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Distinct Temporal Requirements for the Homeobox Gene *Gsx2* in Specifying Striatal and Olfactory Bulb Neuronal Fates

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SUMMARY

The homeobox gene *Gsx2* (formerly *Gsh2*) is known to be required for striatal and olfactory bulb neurogenesis; however, its specific role in the specification of these two neuronal subtypes remains unclear. To address this, we have employed a temporally regulated gain-of-function approach in transgenic mice and found that misexpression of *Gsx2* at early stages of telencephalic neurogenesis favors the specification of striatal projection neuron identity over that of olfactory bulb interneurons. In contrast, delayed activation of the *Gsx2* transgene until later stages exclusively promotes olfactory bulb interneuron identity. In a complementary approach, we have conditionally inactivated *Gsx2* in a temporally progressive manner. Unlike germline *Gsx2* mutants, which exhibit severe alterations in both striatal and olfactory bulb neurogenesis at birth, the conditional mutants exhibited defects restricted to olfactory bulb interneurons. These results demonstrate that *Gsx2* specifies striatal projection neuron and olfactory bulb interneuron identity at distinct time points during telencephalic neurogenesis.

INTRODUCTION

The telencephalon represents the largest and most complex region of the mammalian brain. This region is charged with the task of complex neural processing that controls all cognitive processes and purposeful actions. Accordingly, the telencephalon exhibits the greatest amount of neuronal diversity of any portion of the CNS. Previously, many groups have focused on the generation of neuronal diversity within the telencephalon (reviewed in Marin and Rubenstein, 2003). While neuronal progenitors in the dorsal telencephalon (also termed the pallium) are thought to give rise to the excitatory cortical projection neurons, the vast majority of cortical interneurons originate from progenitor domains located in the ventral telencephalon. Although limited pallial contributions to ventral telencephalic neuronal subtypes have recently been proposed (Willaime-Morawek et al., 2006; Kohwi et al., 2007; Young et al., 2007; Willaime-Morawek and van der Kooy, 2008), most of the neuronal diversity found in the mature telencephalon appears to derive from progenitor cells positioned in the ventral telencephalon during embryogenesis (Rallu et al., 2002; Campbell, 2003; Marin and Rubenstein, 2003).

The lateral ganglionic eminence (LGE) represents one such ventral telencephalic progenitor region, which has been shown to generate the projection neurons of the striatum as well as interneurons in the olfactory bulb (Deacon et al., 1994; Olsson et al., 1995, 1998; Wichterle et al., 2001). Despite the fact that both striatal projection neurons and olfactory bulb interneurons derive from the LGE, they exhibit different temporal profiles of neurogenesis in the rodent; the striatal neurons are generated almost exclusively at embryonic time points, whereas the olfactory bulb interneurons begin their genesis at later embryonic time points and continue into the early postnatal period, when the vast majority are born (Hinds, 1968; Bayer and Altman, 2004; Batista-Brito et al., 2008). Recent studies have suggested that these two neuronal subtypes derive from separate progenitors located in distinct regions within the LGE, termed the ventral (v)LGE and dorsal (d)LGE, respectively (Yun et al., 2001, 2003; Stenman et al., 2003a; Waclaw et al., 2006). Yun et al. (2001) first described these two LGE subdivisions based on gene expression patterns at midgestation stages. The dLGE was characterized by high levels of *Gsx2* and *Er81* in progenitors of the ventricular zone (VZ), while the vLGE lacks *Er81* expression and displays lower levels of *Gsx2*. These compartments can also be identified in the subventricular zone (SVZ) and mantle regions of the LGE. Islet1 (Isl1) is expressed in the vLGE SVZ and transiently in its striatal projection neuron derivatives whereas Er81 and Sp8 mark the dLGE SVZ and remain expressed in distinct subtypes of olfactory bulb interneurons (Stenman et al., 2003a; Waclaw et al., 2006; Allen et al., 2007; Saino-Saito et al., 2007). These two LGE progenitor domains are bordered dorsally by the ventral pallium (marked by Dbx1) and ventrally by the interganglionic sulcus, which is marked by Nkx6.2 expression (Stenman et al., 2003b). *Dbx1*- and *Nkx6.2*-expressing progenitors have been shown to

contribute to amygdalar projection neurons and cortical interneurons, respectively (Hirata et al., 2009; Sousa et al., 2009).

Correct patterning of the vLGE and dLGE requires *Gsx2* and *Pax6* gene function. The loss of the pallial regulator *Pax6* results in a dorsal expansion of dLGE markers (Toresson et al., 2000; Stoykova et al., 2000; Yun et al., 2001; Stenman et al., 2003a; Kroll and O'Leary, 2005; Waclaw et al., 2006), suggesting a role for the paired homeodomain factor in repressing dLGE identity within pallial progenitors. In the absence of *Gsx2*, the vLGE and dLGE as well as their derivatives, the striatal projection neurons and olfactory bulb interneurons, are severely reduced (Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001, 2003; Stenman et al., 2003a; Waclaw et al., 2004, 2006). The specific role of *Gsx2* in patterning and specification of the vLGE and dLGE, however, remains somewhat unclear. So far, no evidence has been provided to support a role for Gsx2 in directly ventralizing telencephalic progenitors (Corbin et al., 2000). Loss-of-function studies, however, suggested that Gsx2 indirectly controls LGE specification by repressing the expression of dorsal telencephalic regulators such as *Pax6* in LGE progenitors (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001). This appears to be a conserved function of Gsx2 since the *Drosophila* homolog Ind (intermediate neuroblasts defective) has also been shown to repress *eyeless*, the *Pax6* homolog, in fly CNS development (Von Ohlen et al., 2007).

We have reexamined the role of Gsx2 in LGE specification using conditional gain-of-function and loss-of-function approaches in mice. These models afforded the analysis of temporally distinct roles for Gsx2 in the specification of vLGE and dLGE. Our results demonstrate that Gsx2 can directly ventralize pallial progenitors and, depending on the developmental stage, specifies different neuronal fates. In particular, at early stages of telencephalic development, Gsx2 is necessary and sufficient to correctly specify the vLGE and its major derivatives, the striatal projection neurons; however, at later stages, high levels of Gsx2 specify LGE progenitors toward dLGE fates including olfactory bulb interneurons.

