Sox14 is essential for initiation of interneuron differentiation in the chick spinal cord

Taiki Katsuyama[1], Minori Kadoya[1], Manabu Shirai[2] and Noriaki Sasai[1]*

[1]Developmental Biomedical Science, Nara Institute of Science and Technology, 8916-5, Takayama-cho, Ikoma 630-0192, Japan

[2]Omics Research Center (ORC), National Cerebral and Cardiovascular Center, 6-1 Kishibe Shinmachi, Suita, Osaka 564-8565, Japan

*Author to whom correspondence should be addressed.

e-mail: noriakisasai@bs.naist.jp

Abstract

The neural tube comprises several different types of progenitors and postmitotic neurons that co-ordinately act with each other to play integrated functions. Its development consists of two phases: proliferation of progenitor cells and differentiation into postmitotic neurons. How progenitor cells differentiate into each corresponding neuron is an important question for understanding the mechanisms of neuronal development.

Here we introduce one of the Sox transcription factors, Sox14, which plays an essential role in the promotion of neuronal differentiation. Sox14 belongs to the SoxB subclass and its expression starts in the progenitor regions before neuronal differentiation is initiated at the trunk level of the neural tube. After neuronal differentiation is initiated, Sox14 expression gradually becomes confined to the V2a region of the neural tube, where *Chx10* is co-expressed. Overexpression of Sox14 restricts progenitor cell proliferation. Conversely, the blockade of Sox14 expression by the *RNAi* strategy inhibits V2a neuron differentiation and causes expansion of the progenitor domain. We further found that Sox14 acted as a transcriptional activator. Taken together, Sox14 acts as a modulator of cell proliferation and an initiator protein for neuronal differentiation in the intermediate region of the neural tube.

Abbreviations

HH stage: Hamburger and Hamilton's stage
pMN: motor neuron progenitor region
FP: floor plate
hpt: hours post transfection
sh-Sox14: *short-hairpin RNA* targeting *Sox14*
IdU: 5-Iodo-2'-deoxyuridine
Shh: Sonic Hedgehog
RT-qPCR: reverse transcription and quantitative polymerase chain reaction
TUNEL: TdT-mediated dUTP nick-end labelling

Introduction

The neural tube is the embryonic organ of the central nervous system, and several distinct types of progenitor cells and neurons are arrayed along the dorsal-ventral axis in an orderly manner [1]. The cells of each progenitor domain differentiate into their corresponding neurons, and the combination of different types of neurons exerts integrated functions as a whole [2].

Neural tube development is largely divided into two phases: the assignment and proliferation of neural progenitor cells and their differentiation into postmitotic functional neurons [3, 4].

During the first phase, at the trunk level of the neural tube, a variety of neural progenitor cells are patterned depending on the positional information provided by the signal molecules, collectively called morphogens. In the ventral part of the neural tube, the assignment of the neural domains is mainly governed by sonic hedgehog (Shh), which is expressed in the notochord and later in the floor plate (FP) cells. The ventral neural tube is consequently divided into six regions: p0-p2, motor neuron progenitor region (pMN), p3, and the FP, along the intermediate region to the ventral, whose assignment is dependent on the concentration of Shh. The cells proliferate to specific numbers in a region-specific manner [5, 6].

During the second phase of neural tube development, progenitor cells differentiate into postmitotic neurons. This process involves proneural genes, a group of basic helix-loop-helix (bHLH) transcription factors, including neurogenins, Atoh (atonal homolog), and NeuroDs, whose expression is regulated by the Notch/Delta and retinoic acid signals [7, 8]. Combination of the transcription factors of proneural genes, including position-specific transcription factors, promote differentiation [9, 10]. Consequently the transcription factors that characterise each neuronal region start to be expressed and each region is established. For instance, the homeodomain transcription factor Chx10/Vsx2 is expressed and is required for the V2a interneuron. In the absence of Chx10, progenitor cells acquire different neuronal identities of the motor neuron (MN), which is located in the adjacent domain of the neural tube [11, 12]. As such, there are many transcription factors that regulate neuronal differentiation and/or acquisition of specific functions. Important questions are how each transcription factor functions and how the gene regulatory networks are formed among them.

