Sox9 overexpression exerts multiple stage-dependent effects on mouse spinal cord development

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Abstract
The high-mobility-group (HMG)-domain protein Sox9 is one of few transcription factors implicated in gliogenesis in the vertebrate central nervous system. To further study the role of Sox9 in early spinal cord development, we generated a mouse that allows expression of Sox9 in a temporally and spatially controlled manner. Using this mouse, we show that premature Sox9 expression in neural precursor cells disrupted the neuroepithelium of the ventricular zone. Sox9 also compromised development and survival of neuronal precursors and neurons. Additionally, we observed in these mice substantial increases in oligodendroglial and astroglial cells. Reversing the normal order of appearance of essential transcriptional regulators during oligodendrogenesis, Sox10 preceded Olig2. Our study reinforces the notion that Sox9 has a strong gliogenic activity. It also argues that Sox9 expression has to be tightly controlled to prevent negative effects on early spinal cord structure and neuronal development.

Key words
astrocyte, gliogenesis, HMG, oligodendrocyte, Sry Sox

1 | INTRODUCTION

In the central nervous system (CNS) gliogenesis follows neurogenesis. Whereas the mechanisms that are responsible for commitment to the neuronal lineage are fairly well studied, much less is known about the corresponding mechanisms for glial commitment. One of the few factors that has been implicated in the process is the high-mobility-group (HMG) domain-containing transcription factor Sox9. An initial study had shown that in the absence of Sox9, glial cells were generated in much fewer numbers in the mouse spinal cord (Stolt et al., 2003). This concerned the generation of oligodendrocyte precursor cells (OPCs) from the ventral pMN-domain of the ventricular zone (VZ) as well as the generation of astroglial cells from the adjacent p2 domain. A simultaneous prolonged production and increase in the number of those neurons that are generated at earlier times from these domains (i.e., motoneurons and V2 interneurons) was taken as evidence that Sox9 is part of a switch mechanism from neuro- to gliogenesis. In its gliogenic function, Sox9 appears to interact with the Notch signaling pathway (Taylor, Yeager, & Morrison, 2007). It induces Nfia as a second gliogenic factor whose temporal expression pattern and interaction with other transcription factors decides over the exact glial fate (Glasgow et al., 2014; Kang et al., 2012). A transient expression of Nfia in the presence of Sox10 causes commitment to the oligodendroglial lineage. In contrast, prolonged expression in the absence of Sox10 allows Nfia to interact with its inducer Sox9, synergistically activate an astrocyte-specific regulatory cascade and thereby induce astrogliogenesis (Glasgow et al., 2014).

In addition to a role in glial commitment, Sox9 has also been reported to be already required in neuroepithelial precursor cells (NEPs) to induce glial competence and thereby transform NEPs into
multipotent neural stem cells (Scott et al., 2010). For the cerebellum it has furthermore been postulated that Sox9 is more important for the suppression of neurogenesis than for the initiation of gliogenesis (Vong, Leung, Behringer, & Kwan, 2015). Thus, multiple functions have been proposed for Sox9, including functions as a glial competence and multipotentiality factor, a neuron–glia switch molecule and an inhibitor of neurogenesis.

All of these studies primarily relied on loss-of-function approaches. If at all performed, gain-of-function experiments were largely carried out in vitro. Therefore we decided to assess Sox9 function in the early developing spinal cord by overexpressing the protein from a tetracycline-controlled transgene. These studies provide evidence for multiple stage-dependent and cell type-specific functions and show that they are at least in part separable from each other.

2 | MATERIALS AND METHODS

2.1 | Generation, husbandry, and analysis of transgenic animals

For the generation of Sox9 overexpressing mice, a transgenic construct was generated based on pBI-EGFP, a plasmid with bidirectional tetracycline-controlled promoter (TRE) that drives enhanced green fluorescent protein (EGFP) expression in one direction and contains a multiple cloning site on the other side of the TRE for insertion of an expression cassette. In pBI-EGFP, EGFP sequences were replaced by tdTomato sequences and an aminoterminal FLAG- and HA-tagged Sox9 coding sequence was inserted in the multiple cloning site on the other side of the TRE (Figure 1a). This TRE::Sox9 construct was micro-injected after linearization into male pronuclei of fertilized oocytes derived from C57Bl6/J × CBA matings according to standard techniques, and a stable line with transgene insertion on chromosome 12qD1 was established from the obtained founders. Sox9 expression was achieved by combining TRE::Sox9 in the heterozygous state with a Rosa26stopflox-tTA allele (Wang et al., 2008) and Brn4::Cre (Ahn, Mishina, Hanks, Behringer, & Crenshaw, 2001) or Nestin::Cre (Tronche et al., 1999) transgenes. Genotyping for the Rosa26stopflox-tTA allele (Wang et al., 2008) and the Cre transgenes was as described in the original reports. The TRE::Sox9 transgene was detected by genomic PCR as a 465 bp fragment using 5′-ACCACAACTAGAA-TGCAGTGAAAA-3′ and 5′-GATCCCAGAATGACCACCAC-3′ as primers. For experiments, a mixed C57Bl6/J × C3HeB/FeJ background was used. Mice were kept under standard housing conditions with 12:12 hr light–dark cycles and continuous access to food and water.
water in accordance with animal welfare laws and under observance of all ethical regulations. For delaying transgenic Sox9 expression, 0.2 mg/ml tetracycline was added to sucrose-containing (0.05 g/ml) drinking water and given to pregnant mice from Day 7.5 until Day 10.5 or Day 12.5 of pregnancy. For proliferation studies, 100 μg BrdU (Sigma, #B5002) per gram body weight were injected intraperitoneally into pregnant females 1 hr before tissue preparation. Embryos and tissues were obtained at embryonic days (E) 9.5, 10.5, 11.5, 12.5, 14.5, and 18.5 and underwent fixation in 4% paraformaldehyde, cryoprotection in 30% sucrose, embedding and freezing at −80°C. Cryotome sections were prepared at 10 μm thickness (Weider et al., 2015). Both male and female embryos were used for the study. Littermates without Rosa26stopSox1TA allele or Brn4::Cre were interchangeably used as controls after confirming that both were comparable to age-matched wild-type embryos.