RESULTS

Dynamic Expression of Gsx2 in LGE Progenitors

The homeobox gene *Gsx2* is first expressed in progenitors of the presumptive LGE between embryonic days (E)9 and E10 (Toresson et al., 2000; Corbin et al., 2003). Initially, this factor is expressed at high levels in cells throughout the presumptive LGE, with an apparent ventral-to-dorsal gradient in Gsx2-positive cell numbers (Figure 1A). Gsx2-positive cells are also scattered somewhat uniformly throughout the adjacent medial ganglionic eminence (MGE), as marked by Nkx2.1 expression (Figure 1A). To detect Isl1, we used an Isl1/2 antibody (Tsuchida et al., 1994). Although Isl1-expressing cells are found in ventral telencephalon throughout development, the closely related Isl2 is not expressed in this brain region (Wang and Liu, 2001). At early stages (e.g., E11), Isl1 cells appear to emerge from a broad portion of the presumptive LGE (Figure 1B); however, Sp8 is seen in scattered cells adjacent to the dorsal-most portion of the LGE (Figure 1B). At later embryonic stages (e.g., E12.5 onward), Gsx2 exhibits a clear graded pattern of expression, with low levels found in cells located ventrally and the highest levels confined to those in the most dorsal portion of the LGE (Figures 1C and 1H). Previously, Yun et al. (2001) used this high level of *Gsx2* along with other markers to define the dLGE. Indeed, Sp8-positive cells that mark the dLGE SVZ adjoin the high-level Gsx2-expressing cells in the VZ (Figure 1D), while the Isl1 (vLGE) SVZ cells are positioned adjacent to the low-level Gsx2-expressing cells of the VZ (Figure 1E). This dynamic expression pattern might suggest that high levels of Gsx2, at early stages of forebrain development (i.e., E9–E11), are capable of specifying both vLGE and dLGE identity with a predominance for vLGE and thus striatal projection neuron fate. At later stages (i.e., E12 onward), however, high levels of Gsx2 in LGE progenitors may primarily promote dLGE fates, including olfactory bulb interneurons.

Conditional Misexpression of *Gsx2*

In order to test this temporal specification model, we developed a doxycyclin (Dox)-regulated binary transgenic system for the spatially and temporally controlled misexpression of Gsx2 in the embryonic mouse telencephalon (Figure 1F). We generated *tetO-Gsx2-IRES-EGFP (IE)* mice to use with *Foxg1*$^{tTA/+}$ mice (Hanashima et al., 2002) to drive expression of Gsx2 in the embryonic telencephalon. *Foxg1*$^{tTA/+}$*;tetO-Gsx2-IE* embryos expressed EGFP throughout the telencephalon (Figure 1G) and this expression was detected as early as E9.5 (data not shown). In addition to EGFP, Gsx2 was also expressed in telencephalic progenitors rather uniformly both in the ventral and dorsal telencephalon (Figures 1I and 1J). Multiple lines of *tetO-Gsx2-IE* mice were identified that were responsive to *Foxg1*$^{tTA/+}$, with broad telencephalic expression of the transgenes. While most of these lines responded robustly to tTA, the *tetO-Gsx2-IE* line, which showed the most reliable and robust response with no leaky expression of the transgenes, was used in the present study. All controls shown are *tetO-Gsx2-IE* single transgenic embryos; however, the *Foxg1*$^{tTA/+}$ single transgenic embryos were identical with respect to the markers examined despite the fact that they only have one functional allele of *Foxg1* (data not shown).

As mentioned above, EGFP could be detected as early as E9.5 in the *Foxg1*$^{tTA/+}$*;tetO-Gsx2-IE* embryos, and thus these animals represented an early misexpression of Gsx2 throughout the telencephalon. To determine whether ectopic Gsx2 could alter dorsal-ventral patterning, we examined the expression of the ventral telencephalic regulators Ascl1 (Mash1) and Dlx proteins at E12.5 (Anderson et al., 1997; Casarosa et al., 1999; Horton et al., 1999). Both Ascl1 (Figure 2B) and Dlx proteins (Figure 2E) were found ectopically expressed (as marked by EGFP) within the dorsal telencephalon (Figures 2C and 2F). Ascl1 was upregulated in the VZ of the pallium while the Dlx proteins were most highly expressed in the SVZ of the pallium, similar to their patterns of expression in the ventral telencephalon (Figures 2A and 2D). In contrast, the pallial regulators Pax6 (Figures 2H and 2I) and Tbr1 (Figure 2K and 2L) were both reduced in the dorsal telencephalon as compared to levels in the control embryos (Figures 2G and 2J). All *Foxg1*$^{tTA/+}$*;tetO-Gsx2-IE* embryos examined showed similar expression patterns of Ascl1, Dlx, Pax6, and Tbr1 proteins, even at later stages (i.e.,

