

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

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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 number 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.

KEY WORDS: Sox2, Neural stem cells, Neurogenesis, Transcription factors

INTRODUCTION

Sox genes (Gubbay et al., 1990) encode transcription factors that regulate crucial developmental decisions (Kamachi et al., 2000; Wilson and Koopman, 2002; Wegner and Stolt, 2005). In mouse, Sox2 is expressed in, and is 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 anophthalmia (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/signaling 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 1.5×10^4 cells/cm² (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% fetal 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 immunofluorescence quantitation, see Fig. S2 in the supplementary material. Apoptosis was assayed by the DeadEnd 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 μ m; one every 16) were counted per animal.

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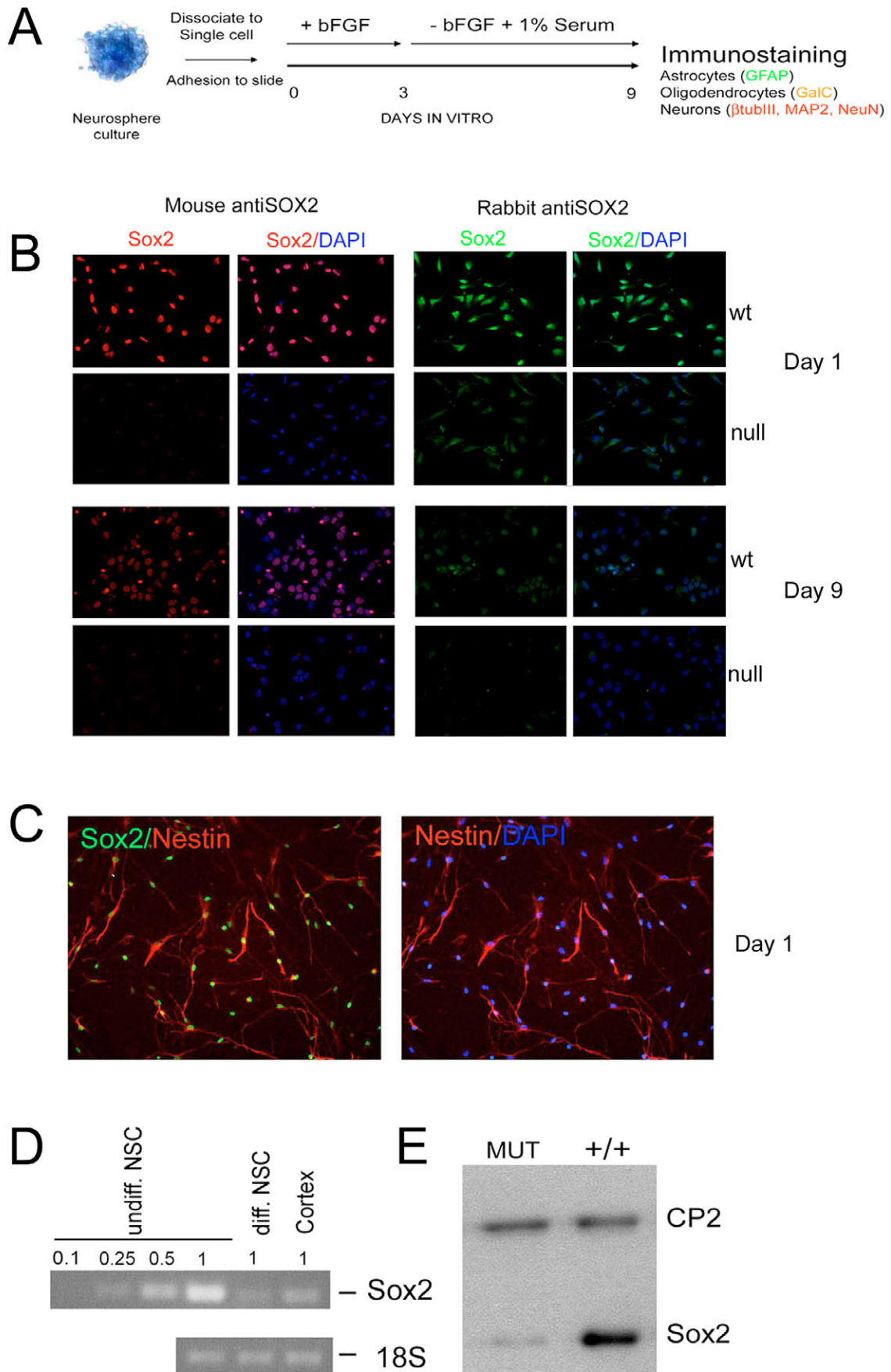


Fig. 1. See next page for legend.

Fig. 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.

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 (Biomedica 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, then 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'TTTCGGAAGTGGCCATGATTAAG3' and 5'AGTTTCAGCTTTCGAACCATACTCC3'.

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'AAAGAATTCCTGTGTAGTACAGGGTCTCTAG3' and 5'AAACTCGAGTACAGT-

GAATGGGTAATAAAAATA3'. 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 Gfp 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 (5×10^5), plated the previous day in 3 cm dishes, were transfected with 0.5 μ g luciferase reporter and 0.5 μ g 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 (*XhoI-Bsu36I* 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 3 hours, the plating medium was replaced with Neurobasal medium with B27, 1 mM glutamine, 5 ng/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* ^{β -geo}) together with a 'knockdown' allele (*Sox2* ^{Δ 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* ^{β -geo/ Δ 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