For immunohistochemistry, the following primary antibodies were applied: rat anti-Mbp monoclonal (Bio-Rad, #MCA409S, Lot #210610, 1:500 dilution), mouse anti-NeuN monoclonal (Chemicon, #MAB377, Lot #19040027, 1:600 dilution), mouse anti-Nestin monoclonal (Developmental Studies Hybridoma Bank, University of Iowa, 1:20 dilution), mouse anti-Nkx2.2 monoclonal (Developmental Studies Hybridoma Bank, University of Iowa, clone 74.5A5, 1:50 dilution), mouse anti-Tfap2a monoclonal (Santa Cruz #sc-12726, Lot #J1617, 1:500 dilution), mouse anti-Hb9 monoclonal (Developmental Studies Hybridoma Bank, University of Iowa, clone 81.5C10, 1:50 dilution), guinea pig anti-Sox10 antiserum (home-made, 1:50 dilution; Stolt et al., 2003), guinea pig anti-Sox11 antiserum (home-made, 1:1,000 dilution; Hoser et al., 2008), guinea pig anti-Lbx1 antiserum (gift of C. Birchmeier, MDC, Berlin, Germany, 1:20,000 dilution), goat anti-Sox2 antiserum (Santa Cruz, sc-17319, Lot # G1210, 1:50 dilution), rabbit anti-Sox2 antiserum (homemade, 1:500 dilution), rabbit anti-Lmx1b antiserum (gift of C. Birchmeier, MDC, Berlin, Germany, 1:10,000 dilution), rabbit anti-Gfap monoclonal (Neomarkers, #RB-087-A, Lot #087A509F, 1:500 dilution), rabbit anti-Nfia antiserum (Active Motif, #39329, Lot #14407002, 1:1,000 dilution), rabbit anti-Nfib antiserum (Sigma, HPA003956, Lot: A114436, 1:200 dilution), rabbit anti-Pdgfra antiserum (Santa Cruz, #sc-338, Lot # E-1210, 1:300 dilution), rabbit anti-Olig2 antiserum (Millipore, #AB9610, Lot # 2060464, 1:1,000 dilution), affinity-purified rabbit anti-Sox9 antibodies (home-made, 1:1,000 dilution; Stolt et al., 2003), rabbit anti-S100b antiserum (NeoMarkers/Thermo, #RB-9018-P, Lot #9018P907A, 1:300 dilution), rabbit anti-Aldh1l1 antiserum (Abcam, ab87117, Lot #GR175128, 1:1,000 dilution), rabbit anti-cleaved caspase 3 antiserum (Cell Signaling Technology, #9661, Lot #0043, 1:200 dilution), rabbit anti-Ki67 antiserum (Thermo Fisher Scientific, #RM-9106, Lot #91065906D, 1:500 dilution), Secondary antibodies were coupled to Cy5 (Dianova) or Alexa488 (Molecular Probes) fluorescent dyes. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Incorporated BrdU was visualized by rat anti-BrdU monoclonal antibodies (Abcam, #ab6326, Lot #GR3173637-7, 1:100 dilution). TUNEL was performed according to the manufacturer’s protocol (Chemicon). Stainings were documented with a DMI6000 B inverted microscope equipped with a DFC 360FX camera (both Leica).

For extract preparation, brains were dissected from E18.5 embryos. Meninges were removed and brain tissue was homogenized in PBS by up and down pipetting. After removal of undissociated material by short and low-speed centrifugation, extracts were prepared from the supernatant as described (Bischof, Weider, Küspert, Nave, & Wegner, 2015). For Western blotting, rabbit anti-Sox9 antiserum (home-made, 1:10,000 dilution; Stolt et al., 2003) and rabbit anti-Gapdh antibodies (Santa Cruz Biotechnology, 1:3,000 dilution) were used in combination with protein A coupled to horseradish peroxidase (Bio-Rad) and Luminol reagent for detection (Bischof et al., 2015; Kuhlbrodt, Herbarth, Sock, Hermans-Borgmeyer, & Wegner, 1998).

Experiments were approved by the responsible local committees and government bodies (Veterinäramt Stadt Erlangen TS—00/12 Biochemie, Regierung von Unterfranken 55.2—2532.1—43/13).

