Genetic ablation of Rest leads to in vitro-specific derepression of neuronal genes during neurogenesis

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SUMMARY
Rest (RE1-silencing transcription factor, also called Nrsf) is involved in the maintenance of the undifferentiated state of neuronal stem/progenitor cells in vitro by preventing precocious expression of neuronal genes. However, the function of Rest during neurogenesis in vivo remains to be elucidated because of the early embryonic lethal phenotype of conventional Rest knockout mice. In the present study, we have generated Rest conditional knockout mice, which allow the effect of genetic ablation of Rest during embryonic neurogenesis to be examined in vivo. We show that Rest plays a role in suppressing the expression of neuronal genes in cultured neuronal cells in vitro, as well as in non-neuronal cells outside of the central nervous system, but that it is dispensable for embryonic neurogenesis in vivo. Our findings highlight the significance of extrinsic signals for the proper intrinsic regulation of neuronal gene expression levels in the specification of cell fate during embryonic neurogenesis in vivo.

KEY WORDS: Rest (Nrsf), Mouse model, Neurogenesis

INTRODUCTION
The establishment and maintenance of neuronal identity underlie the core of neuronal development. The transcriptional repressor RE1-silencing transcription factor [Rest; also known as neuron-restrictive silencer factor (Nrsf)], was initially discovered as a negative regulator of neuron-specific genes in non-neuronal cells (Chong et al., 1995; Schoenherr and Anderson, 1995). Rest is expressed throughout early development, where it represses the expression of neuronal genes and is involved in the transcriptional silencing of neuronal promoters in conjunction with CoRest (Rcor1/2) (Ballas et al., 2001), which recruits additional silencing machinery, including the methyl DNA-binding protein MeCP2, histone deacetylase (HDAC) and the histone H3K9 methyltransferase G9a (Ehmt2) (Andres et al., 1999; Lunyak et al., 2002; Roopra et al., 2004; Shi et al., 2003; You et al., 2001). Rest targets include a number of genes encoding ion channels, neurotransphins, synaptic vesicle proteins and neurotransmitter receptors (Bruce et al., 2004; Johnson et al., 2006; Otto et al., 2007). Indeed, a targeted mutation of Rest in mice caused derepression of neuron-specific tubulin in a subset of non-neuronal tissues, leading to embryonic lethality (Chen et al., 1998).

Mosaic inhibition of Rest in chicken embryos using a dominant-negative form of Rest also caused derepression of neuronal tubulin, as well as several other neuronal target genes, not only in non-neuronal tissues but also neuronal progenitors (Chen et al., 1998). These results suggest that Rest is required to repress the expression of neuronal genes in undifferentiated neuronal tissue. Expression of Rest is highest in embryonic stem cells (ESCs) and is downregulated as ESCs differentiate into neuronal stem cells (NSCs), and it is completely silenced in mature adult neuronal cells (Ballas et al., 2005). Given the fact that Rest represses the expression of a large number of neuronal genes, it is reasonable to expect that it plays a central role in the inhibition of the precocious expression of neuronal genes in NSCs, and that its downregulation upon receipt of neuronal differentiation cues permits the robust expression of differentiation-related neuronal genes, resulting in terminal differentiation (Ballas et al., 2005).

In addition to the involvement of Rest in neurogenesis, recent studies have demonstrated that Rest modulates glial lineage elaboration (Abrajano et al., 2009; Kohyama et al., 2010), suggesting that it also mediates the coupling of neurogenesis and gliogenesis, which might contribute to the neuronal-glial interactions that are associated with synaptic and neuronal network plasticity and homeostasis in the brain. Despite the expectation of a fundamental role of Rest in brain development, the function of Rest in NSCs and neuronal progenitors in the brain in vivo remains to be elucidated. Rest null mice survive to embryonic day (E) 9 without obvious morphological defects, by which time all three germ layers and the neural tube have formed, clearly demonstrating that neuronal progenitors can develop in vivo in the absence of Rest (Chen et al., 1998). However, Rest null mice die by E11.5 accompanied by gross morphological changes starting ~E9.5. This early embryonic lethality has precluded further analysis of the role of Rest in the maintenance and differentiation of NSCs and neuronal progenitor cells (NPCs) in vivo.

In addition to the possible role of Rest in neuronal/glial development, recent studies have indicated that the breakdown of these processes accompanies and promotes neurodegenerative disorders. The disruption of the interaction of Rest with its target genes was reported in epileptic seizures (Bassuk et al., 2008), Huntington’s disease (Zuccato et al., 2007) and Down’s syndrome (Canzonetta et al., 2008; Lepagnol-Bestel et al., 2009). In these disorders, Rest dysfunction is suggested to be a cause of aberrant changes in neuronal gene expression. Considering that abnormal expression of Rest has been seen in a variety of neurological and neurodegenerative diseases, it is important to uncover the...
mechanisms that underlie how Rest suppresses the expression of neuronal genes to control neurogenesis and gliogenesis, and to provide a better understanding of the pathogenesis of such diseases.

