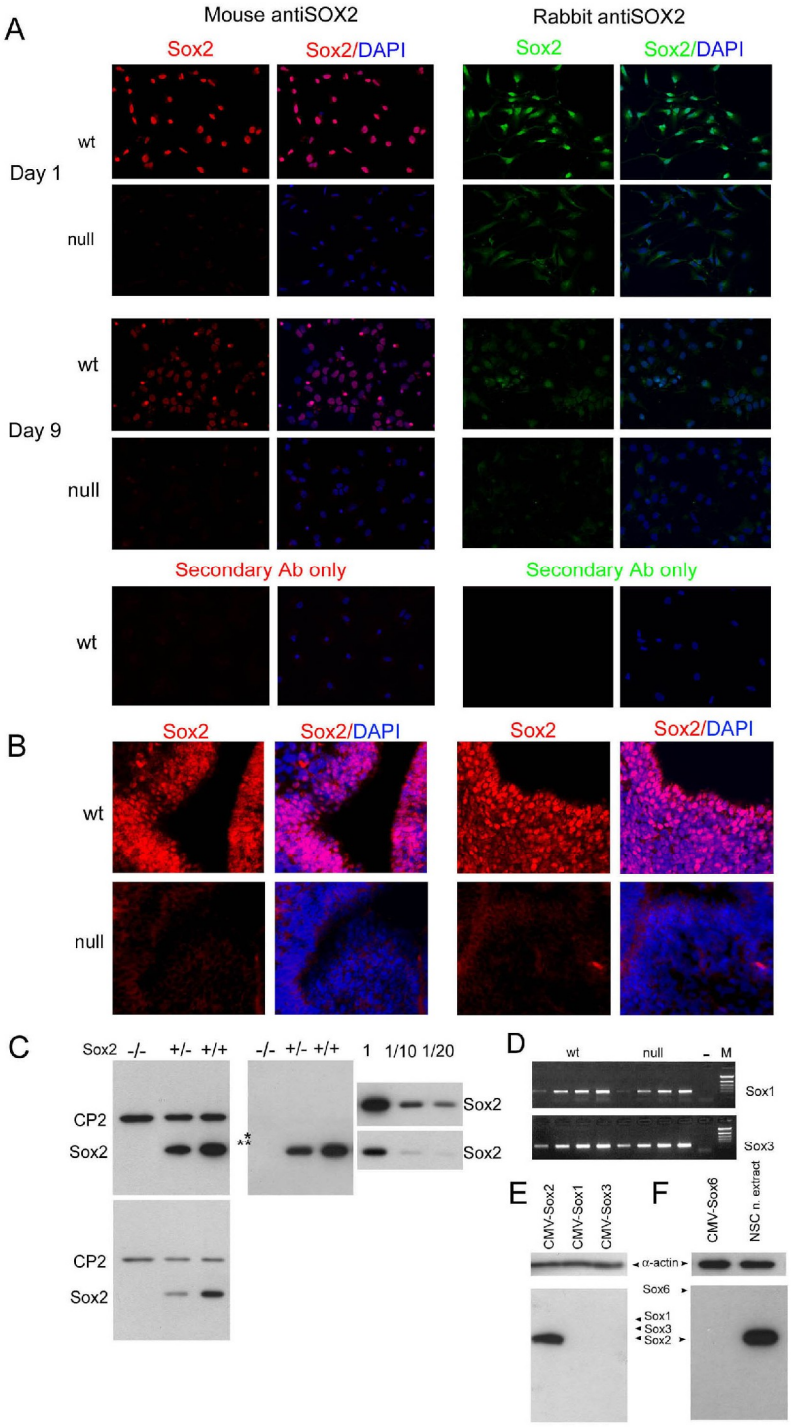


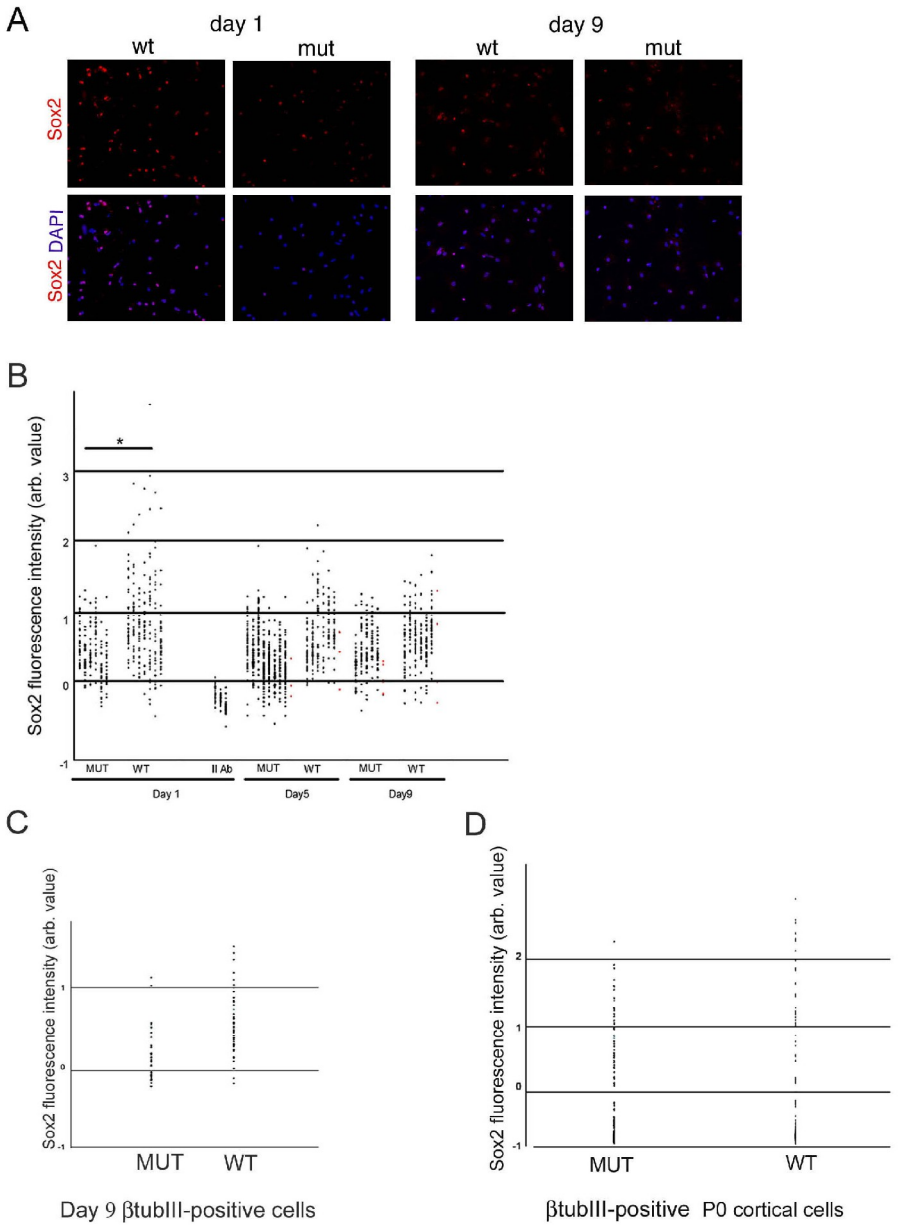
Figure 12 – Impaired neuronal maturation in adult olfactory bulb of Sox2 mutant mice. (**A**) Immunofluorescence of BrdU/NeuN-double positive (red and green, yellow in overlay; first row) and BrdU-single-positive (red only; second row) cells in olfactory bulb sections. Histograms: percentage of BrdU/NeuN 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



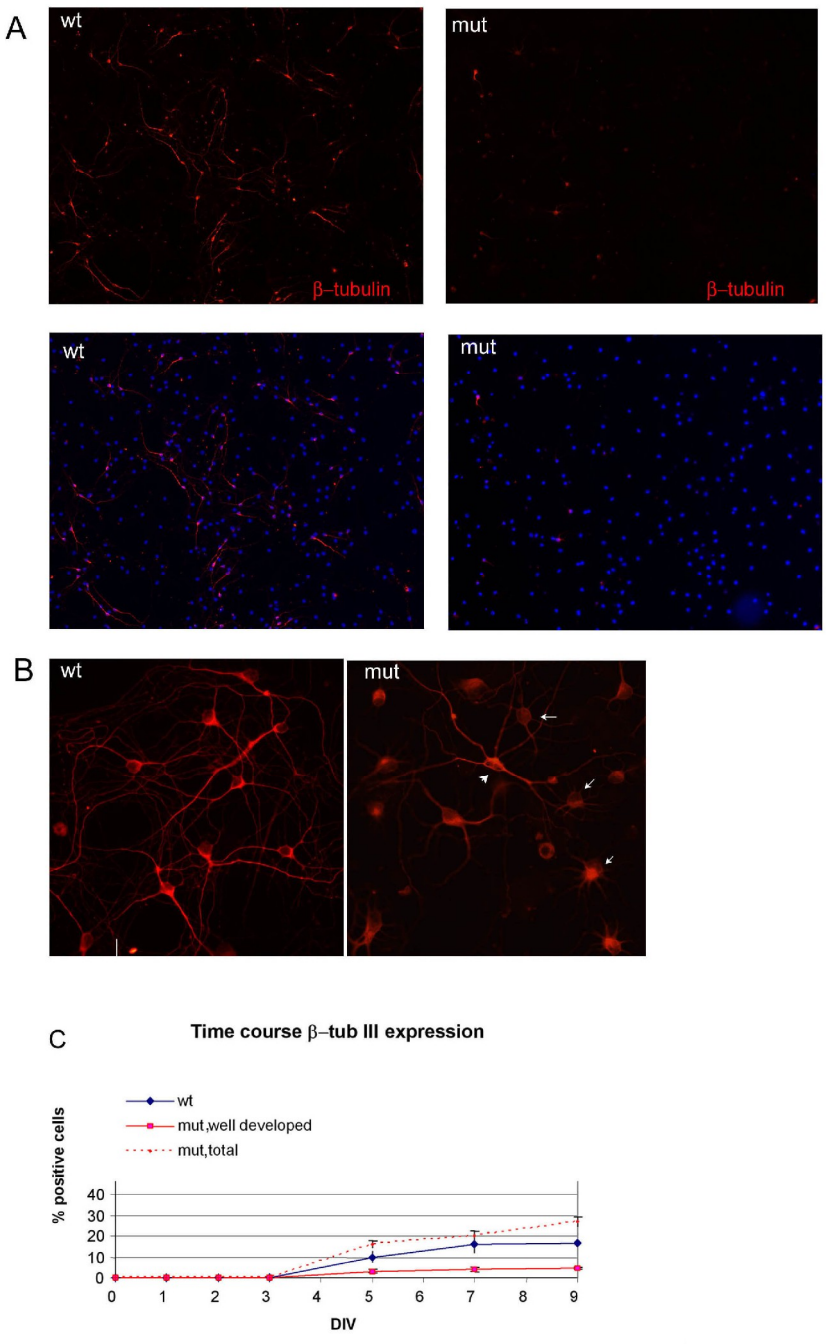
Supplementary Figure 1 – Evaluation of anti-Sox2 antibodies by immunocytochemistry, immunohistochemistry and western blot analysis of wild-type and Sox2-null neural cells, and of recombinant Sox proteins by western blot. We evaluated the Sox2 specificity of two commercial antibodies (R&D, mouse monoclonal; 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.



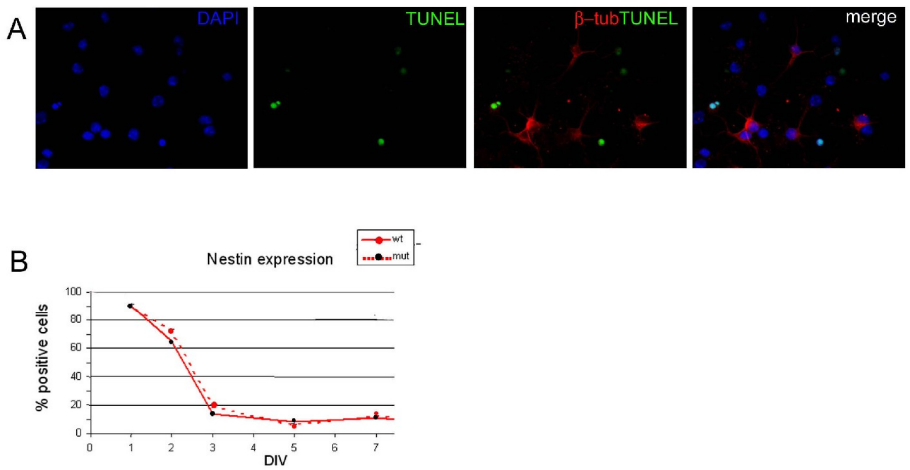
Supplementary Figure 2 – Evaluation of Sox2 immunofluorescence at the single-cell level. To evaluate Sox2 immunofluorescence at the single-cell level, digital images of Sox2 immunofluorescence-labeled nuclei were acquired, and individual nuclei were delimited and evaluated (on the monochromatic image 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.

