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

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ABSTRACT

The transcription factor Sox2 is essential for neural stem cells (NSC) maintenance in the hippocampus and *in vitro*. The transcription factor Emx2 is also critical for hippocampal development and NSC self-renewal. Searching for 'modifier' genes affecting the Sox2 deficiency phenotype in mouse, we observed that loss of one Emx2 allele substantially increased the telencephalic β-geo (LacZ) expression of a transgene driven by the 5′ or 3′ Sox2 enhancer. Reciprocally, Emx2 overexpression in NSC cultures inhibited the activity of the same transgene. *In vivo*, loss of one Emx2 allele increased Sox2 levels in the medial telencephalic wall, including the hippocampal primordium. In hypomorphic Sox2 mutants, retaining a single 'weak' Sox2 allele, Emx2 deficiency substantially rescued hippocampal radial glia stem cells and neurogenesis, indicating that Emx2 functionally interacts with Sox2 at the stem cell level. Electrophoresis mobility shift assays and transfection indicated that Emx2 represses the activities of both Sox2 enhancers. Emx2 bound to overlapping Emx2/POU-binding sites, preventing binding of the POU transcriptional activator Brn2. Additionally, Emx2 directly interacted with Brn2 without binding to DNA. These data imply that Emx2 may perform part of its functions by negatively modulating Sox2 in specific brain areas, thus controlling important aspects of NSC function in development.

INTRODUCTION

The transcription factor Sox2, essential in pluripotent stem cells of the blastocyst (1), is also highly expressed in neural stem cells (NSC) and their early progeny (2–6). Decreased expression of Sox2 in a mouse hypomorphic Sox2 mutant causes important brain and neurologic defects (3,7), which mimic significant aspects of the pathology of Sox2-deficient patients (8,9). In this hypomorphic mutant, we combined the deletion of one Sox2 allele ($Sox2^{\beta\text{-}geo}$ knock-in) with the deletion, on the other allele, of an upstream enhancer of Sox2 ($Sox2^{\Delta Enh}$), important for its expression in telencephalic NSC (3,6,10–13). The hypomorphic mutant, expressing Sox2 at a level about 30% that of the wild-type, shows hippocampal stem cells loss, corpus callosum interruption, parenchymal loss in striatum and thalamus, decreased numbers of GABAergic neurons and neurological defects, including epilepsy (3,7). Recently (14), we showed that Sox2 embryonic deletion leads to complete perinatal loss of hippocampal stem cells. NSC from the forebrain of such mutants become rapidly exhausted in *in vitro* neurosphere culture.

The Emx2 transcription factor is expressed in the developing dorsal telencephalon, including prospective hippocampus and cerebral cortex, from early embryogenesis (15,16). Its expression is maintained postnatally in brain neurogenic regions, the subventricular zone (SVZ) and hippocampus dentate gyrus (DG) (17,18).

Emx2 inactivation in mouse causes delayed hippocampal development, with reduced cerebral cortex and abnormal specification of cortical areas (15,19–21). *In vitro*, mutant $Emx2^{-/-}$ NSC show increased proliferation in long-term neurosphere cultures (17).

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A common aspect of the defects in Sox2 and Emx2 mutants is the abnormal hippocampal development, together with important NSC abnormalities in *in vitro* long-term culture (see above, and 3,14,15,17,21). In this work, we explored potential functional interactions between Sox2 and Emx2 at the molecular level and, *in vivo*, in mouse. We report that Emx2 negatively regulates two Sox2 telencephalic-specific enhancers *in vivo* and in transfection assays, by interfering with binding of positive regulators to their cognate sites within the enhancers. *In vivo*, Emx2 deficiency leads to some increase of Sox2 in the medial wall of the telencephalon, and partially counteracts hippocampal neurogenesis defects observed in Sox2 deficient (hypomorphic) mouse mutants.

MATERIALS AND METHODS

Mouse lines, X-gal staining and immunohistochemistry

The 5′ and 3′ enhancer- β-geo transgenic mice lines were described in (6,11,22,23). The Sox2-hypomorphic (Sox2 $^{\Delta Enh}$) and null (Sox2 $^{\beta\text{-geo}}$) mutant alleles were as in (3). The Emx2 null mutant mice (kindly provided by A. Mallamaci) were described in (15).

X-gal staining, immunohistochemistry (IHC) and histology were as reported (6).

Glial fibrillary acidic protein (GFAP)/nestin and BrdU IHC on hippocampus and all histological analyses were carried out as previously reported (3). IHC with anti-Emx2 antibodies was as described (18). IHC with anti-Brn2 antibody, a SantaCruz goat antibody (22) was used (1:100).

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

Transgenic neurosphere culture and lentiviral transduction

Neurosphere cultures were derived from E15.5 dorsal telencephalon of transgenic brains as described (6,14), expanded (in the presence of 400 μg/ml G418) and transduced (in the absence of G418) with an internal ribosome entry site-green fluorescent protein (Emx2-IRES-GFP) (or GFP-only control)-encoding lentivirus at a multiplicity of infection of 5. To generate the Emx2-transducing virus, the Emx2 coding sequence was cloned, upstream to IRES-GFP, in place of the Sox2 coding sequence, in the lentiviral vector described in refs. 7 and 14.

Two passages after transduction RNA was extracted, reverse transcribed and analysed by real-time PCR (MESA GREEN qPCR Master Mix Eurogentec) for the expression of β-geo (lacZ), Emx2 and Sox2 with the following primers: LacZ-f CTGGATCAAATCTGTCGATCC, LacZ-r CGTATTCGCAAAGGATCAGC, Emx2-f GTC CCAGCTTTTAAGGCTAGA, Emx2-r CTTTTGCCTT TTGAATTTCGTTC, Sox2-f GGCAGCTACAGCATG ATGCAGGAGC, Sox2-r CTGGTCATGGAGTTGTAC TGCAGG; HPRT-f TCCTCCTCAGACCGCTTT, HPRT-r CCTGGTTCATCATCGCTAATC; the dataset are analysed with a 7500 System Software v1.4 (Applied Biosystem). Neurospheres cultured as above from Emx2$^{-/-}$ or wild-type embryonic brains were expanded for one passage, total RNA was extracted and analysed by real-time RT–PCR as above with the same Sox2 and Emx2 primers. Expression levels normalized versus HPRT expression.

Luciferase reporter constructs

The Sox2 5′ telencephalic enhancer core region of 400 bp was PCR amplified from the 0.4a-Sox2 promoter-β-geo vector (22) using the following primers:

Fw: 5′ CGAGGTACCGTCAAATAGGGCCCTTTT CAG 3′; Rv: 5′ TATCTCGAGAAGCCAACTGACA ATGTTGTGG 3′ containing a KpnI and XhoI restriction sites (underlined), for further cloning into the pGL3-based plasmid containing a 215 bp minimal tk promoter (a gift of Hitoshi Niwa) (5′enh-tk-luc).

The reporter plasmid carrying mutations in the ATTA-3 site (ATTA-3 site mut) was obtained as above, starting from 0.4a-MUT Sox2 promoter-β-geo vector (22). All other ATTA-site mut reporter constructs were similarly obtained by PCR-based site-directed mutagenesis.

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

Fw: 5′CGAGGTACCGTCAAATAGGGCCCTTTT CAGATTTTA**AGGA**CAAAATAAA**AGGA**GTCTGC TC3′

Rv: 5′TATCTCGAGAAGCCAACTGACAATGTT GTGG 3′ containing the desidered mutations (in bold) and KpnI/XhoI restriction sites (underlined).

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

Fw: 5′ ACTCTGCAGGTCCCCTGCCGTTCGCCTTC ATTTCCATA**AGGA**GAGGAGGAGAGGAGG 3′

Rv: 5′ CGGGTCGCTGCAGGGTCGCTCGGTGTTC G 3′

PstI restriction site (underlined) in both primers.