2.2 | Luciferase assays

Mouse Neuro2a neuroblastoma (obtained from American Type Culture Collection, authenticated by polymerase chain reaction) were grown in DMEM supplemented with 10% fetal calf serum and transfected with Col2a1 promoter or Sox10 U3 enhancer containing luciferase reporters and pCMV5-based expression plasmids for Sox9 and the tagged version generated in this study for transgenesis (Sock et al., 2003; Werner, Hammer, Wahlbuhl, Böl, & Wegner, 2007). Per 3.5 cm plate, 0.5 μg reporter plasmid and 0.5 μg effector plasmid were used adding empty pCMV5 where necessary. Whole cell extracts were prepared 48 hr after transfection for chemiluminescent measurements of luciferase activities (Hornig et al., 2013).

2.3 | Statistical analysis

To determine whether differences in cell numbers or luciferase activities were statistically significant, a two-tailed Student’s t test or one-way ANOVA with Bonferroni correction was performed (*p ≤ .05; **p ≤ .01, ***p ≤ .001). Results from independent embryos or transfections were treated as biological replicates (n ≥ 3).

2.4 | Data availability

All data generated or analyzed during this study are included in this article and its Supporting Information.

3 | RESULTS

3.1 | Sox9 is efficiently overexpressed throughout the spinal cord of TetSox9Brd4 mice

To allow ectopic Sox9 expression during spinal cord development we generated a transgenic mouse line that carried Sox9 coding sequences under control of a bidirectional tetracycline-responsive TRE promoter (Figure 1a). A tdTomato reporter was additionally under TRE control.
Sox9 carried at its aminoterminal end three copies of a FLAG and three copies of a HA epitope. This tag did not interfere with Sox9 expression nor did it impair the ability of Sox9 to activate reporter genes under control of regulatory regions that had previously been shown to be Sox9-responsive such as the Col2a1 promoter and the U3 enhancer of the Sox10 gene (Figure 1b).

In analogy to a previous study on Sox10 (Weider et al., 2018), we combined the TRE::Sox9 transgene with a Brn4::Cre transgene and a Rosa26stopflox-tTA allele (Figure 1c). Following Cre activation in neuroepithelial cells of the early VZ, tTA started to be expressed after removal of the stopflox cassette in the Rosa26 locus and induced Sox9 expression around E9.0, roughly 1–1.5 days before widespread endogenous Sox9 expression commenced in the mouse spinal cord (Figure 1f,g,l,m; Stolt et al., 2003). Western blot analysis of brain extracts at E18.5 furthermore argued that transgenic Sox9 levels were approximately 2.6-fold higher than those of the endogenous Sox9 in TetSox9Brn4 embryos (Figure 1d,e). Gapdh-normalized amounts of endogenous Sox9 were comparable between TetSox9Brn4 and control extracts. Inspection of spinal cord sections of TetSox9Brn4 embryos revealed that tdTomato was expressed as well and presented an excellent proxy for transgenic Sox9 expression.

In TetSox9Brn4 embryos, Sox9 and tdTomato were widely expressed throughout the spinal cord at early stages (Figure 1m–o,s–u). In contrast, tdTomato expression was not found in Tfxap2a- and Sox9-positive migrating neural crest cells outside the spinal cord (Figure 1a–f). At later stages expression of tdTomato and Sox9 within the spinal cord became more restricted and took on a salt-and-pepper appearance (Figure 1p,q,v,w). At all times, there were many more Sox9 expressing cells in the TetSox9Brn4 spinal cord than in controls (compare Figure 1f–k to Figure 1l–q). Control spinal cords did not express tdTomato.

3.2 | TetSox9Brn4 spinal cords show decreased proliferation and increased apoptosis

TetSox9Brn4 embryos were already recognizable at E14.5 by their smaller head (Figure S1g,h). As evident from tdTomato expression, the transgene was also expressed throughout the brain (Figure S1k,l) where it caused a mild size reduction of the forebrain and a very severe one in mid- and hindbrain regions (Figure S1i,j). These brain defects may explain why TetSox9Brn4 mice did not survive birth, thus restricting our analysis to prenatal times. Already at E11.5, TetSox9Brn4 embryos exhibited a substantially smaller spinal cord (Figure 1h,n,t). Therefore, we performed DAPI staining on control embryos and TetSox9Brn4 littersmates from E9.5 to E18.5 (Figure 2a–l). Quantification of these stainings revealed that cell numbers were similar in control and TetSox9Brn4 spinal cords at E9.5 and E10.5 (Figure 2m). Starting at E11.5, total cell numbers were drastically reduced by 54 ± 3% in TetSox9Brn4 spinal cords and remained significantly lower until E18.5 where the difference between the two genotypes amounted to 59 ± 4%.

To determine the basis for the detected cell number reductions we first analyzed proliferation as 1 hr BrdU incorporation rates in control and TetSox9Brn4 spinal cords (Figure 3a–l). Quantification of the corresponding sections revealed that absolute numbers of BrdU incorporating cells were comparable between genotypes at E9.5 but already started to differ at E10.5 (Figure 3m). The number of BrdU incorporating cells was on average 39% lower in TetSox9Brn4 spinal cords than in controls between E10.5 and E12.5. With the naturally occurring loss of proliferative precursors during spinal cord development from E14.5 onwards, the number of BrdU incorporating cells became again similar between TetSox9Brn4 and control spinal cords. When the number of BrdU incorporating cells was set into relation to total cell numbers, both genotypes were comparable at all-time points.
except E10.5 where proliferation rates were slightly higher in controls (20 ± 2%) than in TetSox9Brn4 (14 ± 1%) spinal cords (Figure 3n). This argues that proliferation rates in TetSox9Brn4 spinal cords are not substantially altered.