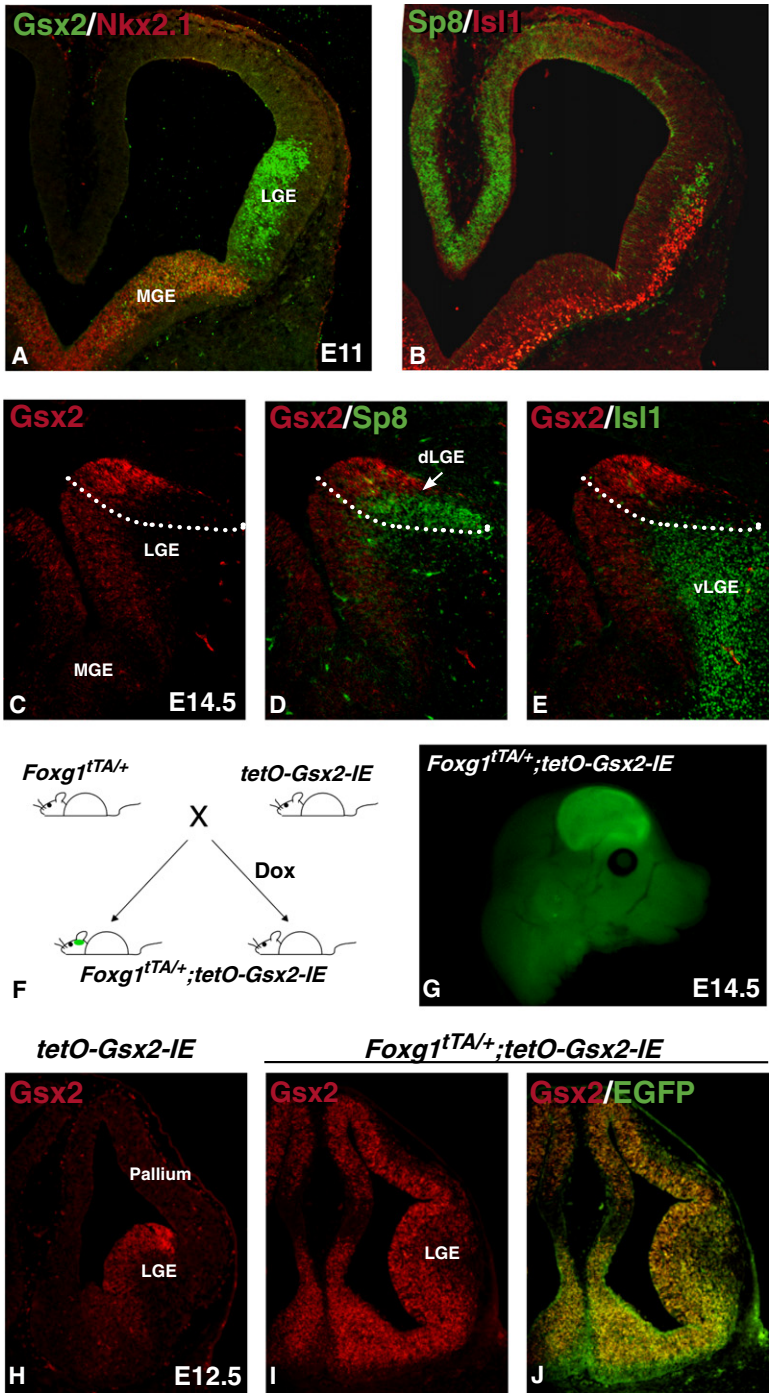

Figure 1. Dynamic Expression of Gsx2 during Development of the LGE

(A) Gsx2 (green) and Nkx2.1 (red) expression in E11 coronal section of the telencephalon. (B) Overlay of adjacent E11 coronal sections stained for Isl1 (red) and Sp8 (green). (C) Gsx2 expression in E14.5 LGE showing a ventral-low to dorsal-high gradient with dLGE and vLGE separated by dotted line (C–E). (D) Overlay of adjacent sections stained with Gsx2 (red) and Sp8 (green). Sp8 cells are adjoining the high Gsx2 domain (i.e., dLGE). (E) Overlay of adjacent sections stained with Gsx2 (red) and Isl1 (green). Isl1-positive cells are neighboring the low Gsx2 domain (i.e., vLGE). (F) Breeding strategy to express Gsx2 throughout the telencephalon. (G) Double transgenic embryo (*Foxg1$^{tTA/+}$;tetO-Gsx2-IE*) expressing EGFP throughout the E14 telencephalon. (H) Gsx2 expression in control (*tetO-Gsx2-IE*) embryo at E12.5. (I) Gsx2 is expressed throughout the telencephalon in an E12.5 double transgenic embryo. (J) Merged image of Gsx2 and EGFP in double transgenic embryo.

E14.5 and E15.5); however, analysis of the double transgenic brains after E15.5 was not performed as the morphology became very disrupted (Figure S1 available online). We never observed ectopic expression of the MGE marker Nkx2.1 and, in fact, found that it was reduced or missing in all the double transgenic embryos examined (Figure S1H). These findings therefore suggest that early misexpression of Gsx2 specifically promotes LGE identity within pallial progenitors.

To study the role of Gsx2 in the specification of the vLGE versus the dLGE, we examined Isl1 and Sp8 expression, respectively. Interestingly, the *Foxg1$^{tTA/+}$;tet-O-Gsx2-IE* embryos were found to express Isl1 throughout the dorsal-ventral aspect of the telencephalon both at E14.5 (Figure 2N) and at E15.5 (Figure S1F). Conversely, only scattered Sp8 cells were detected in the pallium at either stage (Figures 2P and S1G). In all cases, the normal dLGE expression domain of Sp8 in the ventral telencephalon of double transgenic embryos was severely reduced. Taken together with the findings above, our data indicate that early (i.e., from E9.5 onward) misexpression of Gsx2 favors vLGE specification within the majority of telencephalic progenitors.

The widespread overexpression of Gsx2 (and EGFP) observed in the *Foxg1$^{tTA/+}$;tet-O-Gsx2-IE* embryos shown in Figures 1 and 2 does not allow us to conclude whether Gsx2 regulates telencephalic gene expression in a cell autonomous manner or if it induces extrinsic factors, which subsequently control the patterns of gene expression in adjacent progenitors. In this respect, on very rare occasions, we obtained double transgenic embryos that displayed mosaic transgene expression (Figures S2A and S2B). EGFP and Gsx2 were expressed in what appeared as radial clones, and specifically within these clones Pax6 was repressed (Figures S2E and S2F) while Ascl1 and Dlx were cell-autonomously induced (Figures S2C and S2D). As was the case with the uniformly overexpressing embryos (see Figure 2), the mosaic embryos also showed a preferential induction of Isl1 over Sp8 in the EGFP-positive cells (Figures S2G and S2H), indicating that early misexpression of Gsx2 favors vLGE specification within telencephalic progenitors in a cell autonomous manner.

To examine a later role for Gsx2 in the specification of vLGE and dLGE, we made use of the fact that Dox can repress