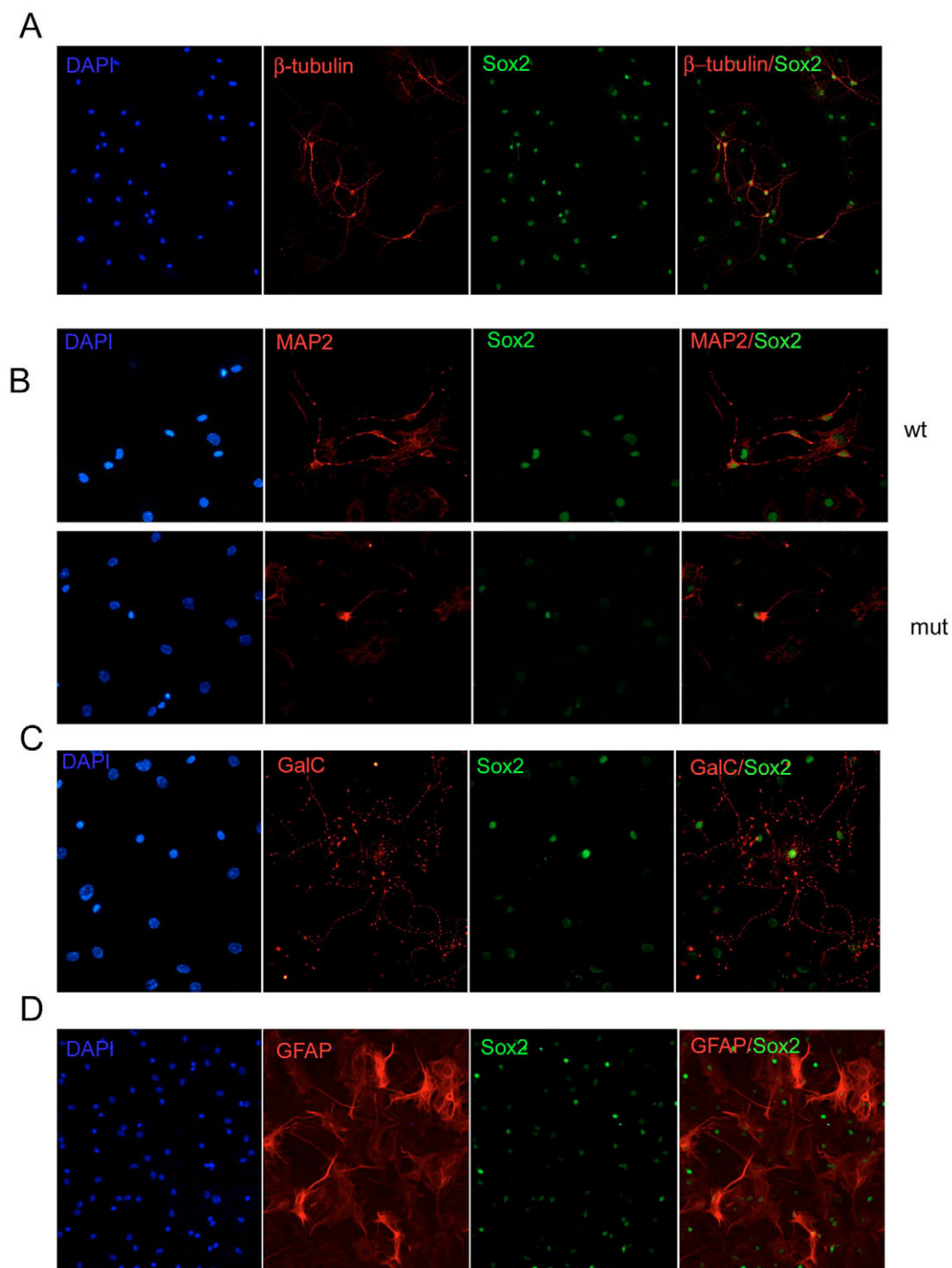


Fig. 2. Immunofluorescence for Sox2, neuronal and glial markers at differentiation day 9. (A) Sox2 and β -tubulin in normal cells. β -Tubulin-expressing cells show relatively high Sox2 positivity. (B) Sox2 and MAP2. Top: normal; bottom: mutant. MAP2-positive cells show significant Sox2 levels in both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

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.