Therefore we turned our attention toward the analysis of dying cells by staining for cleaved caspase 3 (Figure 4a–l). Apart from E9.5 and E18.5, all intermediate time points exhibited substantially increased numbers of cells positive for cleaved caspase 3 in the TetSox9Brn4 spinal cord (Figure 4m). Not only absolute numbers of cleaved caspase 3-positive cells were increased, but also DAPI-adjusted relative numbers (Figure 4n). Qualitatively similar results were obtained when apoptosis was monitored with TUNEL instead of cleaved caspase 3 staining (Figure S2a–n). We conclude from these findings that apoptosis rates are dramatically increased during most of embryogenesis and that increased apoptosis is the primary cause of reduced cell numbers in the TetSox9Brn4 spinal cord.
3.3 | NEPs disappear early during spinal cord development in TetSox9Brn4 mice

To further characterize the phenotype in TetSox9Brn4 mice, we turned our attention to NEPs. These are normally present in substantial numbers until E12.5 and then gradually disappear over the next days (Figure 5a-d). NEPs express the transcription factor Sox2 which is often used as a marker for these cells. However, Sox2 is also expressed by OPCs and astroglial cells (Hoffmann et al., 2014). In contrast to NEPs, both OPCs and APs are localized outside the VZ in the parenchyme. Therefore, we quantified NEPs as Sox2-positive epithelial cells within the VZ.

As evident from representative stainings, absolute NEP numbers were significantly reduced in TetSox9Brn4 spinal cords from E10.5 until E18.5 (Figure 5a-l) with reduction rates ranging between 42 ± 8% for E10.5 to 79 ± 3% for E12.5 and being complete at E14.5 (Figure 5m). In support of strongly reduced NEP numbers, immunohistochemistry with antibodies directed against Nestin revealed a dramatically decreased number of stained NEP-derived radial fibers (Figure S3a–j). Quantification was not feasible. Interestingly, apoptosis was not increased in NEPs (Figure 5n) arguing that these cells may lose their epithelial character and/or develop into a different cell type.

3.4 | Neuronal lineage cells are strongly reduced in TetSox9Brn4 spinal cord

To follow the fate of neuronal cells, we used Sox11 as a marker for cells that are committed to the neuronal lineage but have not yet matured (Sock et al., 2004). These neuronal precursors and immature neurons (from now on referred to as NPs) are found in normal numbers in TetSox9Brn4 spinal cords until E10.5 (Figure 6a,b,g,h). However, differences between genotypes became apparent from E11.5 onwards. Whereas NP numbers rose steeply in controls at E11.5, reached their peak at E12.5 and then gradually declined as neurons mature (Figure 6c–f). NP numbers stagnated in TetSox9Brn4 spinal cords throughout most of embryonic development at E10.5 levels before declining at E18.5 (Figure 6h–l). Quantifications corroborated the dramatic reduction of NPs in TetSox9Brn4 spinal cords after E10.5 (Figure 6m).

Considering the significantly reduced NP numbers, we expected a similarly dramatic decrease in mature neurons. This was indeed confirmed in NeuN stainings. Again, no dramatic differences were observed between control and TetSox9Brn4 spinal cords until E10.5 (Figure 7a,b,g,h). In both genotypes, neurons were present in comparable numbers and predominantly found in the ventral spinal cord. Additional stainings with Nkx2.2 for V3 neurons and Hb9 for moto-neurons confirmed this finding and indicated that the identity of ventral neurons was not affected (Figure S4a–d,g–j). These neurons are mostly specified before transgene expression begins in the Tet-Sox9Brn4 spinal cord. At E11.5, the decrease in neurons was detectable, but not dramatic in TetSox9Brn4 spinal cords (Figure 7c,i). This changed from E12.5 onwards (Figure 7d–f,j–l), when neuron numbers dropped to 31 ± 3% of control levels at E12.5 and around 15–20% of...
control levels at E14.5 and E18.5 (Figure 7m). Dorsal neurons were particularly affected as evident from immunohistochemical stainings for Lbx1 and Lmx1b as markers of different populations of dorsal horn interneurons (Müller et al., 2002; Figure S4e,f,k,l). Similar to NPs, NeuN-positive neurons also exhibited an increased apoptosis (Figure 7n).

FIGURE 6 Neuronal precursors in the TetSox9Brn4 spinal cord. (a–l) Immunohistochemical detection of Sox11-positive NPs in control (ctr, a–f) and TetSox9Brn4 (tg, g–l) spinal cords at E9.5 (a, g), E10.5 (b, h), E11.5 (c, i), E12.5 (d, j), E14.5 (e, k), and E18.5 (f, l). Spinal cords are demarcated by a stippled line and were placed on a black background. Scale bar: 100 μm. (m) Determination of neuronal precursors in control (gray dots) and TetSox9Brn4 (open triangles) spinal cords as mean ± SEM per section (n = 3 spinal cords per genotype, counting three separate sections each). (n) Quantification of cleaved caspase 3-positive apoptotic neuronal precursors in control and TetSox9Brn4 spinal cords at E11.5 as mean ± SEM per section (n = 3). Statistical significance was determined by two-tailed Student’s t test (**p ≤ .01; ***p ≤ .001).