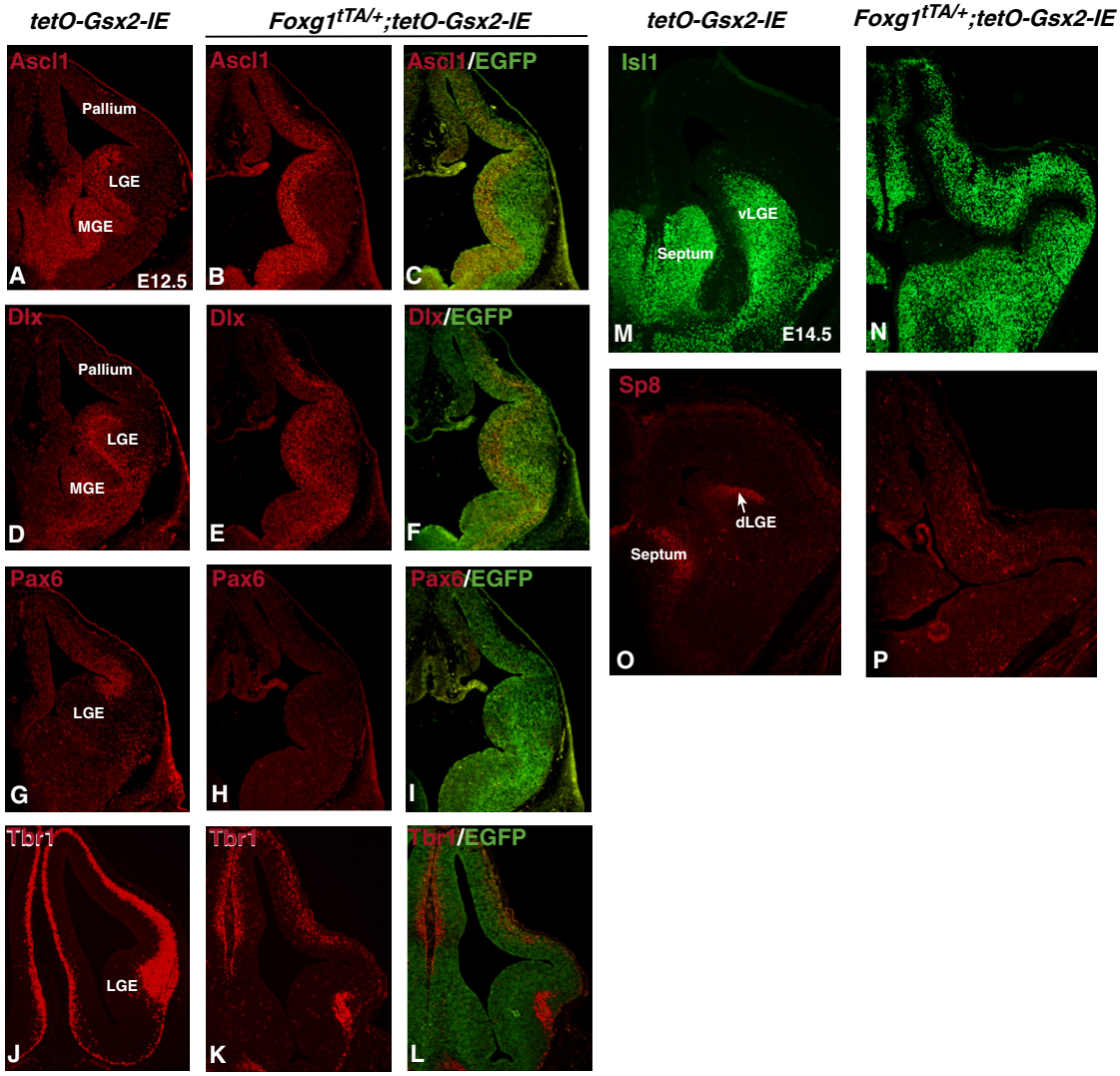

Figure 2. Misexpression of *Gsx2* Results in Increased Expression of Ventral Telencephalic Markers throughout the Pallium

(A–L) Coronal sections through the telencephalon of E12.5 control (*tetO-Gsx2-IE*) (A, D, G, and J) and double transgenic (*Foxg1$^{tTA/+}$;tetO-Gsx2-IE*) (B, C, E, F, H, I, K, and L) embryos. The ventral markers Ascl1 and Dlx proteins are induced in EGFP-positive cells in the pallium of double transgenic embryos (compare B and C to A and E and F to D). The pallial markers Pax6 and Tbr1 are severely reduced in double transgenic embryos (H and K) compared to control embryos (G and J). Note the EGFP transgene is highly expressed in Pax6- and Tbr1-negative regions (I and L).

(M–P) Coronal sections of E14.5 control (M and O) and double transgenic (N and P) embryos. The vLGE marker Isl1 is ectopically expressed throughout the telencephalon of double transgenic embryos (compare N to M), while the dLGE marker Sp8 is only observed in scattered cells within the pallium (P) and is reduced in the normal expression domain of the dLGE.

transgene expression in the *Foxg1$^{tTA/+}$;tetO-Gsx2-IE* embryos (Figure 1F). By adding Dox (0.02 mg/ml) in the drinking water of the pregnant dams from E7 to E9, we found that transgene expression was repressed until around E12.5 (Figure 3F), and not until E13.5 was the transgene expressed throughout the telencephalon (Figure 3I), in a manner similar to endogenous *Foxg1*. As mentioned above, when no Dox is administered the transgene is first detected at E9.5, with robust expression between E10.5 and E13.5 (Figures 3B, 3E, and 3H). The *tetO-Gsx2-IE* embryos served as controls for these studies and do not show ectopic expression at any stage examined (Figures 3A, 3D, and 3G). At E13.5, the level of Gsx2 and EGFP expression is considerably lower in the Dox-treated embryos (Figures 3I, 3L, and 3M) than in the double transgenic embryos that were not administered Dox (Figures 3H, 3J, and 3K). By E15.5, however, the Gsx2 transgene appears to be expressed at similar levels to that in non-Dox-treated embryos (Figures S1E and S1I). A dramatic improvement in the morphology of brains was observed at later stages in the Dox-treated double transgenic embryos (Figures 4 and S1I–S1L) as compared to the untreated double transgenic embryos (Figures 2N and 2P and Figures S1E–S1H).

