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Gsx transcription factors control neuronal versus glial specification in ventricular zone progenitors of the mouse lateral ganglionic eminence

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Abstract

The homeobox gene *Gsx2* has previously been shown to inhibit oligodendroglial specification in dorsal lateral ganglionic eminence (dLGE) progenitors of the ventral telencephalon. The precocious specification of oligodendrocyte progenitor cells (OPCs) observed in *Gsx2* mutants, however, is transient and begins to normalize by late stages of embryogenesis. Interestingly, this normalization correlates with the expansion of *Gsx1*, a close homolog of *Gsx2*, in a subset of progenitors in the *Gsx2* mutant LGE. Here, we interrogated the mechanisms underlying oligodendroglial specification in *Gsx2* mutants in relation to *Gsx1*. We found that *Gsx1/2* double mutant embryos exhibit a more robust expansion of Olig2⁺ cells (i.e. OPCs) in the subventricular zone (SVZ) of the dLGE than *Gsx2* mutants. Moreover, misexpression of *Gsx1* throughout telencephalic VZ progenitors from E15 and onward resulted in a significant reduction of cortical OPCs. These results demonstrate redundant roles of *Gsx1* and *Gsx2* in suppressing early OPC specification in LGE VZ progenitors. However, *Gsx1/2* mutants did not show a significant increase in adjacent cortical OPCs at later stages compared to *Gsx2* mutants. This is likely due to reduced proliferation of OPCs within the SVZ of the *Gsx1/2* double mutant LGE, suggesting a novel role for Gsx1 in expansion of migrating OPCs in the ventral telencephalon. We further investigated the glial specification mechanisms downstream of *Gsx2* by generating *Olig2/Gsx2* double mutants. Consistent with the known essential role for Olig2 in OPC specification, ectopic production of cortical OPCs observed in *Gsx2* mutants disappeared in *Olig2/Gsx2* double mutants. These mutants, however, maintained the expanded expression of gliogenic markers Zbtb20 and

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Bcan in the VZ of the LGE similarly to *Gsx2* single mutants, suggesting that *Gsx2* suppresses gliogenesis via Olig2-dependent and -independent mechanisms.

Keywords

Gliogenesis; Neurogenesis; Oligodendrocyte progenitor cell (OPC); Telencephalon

1. Introduction

During mammalian central nervous system (CNS) development, neural progenitors first give rise to neurons and subsequently generate glial cells, including oligodendrocytes and astrocytes (Bayer and Altman, 1991). This neuronal to glial switch occurs in a ventral-to-dorsal manner and has been shown in anterior (telencephalon) and posterior (hindbrain and spinal cord) CNS regions (Kessarar et al., 2001; Tekki-Kessarar et al., 2001; Fogarty et al., 2005; Vallstedt et al., 2005; Kessarar et al., 2006). In the telencephalon, a regional fate-mapping approach revealed that embryonic oligodendrocyte progenitor cells (OPCs) are first generated from ventral progenitor regions, including the medial ganglionic eminence (MGE) at approximately E12.5 and lateral ganglionic eminence (LGE) at approximately E15, while postnatal OPCs are largely generated from progenitors derived from the dorsal telencephalon (i.e. cortex) (Kessarar et al., 2006). However, the mechanisms controlling the temporal dynamics of the neuronal to glial fate change in VZ progenitor cells of the telencephalon remain elusive.

Gsx2 is one factor which has been shown to play a role in controlling the switch from neurogenesis to gliogenesis (Chapman et al., 2013). *Gsx2* is expressed in a gradient within early ventricular zone (VZ) progenitors of the LGE and MGE, with the highest levels in the dorsal (d)LGE and lowest levels within the MGE. However, by late stages of embryogenesis, *Gsx2* has downregulated within most cells of the MGE and ventral (v)LGE, and high level expression is limited to dLGE progenitors (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001; Waclaw et al., 2009). In accordance with this expression pattern, *Gsx2* both inhibits oligodendroglial specification and promotes the specification of LGE-derived neuronal fates in a temporal manner (Chapman et al., 2013; Waclaw et al., 2009). Specifically, striatal projection neurons are generated from the ventral vLGE during early stages of neurogenesis (E9–11), and olfactory bulb interneurons from the dLGE at late stages of neurogenesis (E12 onward) (Waclaw et al., 2009). Parallel to this, *Gsx2* begins to downregulate in MGE and vLGE progenitors, which corresponds with the timing of the ventral (early) to dorsal (late) generation of OPCs in these regions (Kessarar et al., 2006).

The homeobox gene *Gsx1* is closely related to *Gsx2*, sharing 97% identity between their homeodomains (Valerius et al., 1995). However, its expression pattern is in a gradient largely opposite to that of *Gsx2* expression, with high levels of *Gsx1* expression within the MGE and much lower levels within the vLGE (Valerius et al., 1995; Pei et al., 2011; Qin et al., 2017). In the absence of *Gsx2*, *Gsx1* expands dorsally, encompassing the entire extent of the LGE by E16.5 (Toresson and Campbell, 2001; Yun et al., 2003; Wang et al., 2003). Corresponding to this dorsal expansion of *Gsx1* in *Gsx2* mutant LGE progenitors, the severe

depletion of LGE- derived neuronal subtypes begins to recover by later embryonic stages (Szucsik et al., 1997; Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001; Stenman et al., 2003; Chapman et al., 2013). Thus, *Gsx1* is thought to specify progenitors towards LGE neuronal fates in a manner similar to *Gsx2*, and therefore its dorsal expansion has a compensatory effect and is responsible for the partial restoration of LGE neuronal fates in *Gsx2* mutants (Toresson and Campbell, 2001; Yun et al., 2003; Pei et al., 2011; Chapman et al., 2013). The loss of *Gsx1* alone, however, does not result in a robust phenotype in the LGE, likely due to the remaining *Gsx2* expression within VZ progenitors which correctly specifies these cells (Li et al., 1996; Toresson and Campbell, 2001; Yun et al., 2003; Wang et al., 2013).

Here we explored the molecular mechanisms underlying the OPC phenotype in the *Gsx2* mutant LGE. The precocious oligodendroglial specification in the dLGE of *Gsx2* mutants begins to recover at late embryonic stages, which temporally correlates with the expansion of *Gsx1* (Pei et al., 2011; Chapman et al., 2013). By analyzing OPCs within *Gsx1/2* double mutants as well as *Gsx1* over-expressing embryos, we demonstrate that *Gsx1* negatively regulates the specification of OPCs in telencephalic VZ progenitors similar to *Gsx2* and is required for the partial normalization of neuronal fates within *Gsx2* mutants. Specifically, *Gsx1/2* double mutants display further increased OPC specification than *Gsx2* mutants that persists throughout late stages of embryogenesis. These expanded OPCs, however, exhibit defective proliferation. As a result, the numbers of LGE-derived OPCs in the adjacent cortex of *Gsx1/2* mutants are not as large as those in the *Gsx2* mutants. Complementing these results, misexpression of *Gsx1* throughout late-stage (i.e. E15 onward) telencephalic VZ progenitors suppresses oligodendroglial specification, leading to reductions of OPCs in surrounding mantle regions. Despite that *Gsx1* expression expands throughout the *Gsx2* mutant LGE, it is not expressed throughout the apical-basal extent of the mutant VZ, as is the case for *Gsx2* (Wang et al., 2009). Instead, *Gsx1* expression marks the VZ/SVZ boundary of the *Gsx2* mutant LGE. In contrast, factors associated with glial cell development (*Olig2*, *Bcan*, and *Zbtb20*) are expanded in the *Gsx2* mutant LGE and are expressed in more apical VZ cells as compared to the expanded *Gsx1* expression. Moreover, analyzing *Olig2/Gsx2* double mutants revealed that *Olig2* is crucial for the expansion of the OPCs in the *Gsx2* mutant cortex. However, the glial associated markers (*Bcan* and *Zbtb20*) remain expanded in *Olig2/Gsx2* double mutant VZ progenitors similar to *Gsx2* mutants. Altogether, these studies reveal that *Gsx1* plays a repressive role on embryonic oligodendroglial specification within LGE VZ progenitors, comparable to that of *Gsx2* and *Olig2*-dependent and -independent mechanisms exist downstream of *Gsx* factors in the specification of glial progenitors.

2. Materials and methods

2.1 Animals

Olig2^{cre/+} mice (Dessaud et al., 2007) were used in combination with *Gsx2^{EGFP/+}* (Wang et al., 2009) for the generation of *Gsx2;Olig2* double mutant embryos (*Gsx2^{EGFP/EGFP};Olig2^{cre/cre}*). *Gsx2^{EGFP/+}* mice were crossed with *Gsx2^{RA/+}* mice (“RA” refers to the germline recombined allele from the *Gsx2* conditional mice, resulting in a null

allele) (Waclaw et al., 2009) to generate $Gsx2^{EGFP/RA}$ mutant embryos. $Gsx1^{+/-}$ mice (Li et al., 1996) were genotyped according to previous studies (Toresson and Campbell, 2001). $Gsx1^{+/-}$ and $Gsx2^{EGFP/+}$ heterozygous mice were crossed, as well as $Gsx1^{+/-}$ and $Gsx2^{RA/+}$ heterozygous mice, resulting in $Gsx2^{EGFP/+}$; $Gsx1^{+/-}$ and $Gsx2^{RA/+}$; $Gsx1^{+/-}$ double heterozygotes. These double heterozygotes were then cross-bred with each other to generate $Gsx2^{EGFP/RA}$; $Gsx1^{-/-}$ ($Gsx1/2$) double mutant embryos. $Foxg1^{Tta/+}$ (Hanashima et al., 2002), and $tet-O-Gsx1-IRES-EGFP(IE)$ (Pei et al., 2011) mice were genotyped as previously described (Waclaw et al., 2009; Pei et al., 2011). $Foxg1^{Tta/+}$ mice were crossed with $tet-O-Gsx1-IRES-EGFP(IE)$ mice to obtain $Foxg1^{Tta/+}$; $tet-O-Gsx1-IRES-EGFP(IE)$ double transgenic embryos. 0.02 mg/ml of doxycycline hyclate (Dox, Sigma) was added to the drinking water of pregnant females beginning on E7 and taken away 4 days later (i.e. E11), which results in repression of the transgene until approximately E15 (Chapman et al., 2013).

For staging of embryos, the morning of vaginal plug detection was designated as embryonic day (E)0.5. For every stage and marker used at least three embryos of each genotype were analyzed. Animal protocols were approved by the Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee in accordance with NIH guidelines.