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

Fw1: 5′ GCATCAACCTAGTAAGATGCTTGGCTAG TTCTCGCTA**AGGT**CTGCAAC 3′

Rv1 (XhoI-external primer): 5′ TATCTCGAGAAGCCA ACTGACAATGTTGTGG 3′

Fw2 (KpnI-external primer): 5′ CGAGGTACCGTCAA ATAGGGCCCTTTTCAG 3′

Rv2: 5′ GTTGCAGACCTTAGCGAGAACTAGCCAA GCATCTTACT**AGGT**TGATGC 3′

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

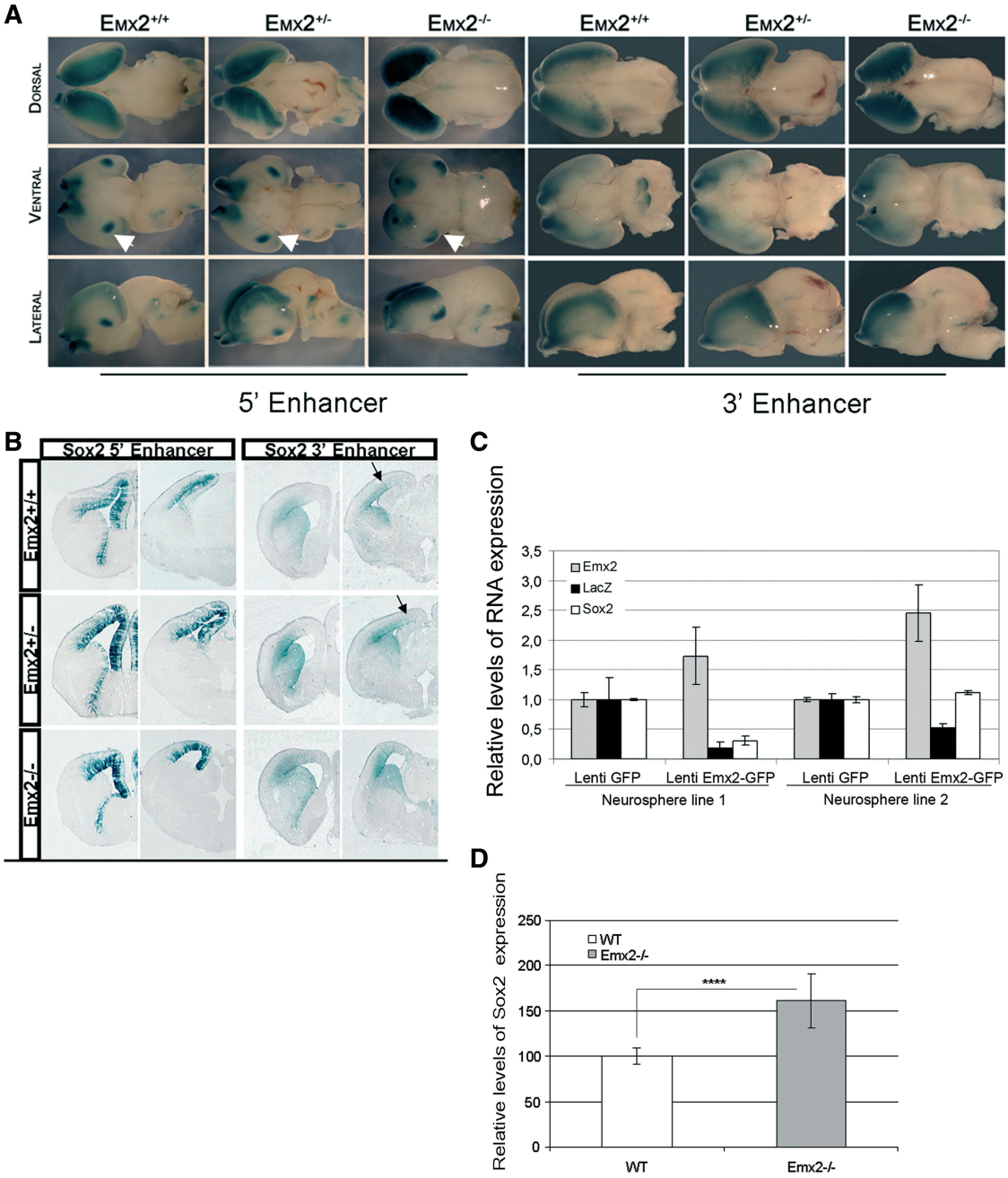

Figure 1. Emx2 deficiency increases activity of Sox2 telencephalic enhancers-driven lacZ transgenes. (**A**) X-gal stained E15.5 brains carrying β-geo transgenes driven by the 5′ Sox2 telencephalic enhancer (left) or by the 3′ enhancer (right), of $Emx2^{+/+}$, $Emx2^{+/-}$, or $Emx2^{-/-}$ genotype, as indicated. Dorsal (top row), ventral (middle row) and lateral (bottom row) views are shown. Increased X-gal staining is seen, most clearly in dorsal views, in $Emx2^{+/-}$ as compared to $Emx2^{+/+}$ brains, and in $Emx2^{-/-}$ as compared to $Emx2^{+/-}$ brains. In the 5′enhancer-transgenic brains, an X-gal-positive spot on the ventral telencephalic vesicles, visibile in the ventral (arrow) and lateral views, has comparable intensity in $Emx2^{+/+}$ and $Emx2^{+/'}$ brains, acting as an internal control for staining. Overall, 7/7 $Emx2^{+/-}$ transgenic embryos (5′ construct, E15.5) showed increased lacZ expression relative to $Emx2^{+/+}$ from the same litter (4 embryos). Similarly, 7/8 $Emx2^{+/-}$ embryos carrying the 3′ transgene showed increased lacZ activity relative to $Emx2^{+/+}$ controls (4 embryos). Homozygous $Emx2^{-/-}$ 5′ transgenic embryos were always (7/7) more intensely stained than their control heterozygotes ($Emx2^{+/-}$) littermates (11 embryos); 7/7 of the $Emx2^{-/-}$ 3′ transgenics were more stained than their $Emx2^{+/-}$ heterozygous controls (10 embryos). (**B**) X-gal stained brain coronal sections of 5′ or 3′ enhancer-lacZ transgenic forebrains of $Emx2^{+/+}$ (top row), $Emx2^{+/-}$ (middle) and $Emx2^{-/-}$ (bottom) genotype. Arrow in B (3′ enhancer) points to some dorsal expansion of X-gal staining signal in $Emx2^{+/-}$, as compared to $Emx2^{+/+}$ brain. (**C**) Relative RNA levels (real-time RT–PCR) of Emx2, β-geo (lacZ), and endogenous Sox2 in cultured NSC (neurospheres) from Sox2 3′ enhancer-β-geo transgenic brains, transduced with Emx2-GFP or GFP (control)—encoding lentiviruses, as indicated. RNA levels in control (GFP-lenti-transduced) cells are set = 1 (for non-normalized data, see Supplementary Figure S1). The values represent the mean ± SD of $n = 2$ independent RT–PCR experiments on each line performed in triplicate (all RNA levels—LacZ, Emx2, Sox2—significantly differ between Lenti-Emx2 and Lenti-GFP transductions ($P < 0.003$ by Student's t-test), except for endogenous Sox2 levels in line 2, which are comparable). (**D**) Relative RNA levels (real-time RT–PCR) of endogenous Sox2 RNA in cultured NSC (neurospheres) from $Emx2^{-/-}$ versus wild type embryonic brains. The values represent the mean ± SD of $n = 3$ independent RT–PCR experiments on two wild-type and three Emx2-mutant independent cultures tested, each performed in triplicate. (****$P = 6.3\,e^{-11}$ by Wilcoxon's one-tailed test).

For constructing the 3X POU/ATTA site 3 plasmid, the combined POU/ATTA site (in bold type characters) was multimerized to three copies, and subcloned into the KpnI/XhoI site of the pGL3-tk luciferase vector, using the following primers:

Fw: 5′CACTGC**TAATTAGCAAT**GCTAGGGTGC**TAA TTAGCAAT**GCTAGGGTGC**TAATTAGCAAT**GCT AGC 3′

Rv: 5′TCGAGCTAGC**ATTGCTAATTA**GCACCCTAG C**ATTGCTAATTA**GCACCCTAGC**ATTGCTAAT TA**GCAGTGGTAC 3′

For constructing the 2X ATTA site 1,2 plasmid, the ATTA site 1,2 core sequence,

5′ TTA**ATTA**CAAAATAAA**ATTA**GTCTGCTCTTC 3′, was dimerized (as a synthetic oligonucleotide) and subcloned into the KpnI/XhoI site of the pGL3-tk luciferase vector.

The Luciferase reporter vectors bearing BamHI/SalI genomic DNA fragments of the 3′enhancer were described (11,23); their core sequence was essentially as in (24): 5′GGATCCCTA**ATTA**ATGCAGAGACTCTA AAAGAATTTCCCGGGCTCGGGCAGCCATTGTGA TGCATATAGG**ATTA**TTCACGTGG**TAAT**GAGCACA GTCGAC 3′

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

The Nestin258-luciferase construct (a gift from H. Kondoh) was previously described (25).

P19 transfection assays

For transfection experiments, P19 cells were grown in MEM-ALPHA medium supplemented with PenStrep, L-glutamine and 10% fetal bovine serum. 2×10^5/well exponentially growing P19 cells were plated in 6-well-plates, and transfected the following day with Lipofectamine 2000 (Invitrogen) according to supplier's instructions. Briefly, medium in each well was replaced with 1 ml of Opti-MEM medium (Invitrogen) with 10 µl of Lipofectamine 2000 (Invitrogen), with DNA. For transfection experiments, we used 1 µg of the luciferase reporter plasmid, and 500 ng of the Emx2 expression vector (pCAGGS-Emx2, a gift from V. Broccoli) per well if not otherwise indicated. In control experiments, equimolar amounts of the Emx2 'empty' vector (pCAGGs) or the Otx2 expression vector (pCAGGS-Otx2, a gift from V. Broccoli), were used. The pBluescript vector was added to each transfection to equalize the total amount of transfected DNA to 2 µg total in each reaction. After 24 h, total cellular extracts were prepared and Luciferase activity was measured according to the Promega Luciferase reporter system protocol.

For cotransfection experiments with Brn2 and Emx2 expression vectors, Brn2 expression vector (a gift from D. Mejiers) (or the 'empty' control) was transfected at the fixed amount of 500 ng/transfection or at increasing amounts (+, ++, +++: 125, 500, 1000 ng) where indicated; in these experiments, Emx2 expression vector was added (100 to 1000 ng) as indicated in the relevant Figures. The 'empty' vector was added to each transfection at the proper concentration to equalize the total amount of DNA transfected in each reaction to 2 µg. Sox2 expression vector (activating the Nestin258-luciferase construct in conjunction with Brn2, ref. 25) was added at the fixed amount of 500 ng/transfection.

In vitro protein expression and purification

Emx2 (in pSG5), Brn2, GATA1 and GATA2 (in pBluescript) were produced using *in vitro* transcription—translation reticulocyte lysate system (TNT, Promega), according to the manufacturer's indication, in a total volume of 50 µl for 1.5 h at 30°C, using 2 µg plasmid template with 25 µl of reticulocyte lysate, and then frozen at −80°C.

The amounts in µl of the TNT reactions used in different experiments are indicated in Figure legends. To use equivalent amounts of *in vitro*-synthethized proteins (Brn2, Emx2, GATA1 or GATA2), TNT reactions were performed in the presence of 35S methionine, the amounts of protein produced were estimated by autoradiography of western blot, normalized for the numbers of methionines in each protein, and equivalent amounts of each recombinant protein were used.

The Emx2 (or CP2 control, ref. 26) cDNAs were cloned in frame into the pGEX2T vector. The *Escherichia coli* BL21 strain cells were transformed with the above plasmid and cultures were grown at midlogarithmic phase (0.6 A_{600}). Protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. The GST-EMX2 protein present in the soluble fraction was bound to GST-Sepharose 4B (Amersham Bioscience) and purified according to the manufacturer's instructions.

Protein was eluted from sepharose, quantitated by Coomassie blue staining in comparison to BSA standards, and 1 µg of total protein (for GST-Emx2, GST-CP2 and GST-only resins) was used for GST-pulldown of ^{35}S Brn2-containing TNT reaction as in (26,27).

Electrophoretic mobility shift assay and Chromatin Immunoprecipitation

Electrophoretic mobility shift assay (EMSA) was performed (28,29) by preincubating TNT-produced proteins or nuclear extract (from the hippocampal stem cell line AHP or from neurosphere cultures) for 30 min on ice in 20 µl of binding buffer (75 mM NaCl, 20% Ficoll, 10 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 10 mM DTT, 1 µg of poly(dI-dC), together with 2 µl (5×10^4 cpm) of ^{32}P-end-labelled oligonucleotide probes. The incubation mixture was resolved by electrophoresis on a 5 or 6% polyacrylamide gel (29:1, acrylamide/bisacrylamide ratio) in 50 mM Tris borate, 1 mM EDTA, pH 8.2 (0.5XTBE) buffer run at 4°C at 150 V for 3 h. Gel were dried and exposed to a Kodak X-AR film at −80°C. For 'supershift' reactions, 1 µl of the 1:10 diluted mouse α-Emx2 antibody (mouse ascites, kindly provided by F. Mavilio) or 8 µl of the goat α-Brn2 antibody (undiluted) (Santa Cruz Biotechnology) were added to the complete binding reaction just prior to the addition of the labelled probe.