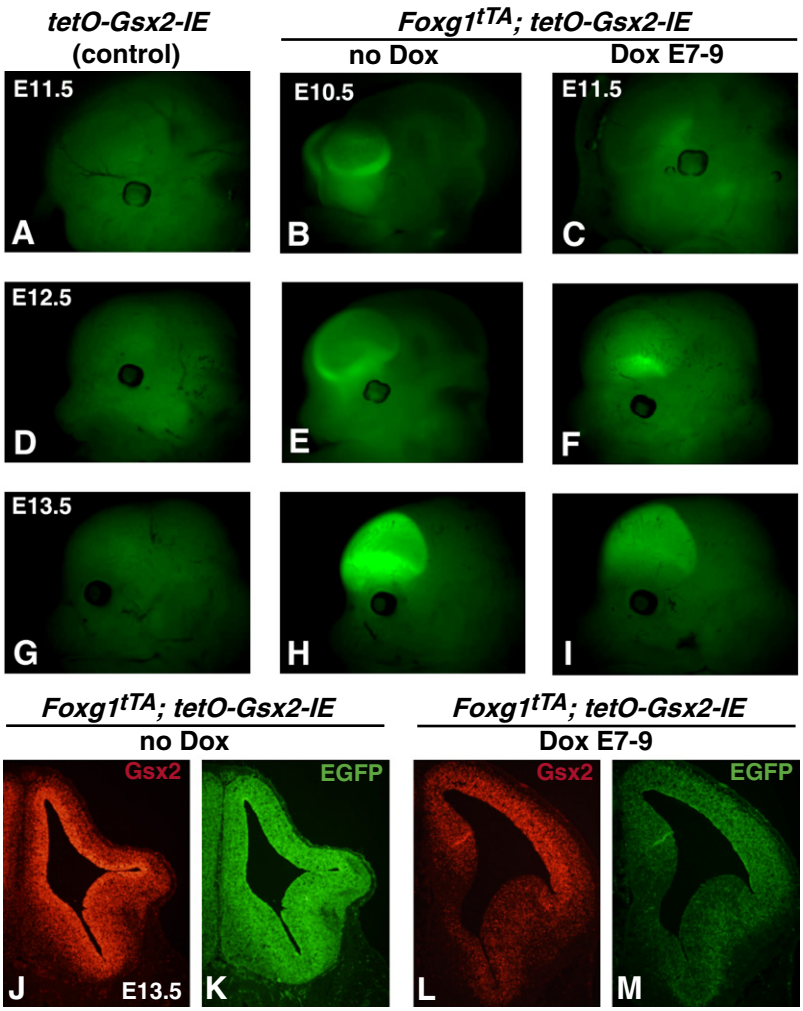

Figure 3. Temporal Control of *Gsx2* Transgene Expression using Doxycycline

(A–I) EGFP expression in the heads of control (*tetO-Gsx2-IE*) (A, D, and G) and double transgenic (*Foxg1$^{tTA/+}$;tetO-Gsx2-IE*) embryos that either did not receive Dox (B, E, and H) or were treated with Dox from E7–E9 (C, F, and I). Double transgenic embryos that were not treated with Dox exhibit EGFP expression in the telencephalon at E10.5 (B), E12.5 (E), and E13.5 (H). Those treated with Dox from E7–E9 exhibit a delay in transgene activation as observed by minimal EGFP expression detected at E11.5 (C) and a progressive increase in EGFP expression at E12.5 (F) and E13.5 (I). Control embryos do not express EGFP and are used for comparison as background fluorescence levels (A, D, and G).

(J–M) Representative coronal sections of double transgenic embryos at E13.5 with no Dox treatment (J and K) and Dox treatment from E7–E9 (L and M). Images were taken at the same exposure. Note that Gsx2 and EGFP are expressed much more highly in the non-Dox-treated embryos (J and K) as compared to the Dox-treated embryos (L and M).

Concomitant with the induction of Gsx2 and EGFP around E13.5 in the Dox-treated *Foxg1$^{tTA/+}$;tetO-Gsx2-IE* embryos, we observed an upregulation of Ascl1 and Dlx proteins in the pallium (data not shown). Tbr1 expression in the Dox-treated double transgenics at E13.5 was only minimally reduced from the control embryos (Figures 4A and 4F), unlike the case in non-Dox-treated embryos (i.e., early misexpression of Gsx2) (Figures 2J and 2K). At E15.5, the domain of Tbr1 expression in Dox-treated embryos is not increased from that seen 2 days earlier (compare Figure 4G with Figure 4F), suggesting that the production of these cells ceases subsequent to Gsx2 misexpression. In fact, many breaks in the Tbr1 expression domain were evident in the *Foxg1$^{tTA/+}$;tetO-Gsx2-IE* embryos and in each case, these Tbr1-negative areas were found to express the EGFP and Gsx2 transgenes at high levels (see inset in Figure 4G). Again, the areas misexpressing Gsx2 and EGFP were found to be Ascl1 and Dlx expressing (data not shown). As was the case in the early misexpression embryos, no ectopic Nkx2.1 was detected. However, in the Dox-treated double transgenic (i.e., delayed misexpression) embryos, much more Nkx2.1 expression remained in the MGE region as compared to their early-misexpression (non-Dox-treated) counterparts (Figure S1L). Thus it appears that even when Gsx2 is expressed in progenitors of the pallium from E13.5, it is capable of respecifying them toward LGE fates.

To determine if the delayed misexpression of Gsx2 differentially regulates the specification of the vLGE versus the dLGE, we examined the expression of Isl1 and Sp8, respectively. At E15.5, Dox-treated embryos showed a dramatic reduction in the domain of Isl1 expression (Figure S1J) and concomitant expansion both ventrally and dorsally of *Sp8* expression (Figure S1K). This was opposite to the trend observed in the double transgenic embryos that were not Dox treated (i.e., early misexpression; Figures S1F and S1G), despite the fact that similar levels of Gsx2 expression were observed in both Dox-treated and non-Dox-treated embryos (Figures S1E and S1I). As mentioned above, the Dox-treated *Foxg1$^{tTA/+}$;tetO-Gsx2-IE* embryos exhibit improved morphology at later stages, and we were thus able to examine vLGE and dLGE development at E18.5. By this stage, Isl1 marks the major vLGE derivative, i.e., the forming striatal complex (Figure 4C). In the Dox-treated double transgenics, Isl1 expression and accordingly the size of the forming striatum is greatly reduced (Figure 4H). Conversely, Sp8 expression is upregulated throughout the developing cerebral cortex, suggestive of dLGE respecification (Figures 4I and 4J). Interestingly, as seen at E15.5 in delayed misexpression embryos, Sp8 expression is expanded ventrally in the LGE also at E18.5. This could indicate that vLGE cells have been respecified to a dLGE identity by the delayed misexpression of Gsx2; however, it is possible that selective proliferation of dLGE progenitors accounts for the enlarged Sp8 expression domain. The *Foxg1$^{tTA/+}$;tetO-Gsx2-IE* brains (with or without Dox treatment) do not form normal olfactory bulbs and therefore it is difficult to fully assess olfactory bulb interneuron development in these animals. We can conclude, however, that the specification of