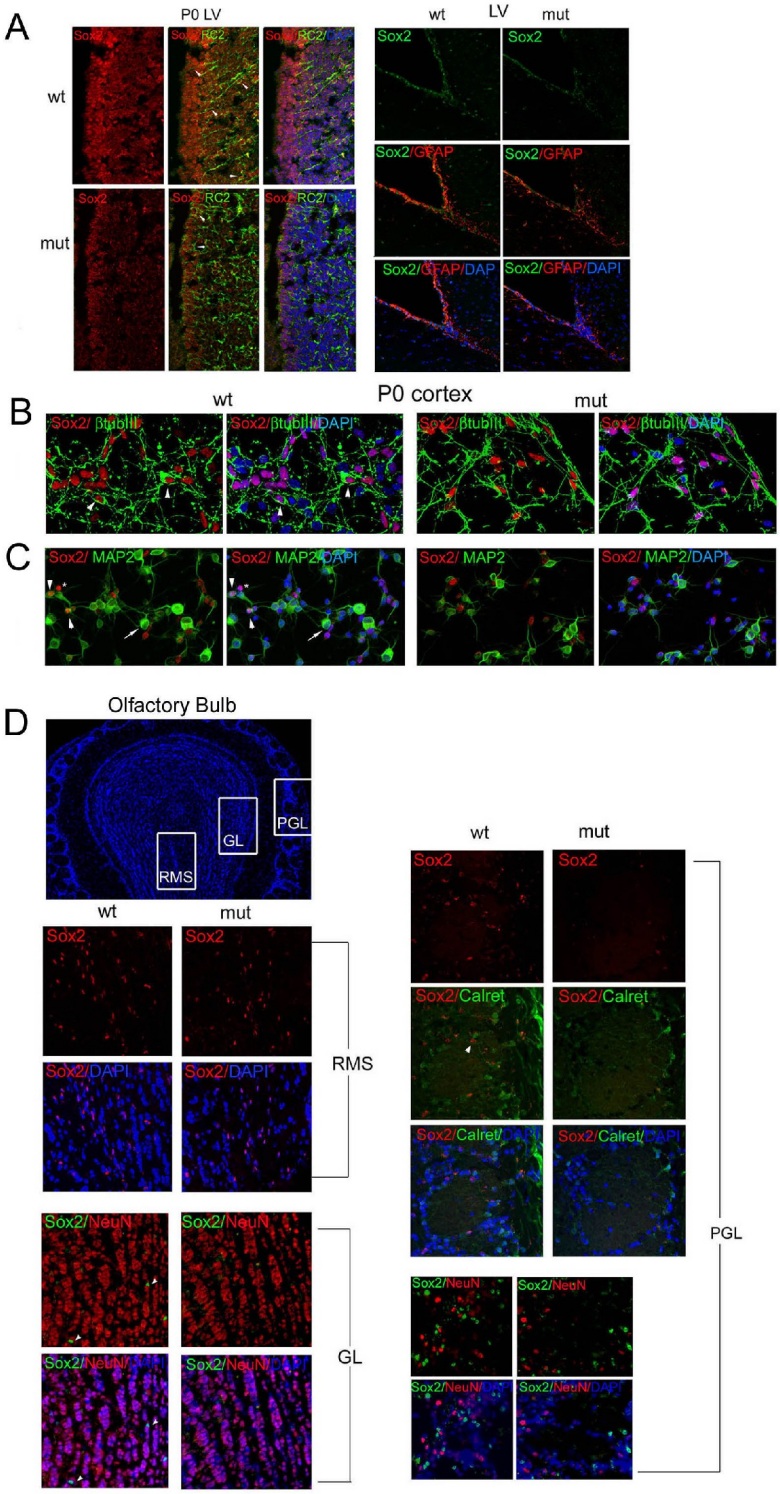


Supplementary Figure 3 – Expression of astrocytic markers S-100 and connexin 43 (CX43) (Nagy and Rash, 2000) in GFAP-positive in vitro differentiated astrocytes (untransduced, or day 1 transduced with Sox2-expressing lentivirus). (A)

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

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

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Hippocampal development and neural stem cell maintenance require *Sox2*-dependent regulation of *Shh*

Rebecca Favaro[1], Menella Valotta[1#], Anna L. M. Ferri[1#], Elisa Latorre[1], Jessica Mariani[1], Claudio Giachino[2], Cesare Lancini[1], Valentina Tosetti[1], Sergio Ottolenghi[1], Verdon Taylor[2] and Silvia K. Nicolis[1]

[1] Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milano, Italy
[2] Department of Molecular Embryology, Max Planck Institute for Immunobiology, Freiburg im Breisgau, Germany
[#] Equally contributing authors

Abstract

Neural stem cells (NSCs) are controlled by diffusible factors. The transcription factor *Sox2* is expressed by NSCs and *Sox2* mutations in humans cause defects in the brain and, in particular, in the hippocampus. We deleted *Sox2* in the mouse embryonic brain. At birth, the mice showed minor brain defects; shortly afterwards, however, NSCs and neurogenesis were completely lost in the hippocampus, leading to dentate gyrus hypoplasia. Deletion of *Sox2* in adult mice also caused hippocampal neurogenesis loss. The hippocampal developmental defect resembles that caused by late *sonic hedgehog* (*Shh*) loss. In mutant mice, *Shh* and *Wnt3a* were absent from the hippocampal primordium. A SHH pharmacological agonist partially rescued the hippocampal defect. Chromatin immunoprecipitation identified *Shh* as a *Sox2* target. *Sox2*-deleted NSCs did not express *Shh in vitro* and were rapidly lost. Their replication was partially rescued by the addition of SHH and was almost fully rescued by conditioned medium from normal cells. Thus,

NSCs control their status, at least partly, through *Sox2*-dependent autocrine mechanisms.

Introduction

Sox2 encodes a transcription factor that is essential for the pluripotency of epiblast, embryonic stem (ES) cells and reprogrammed, induced pluripotent stem cells[1, 2, 3, 4]. *Sox2* is also expressed at early stages of CNS development and marks NSCs[5, 6] and precursors. In humans, rare *SOX2* mutations cause anophtalmia, defective hippocampal development, cognitive defects and seizures[7, 8, 9]. In mice, hypomorphic *Sox2* mutants, expressing 30% of normal *Sox2* levels, show a loss of striatal and thalamic tissue, epilepsy and neurodegeneration[10], which might result from decreased stem cell numbers and defective neuronal differentiation[10, 11].

These observations raise the possibility that *Sox2* is important for controlling self-renewal and multi/pluripotency in several stem cell types. To evaluate this hypothesis and to investigate the molecular mechanisms of *Sox2* function, we developed nervous system–specific conditional *Sox2* knockout mice, bypassing the early embryonic lethality of homozygous *Sox2* mutants. We found that *Sox2* was required for NSC maintenance in the hippocampus and in long-term *in vitro* neurosphere cultures. NSC maintenance in neonatal hippocampus and in neonatal neurosphere cultures required soluble factors, including SHH, which was controlled by *Sox2* itself.

Results

Sox2 deletion causes hippocampal defects with NSC loss

We generated compound heterozygous mice carrying a *beta-geo* knock-in[5, 10] null mutation in *Sox2* and a $Sox2^{loxP}$ allele (Fig. 1a), together with a *nestin-cre* transgene[12], in which *cre* activity is driven by a neural *nestin* enhancer; the selection cassette in the original flox-targeted allele had been removed in previous generations (Fig. 1a). Cre activity, which began at embryonic day 10.5 (E10.5), caused the complete loss of *Sox2* in the CNS by E12.5 (Fig. 1b and data not

shown). Homozygous *Sox2*-deleted mice were born in the expected ratio, but most died by 4 weeks of age.

At birth (postnatal day 0, P0), we detected limited abnormalities in the brain, with the exception of a slightly reduced hippocampus, a moderate lateral ventricle enlargement and slight size reduction of the posterior ventrolateral cortex (Fig. 1c). Subsequently, however, the development of the hippocampus was markedly reduced, particularly caudally, resulting in an almost complete absence of the dentate gyrus at P7 (Fig. 1c).

To understand the causes of this defect, we studied NSCs. During brain development, periventricular NSCs have radial glia morphology and express RC2 and BLBP[13, 14]. Postnatally, radial glia are maintained in the hippocampus from P0 as GFAP and nestin double-positive cells and are the source of continuous neurogenesis in the dentate gyrus throughout life[13, 14]. In *Sox2*-ablated embryos, neither the abundance of radial glia (RC2 and BLBP positive[14]) nor neurogenesis (BrdU incorporation) were substantially altered (BrdU-positive cells, 89 ±6% of normal; Supplementary Fig. 1). In P0 hippocampus, the number of GFAP/nestin-positive cells in the dentate gyrus subgranular layer was only slightly decreased (Fig. 2a). Neurogenesis was almost normal at this stage (Fig. 2b). By P2, however, the number of GFAP/nestin-positive cells was strongly reduced and the cells were completely lost in the dentate gyrus by P7 (Fig. 2a). BrdU labeling showed a similar decrease; at P7, the small residual part of the dentate gyrus showed no BrdU incorporation (Fig. 2b). A transient increase in apoptotic cell death was also evident at P2 by TUNEL staining (Fig. 2b). There were no major changes in the lateral ventricle wall during this limited time window (Supplementary Fig. 1). The loss of neurogenesis and stem/precursor cells observed between P2 and P7 in the hippocampus suggests that *Sox2* is important for the maintenance, but not in the genesis, of hippocampal NSCs.

Sox2 ablation causes early regional loss of *Shh* and *Wnt3a*

The defective hippocampal development in *Sox2*-deleted mice qualitatively mimics that described for mutations of other transcription factors that are part of the *Wnt* signaling pathway (*Lef1*) or of *Wnt3a* itself[15, 16]; moreover, it is similar to the effect of a conditional mutation of *Shh* or of its receptor, *smoothened* (*Smo*)[17]. We analyzed

Wnt3a and *Shh* mRNA expression during development. By E14.5, *Wnt3a* expression in the cortical hem, which includes the hippocampal primordium, was strongly reduced in *Sox2*-deleted mutants, particularly in posterior regions (Fig. 3). We observed this reduction at E17.5 as well, just before hippocampus formation (Fig. 3). We detected no *Wnt3a* expression in normal or *Sox2* mutant hippocampus after birth (data not shown). *Shh* mRNA expression was strongly reduced at E14.5 in telencephalon and diencephalon, but not in midbrain (Fig. 3) and spinal cord (data not shown). This defect was maintained until at least P0, when *Shh* mRNA was easily detectable in normal, but not mutant, telencephalon (Fig. 3) and hippocampus (data not shown). At birth, *Shh* mRNA was absent in the hippocampal hilus of *Sox2* mutants (data not shown). In normal mice, SHH protein was clearly detectable at birth in both hippocampal and lateral ventricle wall neurogenic regions (Fig. 4). In the hippocampus, SHH was abundant in the hilus and marked cells with radial orientation in the dentate gyrus (Fig. 4a); in the lateral ventricle, cells that were positive for both SOX2 and SHH formed a continuous layer along the wall (Fig. 4a). In wild-type mice, confocal microscopy of both regions showed colocalization of SHH and SOX2 in individual cells, with SHH forming a rim around SOX2-positive nuclei (Fig. 4a,b). In contrast, in mutant (*Sox2*-null) mice, SHH was absent in these regions (Fig. 4a,b). Coexpression of SOX2 and SHH was further confirmed in normal NSCs (neurospheres) grown *in vitro*, and *Shh* loss was similarly evident in neurospheres from *Sox2*-deleted brains (see below).

The conditional knockout of *Smo* and *Shh*[17] causes an arrest of postnatal hippocampal development, with partial loss of the dentate gyrus. This phenotype closely resembles that of our *Sox2* knockout mouse, strongly suggesting that *Sox2* controls hippocampal development via regulation of *Shh*. To rescue the mutant *Sox2*-deleted phenotype (Figs. 1b and 2), we administered an SHH pharmacological agonist (SHH-Ag) to pregnant mothers (between E12.5 and P1), which was previously used to rescue embryonic defects of the *Shh* null mutant[18]. SHH-Ag–treated *Sox2*-deleted mutants were analyzed at P2 (TUNEL) or at P7 for GFAP/nestin-positive radial glia and for hippocampal BrdU incorporation; untreated *Sox2*-deleted mutants and normal SHH-Ag–treated P7 mice were also examined. SHH-Ag treatment (Fig. 5) greatly stimulated BrdU incorporation in the hippocampus of *Sox2*-deleted mutants, reaching ~40–50% that observed in wild-type controls (versus 3–4% in untreated *Sox2*-deleted

mutants). In the dentate gyrus, we observed large numbers of nestin and GFAP double-positive radial glia in treated mutants, in contrast with the complete disappearance in untreated mutants (Fig. 5; see also Fig. 2a,b); in addition, the size of the dentate gyrus was increased, particularly in the dorsal blade, in treated mutants (Fig. 5). SHH-Ag also greatly decreased apoptosis at P2 in *Sox2* mutants (Fig. 5). The substantial rescue by SHH-Ag of the postnatal loss of nestin/GFAP-positive NSCs and of neurogenesis in *Sox2*-mutant hippocampus suggests (together with the observed SHH expression defect in the mutant; Figs. 3 and 4) that non–cell-autonomous mechanisms may contribute to the observed phenotype.

Adult *Sox2* deletion causes loss of hippocampal radial glia

The loss of nestin/GFAP-positive radial glia and neurogenesis in postnatal hippocampus might reflect subtle embryonic NSC damage that becomes evident only after birth and would thus be a developmental defect rather than an actual requirement for *Sox2* in early postnatal NSC maintenance. To evaluate this issue, we deleted *Sox2* postnatally in *Sox2*-expressing cells of $Sox2^{loxP\Delta neo/loxP\Delta neo}$ mutants (Fig. 6) by tamoxifen-dependent activation of *creERT2* recombinase[19] driven by the *Sox2* telencephalic-specific promoter and enhancer[5]. This transgene was highly expressed in the hippocampus, as shown by its efficient activation of a transgenic *ROSA26RloxP-stop-loxP lacZ* reporter of Cre activity[20] after tamoxifen administration (Supplementary Fig. 2). We administered tamoxifen for 8 d to 2-month-old $Sox2^{loxP\Delta neo/loxP\Delta neo}$*; Sox2-CreERT2* mice; we used wild-type mice (treated or untreated with tamoxifen) and mice with the same genotype, but without tamoxifen administration, as controls (Fig. 6 and Supplementary Fig. 3). All mice were labeled with BrdU between days 8 and 10. We killed and analyzed the mice 4 d after the last tamoxifen administration (day 12) for SOX2 and for stem/precursor cell markers.

The numbers of SOX2/GFAP-positive cells with radial glia morphology and of GFAP/nestin-positive cells at the base of the dentate gyrus (radial glia stem cells) in tamoxifen-treated hippocampi of $Sox2^{loxP\Delta neo/loxP\Delta neo}$*; Sox2-creERT2* mice were reduced by over 40% relative to controls (Fig. 6a–c). Partial loss of SOX2-positive cells (Fig. 6a,b and Supplementary Fig. 3) was expected, as a result of the

limited efficiency of inducible Cre[19]. These data indicate that stem/precursor cells are lost in connection with *Sox2* deletion; notably, a small proportion of GFAP-positive radial glia had empty SOX2-negative nuclei, suggesting that *Sox2* deletion does not immediately lead to the loss of radial glia cells (Fig. 6a). Consistent with the loss of SOX2/GFAP- and nestin/GFAP-positive cells, the proliferation of precursors downstream to the stem cell was also decreased, as seen by a reduction (40%) in the number of Ki67-positive (that is, cycling) cells and of recently labeled BrdU-positive cells. Doublecortin-positive cells, a more downstream precursor cell population that is partly nonproliferating[21], and NeuN/BrdU-positive cells (early neurons) were decreased, but to a lesser extent (about 20%), suggesting that compensatory mechanisms may operate downstream to early precursors, as has been reported in *Sox2* adult hypomorphic mutants[10]. These data indicate that *Sox2* is required for NSC maintenance in the hippocampus during its early development (Figs. 1 and 2) and during adulthood.