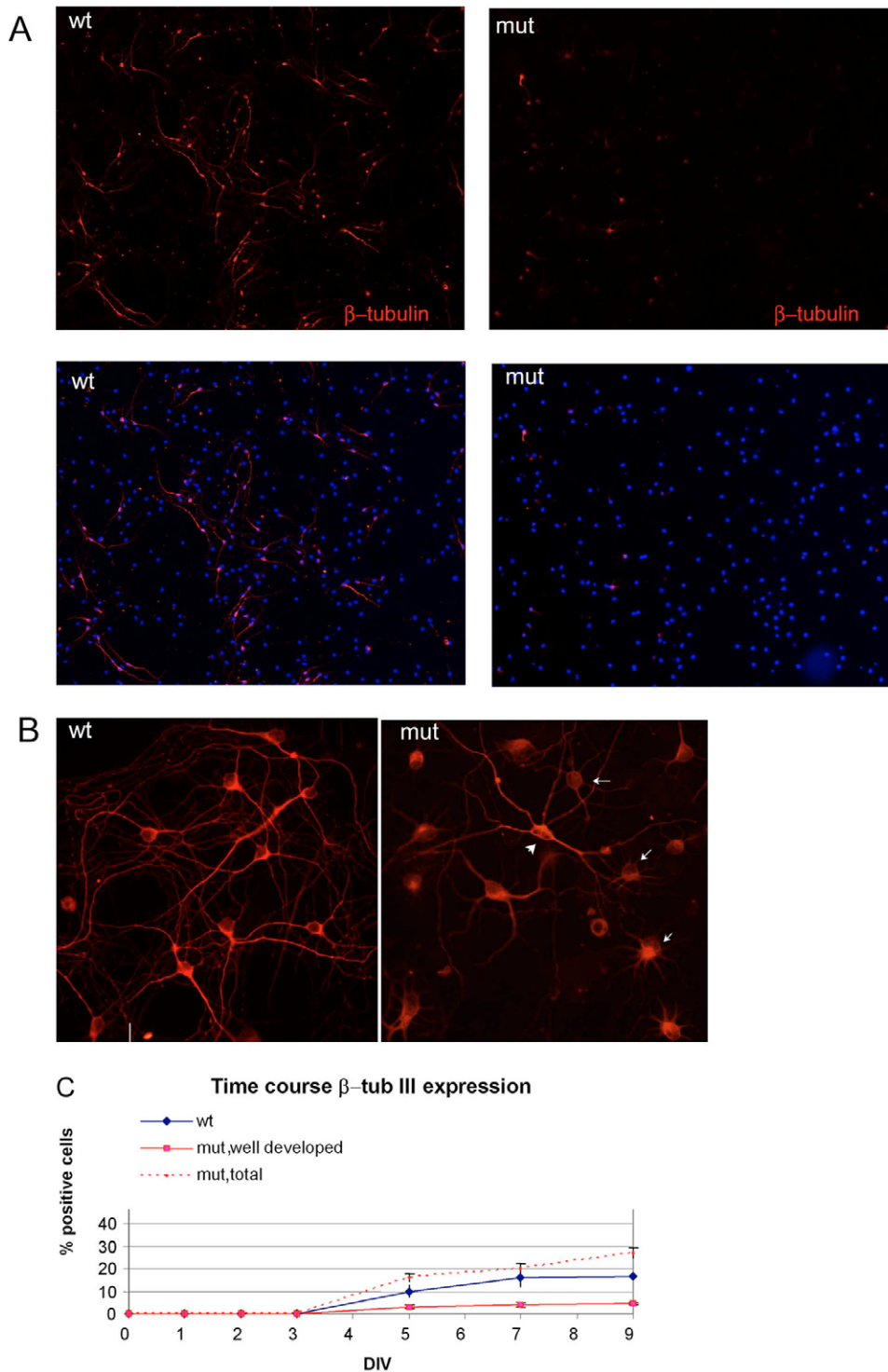


Fig. 3. β -Tubulin-positive cells are abnormal in differentiated Sox2 mutant cell cultures from adult mouse. (A) β -Tubulin

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.

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

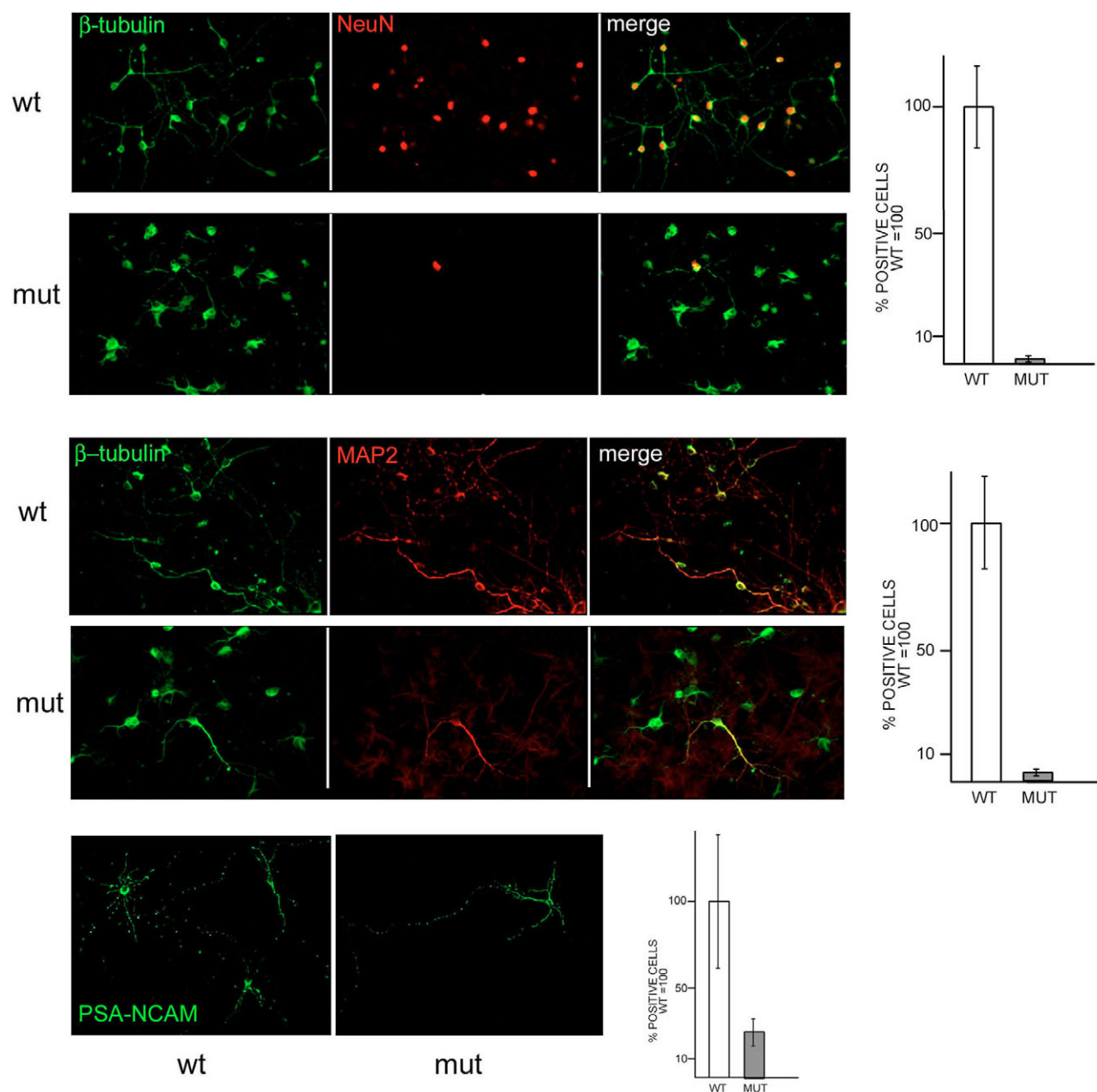


Fig. 4. Cells expressing mature neuronal markers are very reduced in differentiated Sox2 mutant cultures. Neuronal markers in normal and mutant cells at differentiation day 9 (NeuN/ β -tubulin, rows 1, 2; MAP2/ β -tubulin, rows 3, 4; PSA-NCAM, row 5). Most β -tubulin-positive cells in normal are positive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