2.2. Histological analysis

Embryo heads were fixed in 4% paraformaldehyde at 4C overnight, followed by at least 3 washes in PBS. They were then placed into 30% sucrose over multiple days for cryoprotection, prior to sectioning on a cryostat at 12 μ m thickness onto positively-charged slides. Fluorescent immunohistochemical staining was performed on slide-mounted sections, with first a one hour blocking incubation in 10% normal donkey serum (Jackson ImmunoResearch), followed by an overnight incubation in a primary antibody used at the following respective concentrations: chick anti-GFP (1:1000 Aves), goat anti-GFP (1:3000, Abcam), rabbit anti-GFP (1:1000, Invitrogen), guinea pig anti-Gsx1 (1:4000, Qin et al., 2017), rabbit anti-Gsx2 (1:5000, Toresson et al., 2000), rabbit anti-Ki67 (1:1000, Abcam), rabbit anti-Olig2 (1:2000, Millipore), rabbit anti-PDGFR α (1:200, Santa Cruz), goat anti-Sox10 (1:200, Santa Cruz), goat anti-Sp8 (1:8000, Santa Cruz), rabbit anti-Zbtb20 (1:333, Sigma). Subsequently, a two hour incubation in the corresponding fluorescent secondary antibodies was performed using: donkey anti-goat antibodies conjugated to Cy2, Cy3, or Cy5 (1:200, Jackson ImmunoResearch) and donkey anti-rabbit antibodies conjugated to Cy2, Cy3, or Cy5 (1:200, Jackson ImmunoResearch). To detect EGFP protein expressed from the $Gsx2^{EGFP}$ allele, a tyramide amplification kit (ThermoFisher Scientific, T20932) was utilized. Double staining for Olig2 and Gsx2 was done using the tyramide amplification kit to visualize rabbit anti-Olig2 (1:20,000, Millipore) and to quench the first antibody followed by Gsx2 immunostaining as described above.

2.3. In Situ Hybridization

In situ hybridization was essentially carried out as described (Toresson et al., 1999; Kohli et al., 2017). A *Bcan* cDNA fragment (1089 bp) was amplified from E14.5 mouse embryo forebrain cDNA with the following primer pair containing SalI and SpeI sites: CCGTCGACCTTCAATGTCTACTGCTTCC and CCACTAGTAGATGCTGGTCAGATGAG.

The *Bcan* cDNA fragment was cloned into Spe1/Sal1 sites of pBluescript KS. Digoxigenin-labeled antisense probes against *Bcan* were generated by linearizing with Sal1 (New England Biolabs) and using T7 RNA polymerase (Roche). Combination situ hybridization/IHC was carried out as previously described (Kohli et al., 2017).

2.4. Quantification

To quantify PDGFR α , Olig2, and Sp8 expression within the dLGE of E15.5 embryos, thresholding and measuring tools were utilized in ImageJ to determine the area of immunopositive cells in three adjacent rostral to caudal sections. PDGFR α expression was quantified in the VZ/SVZ region due to the cytoplasmic nature of the staining that leads to labeling of the radial glia and robust staining in the VZ/SVZ regions. PDGFR α and Sp8 expression were quantified in the same manner within the E18.5 dLGE, however Olig2 expression within this region is sparse enough for cellular resolution, and thus was quantified manually with a cell counter. For the quantification of E18.5 cortical OPCs, two high-powered images were taken within the cortex of three adjacent rostral-caudal sections, and Sox 10, Olig2 and PDGFR α . OPCs were quantified manually using a cell counter. To account for any variation in cortical size, the volumes of regions quantified were calculated using ImageJ and OPC numbers were normalized by comparing cells per millimeter squared (mm²). OPCs double labeled with EGFP and PDGFR α or Olig2 or Sox 10 were quantified in the same manner and as described in Chapman et al. (2013). For the quantification of Olig2 and Ki67 at E15.5, three images were taken in the dLGE SVZ region and quantified for Olig2 positive cells and Olig2;Ki67 double positive cells per field. At least three embryos were analyzed for each genotype.

3. Results

3.1. Increased oligodendroglial specification in *Gsx1/2* double mutants

In the absence of *Gsx2*, dLGE progenitors are precociously directed towards an oligodendroglial fate at mid-embryonic (i.e. E15) stages, but this expansion of OPCs is transient and improves by birth (Chapman et al., 2013). *Gsx1* is known to expand in the absence of *Gsx2* in a subset of LGE progenitors by mid-embryonic stages (Toresson et al., 2001; Yun et al., 2003; Wang et al., 2009; Pei et al., 2011). As previously described (Wang et al., 2009; Pei et al., 2011), expanded *Gsx1* gene expression in the *Gsx2* mutant LGE marks the VZ/SVZ boundary and does not occupy the apical half of the mutant LGE VZ, where the most primitive progenitors exist. Using a new *Gsx1* specific antibody (Qin et al., 2017), we observe the same staining pattern (Suppl. Fig. 1A-D). To determine if the dorsal expansion of *Gsx1* in the LGE, observed in *Gsx2* mutants (Suppl. Fig. 1C-D; see also Toresson et al., 2001; Yun and Rubenstein, 2003), plays a role in the normalization of OPC specification in *Gsx2* mutants, we generated *Gsx1/2* double mutant embryos (*Gsx2*^{EGFP/RA}; *Gsx1*^{-/-} embryos). The dorsal expansion of *Gsx1* along the VZ/SVZ border reaches the vLGE by E12.5 and is fully extended throughout the dLGE prior to E16.5 (Wang et al., 2009; Suppl. Fig. 1A-D'). Thus, to determine any compensatory effect of *Gsx1* on OPC specification, we examined the expression of the OPC markers PDGFR α and Olig2 within the dLGE of *Gsx1/2* double mutants at E15.5. The dLGE SVZ normally contains Sp8-positive olfactory bulb and amygdalar interneuron progenitors (Fig. 1C) (Waclaw et al., 2006; Waclaw et al.,

2010). In fact, the dLGE SVZ of control embryos completely lacks PDGFR α staining (Fig. 1A) and contains only a very small number of Olig2-positive cells that do not overlap with Sp8 positive cells in the same region (Fig. 1B, 1D). In *Gsx2* mutants, however, the stream of Sp8-positive neuroblasts is drastically decreased and replaced with PDGFR α - and Olig2-positive OPCs (Fig. 1E-H) (Waclaw et al., 2006; Chapman et al., 2013). Interestingly, Sp8 expression in *Gsx1/2* double mutants is more severely diminished and largely absent from the dLGE SVZ as compared to *Gsx2* mutants (compare Fig. 1G to K; see also Fig. 1S). Complementing these results, *Gsx1/2* double mutant embryos display a greater increase in PDGFR α and Olig2 cells within the VZ and SVZ (Fig. 1I,J) as compared to *Gsx2* mutants (Fig. 1E,F). These findings suggest that *Gsx1*, similar to *Gsx2*, negatively regulates OPC specification of LGE progenitors. Indeed, the loss of both genes leads to a further increase in precocious OPCs within the double mutant LGE (Fig. 1Q,R).

Gsx1 has been shown to regulate the specification of LGE neuronal subtypes in a similar manner as *Gsx2* (Pei et al., 2011; Toresson and Campbell, 2001; Yun et al., 2003), however, the loss of *Gsx1* alone has no apparent effect on LGE neuronal specification. This is not surprising given the expression pattern of *Gsx1*, displaying highest levels of expression within the MGE and only scattered cells within the vLGE along the VZ/SVZ border in wild type embryos (Suppl. Fig. 1A-D'). Furthermore, *Gsx1* mutants still have functioning *Gsx2* within the VZ, and even slightly increased levels in the vLGE at late stages of neurogenesis (E16.5-E18.5), which is therefore able to correctly specify these progenitors (Pei et al., 2011). Accordingly, *Gsx1* mutants display no differences in embryonic oligodendroglial specification, and resemble control embryos with a stream of Sp8-positive cells and little to no PDGFR α or Olig2 expression within the dLGE SVZ (Fig. 1M-P).

By late embryonic stages, the molecular identity of LGE progenitors (i.e. *Dlx*, *Isl1*, *Ascl1*, *Er81* expression) in *Gsx2* mutants has begun to normalize (Toresson et al., 2000; Yun et al., 2001). This recovery is largely dependent on the dorsal expansion of *Gsx1* (see Supp. Fig. 1), which is evidenced by the fact that *Gsx1/2* double mutants have more severe misspecification of LGE progenitors than *Gsx2* mutants (Stenman et al., 2003; Toresson and Campbell, 2001; Yun et al., 2003). The expression of Sp8 in the dLGE SVZ of *Gsx2* mutants is improved by E18.5, however, not to the same level as observed in controls (Fig. 2F) (Chapman et al., 2013). Conversely, in *Gsx1/2* double mutants, only a few scattered cells express Sp8 in this region, further supporting that this recovery is largely dependent on *Gsx1* (Fig. 2I,L). To determine if *Gsx1* is similarly required for the normalization of precocious OPC specification seen in *Gsx2* mutants, we examined the OPC markers PDGFR α and Olig2 in E18.5 *Gsx1/2* double mutants.

As expected, *Gsx1/2* double mutants express increased PDGFR α and Olig2 expression within the VZ/SVZ of the dLGE (Fig. 2G,H,J,K) compared to *Gsx2* mutants (Fig. 2D,E). This indicates that in *Gsx2* mutants, the dorsal expansion of *Gsx1* into the LGE not only restores neurogenesis but also prevents ectopic misspecification of OPCs.

3.2. OPCs in adjacent mantle regions are not further expanded in *Gsx1/2* double mutants due to reduced LGE SVZ proliferation

Although the precocious specification of dLGE progenitors toward an oligodendroglial fate in *Gsx2* mutants is transient, this still leads to increased numbers of LGE-derived OPCs in surrounding cortical mantle regions at E18.5 (Fig. 3C,D) (Chapman et al., 2013). Given that *Gsx1/2* double mutants display an even further increase in precocious OPC specification throughout late embryonic stages, as compared to *Gsx2* mutants, we expected these embryos to exhibit a larger expansion of OPCs in the adjacent cortex. Surprisingly, *Gsx1/2* double mutants (Fig. 3E,F) generate significantly fewer OPCs within the cortex than *Gsx2* mutants (Fig. 3C,D), however, they still exhibit significantly more cortical OPCs than in control embryos (Fig. 3A,B). While *Gsx2* mutants exhibited a 43% and 59% increase in Sox10- and PDGFR α -positive cells in the cortex compared to control embryos, *Gsx1/2* double mutants only displayed a 27% and 37% increase in Sox10- and PDGFR α -positive cells, respectively (Fig. 3G). Using the EGFP knock-in allele of *Gsx2* to follow *Gsx2*-expressing progenitors into surrounding mantle regions, we found that the increased cortical OPCs in *Gsx2* mutants are largely, if not exclusively, derived from *Gsx2* mutant progenitors (Chapman et al., 2013). Considering only the EGFP-positive population of OPCs, we again observed a much larger increase, 120% and 108%, in *Gsx2*-derived Sox 10-positive and PDGFR α -positive OPCs, respectively, verifying that the increased cortical OPCs are largely derived from *Gsx2*-mutant progenitors (Fig. 3H). When examining the EGFP positive cells in the cortex of *Gsx1/2* double mutants, we found extensive co-labeling of EGFP/Sox10 and EGFP/PDGFR α (Fig. 3E,F) and a much larger increase than total OPCs, however there are still fewer than in *Gsx2* mutant embryos. *Gsx1/2* double mutants displayed a 80% increase in EGFP/Sox10-positive and an 83% increase in EGFP/PDGFR α -positive OPCs (Fig. 3H). This suggests that, similar to *Gsx2* mutants, the overall increase in OPCs in *Gsx1/2* double mutants is largely derived from *Gsx1/2*-mutant progenitors.