Among the transcription factors expressed in specific neuronal regions, we focused on one of the Sry-type transcription factors, Sox14. Sox14 belongs to the SoxB subclass, encompassing Sox1-3, Sox14, and Sox21 [13, 14]. In previous studies, the expression of Sox14 has been shown in the V2a region, accompanied by that of Chx10, Lhx3, and Sox21 in the developing spinal cord area, and this profile is supported by single-cell transcriptomic analysis [15, 16]. Sox14 is also expressed in a part of the diencephalon, called the subcortical visual shell, and is required for normal circadian rhythm [17]. In humans, mutations in the *Sox14* gene cause congenital disorders, including deformation of the eyelids and severe mental retardation [18, 19]. However, the functions of Sox14 in the spinal cord are not fully understood.

In this study, we used chick embryos to explore the expression and function of Sox14 in neural tube development. Analyses revealed that Sox14 is expressed not only during neuronal differentiation stages, but also in the earlier stages, and it regulates cell cycle progression and neuronal differentiation. Together, we suggest that various transcription factors share functions during differentiation into specific neurons of the V2a region.

Results

Sox14 expression begins before neuronal differentiation starts, and its expression is gradually confined to the intermediate neuronal domains

During analysis of genes that induce domain-specific neuronal differentiation [1], we focused on the Sry-type transcription factor Sox14. Sox14 is reportedly expressed in the V2a ventral neuronal domain [11, 20], and acts downstream of the homeodomain transcription factor Vsx2/Chx10 [11].

As most transcription factor expression starts after proneural gene expression is initiated, we attempted to identify the onset of Sox14 expression. We performed *in situ* hybridisation analysis of sections of the neural tube at various embryo stages. As a result, we found that Sox14 is already expressed in the neural tube at HH stage 14 (Fig. 1a) before neuronal differentiation starts. Moreover, higher expression was observed in the intermediate region of the neural tube. Once neuronal differentiation started, Sox14 expression became confined to the intermediate regions (Fig. 1b), and was found in the V2 region at HH stage 22 (Fig. 1c).

Next, we investigated when *Chx10* expression began. *Chx10* expression was not observed before the initiation of neuronal differentiation (Fig. 1d). The initial expression was found at the V2a region after neuronalisation started (Fig. 1e), and a stronger expression was found at HH stage 22 (Fig. 1f). These findings suggest that *Sox14* has upstream factors other than Chx10 for its initial expression [11].

As *Sox14* encodes a member of the SoxB subfamily, the *Sox14* RNA probe for *in situ* hybridisation might have been crossed with other SoxB family members. To examine this possibility and to quantitatively evaluate the expression, we conducted an intermediate neural explant assay using the intermediate region of the neural plate. The explants were treated with different concentrations of Shh, and gene expression was evaluated using RT-qPCR every 12 h. As a result, *Sox14* was already expressed at 12 h in the presence of a low concentration of Shh (Shh^{L}; see Methods for the definition), and the expression increased continuously (Fig. 1g). In contrast, in the presence of a high concentration of Shh (Shh^{H}), *Sox14* expression was not induced (Fig. 1g), supporting the finding that *Sox14* is expressed in the intermediate region of the neural tube, as obtained by *in situ* hybridisation (Fig. 1b).

The same concentration of Shh was used to induce *Chx10*; however, expression began 24 h after the start of culture, suggesting that the onset of *Chx10* expression was later than that of *Sox14* (Fig. 1h). Therefore, *Sox14* and *Chx10* are expressed in the same lineage of cells; however, *Sox14* expression precedes that of *Chx10*.

Taken together, the collective expression profiling analyses suggest that the two transcription factors, Sox14 and Chx10, have distinct regulation of their expression.

Sox14 inhibits neural progenitor cells and promotes transition from the progenitor to the postmitotic states

Next, we attempted to determine the activity of Sox14 in neural tube development. For this purpose, we performed a forced expression of the control vector or the expression plasmid conveying the *Sox14* gene by electroporation in the neural tube, and analysed its effects at 48 h post-transfection (hpt).