In the present study, we have generated Rest conditional knockout mice that allow the effects of genetic ablation of Rest on brain development to be examined in vivo. We also examined the effect of Rest ablation in cells outside of the nervous system at different developmental stages.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the Animal Research Committee of the Gifu University Graduate School of Medicine. Rest\textsuperscript{lox/lox} mice were generated from the Rest\textsuperscript{lox/lox} ESC line as described previously (Yamada et al., 2010). Rosa26::rtTA; Col1a1::tetO-Cre mice (Yamada et al., 2010) and Sox1-Cre\textsuperscript{+/} mice (Takashima et al., 2007) were bred with Rest\textsuperscript{lox/lox} mice to generate compound transgenic mice. In order to induce Cre recombination, doxycycline (2 mg/ml) was administered in the drinking water of the mice, supplemented with 10 mg/ml sucrose (Hochdelinger et al., 2005). To induce Cre-loxP recombination in the embryos, pregnant female mice were treated with doxycycline in their drinking water for 5 days, and were sacrificed on the last day of the doxycycline administration. In order to label neuronal stem/progenitor cells in the adult brain, BrdU was administered as a daily intraperitoneal injection of 50 mg/kg body weight 2 days before sacrifice on the last day of the doxycycline administration. In order to examine the effect of Rest deletion in vivo, we generated mice containing floxed Rest alleles and doxycycline-inducible Cre alleles (Rest\textsuperscript{lox/lox}, Rosa26::rtTA; Col1a1::tetO-Cre), in which exon 4, which encodes the CoRest binding site, can be removed upon treatment of mice with doxycycline (Fig. 1A) (Andres et al., 1999; Beard et al., 2006; Fink et al., 1999; Hatano et al., 2011; Yamada et al., 2010). Rest contains two repressor domains (Tapia-Ramirez et al., 1997): an N-terminal domain that associates with HDACs and Sin3; and a C-terminal domain that interacts with CoRest (Andres et al., 1999). Importantly, although our recombined Rest knockout (KO) allele (Rest\textsuperscript{lox/lox}) still contains exons 1-3, which encode the N-terminal domain of Rest, altered Rest transcript was not detected in our Rest\textsuperscript{lox/lox} mouse ESCs, suggesting that the Rest\textsuperscript{lox} allele in this system is equivalent to the conventional KO allele (Yamada et al., 2010). We further demonstrated that Stmn2 (SCG10), a CoRest-independent target of Rest-mediated repression (Jepsen et al., 2000; Lunyk et al., 2002),

Antibodies

The primary antibodies used in this study were: anti-mouse neuronal class III beta-tubulin (Tuj1; 1:5000; BabCO); anti-mouse gliarial fibbrillary acidic protein (Gfap; 1:1000; Dako-Cytomation, Glostrup, Denmark); anti-human nestin (1:100; IBL, Gunma, Japan); anti-mouse nestin (1:1000; Chemicon); anti-mouse NeuN (1:1000; Chemicon); anti-BrdU (1:500; Dako-Cytomation); anti-doublecortin (Dex; 1:500; Santa Cruz); anti-Proxl (1:5000; Millipore); anti-radial glial cell marker 2 (clone RC2; 1:300; Millipore); anti-trimethyl histone H3 (Lys27) (1:200; Monoclonal Institute, Hokkaido, Japan).

Gene expression analysis

Total RNA was prepared using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s instructions. The first-strand cDNA was synthesized from 1 µg total RNA using the SuperScript First-Strand Synthesis System (Takara, Shiga, Japan) with oligo(dT) primers. Real-time PCR was performed with SYBR Premix EX Taq (Takara) using Thermal Cycler Dice (Takara) for each gene of interest, and a β-actin endogenous control primer set was used for normalization. The primer sequences used in qRT-PCR analyses were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