Although *Gsx1/2* double mutants exhibit a significant upregulation in both *Gsx2*-derived OPCs as well as total number of OPCs within the cortex compared to control embryos, there are still significantly more OPCs within the cortex of *Gsx2* mutants (Fig. 3G,H). This was unexpected, since *Gsx1/2* double mutants have increased PDGFR α - and Olig2-expressing cells within the dLGE SVZ, as compared to *Gsx2* mutants (see Figures 1 and 2). One possibility could be that the increased OPCs in *Gsx1/2* double mutants are migrating and expanding into different regions of the telencephalon instead of the cortex. To determine if these OPCs were migrating ventrally instead, we analyzed the OPC markers Olig2, PDGFR α , and Sox 10 within mantle regions of the subpallium, however no significant increases were detected (data not shown). We also examined *Plp1* expression at E18.5 to address OPC differentiation. No precocious expression of *Plp1* was found in the embryonic cortex of *Gsx1/2* double mutants (data not shown). Moreover, since *Gsx1/2* double mutants are lethal after P0, we could not address OPC differentiation in the postnatal cortex. Another possibility could be that *Gsx1* plays additional roles outside of the VZ/SVZ of the LGE similar to *Ascl1*, which plays a role in migrating OPCs (Nakatani et al., 2013) and is required for the expansion and/or maintenance of OPCs in the *Gsx2* mutant cortex (Chapman et al., 2013). In line with this, a recent gene expression catalog of early perinatal

cortex cell types showed *Gsx1* as one of several new transcription factors expressed in OPCs (Zhang et al., 2014).

Gsx2 has been shown to play a key role in proliferation in the embryonic progenitors and postnatal neural stem cells from telencephalic regions *in vivo* and *in vitro* (Pei et al., 2011; Mendez-Gomez and Vicario-Abejon, 2012; Lopez-Juarez et al., 2013). In fact, *Gsx2* mutants have reduced proliferation within SVZ progenitors at E12.5, which, similar to the molecular identity of the LGE, has largely recovered by E16.5 (Toresson et al., 2000; Yun et al., 2001), suggesting a role for *Gsx1/2* in progenitor proliferation. This recovery is also dependent on *Gsx1*, as *Gsx1/2* double mutants have been shown to have decreased SVZ proliferation persisting throughout E16.5 (Toresson and Campbell, 2001). OPCs are also highly proliferative, and thus we analyzed the expression of the cell cycle marker Ki67 (Schlüter et al., 1993) within the dLGE SVZ together with Olig2 at E15.5 in order to determine whether the increased OPCs in *Gsx1/2* double mutants exhibit defective proliferation. By E15.5, Ki67 expression has recovered in *Gsx2* mutant SVZ progenitors (Fig. 4C), appearing similar to control embryos (Fig. 4A). Consistent with previous studies (Toresson and Campbell, 2001), *Gsx1/2* double mutant embryos continue to display reduced Ki67 expression within SVZ progenitors (Fig. 4E). Control embryos contain only a few scattered Olig2+ OPCs within the dLGE SVZ, and some of these co-express Ki67 indicating they are actively proliferating (arrows point towards SVZ region in Fig. 4B and 4G).

In contrast, *Gsx2* mutants have significantly more Olig2-positive cells within the SVZ, many of which co-label with Ki67 (Fig. 4D and 4G). *Gsx1/2* double mutant embryos contain even more Olig2-positive cells within the dLGE SVZ, however, a significantly smaller fraction of the total Olig2 population co-labeled with Ki67 compared to *Gsx2* mutants (Fig. 4F-H), which is in line with the reduced Ki67 in the SVZ of the LGE (Fig. 4E and Toresson and Campbell, 2001). In fact, *Gsx1/2* double mutants show only 32% of Olig2 positive cells are Olig2;Ki67 double positive cells compared to the 58% observed in *Gsx2* mutants (Fig. 4G-H). This suggests that although *Gsx1/2* double mutants have an overall increase in OPC specification from dLGE progenitors (Olig2-positive cells), a smaller percentage of the total Olig2 positive cells are actively dividing within the SVZ than those in *Gsx2* single mutants, which may contribute to less of an expansion of OPCs in surrounding cortical regions as compared to *Gsx2* mutants (see Fig. 3).

3.3. *Gsx1* is sufficient to repress the specification of OPCs in late telencephalic progenitors

To over-express *Gsx1* at distinct stages of development, we utilized a binary transgenic system that can be temporally regulated with doxycycline (Dox). This system utilizes a *Foxg1^{tTA/+}* knock-in mouse that expresses the tetracycline transactivator in the *Foxg1* lineage (Hanashima et al., 2002) and *tetO-Gsx1-IE* transgenic mice which express *Gsx1* only after tTA expression (Pei et al., 2011). We analyzed *Foxg1^{tTA}; tetO-Gsx1-IE* double transgenic (*Gsx1* DT) embryos at E18.5 following an E7–11 Dox treatment, which has been shown to delay Dox responsive transgene expression to E15.5 onward in the telencephalon (Chapman et al., 2013). To evaluate transgene activation after DOX treatment, we analyzed GFP expression from the *Gsx1* transgene (*tetO-Gsx1-IRES-eGFP*). We detect GFP

expression in both progenitor and mantle zone areas of the telencephalon at E18.5 (Fig. 5C,F). However, we observe stronger GFP expression in the VZ/SVZ of the dorsal telencephalon compared to the VZ/SVZ of the LGE in the ventral telencephalon and almost no transgene activation in medial/septal regions (Fig. 5C,F). Delayed over-expression of *Gsx1* results in the respecification of progenitors to a dLGE neuronal identity (Fig. 5), similar to what was previously observed with delayed over-expression of *Gsx2* (Chapman et al., 2013). Both *Ascl1* (Fig. 5B,C) and *Sp8* (Fig. 5E,F) are upregulated within the dorsal telencephalon (i.e. cortex) of these *Gsx1* DT embryos at E18.5. This misspecification, though similar to the *Gsx2*DT, appears less robust, which is consistent with results from previous early-stage *Gsx1* over-expression studies (Pei et al., 2011).

*Gsx2*DT embryos with delayed Dox treatment from E7–11 resulted in a significant reduction of OPCs within the cortex at E18.5 (Chapman et al., 2013). To determine whether *Gsx1*, like *Gsx2*, is sufficient to suppress oligodendroglial specification in LGE progenitors, we analyzed OPCs in the cortex of *Foxg1^{TA}; tetO-Gsx1-IE*DT embryos with the same delayed Dox treatment from E7–11. Both *Gsx1* and *Gsx2* DT embryos have cortical morphological defects that are improved with Dox treatment (Waclaw et al., 2009, Pei et al., 2011, and Chapman et al., 2013). However, *Gsx1* DT embryos still show an enlarged ventricle and slightly thinner cortex (Fig. 5 and 6), which is similar to *Gsx2* DT embryos (Chapman et al., 2013). *Gsx1* DT embryos showed a significant reduction in OPCs within the cortex as indicated by *Sox10*, *PDGFR α* , and *Olig2* expression (Fig. 6D-F) as compared to controls (Fig. 6A-C). Specifically, there was a 39% reduction in *Sox10*-expressing cells, a 43% reduction in *PDGFR α* -expressing cells, and a 30% reduction in *Olig2*-expressing cells (Fig. 6G). Consistent with the reduced ability of *Gsx1* to specify progenitors towards neuronal fates as compared to *Gsx2*, this suppression of OPCs is slightly less robust than in *Gsx2*DT embryos, which had a 46%, 47%, and 37% decrease in *Sox10*-, *PDGFR α* -, and *Olig2*-expressing OPCs, respectively (Chapman et al., 2013). When examining EGFP expression (a surrogate of *Gsx1* overexpression) with these OPC markers, we observed little to no co-localization (Fig. 6H-J), indicating that the remaining OPCs were not generated from progenitors that over-express *Gsx1*. Overall, these results indicate that *Gsx1* is able to inhibit oligodendroglial specification within late (i.e. E15 onward) LGE progenitors, analogous to the role of *Gsx2* in OPC specification.

3.4. Increased expression of glial markers in LGE progenitors of *Gsx2* mutant

Gsx2 has been shown to negatively regulate OPC generation from LGE progenitors (Chapman et al., 2013). In particular, dLGE progenitors in the SVZ of *Gsx2* mutants were shown to be precociously misspecified to an oligodendroglial fate (i.e. *Olig2*⁺) at mid-embryonic stages (e.g. E15.5) (Chapman et al., 2013). We analyzed the normal expression of *Olig2* in the VZ of the LGE at E13.5 and compared it to the high dorsal to low ventral gradient of *Gsx2* expression. Interestingly, *Olig2* appears to be expressed in an opposing gradient to *Gsx2* with very few *Olig2* positive cells present in the high *Gsx2*-expressing dLGE domain (Fig. 7A-B). Further examination of *Gsx2* mutants reveals that expanded *Olig2* expression is also observed in VZ progenitors throughout the apical-basal extent of the LGE at E16.5 (compare Suppl. Fig. 1F to E). Notably, the expression of *Olig2* is observed in the more apical VZ cells where the patterning and specification of glial cells takes place

compared to increased Gsx1 positive cells at the VZ/SVZ boundary in the *Gsx2* mutant LGE (Suppl. Fig. 1G-J).