NSC exhaustion *in vitro* is rescued by conditioned medium

To directly confirm that *Sox2* is involved in NSC maintenance, we established neurosphere long-term cultures[22] from *in vivo Sox2*-deleted brains (Fig. 7). SOX2 protein is completely absent from these mutant cells[11]. We expanded large numbers of *Sox2*-deleted (or wild-type controls from the same litter) NSC populations briefly (1–2 passages) in basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF)[22]. Subsequently, we plated (passage 0 in EGF; Fig. 7) aliquots of *Sox2*-deleted or wild-type cells in EGF alone (or in EGF and bFGF) and counted both the number of neurospheres and total cells at each passage. Using P0 cultures (Fig. 7), we found little or no difference between mutant and wild-type cells during the initial expansion; however, by passage 2–4 in EGF, neurospheres and cell numbers were markedly decreased in mutant cultures (Fig. 7a). Subsequently, we continued to replate equal numbers of wild-type and *Sox2*-deleted cells; however, *Sox2*-deleted cultures were completely exhausted and died out by passages 5 or 6 (Fig. 7a). In EGF and bFGF, we obtained similar results, except that mutant cells would adhere to the plate at early passages (2–5) before becoming exhausted (data not shown). P7 cultures were similarly exhausted by passage 3 or 4 (Supplementary Fig. 4). E14.5 cultures were more variable, as

some stopped growth around passage 4, whereas others continued to grow at a low rate (Supplementary Fig. 4). The observed exhaustion differs from that seen in a previous study[23], which reported minor differences between wild-type and *Sox2*-mutant NSCs; however, these experiments (in FGF and EGF) did not extend the culture beyond passage 2, at which point little or no decay of proliferation was observable in our cultures (Fig. 7).

The pronounced defect of NSC maintenance was accompanied by a strong decrease in neurosphere size (Fig. 7b) and the loss of *lacZ* staining (Fig. 7c), reflecting the activity of the *beta-geo* knock-in inserted into the *Sox2* locus. We[5] and others[6] have previously shown that *lacZ* expression, reflecting the activity of the *Sox2* gene, is retained in stem cells/early precursors, but is lost in more differentiated progeny; thus, loss of *lacZ* staining in $Sox2^{null}$ neurospheres suggests a loss of NSC properties. We also observed increased numbers of *beta-tubulin*–expressing cells (that is, cells spontaneously differentiating into neurons) in mutant neurospheres (Fig. 7d). At the same time, BrdU incorporation was decreased by at least 50%; furthermore, we detected increased apoptotic cell death in mutant cells (Fig. 7d), consistent with *in vivo* results (Fig. 2b). These data indicate that NSCs progressively lose their status, changing into more differentiated cell types.

To evaluate the role of secreted molecules in wild-type versus mutant NSCs, we supplemented mutant cells with conditioned medium from wild-type NSC cultures. The conditioned medium efficiently rescued the proliferation defect of *Sox2*-deleted cells (Fig. 7c,e,f), as well as *lacZ* staining (Fig. 7c). In contrast, conditioned medium from *Sox2*-deleted cells had no effect on wild-type NSCs (data not shown). These experiments indicate that wild-type cells release molecule(s) into the medium that maintain the stem cell status and are not produced by mutant cells.

Shh and *Wnt3a* are downregulated *in vivo* in $Sox2^{null}$ mutants (Figs. 3 and 4). *In vitro*, *Shh* (but not *Wnt3a*) was expressed in wild-type neurospheres, but was extremely downregulated in *Sox2*-deleted cultures (Fig. 7g). Transduction of *Sox2*-deleted cultures with a *Sox2-IRES-gfp*–expressing lentivirus[11] at passage 1 (in EGF), just before the decline of self-renewal (see Fig. 7a), induced *Shh* re-expression by 36 h (Fig. 7h) and rescued the formation of neurospheres, which continued to grow, in contrast with parallel cultures of nontransduced mutant cells (Fig. 7i). SHH is a NSC mitogen in brain and in NSC grown *in vitro*[17, 24, 25, 26, 27, 28]. Addition of SHH (alone or with

oxysterols, acting on the same pathway[29]; Fig. 7f) rescued the ability of mutant NSCs to continue to proliferate, albeit at substantially lower rate than cells treated with conditioned medium, without showing any effect on wild-type NSCs. Doubling times for wild-type cells were 33.8 ±5.5 h, with no difference following treatment with conditioned medium or SHH (32 ±1.8 and 33 ±2.4 h, respectively); for mutant cells, doubling times in conditioned medium and SHH were 45.3 ±3.8 and 60.7 ±10 h, respectively. Bio[30], a drug that generically stimulates the Wnt pathway, had no effect (data not shown). Other cytokine genes that are known to affect neural cells growth (*Egf*, *Fgf2* and *Fgf8*) were expressed in neurosphere cultures at normal levels (by RT-PCR), as were their receptors and downstream effector molecules (for example, *Egfr*, *Fgfr1*, *Notch1*, *Ctnnb1*, *Bmi-1*, *p21*, *p16*, *Hes5*, *Rbpj*, etc; data not shown).

The *Shh* gene is a direct target of *Sox2*

Shh expression was lost both *in vivo* in brain and *in vitro* in *Sox2*-deleted NSC cultures (Figs. 4 and 7g). *Shh* expression is controlled by multiple functionally characterized regulatory regions, which are specifically active in different neural tube regions[31]. Several conserved (mouse versus human) potential SOX2-binding sites are present in three of the described regions (T3 and T4, active in telencephalon, and Die, active in the diencephalon) and 5 kb downstream of the gene (P) (Fig. 8a). In T3, one such site is centered on the regulatory region, whereas two further sites are 5 and 35 nucleotides from the described region[31]. Using electrophoresis mobility shift assays (EMSAs), we found that SOX2 bound to sites in T3, Die and P, but not in T4 (Fig. 8).

To confirm that SOX2 is directly involved with these sites *in vivo*, we carried out chromatin immunoprecipitation (ChIP) experiments on embryonic brain cells. SOX2 bound three out of four of the tested regions (T3, Die and P) *in vivo* (Fig. 8b–d), whereas T4 sequences were not immunoprecipitated. Notably, we did not detect enrichment in any of these sequences in SOX2 ChIPs that were carried out in parallel using $Sox2^{null}$ control brain chromatin (Fig. 8c), further demonstrating the SOX2 specificity of the immunoprecipitation. These data indicate that *Shh* is a direct target of SOX2.

Discussion

Our results suggest that *Sox2* is important during development for controlling NSC maintenance, at least in part via non–cell-autonomous autocrine mechanisms. *In vivo*, neural *Sox2* ablation leads to a loss of SHH and Wnt3a, two signaling molecules that are active in NSC proliferation[15, 16, 17, 24, 25, 26], in some (but not all) of the embryonic/neonatal neural domains that normally express them (Figs. 3 and 4), followed by a failure of postnatal development and NSC maintenance in the hippocampus (Fig. 2). Pharmacological activation of the *Shh* signaling pathway substantially rescued the maintenance of hippocampal stem cell development during early postnatal morphogenesis (Fig. 5). *In vitro* experiments further suggest that it is NSCs themselves, or some of their early progeny, that control NSC maintenance via *Sox2*-dependent secretion of growth factors (Fig. 7).

Sox2 is required for NSC maintenance

The complete loss of hippocampal nestin/GFAP-positive stem/progenitor cells starting at early stages of postnatal development (Fig. 2) might be viewed as a developmental defect resulting from damage to the embryonic NSCs. Do normally born adult stem cells still require *Sox2* for their functions? Deletion of *Sox2* in *Sox2*-expressing cells of adult mice caused a rapid loss of GFAP/nestin-positive stem/precursor cells and of cell proliferation in the dentate gyrus, indicating that *Sox2* is still required in normally generated NSCs of the adult mouse hippocampus (Fig. 6). The only partial *Sox2* deletion obtained in adult hippocampal NSCs did not allow us to carry out a rescue experiment with SHH-Ag, as this would stimulate the nondeleted cells. Although SHH-Ag stimulates NSCs in the adult brain[17], it is not known whether *Shh* is continuously required postnatally in hippocampus[27]. Thus, it remains possible that *Sox2* controls hippocampal neurogenesis purely by cell-autonomous mechanisms at this stage.

Our *in vivo* evidence for a role of *Sox2* in NSC maintenance was further corroborated by our observation of a progressive and complete loss of *in vitro* NSC renewal in *Sox2*-deleted neurosphere cultures at P0 and P7 (Fig. 7 and Supplementary Fig. 4) and by the rescue of

neurosphere formation obtained using *Sox2* lentiviral transduction of mutant neurospheres (Fig. 7h, 7i).

Shh and *Wnt3a* expression depend on *Sox2*

The expression of *Wnt3a* and of *Shh*, two critical regulators of brain development, depends on *Sox2* (Figs. 3, 4 and 7g, 7h). This regulation is context dependent. In fact, *Shh* expression was deficient in telencephalon and diencephalon, but not in the midbrain and spinal cord of *Sox2*-deleted mutants; moreover, *Shh* and *Wnt3a* were expressed only in specific cell subsets of the *Sox2*-expression domain. Our ChIP results (Fig. 8) suggest that *Sox2* might contribute to the activation of distant regulatory elements of the *Shh* locus. Whether *Wnt3a* is directly regulated by SOX2 remains to be defined, as regulatory regions have not been identified.

The Notch pathway is strongly repressed in the eyes of mice by retina-specific *Sox2* deletion[32], but it was only moderately, if at all, decreased in the hippocampus of our mutants, as evaluated by the expression of the downstream effector *Hes5* (Supplementary Fig. 5). Effector genes that mediate *Sox2* function may thus differ in different neural tube regions and/or developmental stages (see below).

Non–cell-autonomous effects of *in vivo* S*ox2* ablation

The almost complete postnatal loss of the dentate gyrus that we observed in *Sox2*-ablated brains (Figs. 1c and 2) closely resembles the effects of the *Smo* and *Shh* conditional knockout mice[17]. These knockouts have the same *nestin-cre* transgene that we used in this study and developed the expression defect with kinetics that are similar to those of *Sox2* in our mutants. Thus, our data strongly suggest that the loss of *Shh* in the hippocampal primordium and the neonatal hilus of *Sox2*-deleted mutants (Figs. 3 and 4) may be sufficient to affect hippocampal development. This hypothesis is directly supported by the substantial rescue of stem/progenitor cells and neurogenesis (over tenfold increase in BrdU incorporation; Fig. 5) in *Sox2*-deleted P7 hippocampus following activation of the *Shh* pathway by a chemical agonist; the marked stimulation of BrdU incorporation stands in contrast with the limited effect reported[17] in wild-type mice (see also the similar BrdU incorporation in untreated

versus treated wild-type mice in Figs. 2 and 5, respectively). Thus, these data indicate that limiting *Shh* activity in *Sox2*-deleted mutants contributes to the hippocampal NSC defect.

In addition, the concomitant late loss in *Sox2* mutants (from E14.5) of *Wnt3a* expression (Fig. 3) might justify the somewhat increased severity of the *Sox2* deletion phenotype compared with that of the *Smo* and *Shh* knockout mice[17]. Indeed, total ablation of *Wnt3a* (normally expressed in the cortical hem by E10) by conventional knockout leads to the complete loss of the hippocampus[15, 16].

In vivo, loss of stem cells and of neurogenesis was observed, in our *Sox2* deletion model, mainly in postnatal hippocampus, but at much lower levels in other brain regions, such as the subventricular zone (Supplementary Fig. 1), indicating that the hippocampus is particularly sensitive to *Sox2* deletion. The preferential hippocampal localization was consistent with its relatively high dependence on *Wnt3a*[15, 16] and *Shh* expression (at P15; see Fig. 5 in ref. 17), as compared with the subventricular zone, and with the increased responsiveness to SHH of brain NSCs at late stages of embryogenesis[28] when most hippocampus development occurs.

Moreover, the conditional deletion of *Sox2* is complete only by E12.5 (Fig. 1), whereas *Sox2* expression begins, in the nervous system, by E7.5 or earlier. It is possible that there is a restricted time window, during which the requirement for *Sox2* in NSC is critical. Indeed, we found (unpublished data, A.L.M.F., R.F. and S.K.N.) that early *Sox2* deletion (E9.5 instead of E12.5) with other *cre*-expressing transgenes causes marked neural cell loss in several fore-, mid- and hindbrain regions. This is consistent with the embryonic neural tissue loss that we observed previously in the germline *Sox2* hypomorphic mutant[10]. An additional possibility is that exhaustion of the proliferative ability of the stem cell in the subventricular zone, following *Sox2* deletion, requires a critical number of cell divisions, leading to a late defect (as in other mutants[33, 34]). It should be noted that exhaustion of stem cell renewal in the mutant requires several passages *in vitro* and multiple stem cell divisions (Fig. 7).

Recently, another study reported conditional *Sox2* deletion using a different *nestin-cre* transgene[23]. The phenotype observed in that study was more severe than that reported here, as the mice died just before birth, precluding the study of the hippocampus. Brain morphology, however, was essentially normal; BrdU incorporation was substantially decreased in the ganglionic eminence, but not in the cortex. Overall, these data are consistent with ours, in the examined

time window, indicating that late embryonic *Sox2* deletion has only minor effects on brain development in general.

Sox2-dependent NSC autocrine mechanisms *in vitro*

In vitro, NSC cultures from *Sox2*-deleted forebrain progressively failed to grow and became exhausted, but exponential growth was almost fully rescued by medium conditioned by wild-type NSC cultures (Fig. 7e, 7f) together with full expression of a *beta-geo* reporter knocked-in at the *Sox2* locus (Fig. 7c), reflecting NSC status[5, 10]. Thus, *in vitro*, as in *vivo*, *Sox2* has an important non–cell-autonomous role in controlling NSC growth through diffusible products. One such product was SHH, a direct *Sox2* target (Fig. 8). Endogenous Hedgehog signaling is important for normal NSC growth *in vitro*[25, 26]. *Shh* was widely expressed in wild-type neural cells in culture (Fig. 7g), as was *Sox2* (ref. 11 and Fig. 7g), and was lost in $Sox2^{null}$ cells *in vitro* (Fig. 7g), consistent with our *in vivo* observations (Fig. 4). Addition of SHH to mutant NSCs *in vitro* rescued cell growth (Fig. 7f), although much less than conditioned medium, indicating that SHH is but one of the *Sox2*-dependent secreted factors. Indeed, an antibody to SHH slightly inhibited the effect of added conditioned medium on *Sox2*-deleted cells (as well as on wild-type cells; Supplementary Fig. 6). Furthermore, when *Sox2* was re-expressed in mutant cells by lentiviral *Sox2* transduction, just before the beginning of the growth decline, *Shh* expression was reactivated, together with neurosphere formation (Fig. 7h, 7i). These results indicate that the effect of *Sox2* on NSCs growth and maintenance *in vitro* is partially mediated by SHH secretion, although other undefined factors are probably important. This conclusion complements our *in vivo* finding that SHH, in the hippocampus, was an important mediator of *Sox2* function on NSC (Fig. 5).

Neural cells are a component of the stem cell niche, which provides the necessary environment for NSC maintenance and expansion. The niche includes astrocytes, ependyma and non-neural cell types, such as endothelial cells[35, 36]; indeed, it has been viewed as a specialized cell function separated from neural stem/precursor cells. The role of *Sox2* in the control of *Shh* (and *Wnt3a*) in developing brain *in vivo* (Figs. 3 and 4) and in cells *in vitro* (Fig. 7), and evidence for direct SOX2 activity on the *Shh* gene (Fig. 8), suggest that there is a

previously unknown self-regulatory loop for stem cell regulation in the neural niche during development.

