Sox2 is required for embryonic development of the ventral telencephalon through the activation of the ventral determinants Nkx2.1 and Shh

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SUMMARY
The Sox2 transcription factor is active in stem/progenitor cells throughout the developing vertebrate central nervous system. However, its conditional deletion at E12.5 in mouse causes few brain developmental problems, with the exception of the postnatal loss of the hippocampal radial glia stem cells and the dentate gyrus. We deleted Sox2 at E9.5 in the telencephalon, using a Bf1-Cre transgene. We observed embryonic brain defects that were particularly severe in the ventral, as opposed to the dorsal, telencephalon. Important tissue loss, including the medial ganglionic eminence (MGE), was detected at E12.5, causing the subsequent impairment of MGE-derived neurons. The defect was preceded by loss of expression of the essential ventral determinants Nkx2.1 and Shh, and accompanied by ventral spread of dorsal markers. This phenotype is reminiscent of that of mice mutant for the transcription factor Nkx2.1 or for the Shh receptor Smo. Nkx2.1 is known to mediate the initial activation of ventral telencephalic Shh expression. A partial rescue of the normal phenotype at E14.5 was obtained by administration of a Shh agonist. Experiments in Medaka fish indicate that expression of Nkx2.1 is regulated by Sox2 in this species also. We propose that Sox2 contributes to Nkx2.1 expression in early mouse development, thus participating in the region-specific activation of Shh, thereby mediating ventral telencephalic patterning induction.

KEY WORDS: Brain development, Sox2, Ventral telencephalon, Mouse, Neurogenesis, Sonic hedgehog, Nkx2.1

INTRODUCTION
The transcription factor Sox2 is necessary for the maintenance of pluripotency in epiblast and embryonic stem cells; its knockout is early embryonic lethal (Avilion et al., 2003; Masui et al., 2007). Later in development, Sox2 is required in various tissue stem cells and early progenitors, in particular in the nervous system (Que et al., 2009; Basu-Roy et al., 2010; Pevny and Nicolis, 2010). Throughout vertebrate evolution, Sox2 is expressed in the developing neuroectoderm from its earliest stages (Wegner and Stolt, 2005). In the embryonic nervous system, Sox2 marks undifferentiated neural precursor cells, including neural stem cells (NSCs). Postnatally, Sox2 is expressed in NSCs within the neurogenic niches of the subventricular zone (SVZ) and hippocampus dentate gyrus (DG) (Zappone et al., 2000; Ellis et al., 2004; Ferri et al., 2004; Suh et al., 2007). Sox2 is also expressed in some differentiating neural cells and neurons (Ferri et al., 2004; Taranova et al., 2006; Cavallaro et al., 2008).

Interestingly, heterozygous Sox2 mutations in humans cause a characteristic spectrum of CNS abnormalities, including eye, hippocampus, hypothalamus and basal ganglia defects, with neurological pathology including epilepsy and motor control problems (Fant et al., 2003; Kelberman et al., 2008; Sisodiya et al., 2006).

Sox2 gain-of-function and dominant-negative experiments established roles for Sox2 in the maintenance of NSC/progenitor cells in chicken and frog (Kishi et al., 2000; Bylund et al., 2003; Graham et al., 2003). Moreover, neonatal and embryonic NSCs grown in vitro from mice with a nestin-Cre-driven conditional ablation of Sox2 in the neural tube at embryonic day of development (E) 12.5 became prematurely exhausted in long-term culture experiments (Favaro et al., 2009).

Despite the severe in vitro defects of NSC maintenance, in vivo embryonic brain abnormalities in Sox2-nestin-Cre mutants are rather limited (Miyagi et al., 2008; Favaro et al., 2009); the only prominent defect is early postnatal failure to maintain hippocampal NSCs (radial glia) and neurogenesis, followed by loss of the hippocampal dentate gyrus. These defects were preceded by embryonic-perinatal loss of sonic hedgehog (Shh) expression in the telencephalon (but not in midbrain and in spinal cord), and could be rescued by a chemical Shh agonist (Favaro et al., 2009).

The reasons for the limited effects of Sox2 deletion on brain development remain unclear. Other Sox proteins, such as Sox1 and Sox3, which play roles similar to those of Sox2 (Bylund et al., 2003; Graham et al., 2003), might compensate in vivo for Sox2 absence. Alternatively, the timing of embryonic Sox2 deletion in previous experiments (Favaro et al., 2009) might have been too late, thus failing to uncover essential earlier functions of Sox2.