The microarray analysis was performed according to the manufacturer’s instructions (materials for Agilent unless otherwise stated). Briefly, cyanine-3 (Cy3)-labeled cRNA was prepared from 100 ng RNA using the One-Color Low RNA Input Liner Amplification Kit, followed by RNeasy column purification (Qiagen). Dye incorporation and cRNA yield were checked with a NanoDrop ND-1000 spectrophotometer. A total of 1.5 µg of Cy3-labeled cRNA (specific activity >10.0 pmol Cy3/mg cRNA) was fragmented at 60°C for 30 minutes in a reaction volume of 50 µl containing 1× fragmentation buffer and 2× blocking agent following the manufacturer’s instructions. On completion of the fragmentation reaction, 50 µl 2× HI-RPM Hybridization Buffer was added and hybridized to Whole Mouse Genome Oligo Microarrays (G4122F) for 17 hours at 65°C in a rotating hybridization oven. After hybridization, microarrays were washed for 1 minute at room temperature with GE Wash Buffer 1 and 1 minute at 37°C with GE Wash buffer 2, then dried immediately by brief centrifugation. Slides were scanned immediately after washing on a DNA microarray scanner (G2565B) using the one-color scan setting for 4×44k array slides [scan area 75×25 mm, scan resolution 5 µm, dye channel set to green and green PMT set to 10-100% (XDR)]. The scanned images were analyzed with the Feature Extraction Software package version 9.5.3.1 using default parameters (protocol GE1+5 95_Feb07 and Grid: 014868_D_F_20101102) to obtain background-subtracted and spatially detrended processed signal intensities. Data were analyzed using GeneSpring software.

RESULTS

Conditional ablation of the CoRest binding site in developing embryos results in embryonic lethality

In order to examine the effect of Rest deletion in vivo, we generated mice containing floxed Rest alleles and doxycycline-inducible Cre alleles (Rest\textsuperscript{lox/lox}, Rosa26::rtTA; Col1a1::tetO-Cre), in which exon 4, which encodes the CoRest binding site, can be removed upon treatment of mice with doxycycline (Fig. 1A) (Andres et al., 1999; Beard et al., 2006; Fink et al., 1999; Hatano et al., 2011; Yamada et al., 2010). Rest contains two repressor domains (Tapia-Ramirez et al., 1997): an N-terminal domain that associates with HDACs and Sin3; and a C-terminal domain that interacts with CoRest (Andres et al., 1999). Importantly, although our recombined Rest knockout (KO) allele (Rest\textsuperscript{lox/lox}) still contains exons 1-3, which encode the N-terminal domain of Rest, altered Rest transcript was not detected in our Rest\textsuperscript{lox/lox} mouse ESCs, suggesting that the Rest\textsuperscript{lox} allele in this system is equivalent to the conventional KO allele (Yamada et al., 2010). We further demonstrated that Stmn2 (SCG10), a CoRest-independent target of Rest-mediated repression (Jepsen et al., 2000; Lunyk et al., 2002),

Histology and immunohistochemistry

The brains were enucleated and fixed by immersion overnight in 10% formalin in phosphate buffer (pH 7.2). Specimens were dehydrated with 100-µm slices and cultured in DMEM/F12 supplemented with 10% fetal calf serum (FCS) (Nichirei Bioscience, Tokyo, Japan). MEFs were derived from small pieces of the outer part of the trunk region prepared as described above. The cells were seeded in 100-mm dishes and cultured in DMEM supplemented with 10% FCS. In order to induce Rest recombination in vitro, cultured cells were treated with doxycycline at 2 µg/ml. The cells were analyzed for GFP signals using a FACS Aria dual-laser flow cytometer (Becton-Dickinson).
is upregulated in Rest<sup>lox/lox</sup> mouse ESCs (supplementary material Fig. S1), indicating again that our Rest KO cells are equivalent to the Rest null cells.

A previous study using conventional KO mice revealed that mice lacking the Rest gene die during early embryonic development (Chen et al., 1998). When we administered doxycycline to the Rest conditional KO embryos to delete the Rest gene in utero (E1.5-5.5), we observed lethality of the embryos carrying the tetO-Cre allele at ~E10.5 with a growth retardation phenotype, which was accompanied by the loss of GFP signals, indicating that the phenotype of the conventional KO mice could be recapitulated in our Rest conditional KO mice (Fig. 1B).

**Genetic ablation of Rest in non-neuronal cells outside of the central nervous system in vitro**

Previous studies suggest that Rest is expressed in a variety of non-neuronal cells to suppress the neuronal differentiation of these cells. Indeed, the conventional Rest KO mice showed ectopic expression of Rest target genes, such as Tuj1 (Tubb3), in non-neuronal cells outside of the brain (Chen et al., 1998). Therefore, to elucidate whether Rest ablation can induce the expression of Rest target genes in non-neuronal cells, we used mouse embryonic fibroblasts (MEFs) containing floxed Rest alleles and doxycycline-inducible Cre alleles (Rest<sup>fl/fl</sup>; Rosa26:rtTA; Col1a1::tetO-Cre). The Rest conditional KO MEFs were treated with doxycycline for 3 days starting 1 day after the seeding of the MEFs (passage 1). Seven days after the seeding of the MEFs, the MEFs were examined for GFP expression by FACS analysis. Three weeks after the seeding of the MEFs, they were analyzed by immunocytochemistry with a Tuj1 antibody to detect expression of the neural cell marker. The expression of Rest target genes was also examined by real-time RT-PCR 3 weeks after the seeding of the MEFs.