The following double-stranded oligonucleotides were used as probes for EMSA (only the top strand is shown)

(underlined sequences correspond to mutated nucleotides):

ATTA-site 3: 5′-TCGTCAAACTCTGCTA**ATTA**GCAATGCTGAGAAA-3′;
ATTA-site 3 mut1: 5′-TCGTCAAACTCTGCATCCTTGCAGAGCTGAGAAA-3′;
ATTA-site 3 mut2: 5′-TCGTCAAACTCTGCTACGGCGCAATGCTGAGAAA-3′;
3′-Enh: 5′-GGCAGGTTCCCCTCTAATTAATGCAGAGACTC-3′;

ATTA-1/2 sites:

5′-GGGCCCTTTTCAGATTTTAATTACAAAATAAAATTAGTCTGCTCTTCCTCGG-3′;

ATTA-1/2 sites mut:

5′-GGGCCCTTTTCAGATTTTAAGGACAAAATAAAAGGAGTCTGCTCTTCCTCGG-3′;

Delta1-Enh: 5′-AGAGAGCAGGTGCTGTCTGCATTACCATACAGCTGAGCGC-3′;
Nestin-Enh: 5′-GTGTGGACAAAAGGCAATAATTAGCATGAGAATCGGCCTC-3′.

Chromatin immunoprecipitation (ChIP) was as described (13).

RESULTS

Emx2 negatively regulates transgenic Sox2-β-geo reporters

We initially bred mice carrying transgenic β-geo reporters driven by Sox2 regulatory elements to Emx2 mutant ($Emx2^{+/-}$) mice. The Sox2-β-geo transgene (6) is driven by 5.7 kb of the Sox2 promoter/enhancer, and its neural expression is progressively confined to the telencephalon, after E11.5. The SRR2 transgene (11,23) is driven by the tk-promoter linked to an enhancer normally located immediately 3′ to the Sox2 coding region (these mouse lines are denominated 5′ and 3′ enhancer lines, respectively; alternative names in the literature for the 5′ and 3′ enhancers include SRR1 and N2, and SRR2, respectively, 11,24,30). Breeding with Emx2-mutant mice, we obtained E15.5 progeny consisting of embryos carrying the transgene in the heterozygous state, together with the three possible Emx2 genotypes (wild-type, +/+; heterozygote, +/−; homozygote, −/−).

For both constructs, loss of one Emx2 allele is associated to significantly increased β-geo expression (evaluated by classical X-gal staining) (Figure 1A); a further strong increase is observed in $Emx2^{-/-}$ mice (note, however, that the $Emx2^{-/-}$ brain is abnormal, as expected (15).

We confirmed these results by X-gal staining of brain sections (Figure 1B). The 5′enhancer construct is expressed in dorsal and medial areas of the telencephalic ventricular zone and, to lower levels, ventrally, along the ganglionic eminence, whereas the 3′ enhancer construct is more active in ventrolateral areas. In $Emx2^{+/-}$ heterozygotes, the respective domains of expression were more intensely stained, both anteriorly and posteriorly; additionally, the extension of the X-gal-positive region was somewhat increased towards the midline, in mice carrying the 3′enhancer construct (arrows). As expected, homozygous $Emx2^{-/-}$ mutants showed increased β-geo expression, although matching the different areas is problematic due to morphological abnormalities. We conclude from these Emx2 loss-of-expression experiments that Emx2 negatively modulates two different telencephalic enhancers of Sox2 in the developing mouse brain.

We further asked whether abnormally increased levels of Emx2 could inhibit Sox2 telencephalic enhancers in neural cells. To this end, we derived independent NSC cultures from the dorsal telencephalon of two E15.5 mice carrying the 3′enhancer-β-geo construct, and we transduced them with an Emx2-GFP-expressing lentivirus (or with control GFP-expressing virus). In both cell populations, the β-geo reporter expression was strongly inhibited by the Emx2-expressing virus, as compared to the control virus (Figure 1C). In one of the two lines, which expressed moderate levels of Sox2, also the endogenous Sox2 level was significantly decreased; in the second line, which showed a much higher expression of endogenous Sox2, no significant inhibition could be observed (Figure 1C and Supplementary Figure S1); it is possible that the 'isolated' enhancer guiding β-geo more readily responds to acute, exogenous variations of Emx2 levels than the 'full' Sox2 locus, which is controlled by various different regulatory regions. Taken together, the above results indicate that Emx2 negatively regulates, *in vivo* and in *ex vivo* derived neural progenitors, the activities of Sox2' telencephalic enhancers.

We also wished to address whether Emx2 deficiency would affect endogenous Sox2 levels in NSC cultures, as it does *in vivo* in the developing brain. To this end, we measured endogenous Sox2 mRNA levels in NSC cultures derived from Emx2-mutant telencephalon, as compared to wild-type littermates cultures, by real-time RT–PCR (Figure 1D). Emx2 ablation led to a significant (average 50%) increase in endogenous Sox2 levels in three independent mutant cultures tested (Figure 1D and Supplementary Figure S2), confirming that expression levels of the resident Sox2 gene are modulated by Emx2 in cultured neural stem/progenitor cells.

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

To begin to address whether the Emx2-dependent inhibition of Sox2 telencephalic regulatory elements has any *in vivo* effects on Sox2-dependent brain phenotypes, we selected for further studies the hippocampus neural stem/progenitor cells of the hypomorphic $Sox2^{\beta\text{-geo}/\Delta Enh}$ mutant (3,7), that expresses Sox2 (from the single residual knock-down allele) at low levels (30% relative to normal, whereas heterozygotes express 65%) (3,7). In these mice, postnatal neurogenesis is strongly diminished, particularly in the hippocampus. In particular, the number of nestin/GFAP double-positive radial glia cells (a stem/

progenitor cell expressing Sox2 (3,5) is drastically decreased (3).

In Sox2 hypomorphic mutants, heterozygosis for a mutated Emx2 allele was sufficient to substantially rescue the number of GFAP/nestin stem/progenitor cells from about 20% to 60% of wild-type levels (Figure 2A and B); additionally, the radial glia was converted from a thin, poorly-developed appearance typical of cells of the hypomorphic mutant, to quasi-normal morphology (Figure 2A). In agreement, BrdU incorporation (Figure 2B) was increased to about 45% of wild-type levels in $Sox2^{\beta\text{-geo}/\Delta Enh}$; $Emx2^{+/-}$, versus about 30% in $Sox2^{\beta\text{-geo}/\Delta Enh}$; $Emx2^{+/+}$ controls (even if loss of a single Emx2 allele, per se, causes some decrease of BrdU incorporation (Figure 2B, ref. 31, see section 'Discussion').

To interpret this result, we examined Sox2 expression in wild-type mice in the prospective hippocampal area during development. In this area, Sox2 and Emx2 are coexpressed in a large proportion of cells (Figure 2C). At E 15.5, both the medial and lateral walls of the telencephalon expressed Sox2; however the medial wall of the lateral ventricle, from which the hippocampus will originate, expressed Sox2 at comparatively lower levels than the lateral wall in the wild-type (Figure 2C, filled versus empty arrowheads). On the other hand, the Emx2 level was higher in the medial as compared to the lateral wall (Figure 2C, arrowheads see also 15,20), pointing to an inverse relation between Sox2 and Emx2 expression. Within the medial telencephalic wall (prospective hippocampus region), an inverse Sox2/Emx2 relation is also seen with an Emx2-high, Sox2-low region developing adjacent to a comparatively Emx2-low, Sox2-high region (Figure 2C, thin arrowheads; Supplementary Figure S3).

In $Emx2^{+/-}$ heterozygotes we noted a significant upregulation of Sox2 expression in the medial telencephalic, relative to the lateral wall, when compared to wild-type mice (Figure 2C, arrowheads). This inverse correlation suggests that, within the area from which the hippocampus will arise, Emx2 may negatively modulate Sox2 levels. This result is consistent with the possibility that the loss of a single Emx2 allele in Sox2 hypomorphic / $Emx2^{+/-}$ double mutants contributes, by upregulating the deficient Sox2 expression, to the observed radial glia rescue.

Emx2 transfection in Sox2-positive P19 teratocarcinoma cells inhibits the activity of reporter genes driven by the 5′ or 3′ Sox2 enhancer

The previous *in vivo* results, indicating that Emx2 somehow negatively modulates the Sox2 enhancers, raise the question whether Emx2 effects on Sox2 are direct or mediated by other factors. The 5′- and 3′-enhancers 'core' elements were previously defined *in vivo* by transgenic assays and, *in vitro*, by transfection in Embryonic Stem (ES) Cells (11,22,23). Both elements contain POU sites, known to be functionally important in ES and brain cells, which bind specific transcription factors (Oct4 in ES, Brn1 and Brn2 in neural cells) (11,22,23). In transgenic mice, ~400 nt of the 5′ enhancer recapitulate full expression, but as little as 120 nt are sufficient for some specific activity (22). The 400 nt enhancer contains, in addition to the two POU sites, several ATTA sites (referred to as ATTA-1 to ATTA-6, Figure 3A), which represent the core of potential homeobox transcription factor-binding motifs (22), including Emx2. The more 5′ POU site is combined with ATTA-3 site within a single overlapping sequence. The 3′ enhancer similarly contains several ATTA sites, together with a previously characterized POU-binding element (23) (Figure 3A).

To address the possibility that Emx2 directly affects Sox2 enhancer function, and to investigate its molecular mechanisms of action, we developed a simplified *in vitro* transfection system. We performed transfection experiments in P19 teratocarcinoma cells, using the 5′ and 3′ enhancer 'core' regions linked to a luciferase reporter. P19 cells express Sox2 at high levels, but are negative for both Emx2 and the putative neural Sox2 activators Brn1 and Brn2 (11,22), although they express the related POU factor Oct3/4, an activator of Sox2 in ES cells (22,23); this allows us to test for the effects of adding these exogenous factors in appropriate combinations and dosage, and to evaluate the effects on enhancer functions of different, specific point mutations within transcription factors recognition sites.