Olig2 and PDGFR α represent markers of early stage OPCs (reviewed in Rowitch and Kriegstein, 2010; Emory and Lu, 2015) and are ectopically and robustly expressed in the *Gsx2* mutant LGE progenitors of both the VZ and SVZ from E12.5 onward (Chapman et al., 2013; Fig. 1). However, other OPC markers like Sox10 were not detected in mutant LGE progenitors but only in the migrating OPCs located within the brain parenchyma (i.e. cortex) of *Gsx2* mutants (Chapman et al., 2013). To better assess the extent of glial specification of VZ progenitors in the *Gsx2* mutant LGE, we identified additional candidate glial markers previously associated with gliogenesis or astrogliogenesis that are increased in *Gsx2* mutant LGE progenitors. We found that the glial associated transcription factor Zbtb20 (Nagao et al., 2016) and proteoglycan *Brevican/Bcan* (Jaworski et al., 1995) display somewhat complimentary expression to Gsx2 in the developing LGE of wild type embryos (Fig. 7C-E). In fact, in situ hybridization for *Bcan* and IHC for Gsx2 show that the high Gsx2 domain in the dLGE is weak for *Bcan* which is more robustly expressed in vLGE at E18.5 (Fig. 7D-E). Importantly, *Gsx2* mutants show increased Zbtb20 (Compare Fig. 7G to F) and *Bcan* (compare Fig. 7I to H) in the dLGE region of the VZ at E16.5. Moreover, it appears that the increased expression of these glial markers is found in VZ cells that are closer to the apical surface (Fig. 7J-M) than the expanded Gsx1 positive cells located basally at the VZ/SVZ boundary in the *Gsx2* mutant LGE (Fig. 7K',M'). The increase of Zbtb20 and *Bcan* at E16.5 is quite interesting given that these are genes originally associated with gliogenesis and that they are also expressed in the LGE at times where the LGE is suggested to be both neurogenic and gliogenic. Moreover, the pallial genes (Pax6/Ngn2) that are abnormally expressed in *Gsx2* mutant LGE are largely recovered after E14.5 when Gsx1 has expanded into the dorsal regions of the LGE (Corbin et al., 2000; Toresson and Campbell, 2000; Toresson et al., 2001; Yun et al., 2001 and 2000). Therefore, it is possible that in the absence of *Gsx2*, dLGE VZ progenitors acquire molecular identity associated with glial specification at a stage earlier than normal, which leads to precocious generation of OPCs (until E15, before the compensatory expansion of Gsx1 expression into the dLGE).

3.5. Role of Olig2 in regulating glial identity of VZ progenitors in the *Gsx2* mutant LGE

Olig2 is required for the generation of OPCs throughout the CNS (Lu et al., 2002; Zhou and Anderson, 2002). To determine a role for Olig2 in regulating glial specification of VZ progenitors in *Gsx2* mutants, we generated *Gsx2;Olig2* double mutant embryos (*Gsx2*^{EGFP/EGFP};*Olig2*^{cre/cre}). Since germline *Olig2* mutants are not viable at postnatal stages (Lu et al., 2002; Zhou and Anderson, 2002), we analyzed embryos at E18.5 when *Gsx2* mutants show a robust increase in PDGFR α positive OPCs in the brain parenchyma (cortex) (Fig. 3) as well as in VZ progenitors of the mutant LGE (compare Fig. 8G to A). Predictably, the increased cortical PDGFR α expression observed in *Gsx2* mutants is absent in *Gsx2;Olig2* double mutants (compare asterisk in Fig. 8J to G). In fact, the apparent loss of OPCs is identical to the phenotype observed in the *Olig2* single mutant cortex (compare cortex in Fig. 8J,D to A). Interestingly, however, the ectopic PDGFR α expression in VZ progenitors of the *Gsx2* mutant LGE remained in *Gsx2;Olig2* double mutants (compare arrows in Fig. 8J to G) suggesting that the *Gsx2* mutant VZ progenitors are still biased

towards an oligodendroglial cell fate despite the loss of *Olig2*. Complementing this result, two other glial progenitor markers *Zbtb20* and *Bcan* also remain expanded throughout the LGE VZ in *Gsx2;Olig2* double mutants which is indistinguishable from *Gsx2* mutants at E18.5 (compare arrows in Fig. 8K,L to H,I) and in contrast to the weak or absent expression detected in the dLGE VZ of both WT (Fig. 8B,C) and *Olig2* mutants (Fig.8E-F). Collectively, these data suggest that *Olig2* is required for the OPC expansion phenotype in the LGE SVZ and cortex of *Gsx2* mutants but the upregulation of a set of glial markers (e.g. PDGFR α , *Zbtb20*, and *Bcan*) in VZ progenitors of the *Gsx2* mutant LGE is independent of *Olig2* function. Moreover, we detect ectopic *Bcan* in *Gsx1/2* double mutants (data not shown) identical to *Gsx2* mutants, further supporting the idea that the ectopic expression of *Bcan/Zbtb20* in the apical VZ is independent or upstream of the compensatory *Gsx1* expression in the VZ/SVZ boundary of the *Gsx2* mutant LGE. These results suggest that *Gsx* factors inhibits gliogenesis in VZ progenitors via two separate mechanisms; one through the suppression of *Olig2* and a second manner through the inhibition of other gliogenic factors.

4. Discussion

The complex molecular mechanisms underlying the neurogenic to gliogenic fate change in neural progenitors are currently being elucidated and involve specific spatial and temporal regulations. *Gsx2* has been implicated in regulating the timing of the neuronal to glial switch within LGE progenitors by simultaneously promoting neuronal fates and suppressing oligodendroglial fates in the developing ventral telencephalon (Waclaw et al., 2009; Chapman et al., 2013). *Gsx1* has been shown to specify progenitors towards LGE neuronal fates in a manner similar to *Gsx2* (Pei et al., 2011), however it is unknown whether *Gsx1* is also able to repress the specification of OPCs from embryonic VZ progenitors. Analyzing oligodendroglial markers within *Gsx1/2* double mutants as well as *Gsx1* gain-of-function embryos, we have demonstrated that *Gsx1* acts in the same manner as *Gsx2* in repressing embryonic OPC specification within LGE VZ progenitors. We also show complementary domains of expression within the LGE between *Gsx2* and the glial progenitor genes (*Olig2*, *Zbtb20*, and *Bcan*). In addition, we show that *Gsx2* is required to restrict the expression of these genes in more apical VZ progenitors compared to the compensatory *Gsx1* expression in the *Gsx2* mutants at the VZ/SVZ boundary in the dLGE region. Moreover, despite that *Olig2* has been shown to be a major regulator of OPC generation, its increased expression in the LGE VZ progenitors of *Gsx2* mutants, is only required for the increased expansion of OPCs in the LGE SVZ and surrounding parenchymal regions observed in *Gsx2* mutants but not for the abnormal expression of glial markers in primary VZ progenitors seen in these mutants.

Gsx1 is normally expressed in progenitors along the VZ/SVZ boundary in a gradient of expression with highest levels in cells of the MGE compared to only scattered cells within the vLGE and limited expression in cells of the dLGE (Toresson and Campbell, 2001; Yun et al., 2003; Pei et al., 2011; see also Suppl. Fig. 1). The loss of *Gsx1* alone does not overtly affect the specification of LGE progenitors. In contrast, in the absence of *Gsx2*, *Gsx1* expands throughout the dorsal extent of the LGE at the VZ/SVZ boundary and is then required for much of the normalization of gene expression that occurs in late stage mutant

LGE progenitors (Toresson and Campbell, 2001; Yun et al., 2003). In fact, *Gsx2*-expressing progenitors normally generate striatal projection neurons and olfactory bulb interneurons from the vLGE and dLGE, respectively (Waclaw et al., 2009). In *Gsx2* mutants, these neuronal subtypes are severely diminished, but begin to recover at later stages (E14.5 onward). This restoration of neurogenesis is due to the dorsal expansion of *Gsx1* and its ability to specify LGE progenitors in a similar manner to *Gsx2* (Toresson and Campbell, 2001; Yun et al., 2003; Pei et al., 2011).

Gsx2 mutants also display a precocious increase in oligodendroglial specification within dLGE SVZ progenitors at E15.5, however by E18.5, this misspecification begins to normalize (Chapman et al., 2013). Our results indicate that, similar to the partial restoration of LGE neuronal specification, this recovery in precocious oligodendroglial specification is dependent on *Gsx1*. At E15.5, *Gsx1/2* double mutants exhibit a more robust increase in OPC specification within progenitors of the dLGE SVZ, as compared to *Gsx2* mutants. Fittingly, this expansion of OPCs is in more ventral regions of the double mutant dLGE (Fig. 1I,J compared to E,F), which is in accordance with the dorsal expansion of *Gsx1* approaching from the vLGE. Additionally, *Gsx1/2* double mutants continue to display increased levels of OPC markers in dLGE SVZ progenitors at E18.5, which is distinct from the recovery observed at this time in *Gsx2* single mutants. This suggests that the dorsal expansion of *Gsx1* in *Gsx2* mutants not only restores neurogenesis, but also negatively regulates the specification of OPCs to inhibit precocious oligodendrogenesis.

We were surprised to find, however, that even though *Gsx1/2* double mutants have increased oligodendroglial specification within SVZ progenitors of the dLGE as compared to *Gsx2* mutants, it does not lead to more of an expansion of OPCs in surrounding mantle regions as seen in *Gsx2* mutants. In fact, there are significantly fewer OPCs within the cortex of *Gsx1/2* double mutants than in *Gsx2* mutants. Nevertheless, *Gsx1/2* double mutants still generate significantly more cortical OPCs than control embryos. This discrepancy in numbers of OPCs between the LGE SVZ and cortical mantle zone can be partially explained by defective proliferation of OPCs within the SVZ of *Gsx1/2* double mutants (Toresson and Campbell, 2001). Thus, even though there are more cells exhibiting OPC characteristics (e.g. Olig2) within the dLGE, a reduced percentage of these are dividing, resulting in fewer OPCs within the adjacent mantle regions. Another partial explanation for the fewer cortical OPCs could simply be due to the severe disruption in the morphology and patterning of the double mutant telencephalon (Toresson and Campbell, 2001; Yun et al., 2003). *Gsx1/2* double mutants have a much smaller LGE/ striatal size than the *Gsx2* mutant, which is also smaller than controls, and accordingly double mutants have fewer VZ progenitor cells surrounding the lateral ventricle. Perhaps these decreased numbers of early VZ progenitors in combination with more severe molecular alterations also contribute to the lack of further expansion of OPCs into adjacent mantle regions. In line with this possibility, we cannot rule out a change in fate of the *Gsx* lineage in the cortical cells originating from the LGE/MGE. However, previous studies have shown that no abnormalities in the generation of cortical interneurons were detected in *Gsx1/2* double mutants (Yun et al., 2003). A final possibility could be that *Gsx1* may play additional roles beyond embryonic LGE VZ progenitors, more specifically in the postnatal oligodendrocyte lineage. In fact, *Gsx1* was recently identified as one of several novel markers in a gene expression catalog of different stages of

oligodendrocyte development in the perinatal cortex (Zhang et al., 2014). In any case, *Gsx1* is required for the full expansion potential of the precocious dLGE-derived OPCs generated in the *Gsx2* mutant. Given that both *Gsx2* and *Gsx1/2* mutants are lethal at birth before OPC differentiation, our future experiments will utilize conditional mutagenesis to overcome the lethality and address mutant OPC expansion, differentiation, and myelination.