Here, we have used an early-acting Bf1 (Foxg1)-Cre transgene, which completely ablated Sox2 by E9.5 in the developing telencephalon, two days earlier than the deletion with nestin-Cre (Miyagi et al., 2008; Favaro et al., 2009). This caused defects much more severe than those observed with nestin-Cre (Miyagi et al.,...
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2008; Favaro et al., 2009). Unexpectedly, these defects were markedly region specific, with much more pronounced ventral than dorsal telencephalic alterations. The medial ganglionic eminence (MGE) was completely lost at E12.5, preceded by an earlier failure to express the ventral determinants Nkx2.1 (Nkx2-1) and Shh. Treatment with a Shh agonist (Shh-ag) in vivo was sufficient to rescue the ventral (MGE) phenotype to a significant, but not complete, extent. Furthermore, we show that Sox2 regulates Nkx2.1, a known direct activator of Shh (Jeong et al., 2006).

**MATERIALS AND METHODS**

**Mouse strains**

Sox2fl/fl mice (Favaro et al., 2009) were bred to Bfl-Cre mice (Hébert and McConnell, 2000) to obtain compound Sox2fl/fl; Bfl-Cre heterozygotes, which were bred to Sox2fl/fl mice to generate Sox2-deleted embryos. Bfl-Cre mice were maintained by brother-sister mating, and subsequently on a 129 background (Hébert and McConnell, 2000).

**Histology, in situ hybridisation (ISH), immunohistochemistry and Shh agonist treatment**

Histology, ISH and immunohistochemistry were carried out as previously described (Ferri et al., 2004; Favaro et al., 2009). Antibodies used were: anti-SOX2, anti-SOX1, anti-SOX3, anti-SOX9 mouse monoclonals (R&D Systems); anti-Nkx2.1 rabbit polyclonal (BIOAT); anti-SHH rabbit polyclonal (Santa Cruz); and anti-SHH mouse monoclonal [Developmental Studies Hybridoma Bank (DSHB)]. BrdU (Sigma B5002, 15 µg/ml in PBS) was administered to pregnant females at 6 µg/g body weight; females were sacrificed after 30 minutes. BrdU immunofluorescence and TUNEL analysis were carried out as described by Favaro et al. (Favaro et al., 2009) and Ferri et al. (Ferri et al., 2004), respectively.

Shh agonist #1 (Frank-Kamenetsky et al., 2004) was administered to pregnant females at E8.5 and E10.5, by oral gavage of a 1.5 mg/ml solution in 0.5% methylcellulose/0.2% Tween 80 at 100 µl/g body weight; females were sacrificed after 30 minutes. BrdU immunofluorescence and TUNEL analysis were carried out as described by Favaro et al. (Favaro et al., 2009) and Ferri et al. (Ferri et al., 2004), respectively.

**RESULTS**

**Sox2 early deletion severely impairs embryonic brain development**

To ablate Sox2 in the early embryonic brain, we bred mice carrying a Sox2fl/fl conditional mutation (Favaro et al., 2009) to mice expressing the Cre-recombinase gene under the control of the Bfl regulatory regions, specifically active in the developing telencephalon from embryonic day (E) 9.5 of development (Bflcre ‘knock-in’) (Hébert and McConnell, 2000). In Sox2fl/fl; Bflcre embryos, Sox2 protein was completely ablated by E9.5 in the telencephalon, though not in more posterior neural tube regions, as expected (Fig. 1A). This caused early morphological defects: at E12.5, telencephalic vesicles were reduced and the eyes were abnormal (Fig. 1B,C). Interestingly, although the whole telencephalon was affected, the ventral part was much more severely compromised than the dorsal one (Fig. 1C,F); histological sections (Fig. 1F) showed that the ventral primordia of the medial ganglionic eminence (MGE), involved in the generation of the basal ganglia (Sur and Rubenstein, 2005; Hébert and Fishell, 2008), were severely reduced (Fig. 1F, arrowhead). These initial defects developed into profoundly abnormal development, leading to death just after birth. At E18.5, mutant pups had a smaller head (Fig. 1E) and the telencephalon was smaller than in wild type (Fig. 1D,G: compare with the almost unaffected midbrain); also, the olfactory bulbs and the midline ventral structures were absent (Fig. 1D, black arrowhead pointing to ventral ‘hole’). In tissue sections, the ventral midline and the immediately adjacent territories were missing (Fig. 1G, arrowheads).

In agreement with the early MGE abnormalities, GABAergic cortical interneurons, which originate in the MGE and then migrate to more dorsal locations (Sur and Rubenstein, 2005; Hébert and Fishell, 2008; Elias et al., 2008), were strongly decreased in mutants, as indicated by the almost complete loss of somatostatin (SS)-positive and the strong reduction of the neuropeptide Y (NPY)-
positive subsets of neurons (Markram et al., 2004; Toledo-Rodriguez et al., 2005; Elias et al., 2008; Hébert and Fishell, 2008) (Fig. 1H). SS-positive interneurons originate from the (dorsal) MGE progenitors and require the Nkx2.1 transcription factor for their development (see below) (Hébert and Fishell, 2008; Butt et al., 2008; Flandin et al., 2011). NPY-positive neurons originate from the progenitor domain of the adjacent preoptic area (Gelman et al., 2009), which may be somewhat less severely affected.