We first transfected into P19 cells a luciferase reporter gene, driven by the minimal tk promoter linked to the core 5′Sox2 enhancer, in the absence or presence of an Emx2-expression vector.

Emx2 cotransfection strongly repressed the activity of the enhancer, to a level just above that of the control enhancer-less tk-luciferase vector (Figure 3B). Cotransfection with a vector expressing Otx2, a related homeobox gene, or with empty vector gave no significant repression. Similarly, Emx2 repressed the activity of the 3′Sox2 telencephalic enhancer (11,23), when assayed with both a full size and a 'core' enhancer (22) construct (Figure 3C), though the observed repression was less pronounced than that observed with the 5′ enhancer. The repression caused by Emx2 was dose-dependent for both the 5′ and 3′ enhancers (Figure 3D).

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

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

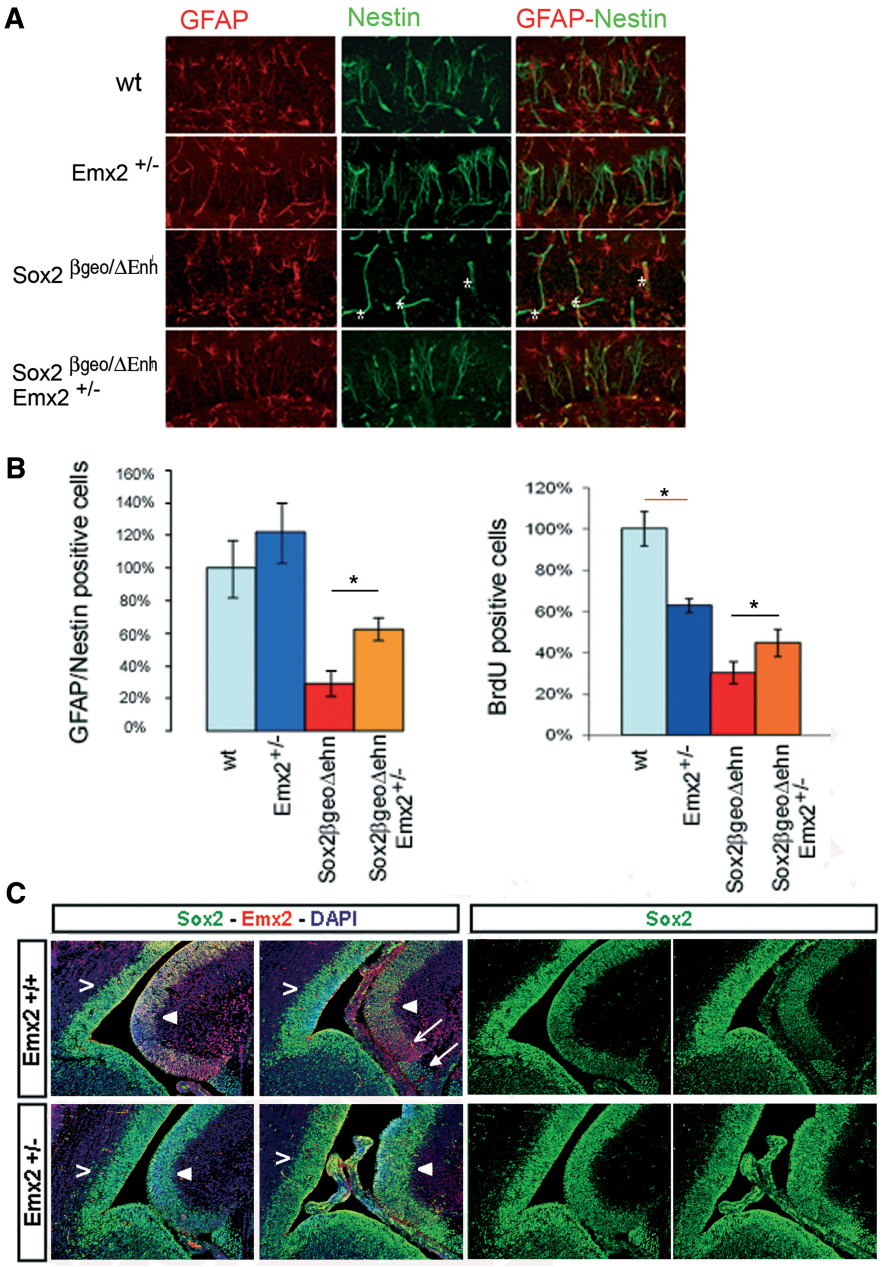

Figure 2. Emx2 deficiency ($Emx2^{+/-}$) rescues GFAP/nestin stem cells impairment in the hippocampus of Sox2-deficient ($Sox2^{\beta\text{-geo}/\Delta Enh}$) mutant mice. (**A**) GFAP/nestin double immunofluorescence of hippocampus DG in the indicated genotypes. GFAP/nestin-positive cells, strongly depleted in Sox2-hypomorphic ($Sox2^{\beta\text{-geo}/\Delta Enh}$) mutants, recover to a significant extent in $Sox2^{\beta\text{-geo}/\Delta Enh}$; $Emx2^{+/-}$ double mutants (asterisks mark vessels, showing non-specific fluorescence). (**B**) GFAP/nestin-positive cells and BrdU-positive cells. Wild-type is set = 100%. $n = 5$ mice per genotype; $^{*}P \leq 0.002$ by Student's t-test. (**C**) Double immunofluorescence with anti Emx2 (red) and anti Sox2 (green) antibodies on E15.5 telencephalic sections (confocal microscopy), in wild-type ($Emx2^{+/+}$, top) and $Emx2^{+/-}$ heterozygotes (two different representative mice per genotype, out of $n = 5$ mice analysed). The Sox2 (green) channel is also separately shown on the right panels. In $Emx2^{+/-}$ brains, compared to $Emx2^{+/+}$ controls, a comparative increase in the intensity of Sox2 staining is seen in the medial telencephalic wall (filled arrowhead) (comprising the hippocampal primordium region, arrows), as compared with the outer/lateral wall (empty arrowhead) within the same section. In the lower medial telencephalic wall, in the region of the prospective hippocampus, a boundary can be appreciated between two regions showing Sox2-high/Emx2 low (filled arrow) and comparatively Sox2-low/Emx2 high levels (non-filled arrow) (see also magnification and channel separation in Supplementary Figure S3).

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

We characterized by EMSA the binding of recombinant Emx2 to all of the ATTA sites in the core 5′ enhancer. ATTA-3 resembles (Figure 4A) one of the few characterized Emx2-binding sites, that of the Wnt1 gene (32,33); furthermore, a similar site is located in the 3′ enhancer (ATTA-4) just upstream to the already studied (11,23), functionally important, POU site. In EMSA,

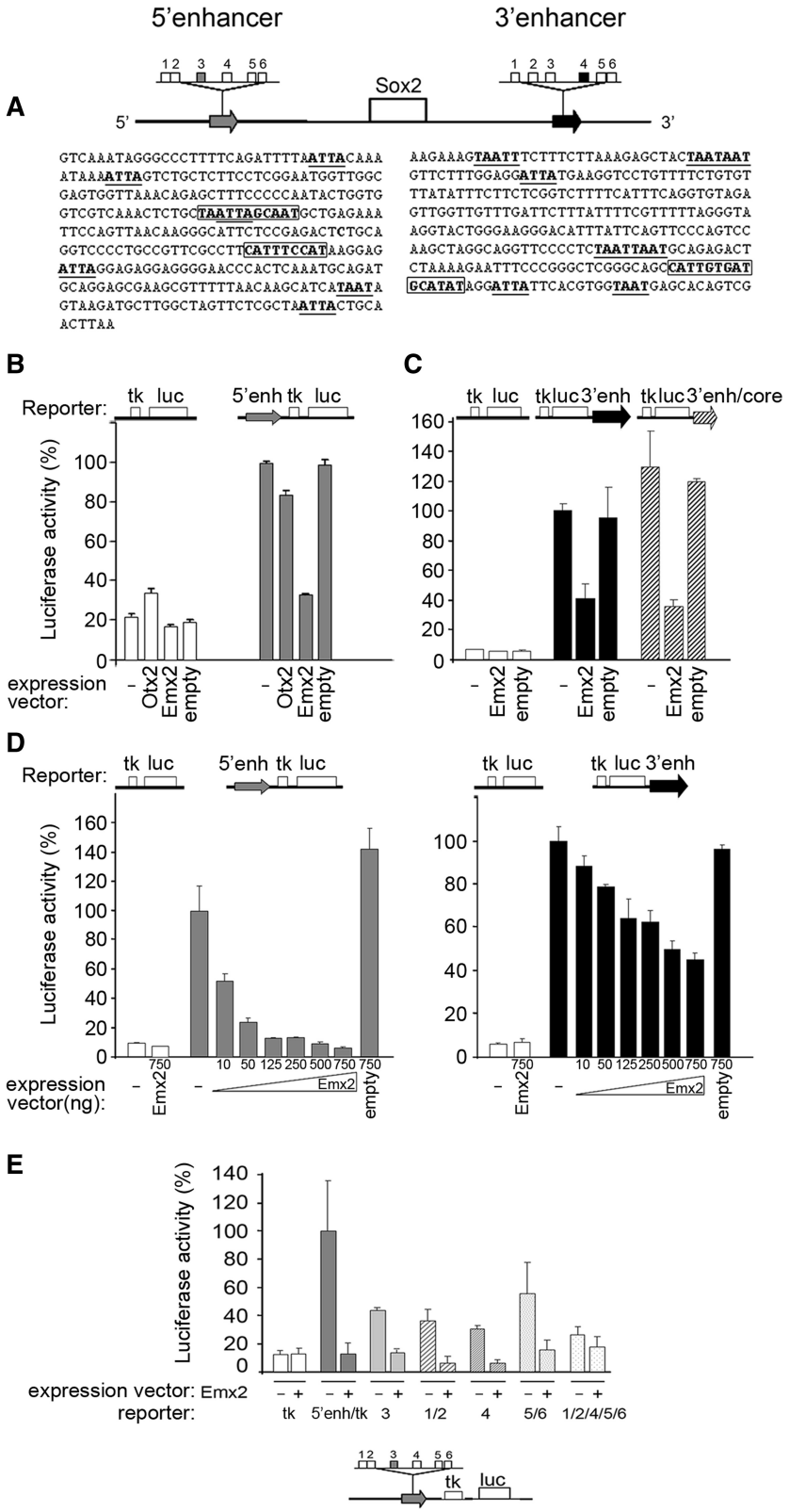

Figure 3. Emx2 represses the activity of the 5′ and 3′ Sox2 telencephalic enhancers in transfection assays. (**A**) 5′ and 3′ Sox2 telencephalic enhancers. Numbered squares: ATTA sites, underlined and bold in the sequences below. Boxed bold sequences: POU sites (11,22,23) in 5′ and 3′ enhancers (**B** and **C**). Cotransfection of 5′ or 3′ enhancer-driven (black bars, full enhancer; striped bars, 'core' enhancer) tk-luciferase vectors, or 'empty' tk-luciferase vector (white bars), with Emx2 or Otx2 expression vectors, or with 'empty' vector. The mean activity of the enhancer-driven constructs (with no cotransfected expression vector) is set = 100% luciferase activity. (**D**) Cotransfection of 5′ and 3′-enh. luciferase constructs with increasing amounts of Emx2-expression vector. (**E**) Luciferase activity of 5′ enhancer constructs carrying mutations in the indicated ATTA sites, and their response to cotransfection of the Emx2 expression vector (500 ng). Values represent the mean ± SD of $n \geq 3$ independent transfection experiments, with each transfection done in triplicate.