FIGURE 7 Neurons in the TetSox9Brn4 spinal cord. (a–l) Immunohistochemical detection of NeuN-positive neurons in control (ctr, a–f) and TetSox9Brn4 (tg, g–l) spinal cords at E9.5 (a, g), E10.5 (b, h), E11.5 (c, i), E12.5 (d, j), E14.5 (e, k), and E18.5 (f, l). Spinal cords are surrounded by a stippled line and were placed on a black background. Scale bar: 100 μm. (m) Determination of neurons in control (gray dots) and TetSox9Brn4 (open triangles) spinal cords as mean ± SEM per section (n = 3 spinal cords per genotype, counting three separate sections each). (n) Quantification of cleaved caspase 3-positive apoptotic neurons in control and TetSox9Brn4 spinal cords at E11.5 as mean ± SEM per section (n = 3). Statistical significance was determined by two-tailed Student’s t test (**p ≤ .01; ***p ≤ .001). VZ, ventricular zone.
Oligodendroglial lineage cells are strongly increased in TetSox9Brn4 spinal cord

We next studied the oligodendroglial lineage. Olig2 is continuously expressed in these cells and can serve as a lineage marker (Lu et al., 2000; Zhou, Wang, & Anderson, 2000). However, Olig2 also labels the NEPs in the pMN domain of the ventral VZ that gives rise to motoneurons and most spinal cord oligodendrocytes. For quantifications of oligodendroglial cells, we therefore excluded the pMN NEPs and only counted the Olig2-positive cells in the mantle zone.

At E9.5, Olig2 stainings were comparable between wild type and TetSox9Brn4 spinal cords (Figure 8a,g). Almost all Olig2-positive cells
were restricted to the pMN domain and represent NEPs. The same localization was still observed in control spinal cords at E10.5 and E11.5 in line with the first appearance of OPCs in the mantle zone of the spinal cord around E12.0 (Figure 8b–d). In the TetSox9<sup>Brom</sup> spinal cord, however, Olig2-positive cells were found at these time points already in the mantle zone (Figure 8h,i). At E10.5, most of these Olig2-positive cells were still in the ventral spinal cord where OPCs usually arise. At E11.5, Olig2-positive cells were present in dorsal regions as well. Thus, OPCs seem precociously generated in the TetSox9<sup>Brom</sup> spinal cord.

At the onset of OPC generation in the control spinal cord at E12.5, Olig2-positive cells were already spread in large numbers throughout the TetSox9<sup>Brom</sup> spinal cord (Figure 8d,j). This dramatic difference persisted at E14.5 (Figure 8e,k). However, at E18.5, wild-type OPCs had caught up and were present in similar numbers in control spinal cords as Sox9-overexpressing OPCs in the TetSox9<sup>Brom</sup> spinal cords (Figure 8f,l). Quantification confirmed the increase of oligodendroglial cells from E10.5 onwards in TetSox9<sup>Brom</sup> spinal cords both in absolute numbers and even more impressively when normalized to total cell numbers (Figure 8m,n). Olig2-positive cells furthermore exhibited no substantial increase in cleaved caspase 3 staining (Figure 8o).

To corroborate our findings, we employed additional markers. Stainings for Pdgfra as an OPC marker (Pringle & Richardson, 1993) and for Sox10 as an oligodendroglial lineage marker (Kuhlbrodt et al., 1998) confirmed the precocious appearance of OPCs and the drastic increase in oligodendroglial numbers (Figure S5a–h and Figure S6a–l). Co-stainings with Ki67 at E11.5 and E12.5 furthermore pointed to a high rate of proliferation among parenchymally localized OPCs (Figure 8p).

Closer inspection of the numbers of Sox10-, Olig2-, and Pdgfra-positive cells revealed that there were more Sox10- than Olig2- or Pdgfra-positive cells particularly at early stages in TetSox9<sup>Brom</sup> spinal cords (Figure 8q; Figure S5j), whereas their numbers closely matched in control spinal cords (Figure 8r). Co-immunohistochemistry furthermore confirmed that a substantial fraction of Sox10-positive cells did not express Olig2 or Pdgfra (Figure 8s; Figure S5i). This fraction was particularly high between E10.5 and E12.5 and leveled out at later time points. Within the spinal cord, Sox10-positive cells without Olig2 and Pdgfra are unlikely to represent neural crest cells as they did not express the neural crest marker Tfap2a (Figure S1a–f).

In line with increased and precocious generation of OPCs in the TetSox9<sup>Brom</sup> spinal cord, we also observed a premature appearance of Mbp-expressing differentiating oligodendrocytes at E14.5 (Figure 8t,u,w). These cells were predominantly localized in the dorsal spinal cord, whereas Mbp-expressing cells normally appear first in ventral regions. Their number remained increased at E18.5 (Figure 8t,v,x). However, even at E18.5 only a minor fraction of oligodendroglial cells in the TetSox9<sup>Brom</sup> spinal cord had started to express Mbp (Figure 8t).