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,

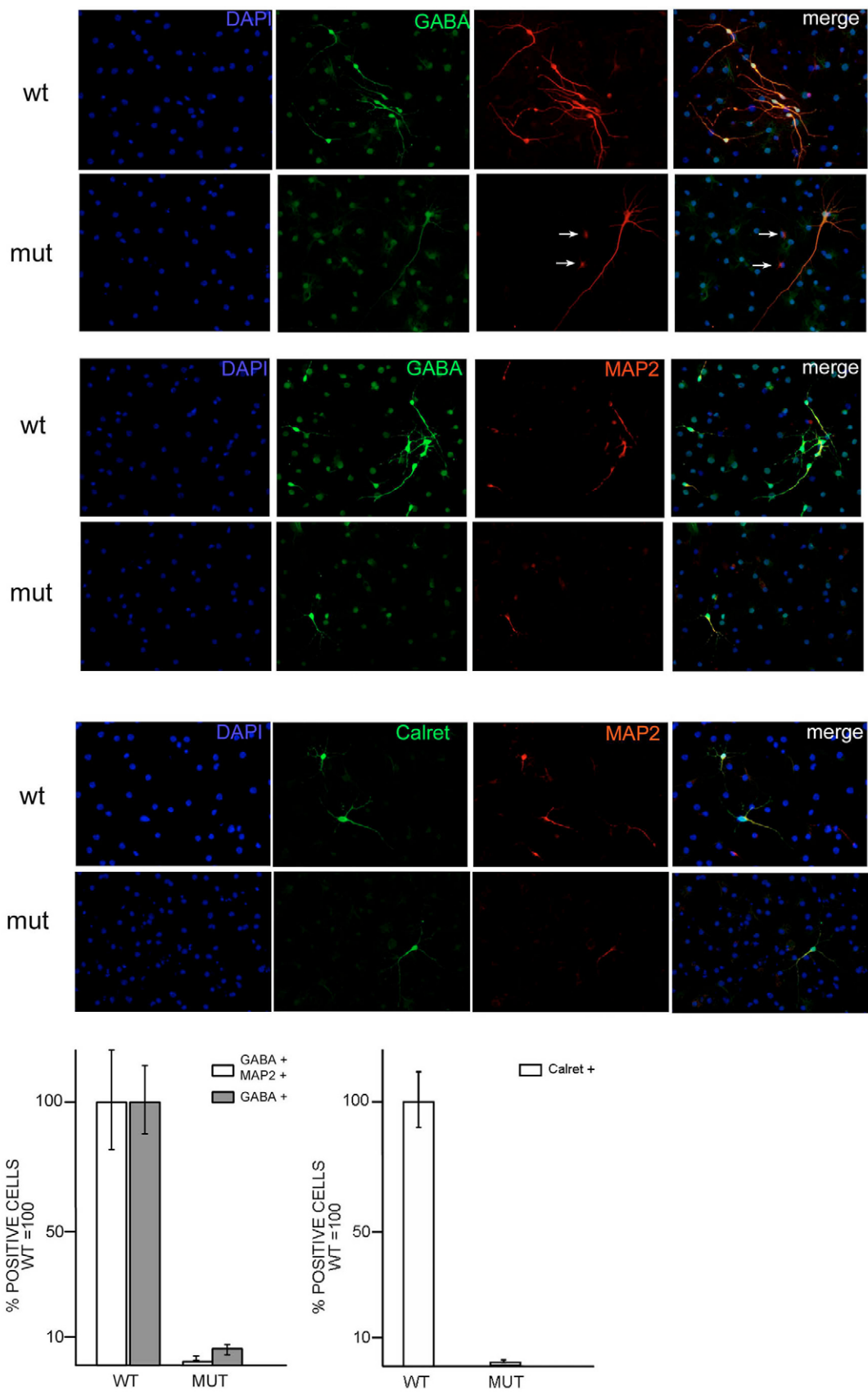


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Fig. 5. Cells expressing GABAergic markers are very reduced in differentiated Sox2 mutant cultures. Double-immunofluorescence with general neuronal markers (β -tubulin, rows 1, 2; MAP2, rows 3, 6; red), GABA (rows 1-4) and calretinin (5-6), in normal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