Perspectives

It will be interesting to evaluate whether *Sox2* contributes to ES cell pluripotency, in part, by non–cell-autonomous mechanisms. A role for *Wnt3a* (a *Sox2* target in neural cells; Fig. 3) in reprogramming somatic cells to pluripotency was recently described[37]. Furthermore, *Sox2* is expressed in many neural brain tumor cells, particularly in the stem cell component[38, 39, 40]. As the *Shh* pathway is an important target of both tumorigenic mutations and experimental pharmacological treatments[39, 41], a role of *Sox2* in cancer stem cell maintenance should be considered.

Online Materials and Methods

Generation of $Sox2^{loxP}$ and $Sox2^{loxP\Delta neo}$ alleles and mice

The targeting vector (Fig. 1a) was generated with Sox2 genomic fragments from a 129/SvJ mouse genomic library, cloned into the pLM-FLRT-3 vector (gift from S. Dymecki, Harvard Medical School). The vector (Fig. 1a) includes the 5' homology arm (10,942 bp, a HindIII fragment, position 59,108–70,060 in BAC, Genbank accession number AL606746), the 5' loxP site, the HindIII-SalI fragment containing the Sox2 gene (position 70,060–76,650), the 3' loxP site, a neomycin-resistance cassette flanked by FRT sites, substrate of FLP recombinase[42], the 3' homology arm, a 2.5 kb SalI fragment (position 76,650–79,180 in AL606746), and a dyphteria toxin–encoding (DTA) cassette (a 2-kb SalI-XhoI PGK-DTA fragment). To allow stable replication in E. coli, we changed the vector backbone to pBR322 carrying a SalI-AatII deletion. To improve stability, we grew the construct in a pcnB mutant E.coli (pcnB::Tn10 recA::cat C-5507 E. coli C; a gift of S. Zangrossi and G. Dehò, University of Milano) that reduces the replication efficiency of ColE1 origins. The vector was linearized with KpnI for transfection.

Gene targeting was carried out in CJ7 ES cells. G418-resistant clones were analyzed by PCR (primers 1–4; Fig. 1a) and Southern

blotting; EcoRI-digested genomic DNA was probed with a 2.6-kb SalI-EcoRI fragment (Fig. 1a). In the wild-type Sox2 locus, this hybridized to a 15.5-kb fragment and to a 7.5-kb fragment in the $Sox2^{loxP}$ allele. The Sox2 internal probe (Fig. 1a) hybridized to a 15.5-kb fragment in the wild type or to a 6.5-kb in the $Sox2^{loxP}$ allele.

Chimeras were bred to C57BL/6J or B6D2F1 females to obtain mice carrying the $Sox2^{loxP}$ allele, which were bred to FLPeR (FLP recombinase–expressing) mice[42] to remove the neomycin-resistance cassette. nestin-cre mice[12] were crossed with mice carrying the $Sox2^{\beta geo}$ null allele[1, 5] to obtain double heterozygotes; experimental mice were obtained crossing $Sox2^{loxP\Delta neo/loxP\Delta neo}$ with $Sox2^{\beta geo/+}$; nestin-cre mice. Experimental procedures involving mice were approved by the Italian Ministry of Health.

Immunohistochemistry and *in situ* hybridization of brain sections

Embryos or dissected brains were fixed overnight at 4 °C in 4% paraformaldehyde (PFA, wt/vol) in phosphate-buffered saline (PBS), cryoprotected with 30% sucrose in PBS and cryostat sectioned onto slides (SuperFrost Plus).

For immunohistochemistry, sections were incubated overnight at 4 °C with primary antibody diluted in 0.1% BSA (wt/vol) in PBS, extensively washed in PBS containing 0.1% BSA, incubated for 1 h at 20–25 °C with a secondary antibody conjugated with a fluorochrome (Molecular Probes), washed in PBS and mounted in FluorSave reagent (Calbiochem 345789). For primary antibodies, we used mouse antibody to SHH (1:10, Developmental Studies Hybridoma Bank, 5E1), rabbit antibody to SHH (1:100, Chemicon), rabbit antibody to SOX2 (1:500, AB5603, Chemicon), mouse antibody to SOX2 (1:50, R&D MAB2018)[11], mouse antibody to nestin (1:500, MAB353, Chemicon), rabbit antibody to GFAP (1:400, 18-0063, Zymed). Jagged-1 immunohistochemistry was carried out as described previously[43]. For SHH/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 oven before blocking. All images were collected on a Zeiss Axioplan 2 microscope and processed with Adobe Photoshop 7.0 software (Adobe Systems). We used five mice of each genotype and stage for the experiments shown in Figures 2, 3, 4.

For *in situ* hybridization, embryos were fixed in 4% PFA overnight, cryopreserved in 30% sucrose, frozen in OCT compound and sectioned onto slides. *In situ* hybridization was performed as described previously[44], on fixed tissue with digoxigenin-labeled single-stranded RNA probes at 65 °C, followed by incubation with BM Purple AP substrates (Roche). We used antisense RNA probes to *Shh*[45], *Wnt3a*[16] and *Hes5* (ref. 43).

TUNEL and BrdU labeling

Cryostat sections were washed for 10 min in PBS containing 0.1% Triton X-100 (vol/vol), fixed for 5 min in 4% PFA and washed again in PBS. The enzymatic reaction was then performed at 37 °C according to the manufacturer's protocol (G3250, Promega). NSCs, dissociated and attached to slides, were treated the same way. Quantitative immunocytochemical data (for sections) represent mean±s.d. for cell counts of the hippocampus in consecutive sections through its entire length, every 150 μm. For P0, P2 and P7 labeling, BrdU was injected at 100 μg per g of body weight 2 h before the mice were killed. Proliferating cells were revealed by BrdU immunohistochemistry on frozen sections (or dissociated cells attached to slides). Sections (or cells) were denaturated with 2N HCl in H_2O at 37 °C for 1 h (30 min for cells), neutralized with 0.1 M borate buffer (pH 8.5) for 10 min, blocked in 1% BSA in PBS (with 0.2% Triton X-100 for cells) for 1 h at room temperature (20–25 °C), and probed with a Harlan rat monoclonal antibody to BrdU (1:500 in 0.1% BSA in PBS) overnight at 4 °C. All images were collected on a Zeiss Axioplan 2 microscope and processed with Adobe Photoshop 7.0 software. Quantitative immunocytochemical data represent mean±s.d. for cell counts of the hippocampus, obtained by reacting and counting 1 every 4 sections through the entire hippocampus, every 150 μm. We assayed five mice for each genotype and stage.

In vivo rescuing and adult deletion

For the *in vivo* rescuing experiment, *Shh* agonist #1.2 (a gift from Curis)[17, 18] was administered to pregnant mothers by oral gavage at a concentration of 1.5 mg ml^{-1} in 0.5% methylcellulose (wt/vol)/0.2% Tween 80 (vol/vol), 100 μl per 10 g of body weight, as described

previously[18], on E12.5, 14.5, 17.5 and P1. Newborns were then analyzed at P2 or P7 (as for Fig. 2).

For adult *Sox2* deletion, we generated mice carrying a transgene with the 5.7-kb *Sox2* 5' telencephalic enhancer/promoter[5] driving *creERT2* (ref. 19; a gift from P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire)). Six transgenic lines were obtained and each had the expected tamoxifen-dependent Cre activity[5], as assayed by breeding the mice to a transgenic mouse with a *loxP-lacZ* reporter of Cre activity[20]; the most efficient line was used (deletion efficiency was about 50% in embryonic and adult neural precursors *in vivo*).

Adult mice (2.5 months) were treated with tamoxifen (20 mg ml^{-1} in ethanol/corn oil 1:10, 0.1 mg per g of body weight) by intraperitoneal injection, one injection per d for 8 d (day 1–8), treated with BrdU on days 8, 9 and 10, perfused 4 d later with 4% PFA, and cryostat sectioned (20-μm sections). We analyzed 5–7 sections that were representative of the whole hippocampus length (1 every 8 for P2–P7; 1 every 10 for adults) for GFAP, nestin, SOX2, BrdU (as for Fig. 2, see above), doublecortin[21] (Santa Cruz goat SC8066, 1:200), Ki67 (NovoCastra rabbit polyclonal, 1:500) and NeuN (Chemicon mouse MAB377, 1:500) immunofluorescence.

Statistical analysis was performed by Student's *t* test (two-tailed), comparing experimental groups of mice of the same genotype. In all histograms, values are shown as mean±s.d. from a number of independent samples (*n* indicated).

NSC culture

P0 brain cells for NSC (neurosphere) cultures from wild-type and *Sox2*-deleted (*Sox2*$^{loxP/\beta\text{-}geo}$*; nestin-cre*) mice were obtained as described previously[21, 41], plated at 20,000 cells per ml in 25-ml flasks and cultured to expand their number in complete medium (2% B27 (vol/vol) in DMEM F12 with Glutamax[43]), supplemented with 10 ng ml^{-1} of EGF, 10 ng ml^{-1} of bFGF with 0.2% heparin, vol/vol), and passaged (by dissociation and dilution) two or three times every 5 d. After sufficient cell numbers had been obtained, cells dissociated from neurospheres (10,000 cells per 0.5 ml in triplicate, in 24-well plates) were passaged into complete medium with EGF, but not bFGF; this initial passage is taken to represent passage 0 (Fig. 7a, 7e, 7f). In some experiments, SHH (200 ng ml^{-1}, R&D Systems, 464-SH), oxysterols

(22(S)-and 20α-hydroxycholesterol, Sigma H5884 and H6378, 0.2 μM each) or conditioned medium from normal cells were added (Fig. 7c,e,f). No substantial difference was found between treatments with SHH and oxysterols and SHH only (Fig. 7f and data not shown). Conditioned medium was the supernatant obtained by growing wild-type cells in complete medium (with EGF) for 2 d, followed by removal of the cells by centrifugation. Complete removal of the wild-type cells was assessed by routine microscopy. Note that cells grown from mutant cell cultures treated with conditioned medium were all X-gal positive, confirming that they originated from *Sox2*$^{\beta\text{-}geo/loxP}$ (mutant), but not (potentially contaminating) *Sox2*$^{+/+}$ (wild type), cells (Fig. 7c). In some experiments, conditioned medium from mutant cells was added to wild-type cell cultures, but no effect was observed (data not shown).

Sox2-GFP lentivirus transduction

Sox2^{null} neurosphere cells grown for three passages in EGF and bFGF were passaged once in EGF, dissociated, plated in EGF at 40,000 cells per well in 4-well chambered slides and transduced 4 h later with a *Sox2-gfp*–expressing lentivirus as described previously[11]. Virus was removed by medium change at 24 h; nontransduced controls were treated equivalently (without virus). For RT-PCR (Fig. 7h), cells were harvested 36 h after infection and lysed in Tryzol, RNA was extracted from two transduced (or nontransduced) wells, DNAase treated (RQ1 DNAase, Promega) and reverse transcribed with random hexamer primers using an Applied Biosystems cDNA reverse transcription kit (with a reverse transcriptase–negative control). About 5 μl. of a 1:10 dilution (adjusted following normalization by *Hprt* expression) were used for the RT-PCR shown in Figure 7h. In parallel, for the cultures shown in Figure 7i, 20,000 cells per well were plated in suspension (to allow for subsequent neurosphere formation) in a 24-well plate and transduced as described above (4 h after plating, changing medium after 24 h). Small amounts of medium were added every 3 d; cells were passaged first after 1 week and re-passaged twice before freezing (nontransduced controls were exhausted at passage 1). Images showing neurosphere formation by transduced cells were taken 7 days after transduction. For RT-PCR, we used two primers for *Shh*, *Shh* forward (GGA AAG AGG CGG

CAC CCC AAA AAG) and *Shh* reverse (CTC ATC CCA GCC CTC GGT CAC TCG). Annealing was carried out at 65 °C for 45 cycles and we obtained a 278-bp reaction product. RT-PCR for *Sox2* and *HPRT* was as described previously[11].

ChIP and EMSA

ChIP was carried out as described previously[46]. Briefly, BDF1 E14.5 brains were fixed in 1% PFA in DMEM cell culture medium (with 10% fetal calf serum (vol/vol) and penicillin-streptomycin) for 15 min at 37 °C, then crosslinking was blocked treating with 125 mM glycine for 15 min at 37 °C. Tissue was digested with 10 mg ml^{-1} collagenase at room temperature (20–25 °C), centrifuged at 800*g* and harvested in ice-cold RIPA Lyses Buffer (0.1% SDS, 1% DOC, 150 mM NaCl, 2 mM EDTA, 1% NP-40 (vol/vol), protease inhibitor cocktail; Complite Roche) at a final concentration of about 10^4 cells per ml. Keeping samples ice cold, we passed the cells through a syringe needle (U-100 insulin syringe, four times) and sonicated them to shear chromatin into DNA fragments of about 1,000 base pairs (six intervals of 25 s, 2-min 'rest on ice' between intervals, power setting 3, Branson 150 cell disruptor). Cell debris were removed by a 15-min centrifugation at 9,000*g*. Prior to immunoprecipitation, 1 μg μl^{-1} of yeast tRNA was added as a blocking agent to the sonicated samples. Sepharose nProt A 4fast flow (Amersham #17.5280.01) beads were pre-blocked in RIPA buffer with low molecular weight salmon sperm DNA, and the antibody of interest was added. We used 10 mg of antibody-coated beads to immunoprecipitate chromatin from 10^6 cells (antibody to SOX2, R&D MAB2018; IgG, Santa Cruz sc-2027; SV40 T-Ag, Santa Cruz sc-147). Immunoprecipitation was carried out for 2 h at 4 °C and beads were then washed three times in an equal volume of ice-cold washing buffer (100 mM Tris-HCl (pH 8.0), 500 mM LiCl, 1% NP-40). DNA-protein complexes were eluted by ice-cold elution buffer (1% SDS, 50 mM $NaHCO_3$) and treated with 200 mM NaCl, 100 ng μl^{-1} RNAse A and 200 ng μl^{-1} proteinase K at 52 °C for 3 h. DNA was purified by phenol-chloroform-isoamylic alcohol (25:24:1, pH 8) equal volume extraction and ethanol precipitated. DNA was resuspended in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA and used as template for PCR. Zero (background) values (Fig. 8e) were measured as the average of the values (PCR band intensities) obtained with the three negative controls in Figure 8c (no antibody or unrelated

antibody, antibody to SV40T antigen; pre-immune IgG), as described previously[47].

We used five primer sets for PCR, P_fw: CCAGGTACATCTTTGATTGACATTCAGC and P_rev: TGTTTCTGAACTAAGTTGGTGTTGCGTT, die_f (AAA ATA AAC CCC AGC CAG ACG CAA CC) and die_r (TTC ATC TGA TCC CCT GCT TTT AGC), T3_f (GGA AAT GGC ACT GAG AGT AAG AAC) and T3_r (TTT CCA AAT CAG CAG AGT GGC TCC G), T4_f (CTT TAA TTT TGC GTT ATT TCC AGC C) and T4_r (TCC GCT TAA ATC TTA GAG AGC G), and actin_f (GGT CAG AAG GAC TCC TAT GT) and actin_r (ATG AGG TAG TCT GTC AGG TC). The *Gfap* and *Srr2* primer sequences were described previously[11].