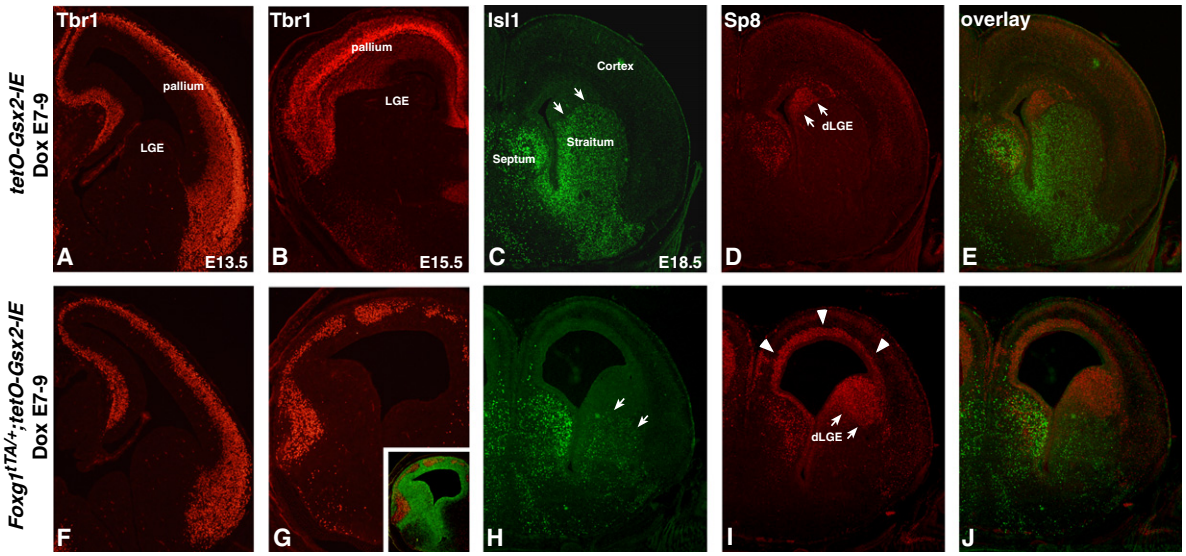

Figure 4. Delayed Expression of the *Gsx2* Transgene Results in an Expansion of the dLGE

Coronal sections of control (*tetO-Gsx2-IE*) (A–E) and double transgenic (*Foxg1$^{tTA/+}$;tetO-Gsx2-IE*) (F–J) embryos treated with Dox from E7–E9. At E13.5, Tbr1 expression in double transgenic embryos is similar (albeit slightly reduced) to that of control embryos (compare F to A). By E15.5, however, Tbr1 expression in double transgenic embryos is greatly reduced as compared to that in control embryos (compare G to B). Inset in (G) shows a merged image of Tbr1 expression with complementary expression of EGFP from the transgene. Isl1 expression is severely reduced in the ventral telencephalon (i.e., forming striatum) and is not expressed in the pallium of double transgenic embryos at E18.5 (compare H to C). Conversely, Sp8 expression is increased throughout the pallium (arrowheads in I) and even expanded ventrally in the LGE (arrows in I) of double transgenic embryos, as compared to control embryos (D). Overlays of adjacent sections clearly show the expansion of Sp8 (i.e., dLGE) into the normal Isl1 (i.e., vLGE) expression domain of the LGE (compare J to E).

these dLGE derivatives is exclusively promoted by delayed misexpression of Gsx2. In summary, early misexpression (i.e., E9–E10) of Gsx2 appears to favor vLGE specification while later misexpression (i.e., E13 and onward) promotes dLGE specification, apparently at the expense of the vLGE.

Conditional Mutagenesis of *Gsx2*

To complement the gain-of-function experiments described above, we have taken a conditional loss-of-function approach to study the temporal role of *Gsx2* in the specification of LGE fates. We generated a conditional mutant allele (*Gsx2^{flox}*) by engineering *loxP* sites flanking exon 2, which includes the entire homeodomain (Figures 5A and S3). *Gsx2$^{flox/flox}$* mice appear normal and express wild-type levels of Gsx2 protein (data not shown), suggesting the floxed allele acts in a manner similar to that of the wild-type *Gsx2* allele. To determine if our recombined conditional allele produced a loss-of-function mutation of Gsx2, we crossed *Gsx2$^{flox/+}$* mice with mice carrying an *EIIA-cre* transgene (Lakso et al., 1996), which recombines in the germline, to generate mice carrying a globally recombined allele (RA) of *Gsx2* (*Gsx2$^{RA/+}$*). *Gsx2$^{RA/RA}$* mutant embryos do not express Gsx2 protein (Figure S3D). In addition, *Gsx2$^{RA/RA}$* mutant embryos displayed a phenotype in the telencephalon (data not shown) identical to that of the previously described mutation of *Gsx2* (Szucsik et al., 1997; Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001).

To conditionally inactivate *Gsx2*, we crossed *Gsx2$^{flox/flox}$* mice with mice that were double heterozygous for the floxed allele (*Gsx2$^{flox/+}$*) and a cre knockin to the *Emx1* locus (*Emx1$^{cre/+}$*) (Gorski et al., 2002). The *Emx1^{cre}* mice are widely used to recombine floxed genes in the dorsal telencephalon; however, these mice also induce recombination in scattered cells of the LGE (see Figure 1F in Gorski et al., 2002). Our initial plan was to use the *Emx1$^{cre/+}$* mice to produce a mosaic inactivation of *Gsx2* in the LGE in order to study cell autonomous versus non-cell-autonomous requirements as a complement to our mosaic misexpression (Figure S2). Indeed, recombination of the ROSA26 reporter (R) (Mao et al., 1999) was detected as early as E10.5 in the presumptive LGE (insets in Figures 5B and 5C), and at that stage Gsx2 showed a slight mosaic recombination pattern in the conditional mutants (*Emx1$^{cre/+}$;Gsx2$^{flox/flox}$;R26R*) (compare Figures 5C to 5B). A clear mosaic inactivation of Gsx2 within the LGE was evident by E12.5 when nearly half of all Gsx2 staining was lost (compare Figures 5E to 5D, see also S4). Surprisingly, however, by E18.5, nearly 80% (430.3 ± 6.7 versus 96.3 ± 3.9 cells/section, $p < 0.001$; $n = 3$) of Gsx2-expressing cells were lost in the LGE of conditional mutants (Figure 5I) compared to controls (Figure 5H). This finding is quite significant because *Emx1$^{cre/+}$* mice have been largely characterized as pallial specific for cre recombination (Gorski et al., 2002; Willaime-Morawek et al., 2006; Willaime-Morawek and van der Kooy, 2008; Kohwi et al., 2007). Our data provide clear evidence that *Emx1^{cre}* induces recombination of the *Gsx2* floxed allele in the LGE beginning around E10.5. In addition, the recombination is progressively more severe in the LGE later in development, resulting in an extensive loss of Gsx2 protein expression at perinatal stages.