Consistent with the recombination, FACS analysis revealed a decreased GFP signal in the Rest conditional KO MEFs treated with doxycycline (Fig. 2A). As demonstrated in a previous study using conventional KO mice, deletion of Rest caused an increase in the expression of Tuj1 in MEFs (Fig. 2B) (Chen et al., 1998). The real-time RT-PCR revealed that MEFs treated with doxycycline expressed a significantly reduced level of GFP and Rest (Fig. 2C). We found that this was associated with increased expression of Syt4, Tubb3 and Calb1, which contain RE1 sites and are targets of the Rest repressor complex (Chong et al., 1995; Johnson et al., 2008; Schoenherr and Anderson, 1995; Schoenherr et al., 1996) (Fig. 2C). We also found that Simn2, a CoRest-independent target of Rest-mediated repression, was also derepressed in MEFs by doxycycline exposure (Fig. 2C). These results indicate that Rest target genes are rapidly derepressed upon the loss of Rest in MEFs. However, Bdnf, which also contains an RE1 site and is a target of the Rest repressor complex in ESCs/NSCs (Johnson et al., 2008; Yamada et al., 2010), did not show any detectable derepression in doxycycline-treated MEFs (Fig. 2C).

Although we confirmed that removal of the Rest CoRest binding site induces ectopic neuronal gene expression in non-neuronal cells outside of the brain, it remains unclear whether Rest ablation can actually induce neuronal differentiation in non-neuronal cells. In the present study, despite the observed increase in the expression of neuronal genes such as Syt4, Tubb3, Calb1 and Simn2 after ablation of Rest in MEFs, the morphology of the Tuj1-expressing cells did not change (Fig. 2B). In addition, the expression of Fsp1 (S100a4), a marker for fibroblasts (Strutz et al., 1995), was not decreased in the Tuj1-expressing MEFs (supplementary material Fig. S2). These findings suggest that Rest ablation in non-neuronal cells leads to ectopic neuronal gene expression, but that its ablation is not sufficient to induce transdifferentiation into neuronal cells (Vierbuchen et al., 2010).

We also examined the effect of Rest ablation in adult non-neuronal cells in vitro using tail tip fibroblasts (TTFs) containing the floxed Rest alleles and doxycycline-inducible Cre alleles. After exposure to doxycycline, we detected significant upregulation of the Rest target genes Syt4, Tubb3, Calb1 and Simn2 in the TTFs, which was accompanied by the downregulation of Rest and GFP expression (supplementary material Fig. S3). Consistent with the results in MEFs, we failed to detect derepression of Bdnf or downregulation of Fsp1 in TTFs after Rest ablation (supplementary material Fig. S3). We also conditionally deleted the Rest CoRest binding site in adult mice by the administration of doxycycline in the drinking water, and examined the expression of Rest target genes in the tail tissues. We confirmed the derepression of Rest target genes in the adult tail tissues after genetic ablation of Rest in vivo (supplementary material Fig. S4).
In vitro ablation of Rest in neuronal progenitor cells

Rest is downregulated in the brain as gestation progresses (Ballas et al., 2005). We first examined the expression of Rest in the developing mouse brain. The conditional KO alleles contain IRES-GFP sequences at the 3′ H11032 UTR of the Rest gene, which enable us to detect the expression and distribution of Rest by the GFP signals. By analyzing GFP expression, we confirmed that cells in the brain at E13.5 actually express the Rest gene (Fig. 3A).

In order to investigate the effect of genetic ablation of Rest during neurogenesis in vitro, we generated neurospheres from the brains of E13.5 Rest conditional KO embryos carrying the doxycycline-inducible Cre alleles. The primary neurospheres were passaged to form secondary neurospheres. Doxycycline was administered for 3 days starting 1 day after the passage of the primary neurospheres (passage 1). When we measured the number of secondary neurospheres in order to compare the formation of neurospheres in the presence and absence of doxycycline, the number of neurosphere cells was not significantly different 1 week after passage, regardless of doxycycline exposure (Fig. 3B). By contrast, the number of cells constituting the neurospheres exposed to doxycycline was significantly decreased after long-term culture of the neurospheres (Fig. 3C), suggesting that the ablation of Rest inhibited the growth of the neurospheres. Since a recent study demonstrated that Rest ablation in cultured neurosphere cells actually results in decreased proliferation (Gao et al., 2011), the decreased proliferative activity might be responsible for the decreased number of cultured cells upon doxycycline treatment in vitro.