Results indicated that expression of Sox2, a neural progenitor marker, was reduced in the electroporated cells, while no change was found by electroporation of the control vector (Fig. 2a-b'; n=5 for control and n=8 for *Sox14*). We reasoned that the cells might have precociously differentiated into neurons; however, Sox14 overexpression also reduced $p27^{KIP1}$ expression, suggesting that neuronal maturation was also perturbed by *Sox14* overexpression (Fig. 2c-d'). Therefore, we hypothesised that the initial stage of the neurons was induced by Sox14, and investigated the expression of $p57^{KIP2}$, which appears immediately after neuronal birth [21, 22], using *in situ* hybridisation. As a result, we found the expressing area expanded into the progenitor regions, as well as the upregulated expression level (Fig. 2e-f'; n=5 for control and n=6/8 for *Sox14*).

To quantify the induction of genes involved in neurogenesis [9, 10], we analysed the expression of genes involved in neurogenesis using RT-qPCR in explants electroporated with *Sox14*. We observed selective expression of $p57^{KIP2}$ through the overexpression of Sox14, whereas other proneural genes, including *Ngn1*, *Ngn2*, and *NeuroD4*, remained unchanged in their expression (Fig. 2g).

Taken together, Sox14 induces $p57^{KIP2}$-expressing early-stage neurons.

Sox14 restricts proliferation of neural progenitor cells

We found that the cell number tended to be reduced by Sox14 overexpression (Fig. 2b,b',d,d',f,f'), and supposed that this may be due to the blockade of cell proliferation or programmed cell death. To test these possibilities, we performed a 5-Iodo-2'-deoxyuridine (IdU) incorporation assay to label S-phase cells at 24 hpt of control or *Sox14*. As a result, the number of cells in the S-phase was greatly reduced in the Sox14-electroporated side (Fig. 3a-c; n=5 for control, n=6 for *Sox14*). In addition, immunofluorescence with phospho-histone 3 (pHH3) to identify the M-phase cells revealed a dramatically reduced number of pHH3-positive cells in the electroporated side (Fig. 3d-f; n=5 for control, n=6 for *Sox14*).

Next, we investigated whether programmed cell death occurs in the *Sox14*-electroporated cells and performed a TdT-mediated dUTP nick-end labelling (TUNEL) assay, which detects fragmented genomic DNA. However, we did not find a significant increase in the positive signals in the *Sox14*-electroporated side (Fig. 3g-h',j; n=5 for control, n=6 for *Sox14*), while *PtchΔ*-overexpressing neural cells showed positive signals (Fig. 3i,i',j; n=7) [23, 24], suggesting that the experiment *per se* was successful.

These findings indicate that cell cycle progression was blocked by Sox14.

Sox14 is required for neuronal differentiation

To reveal the essential roles of Sox14 in neural tube development, we attempted to disrupt *Sox14* expression using an RNA interference (RNAi) strategy [25]. We designed the *sh-RNA* construct targeting chick Sox14 (*sh-Sox14*) and electroporated either the control vector (*sh-control*) or the plasmid conveying *sh-Sox14* into the neural tube. At 48 hpt, inhibition of *Sox14* expression by *sh-Sox14* was confirmed, while *sh-control* electroporation maintained expression (Fig. 4a-b'; none downregulated in control (n=5), 100% downregulated in *sh-Sox14* (n=6)). We further found that *Chx10* expression was blocked by *sh-Sox14* (Fig. 4c-d'; none downregulated in control (n=5), 100% downregulated in *sh-Sox14* (n=6)), suggesting that Sox14 is required for *Chx10* expression.

In contrast, the motor neuron domain, characterised by Islet1, was not affected by *sh-Sox14* (Fig. 4e-f'; n=5 for control, n=6 for *sh-Sox14*), and the Sox2-expressing neural progenitor domain was laterally expanded (Fig. 4g-h'; no ectopic expression in control (n=5), 83% displayed ectopic expression in *sh-Sox14* (n=6)), suggesting that *Sox14* knockdown inhibited neuronal differentiation.

Therefore, Sox14 is required for the progression of the neuronal differentiation, particularly for the V2a identity.