To complement these loss-of-function studies, we also over-expressed *Gsx1* from E15 onward throughout telencephalic progenitors and found significant reductions in OPCs within the adjacent cortex three days later, at E18.5. Very few, if any of the progenitors that overexpressed *Gsx1*, gave rise to OPCs, suggesting that *Gsx1*, like *Gsx2*, is sufficient to suppress oligodendroglial specification in embryonic telencephalic VZ progenitors. However, we did not observe a complete loss of OPCs in the cortex at E18.5 after delayed misexpression of *Gsx1*. In fact, nearly all of the remaining OPCs in the cortex were negative for GFP, a surrogate for *Gsx1* transgene expression. One possible explanation could be the mosaic and weaker expression of the transgene in LGE progenitors compared to pallial progenitors after DOX treatment. In fact, it is proposed the LGE is the progenitor source for OPCs after E14 (Kessar et al., 2006), which is in line with the transgene activation after E7-E11 DOX treatment (Chapman et al., 2013). These delayed over-expression studies confirm a similar temporal effect of *Gsx1* to *Gsx2* in promoting LGE molecular identity (Pei et al., 2011) since this delayed over-expression of *Gsx1* also results in misspecification of progenitors toward dLGE neuronal fates, as indicated by ectopic upregulation of *Sp8* and *Ascl1* within the cortex.

It is interesting to note that despite the ability of *Gsx1* to specify telencephalic progenitors in the same manner as *Gsx2*, it does so in a less robust way. This is consistent with results from previous early-stage *Gsx1* over-expression studies (Pei et al., 2011). We discovered that this was the case, in regard to both neuronal and oligodendroglial specification, with increased cortical *Ascl1* and *Sp8* expression and fewer OPCs in the cortex in *Gsx2*DT embryos as compared to *Gsx1* DT embryos. This may not be unexpected, considering *Gsx1* is normally only expressed in scattered cells at the VZ/SVZ boundary of the vLGE and very sparingly in the dLGE.

Combined fate mapping approaches with *Gsx2-cre* and *Nkx2.1 cre* have suggested that the LGE produces OPCs at midgestation stages (around E15.5) (Kessar et al., 2006). However, it remains unknown if the entire span of the LGE VZ or a specific dorsal or ventral subdomain within it is responsible for gliogenesis. In the absence of *Gsx2*, the dLGE SVZ, which is normally occupied by the neurogenic markers (*Sp8* and *Er81*), ectopically expresses OPC markers (*PDGFR α* and *Olig2*), suggesting that *Gsx2* expression in the dLGE VZ promotes neurogenic genes and also controls the timing of OPC generation from the LGE (Chapman et al., 2013). We show here that *Gsx2* and *Olig2* are expressed in largely opposing gradients in the LGE VZ with highest expression of *Gsx2* in the dLGE and *Olig2* largely absent in the dLGE with higher levels in the vLGE. In line with the role of *Gsx2* controlling OPC timing, the absence of *Gsx2* results in an expansion of *Olig2* and other candidate gliogenic markers (*Zbtb20* and *Bcan*) into the dLGE region in the apical part of the VZ. This is in contrast to the compensatory expansion of *Gsx1* that is found at the VZ/SVZ boundary region of the *Gsx2* mutant LGE (Pei et al., 2011 and Suppl. Fig. 1).

Despite the role of *Olig2* as a major regulator of oligodendrocyte specification and differentiation (Lu et al., 2002; Zhou and Anderson, 2002; Yue et al., 2006; Zhu et al., 2012; Yu et al., 2013), some gliogenic genes such as *Zbtb20*, *Bcan*, and *PDGFR α* remain expressed in the dLGE VZ of *Olig2;Gsx2* double mutants (Figure 8), indicating that *Olig2* is not required for their expression in the LGE VZ.

Altogether, our results indicate a novel role for *Gsx1* in specifying LGE progenitors in *Gsx2* mutants. In addition to promoting LGE neuronal subtypes, *Gsx1* is also able to suppress oligodendroglial specification from LGE VZ progenitors. This results in a compensatory effect of *Gsx1* that is able to partially restore neurogenesis and repress precocious oligodendroglial specification that occurs in the dLGE of *Gsx2* mutants. Our findings in *Gsx1/2* mutants suggest that *Gsx* genes are required for the full expansion potential of LGE-derived OPCs. Furthermore, our results suggest *Gsx* factors suppresses gliogenesis via *Olig2*-dependent mechanisms in the expansion of cortical OPCs and *Olig2*-independent mechanisms in expansion of glial specification markers in the apical VZ upstream of the VZ/SVZ expression of *Gsx1*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Loss of Gsx1 and Gsx2 results in increased OPC specification in LGE progenitors.
- Misexpression of Gsx1 in telencephalic VZ progenitors resulted in a reduction of cortical OPCs.
- Candidate gliogenesis genes (Olig2, Bcan, and Zbtb20) are expanded in the Gsx2 mutant LGE and expressed in more apical VZ cells as compared to Gsx1 expression.
- Olig2-dependent and -independent mechanisms exist downstream of Gsx factors in the specification of glial progenitors in the LGE.

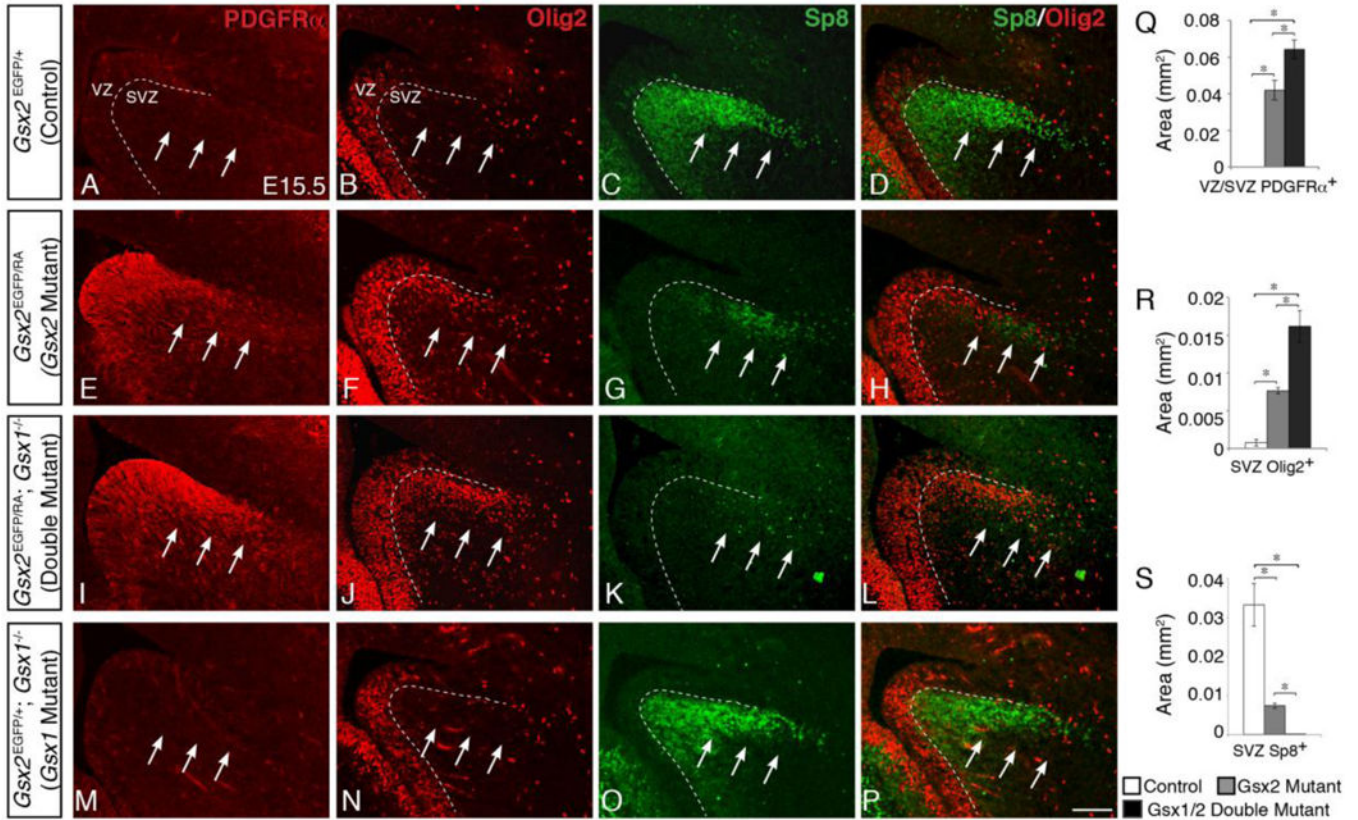


Fig. 1. *Gsx1/2* double mutants display a greater increase in OPC specification from dLGE progenitors than *Gsx2* mutants.

At E15.5, control dLGE progenitors generate many Sp8- positive neuroblasts (C) and very few OPCs, as indicated by lack of PDGFR α expression (A) and only scattered Olig2 expression within the SVZ (B). *Gsx2* mutants conversely express much lower levels of Sp8 (G) and instead ectopically express high levels of PDGFR α (E) and Olig2 (F) within the dLGE. This loss of Sp8 corresponds with the expanded OPCs, indicated in an overlay of Sp8 and Olig2 (H, compared to D). *Gsx1/2* double mutants display even higher levels of PDGFR α (I) and Olig2 (J,L) expression within the dLGE. Accordingly, only a few cells express Sp8 within this region of double mutants (K,L). Quantification of these cells indicate that *Gsx1/2* double mutants have significantly more PDGFR α expression within the VZ/SVZ and Olig2 expression within the SVZ than *Gsx2* mutants, which have significantly more than control (Q,R). Correspondingly, *Gsx1/2* double mutants have significantly fewer Sp8-positive cells within the dLGE SVZ than *Gsx2* mutants, which have substantially fewer than control embryos (S). *Gsx1* mutant embryos display no defects in oligodendroglial or neuronal specification, and resemble control embryos with no PDGFR α expression (M), very little Olig2 expression (N,P), and normal levels of Sp8 (O,P) within the dLGE. Data represent the mean \pm SEM. * $p < 0.01$, as determined by a one-way ANOVA followed by a Tukey HSD post-hoc test. Scale bar: P = 100 μ M