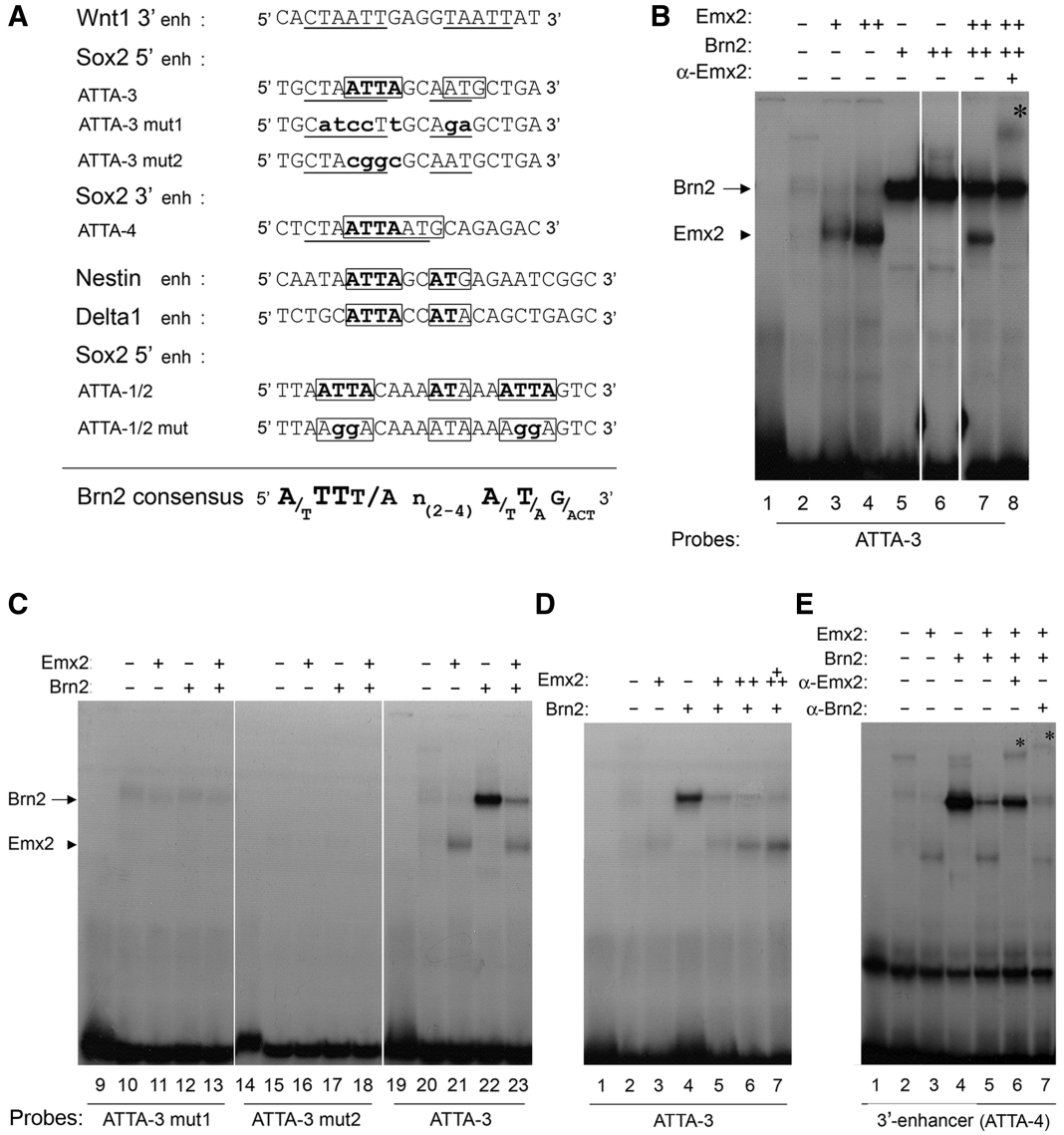

Figure 4. Emx2 binds to ATTA sites within the Sox2 5′ and 3′ enhancers, and antagonizes binding of the activator Brn2. (**A**) ATTA sequences binding Emx2 and/or Brn2. Lowermost line: Brn2/POU consensus based on TFBS cluster (http://hscl.cimr.cam.ac.uk/TFBScluster) and our data. Letter size is proportional to nucleotide frequency. The spacer (n) is 2–3 nt in previously validated sites (34,35). For the interaction of a POU factor with its binding site, and spacer length, see (27). Boxed sequences are homologies to the Brn2 consensus. Underlined sequences correspond to the previously reported Emx2 binding sequence (footprint) in the Wnt1 enhancer (32,33), and to homologous sequences within the 5′ and 3′ Sox2 enhancers. (**B**) EMSA with an ATTA-3 site probe (5′ enhancer) and recombinant Emx2 and Brn2 proteins (as indicated above the lanes). Anti-Emx2 antibody was added in lane 8. Asterisk: supershifted band. Amounts of TNT product used (see section 'Materials and Methods'): Emx2: + = 3 µl; ++ = 6 µl. Brn2: + = 3 µl; ++ = 6 µl. α-Emx2 antibody: + = 1 µl (of a 1:10 dilution of ascites fluid). (**C**) EMSA with wild-type (lanes 19–23) and two different mutated (lanes 9–13; 14–18) ATTA-3 site probes (5′ enhancer). Amounts of TNT product used: Emx2: + = 6 µl; Brn2: + = 1.5 µl. (**D**) Addition of increasing amounts of Emx2 (lanes 5–7) to ATTA-3 site probe (5′ enhancer) together with a fixed amount of Brn2 (as in lane 4). An Emx2 retarded band appears, while the Brn2 band progressively disappears. Amounts of TNT product used: Emx2: + = 4 µl; ++ = 8 µl; +++ = 12 µl. Brn2: + = 1 µl. (**E**) EMSA with a probe from the 3′ enhancer ATTA-4 site, showing ability to bind Emx2 or Brn2. Addition of Emx2 together with Brn2 (lane 5) antagonizes Brn2 binding. Asterisks indicate bands supershifted by antibodies (lanes 6 and 7). Amounts of TNT product used: Emx2: + = 5 µl. Brn2: + = 2 µl. α-Emx2 antibody: + = 1 µl (of a 1:10 dilution of ascites fluid). α-Brn2 antibody (Santa Cruz): + = 8 µl.

recombinant Emx2 (Supplementary Figure S4, panel A) bound to the Wnt-1 oligonucleotide (originally characterized only by foot-printing) generating a complex, that was super shifted by an anti-Emx2 antibody (Supplementary Figure S4, panel B). Similarly, ATTA-3 was efficiently bound by Emx2 (Figure 4B, lanes 3–4; Figure 4C, lane 21); two different mutations of ATTA-3 abolished Emx2 binding (Figure 4C, lanes 11 and 16, versus lane 21). Further, the ATTA-3/Emx2 binding was efficiently competed by excess unlabelled Wnt-1 or ATTA-3 oligonucleotides, with similar kinetics (Supplementary Figure S4B). We conclude that ATTA-3 can be bound, *in vitro*, by Emx2.

An oligonucleotide including the combined ATTA/POU site (ATTA-3) binds (21) the ES cell factor OCT4 and its brain homologues Brn1 and Brn2. As Emx2

negatively modulates the activity of Sox2 telencephalic enhancers in brain (Figure 1), we asked if Emx2 binding to the POU sites in brain cells might interfere with the binding of Brn factors. Brn2 bound, as expected, the composite POU/ATTA-site 3 (ATTA-3) of the 5′enhancer, that was shown to bind Emx2 (Figure 4B, lanes 5 and 6). When Brn2 and Emx2 were added together, no ternary Emx2-Brn2-probe complex was detected, suggesting that the binding was mutually exclusive. Addition of anti-Emx2 antibody caused the loss of the Emx2 band and its supershift, but did not affect the Brn2 band (Figure 4B, lanes 7 and 8). Importantly, Brn2 binding was abolished (Figure 4C, lanes 12 and 17 as compared to lane 22) by the same mutations that caused loss of Emx2 binding.

Adding increasing amounts of Emx2, in the presence of a fixed amount of Brn2, proportionally increased Emx2 binding, whereas Brn2 binding was strongly decreased (Figure 4D, lanes 5–7). The repression of Brn2 binding was observed already at relatively low levels of added Emx2 (and Emx2 binding), and under conditions of a large excess of labelled oligonucleotide probe; this suggests that the repression of Brn2 binding is not simply the result of a direct competition on the same DNA molecule, but rather entails other indirect mechanisms (see below).

In the 3′enhancer, a motif (ATTA-4) similar to the Emx2-binding ATTA-3 site is located just upstream to an already studied (11,22), functionally important, POU site. We performed EMSA experiments with Emx2 and Brn2 using the 3′ enhancer ATTA-4-site. Again, ATTA-4 (Figure 4A) bound both Brn2 and Emx2 (Figure 4E), and addition of Emx2 greatly decreased the binding of Brn2 (Figure 4E, lanes 4 and 5). Similarly, to the 5′ site, mutation of this site abolished the binding of both Emx2 and Brn2 (data not shown).