Sox14 acts as a transcriptional activator

Based on the amino acid sequences, SoxB transcription factors have been categorised into two subgroups, SoxB1 and SoxB2, where SoxB1 includes Sox1-3, while SoxB2 is composed of Sox14 and Sox21 [13]. While Sox1-3 are transcriptional activators [26], Sox14 and Sox21 are thought to act as transcriptional repressors, as judged from their amino acid sequences [13]. Therefore, we attempted to reveal the mode of action of Sox14 and generated chimeric constructs of the DNA-binding region of Sox14 fused either with the transactivating domain of VP16 of the herpes simplex virus ($Sox14_{DBD}$-VP16) or the Drosophila *Engrailed* repressor domain ($Sox14_{DBD}$-EnR) so that each construct mimics transcriptional activation or repression (Fig. 5a). By the electroporation of $Sox14_{DBD}$-*VP16*, the electroporated cells abolished the expression of the neural progenitor Sox2 (Fig. 5b,b'; n=10), and $p57^{KIP2}$ was found to be expanded (Fig. 5c,c'; n=10). Moreover, injection of IdU into the $Sox14_{DBD}$-*VP16*-electroporated embryos revealed that the number of S-phase cells was reduced in the electroporated cells (Fig. 5d,d'; n=7), suggesting that $Sox14_{DBD}$-VP16 mimics the activity of the wild-type Sox14 (Fig. 3b,c).

In contrast, the electroporation of $Sox14_{DBD}$-*EnR*, a mimetic construct for transcriptional repressor activity, induced the ectopic expression of Sox2 (Fig. 5e,e'; n=12) at the expense of $p57^{KIP2}$ (Fig. 5f,f'; n=12), suggesting that $Sox14_{DBD}$-EnR exerts opposing functions compared to wild-type Sox14. In addition, the IdU injection did not change the rate of incorporation as in the control (Fig. 5g,g'; n=8), which differs from the phenotype observed in wild-type Sox14 (Fig. 3b,c).

Taken together, these results suggest that (i) Sox14 acts as a transcriptional activator and the EnR-fused construct acts in an antimorphic manner, and (ii) Sox14 is essential for promoting the differentiation of neural progenitor cells.

Discussion

Sox14 expression begins earlier than initiation of neuronal differentiation.

In this study, we analysed the function of one of the Sox transcription factors, Sox14, and demonstrated that Sox14 is required for the progression of neuronal differentiation (Fig. 4). In addition, we demonstrated that Sox14 acts as a transcriptional activator, as the VP16-chimeric construct mimics wild-type Sox14 (Fig. 5).

Sox14 was initially identified as a SoxB subclass gene, which encompasses Sox1-3, Sox14, and Sox21 [13]. A subsequent study demonstrated that Sox14 expression was found in the V2a neuronal region, and its expression was overlapped, at least in part, with that of Chx10 and Lhx3/Lim3 [20]. Moreover, a recent study showed that electroporation of Chx10 induces ectopic expression of Sox14, indicating that Chx10 is a sufficient upstream factor for *Sox14* [11]. In contrast, our analysis revealed that Sox14 expression onset occurred earlier than that of *Chx10* (Fig. 1), suggesting that Chx10 is not a single upstream regulator for the initiation of *Sox14* expression. One possible upstream factor is retinoic acid (RA), as Sox14 expression is upregulated by RA during neural differentiation in embryonic carcinoma cells [27]. In addition, RA has been shown to play an important role in the intermediate region of neural progenitor cells [28], and Pax6 expression was upregulated by RA, as indicated by RT-qPCR analysis of neural explants. However, Sox14 expression remained unchanged after RA treatment (Supplementary Fig. S1), suggesting that Pax6 and Sox14 expression is initiated by different mechanisms.

In addition, it is notable that there are multiple binding sites for Nkx2.2 and Olig2 in the regulatory region of *Sox14* [29]. Considering that Nkx2.2 and Olig2 are induced by Shh [30] and are transcriptional repressors [31], Sox14 may be expressed in neural progenitor cells as a default, and its expression is restricted by Nkx2.2 and Olig2.