Germline *Gsx2* mutants display abnormalities in dorsal-ventral patterning of the telencephalon as observed by a ventral expansion of pallial regulators into the LGE and a concomitant

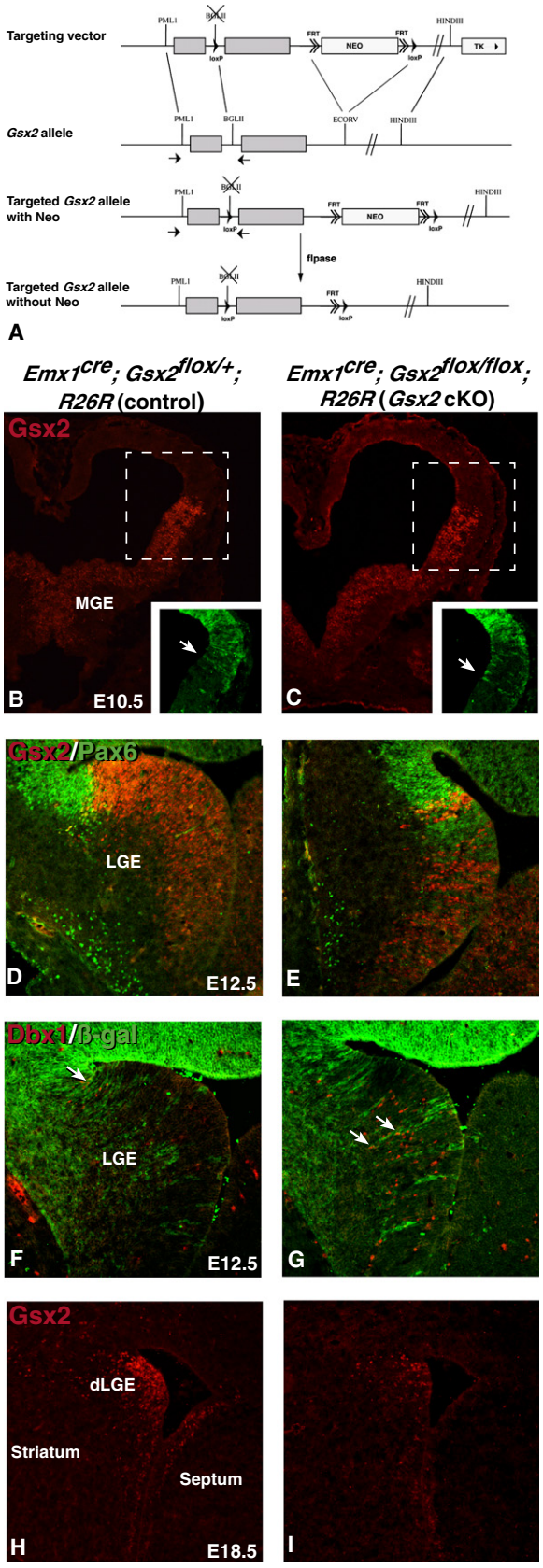

Figure 5. Generation of a Conditional Allele of *Gsx2* and Temporal Deletion using *Emx1*$^{cre/+}$ Mice

(A) Diagram of gene targeting scheme to generate a conditional *Gsx2* allele. (B–I) Representative coronal sections of control and *Emx1*^{cre};*Gsx2*$^{flox/flox}$ (i.e., *Gsx2* cKO) embryos at E10.5 (B and C), E12.5 (D–G), and E18.5 (H and I). Deletion of floxed *Gsx2* allele with *Emx1*$^{cre/+}$ mice results in a slight mosaic loss of Gsx2 in the LGE at E10.5 (C) compared to control (B). *Emx1*$^{cre/+}$ recombined cells were found scattered in the presumptive LGE, as visualized by β-gal expression from R26R allele, in both the control and cKO (insets of dashed boxes in B and C, respectively). Two days later (E12.5), the loss of Gsx2 in cKO embryos is more apparent, manifesting as a clear mosaic deletion pattern in LGE (compare E to D). The pallial gene Pax6 (D) expands into the *Gsx2* null regions, predominantly in the dorsal half of the *Gsx2* cKO LGE (E). The ventral pallial marker Dbx1 (F) expands throughout the dorsal-ventral aspect of the LGE in the *Gsx2* null areas (G), some of which double label with β-gal from R26R locus (arrows in F and G). Remarkably, by E18.5 the vast majority of Gsx2-expressing cells are lost in the cKO LGE (compare I to H).