Additional abnormalities included absence of the olfactory epithelium [Fig. 1F, asterisk in wild type (wt)] and face abnormalities: the nasal plate, normally developing a characteristic bilateral symmetry, was consistently centrally fused (Fig. 1E, arrows) and underdeveloped. Furthermore, the eyes were abnormal and extremely reduced in size (Fig. 1B,E,F) (see also Taranova et al., 2006); maxillary structures, e.g. the palate, were also abnormal (Fig. 1G); the cortex (Fig. 1B,D,G) was reduced; and the
hippocampus (at E18.5) was severely underdeveloped (not shown). None of the defects described above was seen in control mice (Sox2^flox/+; Bf1-Cre; Sox2^flox/+; Sox2^flox/flox) (not shown).

Early expression of ventral forebrain determinants is impaired in Sox2 mutants

We focused on the most severely affected region, the ventral telencephalon, to study genes known to be involved in its specification and development. We first analysed embryos by ISH at E12.5, when the morphological defect becomes overt, and at E11.5, when the defective morphology can first be appreciated. The Shh gene is expressed in the developing ventral telencephalon, and is crucial at early stages for the development of this region (Fuccillo et al., 2004; Sousa and Fishell, 2010). Furthermore, we had previously found that Shh is a Sox2 target, acting as its functional effector in postnatal hippocampal development (Favaro et al., 2009). By E12.5, Shh mRNA is completely absent in the midline region following the loss of the tissue expressing it, and is strongly downregulated in the amygdala region (Fig. 2A); in E11.5 mutant embryos, Shh is already severely downregulated in the medial ventral telencephalon (Fig. 2A). Indeed, deletion of the Shh gene, or that of its receptor Smo, from the early ventral telencephalon using the same Bf1-Cre transgene (Fuccillo et al., 2004) produces abnormalities very similar to those of our mutants. Importantly, these abnormalities are less severe than those seen in the complete Shh knockout, in which Shh expression in the prechordal plate mesoderm is also lost (Chiang et al., 1996).

The transcription factor Nkx2.1, a direct regulator of Shh (Sussel et al., 1999; Jeong et al., 2006), is specifically expressed in the MGE within the developing brain, and is absolutely required for its development (Sussel et al., 1999; Butt et al., 2008; Nóbrega-Pereira et al., 2008). In Sox2 mutants, Nkx2.1 expression was already undetectable at E11.5 in the telencephalon (Fig. 2B), but still observed in the non-Sox2-deleted diencephalon (Fig. 2B).

Six3, another transcription factor essential for ventral telencephalic development (Lagutin et al., 2003; Geng et al., 2008),
is also a direct activator of Shh (Jeong et al., 2008); expression of Six3 was only slightly reduced at E11.5, in coincidence with the initial tissue loss (Fig. 2C). Expression of the gene encoding Mash1 (Ascl1 – Mouse Genome Informatics), a transcription factor expressed in the MGE and lateral ganglionic eminence (LGE) and important for GABAergic interneuron development (Guillenot 2007), was essentially lost in regions close to the midline, and reduced more laterally (Fig. 2C). The genes encoding Dlx2 and Olig2, two transcription factors expressed in the MGE and LGE, downstream of Shh activity (Fuccillo et al., 2004), and required for ventral telencephalic development (Sur and Rubenstein, 2005; Hébert and Fishell, 2008), were similarly reduced (Fig. 2C). The Ebf1 transcription factor is expressed within the developing LGE, but not the MGE (Fuccillo et al., 2006; Geng et al., 2008); expression of Ebf1 was maintained, to some extent, in mutants (supplementary material Fig. S2). These data are consistent with a severe loss of MGE, but some degree of maintenance of LGE primordia.

In contrast to the strong reduction of the 'ventral' effectors described above, expression of transcription factor genes marking the dorsal brain and required for its development, such as Pax6, Ngn2 (Neurog2 – Mouse Genome Informatics) and Gli3, was maintained at E11.5-12.5 in mutants, with a clear tendency for dorsal-specific expression to spread ventrally (Fig. 2D), particularly at E12.5.

Expression of the gene encoding Bf1, a transcription factor expressed both dorsally and ventrally, but required mainly in ventral regions (Gutin et al., 2006; Hébert and Fishell, 2008), was maintained in lateral and dorsal regions, though it was severely reduced in the area affected by initial tissue loss (Fig. 2D, lower-right panel).