EMSAs were carried out as described[11]. Nuclear extracts were obtained from COS cells transfected with *Sox2-IRES-GFP*[11] expression vector or backbone alone. For probes, we used T3s (AAG AAC AAA GAG CTG TTC GGA GCA AGC AGC ACA CTT), T3r (GTG AAC AAA GTG TGC TGC TTG CTC CGA ACA GCT CTT TGT TCT T), T3smut (AAG CCC CCC GAG CTG TTC GGA GCA AGC AGC ACA CCC CGC CC), T3rmut (GTG GGC GGG GTG TGC TGC TTG CTC CGA ACA GCT CGG), Dies (CAA CCT GCC TTT GTT CCC TAA GCT GCT T), Dier (AAG CAG CTT AGG GAA CAA AGG CAG GTT G), Die_mutr (CAA CCT GCC CCC GCC CCC TAA GCT GCT T), Die_muts (AAG CAG CTT AGG GGG CGG GGG CAG GTT G), T4s (TCT CTA CAG AAC AAA GTG GGC TTT ACC T), T4r (AGG TAA AGC CCA CTT TGT TCT GTA GAG A), T4_mutr (TCT CTA CAG CCC CCC GTG GGC TTT ACC T), T4_muts (AGG TAA AGC CCA CGG GGG GCT GTA GAG A), Pr (AGG GAG GGG GGC ATT GTG TAC AAG CCC TG), Ps (CAG GGC TTG TAC ACA ATG CCC CCC TCC CT), P_mutr (AGG GAG GGG GGC CCC GCG TAC AAG CCC TG) and P_muts (CAG GGC TTG TAC GCG GGG CCC CCC TCC CT).

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the 1.2 SHH agonist[18], S. Lugert for help with the Notch pathway expression studies, J. Cozzi and GenOway for help with ES cell targeting, C. Tiveron and Layline Genomics for blastocyst injections, C. Tiveron and L. Tatangelo for *Sox2-creERT2* pronuclear injections, R. Caccia for help with *in situ* hybridization, E. Bagnaresi and A. Marinelli for help with constructs, A. Ronchi for advice on EMSAs and ChIP and D. Santoni for animal care. The monoclonal antibody to SHH developed by T.M. Jessell was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the US National Institute of Child Health and Human Development and maintained by the University of Iowa. This work was supported by Telethon (GGP05122), the European Economic Community (STEMBRIDGE), Fondazione Cariplo, Associazione Italiana Ricerca sul Cancro, Fondazione Banca del Monte di Lombardia, the Ministero Istruzione Università e Ricerca (Cofin) and FAR 2004-7 grants to S.K.N., and by the Max Planck Gesellschaft through V. Taylor.

Figures

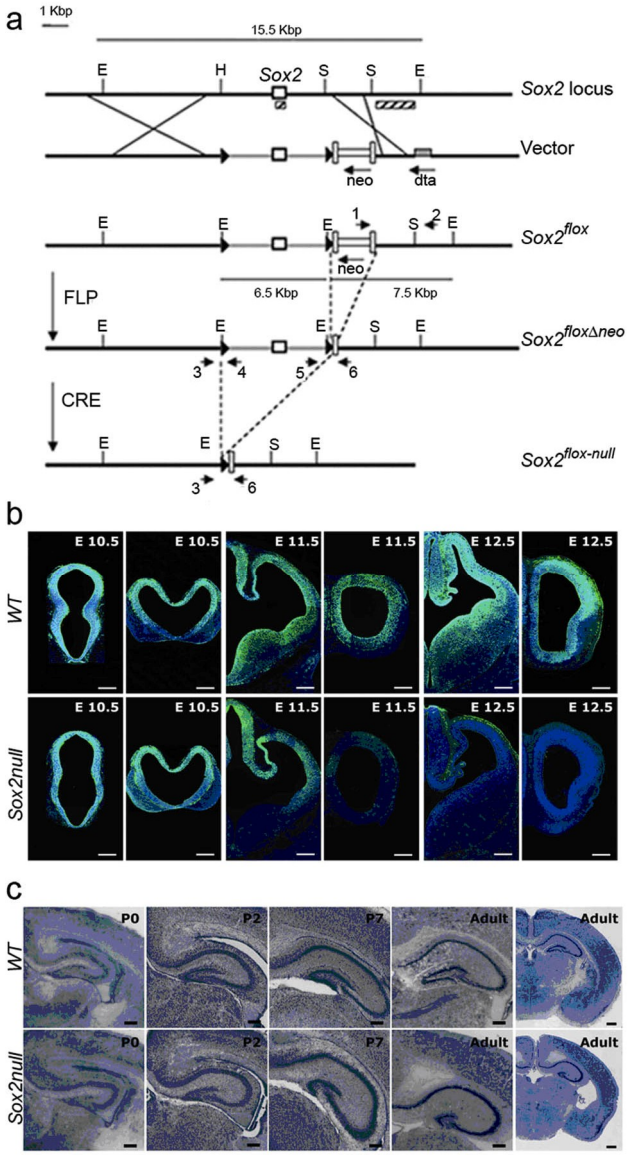


Figure 1 – *Sox2* conditional null allele, SOX2 protein ablation by *nestin-Cre* in mutant embryonic brain and *in vivo* morphological defects of *nestin-cre Sox2*-deleted mutants ($Sox2^{null}$). (**a**) Top to bottom, the *Sox2* locus and targeting vector, the $Sox2^{loxP}$ allele obtained by homologous recombination, the $Sox2^{loxP\Delta neo}$ allele obtained by subsequent FLP recombinase–mediated excision of the *neo* cassette, and the $Sox2^{loxP\text{-}null}$ allele, resulting from Cre-mediated *Sox2* excision. Filled triangles represent *loxP* sites (Cre recombinase substrate) and open vertical rectangles

represent FRT sites (FLP recombinase substrate). Note that the *Sox2*$^{loxP\Delta neo}$ allele is identical to the wild-type locus, except for the insertion of the *loxP* and FRT sites. The dashed boxes under the *Sox2* locus represent probes used for Southern analysis. E, *Eco*RI site; H, *Hin*dIII site; S, *Sal*I site. (**b**) SOX2 immunohistochemistry on wild-type (WT) and *Sox2*$^{loxP\Delta neo/loxP\Delta neo}$*; nestin-cre* (*Sox2*^{null}) embryonic brain at E10.5, 11.5 and 12.5. At E10.5, wild-type and mutant were still undistinguishable; SOX2 ablation was seen at E11.5, particularly ventrally, and was essentially complete by E12.5. Scale bar represents 200 μm. (**c**) Thionine staining of sections of P0, P2, P7 and adult hippocampus (left four columns, scale bar represents 500 μm) and adult forebrain (far right column, scale bar represents 200 μm). The mutant hippocampus, in particular the dentate gyrus, remained underdeveloped after P0 compared with wild type. We observed a smaller cortex and a prematurely interrupted corpus callosum in adult forebrain, along with the smaller hippocampus.

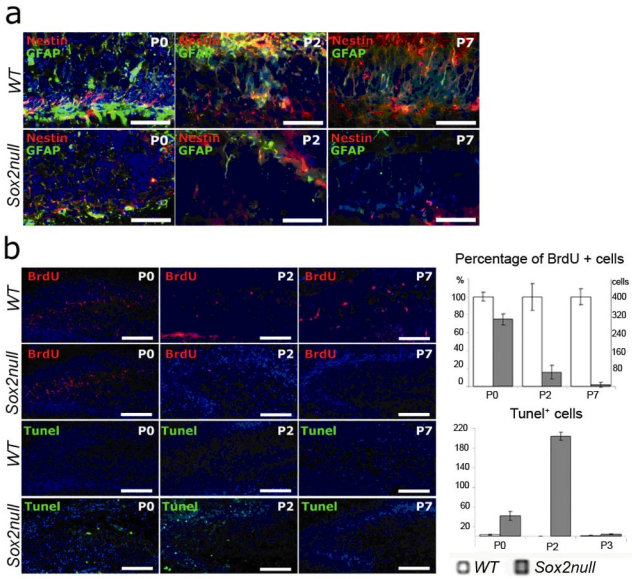


Figure 2 – Cellular defects of *nestin-cre Sox2*-deleted mutant brain. (**a**) Nestin/GFAP immunohistochemistry of hippocampus dentate gyrus, labeling postnatal NSCs[13, 14]. Abundant nestin/GFAP-positive radial cells developed in normal brain between P0 and P7, but were lost in the mutant. Scale bars represent 50 μm. (**b**) Left, BrdU (top) and TUNEL (bottom) dentate gyrus labeling at P0, P2 and P7 (scale bars represent 100 μm). Right, quantification of BrdU-positive cells (top, wild-type set = 100%) or TUNEL-positive cells (bottom) (absolute numbers on *y* axes are total number of cells counted). Histograms represent mean±s.d. values for *n* = 5 mice per genotype assayed for each stage.

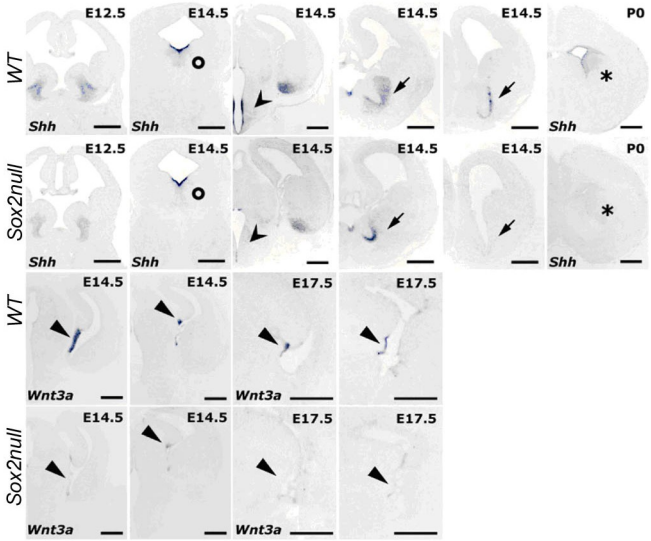


Figure 3 – *Sox2*-deleted mutant brains have defective *Shh* and *Wnt3a* mRNA expression. *Shh* (top) and *Wnt3a* (bottom) *in situ* hybridization in E12.5–P0 brain sections. At E12.5, *Shh* expression in ventral forebrain was reduced in the mutant. At E14.5, expression was unmodified in midbrain (circle), but strongly reduced in diencephalon (arrowhead) and telencephalon (arrow), particularly anteriorly (arrow, right). At P0, no expression was detected in the mutant lateral ventricle (asterisk). At both E14.5 and E17.5, the *Wnt3a* signal (arrowhead) was severely decreased in the mutant cortical hem. Scale bars represent 500 μm.

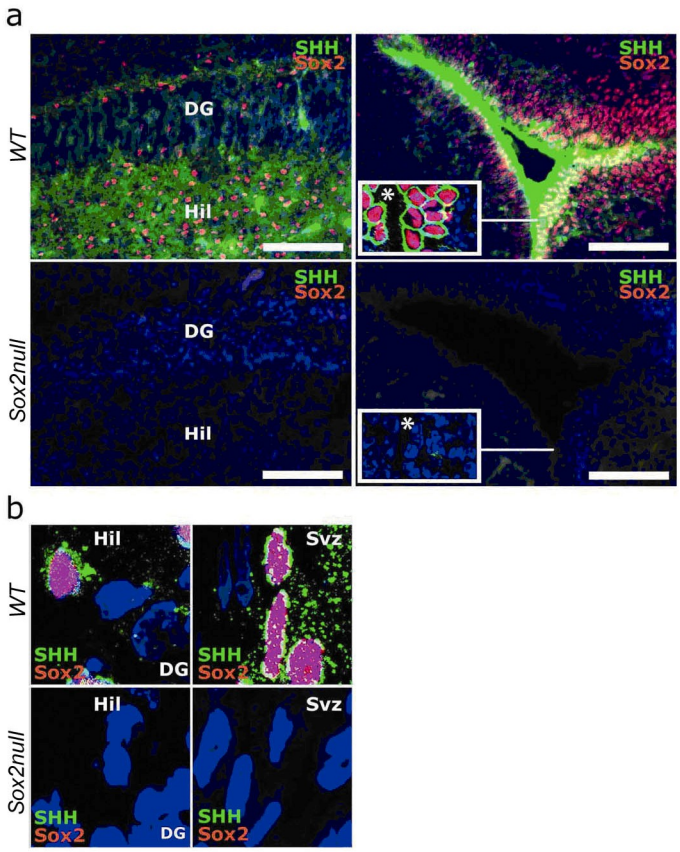


Figure 4 – SHH colocalizes with SOX2 in neural cells in postnatal neurogenic regions and its expression is lost in *Sox2*^{null} mutants. (**a**) SHH and SOX2 immunofluorescence on P0 neurogenic regions, dentate gyrus (left) and lateral ventricle (right). In normal dentate gyrus, SHH (green) was expressed along radial processes in dentate gyrus (DG) and in the underlying hilus (Hil), where abundant SOX2-positive nuclei (red) were seen. In the lateral ventricle, SHH was expressed in periventricular cells, together with SOX2. Expression was lost in *Sox2*^{null} mutants at both locations. Inserts, higher magnification details, by confocal microscopy, of lateral ventricle walls from the lower ventricle region (see connecting line), showing chains of SOX2/SHH double-positive cells lining the ventricular space (asterisk) in wild type, and loss of SHH and SOX2 in mutant. Scale bars represent 100 μm. (**b**) Colocalization of SHH (green) and SOX2 (red) in cells of wild-type dentate gyrus, lateral ventricle subventricular zone (Svz), and absence of SHH in *Sox2*^{null} mutant cells (confocal microscopy; original magnification was 100x).