We next cultured Rest conditional KO neurospheres (Rest2lox/2lox, Rosa26::rtTA; Col1a1::tetO-Cre) under differentiation conditions. To examine the effects of Rest ablation on neuronal differentiation, the...
doxycycline treatment was started 1 day after seeding the neurospheres in adherent culture, and the cells were treated with doxycycline for an additional 3 days. The adherent spheres were stained with anti-Tuj1 and anti-Gfap antibodies 1, 2 and 3 weeks after doxycycline exposure (Fig. 3D and supplementary material Fig. S5) and we counted the number of Tuj1-positive or Gfap-positive cells and DAPI-positive (total) nuclei in three independent areas of 1.5 mm² to calculate the proportion of Tuj1-positive or Gfap-positive cells. The doxycycline-treated cells contained a significantly increased percentage of Tuj1-positive cells among total cells than the control non-treated cells after 2 and 3 weeks of the treatment (Fig. 3D). In addition, a real-time PCR analysis revealed that the expression levels of Syt4 and Calb1 increased in the neurosphere adherent culture after genetic ablation of Rest (Fig. 3E). By contrast, the percentage of Gfap-positive glial cells among total cells was not altered (Fig. 3F), suggesting that ablation of Rest does not have a significant effect on glial differentiation in vitro in this experimental condition.

Because the Tuj1 and Gfap double-negative cells in the adherent spheres decreased after doxycycline treatment, Rest ablation may induce Tuj1 expression in such Tuj1 and Gfap double-negative cells. Immunocytochemical analysis of doxycycline-treated neurosphere cells revealed that a subset of non-neuron-like cells expresses Tuj1 and/or calbindin, whereas non-neuron-like cells in the control neurospheres did not express these markers (supplementary material Fig. S6A,B). Consistent with a previous study (Gao et al., 2011), we observed a small number of cells that express both Tuj1 and Gfap, suggesting the misexpression of Rest target genes (supplementary material Fig. S6C). Collectively, these results suggest that derepression of Rest target genes occurred in the adherent neurosphere cells upon Rest ablation, and that this derepression might play a role in the promotion of neuronal differentiation.

The in vivo effects of Rest ablation on gene expressions in non-neuronal and neuronal cells of the developing embryo

In the E13.5 mouse embryo the expression level of Rest in the limb was higher than that in the brain (supplementary material Fig. S7). By contrast, the expression level of Rest target genes was higher in the brain than in the limb (supplementary material Fig. S7).
Rest and in vivo neurogenesis

Fig. 4. In vivo genetic ablation of Rest in developing embryos. (A) Neuronal gene expression levels in the brains of E13.5 embryos and adult mice. The expression of Syt4 and Calb1 was significantly higher in the adult brain, suggesting that Rest neuronal target genes are still repressed in the E13.5 brain. The transcript levels were normalized to β-actin. The data are presented as average values with s.d. of six independent samples. (B) The experimental protocol for recombination of the Rest alleles in vivo. Pregnant mice with Rest conditional KO embryos were treated with doxycycline for 5 days, and embryos were sacrificed at E13.5. GFP fluorescence was decreased in embryos with the tetO-Cre allele, as compared with control embryos without the tetO-Cre allele. (C) The in vivo expression of Rest target genes in the brain. Although the expression levels of Rest and GFP were significantly downregulated, the expression levels of most Rest target genes were not derepressed in the brains of Cre+ embryos. Transcript levels were normalized to β-actin. The data are presented as average values with s.d. of four independent samples. (D) The expression of Rest target genes in the peripheral tissues (limb) in vivo. The expression of Syt4, Tubb3, Calb1 and Stmn2 was derepressed after genetic deletion of Rest. Transcript levels were normalized to β-actin. The data are presented as average values with s.d. of four independent samples. (E) A microarray analysis of E13.5 brain and non-neuronal (limb) tissue after genetic ablation of Rest. Rest binding genes in neuronal stem cells (Johnson et al., 2008) are shown as green dots. Rest target genes were significantly upregulated in the Rest-deleted non-neuronal tissue (limb). By contrast, the derepression of Rest target genes in the brain was not observed following genetic ablation of Rest. *, P<0.01; **, P<0.005; ***, P<0.05.
However, the expression levels of \textit{Syt4} and \textit{Calb1} in the E13.5 brain were significantly lower than those in the adult brain (Fig. 4A). These observations are consistent with the hypothesis that the expression of Rest target genes is still repressed in the E13.5 brain in vivo. Since our in vitro experiments revealed that the genetic ablation of Rest results in the increased expression of Rest target genes in both non-neuronal and neuronal cells, we next tried to dissect the effects of Rest ablation on the non-neuronal and neuronal cells in vivo using embryos with floxed Rest genes and doxycycline-inducible \textit{Cre} alleles. The Rest conditional KO embryos were treated with doxycycline in utero (E8.5-13.5) to induce \textit{Cre}-mediated recombination in both non-neuronal and neuronal cells, and the mice were sacrificed at E13.5 (Fig. 4B). In accordance with the recombination, E13.5 embryos with a \textit{tetO-Cre} allele had decreased signals for GFP when compared with embryos without a \textit{tetO-Cre} allele (Fig. 4B). We also collected the brains and limbs from \textit{Rest}-deleted embryos and their control littermates without the \textit{tetO-Cre} allele. Consistent with the decreased GFP signals, real-time RT-PCR analysis revealed that the expression of Rest was significantly downregulated in both the brain and limbs from embryos with a \textit{tetO-Cre} allele compared with those from control littermates (Fig. 4C,D).