**Early downregulation of Nkx2.1 precedes ventral tissue loss**

As morphological abnormalities are already evident at E11.5, we investigated whether any gene expression defects precede their development. At E10.5 and E9.5, Nkx2.1 expression was clearly detectable in the ventral telencephalon of the wild type, but was strongly downregulated or absent in the mutant (Fig. 3A). Consistent with a relationship between Sox2 and Nkx2.1 expression, the latter was clearly present in diencephalon (Fig. 3A), where Sox2 was normally expressed (Fig. 1A). Similarly, Shh expression, which largely overlaps with that of Nkx2.1, was absent or weak in a few of the mutant embryos at E10.5 (not shown). Six3 expression was only slightly decreased in mutants at E10.5 and E9.5 (Fig. 3B,C). By contrast, the gene encoding Bf1, which acts in parallel with Shh (Hébert and Fishell, 2008), was normally expressed in Sox2 mutants, compared with controls (Fig. 3C). Sox1 and Sox3, members of the same Sox transcription subfamily as Sox2, are widely co-expressed with Sox2 in the telencephalon; they do not show major variations in mutant embryos at these stages (Fig. 3C). Sox9, which stimulates NSC growth after E10.5-11.5 (Scott et al., 2010), was normally expressed at these early stages (supplementary material Fig. S1).

We conclude that Sox2 deletion affects the expression of early, important determinants of brain development, in a region-specific manner: several ventral fate genes are severely affected, whereas activity of dorsal genes is maintained. Notably, one essential effector of ventral telencephalon and MGE development, and activator of Shh, Nkx2.1, is downregulated at early stages.

**Increased apoptotic cell death in early Sox2-mutant ventral telencephalon**

We investigated whether ventral tissue loss in Sox2 mutants was due to impaired cell proliferation and/or increased cell death. Cell proliferation, assessed by BrdU labelling at E9.5 and E10.5 just prior to the appearance of morphological defects, was not decreased overall in mutant telencephalon or specifically in the ventral region (Fig. 4A). Apoptotic cell death, assayed by TUNEL, was comparable between normal and mutant embryos at E9.5, but a threefold increase in TUNEL-positive cells was observed in the ventral telencephalon of E10.5 mutants (Fig. 4B).

Thus, increased cell death could directly cause ventral tissue loss in the mutants. Apoptotic death is a possible consequence of impaired ventral gene expression (e.g. loss of Shh, which has anti-apoptotic activities) (Cayuso et al., 2006), which precedes by at least one day the increase in cell death.

**Defective expression of ventral genes and morphological abnormalities of Sox2 mutants are rescued by a Shh agonist**

The ventral defects observed in Bf1-cre-deleted Sox2 mutants are very similar to those observed in mutants of the sonic hedgehog pathway [in which the Shh receptor smoothed (Smo) is conditionally ablated with the same deleter, Bf1cre] (Fuccillo et al., 2004), as well as to that of Nkx2.1 mutants (Sussel et al., 1999). Indeed, Sox2 mutants show (Figs 2, 3) severely impaired expression of both Shh and Nkx2.1, a direct activator of Shh (Jeong et al., 2006).
Hence, we tested whether Shh signalling was involved in the Sox2 mutant phenotype, by administering mice an agonist (Shh-ag) that activates the Shh co-receptor smoothened (Frank-Kamenetsky et al., 2002). Shh-ag was administered at E8.5 (just prior to Sox2 ablation) and E10.5. Already at E14.5, expression of ventral determinants Mash1 and Dlx2, which is impaired in the untreated mutants, recovered to a significant, albeit not complete, extent (Fig. 5); morphologically, the ventral brain also recovered a somewhat more normal shape, with ventral bulges reminiscent of wild-type ganglionic eminences (Fig. 5). By contrast, no major effect was observed on brain morphology or gene expression of treated wild-type littermates (Fig. 5).

We conclude that failure to activate Shh signalling is an important cause of the defects observed in Sox2-mutant embryonic telencephalon.

**Sox2 activates Nkx2.1 cell-autonomously**

Nkx2.1 is a direct activator of the Shh gene and is required for its expression in vivo (Sussel et al., 1999; Jeong et al., 2006; Sousa and Fishell, 2010); however, Nkx2.1 expression is also stimulated in response to Shh signalling (Fuccillo et al., 2004; Xu et al., 2005; Gulacsi and Anderson, 2006; Sousa and Fishell, 2010). Nkx2.1 expression failed to be established early in Sox2 mutants, and remained absent at later stages (Figs 2, 3); we thus investigated whether loss of Nkx2.1 expression depends on Sox2 cell-autonomously or is secondary to the loss of Shh expression. To this end, we used a Sox2CreERT2 transgene, encoding the tamoxifen-activatable Cre guided by the Sox2 telencephalic enhancer/promoter (Favaro et al., 2009). Tamoxifen treatment at E8.5 and E10.5 caused a ‘salt-and-pepper’ deletion of Sox2, as seen by immunofluorescence at E14.5 (Fig. 6); some Shh expression, presumably arising from non-deleted cells, was maintained (Fig. 6), and no major abnormality was noticed in these mosaic-deleted embryos. We analysed Nkx2.1 and Sox2 expression by immunofluorescence in the ventral telencephalic ventricular zone. In control embryos, most cells co-expressed Sox2 and Nkx2.1 (Fig. 6). In tamoxifen-treated embryos, Sox2-expressing cells were strongly reduced; Nkx2.1 expression was retained in cells in which Sox2 was still expressed, but was rarely, if ever, seen in cells that did not express Sox2 (Fig. 6). We conclude that expression of Nkx2.1 requires Sox2 cell-autonomously.
Regulation of Nkx2.1 by Sox2

The early loss of Nkx2.1 following Sox2 telencephalic ablation (Figs 2, 3) raised the possibility that Nkx2.1 expression is directly controlled by Sox2, within a specific subregion of the Sox2 pan-neural expression domain.