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

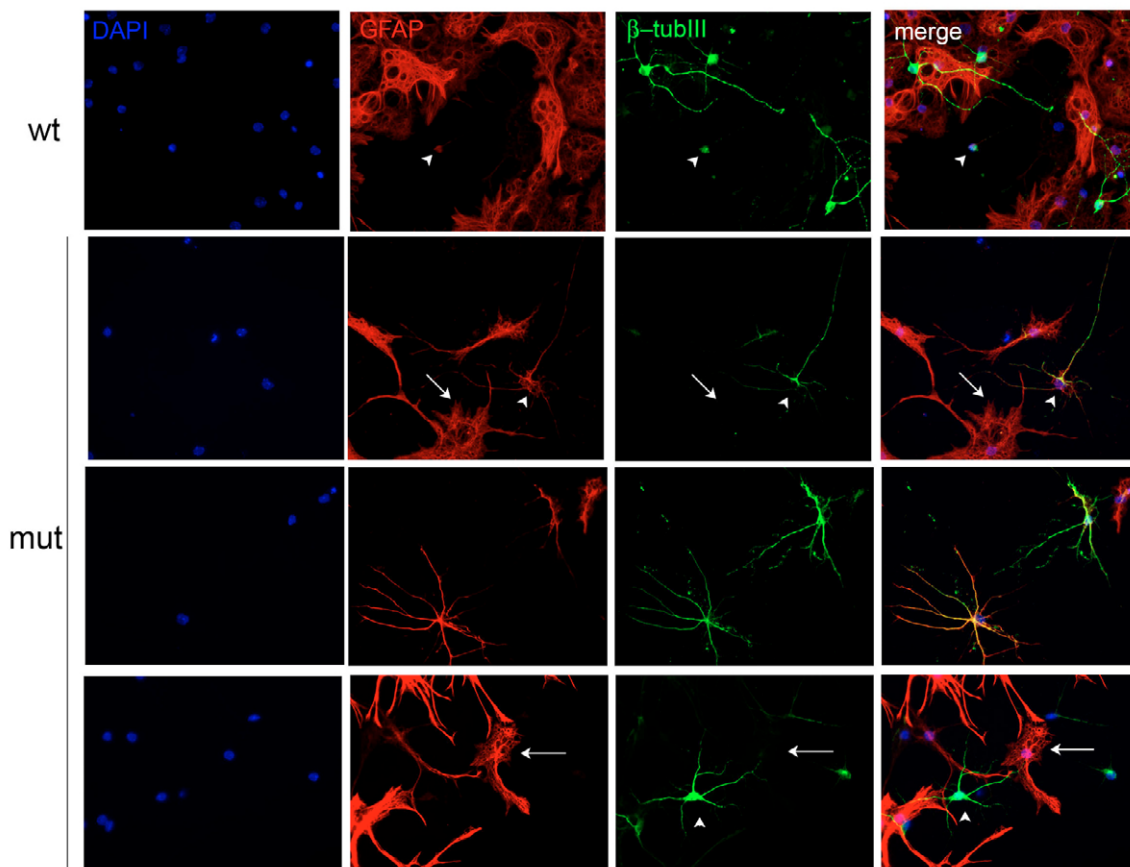


Fig. 6. Co-expression of neuronal and glial markers in individual cells in Sox2 mutant cultures. Double immunofluorescence (β -tubulin and GFAP) of normal (wt) and mutant (mut) day 9-differentiated cells. Typical wild-type neurons (β -tubulin positive) show extensive arborization, are closely associated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

Fig. 7. Rescue of neuronal maturation in mutant cells by lentiviral Sox2 expression at early stages of in vitro differentiation.

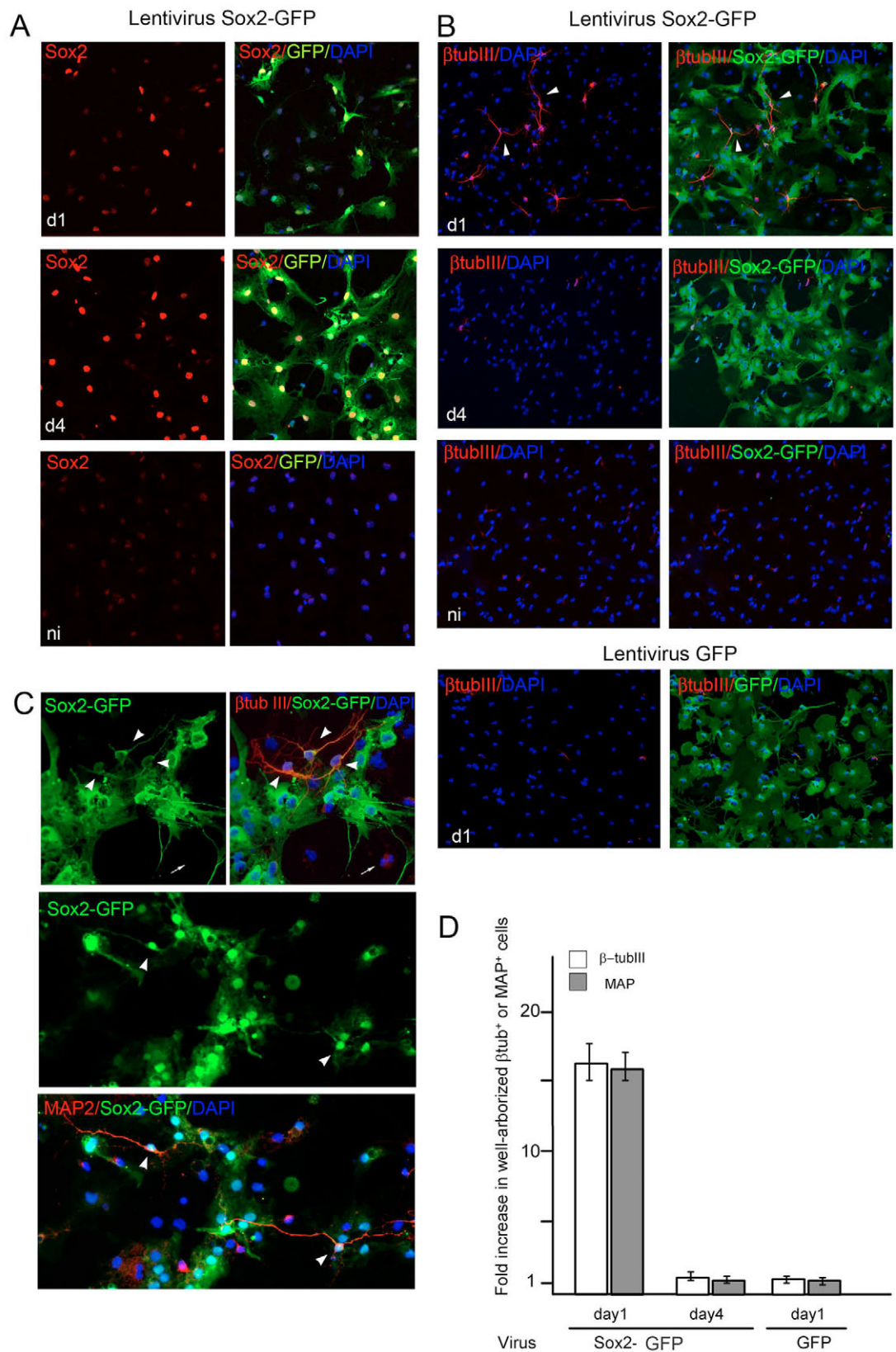
(A) Immunofluorescence for Sox2 (red) (R&D) and GFP (green), encoded by Sox2-IRES-GFP lentivirus, in cells infected at day 1 (d1) or day 4 (d4), compared with non-infected (ni) control.

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.