### 3.6 Astroglial cells are increased in TetSox9<sup>Brom</sup> spinal cord

Astroglial cells represent the second main type of neuroectodermal, macroglial cells in the CNS. However, few specific markers are available and none of the markers typically used to identify astroglial cells, such as Glast, Fgfr3, Nfia, or Nfib, is able to unambiguously identify this cell type during early spinal cord development. Glast and Fgfr3, for instance, label NEPs in addition to astroglial cells (Stolt et al., 2003). Therefore, they cannot distinguish between an astroglial cell and a displaced former NEP cell in the TetSox9<sup>Brom</sup> spinal cord. Nfia and its relative Nfib are expressed even more broadly, as they label NEPs, astroglial cells, OPCs, and subsets of neurons (Figure S7a–l).

Therefore, we used a combination of S100b and Sox10. From E12.5 onwards astroglial cells are S100b-positive (Raponi et al., 2007). In contrast to other astroglial markers, S100b does not stain NEPs. However, S100b is expressed in a subset of Sox10-positive oligodendroglial cells. We therefore defined astroglial cells as S100b-positive cells that do not express Sox10.

Such cells were undetectable in control and TetSox9<sup>Brom</sup> spinal cords before E12.5 and increased steadily thereafter (Figure 9a–h). Quantification showed that the increase was much stronger in TetSox9<sup>Brom</sup> spinal cords than controls (Figure 9i) arguing that Sox9 overexpression also promotes the generation of astrocytes as the second macroglial cell type. However, the increase in astroglial numbers was less pronounced than the one in oligodendrocytes at any given time and we did not find evidence for a precocious generation of astroglial cells.

To corroborate the increase in astroglial cells, we performed additional stainings for the astroglial marker Aldh1a1. Although difficult to...
quantify, these staining revealed a strong increase of Aldh1l1-positive cells at E18.5 in the TetSox9Brn4 spinal cord over control (Figure 9j,k). These cells are normally regarded as astroglial cells. Similarly, Gfap staining was increased and found in mantle and marginal zones of TetSox9Brn4 spinal cords at E18.5, whereas its occurrence was restricted in age-matched control spinal cords to the marginal zone (Figure 9l,m).

**FIGURE 9**  Astroglial cells in the TetSox9Brn4 spinal cord. (a–h) Immunohistochemical detection of cells that were positive for S100b (green) and negative for Sox10 (magenta) in control (ctr, a–d) and TetSox9Brn4 (e–h) spinal cords at E10.5 (a, e), E12.5 (b, f), E14.5 (c, g), and E18.5 (d, h). (i) Determination of astroglial cells in control (gray dots) and TetSox9Brn4 (open triangles) spinal cords. Numbers of S100b-positive and Sox10-negative cells are shown and presented as mean ± SEM per section (n = 3 spinal cords per genotype, counting three separate sections each). (j–m) Immunohistochemical staining of control (j, l) and TetSox9Brn4 (k, m) spinal cords section at E18.5 with antibodies directed against Aldh1l1 (j, k) and Gfap (l, m). For documentation of immunohistochemical stainings, spinal cords are surrounded by a stippled line and were placed on a black background. Scale bars: 100 μm (h, m). Statistical significance was determined by two-tailed Student’s t test (*p ≤ .05; ***p ≤ .001)
3.7 Effects of Sox9 transgene induction by Nestin::Cre resemble those by Brn4::Cre

To investigate the robustness of the effects observed in Tet-Sox9Brn4 spinal cords on various cell populations, we repeated the key analyses in embryos in which the Sox9 transgene was under control of a Nestin::Cre instead of a Brn4::Cre transgene at E12.5 and E14.5. These experiments confirmed that Sox9 transgene expression leads to an overall reduction of cells in the spinal cord (Figure S8a) with more apoptotic cells (Figure S8b) and a substantial increase of oligodendroglial (Figure S8c,d) and astroglial (Figure S8e) cells. Although effects were qualitatively similar in mice harboring Brn4::Cre or Nestin::Cre transgenes, there were differences in the size of the observed effects. These may be attributable to different onsets of transgene induction in the two mouse lines.

3.8 Delayed Sox9 overexpression leaves NEPs and neuronal cells unaffected

As Sox9 expression in transgenic mice was under control of tTA, we were able to alter its onset by treatment with tetracycline or derivatives such as doxycycline. We chose tetracycline over doxycycline because resumption of tTA activity has been reported to be faster after the end of tetracycline application and thus easier to control (A-Mohammadi, Alvarez-Vallina, Ashworth, & Hawkins, 1997). We employed two different schemes using TetSox9Brn4 mice. For one, pregnant mice received tetracycline from E7.5 until E10.5 and embryos were analyzed at E12.5 (Figure 10a). In the second paradigm, treatment was from E7.5 until E12.5 before analysis at E14.5. As a consequence, Sox9 overexpression in tetracycline-treated TetSox9Brn4 embryos was restricted to the 2 days before analysis. Detection of Sox9 and tdTomato confirmed a much more restricted transgene expression. Many cells in the mantle zone, especially the more lateral ones did not express Sox9 or tdTomato (Figure S9a–h).