In a survey for evolutionarily conserved regions in the Nkx2.1 genomic locus, we detected a small conservation peak just upstream to the second Nkx2.1 exon (Fig. 7A,B). Evolutionary conservation within this region was present across vertebrate evolution (Fig. 7A). This region included a single and a twin potential Sox2-binding sites; both sites are conserved in mammals, and at least one site is conserved in vertebrates, including teleostean fishes (Fig. 7A). The Nkx2.1 gene has two promoters, one upstream to the first exon (‘distal’ promoter), the other in the intron between exon 1 and 2 (‘proximal’ promoter), both of which are functional in vitro and in vivo (including E10.5 and E14.5 telencephalon; supplementary material Fig. S3), though the latter might be the stronger (Pan et al., 2004; Hamdan et al., 1998). The Sox2-binding sites (Fig. 7B) lie in the region between the ‘distal’ and ‘proximal’ transcriptional start sites. ChIP from E14.5 embryos gave a moderate (2.5-fold) enrichment for this region (not shown). To develop a functional reporter assay for promoter sequences, we cloned a fragment encompassing the conserved Sox2 sequences from the Nkx2.1 region upstream to a green fluorescent protein (GFP) gene (Fig. 7B), and tested it in Medaka embryos. These sequences drove GFP activity in forebrain regions superimposable with those showing endogenous Nkx2.1 expression (Fig. 8A,B). In line with these observations, ChIP from stage 16-18 Medaka embryos with anti-Sox2 antibody revealed a 30-fold enrichment (relative to ChIP with a non-related IgG) of the Nkx2.1 intronic conserved element, which was not observed in negative control regions (a different region of the Nkx2.1 gene devoid of Sox2 consensus sites and the 18S RNA-encoding gene) (Fig. 8C), indicating that Sox2 binds to the Nkx2.1 promoter/enhancer in vivo.

Co-injection of Sox2 mRNA enhanced expression of the Nkx2.1-GFP transgene (Fig. 8F,J, compare with 8D,H), as well as of the endogenous Nkx2.1 gene, which is both increased and expanded anteriorly, as detected by ISH (Fig. 8N, compare with 8L). Conversely, co-injection of anti-Sox2 morpholino (Sox2 MO) (Beccari et al., 2012) (Fig. 8H,I) antagonised the activity of the co-injected Nkx2.1-GFP transgene (Fig. 8E,I, compare with 8D,H), as well as endogenous Nkx2.1 expression, the domain of which was also reduced (Fig. 8M, compare with 8L).

To evaluate the importance of a direct action of Sox2 on transgene regulation, we mutated the conserved Sox2 sites within the Nkx2.1-GFP transgene. GFP expression required the integrity of the Sox2-consensus sites, as their mutation caused a substantial loss of transgene activity (Fig. 8G,K, compare with 8D,H). This result is consistent with experiments showing that mutation of the same Sox2 sites in a luciferase-reporter gene driven by the ‘proximal’ promoter abolishes the response to co-transfected Sox2 in P19 teratocarcinoma cells (Fig. 7C).

These experiments show that Sox2 is an important regulator of Nkx2.1 expression in Medaka fish. In Medaka fish, the telencephalon is substantially reduced in size and lacks detectable endogenous Nkx2.1 expression (Fig. 8A,L). This raises the question of whether the Nkx2.1 regulatory elements studied here are sufficient to drive expression in the ventral telencephalon of the mouse. We thus tested the GFP construct described above in E13.5 mouse telencephalon by transient electroperoration. Supplementary material Fig. S4 shows that two days after electroporation the transgene is expressed in the ventral telencephalon. At E13.5-15.5, mutation of the Sox2 sites had little effect on telencephalic expression, indicating that, at this developmental stage, other transcription factor-binding sites play a role in the regulation of this construct in the telencephalon (see Discussion).