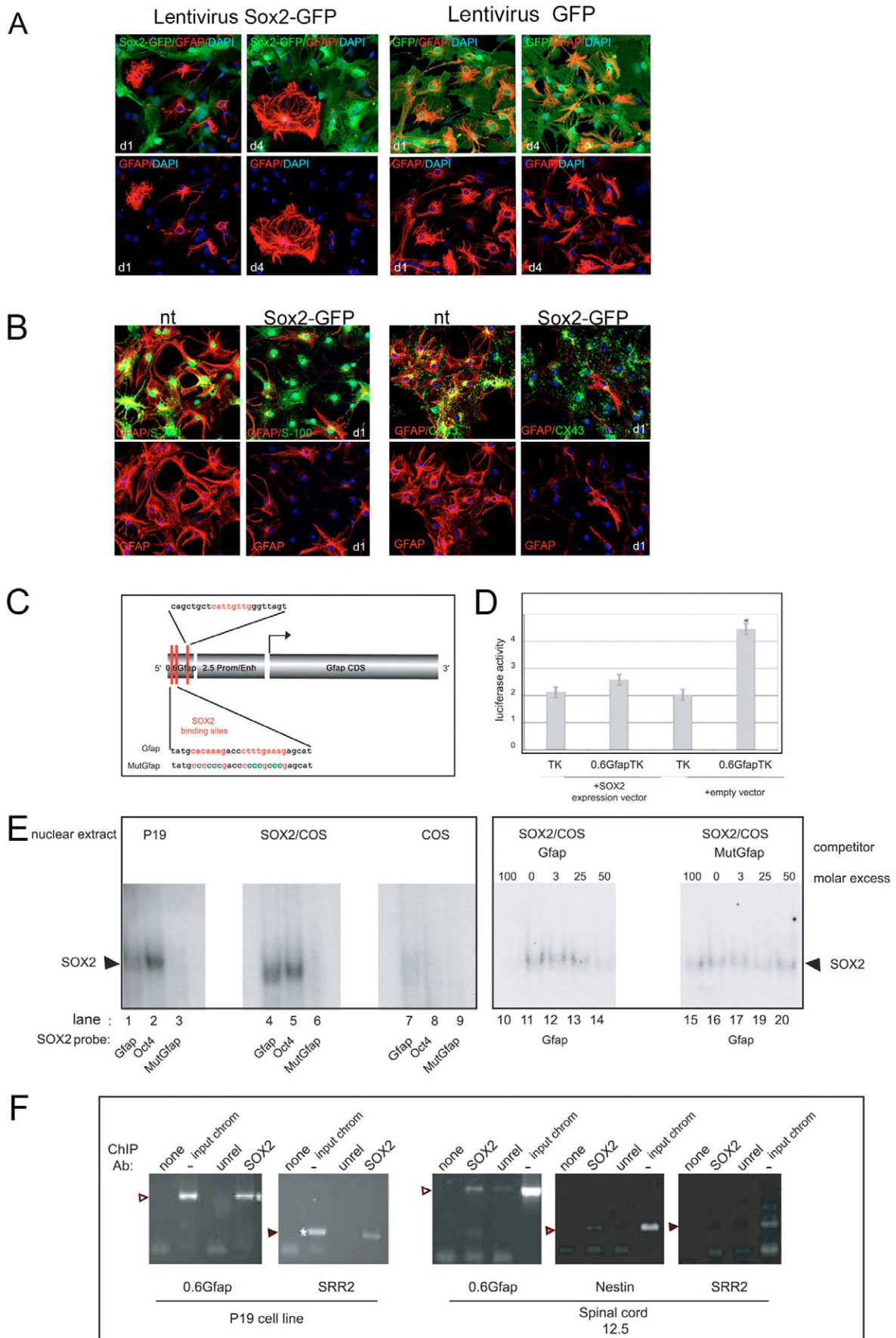


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Fig. 8. Sox2 regulates GFAP expression and directly interacts with upstream regulatory DNA sequences of the GFAP gene in vitro and in neural cells chromatin. (A) Sox2 overexpression in differentiating cells represses endogenous GFAP expression. Double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