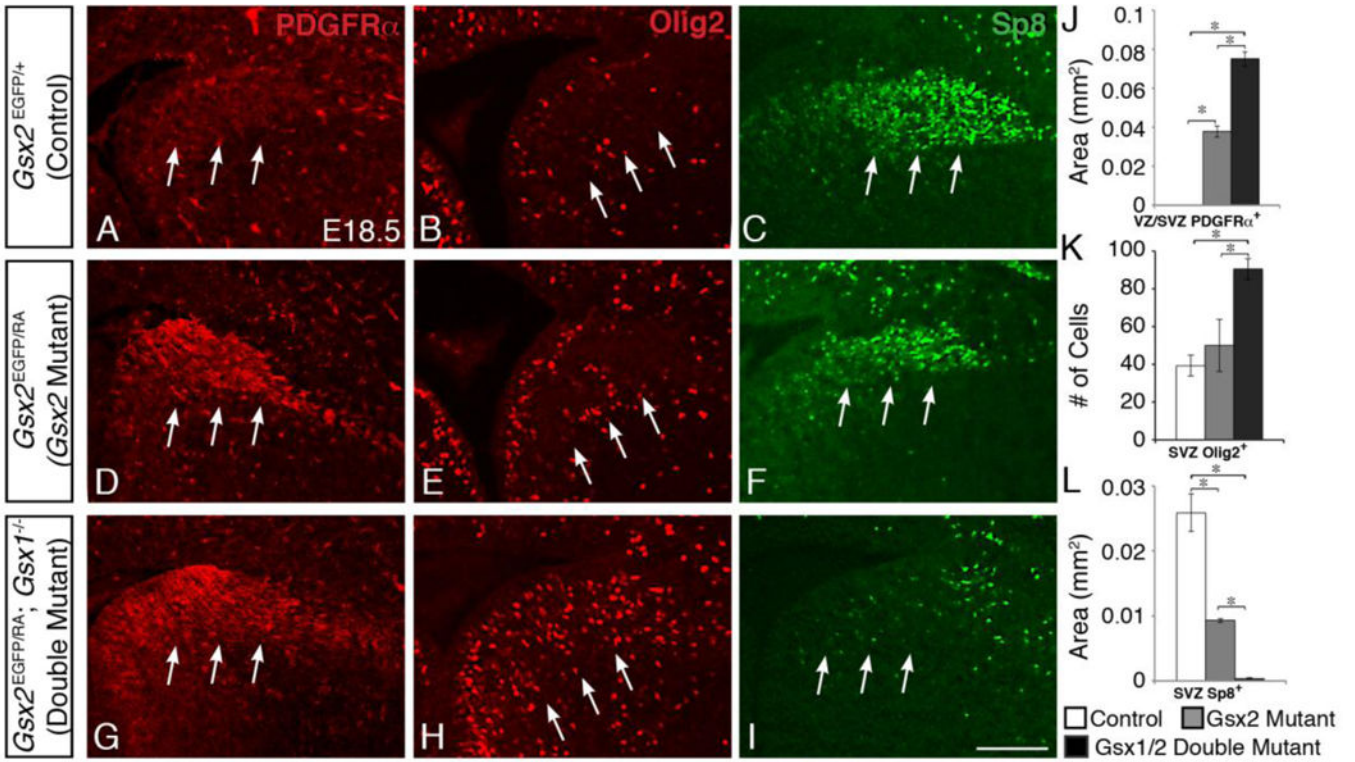


Fig. 2. Expanded OPC specification from dLGE progenitors persists up until birth in *Gsx1/2* double mutants, despite recovering in *Gsx2* mutants.

By E18.5, the increased oligodendroglial specification from the dLGE is beginning to recover in *Gsx2* mutants, with only slightly increased PDGFR α expression in the VZ/SVZ (D, compared to A) and no difference in Olig2-positive cells (E, compared to B) within the SVZ. Accordingly, Sp8 expression is also returning to the dLGE (F, compared to C). In contrast, *Gsx1/2* double mutants continue to express highly increased levels of PDGFR α (G) and Olig2 (H), while only few scattered Sp8-positive cells remain within the dLGE (I). The number of Olig2-positive cells as well as the area of PDGFR α - and Sp8-immunostained cells within the dLGE are quantified in J-L. Data represent the mean \pm SEM. * $p < 0.01$, as determined by a one-way ANOVA followed by a Tukey HSD post-hoc test. Scale bar: I = 100 μ M

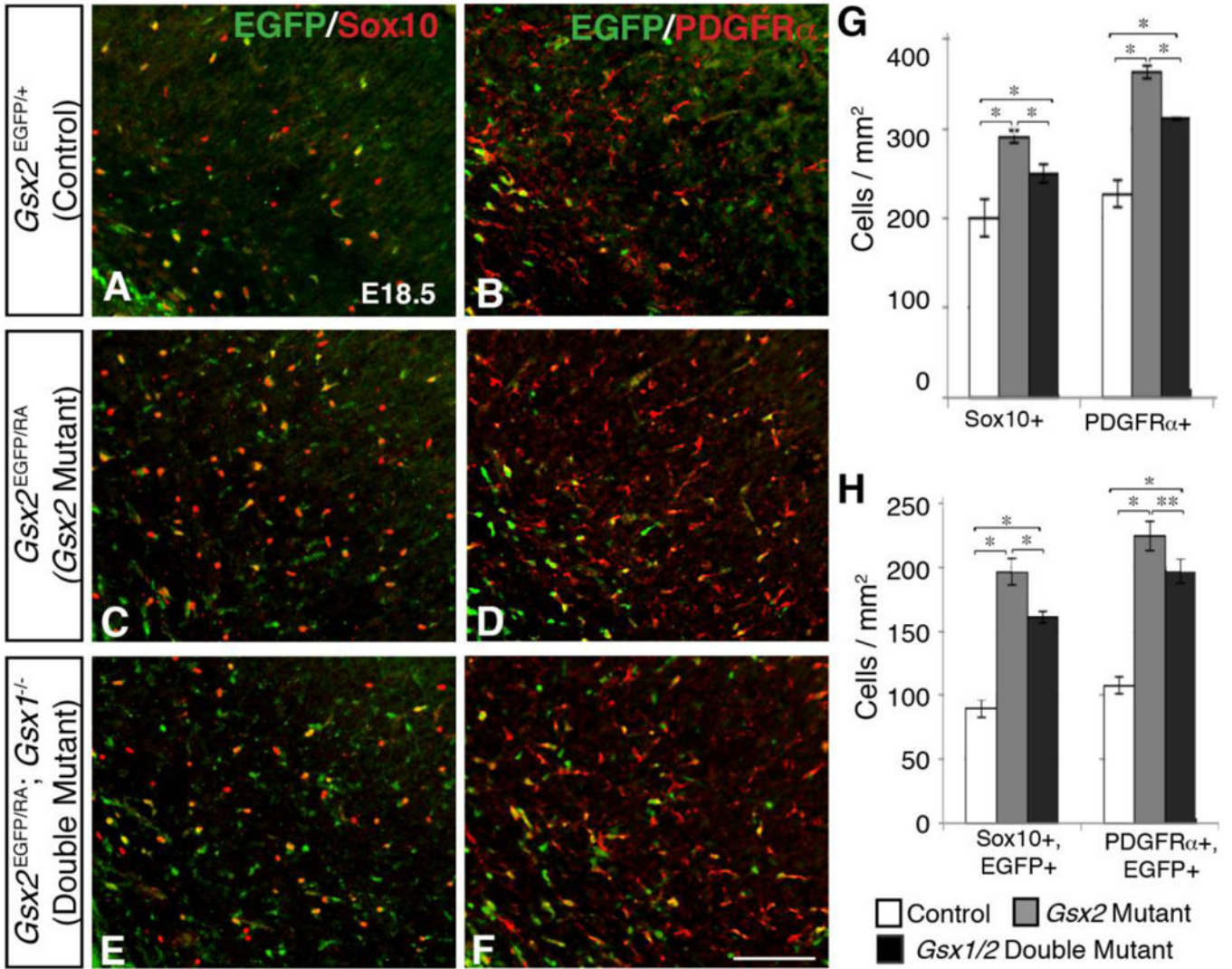


Fig. 3. Increased oligodendroglial specification in *Gsx1/2* double mutants does not lead to a further expansion in adjacent cortical OPCs as compared to *Gsx2* mutants.

Gsx2 mutants display increased Sox10 (control 203.2±28.7; mutant 290.3±6.4 cells/mm²) and PDGFR α (control 227.4±15.2; mutant 362.6±7.0 cells/mm²) OPCs within the cortex at E18.5 (C-D compared to A-B; G). *Gsx1/2* double mutants also exhibit increased cortical Sox10 (258.7±7.8) and PDGFR α (310.8±1.1) OPCs compared to controls (E-F), however *Gsx1/2* double mutants have significantly fewer OPCs than *Gsx2* mutants (G). When considering only cortical OPCs that originated from G.vx2-expressing progenitors (co-expressing EGFP), *Gsx1/2* double mutants again generated significantly more Sox10 (control 89.5±6.9; double mutant 161.0±4.5 cells/mm²) and PDGFR α (control 107.8±6.6; double mutant 196.8±9.3 cells/mm²) OPCs than controls, yet significantly fewer than *Gsx2* mutants (196.6±10.5 Sox10⁺ cells/mm²; 224.5±11.6 PDGFR α ⁺ cells/mm²) (H). Both *Gsx2* mutants and *Gsx1/2* double mutants contained significantly more *Gsx2*-derived OPCs within the cortex, indicating that the vast majority of increased OPCs arose from *Gsx2* mutant progenitors (H). Data represent the mean ±SEM. *p<0.01 and **p<0.05, as determined by a one-way ANOVA followed by a Tukey HSD post-hoc test. Scale bar: F = 100 μ M