DISCUSSION

The Sox2 transcription factor is crucial for the maintenance of several types of stem cells, including pluripotent, neural and osteogenic stem cells (Masui et al., 2007; Favaro et al., 2009; Basu Roy et al., 2010). Despite the importance of Sox2 in NSCs in vitro, major abnormalities in brain development were not detected by conditional ablation of Sox2 at midgestation (E12.5) in mouse, with the exception of defects in postnatal development of the hippocampus dentate gyrus and of the retina (Taranova et al., 2006; Miyagi et al., 2008; Favaro et al., 2009). Here, we examined the hypothesis that Sox2 is required in the developing telencephalon at early developmental stages. By conditionally deleting Sox2 by E9.5 in all the developing telencephalon, we discovered that Sox2 deletion strongly affects embryonic development of the ventral telencephalon. Patterning of the ventral telencephalon is crucially dependent on the induction of the diffusible factor Shh, which is mediated by the transcription factor Nkx2.1 (Sousa and Fishell, 2010). The crucial role of Shh is highlighted by the severe abnormalities in patients affected with holoprosencephaly, a developmental defect of the brain ventral midline, caused by SIHH mutations (Dubourg et al., 2004; Roessler and Muenke, 2010). Here, we show that Sox2 is required for the early expression of Nkx2.1, thus controlling downstream ventral patterning genes such as Shh.
Early Sox2 loss affects Nkx2.1 and Shh expression

In Bf1-cre Sox2-deleted embryos, extensive ventral tissue loss occurs starting at ~E11.5, developing into major abnormalities of the ganglionic eminences (particularly the MGE) and of MGE-derived GABAergic neurons at later stages (Fig. 1); the expression of the dorsal markers Pax6 and Ngn2 (Fig. 2) also tends to spread ventrally, pointing to abnormalities of the ventral versus dorsal specification of the telencephalon. These defects strongly resemble those observed in Nkx2.1 germ-line deletion and in the conditional ablation (via Bf1-cre) of the Shh receptor smoothened (Sussel et al., 1999; Fuccillo et al., 2004; Sousa and Fishell, 2010). We confirmed the connection to Shh by showing that Shh expression is strongly diminished in the ventral region of the mutant telencephalon concomitantly and prior to the onset of tissue loss (Figs 2, 3). Moreover, treatment of the embryos with a Shh agonist substantially rescued ventral development in the mutant brain (Fig. 5), though prenatal lethality still occurred. This rescue is reminiscent of that of the hippocampal dentate gyrus stem cells and postnatal growth by the same drug, in nestin-cre Sox2-deleted mice (Favaro et al., 2009). Local cell death in the ventral telencephalon is detected just prior to the onset of tissue loss (Fig. 4); this might also relate to loss of Shh signalling, which activates the anti-apoptotic gene Bcl2 (Cayuso et al., 2006). These data, together with those of Favaro et al. (Favaro et al., 2009), highlight an unexpected role of Sox2 in mediating the development of specific brain regions at defined stages via Shh-dependent regulation.

How does Sox2 regulate Shh expression in the ventral telencephalon? Sox2 might regulate genes involved in local specification of ventral regions. A prime candidate target is Nkx2.1, essential for ventral brain development and correct doroventral patterning (Sussel et al., 1999; Sousa and Fishell, 2010). Nkx2.1 is thought to mediate the early ‘homogenetic’ induction of Shh in the ventral telencephalon, in response to the gradient of mesendoderm-derived Shh (Sousa and Fishell, 2010). Indeed, Nkx2.1 mutant mice fail to express Shh in the ventral region (Sussel et al., 1999), and their phenotype resembles both that of Shh or smoothened mutants.

Fig. 7. Nkx2.1 regulation by Sox2. (A) The Nkx2.1 intronic promoter/enhancer is evolutionary conserved in vertebrates. The genomic Nkx2.1 sequences from the different vertebrate species were retrieved from the UCSC genome browser and aligned using the Shuffle-LAGAN of Vista. Pink, pale-blue and violet peaks represent conserved non-coding elements (75% conservation over 100 bp), mRNA untranslated sequence and coding sequence, respectively. Putative Sox2-binding sites, indicated as S1, S2 and S3, localise to a conserved element in the first intron. S1 binding site is conserved among mammals but not in other vertebrates. S2 and S3 binding sites were conserved among most vertebrates. The indicated Consensus within the alignment was derived with Simple Consensus Maker (http://www.hiv.lanl.gov/content/sequence/CONSENSUS/SimpCon.html). The general Sox2 consensus is from Engelen et al. (Engelen et al., 2011). (B) Schematic of the Nkx2.1 gene (Sox2 sites indicated by red dots) with constructs used for transgenic (EGFP) and transfection experiments (luciferase); sequence shows the Sox sites and the mutations introduced. EGFP constructs: wt-long: a region from nucleotide −495 (5’ to exon 1) to nucleotide +1942 in the second exon was cloned in frame with the EGFP reporter (green box); this fragment comprises the conserved elements in the first intron. wt-long-mut: carries mutations in the Sox2 sites, shown below (underlined). control: promoter-less EGFP construct. Luciferase constructs: wt-long: same region as in the EGFP construct, here linked to a luciferase reporter. wt: a shorter region encompassing the two Sox2 sites, from +1172 to +1757. S1/S2/S3 mutant: same as wt, with the same Sox2 mutations as in the wt-long-mut-EGFP reporter. (C) Co-transfection in P19 cells of Nkx2.1 promoter with luciferase vectors (1 μg) and their mutant versions (shown in B), with increasing amounts (+, 0.125 μg; ++, 0.25 μg; ++++, 0.5 μg) of Sox2-expressing vector (Sox2), or with the corresponding ‘empty’ vector. Results are the mean of at least three independent transfections, in triplicate.
Two prototypic embryos are shown for each experimental condition. Scale bars: 50 μm in A-K; 40 μm in L-N.