Sox14 is required for the progression of neuronal differentiation of intermediate neurons.

Sox14 and Chx10 are expressed in the same region of the V2a intermediate neuronal area. *Chx10* is induced by Lhx3 and consolidates V2a identity by repressing the genes that characterise the adjacent neuronal regions of non-V2a interneurons or MN [11]. Attenuation of the *Chx10* gene causes aberrant upregulation of the *MN* gene, suggesting that Chx10 determines the direction of neuronal identity [11].

In contrast, our analyses revealed that Sox14 is required for the promotion of neuronal differentiation. Knockdown of the *Sox14* gene caused the abolishment of neuronal differentiation, rather than producing different types of neurons (Fig. 4). Therefore, it can be said that Chx10 and Sox14 play different functions during neuronal differentiation, Sox14 promotes differentiation, and Chx10 determines the direction.

Previous studies have shown that Sox14 plays multiple roles in neural and neuronal development. For instance, Sox14 is required for terminal differentiation of dorsal midbrain GABAergic neurons [32]. In addition, Sox14 activates the p53 pathway to induce cell death in carcinoma cells [17]. While the context in which we analysed the Sox14 function is different from these previous reports, the activities of Sox14 revealed from our analyses are consistent with these previous results; Sox14 is required for the promotion of neuronal differentiation.

In our analyses, Sox14 acted as a transcriptional activator (Fig. 5). We recognise that Sox14 has been suggested as a transcriptional repressor, which is controversial for its mode of action. The repressor assumption was based on its amino acid sequence [13], and we predicted that the mode of action would change in a context-dependent manner.

In this study we demonstrated that Sox14 induces the *p57^{KIP2}* expression; however, whether this induction is direct is still elusive. Thus future studies will focus on searching for the direct target genes of Sox14 using chromatin immunoprecipitation. Moreover, it is highly possible that the target genes of Sox14 in neural progenitor cells and those in V2a neurons are distinct. Therefore, in addition to searching for different target genes depending on the neural differentiation steps, it would be useful to identify cofactors that bind to Sox14. These cofactors would include the general proneural genes, such as Neurogenin1/2 or NeuroD2/4/6, and would modulate the functions of Sox14.

Understanding the function of each transcription factor is useful for generating specific functional neurons from stem cells [33]. We envision that the findings of this study will partly contribute to regenerative medicine in the future.

Methods

Animal experiments

All animal experiments were performed under the approval of national and internal legislation; approval number 1636 was obtained from the review panel of the animal experiments of the Nara Institute of Science and Technology.

Manipulations and histological analyses on chick embryos

Chicken eggs were purchased from Yamagishi Farm (Wakayama prefecture, Japan). The development of chick embryos was evaluated using the Hamburger and Hamilton criteria [34]. Embryos were electroporated at HH stage 12 with the electroporator BTX830 and were incubated at 38 °C for the indicated periods.

For the overexpression analyses, the coding regions of the genes of interest were subcloned into the *pCIG* expression vector [35], which contains the chicken β-actin promoter and the *GFP* gene downstream of the internal ribosomal entry site. *Drosophila* EnR [36] and herpes-simplex virus-derived VP16 transactivation domain [37] chimeric constructs were generated by fusing the amino-acids 1-90 of chicken Sox14.

To construct *sh-Sox14*, the sequence AAGCCTCCGGTTGCCTACATATTAT was subcloned into the vector *pRFPRNAi* [25], in which the electroporated cells were identified by RFP expression.

For tissue analyses, the harvested embryos were fixed with 4% paraformaldehyde for 90 min, and were replaced with 15% (w/v) sucrose overnight with rotating incubation. These embryos were embedded in OCT compound (Sakura) and cryosectioned to a thickness of 12 μm using a Polar cryostat (Sakura Finetek). The following antibodies were used in this study: Sox2 (rabbit; Millipore, #5603), p27 (mouse; BD Transduction Laboratories, #610241), Olig2 (rabbit; Millipore; #9610), Nkx2.2 (mouse; DSHB; #74.5A5), Islet1 (mouse; DSHB; #39.4D5), Pax7 (mouse; DSHB), and GFP (sheep; AbD Serotec; #4745-1051). The secondary antibodies used were rabbit (Jackson Laboratories; #711-166-152 for Cy3; #711-606-152 for Cy5), mouse (#715-166-151 for Cy3; #715-606-150 for Cy5), and sheep (#713-096-147 for FITC).