(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

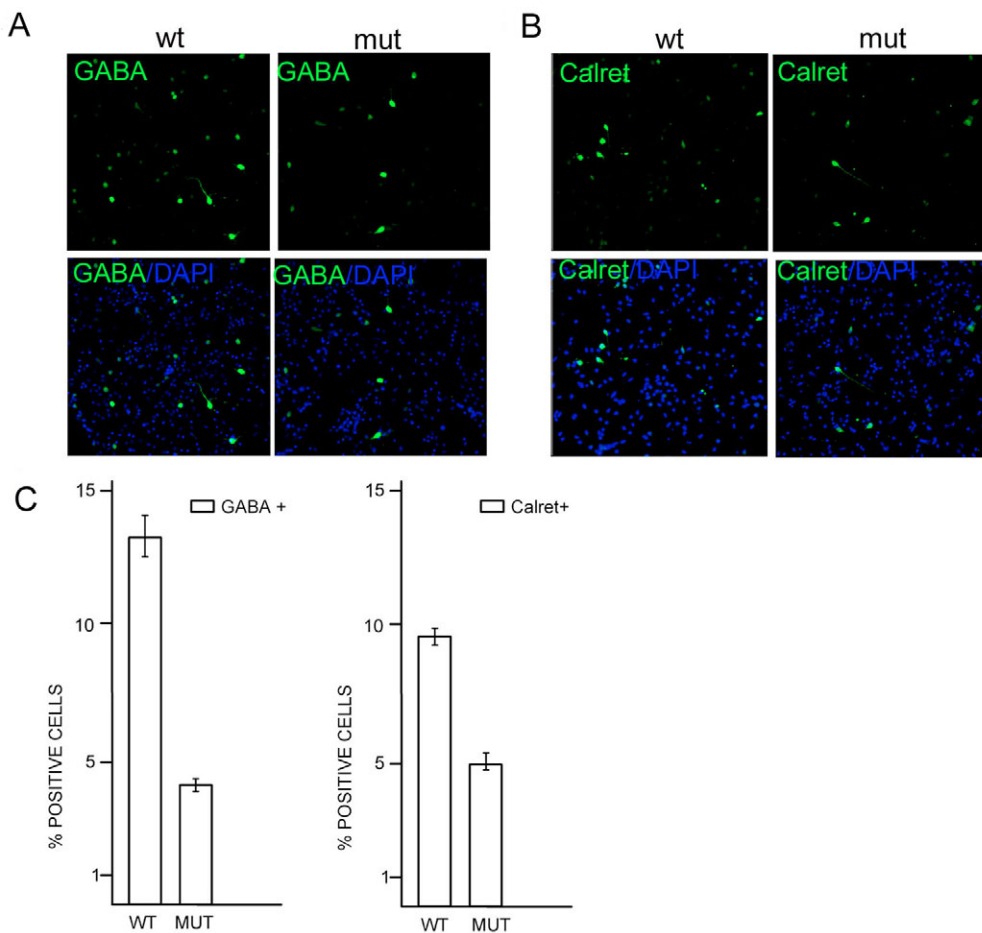


Fig. 9. Neurons expressing GABAergic markers are reduced in Sox2 mutant neonatal brains. (A, B) GABA (A) and calretinin (B) immunofluorescence of P0 cortical neurons (normal, left; mutant, right). Lower panels are counterstained with DAPI. (C) Percentage of GABA- or calretinin-positive cells in normal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

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 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 (Makram et al., 2004; Wonders and Anderson, 2006). In adult mutant cortex, calretinin-positive cells showed significant abnormalities, such as reduced

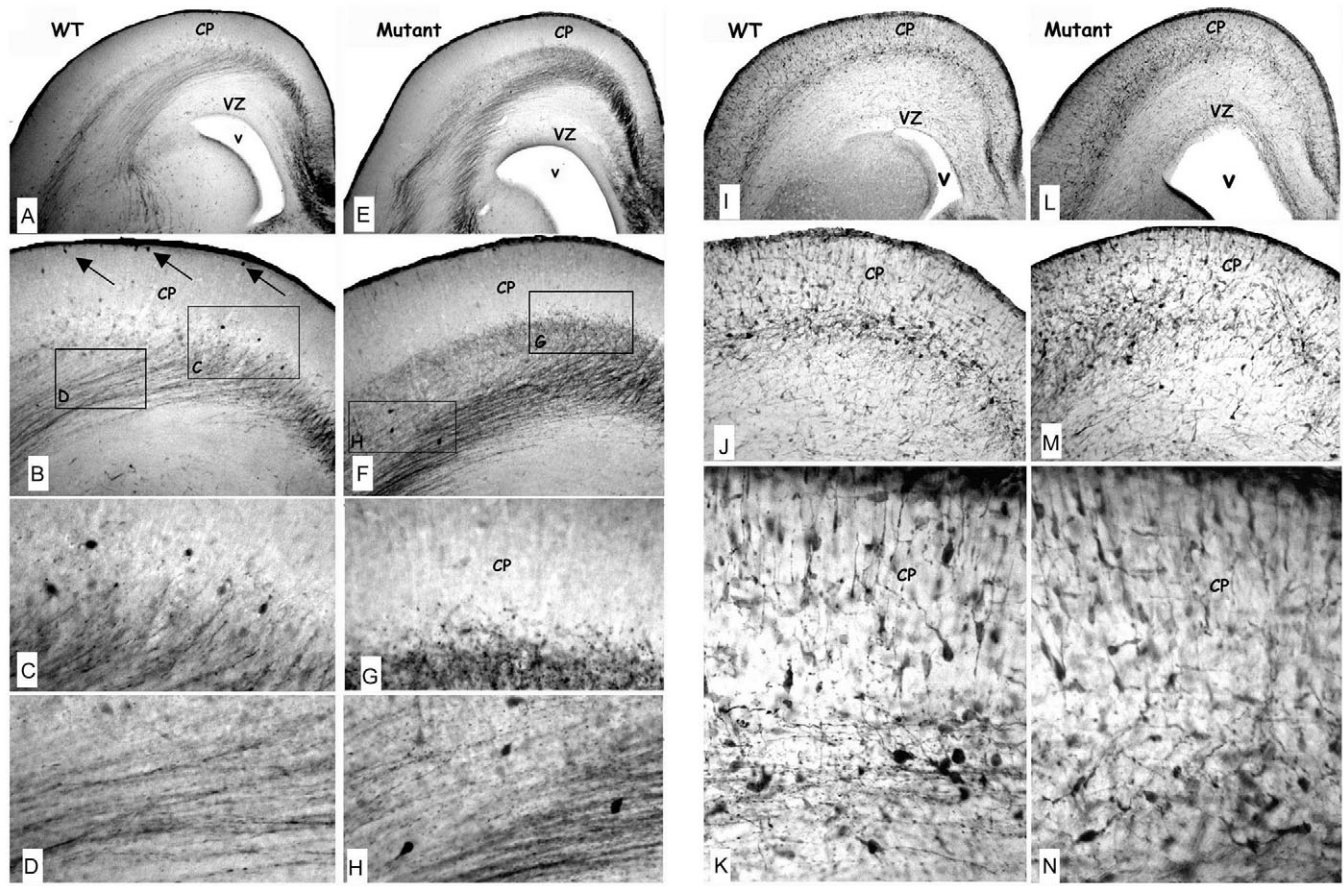


Fig. 10. Abnormal calretinin- and GABA-positive neurons in E17.5 mutant brain. Calretinin (A-H) or GABA (I-N) immunohistochemistry in sections from normal (A-D,I-K) and mutant (E-H,L-N) forebrains. (A,E,I,L) General views of normal and mutant forebrain sections (dorsal region). Lower panels show progressively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