The shape of the embryos and of the eye (e) are outlined by dashed white lines. The hypothalamic mRNA distribution of

The early severe impairment of Nkx2.1 expression in Sox2 mutants already by E10.5 (Fig. 3), and the absence of Shh at least from E10.5/11.5 onwards (Fig. 2), are consistent with the hypothesis that a large part of the phenotypic effects of Sox2 ablation is initially mediated by Nkx2.1 deficiency.

Do other transcription factors mediate the effects of Sox2 deficiency?

Presently, we can neither rule out nor implicate other genes besides Nkx2.1 (and Shh) in the early effects of Sox2 ablation. SIX3 mutations are found in some human patients affected with holoprosencephaly (Jeong et al., 2008), and Six3 haploinsufficiency caused by the ‘knock-in’ of a human mutant SIX3 gene impairs Shh expression and MGE development in mouse, recapitulating features of the human phenotype (Geng et al., 2008). Moreover, a mutation in a SIX3-binding site, within a SHH long-range acting enhancer, has been detected in a human holoprosencephalic patient (Jeong et al., 2008). In our mutants, Six3 expression was only slightly diminished in the ventral region at early stages, when Nkx2.1 expression was already substantially affected (Figs 2, 3), making it unlikely that the effects of Sox2 ablation are mediated by Six3 deficiency. Interestingly, in the Medaka telencephalon, Sox2 activates Six3, but the two genes seem to have antagonistic function in the hypothalamus (Beccari et al., 2012). The expression of Bf1, another candidate gene (Gutin et al., 2006; Hébert and Fishell, 2008), was also not significantly affected at these early stages, despite the Bf1 hemizygosity due to cre ‘knock-in’ (Fig. 3).

After Nkx2.1 (and thus Shh) expression is initially activated by mesendoderm-derived Shh, its activity is normally maintained, at later stages, by Shh itself (Xu et al., 2005; Xu et al., 2010; Sousa and Fishell, 2010). Following mosaic Sox2 deletion at E8.5 (Fig. 6), Nkx2.1 is poorly expressed later on in Sox2-deleted cells, even in the presence of Shh. These data do not contradict the notion that Shh maintains later expression of Nkx2.1 (Xu et al., 2005; Xu et al., 2006). Furthermore, mutations destroying a consensus Nkx2.1-binding site in a distant Shh enhancer, active in telencephalon, impair the transcription of reporter constructs in transgenic mice (Jeong et al., 2008), and SIX3 gene impairs Shh expression and MGE development in mouse, recapitulating features of the human phenotype (Geng et al., 2008). Moreover, a mutation in a SIX3-binding site, within a SHH long-range acting enhancer, has been detected in a human holoprosencephalic patient (Jeong et al., 2008). In our mutants, Six3 expression was only slightly diminished in the ventral region at early stages, when Nkx2.1 expression was already substantially affected (Figs 2, 3), making it unlikely that the effects of Sox2 ablation are mediated by Six3 deficiency. Interestingly, in the Medaka telencephalon, Sox2 activates Six3, but the two genes seem to have antagonistic function in the hypothalamus (Beccari et al., 2012). The expression of Bf1, another candidate gene (Gutin et al., 2006; Hébert and Fishell, 2008), was also not significantly affected at these early stages, despite the Bf1 hemizygosity due to cre ‘knock-in’ (Fig. 3).

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al., 2010), but simply highlight an early requirement for Sox2 in establishing this process.

The ventral telencephalic defects due to Sox2 early ablation point to a marked regional specificity of Sox2 requirement in development (Fig. 1). As an example, Sox2 is required for Nkx2.1 (and Shh) (see also Favaro et al., 2009) expression only within a specific subregion of the Sox2 pan-neural expression domain (Figs 2, 3). This might depend on local Sox2 concentrations, and/or on the presence of additional co-regulators. The transcription factors Sox1 and Sox3 are closely related to Sox2 (Wegner and Stolt, 2005), and recognise similar DNA sequences in vitro (Kondoh and Kamachi, 2010; Wegner, 2010). Thus, in regions in which Sox2 ablation causes few or no defects, Sox1 and/or Sox3 might compensate for Sox2 deficiency. Indeed, embryos doubly mutant for Sox2 and Sox3 (Sox2<sup>−/−</sup>; Sox3<sup>−/−</sup>) develop diencephalic defects, mirroring those observed following early diencephalic deletion of the Shh gene (Zhao et al., 2012); by contrast, no such defects were observed in single mutants. Thus, Sox3 does compensate for some Sox2 functions in the diencephalon. By contrast, in the ventral telencephalon (present paper), Sox2 has some region-specific functions that cannot be complemented by Sox3 and Sox1, in spite of their normal expression levels (as also observed in Medaka) (Beccari et al., 2012). Most sequence diversity between Sox1, Sox2 and Sox3 occurs outside the DNA-binding domain; unique partnerships between Sox2 and co-factors (Kondoh and Kamachi, 2010; Bernard and Harley, 2010; Wegner, 2010) might mediate its specific functions in ventral (and hippocampal) (Favaro et al., 2009) brain development.