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

The ATTA motif is part of a large number of core sequences of distinct transcription factor-binding motifs, which are difficult to identify purely on the basis of the DNA sequence. As the POU/ATTA sequence (ATTA-3) binds both Oct3/4 and Brn1/Brn2 (21), and other sequences containing an ATTA motif bind Brn1 and Brn2 (33,34; Figure 4A), we tested all ATTA sites in the 5′ enhancer for binding to these factors. Brn2 bound (Figure 5A) an oligonucleotide containing both sites 1 and 2 (ATTA-1/2), whereas Emx2 did not bind (the weak band migrating slightly faster than Brn2 in lane 3, arrowhead, is due to a protein contained in the TNT extract used for Brn2 synthesis, see lane 2). We could not detect any binding of Emx2 to the ATTA-1/2 probe even when adding Emx2 in the absence of Brn2, in amounts equal or greater than those able to generate a strong shifted band with the ATTA-3 probe in a control binding run in parallel (Supplementary Figure S5). Mutation of the conserved TT doublet in the ATTA motif abolished Brn2 binding, leaving only the fast TNT-derived band (lanes 10 and 11). The Brn2 band was almost completely ablated by addition of anti-Brn2 antibody (lane 4). Finally, excess unlabelled ATTA-1/2 oligonucleotide competed the binding of the previously validated Brn2-binding site, ATTA-3 in the 5′ enhancer (22 and present article) as efficiently as unlabelled ATTA-3 site oligonucleotide did (Figure 5B, lanes 4 and 5, versus lane 3). In contrast, a mutated ATTA-1/2 site oligonucleotide failed to compete (lane 6). We conclude that Brn2 can bind to the ATTA-1/2 site in a sequence-specific way.

As shown in Figure 4D, Emx2 might inhibit the binding of Brn2 to the POU/ATTA site (ATTA-3) oligonucleotide both by direct DNA binding and by other indirect mechanisms. We tested the effects of Emx2 addition to the ATTA-1/2 site oligonucleotide, in the presence of Brn2. Emx2 addition (Figure 5A, lane 5) almost completely abolished Brn2 binding, already at low Emx2 concentrations. Similar or higher amounts of the haematopoietic transcription factors GATA-1 and GATA-2 did not interfere with Brn2 binding (Figure 5A, lanes 6 and 7), nor did addition of a TNT lysate prepared by transcription/translation of an ‘empty’ vector in control experiments (data not shown).

In additional experiments (Figure 5C) Emx2 prevented Brn2 binding, in a dose-dependent fashion, to two independently characterized Brn2-binding sites (Figure 4A), those in the Delta and Nestin genes neural enhancers (34,35).

ATTA-1/2 and ATTA 3 probes were also tested with nuclear extracts from the neural (adult hippocampal) AHP cell line (Figure 5D and E); endogenous Brn2 bound to both probes (Figure 5E, lanes 1 and 2; Figure 5E, lane 1) generating bands that were supershifted by anti-Brn2, but not anti-GATA-1 (Figure 5D, lanes 3 and 4); and were properly competed by the same unlabelled oligonucleotide, but not by its mutated version (Figure 5D, competitors: lane 5:ATTA-1/2; lanes 6 and 7: mutated ATTA-1/2; lane 8: ATTA-3). Also in this neural cell context, the addition of increasing amounts of Emx2 (but not of GATA-1) caused a sharp decrease of Brn2 binding, already at low Emx2 concentrations (Figure 5E, lanes 2–4, compare to lanes 1 and 5).

Overall, the experiments reported above (Figures 4 and 5) demonstrate that Emx2 prevents the binding of transcription factors (in this case Brn2) to their cognate motifs via mechanisms independent of its binding to DNA; one possible mechanism might be protein–protein interaction between Emx2 and Brn2. In a GST-pull down assay, a GST–Emx2 fusion protein retained *in vitro* synthesized Brn2 (Figure 5F), indicating that Emx2 and Brn2 proteins are able to physically interact.

Emx2 functionally antagonizes Brn2

POU factors, including Oct4 and neural transcription factors Brn1 and Brn2, were characterized as activators of the Sox2 3′ enhancer in co-transfection experiments, and the mutation of the POU/ATTA site (ATTA-3 site) in the 5′enhancer (22) or of the POU site in the 3′enhancer (11,22) substantially decreased the activity of Sox2 transgenic constructs, suggesting that Brn1 and Brn2 factors

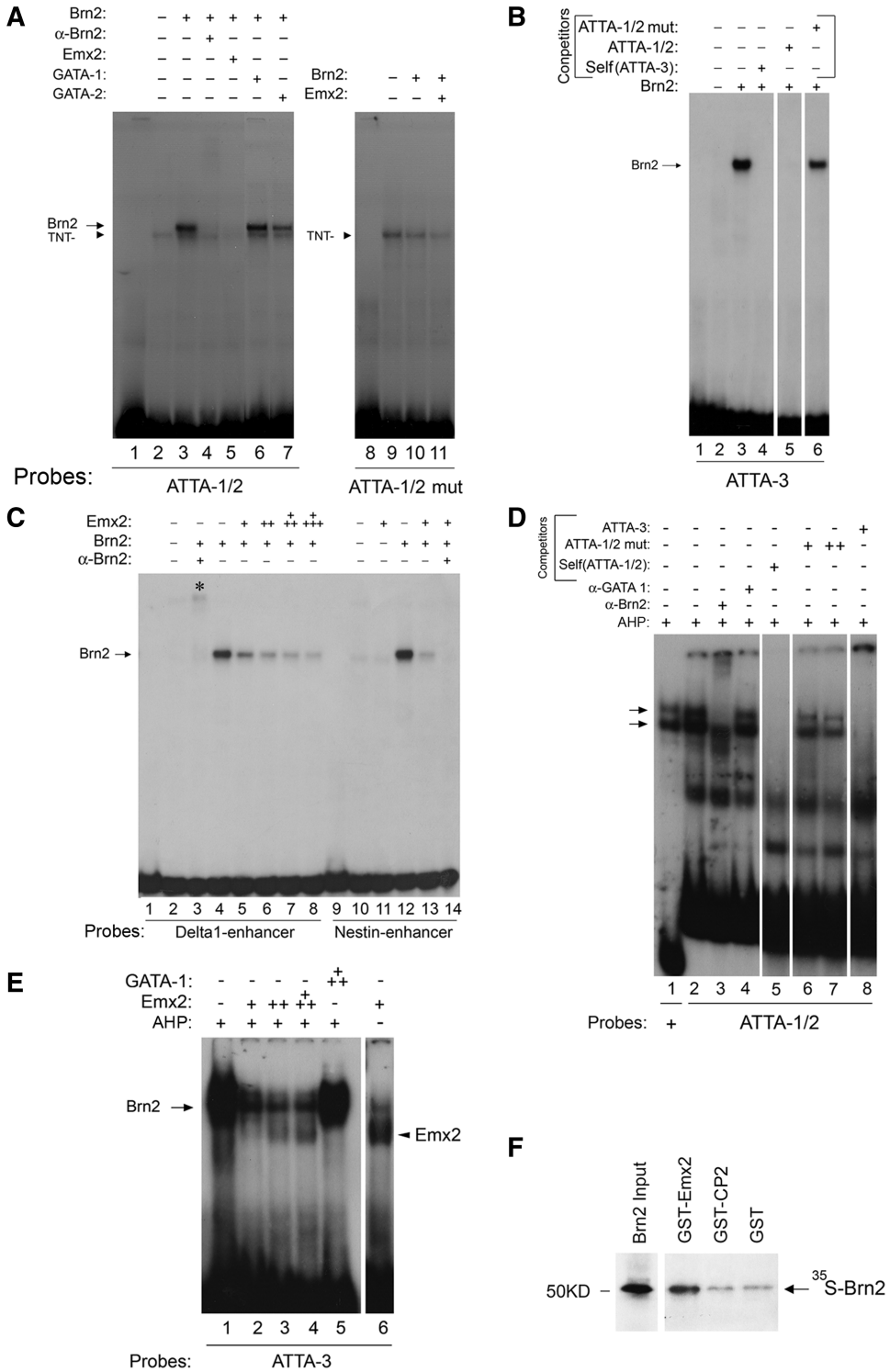

Figure 5. Emx2 antagonizes the binding of Brn2 to ATTA-1/2 sites in the 5′ enhancer, and to previously characterized Brn2 binding sites in other neural enhancers. (**A**) EMSA with a probe containing ATTA sites 1 and 2 (5′ enhancer); added recombinant proteins, and Brn2 antibody, are indicated above the lanes. The probe binds recombinant Brn2 (arrow), but not Emx2 (TNT- arrowhead indicates a non-specific band seen also with TNT extract only). Addition of Emx2 antagonizes Brn2 binding (lane 5). No antagonism is seen upon addition of GATA1 or GATA2 (lanes 6 and 7). Amounts of TNT product used (see section 'Materials and Methods'): Brn2: + = 1.5 µl; Emx2: + = 4 µl; GATA1/2: + = 4 µl. α-Brn2 antibody (Santa Cruz): + = 8 µl. (**B**) EMSA with an ATTA-3 site probe (a previously validated Brn2 binding site in the 5′ enhancer (11,22,23); binding of Brn2 is efficiently competed by wild-type non-labelled ATTA-1/2 sites oligonucleotide (lane 5), but not by its mutated version (lane 6). Competition is as efficient as with the 'self' oligonucleotide (lane 4). Amounts of TNT product used: Brn2: + = 1.5 µl. Competitor amounts: Self (ATTA-3): + = 25X (molar excess of cold competitor oligonucleotide); ATTA-1/2: + = 25X; ATTA-1/2 mut: + = 25X. (**C**) EMSA with probes containing previously validated Brn2 binding sites in the nestin and Delta-1 enhancers. Brn2 binding (arrow) is antagonized by simultaneous Emx2 addition in a dose-dependent way. Asterisk: Brn2 antibody-supershifted band. Amounts of TNT product used for Delta 1-enhancer : Brn2: + = 2 µl; Emx2: + = 1 µl;++ = 2 µl; +++ = 3 µl; ++++ = 4 µl. α-Brn2 antibody: + = 8 µl (Santa Cruz). Amounts of TNT product used for

(continued)