An obvious consequence of the retarded Sox9 overexpression was that reductions in size of the TetSox9Brn4 spinal cord and the total number of cells was less severe than observed after Sox9 overexpression from earlier times (Figure 10b–f). Intriguingly, the VZ with its NEPs remained intact in the spinal cord of these mice and NEP numbers were comparable to those in control spinal cords (Figure 10g–k). Sox11-positive NPs were also more numerous than in

**FIGURE 10** NEPs and neuronal cells in the TetSox9Brn4 spinal cord after delayed Sox9 induction. (a) Tetracycline application scheme for delaying transgenic Sox9 induction in TetSox9Brn4 mice. (b–f) DAPI staining of spinal cords from tetracycline-treated (b, c) and untreated (d, e) TetSox9Brn4 mice at E12.5 (b, d) and E14.5 (c, e) and quantification (f) of cell numbers (untreated TetSox9Brn4, open triangles; treated TetSox9Brn4, blue squares; controls, gray dots). (g–u) Staining of spinal cords from tetracycline-treated (g, h, l, m, q, r) and untreated (i, j, n, o, s, t) TetSox9Brn4 mice at E12.5 (g, i, l, m, q, s) and E14.5 (h, j, n, o, r, t) as well as NeuN-positive neurons (q–r), and quantification of NEPs (k), NPs (p), and neurons (u). Scale bar: 100 μm. All numbers are presented as mean ± SEM per section (n = 3 spinal cords per genotype, counting three separate sections each). Statistical significance was determined by one-way ANOVA with Bonferroni’s multiple comparison (*p ≤ .00332; **p ≤ .0021; ***p ≤ .0002). DAPI, 4′,6-diamidin-2-phenylindole; NEP, neuroepithelial precursor cell.
untreated age-matched TetSox9Brn4 embryos (Figure 10l–p). Curiously, NP numbers remained below control levels with tetracycline treatment until E10.5, but surpassed them when tetracycline treatment was performed until E12.5. This may point to the existence of stage-specific effects of Sox9 in NPs. Number and position of NeuN-positive neurons in tetracycline-treated TetSox9Brn4 embryos were comparable to controls in both paradigms (Figure 10q–u).

3.9 | Even delayed Sox9 overexpression strongly increases gliogenesis

Considering that delayed Sox9 overexpression had only mild effects on NEPs and neurogenesis, it was interesting to study the consequences of tetracycline treatment on gliogenesis in TetSox9Brn4 embryos. Both Olig2- and Sox10-positive cells were still increased in number relative to controls, only slightly less than in untreated

**FIGURE 11** Glial cells in the TetSox9Brn4 spinal cord after delayed Sox9 induction. (a–o) Staining of spinal cords from tetracycline-treated (a, b, f, g, k, l) and untreated (c, d, h, i, m, n) TetSox9Brn4 mice at E12.5 (a, c, f, h, k, m) and E14.5 (b, d, g, i, l, n) for Olig2-positive (a–d), Sox10-positive (f–i), as well as S100b-positive, Sox10-negative cells (k–n), and quantification of oligodendroglial (e, j) and astroglial cells (o). Scale bar, 100 μm. Values for untreated TetSox9Brn4 spinal cords are depicted as open triangles, those for treated TetSox9Brn4 spinal cords as blue squares and those for controls as gray dots. All numbers are presented as mean ± SEM per section (n = 3 spinal cords per genotype, counting three separate sections each). Statistical significance was determined by one-way ANOVA with Bonferroni’s multiple comparison test (*p < .00332; **p < .0021; ***p < .0002)
TetSox9Brn4 embryos (Figure 11a–j). Compared to Olig2, Sox10 was detected in cells closer to the VZ especially at E12.5 arguing that Sox10 was induced earlier in these cells than Olig2. There was also a substantial increase in the number of S100b-positive, Sox10-negative astroglial cells in tetracycline-treated TetSox9Brn4 embryos relative to controls, although the increase did not reach statistical significance (Figure 11k–o). In summary, this argues that Sox9 is able to promote gliogenesis even if induced late during embryonic development.

**DISCUSSION**

Sox9 is one of the few transcriptional regulators that has been associated in the past with commitment to the glial cell lineage in the CNS (Kang et al., 2012; Scott et al., 2010; Stolt et al., 2003). Most of these studies were based on loss-of-function approaches and the few gain-of-function studies were performed ex vivo in cultured cells. Here, we have employed an in vivo gain-of-function approach and selectively overexpressed Sox9 in the CNS using an inducible, tetracycline-responsive tTA-dependent expression system. In the spinal cord as our site of analysis, this strategy allowed Sox9 expression from E9.0 onwards in all NEPs and cells derived from them. However, the system also allowed us to delay Sox9 overexpression until E10.5 or E12.5 by administering tetracycline to pregnant mothers from E7.5 onwards.

In the standard early induction paradigm without a tetracycline-dependent delay, expression of the Sox9 transgene preceded expression of endogenous Sox9 in NEPs. As a consequence of the ectopic expression, NEPs lose their epithelial characteristics and the VZ disappears. This argues that premature Sox9 expression in NEPs is detrimental. As we did not detect increased apoptosis among NEPs, it appears likely that Sox9 interferes with maintenance of the NEP pool either by counteracting the epithelial character or by promoting conversion into different cells. It has previously been reported that Sox9 expression in neural crest precursors promotes epithelial to mesenchymal transition and is essential for their emigration from the roof of the neural tube (Cheung et al., 2005; Cheung & Briscoe, 2003). Inappropriate expression in NEPs may have similar effects and thereby cause resolution of the VZ. However, we have no evidence to assume that Sox9 expression leads to a conversion of NEPs into neural crest cells.