Similar to the results obtained in vitro, we detected a significant increase in the expression of \textit{Syt4}, \textit{Tubb3}, \textit{Calb1} and \textit{Stmn2} in the limbs of embryos with the \textit{tetO-Cre} allele (Fig. 4D). By contrast, the expression level of \textit{Tubb3}, \textit{Calb1} and \textit{Stmn2} in the brains of E13.5 embryos with a \textit{tetO-Cre} allele remained repressed, whereas the expression levels of Rest and GFP itself were downregulated in the same samples (Fig. 4C). Although the expression of \textit{Syt4} was slightly upregulated in the brain of embryos with a \textit{tetO-Cre} allele (Fig. 4C), the effect was only modest when compared with the levels in the adult brain (Fig. 4A). Immunohistochemical analysis confirmed that there was no alteration in the expression pattern of \textit{Tuj1} in the E13.5 brain of embryos with a \textit{tetO-Cre} allele (supplementary material Fig. S8A). We also examined the expression of Rest target genes in the brain or tail of E16.5 embryos with a \textit{tetO-Cre} allele, and found no altered expression levels of these genes in brains, whereas a significant increase in the expression of \textit{Syt4}, \textit{Calb1} and \textit{Stmn2} was observed in the tail (supplementary material Fig. S8B). These results indicate that the Rest target genes are specifically derepressed in non-neuronal cells outside of the brain by the genetic ablation of Rest in vivo.

We next performed a microarray analysis to determine the changes in gene expression after genetic deletion of Rest in E13.5 brain and limb in vivo. Consistent with the results of the real-time RT-PCR analysis, Rest target genes were significantly upregulated in the \textit{Rest}-deleted limb tissue (Fig. 4E; genes interacting with Rest in ESCs and NPCs are represented by green dots) (Johnson et al., 2008). However, the derepression in the limb tissues (upregulated more than 2-fold after Rest ablation) was observed in only a subset of the genes with a Rest binding site (27% of the genes; Fig. 4E, limb), suggesting gene-specific derepression. By contrast, only 2% of the genes with a Rest binding site were upregulated more than 2-fold in the brain, suggesting that the derepression only occurs at a minority of Rest target genes after the genetic ablation of Rest (Fig. 4E, brain).

**In vivo ablation of Rest in progenitor cells of the developing brain**

\textit{Sox1} was shown to be one of the earliest transcription factors expressed in ectoderm cells committed to a neural fate (Pevny et al., 1998; Takashima et al., 2007). The expression of \textit{Sox1} starts at E7.5-8.5 in the neural tube (Takashima et al., 2007). We used a \textit{Sox1-Cre} allele (Takashima et al., 2007) (\textit{Rest}^{2lox/2lox}; \textit{Sox1-Cre}^+/-) to excise the floxed Rest genes in early progenitor cells of the developing mouse brain in vivo. The brains from \textit{Rest} conditional KO mice carrying the \textit{Sox1-Cre} allele (\textit{Rest}^{2lox/2lox}; \textit{Sox1-Cre}^+/-) and control littermates (\textit{Rest}^{2lox/2lox}) were collected at E13.5, E16.5 and postnatal day (P) 0 and the expression levels of Rest target genes were compared by real-time RT-PCR. The brains from
embryos carrying Sox1-Cre had significantly lower levels of both Rest and GFP expression at all time points, reflecting the genetic ablation of Rest (supplementary material Fig. S9). However, consistent with the results in the experiments using doxycycline-inducible Cre mice, the expression levels of Rest target genes such as Syt4, Tubb3, Calb1, Bdnf and Stmn2 (except for Stmn2 at E13.5) were not significantly increased in the brains of developing embryos with the Sox1-Cre allele (supplementary material Fig. S9). These results confirm that the conditional deletion of Rest does not substantially affect the expression of Rest neuronal target genes in the developing brain.