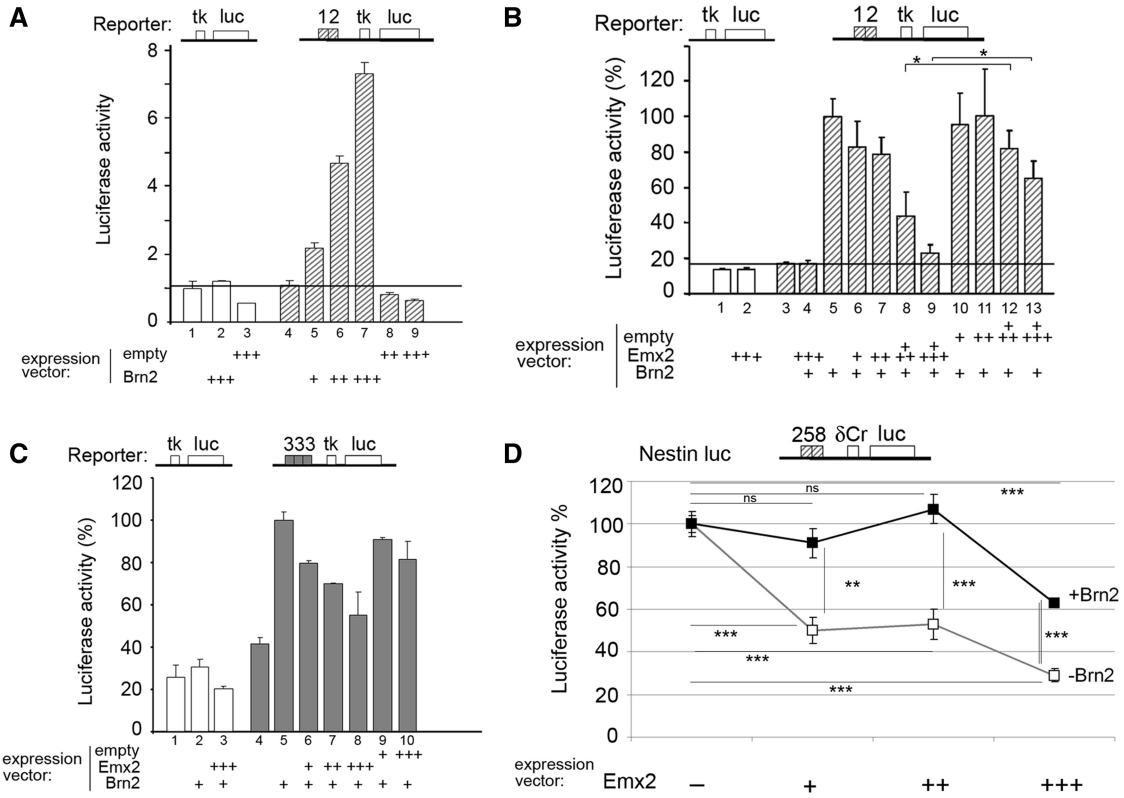

Figure 6. Emx2 represses Brn2-transactivated ATTA-1/2 and ATTA-3 sites—tk luciferase reporter constructs in a dose-dependent way. (**A**) Brn2 dose-dependent transactivation of ATTA-1/2 sites (5′ enhancer) (Brn2: +, ++, +++, 125, 500, 1000 ng) (B and C) Emx2 dose-dependent repression of Brn2-dependent transactivation of ATTA-1/2 sites construct **(B)** and of ATTA site 3 construct (**C**) (Brn2: +, 500 ng; Emx2: +, ++, +++, ++++; 100, 200, 500, 1000 ng). In **A**, luciferase activity is expressed in arbitrary units, where 1 is the activity of the tk luc reporter; in B and C, 100% luciferase activity is set to the maximum observed activity. The horizontal line in A and B represents the background activity of the ATTA-1/2 site construct in the absence of cotransfected Brn2. (*$P < 0.007$ by Student's *t*-test, non-parametric, Welch correction) (**D**) Nestin-enhancer (258 wt)-driven luciferase reporter, transactivated by cotransfected Sox2 expression vector 500 ng (as in 25), is cotransfected with increasing amounts of Emx2 expression vector, in the presence (+Brn2) or absence (−Brn2) of Brn2 expression vector (Brn2: 500 ng; Emx2: +, ++, +++; 125, 250, 500 ng). Brn2 addition antagonizes the repressive effect of Emx2 (**$P < 0.001$; ***$P < 0.0002$ by Student's *t*-test, non-parametric, Welch correction). 100% luciferase activity is set to that observed without Emx2 cotransfection for both +Brn2 and −Brn2 samples. In the absence of cotransfected Brn2, the activity of the construct was about 20% lower (marginally significant, data not shown) than in the presence of Brn2. Note that a Sox2-expression vector was cotransfected in all the experiments, as Sox2 had been reported to increase the activity of the present reporter in other cell types (25). Values represent the mean ± SD of $n \geq 3$ independent transfection experiments, with each transfection in triplicate.

may be positive regulators of Sox2 transcription in the brain (11,22,23).

To evaluate the respective roles of Brn2 and Emx2 in transfection experiments we linked to the minimal tk-promoter the ATTA-1/2 or the POU/ATTA (ATTA-3) site (the latter as a trimer) from the 5′enhancer. We transfected the construct into P19 teratocarcinoma cells (which express Sox2) in the presence of different amounts of Brn2-and/or Emx2 expression vectors (Figure 6). In the absence of Emx2, Brn2 strongly stimulated the activity of the ATTA-1/2 construct in a dose-dependent way and, to a lesser extent, that of the ATTA-3 construct (Figure 6A and C). The Brn2-dependent stimulation of the ATTA-1/2 construct was repressed to basal levels (just above the level of the tk-luc reporter, lane 9 versus lanes 1 and 2), by cotransfection of progressively increasing amounts of the Emx2-expression vector (Figure 6B). Cotransfection of

Figure 5. Continued
Nestin-enhancer: Brn2: + = 2 µl; Emx2: + = 4 µl. α-Brn2 antibody: + = 8 µl (Santa Cruz). (**D**) EMSA with ATTA-1/2 site probe and nuclear extracts from AHP neural cells. Two complexes are generated (arrows) with both ATTA-3 (lane 1, '+' as in ref. 21) and ATTA-1/2 (lane 2), which are supershifted by anti-Brn2 (lane 3), but not anti-GATA1 antibodies (lane 4). Binding of Brn2 to ATTA-1/2 is efficiently competed by unlabelled ATTA-3 (lane 8), by 'self' ATTA-1/2 (lane 5), but not by mutated ATTA-1/2 (lanes 6 and 7) oligonucletides. Unlabeled competitor oligonucleotides were added in 25-fold molar excess (+) or 50-fold molar excess (++). (**E**) EMSA with ATTA-3 probe and nuclear extracts from AHP cells. Added recombinant proteins (Emx2, GATA-1) are indicated above the lanes. The Brn2 retarded complex (lane 1, arrow) (see also ref. 21 and panel D) is sharply decreased following addition of Emx2 (lanes 2–4), but not of control GATA-1 (lane 5). The lower, Emx2-containing complex is progressively increased in parallel with the addition of Emx2. This complex has the same mobility of that generated by direct binding of recombinant Emx2 to the ATTA-3 probe (lane 6). **(F)** Emx2 and Brn2 directly interact in a GST pulldown assay. Brn2 is retained by GST-Emx2, but not by GST-CP2 control resin (which gives a weak signal equivalent to that seen with the 'empty' resin GST).

control 'empty' vector, instead of Emx2^{-} expression vector, yielded a slight inhibition only at the highest tested levels, ensuring specificity of the Emx2 repression observed (Figure 6B, lanes 10–13). Similarly, on the ATTA-3 construct, Brn2-dependent stimulation was inhibited by Emx2 (Figure 6C), though the observed repression is weaker. Thus, Brn2 is an activator at both the ATTA-3 [as previously shown *in vivo* and *in vitro* (22)] and the ATTA-1/2 sites, and Emx2 inhibits the transcriptional activity at the same sites, antagonizing Brn2-dependent stimulation. As Emx2 does not bind to ATTA-1/2 site sequences (Figure 5A), this inhibition may be caused by mechanisms that do not strictly require Emx2 binding to the DNA. The somewhat lower effect of Emx2 in the Brn2-dependent system, as compared to the drastic effect observed with the full 'core' element (in the absence of cotransfected Brn2) (Figure 3), probably reflects the modest enhancer activity of the individual ATTA sites in isolation, as compared with the cooperative activity of the multiple sites active in the full enhancer (Figure 3).

Is the Emx2 inhibitory effect limited to the Sox2 5′ and 3′ enhancers? To evaluate this point, we performed experiments using the nestin enhancer, that is positively regulated by transfection of Brn factors, in conjunction with Sox2 (25). As shown above (Figure 5C), Emx2 addition antagonized Brn2 binding (in EMSA) to this enhancer. In transfection experiments in P19 cells, in the absence of transfected Brn2, Emx2 strongly inhibited the activity of the enhancer already at low concentrations (Figure 6D, '−Brn2', open squares). In this condition, enhancer activity likely depends on the related POU factor Oct3/4, expressed in P19 cells. In contrast, the addition of Brn2 ('+Brn2', filled squares) completely prevented the Emx2 repression at low/intermediate Emx2 levels, and substantially attenuated it at the highest Emx2 level (Figure 6D). Note that, in P19 cells, the addition of Brn2 *per se* stimulates the activity of the enhancer only minimally (~20%, data not shown), indicating that the observed Brn2-dependent attenuation of Emx2-mediated repression is not the result of independent stimulation of gene activity by Brn2; rather, the excess Brn2 may directly 'titrate' Emx2 activity. These results, together with those reported above, suggest that Emx2 and Brn2 antagonize each other's activities.

Emx2 binds to the 5′enhancer *in vivo*

To ascertain if Emx2 interacts in brain cells with the Sox2 regulatory elements, we performed *in vitro* ChIP with anti-Emx2 antibodies, using chromatin from embryonic telencephalon (E14.5), from wild-type and Emx2-null (negative control) embryos. A fragment comprising the ATTA-3 and the adjacent ATTA-1/2 sites was bound by Emx2 in wild-type chromatin, but not in Emx2-null chromatin (Figure 7). No binding was detected in an adjacent region B, comprising ATTA-5 and 6 sites, and lying 3′ to the bound DNA region. We conclude that Emx2 likely functionally interacts with the Sox2 regulatory region *in vivo*.