Ectopic Sox9 expression furthermore continues in all neuronal cells that are generated from these NEPs. Both Sox9 overexpressing neuronal precursors and neurons exhibited increased rates of apoptosis. We therefore conclude that Sox9 compromises survival of neuronal cells during their maturation. Interestingly, ventral neurons were largely spared. This is due to the fact that most ventral neurons in our transgenic animals do not express Sox9 as their specification precedes transgene induction.

In contrast, in the late induction paradigm following tetracycline treatment Sox9 transgene expression was induced in NEPs that already expressed endogenous Sox9 and preferentially give rise to glial cells. Surprisingly, neuronal cells were largely Sox9 negative. One plausible explanation for this unexpected observation may be that the TRE:Sox9 transgene was epigenetically inactivated during neuronal differentiation so that it could no longer be induced once tTA regained activity. As a consequence, transgenic Sox9 is largely expressed in cells that already express Sox9 endogenously, and increases Sox9 levels in these cells. Under these conditions, NEPs and the VZ remain largely unaffected, as do neurons and their precursors.

However, under both conditions, transgenic Sox9 expression causes a dramatic increase in cells with glial identity. This concerns both oligodendroglial and astroglial cells. The changed distribution of cells among NEPs, neuronal, oligodendroglial and astroglial cells is summarized for E12.5 and E14.5 in Figure 12a,b. The pro-gliogenic activity of Sox9 is in agreement with previous conclusions from loss-of-function studies in the mouse (Stolt et al., 2003). According to our studies, surplus oligodendroglial cells are
generated earlier and in greater number than astrogial cells. This may simply reflect the normal situation in the developing spinal cord where oligodendrogenesis precedes astrogliogenesis. However, this result has to be taken with caution because of the dearth of markers that reliably distinguish astrogial cells from NEPs or oligodendroglial cells at early developmental times.

When using Sox10 or Olig2 as oligodendroglial lineage markers, we furthermore observed substantially more Sox10-positive cells than Olig2-positive cells especially at early times. These represent a sizeable population (labeled as "other" in Figure 12a,b). In the late, tetracycline-delayed induction paradigm where the structure of the spinal cord is much better preserved, it is additionally obvious that cells with exclusive Sox10 expression are localized closer to the VZ than double-positive cells. This argues that Sox10 precedes Olig2 expression in a large fraction of the supernumerary oligodendroglial cells induced by Sox9 transgene expression. A likely scenario thus posits that Sox9 induces Sox10 that in turn activates Olig2 expression. This is in line with previous reports that have shown that Sox10 is directly induced by Sox9 at least in the emerging neural crest (Cheung et al., 2005; Cheung & Briscoe, 2003; Wahlbuhl, Reiprich, Vogl, Bosl, & Wegner, 2011), and once present has the ability to stimulate Olig2 expression (Liu et al., 2007; Weider et al., 2015). Currently, we do not know whether all Sox10-positive cells eventually convert into Sox10- and Olig2-positive oligodendroglial cells or not. However, we do know that the Sox10-positive cells without Olig2 do not have a neural crest identity. The eventual convergence of Sox10- and Olig2-positive cell numbers and our failure to detect increased rates of apoptosis among Sox10-positive cells argues that the large majority of Sox10-positive cells become oligodendroglial cells.

The direct induction of Sox10 by Sox9 may also explain why more ectopic oligodendroglial than astrogial cells are generated in our transgenic mice as Sox10 has been reported to promote oligodendrogenesis and inhibit astrogenesis (Glasgow et al., 2014). If this is the case, then effective ways must exist in normal development that suppress Sox10 induction or counteract its activity in those cells that become astrocytes. The transcription factor Nfia has been described as one such factor (Glasgow et al., 2014). It remains to be shown whether Nfia is sufficient or whether other factors contribute.

Finally, it deserves to be noted that most of surplus glia that were generated, remained in the precursor state until birth. Given the premature generation and high number of oligodendroglial cells, a premature maturation may have been expected as well. Our analysis showed that the number of Mbp-expressing cells is indeed increased in the spinal cord shortly before birth. However, they remained fairly low in absolute numbers and represented only a minor fraction of the oligodendroglial cell population. They were furthermore predominantly localized in dorsal regions, whereas differentiating oligodendrocytes under normal conditions appear first in the ventral spinal cord. This may argue that conditions in the spinal cord are not yet conducive for differentiation, that proliferation promoting and anti-differentiation factors in OPCs such as the Hes5, Sox5, Sox6, and Id4 have not yet been sufficiently downregulated and that conditions in ventral regions are even worse than in dorsal ones (Liu et al., 2006; Marin-Husstege et al., 2006; Stolt et al., 2006). Future experiments will have to distinguish between these options.

In any case, our study provides unequivocal evidence for a pro-gliogenic role of Sox9 in the spinal cord. In addition, we show that the onset of Sox9 expression has to be tightly controlled to preserve the integrity of the VZ and prevent detrimental influences on neurogenesis. This dual function of Sox9 as a pro-gliogenic factor and an impediment to neurogenesis may be one of the reasons why gliogenesis sets in after neurogenesis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article and its supplementary information.

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