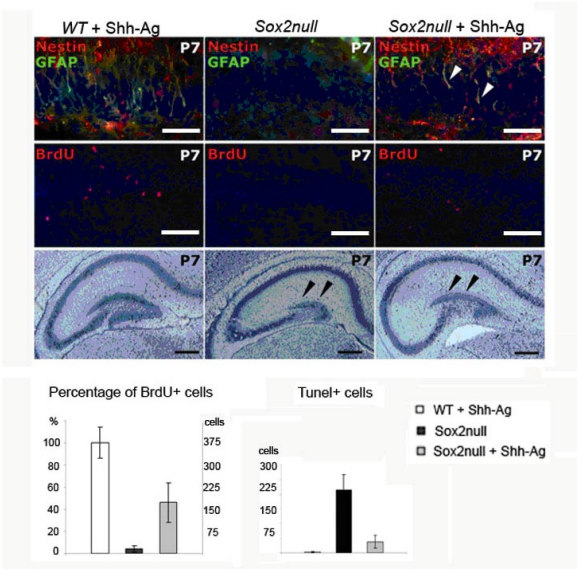


Figure 5 – Stimulation of the *Shh* signaling pathway rescues hippocampal NSC and neurogenesis in *Sox2* mutants. SHH-Ag was administered to *Sox2*^{null} or wild-type controls. Top row, GFAP and nestin double immunofluorescence, showing hippocampal radial glia NSCs; these were lost in *Sox2*^{null} hippocampi compared with wild types (see also Fig. 2a), but were substantially rescued by SHH-Ag (*Sox2*^{null} + SHH-Ag; arrowheads point to GFAP and nestin double-positive radial glia, scale bars represent 50 μm). Middle row, BrdU immunofluorescence. Scale bars represent 100 μm. Bottom row, thionine staining of hippocampus sections. The dentate gyrus of *Sox2*^{null} mutants, very poorly developed compared with wild types (see also Fig. 1), showed visible recovery of size in *Sox2*^{null} SHH-Ag–treated mutants (arrowheads). Scale bars represent 500 μm. Left histogram, quantitation of BrdU-positive cells (wild type + SHH-Ag is set to 100%). Histogram bars represent mean±s.d. values for five mice per genotype assayed for each stage and treatment (+SHH-Ag or untreated). BrdU labeling, essentially lost in *Sox2*^{null} mutant (see also Fig. 2b), recovered to about 50% of wild-type levels in SHH-Ag–treated mutants. Total numbers of BrdU-positive cells for WT + SHH-Ag (right *y* axis) did not differ substantially from those found with untreated wild-type mice (see experiment in Fig. 2b). Right histogram, quantitation of TUNEL-positive cells at P2. SHH-Ag reduced the apoptosis seen in the mutant hippocampus at P2 (see also Fig. 2).

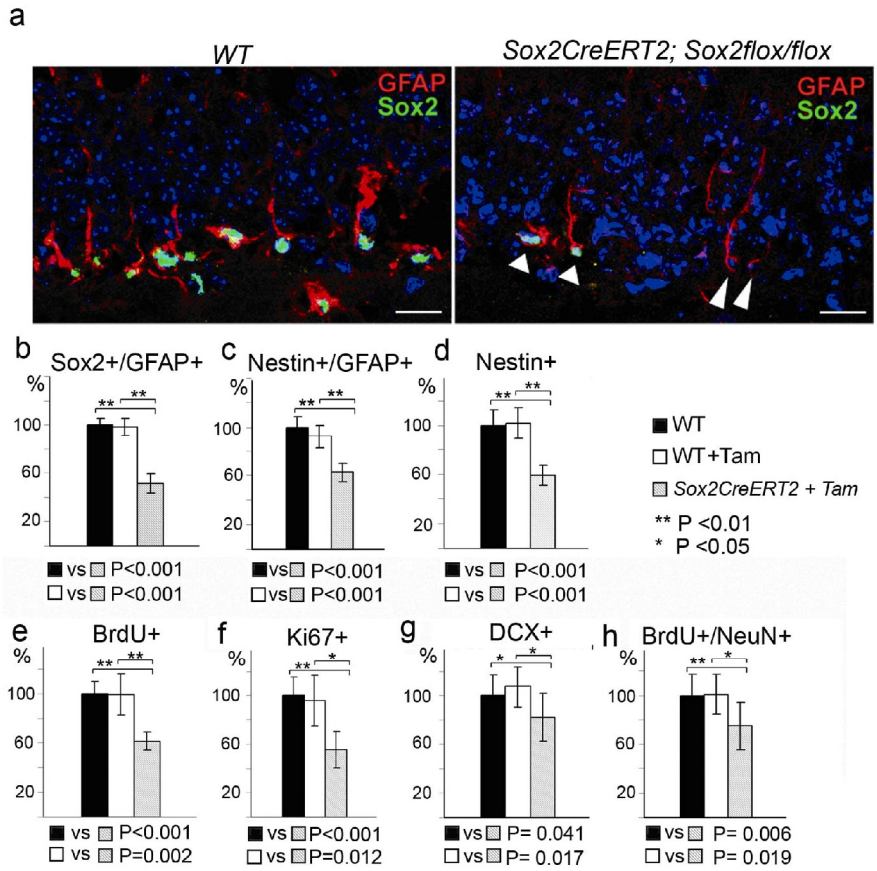


Figure 6 – *Sox2* deletion in adult brain leads to rapid loss of radial glia cells and of cell proliferation in the hippocampus dentate gyrus. *Sox2* was deleted via tamoxifen treatment of *Sox2*$^{loxP\Delta neo/loxP\Delta neo}$ adult mice carrying a *creERT2* transgene driven by the *Sox2* telencephalic enhancer-promoter (*Sox2-creERT2*)[11]. *Sox2-creERT2* induced efficient, tamoxifen-dependent activity in the expected expression domain[11] (in the hippocampus, the cells at the base of the dentate gyrus), as verified by breeding with a *ROSA26RloxP-stop-loxP lacZ* reporter of *Cre* activity (Supplementary Fig. 2 and data not shown)[20]. (**a**) GFAP and SOX2 double immunofluorescence on dentate gyrus of wild-type controls *Sox2*$^{loxP\Delta neo/loxP\Delta neo}$ without the *cre-ERT2* transgene (left) and tamoxifen-treated *Sox2-creERT2; Sox2*$^{loxP\Delta neo/loxP\Delta neo}$ mutants (right) (confocal microscopy). The vast majority of GFAP-positive radial glia cells in wild types were positive for SOX2 (green nuclei); in mutant, together with GFAP/SOX2 double-positive cells (broad arrowheads), some cells were GFAP positive, but SOX2 negative (thin arrowheads, SOX2-negative nuclei surrounded by GFAP-positive cytoplasm; see also Supplementary Fig. 3). Scale bars represent 20 μm. (**b**–**h**) Quantitation of dentate gyrus cells that were positive, by immunofluorescence, for the proteins indicated above each histogram. *P* values (Student's *t* test, two-tailed) are indicated below the histograms. Wild-type values were set to 100%. Histogram bars represent mean±s.d. values for seven mice for each genotype (wild type, wild

type + tamoxifen, *Sox2*$^{loxP\Delta neo/loxP\Delta neo}$*; Sox2-creERT2* + tamoxifen). Further controls (Supplementary Fig. 3 and data not shown), that is, *Sox2*$^{loxP/+}$*; Sox2-creERT2* and *Sox2*$^{loxP\Delta neo/+}$*; Sox2-creERT2* + tamoxifen, did not substantially differ from untreated wild type and wild type + tamoxifen. DCX, doublecortin.

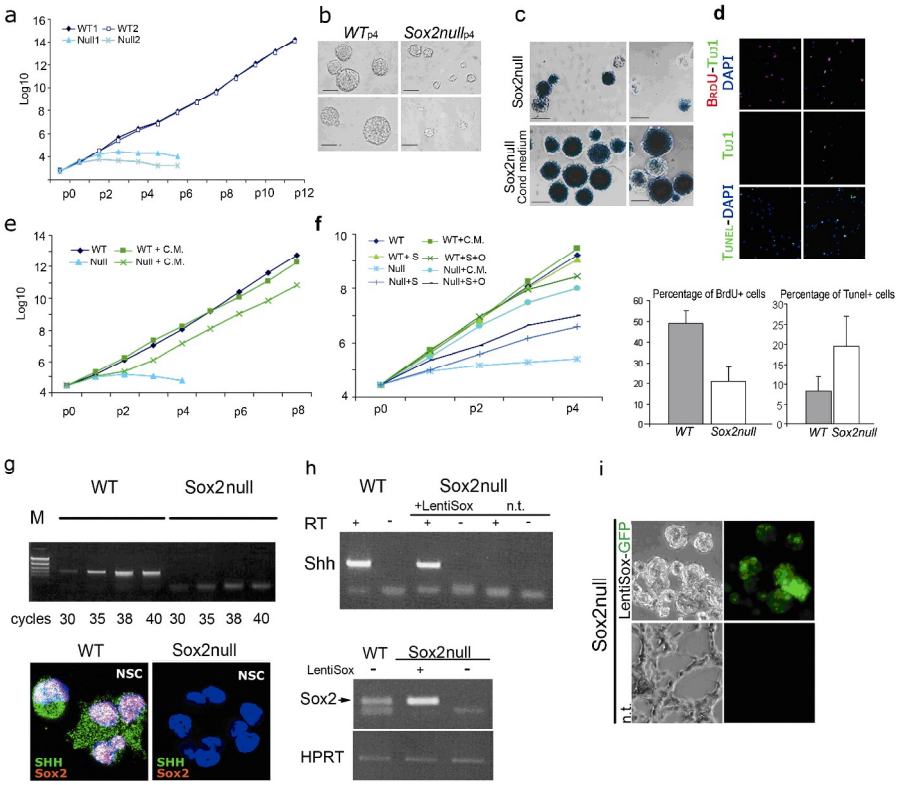


Figure 7 – Impaired maintenance of *Sox2*^{null} NSCs in culture and rescue by extracellular factors. (**a**) Growth in EGF of neurosphere cultures from two wild-type and *Sox2*^{null} P0 brains. Total cell numbers (Log_{10}) are reported over 12 passages (48 d). Data are representative of eight wild-type and eight mutant cultures. (**b**) Neurospheres from two wild-type and two *Sox2*^{null} cultures at passage 4 (p4). (**c**) X-gal staining of two *Sox2*^{null} cultures (p4) in normal medium (top) or in medium conditioned by wild-type cultures. Scale bars in **b** and **c** represent 100 μm. Blue color reflects the activity of *Sox2*$^{\beta\text{-}geo}$, indicating an undifferentiated state[5, 10]. Most *Sox2*^{null} spheres were partly or completely white; conditioned medium restored blue staining in most neurospheres and normal size. (**d**) BrdU (red), TuJ1 (green, β-tubulin) immunofluorescence and TUNEL (green) analysis of (passage 3) dissociated neurospheres and (histograms) percentage of BrdU- or TUNEL-positive cells in wild-type and *Sox2*^{null} cells. Original magnification was 50x. (**e**,**f**) Growth of wild-type and *Sox2*^{null} cultures in standard medium, medium conditioned by wild-type cells (CM) or in medium with SHH or SHH and oxysterols (S, S + O). We

observed a response to CM in 6 out of 6 and to SHH in 4 out of 5 mutant cultures. (**g**) Top, RT-PCR of *Shh* mRNA from wild-type and *Sox2*^{null} neurospheres. We detected no *Shh* mRNA in 2 out of 3 *Sox2*^{null} lines and very low levels in another line. Bottom, SOX2 and SHH immunofluorescence in wild-type and *Sox2*^{null} neurospheres. Original magnification was 200x. (**h**) Top, RT-PCR of *Shh* mRNA in *Sox2*^{null} neurospheres either transduced with *Sox2-gfp*–expressing lentivirus (LentiSox) or not transduced (NT), and harvested after 36 h, versus wild-type control. Bottom, RT-PCR of *Sox2* mRNA and *Hprt* (for normalization) in the same cultures. (**i**) Bright field and GFP fluorescence of *Sox2-gfp* lentivirus–transduced *Sox2*^{null} (top) and nontransduced cultures (bottom) 1 week after transduction. Magnification as in **b** and **c**.

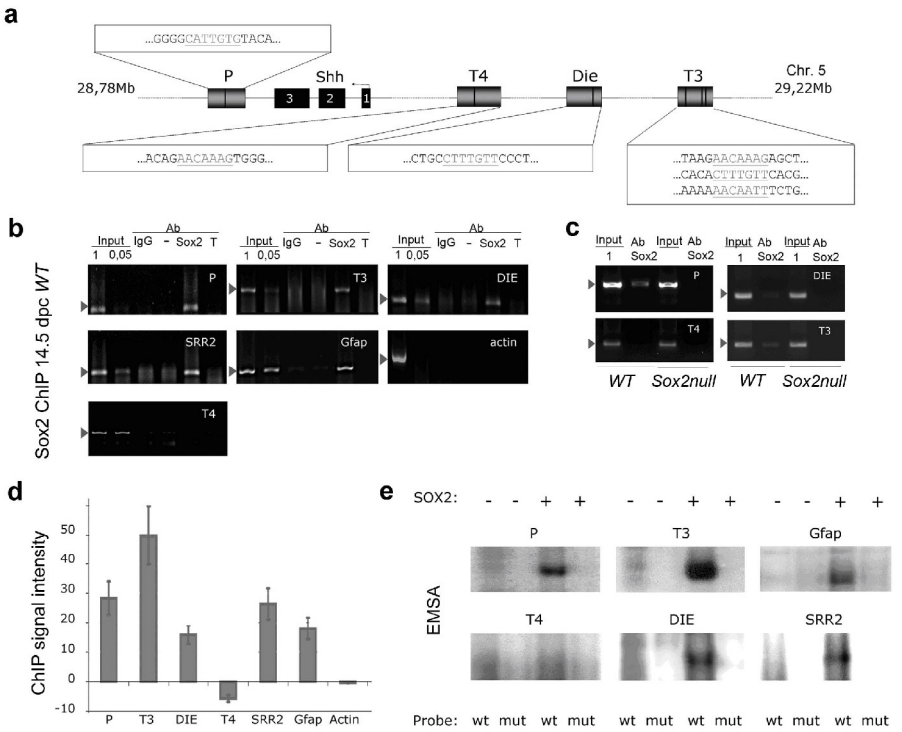
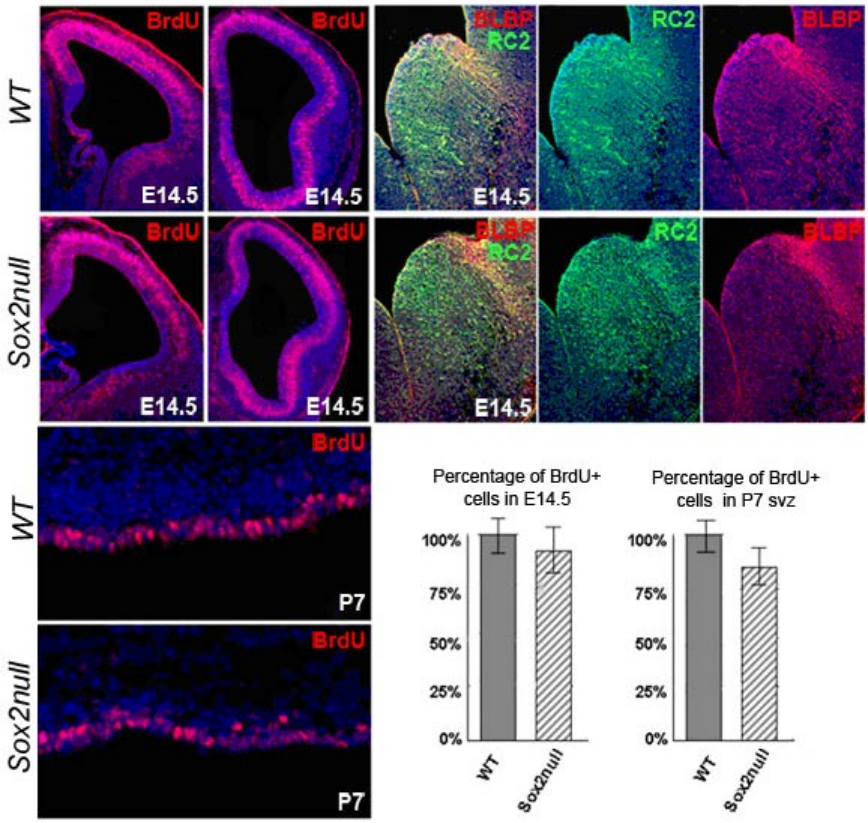