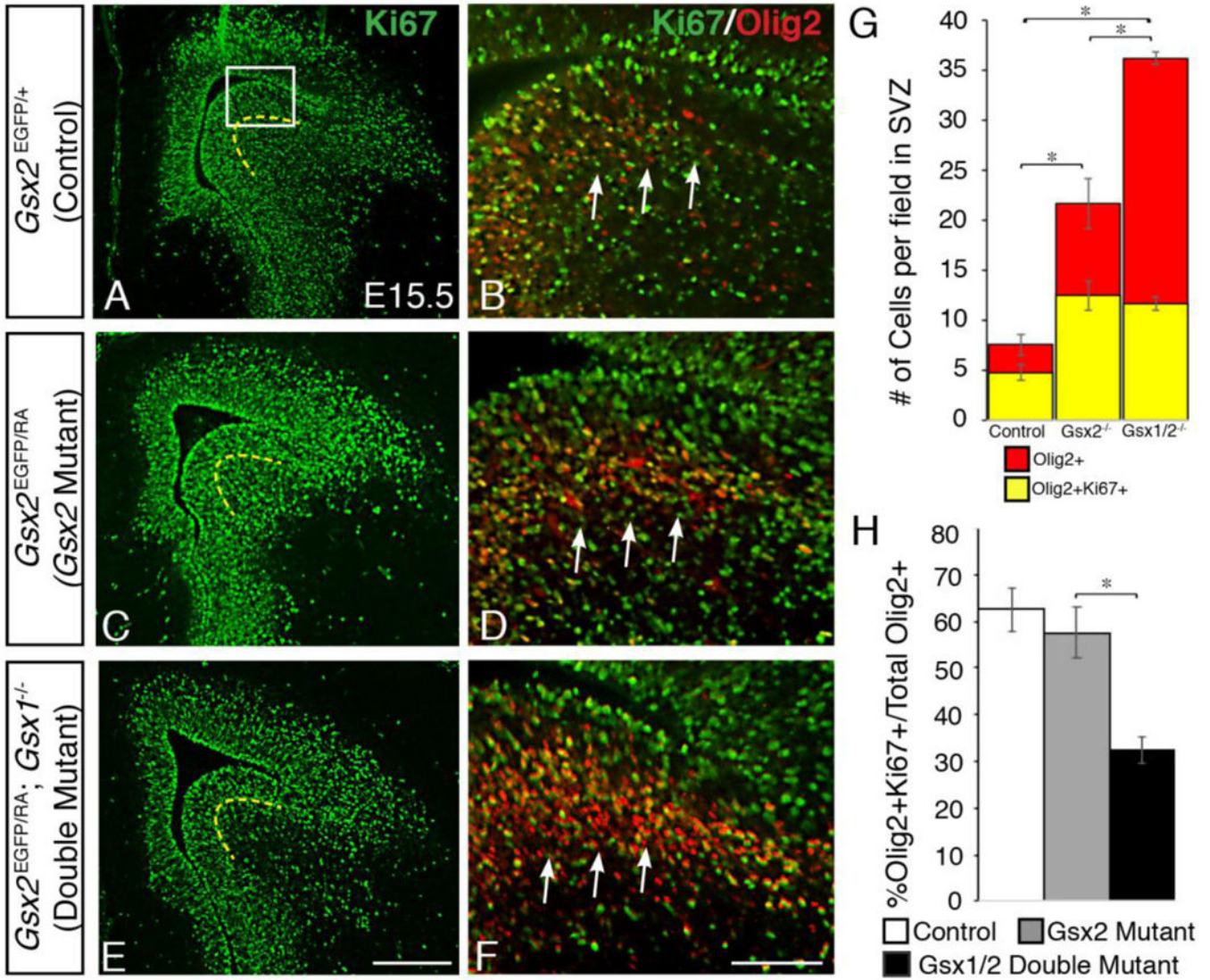


Fig. 4. Defective proliferation of OPCs within the dLGE of *Gsx1/2* double mutant embryos. *Gsx2* mutants have defective proliferation within the SVZ of the LGE at early stages of neurogenesis, however this begins to recover by E15.5 as expression of cell cycle marker Ki67 appears within the SVZ (denoted by dashed line) and looks rather normal in the *Gsx2* mutant (C) compared to controls (A). In contrast, *Gsx1/2* double mutants continue to display reduced levels of proliferation within the SVZ at E15.5 (E). When analyzing the OPC marker Olig2 in combination with Ki67 in high-powered images of the dLGE, there are very few Olig2+ cells within the dLGE SVZ of control embryos and some express Ki67 (B) (4.8 ± 0.8 cells per field). In *Gsx2* mutants, many of the expanded Olig2-positive cells within the SVZ are proliferative, as demonstrated by co-expression with Ki67 (D) (12.5 ± 1.5 cells per field). *Gsx1/2* double mutants show similar numbers of Olig2+Ki67+ cells in this region (11.7 ± 0.7 cells per field) compared to *Gsx2* mutants. However, *Gsx1/2* double mutants show increased total Olig2-positive cells within this region (36.2 ± 0.6 cells per field) compared to control (7.6 ± 1.0 cells per field) and *Gsx2* mutants (21.7 ± 2.5 cells per field) (F,G). When

considering the percentage of 01ig2+Ki67+ cells to total 01ig2+ cells, *Gsx1/2* double mutants (32.3%±2.8) showed reduced numbers compared to control (63.2%±4.7) and *Gsx2* mutants (57.6%±5.6) (**H**). Data represent the mean ±SEM. *p<0.01 in G and *p<0.05 in H as determined by a one-way ANOVA followed by a Tukey HSD post-hoc test. Scale bar: **E** = 200µM, **F** = 100µM

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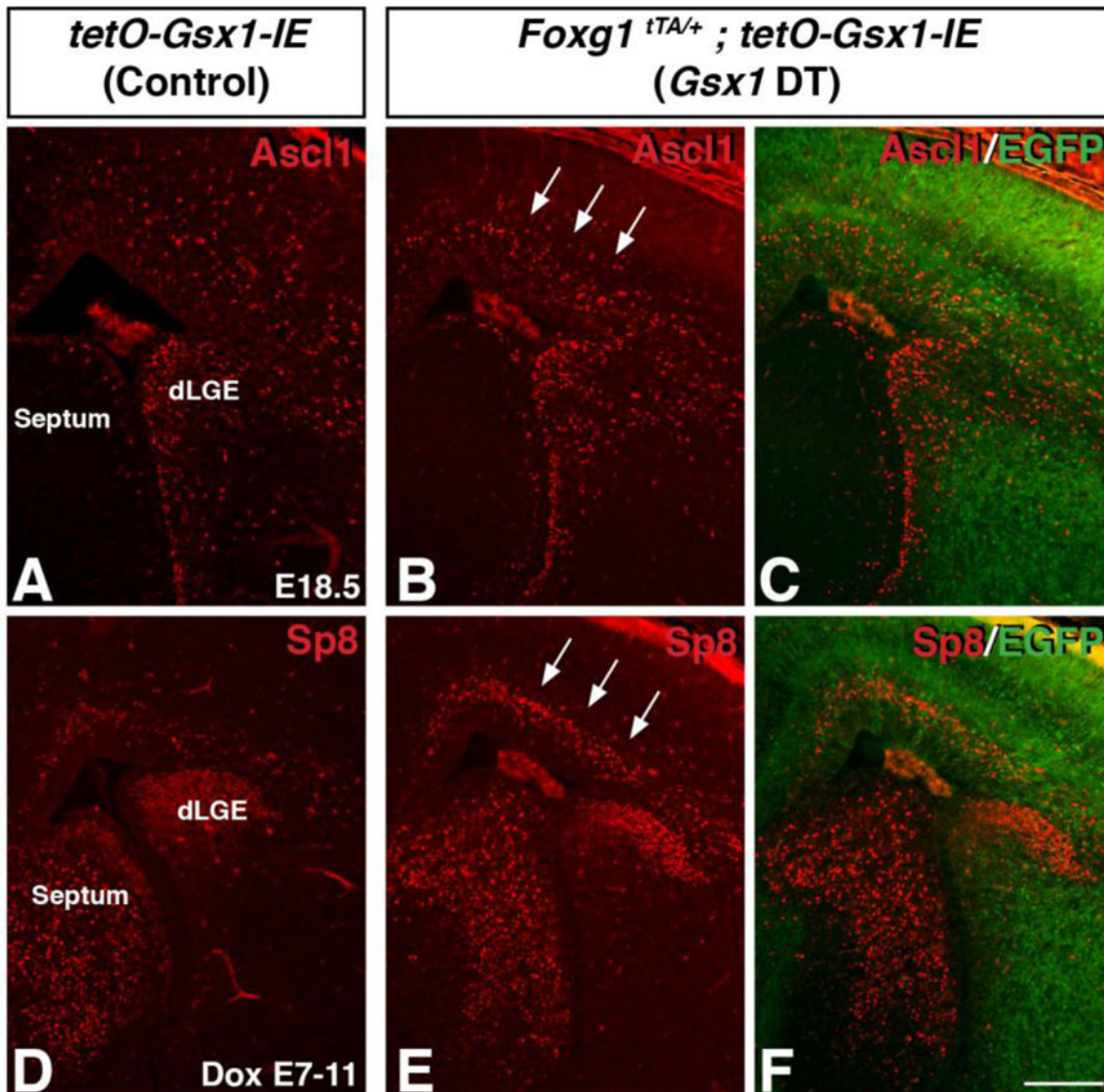


Fig. 5. Over-expression of *Gsx1* from E15 on results in specification of VZ progenitors toward a dLGE fate.

Misexpression of *Gsx1* from E15 onward promotes dLGE neurogenesis, as indicated by the ectopic upregulation of *Ascl1* (**B,C**, arrows) as well as the dLGE marker *Sp8* (**E,F**, arrows) throughout the SVZ of the dorsal telencephalon, as compared to control embryos (**A, D**).