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

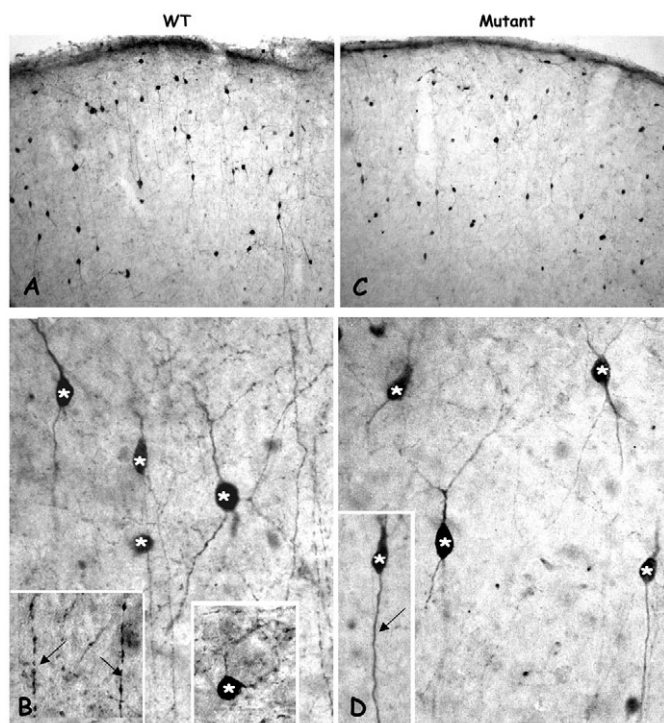


Fig. 11. Decreased frequency and arborization of calretinin-positive neurons in adult mutant somatosensory cortex.

(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).

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).

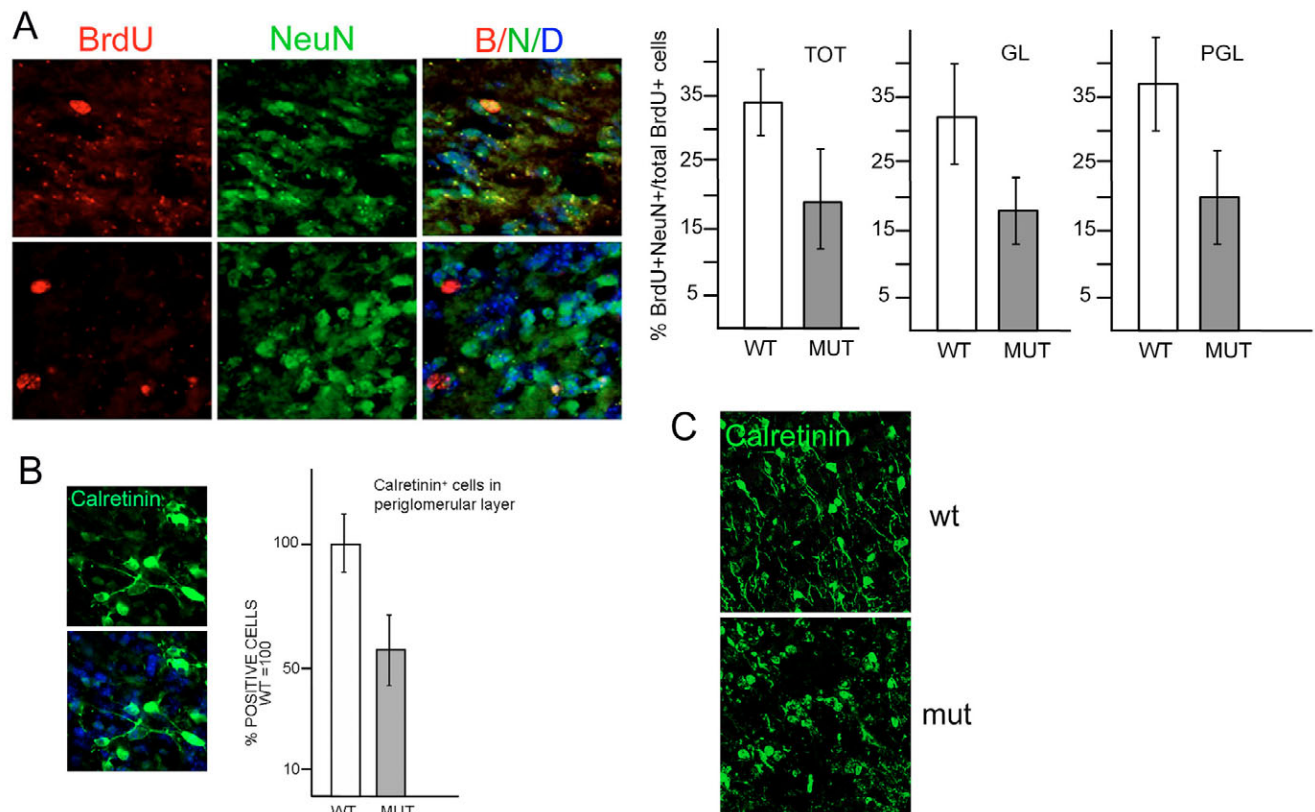


Fig. 12. Impaired neuronal maturation in adult olfactory bulb of Sox2 mutant mice. (A) Immunofluorescence of BrdU/NeuN-double positive (red and green, yellow in overlay; first row) and BrdU-single-positive (red only; second row) cells in olfactory bulb sections. Histograms: percentage of BrdU/NeuN double-positive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

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-Yaghoob et al. (Bani-Yaghoob 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 3-5) and extend preliminary *in vivo* evidence of loss of neural parenchyma and reduced maturation of postnatal neurons (Ferri et al., 2004).

From a quantitative point of view, the overall population in the P0 cortex and postnatal olfactory bulb is not as deeply affected as in the *in vitro* experiments. We suggest several, not mutually exclusive, explanations for this discrepancy.

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

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

Third, *in vitro* culture conditions, while allowing efficient differentiation of normal neural stem cells, might be subtly deficient relative to the *in vivo* environment. This might exaggerate the proportion of mutant Sox2 cells that fail to undergo appropriate differentiation *in vitro*. Indeed, *in vitro* not all differentiated markers are developed, and very few cells express appropriate electrophysiological properties, in contrast to *ex vivo* neurons (Gritti et al., 1996; Gritti et al., 2001).

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

Conclusions

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

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/3/541/DC1>

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