Rest ablation during adult neurogenesis in vivo
To further examine the function of Rest in the maintenance of neurogenesis in adult brain tissue, we analyzed the brains of adult Rest conditional KO mice carrying the Sox1-Cre allele. Contrary to our expectation, the Rest conditional KO mice carrying the Sox1-Cre allele were apparently normal and grew into adults. These mice were viable for more than 1.5 years and were fertile. A Southern blot analysis confirmed that the brains of mice with the Sox1-Cre allele had lost the floxed Rest genes (Fig. 5A). Despite the lack of Rest throughout the entire brain tissue (Fig. 5A), brain weight at 8 weeks of age was not significantly different between the mice with and without the Sox1-Cre allele (Fig. 5B).

Next, we examined the histology of the brains of mice with and without the Sox1-Cre allele at different developmental stages and ages (E16.5, P0, P4, P7, P10, 4 weeks, 8 weeks, 10 weeks, 6 months and 9 months of age). However, we did not find any histological differences in the brains, including in the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ), where NSCs and NPCs reside and generate new neurons and glia (Fig. 5C) (Gage, 2002). We further performed immunohistochemical staining to examine the expression of various markers, including Prox1, Dcx, RC2, TuJ1, NeuN, nestin and Gfap at various time points (E16.5, P4, 8 weeks and 6 months) in the Rest-deficient and control brains. Prox1, Dcx and RC2 were used as markers for intermediate progenitor cells, immature neuronal cells and radial glial cells, respectively (Gao et al., 2011; Misson et al., 1988). Importantly, we did not observe any difference in the staining patterns of these markers between Rest-deficient and control brains (Fig. 6). We also confirmed that nestin-positive cells and Gfap-positive cells did not express TuJ1 in Rest-deficient brain, suggesting that misexpression of TuJ1 does not occur in the Rest-deficient cells in vivo (supplementary material Figs S10, S11). Although a recent study showed that acute Rest ablation in mice leads to a decreased number of Prox1-positive cells at SGZ regions, we did not observe any significant differences in the number of Prox1-positive cells, even in 9-month-old mice (supplementary material Fig. S12).

In order to examine the effect of Rest ablation on the maintenance of adult NSCs, we compared the numbers of BrdU-labeled cells in the SVZ of the adult brain of the Rest conditional KO mice carrying the Sox1-Cre allele with those of control littermates (Doetsch et al., 2002; Lendahl et al., 1990). BrdU was administered as a daily intraperitoneal injection of 50 mg/kg body weight for 12 days starting at the age of 8 weeks, and the brains were fixed 1 day after the last injection as described previously (Shi et al., 2004). We did not find any significant difference in the number of BrdU-positive cells in the SVZ of these mice (Fig. 7A). We also confirmed co-localization of BrdU-positive cells and those positive for Dcx, a marker for premature neuronal cells, in the SVZ of Rest-deficient mice (Fig. 7B), suggesting that adult neurogenesis occurs in these mice. In addition, the localization and the number of differentiated NeuN-positive cells in the adult mouse brain did not differ in the presence or absence of the intact Rest gene (Fig. 6).
A real-time RT-PCR analysis revealed that the expression of Syt4 and Calb1 was not altered in the adult brains lacking the CoRest binding site of Rest (Fig. 7D).

These results indicate that Rest is not required for brain development and suggest that genetic ablation of Rest during the initial stage of neural development does not cause any detectable abnormality in adult neurogenesis in vivo.

DISCUSSION

Differentiation of neuronal progenitors to mature neurons proceeds with loss of the Rest repressor complex from the RE1 site of neuronal genes, which is accompanied by increased expression of the target genes (Ballas et al., 2005). In the present study, using Rest conditional KO mice we confirmed that Rest plays a role in the repression of Rest neuronal target genes in vitro cultured neuronal progenitor cells to inhibit terminal differentiation. By contrast, genetic ablation of Rest in the whole brain in vivo does not result in altered expression of target genes. Furthermore, mice lacking Rest in the brain are apparently normal and grow into adults. These findings suggest that, in contrast to the repressive role of Rest in vitro cultured neuronal cells, Rest is dispensable for embryonic neurogenesis in vivo.