In situ hybridisation and TUNEL assays were performed as previously described [6, 38, 39]. For *in situ* hybridisation analyses, the expression of genes or *si-RNAs* was validated by the tracer (GFP/RFP) expression on the adjacent sections. As a positive control for the TUNEL assay, *pCIG-PtchΔ*, encoding an insensitive mutant to the Hedgehog signal [23], was electroporated, as the blockade of the Hedgehog signal has been shown to induce programmed cell death [24].

For IdU incorporation assays, 100 mM IdU (Wako, Japan) was injected into the neural tube cavity at 22 hpt and incubated for 2 h. To identify the incorporated cells, the sectioned tissues were pre-treated with 1M hydrochloric acid, and the IdU antibody (mouse; Thermo; MA5-24879) was used.

Explants and RT-qPCR

The intermediate neural explants were prepared as described previously [38, 39]. Briefly, HH stage 9 embryos were placed in L-15 medium and the caudal stem zone was excised [40]. After treatment with 10 μg/mL dispase II (Sigma), the intermediate region of the neural plate was further cut out and embedded in the collagen gel (Sigma). The embedded cells were cultured with DMEM/F-12 supplemented with Mitoserum extender (BD Biosciences) and 1× penicillin/streptomycin/glutamine mixture (Wako, Japan). A high concentration of Shh (Shh^{H}) was defined as the concentration at which approximately 80 % of Nkx2.2-positive cells were found, whereas a low concentration of Shh (Shh^{L}) resulted in approximately 60% of Olig2-positive cells at 24 h [41]. For RT-qPCR, RNA was extracted using the Picopure RNA extraction kit (Thermo; #KIT0204) and analysed using a CFX qPCR machine (Bio-Rad). Expression levels were normalised with that of *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*). The primer sequences used are shown in Supplementary Tab. S1.

Image processing and statistics

Images were captured using the LSM710 confocal microscope or AxioVision (Carl Zeiss) and were processed using the software Photoshop (Adobe). Figures were formed using an Illustrator (Adobe). Quantitative data are presented as means ± SEM, and differences were evaluated using the two-tailed Student's *t* test. Statistical significance was set at $p < 0.05$, and *p*-values (*<0.05; **; $p<0.01$, ***; $p<0.001$) are indicated in each graph.

Declarations

Ethics approval and consent to participate

This study does not include human participants, tissues or data.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and material

All relevant data are presented in the text and figures. The plasmids used in this study are available from the corresponding author upon request.

Funding

This work was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (19H04781 and 20H03263) to NS, and by a Sasakawa Scientific Research Grant from the Japan Science Society to MK.

Authors' contributions

NS conceived the project; TK, NS, MS performed experiments and analysed the data; MK provided essential materials; all authors joined the discussion; NS wrote the manuscript.

Acknowledgements

The authors thank Erika Yoshihara for comments, Chikara Kogiso and Atsuki Yatsuzuka for technical guidance, and all other laboratory members for their support and discussions.

Figure legends

Fig. 1 *Sox14* expression begins before initiation of neuronal differentiation. (a-f) Cross-sections of the trunk level of the neural tube at HH stages 14 (a,d), 18 (b,e), and 24 (c,f) of chick embryos were analysed either using *in situ* hybridisation with probes against *Sox14* (a-c) and *Chx10* (d-f). Expression is indicated with a bracket (b) or with arrowheads (c,e,f). Scale bar in (a) = 100 μm for (a-f). (g,h) Expression analysis of *Sox14* (g) and *Chx10* (h) using RT-qPCR on the explants cultured in the control medium, or with Shh^{L} or Shh^{H} for various time periods.