Figure 8 – *Shh* is a direct target of SOX2. (**a**) *Sox2* consensus sites in flanking/regulatory regions of the *Shh* gene. Black boxes, *Shh* exons; gray boxes, regions tested by ChIP with SOX2 antibodies; vertical lines, *Sox2* consensus. T3, Die and T4 overlapped with *Shh* regulatory elements active in telencephalon (T3, T4) or diencephalon (Die)[31]. P is a region downstream of *Shh*. (**b**) SOX2 ChIP, PCR of SOX2 antibody–precipitated E14.5 embryonic brain chromatin with primers amplifying regions shown in **a**. *Srr2* and *Gfap* (positive controls) are identified SOX2 targets[11]. Actin is a negative control[11]. Input: input chromatin aliquots. ChIP with unrelated (antibody to SV40T, or pre-immune IgG) or no antibodies (-) were negative controls. (**c**) Control SOX2 ChIP on *Sox2*^{null} brain chromatin, compared

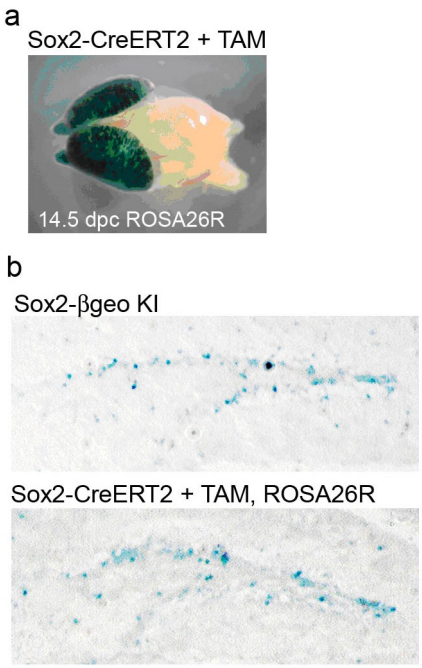
with wild type. Ab, SOX2 antibody; In, input chromatin aliquots. (**d**) Quantitation of SOX2 ChIP experiments. Values are ratios between PCR signal intensity of the SOX2 antibody–precipitated sample and that of the three negative controls (average). Results are average of three independent experiments. (**e**) EMSAs with nuclear extracts from COS cells that were (+) or were not (-) transfected with a *Sox2*-expressing vector[11]. Probes contain the sites indicated in **a** (T3 only the two more downstream sites), as normal (WT) or mutated (mut) versions. *Gfap* and *Srr2* are positive controls[11]. P, Die and T3 were bound by SOX2 and T4 was not, consistent with our ChIP data.

Supplementary figures

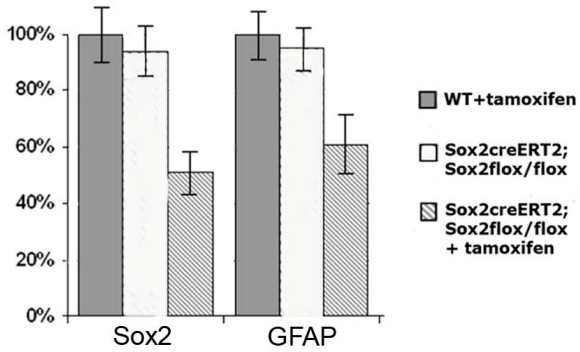


Supplementary Figure 1 – BrdU incorporation and RC2+ and BLBP+ radial glia cells are not significantly altered in E14.5 embryonic brain, nor in early postnatal (P7) subventricular zone, of *Sox2*-null (*nestinCre*-deleted) embryos. Top: BrdU immunofluorescence (left; telencephalon coronal sections at two different levels) and RC2/BLBP double immunofluorescence (right; telencephalon coronal sections, ganglionic eminence region) of E14.5 wild type (WT, first row) and *Sox2*-null (second row) embryos. Quantitation of BrdU-positive nuclei is shown in the

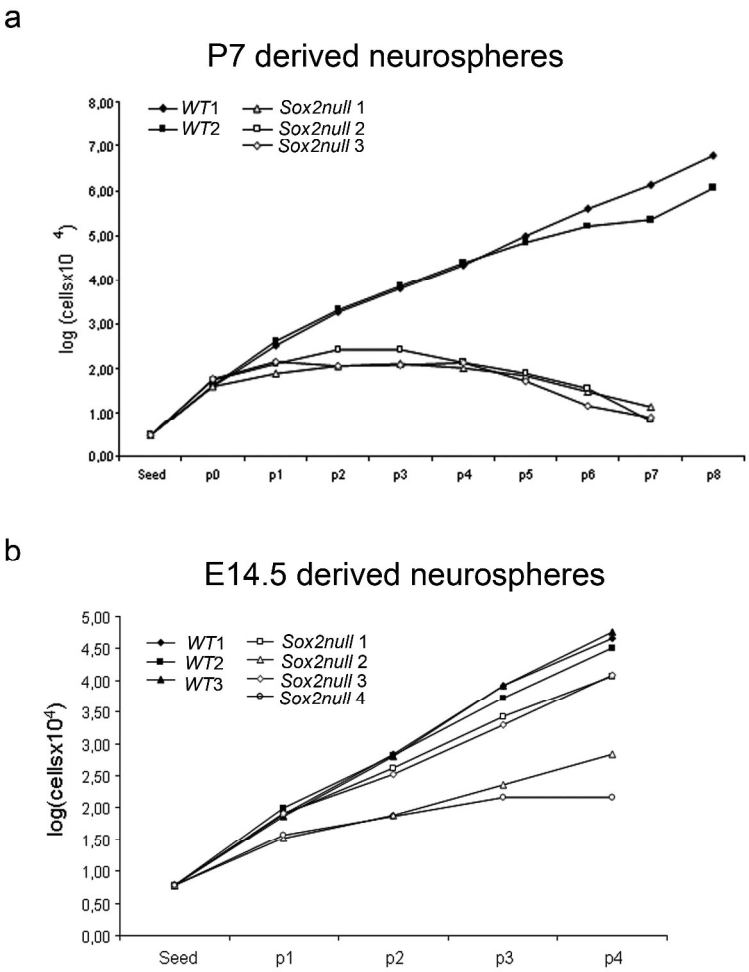
corresponding histogram. Bottom left: BrdU immunofluorescence of P7 WT and *Sox2*-null lateral ventricle region. Quantitation of BrdU-positive nuclei is in the corresponding histogram (bottom right) (histograms represent mean±s.d. values; WT average is set =100%).



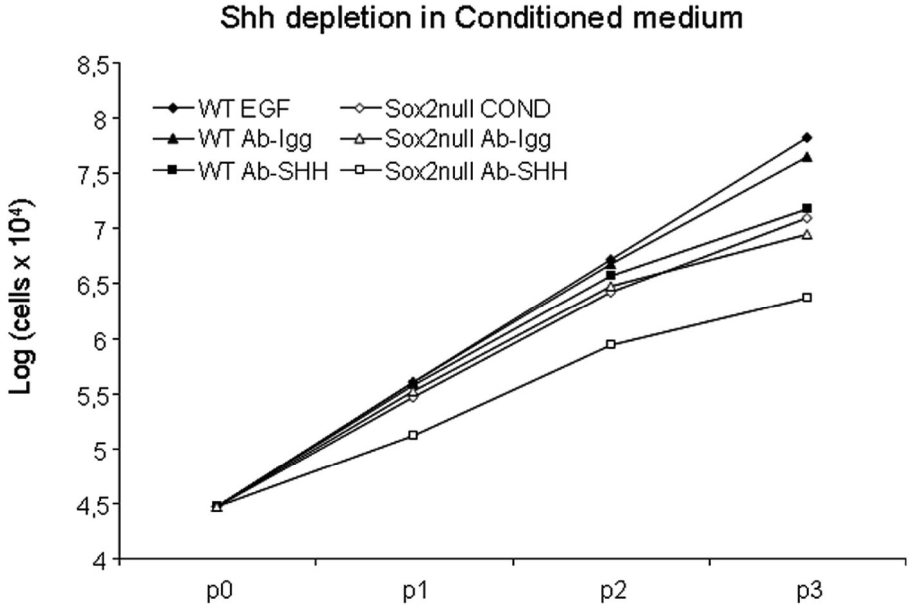
Supplementary Figure 2 – Activity of the *Sox2CreERT2* transgene. Activity was assayed by breeding *Sox2CreERT2* transgenic mice to the *ROSA26R* reporter of *Cre* activity strain, and assaying X-gal staining in the progeny following tamoxifen treatment. (**a**) Activity at E14.5 is specifically seen in the telencephalon, precisely mirroring the reported activity of the *Sox2* promoter/enhancer used to drive the transgene (described in Ref. 5). (**b**) Activity of the *Cre* transgene in the hippocampus dentate gyrus (bottom panel) is essentially superimposable to that of a *beta-geo* transgene "knocked-in" in the *Sox2* locus (top panel), and thus driven by the *Sox2* regulatory sequences[10].



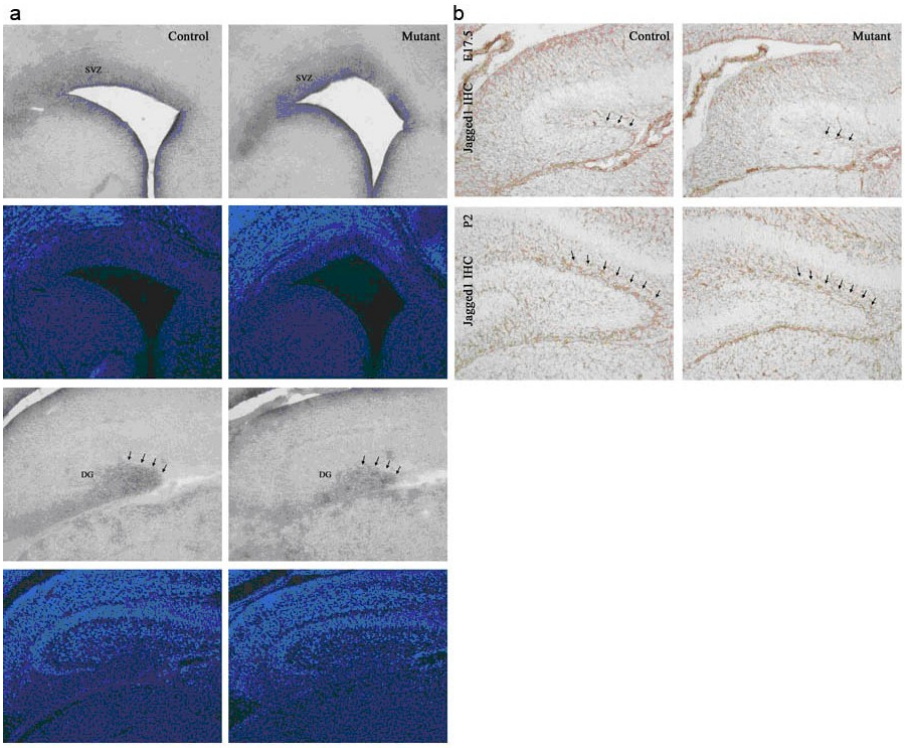
Supplementary Figure 3 – Adult *Sox2* deletion by *Sox2CreERT2* leads to reduction of SOX2-positive and GFAP-positive cells in the dentate gyrus of adult *Sox2*$^{flox/flox}$*; Sox2CreERT2* mice following tamoxifen treatment. Quantitation of hippocampus dentate gyrus immunofluorescence labelling with SOX2 (left) and GFAP (right) antibodies in adult mice of the indicated genotypes. The vast majority of SOX2-positive and GFAP-positive cells are located at the basis of the dentate gyrus (see **Fig. 6a**). SOX2-positive cells are reduced, specifically in *Sox2*$^{flox/flox}$*; Sox2CreERT2* mice, by about 50%, whereas GFAP-positive cells are reduced by about 40%. Results are from n=5 WT (i.e. *Sox2*$^{flox/flox}$ without *CreERT2* transgene) (+Tam), n=6 treated mutants (*Sox2*$^{flox/flox}$*;Sox2CreERT2*+Tam) and two untreated mutants (*Sox2*$^{flox/flox}$*; Sox2CreERT2*). No significant difference is found between any of the controls; further controls (**Fig. 6** and not shown), i.e. untreated WT and *Sox2*$^{flox/+;}$*Sox2CreERT2*+Tam, did not differ from untreated *Sox2*$^{flox/flox}$*;Sox2CreERT2* or WT+Tam.



Supplementary Figure 4 – NSC cultures from P7 and E14.5 *Sox2*-null (*nestinCre*-deleted) mutants show initial growth followed by growth decline. (**a**) Growth curves for P7 cultures show initial growth, followed by exhaustion starting between passages 1– 4, as seen with P0 cultures (**Fig. 7**). (**b**) E14.5 cultures are more variable, as some stop growth around passage 4 while others continue to grow at lower rates than normal for some passages (not shown). This suggests that *Sox2* dependence of *in vitro* growth might become more pronounced between E14.5 and P0.



Supplementary Figure 5 – Anti-SHH antibody reduces the ability of conditioned medium from wild type cells to rescue growth of mutant cells, and causes slight decline also of wild type cells growth. Growth curves of wild type (WT) and *Sox2*-deleted (*Sox2null*) neurosphere cultures; *Sox2null* cells were cultured in medium conditioned by WT cells (see **Fig. 7**) (*Sox2null* COND), in the same conditioned medium with added anti-SHH antibody (4 μg/ml[26] (*Sox2null* Ab-SHH), or indifferent isotype control antibody[26] (*Sox2null* Ab-Igg). WT cells were cultured in EGF-supplemented medium (EGF)(**Fig. 7**), or in the same medium supplemented with anti-SHH or anti-Igg antibody, as for the mutant. A slight though significant growth decrease is seen in mutant, and (to a lesser degree) wild type cultures treated with Ab-SHH, but not with the control Ab-Igg antibody. Toxicity prevented the use of higher antibody concentrations.