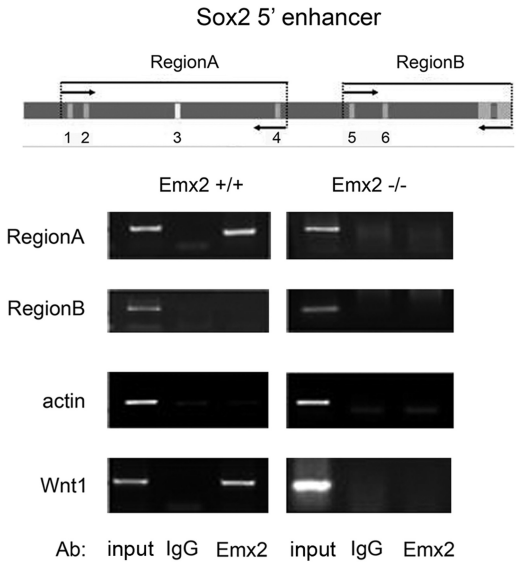

Figure 7. Emx2 is bound to the Sox2 enhancer *in vivo*. ChIP with anti-Emx2 antibodies of E14.5 embryonic brain chromatin from wild-type and Emx2$^{-/-}$ control embryos. Region A, containing ATTA-3 site is immunoprecipitated from wild-type, but not Emx2-null chromatin. The previously described Wnt1 enhancer containing an Emx2 binding site (33) is used as a control (Wnt1), and is similarly precipitated from wild-type, but not mutant, chromatin. Antibodies used are indicated below the lanes. Input: input chromatin. IgG: anti-IgG control antibodies. Emx2: anti-Emx2 antibodies.

DISCUSSION

Emx2 is a transcription factor involved in hippocampal growth and in cortex patterning (19,36). With the exception of the Wnt1 and FGF8 genes (32,37,38), there are few identified neural Emx2 targets. Here we show, by *in vivo* and *in vitro* experiments, that Emx2 negatively regulates two characterized Sox2 enhancers. Loss of a single Emx2 allele increases Sox2 expression in the E15.5 medial telencephalic wall and partially rescues an hippocampal phenotype dependent on Sox2 deficiency. Our results, together with data of the literature, suggest that Emx2 may control aspects of Sox2 expression and brain development by antagonizing the activities of transcriptional activators, such as Brn2.

Emx2 negatively modulates Sox2 telencephalic enhancers *in vivo*

Sox2 neural expression in chick (30) and mouse (6,11,22,23,39) is regulated by multiple enhancers. Among the best characterized mouse enhancers are the 5′ and 3′ Sox2 enhancers studied here, which direct transgenic reporter gene expression to the telencephalon, the 5′ enhancer being more active in dorso-medial regions, and the 3′ enhancer in ventro-lateral regions. Emx2 is expressed in the dorsal telencephalon in a posterior medial to anterior lateral concentration gradient, that intercepts the wide Sox2 expression domain; at the cellular level, the two expression domains substantially overlap within the ventricular zone, allowing for potential cross-regulation (3,19,36). In our experiments (Figure 1), the loss of one or both Emx2 copies substantially

increases the expression of transgenes driven by the 5′ or the 3′ Sox2 enhancers, indicating that the normal levels of Emx2 may inhibit to some extent the activities of enhancers of Sox2. The inhibitory activity of Emx2 is further reflected in the decreased activity of the same enhancers brought about by Emx2 overexpression in NSC cultures (Figure 1C).

Does the altered regulation of Sox2 enhancers by Emx2 modify the levels of endogenous Sox2 *in vivo*? Overall, in developing brain, Sox2 levels are not highly changed in Emx2 +/− embryos, but significant modulation can be appreciated at specific locations. In the late embryo, Sox2 and Emx2 are coexpressed in the prospective hippocampal domain; at this stage, in the lateral ventricle, regions of high Sox2 expression show relatively lower Emx2, and regions of high Emx2 expression have lower Sox2 levels (Figure 2C). This inverse correlation is particularly evident in the medial telencephalic wall, where the hippocampus primordia develop (Figure 2C and Supplementary Figure S3). Loss of a single Emx2 allele results, in this region, in increased Sox2 expression (Figure 2C and Supplementary Figure S3). Thus, effects of Emx2 deficiency on Sox2 may be more evident in specific regions/developmental stages, possibly depending on expression levels of Emx2 itself, or interactions with other factors. This conclusion is in agreement with the rather subtle phenotipic effects of changes in Emx2 levels observed in the cortex as compared to the hippocampus (36).

Heterozygous Emx2 deficiency antagonizes the hippocampal NSC loss of Sox2 hypomorphic mutants

An important question arising from our present results, is whether heterozygous Emx2-deficiency (that, by itself, has little effect on brain development, 15,20,36) has any phenotypic consequences on Sox2-dependent functions.

Both Emx2 homozygous mice and Sox2 mutants (Sox2 hypomorphic and Sox2 conditional-null mice) show severe hippocampal defects, indicating that both genes are essential for hippocampal development (3,14,15). In the hippocampus, Sox2 is required for postnatal NSC survival; complete Sox2 ablation by E12.5 results in the loss of hippocampal neurogenesis and DG severe hypoplasia, between P2 and P7 (14). Moreover, in adult Sox2 hypomorphic ($Sox2^{\beta\text{-geo}/\Delta Enh}$) mutants, expressing 30% of the normal Sox2 RNA, nestin/GFAP radial glia cells (a Sox2-expressing neural stem/progenitor cell) (3,5,40) in the hippocampus are importantly decreased (Figure 2). As Sox2 and Emx2 are coexpressed in the hippocampal primordium (Figure 2) and in the adult hippocampus (as well as in the hippocampal AHP cell line (Supplementary Figures S6 and S7), a reduction in Emx2 dosage may be expected to affect Sox2-dependent functions in this region.

In adult hypomorphic Sox2 mutants, heterozygous Emx2-deficiency strongly increases the number of nestin/GFAP radial glia cells and, to a lesser extent, BrdU incorporation (Figure 2A and B); note that, in wild-type mice, heterozygous Emx2 deficiency, *per se*, only slightly raises the number of nestin/GFAP radial glia cells and decreases, rather than increases, BrdU incorporation (Figure 2A and B; ref. 31). These results demonstrate that Emx2 deficiency rescues, in part, at least one well characterized Sox2-dependent NSC phenotype. These data, taken together with the increased Sox2 expression in the medial lateral ventricle wall (that includes the prospective hippocampus) of heterozygous Emx2-deficient mice (Figure 2C, Supplementary Figure S3), are thus consistent with the hypothesis that Emx2 deficiency may contribute to phenotypic rescue by raising Sox2 expression. Of course, additional mechanisms might also contribute to the observed phenotype.

Emx2 represses telencephalic enhancers in transfection assays, directly binds to enhancer sequences *in vitro* and antagonizes binding of POU transcriptional activators

To begin to address at the molecular level of our *in vivo* data, we performed EMSA and transfection experiments with P19 cells, that express Sox2, and can be manipulated by transfection to express Brn2 and/or Emx2 (absent in the basal state). We propose two mechanisms whereby Emx2 might downregulate Sox2 enhancer activity (Figures 4, 5 and 6; Supplementary Figure S8).

Firstly, Emx2, by directly binding to its cognate sites in DNA, might prevent the activity of other transcription factors which bind to sites overlapping the Emx2 motif; secondly, Emx2 might repress transcription by antagonizing the binding to DNA of transcription factors, without direct DNA binding, through protein–protein interactions. As to the first mechanism, Emx2 directly binds to 5′ (ATTA-3) and 3′enhancer (ATTA-4) sites in the Sox2 gene (Figure 4), which are also bound by the POU factors Brn1 and Brn2; importantly, these factors were previously implicated in Sox2 activity on the basis of *in vivo* experiments (transfection, transgenic mice and ChIP (11,22,23). As mutations at the ATTA-3 site abolish the binding of both Emx2 and Brn2, their binding sites are likely functionally overlapping, and their binding might be mutually exclusive; indeed, we did not detect by EMSA (even at high protein concentrations, data not shown) any band of mobility slower than that of Brn2, that might suggest the formation of a ternary complex of DNA with both factors. Moreover, in EMSA, the addition of increasing amounts of Emx2 resulted in increased binding of Emx2, together with progressive disappearance of the Brn2 band (Figure 4).

The second mechanism is suggested by the following observations: the binding of Brn2 to ATTA-sites in Sox2 enhancers and to other previously validated Brn2 sites (nestin, delta; 22,34,35) is prevented by Emx2 addition, in the absence of any binding of Emx2 itself to the same sequences (Figure 5). Emx2 and Brn2 might be reciprocally antagonistic (Figure 6) through direct protein–protein interaction, which would prevent Brn2 binding to regulatory sequences, and transcriptional activation (Figure 6). Indeed, GST pull-down experiments show that Brn2 and Emx2 may physically interact (Figure 5F). Our present interpretation is in agreement with data reported by other authors in a different experimental system; Sahara *et al*. (37) reported that Emx2

represses SP8 trancription factor-dependent activity of the FGF8 promoter without binding to the promoter itself; moreover, other authors reported that Emx2 and SP8 physically interact (38).

The DNA motif recognized by Brn2 in our experiments is a rather degenerate one, centred on an ATTA motif potentially recognized by many transcription factors (41). It can be hypothesized that, in addition to Brn2, other transcription factors, particularly, among neural factors, the Brn1 homolog, or Oct6, might bind to this sequence, and could thus be antagonized by Emx2. The 5′ enhancer is also bound by Oct3/4 at early developmental stages, and Oct3/4 is required for its activity in ES cells (22) and in the early neural plate (13), leading to the suggestion that an exchange between early Oct3/4 and later Brn/Oct factors binding is important in the early function of this element (13,22). Of note, this enhancer is active in P19 cells, which do not express neural POU factors, but do express Oct3/4; Emx2 addition by cotransfection can antagonize both basal 5′ enhancer activity (presumably dependent on Oct3/4 binding, 13,22), as well as Brn-stimulated activity (Figures 3 and 6). This may, again, reflect a wider ability by Emx2 to antagonize different POU factors, which may differently act on Sox2 (and other genes) regulation at different developmental stages and in different telencephalic regions.

Additional data suggest that these mechanisms do operate *in vivo*. In fact, Emx2 binds to a fragment comprising the 5′ ATTA-3 site in nuclei from normal telencephalon, in ChIP experiments (Figure 7). This fragment lies within a 120 bp DNA region that mediates POU site-dependent reporter gene expression in the telencephalon of transgenic embryos (22).

In conclusion, we propose that Emx2 contributes to the modulation of Sox2 expression by antagonizing Brn2 and possibly other activators able to bind the ATTA core sequence. The mechanism may not be restricted to the Sox2 enhancers, as at least the nestin enhancer may be similarly regulated (Figures 5C and 6D). It provides a wide scope for regulation, depending on the affinities of Emx2 for its DNA target and/or protein interactors, and on the relative ratios between Emx2 and brain transcription factors.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–8.

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