The unsolved question is why derepression of Rest target genes after Rest ablation can be detected in vitro cultured neuronal cells but not in developing brain tissue in vivo. It has been demonstrated that neuronal progenitor cells are competent for extrinsic signals involved in the specification of cell fate during neurogenesis (Edlund and Jessell, 1999). Our findings suggest that the local environment in the brain, which consists of multiple cell types, is likely to provide complementary regulatory mechanisms for the proper intrinsic regulation of neuronal genes in vivo.

Epigenetic mechanisms serve as important interfaces between gene expression and the environment (Jaenisch and Bird, 2003). Given that Rest exerts its repressive effects in conjunction with epigenetic modifiers (Ballas et al., 2005; Naruse et al., 1999; You et al., 2001), it is possible that extrinsic niche signals in the brain compensate for the lack of Rest through epigenetic regulatory mechanisms. Consistent with this hypothesis, we could not detect
any differences in the staining pattern of histone H3K27me3, a mark of epigenetic silencing, between Rest wild-type and Rest-deficient brains in vivo (data not shown).

Another study indicated that MeCP2 and other co-repressors remained on the Rest target promoters even after loss of Rest from the RE1 site, suggesting that Rest co-repressors might be involved in the additional regulatory mechanisms that are responsible for repressing the expression of neuronal genes in neuronal cells in the absence of Rest (Ballas et al., 2005). It is possible that such factors specifically compensate for the effect of Rest ablation in the repression of Rest neuronal target genes during embryonic neurogenesis in vivo. It is also possible that transcriptional activators might be required for the derepression of Rest target genes in the developing brain. In this context, the decreased levels of transcriptional activation might maintain the proper expression levels of Rest target genes in Rest-deficient brains in vivo.

A recent study by Gao et al. demonstrated that the acute deletion of Rest in the adult dentate gyrus (DG) leads to a decreased number of Prox1-positive DG cells (Gao et al., 2011). However, in the present study, we did not observe any significant differences in the number of Prox1-positive DG cells upon Rest ablation, even in 9-month-old mice. A possible explanation for the discrepancy is that the acute deletion of Rest in the adult DG cannot activate the compensatory mechanisms, resulting in premature differentiation of adult NSCs, whereas its deletion at the early embryonic stage, as performed in this experiment, activates the complementary machinery that masks Rest function at adult stages. Therefore, further experiments are still required to determine the role of Rest in the maintenance of adult NSCs in vivo.

The expression of Rest target genes in MEFs/TTFs is upregulated upon the loss of Rest, suggesting that Rest is involved in the active repression of neuronal genes in non-neuronal cells outside of the brain. However, we found that Bdnf, which contains RE1 sites and is repressed by Rest in ESCs (Yamada et al., 2010), was not derepressed after the deletion of Rest in MEFs/TTFs. As reported in a previous study (Chen et al., 1998), these findings suggest that there is cell type specificity of Rest-mediated gene silencing. In addition, a microarray analysis revealed that only a subset of genes with a Rest binding site (27%) is derepressed by more than 2-fold following genetic ablation of Rest in non-neuronal tissues. In addition to the cell type-specific repression, these findings suggest that there is gene-specific repression by Rest (Chen et al., 1998). Since epigenetic silencing occurs through multiple modifications, including DNA methylation and histone modifications (Jaenisch and Bird, 2003; Lunyak et al., 2002; Martinowich et al., 2003), Rest deletion alone might not be sufficient to reactivate the silenced locus once silencing, involving multiple epigenetic modifications, has been completed. It is also possible that the cell type- and gene-specific activity of transcriptional activators is responsible for such different responses to Rest deletion.

The impaired interaction of Rest with its target genes has been reported in various neurological and neurodegenerative diseases. Although we found that mice lacking the CoRest binding site of Rest in the brain had no gross anatomical abnormalities even upon reaching adulthood, it is possible that more detailed analyses might highlight behavioral abnormalities in the Rest KO mice. In this context, these mice might be useful in investigation of the role of altered Rest interactions in neurological and neurodegenerative diseases. It would also be interesting to examine the functional alterations of Rest-deficient neuronal cells in vivo, which eventually might uncover the pathogenesis of such diseases.

In summary, we have generated Rest conditional KO mice and examined the effects of Rest ablation in neuronal and non-neuronal cells in vitro and in vivo. We showed that, in contrast to the role of Rest in the repression of Rest target genes in vitro cultured neuronal cells, as well as in non-neuronal cells outside of the brain, the CoRest binding site of Rest is dispensable for embryonic neurogenesis in vivo.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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