Supplementary Figure 6 – Expression of *Notch* signaling pathway genes is not significantly altered in *Sox2*-deleted E17.5 and postnatal hippocampus and lateral ventricle. (**a**) *Hes5* in situ hybridization (ISH) on E17.5 wild type (control) and *Sox2-deleted* (mutant) lateral ventricle/subventricular zone (SVZ; top) and hippocampus dentate gyrus (DG; bottom). Top pictures of SVZ or DG panels show the ISH signal (purple/violet); below each picture is DAPI nuclear staining of the same section. *Hes5* ISH signal is visible in the SVZ and in the hilus/DG region (arrows), respectively, at similar levels in control and mutant. A very slight reduction is seen in mutant hippocampus. (**b**) *Jagged-1* immunohistochemistry (IHC) (brown) in E17.5 and P2 hippocampus. The signal is seen in the developing control and mutant hippocampus (arrows), at similar levels, though a slight reduction can be appreciated in the mutant.

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DISCUSSION AND FUTURE PERSPECTIVES

§ 1. Why study Sox2?

1.1. Sox2 is required to maintain neural stem cells and for neuronal differentiation

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). Later, in many different species, Sox2 is a marker of the nervous system from the beginning of its development (neural plate). As development proceeds, Sox2 is expressed in neural stem/progenitor cells residing in the ventricular zone of the developing brain and also in the neurogenic regions of the adult brain (Zappone et al., 2000; Ferri et al., 2004). Subsequently, Sox2 is down-regulated in the marginal zone where differentiating neurons reside (Miyagi et al., 2004). This suggest that Sox2 function is related to important aspects of the biology of the neural stem cells (NSC).

Strikingly, Sox2 can also recreate pluripotency in terminally differentiated cells, reprogramming them to iPS (induced pluripotent stem) cells, acting together with a very small number of other TFs (Takahashi and Yamanaka, 2006).

In our laboratory, we investigated the role of Sox2 on brain development and in neural stem cells (in vivo and in vitro) mede use of both hypomorphic (Ferri et al., 2004; Cavallaro et al., 2008) and conditional knock-out (Favaro et al., 2009) mouse models.

We observed that reduced level of Sox2 expression (hypomorphic mice) causes depletion of neural stem/precursor cells and cerebral defects including reduced cortex, and thalamo-striatal parenchymal loss, with epilepsy and motor/neurological problems (Ferri et al., 2004). GABAergic neurones were greatly diminished in number in newborn mouse cortex and in the adult olfactory bulb. By in vitro differentiation studies on NSC cultured from these mutant mice, we demonstrated that normal Sox2 levels are required for the proper neuronal differentiation of NSC: mutant cells produce reduced

numbers of mature neurons (in particular GABAergic neurons), but generate normal glia. (Cavallaro et al., 2008).

These data, by demonstrating a role for Sox2 in neuronal differentiation, will allow the identification of Sox2 targets important for neuronal differentiation, by functional rescue experiments. My preliminary results, point to Mash1 (a proneural basic-helix-loop-helix transcription factor) as an early target of Sox2. In fact, the transduction of Sox2-deficient NSC with a Mash1-IRES-GFP expressing lentivirus increased the number of mature neurones (severely impaired in mutant cells). Parallel work in the lab (V. Tosetti) support this hypothesis and seem to reveal that Mash1 is a direct transcriptional target of Sox2.

Again, rare cases of Sox2 deficiency in man are characterized by hippocampal abnormalities, epilepsy, micro/anophthalmia and motor problems (Fantes et al., 2003; Ragge et al., 2005; Sisodiya et al., 2006; Kelberman et al., 2006), also reported in our mutant mice (Ferri et al., 2004). Loss of GABAergic inhibitory neurones leads to epilepsy in mouse and man. Our observation of GABAergic neuron deficiency in mouse points to a plausible cellular basis for epilepsy in humans with SOX2 mutations (Cavallaro et al., 2008). However, other neuronal subsets remain to be tested for their Sox2 requirement. For instance, due to their primary role in Parkinson's disease, it will be important to evaluate if dopaminergic neurones are impaired in Sox2 hypomorphic mutant mice.

1.2. Sox2 and cell therapy

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.

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.

1.3. The stem cell niche as a target of novel therapies

Whit the complete Sox2 panneural deletion (conditional knock-out mice) we revealed an important and unexpected role of Sox2 in controlling the maintenance of NSC in their niche, via non-cell autonomous autocrine mechanisms (Favaro et al., 2009). In fact, neural ablation of Sox2 leads to loss of Shh and Wnt3a expression (two signaling molecules/growth factors that regulate patterning, proliferation and survival of NSC) from neuroepitelial cells. Further, the in vitro experiments suggests it is NSC themselves, or some of their early progeny, that control NSC maintenance by Sox2-dependent secretion of growth factors.

Stem cell populations are established in “niches”, specialized microenvironments that regulate how they participate in tissue generation, maintenance and repair (Scadden, 2006). The niche may also induce pathogenesis by imposing aberrant function on stem cells. Thus, the “niche” will become a novel target for the development of novel therapies to enhance the regenerative capacity of normal stem cells and limit their aberrant proliferation/differentiation. The fact that Shh is required for cell proliferation in the mouse forebrain’s SVZ stem cell niche (Palma et al., 2004), together with our observation that Sox2 controls Shh in developing brain, highlight a novel self-regulatory loop for stem cell regulation in the neural niche during development that could be deregulated in some diseases.

1.4. Sox2 and human diseases

More in general, the Sox2 mutant mice generated in our laboratory (hypomorphic and conditional knock-out mouse models), reproduce different aspects of the neurological disorders seen in Sox2-deficient patients. Indeed, Sox2 loss-of-function mutations have been detected, in heterozygosity, in patients affected by microphthalmia (small eye)

and anophthalmia (absent eye) (Fantes et al., 2003). A closer inspection revealed that, together whit micro/anophthalmia, these patients showed important central nervous system abnormalities, including epilepsy, hippocampal defects and pitutary defects (Ragge et al., 2005; Sisodiya et al., 2006; Kelberman et al., 2006).

Central nervous system abnormalities, including epilepsy and hippocampal abnormalities, are well mirrored in both mutant mice generated in our lab (Ferri et al., 2004; Cavallaro et al., 2008; Favaro et al., 2009) which are providing crucial tools to understand the cellular and molecular bases for these defects. For instance, the abnormal differentiation of GABAergic neurones in Sox2-deficient mice (Cavallaro et al., 2008) suggest an explanation for the epilepsy of mutant mice and Sox2-deficient patients.

1.5. Sox2 and cancer stem cells in brain tumors

In cancers, a small population of cells with stem cell properties (cancer stem cells) is responsible for the maintenance and growth of the tumor (Reya et al., 2001). In neural tumors, stem cells have been detected in glioblastoma, medulloblastoma and ependymoma. Thus, normal stem cells could be the origin of cancer stem cells or, alternatively, a more differentiated progeny may revert to a "stem-like" status, and give rise to cancer stem cells (Nicolis 2007). In the adult brain, residual stem cells are located in the hippocampus, in the subventricular zone and possibly in the cerebellum. Thus, also the loss of normal stem cell control by their environment ("niche") may be an important component of the emergence of cancer stem cells (Nicolis 2007).

Genes originally identified as important for normal neural stem cells, may be essential also to support cancer stem cells. Often, in brain tumors of man and mouse, mutations target stem cell genes or genes lying in their functional pathway. Interestingly, several stem cell genes are often over-expressed in brain tumors, even if they are not mutated, suggesting that these genes may be important for the generation of cancer stem cells from more differentiated precursors, or for cancer stem cell maintenance.

Genetic alteration have been found in both Sonic hedgehog (Shh) and Wnt pathways, two important pathways for cell proliferation and differentiation during cerebellum development and involved in the

patogenesis of medulloblastoma. Bmi-1 and Sox2 represent an example of neural stem cell genes over-expressed in neural tuomrs, but that are not mutated. Bmi-1, a target gene of Shh, is often over-expressed in medulloblastoma, and the transcription factor Sox2 is over-expressed in primary neural tumors, as well as in tumor-derived stem cells.

Interestingly, Sox2 plays a critical role in neural stem cells and early precursors, and its reduced expression causes neurological defects together with impaired neuronal differenitiation (Ferri et al., 2004; Cavallaro et al., 2008). Importantly, Sox2 deficient cells have an abnormal differentiation, with co-expression of markers normally found in separate cell lineags, resembling the differentiated cells cultured from glioblastoma multiforme neural stem cells. Again, Sox2 reactivation is essential for the ability of oligodendrocyte precursors to reaquire stem cell properties upon PDGF treatment. Thus, Sox2 activation might represent an important step in the genesis of cancer stem cells during the reactivation of a "latent neural stem cell program" that persists in some committed precursors.

The cancer stem cell hypothesis implies a novel point of view on novel therapeutic approaches. Stem cell properties can be extinguished in neural cancer stem cells, together with expression of some stem cell genes. For example, under appropriate conditions, cancer stem cells efficiently differentiate in vitro into essentially normal neurones or glial cells, losing their stem cell properties and tumorigenicity, together with repression of Sox2, Bmi-1 and other stem cell genes (Singh et al., 2003, 2004 a-b; Lee et al., 2006). Also the stem cell niche environment in vivo may be able to prevent extensive stem cell replication, limiting their expansion. Studies on stem cells genes (as a novel target for cancer therapy) and on signaling patways may provide useful information to identify genes and molecules useful for differentiation therapy of neural cancer stem cells or for induce apoptosis of tumor cells (Hallahan et al., 2003). A probelm inherent in the use of drugs affecting stem cell genes functions, is that also normal stem cells can be targeted together with cancer stem cells. Thus, finding key molecular distinctions between normal and malignant stem cells will be essential and of use in designing therapies to specifically target malignant cells (Nicolis 2007).

In this regard, the Sox2 conditional knock-out mouse model, developed in our laboratory, could provide an useful means to address the requirement of Sox2 in vivo, for tumor establishment and

progression. In fact, we observed that the embryonic deletion of Sox2 in the forebrain causes loss of expression of the signaling molecules Shh and Wnt3a (Favaro et al., 2009), two genes that are deregulated in some neural tumors. Thus, the targeted suppression of Sox2 may lead also to the repression of other genes involved in the pathogenesis of brain tumors. We know that Sox2 is over-expressed in neural tumors and Sox2-deleted cells become rapidly exhausted, losing their proliferation and self-renewal properties: these data may suggest that Sox2 could have a role as potential target to be silenced by "differentiating therapies" of neural cancer stem cells. Another gene that may be important in the development of "differentiating therapies" of cancer stem cells is Emx2. Abolishing or, increasing Emx2 expression in adult neural stem cells greatly enhances or reduces their rate of proliferation, respectively. However, no alteration in their ability to give rise to neurones, astrocytes and oligodendrocytes, was observed (Galli et al., 2002). The functional antagonism of Emx2 and Sox2 on neural stem cells proliferation rate, and the observation that Emx2 is a transcriptional repressor of Sox2 expression in the forebrain, highlight a potential role of Emx2 in the exhaustion of the cancer stem cell pool. The over-expression of Emx2 could allow the repression of Sox2, together with the proper differentiation and exhaustion of cancer stem cells.

§ 2. Emx2 as a regulator of Sox2: a novel role of Emx2 in differentiation and cortical patterning?

The defective development of the hippocampus, together with the significant decrease in cotrex growth and patterning defects in Emx2 mutant mice (Grove et al., 1999; Tole et al., 2000; O'Leary et al., 2007 a-b) are the results of complex mechanisms. Although a direct patterning activity of Emx2 was already demonstrated by transgenic Emx2 over-expression (Leingartner et al., 2003), the cortex growth deficiency, failure of hippocampal development and, patterning activity, are at least in part explained by changes in gradients of diffusible factors (Grove et al., 1999; Mallamaci et al., 2000 a-b).

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: Oct4 in embryonic stem (ES) cells and Brn1/2 in neural stem/progenitor cells. We can assume that different POU-homeodomain transcription factors may act on a single binding site in the Sox2 5' and 3' enhancers to regulate their function through development and differentiation. Oct4 may contribute to Sox2 expression in totipotent ES cells acting at the POU sites in the Sox2 regulatory elements. In neural cells the same POU sites control the level of Sox2 expression by binding Brn1/2. 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.

In this work, we did not examine whether Emx2 antagonizes Sox2 functionally in the control of stem cells self-renewal/proliferation and differentiation. The ablation of Emx2 expression in neural stem cells enhances their rate of proliferation, and it is possible that Emx2 deficiency counteracts the deleterious effects of Sox2 deficiency on neural stem cells proliferation ability. Furthermore, we have shown that Sox2 deficiency impairs neuronal differentiation and it is possible that a simultaneous decrease of Emx2 (a Sox2 repressor) may antagonize this defect by rescuing Sox2 levels.

§ 3. Emx2: human diseases and tumorigenesis

Different studies revealed that the homeodomain-containing TF Emx2 is important in developing mouse brain. In fact, mouse Emx2 is expressed in the dorsal telencephalon and mice homozygous for Emx2

mutation have severe abnormalities in brain development, including small cerebral hemispheres and olfactory bulbs (Yoshida et al., 1997).

In humans, EMX2 mutation in the germline have been reported in several patients with schizencephaly, a rare disorder characterized by a thick cleft within the cerebral hemispheres, usually accompained by the absence of larg portions of the cerebral hemispheres (Brunelli et al., 1996; Faiella et al., 1997; Granata et al., 1997).

Studies in mouse have demonstrated that in addition to its function in the CNS, Emx2 is involved in the development of the urogenital system. Emx2 is expressed in the tissues that give rise to the kidneys, gonads, and genital tracts in both male and fimale mice. Mice with homozygous deletion of Emx2 lack kidneys and reproductive gonads, and die within a few hours of birth (Yoshida et al., 1997).

There are evidences that Emx2 also functions in adult vertebrates. In mouse, Emx2 is expressed in the two main neurogenic regions of the adult brain, regulating proliferation/differentiation of neural stem/precursor cells (Galli et al., 2002; Gangemi et al., 2001, 2006). In the adult newt, Emx2 is expressed during limb regeneration (Beauchemin et al., 1998).

Importantly, human Emx2 is expressed at high levels in the adult uterus, playing important roles during endometrial development. In particular, expression studies revealed that Emx2 level seems to be inversely correlated with the endometrial proliferation (Noonan et al., 2001). EMX2 mutation analysis in endometrial cancers revealed a few mutations within the three exons of the protein, and allelic deletions in the coding sequence region or in the 3'UTR of the Emx2 gene. Expression analysis in primary endometrial tumors and cancer cell lines showed that EMX2 levels were substantially lower in tumors than in normal endometrium, and that most of the cancer cell lines failed to express EMX2 (Noonan et al., 2001). The expression pattern of Emx2 in the normal endometrium (more abundant in non-proliferative than in proliferative endometrium) and in endometrial tumors (lower levels in malignant vs. normal endometrium) suggest an involvement of Emx2 in the control of differentiation and possibly in the tumor suppression, functioning as a negative regulator of cellular proliferation and as a tumor suppressor gene in the uterine endometrium.

As Emx2 over-expression has an anti-proliferative effect also on adult neural stem cells, would be important to evaluate if this gene has a more general function in tumorigenesis, acting as a tumor suppressor gene also in brain tumors.

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Università degli Studi di Milano-Bicocca

CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

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

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