Scale bar: **F** = 200 μ M

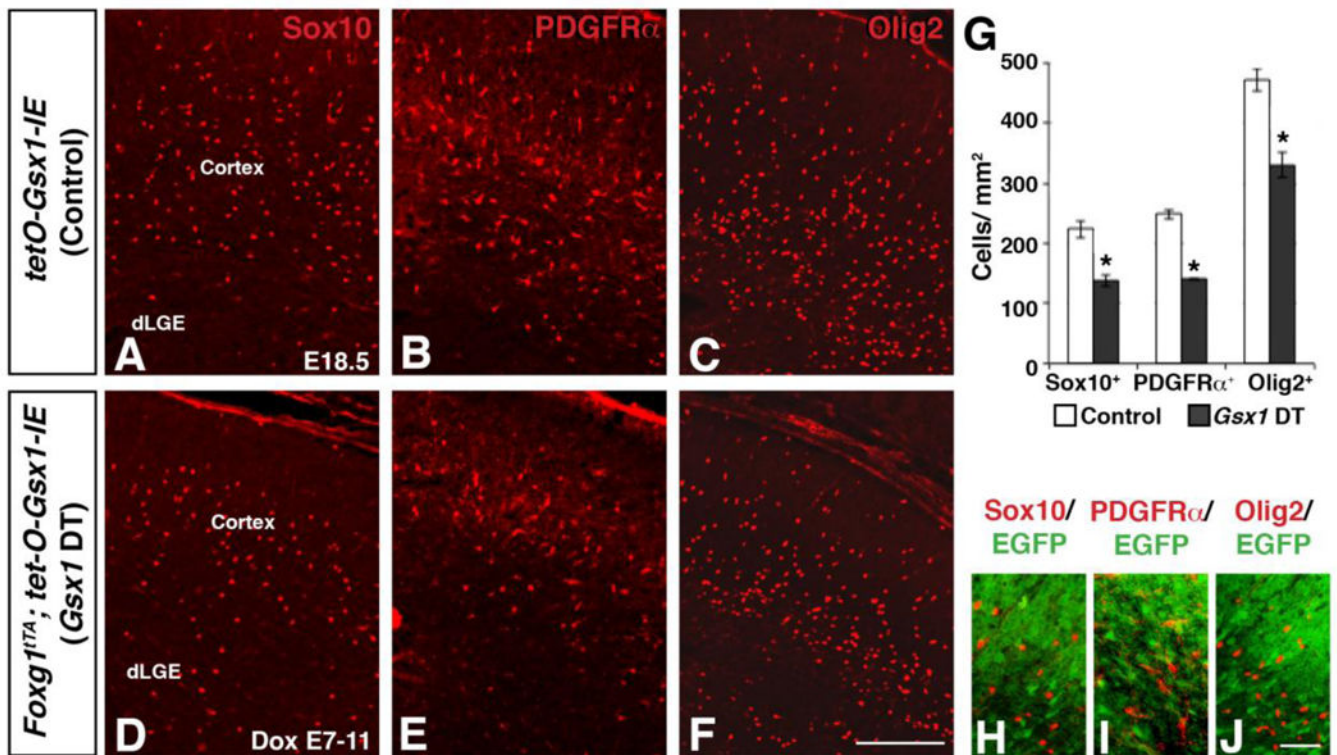


Fig. 6. *Gsx1* is sufficient to repress the specification of OPCs within telencephalic VZ progenitors. The over-expression of *Gsx1* in telencephalic progenitors results in severely reduced Sox10 (control 223.5 ± 14.4 ; DT 137.2 ± 9.8 cells/mm²), PDGFRα (control 248.3 ± 7.9 ; DT 140.6 ± 1.9 cells/mm²), and Olig2 (control 471.8 ± 19.0 ; DT 330.8 ± 21.6 cells/mm²) OPCs within the cortex (**D-F**) as compared to control embryos (**A-C**; quantification in **G**). Immunostaining for EGFP in combination with OPC markers (**H-J**), revealed little to no co-expression, suggesting that *Gsx1* is sufficient to inhibit OPC specification. Data in **G** represent the mean \pm SEM. * $p < 0.005$, significance determined by Student's t-test. Scale bar: **F** = 100 μ M, **J** = 50 μ M

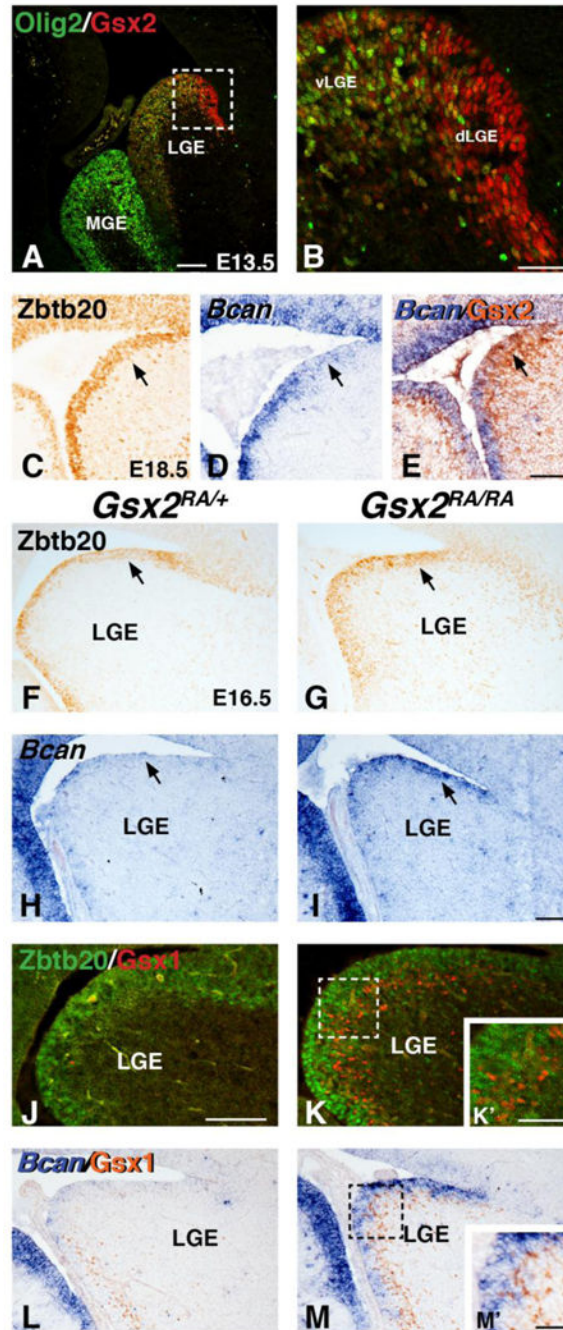


Fig. 7. Candidate glial progenitor genes, *Zbtb20* and *Bcan* are increased in VZ progenitors of *Gsx2* mutants.

Immunostaining for *Olig2* and *Gsx2* reveals opposing patterns of expression in the E13 LGE (A-B). Dashed box in A depicts the high magnification view of the LGE in B. *Gsx2* positive cells in the dorsal LGE region (dLGE) are largely *Olig2* negative, whereas double positive cells are throughout the ventral LGE (vLGE). Immunostaining for *Zbtb20* (C) and *in situ* staining for *Bcan* (D) show weak expression in the dLGE at E18.5 (arrows in C-D). Double labeling with *in situ* for *Bcan* and IHC for *Gsx2* show the dLGE is high for *Gsx2* and weak

for *Bcan* (arrow in **E**). *Gsx2* mutants display increased *Zbtb20* (compare arrows in **G** to **F**) and *Bcan* (compare arrows in **I** to **H**) expression in the dLGE region at E16.5. Immunostaining for *Zbtb20* and *Gsx1* show the increased *Zbtb20* is distinct from the expanded *Gsx1* positive cells in VZ progenitors of *Gsx2* mutants (compare **K** to **J**, see also inset **K'**). Double labeling with in situ for *Bcan* and IHC for *Gsx1* (**L**) also shows little overlap in the *Gsx2* mutant LGE progenitors (**M** and **M'**). Scale bar: **A** = 200 μ M, **B**, **K**, **M'** = 50 μ M, **E** 100 μ M for C-E, **I** = 100 μ M for F- I,L-M, **J** = 100 μ M for J-K

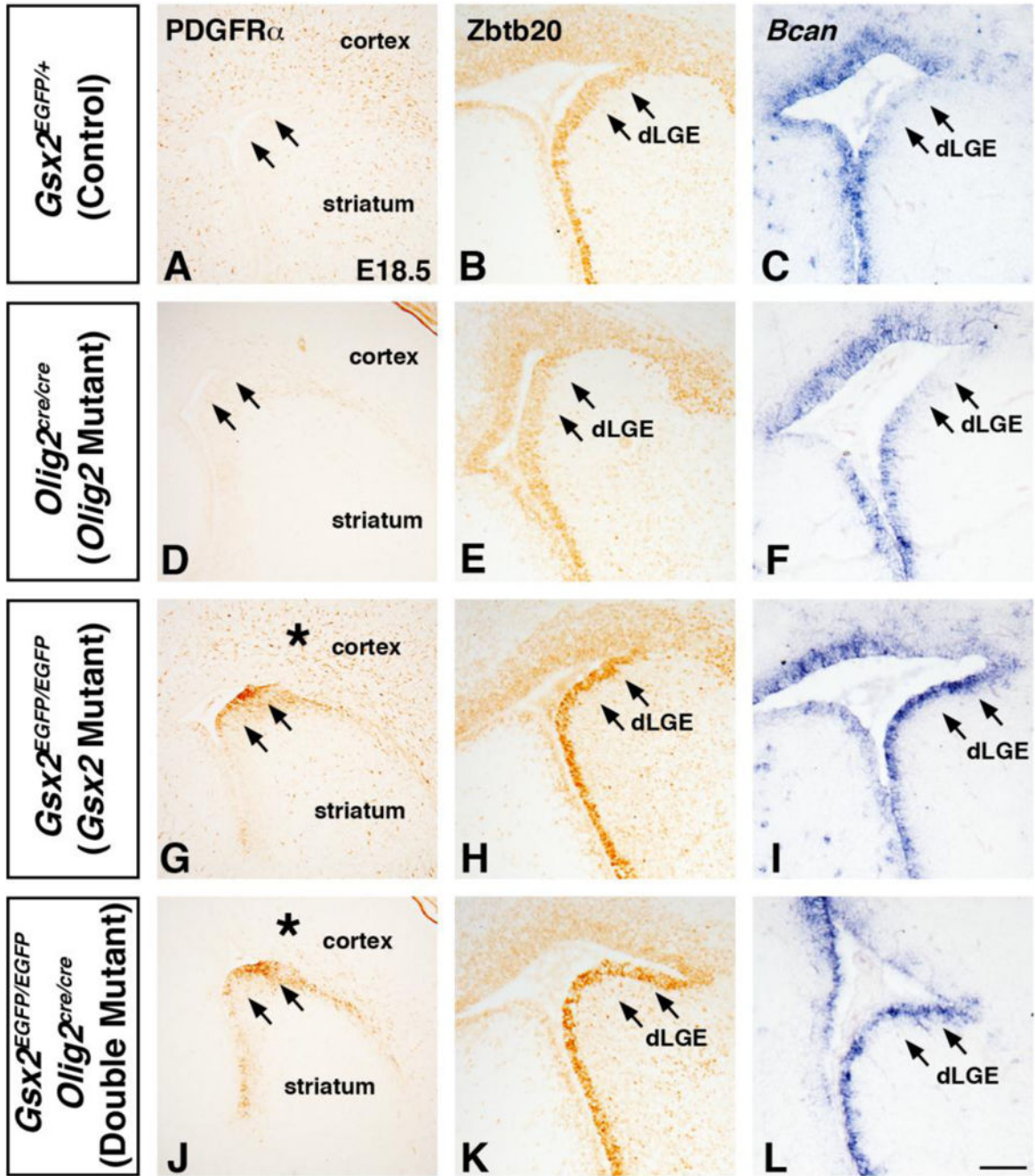


Fig. 8. *Gsx2*;*Olig2* double mutants display expanded glial progenitor marker in the dLGE region. At E18.5, *Gsx2* mutants show increased PDGFR α positive cells in the cortex and LGE (compare **G** to **A**). *Gsx2*;*Olig2* double mutants show a loss of PDGFR α expression in the cortex (compare asterisk in **J** to **G**) which is similar to *Olig2* mutants (compare **J** to **D**). However, PDGFR α expression remains expanded in the LGE of *Gsx2*;*Olig2* double mutants similar to *Gsx2* mutants (compare arrows in **J** to **G**). Both *Zbtb20* and *Bean* remain increased in the dLGE of *Gsx2*;*Olig2* double mutants similar to *Gsx2* mutants (compare arrows in **K,L** to **H,I**). *Olig2* mutants do not show changes in the LGE expression gradient

(low in dLGE) of *Zbtb20* (compare **E** to **B**) or *Bcan* (compare **F** to **C**). Scale bar: **L** = 100 μ M.

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