Among Sox2-specific interactors/DNA-binding proteins, CHD7 is known to activate a set of common targets relevant for anophtalmia (caused by SOX2 mutations in humans and mice) and CHARGE syndrome (caused by CHD7 mutations) (Engelen et al., 2011). The specific expression of different Sox2 interacting/cooperating factors in various tissues might impart regional specificity to the defects caused by the absence of Sox2; indeed, an important (antagonistic) relationship of Sox2 with Pax6 was reported in a study of the development of neural competence in the optic cup (Matsumisha et al., 2011).

How does Sox2 regulate Nkx2.2.1? In Medaka, modulation of Sox2 levels correlates with changes in endogenous Nkx2.1 expression intensity and spatial distribution (Fig. 8L-N). Moreover, the Nkx2.1-GFP construct faithfully recapitulates endogenous Nkx2.1 expression, and requires intact Sox2-binding sites for activity (Fig. 8B-D-K), consistent with transfection results in P19 cells (Fig. 7C). These data identify Nkx2.1 as a Sox2 target in Medaka. In Medaka, unlike in mouse, Nkx2.1 is not active in the telencephalon, and we thus cannot directly extrapolate from the Medaka results to infer direct regulation of Nkx2.1 by Sox2 in the telencephalon. We tested the same Nkx2.1 construct in mouse by electroporation in the telencephalon at E13.5-15.5, showing that it is active in the telencephalon, preferentially in the MGE and other ventral regions (supplementary material Fig. S4); however, the mutation of the Sox2 sites did not actually affect the activity of the construct (not shown). These results formally rule out the possibility that, at the E13.5-15.5 stage, the Sox2-binding sites, per se, are required for activity of the Nkx2.1 promoters in the telencephalon. As a consequence, it remains unclear whether, in Sox2 mutant mouse, the observed loss of Nkx2.1 expression depends on the loss of a direct activity of Sox2 on the Nkx2.1 promoter (so far unproven), on additional effects on other regulatory elements, or on indirect effects mediated by other Sox2-dependent factors.

How do we reconcile the data obtained by electroporation in mouse telencephalon with the low activity of the Sox2-mutated reporter in Medaka (and in in vitro transfected P19 cells)? We speculate that the requirement for Sox2 binding to the Nkx2.1 promoter (if any) might be limited to the early stages of development. We know that late Sox2 ablation (E12.5) has little effect on ventral telencephalic development and gene expression (Favaro et al., 2009; unpublished data), whereas early ablation (E9.5) causes important defects. We therefore hypothesise that Sox2-binding sites in the Nkx2.1 promoter might be required for Nkx2.1 regulation in mouse telencephalon at early (~E9.5), but not later, stages of development. Once established, Nkx2.1 expression might be maintained, at E14.5, by transcription factors other than Sox2, and additional regulatory regions might be involved in controlling Nkx2.1 expression. Unfortunately, the present constructs show very low activity following electroporation at E9.5, and similar transgenic constructs were not expressed in embryonic ventral telencephalon (Pan et al., 2004), making it difficult to test this hypothesis. Several regions adjacent to Nkx2.1 bind Sox2 in ChIP experiments, and distal intergenic regions exhibit long-range interactions with the Nkx2.1 gene (not shown), and might potentiate the promoter. These sequences will be investigated in the future in order to determine their regulatory potential.

Conclusions Sox2, despite its ubiquitous expression in neural stem/progenitor cells at all levels of the developing central nervous system, is absolutely required, in a stage- and region-specific way, in a limited set of locations, here exemplified by the early ventral telencephalon and by the hippocampus (Favaro et al., 2009). In the ventral telencephalon, Nkx2.1 is likely to be the main (although not necessarily the exclusive) mediator of Sox2 effects; other factors might mediate Sox2 activities in different regions. Expression defects of Shh (a target of Nkx2.1 in the ventral telencephalon), are common to both territories affected by Sox2 loss (ventral telencephalon and hippocampus); it will be interesting to examine other embryonic brain sites expressing Shh for defects caused by Sox2 ablation at different developmental stages.

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Brain development requires Sox2