Fig. 2 Sox14 induces early neuronal differentiation during the neural tube development. *pCIG* (a,a',c,c',e,e') or *pCIG-Sox14* (b,b',d,d',f,f') were electroporated at HH stage 12 and embryos were harvested at 48 hpt. Expression levels of Sox2 (red; a-b') and $p27^{KIP1}$ (red; c-d') were analysed using immunofluorescence, and the $p57^{KIP2}$ (e-f') was analysed using *in situ* hybridisation. Scale bar in (a) = 50 μm for (a-f'). (g) The effect of the electroporation of *pCIG* or *pCIG-Sox14* was analysed using RT-qPCR at 36 hpt on the explants electroporated with *pCIG* (blue bars) or *pCIG-Sox14* (red bars). Note that, in (e',f'), the GFP expression was taken from the adjacent sections.

Fig. 3 Sox14 inhibits cell proliferation without inducing programmed cell death. Either *pCIG* (control; a,a',c,d,d',f,g,g',j) or *pCIG-Sox14* (b,b',c,e,e',f,h,h',j) were electroporated into HH stage 12 and embryos were subjected to the following assays. (a-c) IdU incorporation assay to detect the S-phase cells. IdU was injected into the cavity of the neural tube at 22 hpt for 2 hours, and embryos were harvested for immunofluorescence with the IdU antibody. (c) Quantification of the IdU-positive cells in the total GFP-positive cells. (d-f) Immunofluorescence with the M-phase marker phospho-Histone 3 (pHH3). Embryos were harvested at 24 hpt and were subject to immunofluorescence with pHH3 antibody. (f) Quantification of the pHH3-positive cells over the GFP-positive cells on the apical side. (g-j) TUNEL assay to detect programmed cell death. Embryos were harvested at 24 hpt as in (d-f) and were analysed using a TUNEL assay. (i,i') For the positive control of the TUNEL assay, *pCIG-PtchΔ* was electroporated and the same assay as in (g-h') was performed. (j) Quantification of the TUNEL-positive cells in all GFP-positive cells. Scale bar in (a) = 50 μm for (a-b',d-e',g-i').

Fig. 4 Sox14 is required for neuronal differentiation. Embryos were electroporated either with *pRNAi* (a,a',c,c',e,e',g,g') or *pRNAi-Sox14* (b,b',d,d',f,f',h,h') and were analysed at 48 hpt. *Sox14* (a-b') and *Chx10* (c-d') expression levels were analysed using *in situ* hybridisation, and Islet1 (e-f') and Sox2 (g-h') expression was analysed using immunofluorescence. Normal (a,b,c,d) or ectopic (h) expression is indicated by filled arrowheads; reduced expression (b,d) by outlined arrowheads. Scale bars in (a) for (a-d') and in (e) for (e-h') = 50 μm. Note that the RFP expression in (a',b',c',d') was taken from the adjacent sections.

Fig. 5 Sox14 acts as a transcriptional activator. (a) Schematic of the $Sox14_{DBD}$-VP16 and $Sox14_{DBD}$-EnR. DBD; DNA-binding domain of Sox14 (amino acid numbers 1-90 of chicken Sox14). (b-h) Either *pCIG-Sox14$_{DBD}$-VP16* (b-d') or *pCIG-Sox14$_{DBD}$-EnR* (e-g') was electroporated into the neural tube and the embryos were analysed using immunofluorescence with antibodies against Sox2 (b,b',e,e') and $p57^{KIP2}$ (c,c',f,f') at 48 hpt, or were subjected to an IdU incorporation assay at 24 hpt, as in Fig. 3a-c. Ectopic (e,f) and reduced (b,c) expression is indicated by filled and outlined arrowheads, respectively. Note that the GFP expression in (c',f') was taken from the adjacent sections. Scale bars in (b) for (b-c',e-f') and in (d) for (d,d',g,g') = 50 μm. (h) Quantification of the IdU-positive cells in all GFP-positive cells. The ratio of the control condition is taken from Fig. 3c.

Supplementary Figures

Supplementary Fig. S1 Sox14 expression is not upregulated by retinoic acid (RA). Intermediate neural explants were cultured either in the control medium or the medium with RA (100 nM) for 24 hours to be subjected for an RT-qPCR.

Supplementary Table

Supplementary Tab. S1 The RT-qPCR primers used in this study.

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