downregulation of ventral telencephalic genes (Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001, 2003). As mentioned above, conditional deletion of *Gsx2* using *Emx1*$^{cre/+}$ mice results in a mosaic loss of Gsx2 protein in the LGE at E12.5 (Figure 5E), which allows for the assessment of the *Gsx2* mutant phenotype in a partial wild-type environment. To compare mutant cells to wild-type cells, we crossed the *R26R* mouse onto our conditional mutant breeding scheme (*Gsx2*$^{flox/+}$;*Emx1*$^{cre/+}$ × *Gsx2*$^{flox/flox}$;*R26R*$^{+/-}$). These experiments clearly revealed that β-gal expression from the *R26R* mouse underrepresents cre recombination in the LGE because the reduction in Gsx2 protein expression in conditional mutants is considerably more extensive (Figure 6B). Interestingly, the Gsx2 null regions of the LGE, particularly in the dorsal half, showed ectopic expression of pallial markers. As is the case in germline *Gsx2* mutants (Yun et al., 2001, 2003; Stenman et al., 2003b), the expression of the ventral pallial marker Dbx1 (Figure 5F) was expanded throughout the LGE in *Emx1*^{cre} recombined areas of *Gsx2* conditional mutants at E12.5. Moreover, a number of the fate-mapped cells colocalized Dbx1 (Figure 5G). High levels of Pax6 expression, normally observed at the pallio-subpallial boundary (Figure 5D), were expanded within the dorsal half of the LGE, specifically in the *Gsx2* null areas (Figure 5E). In germline *Gsx2* mutants, the pallial restricted markers Tbr2 and Tbr1 (Figures 6C and 6G) have both been shown to be ectopic within the mutant LGE (Yun et al., 2001). Accordingly, in the *Gsx2* conditional mutant LGE, ectopic Tbr2- and Tbr1-expressing cells were observed in the SVZ and mantle zone of the dorsal half of the LGE, respectively, and many of these corresponded to *Emx1*^{cre} fate-mapped cells (Figures 6E, 6F, 6I, and 6J). It should be noted that there were clear *Gsx2* null regions in the vLGE (Figure 6B and S4), but only weak Tbr2 cells (Figures 6E and 6F) and no Tbr1 cells (Figures 6I and 6J) were observed in this region. These results indicate that Gsx2 is required cell autonomously to repress dorsal telencephalic identity in LGE progenitors (predominantly in the dLGE) until late embryonic stages.

Previous studies have shown that germline deletion of *Gsx2* results in reductions in the expression of dLGE and vLGE genes that eventually lead to abnormalities in the production of olfactory bulb interneurons and striatal projection neurons (Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell,

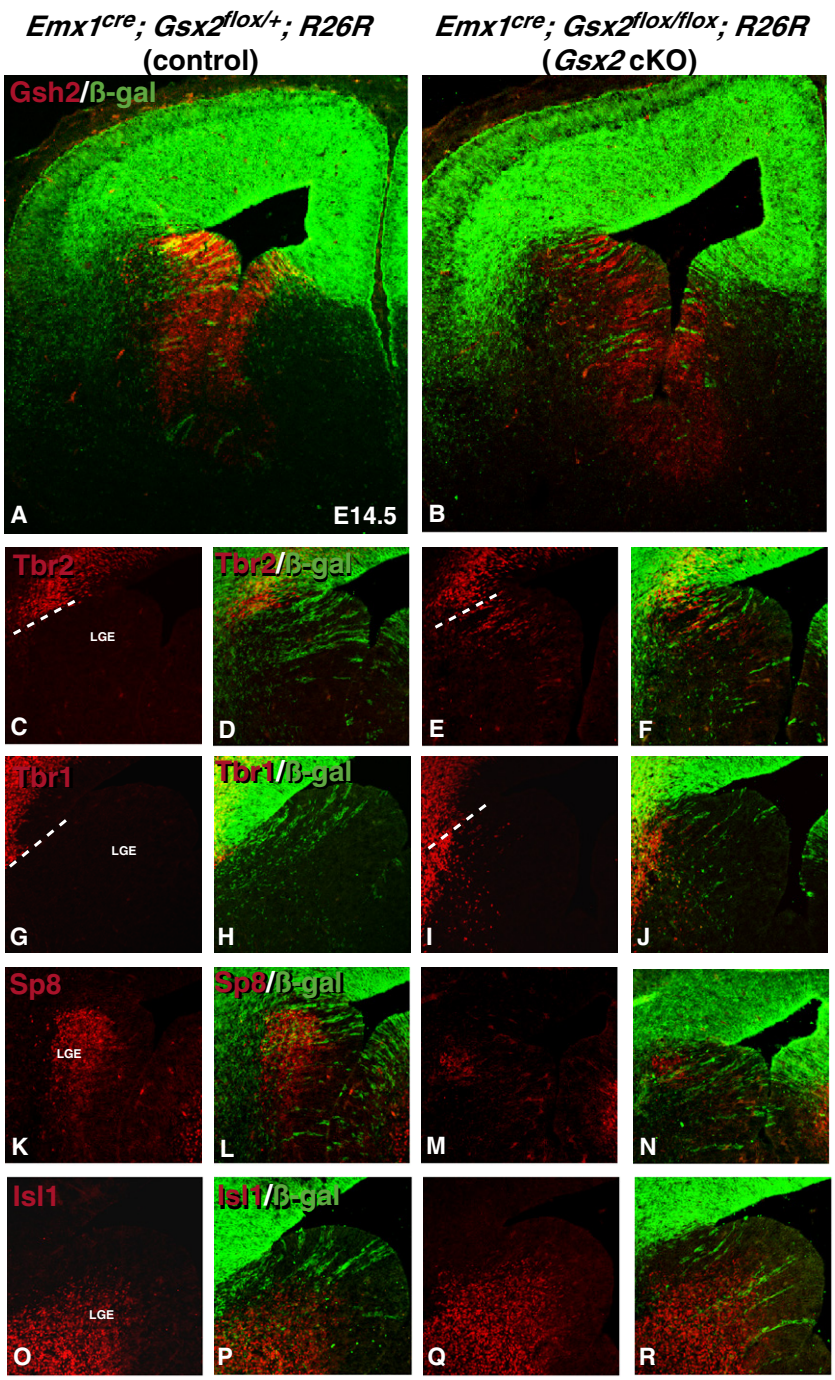

Figure 6. Respecification of *Gsx2* Null Areas in the Dorsal Portion of the LGE toward a Pallial Cell Fate

Confocal images of E14.5 control (A, C, D, G, H, K, L, O, and P) and *Gsx2* cKO (B, E, F, I, J, M, N, Q, and R) embryos.

(A and B) In control embryos there is significant colocalization of Gsx2 and β-gal (i.e., recombined cells) in the LGE (A); however, in the cKO embryos Gsx2 expression is dramatically reduced and the β-gal-expressing cells do not coexpress Gsx2 (B).

(C–J) Tbr2 and Tbr1 are normally expressed in the developing pallium with a sharp boundary at the pallio-subpallial border (dashed line in C and G). Indeed, no β-gal-expressing (i.e., recombined) cells in the LGE were observed to colocalize either of these factors in the control embryos (D and H). In cKO embryos, both Tbr2- (E) and Tbr1- (I) expressing cells are found ectopically in the dorsal portion of the LGE (the pallio-subpallial boundary is indicated by dashed lines). A number of these ectopic cells were derived from *Gsx2* mutant cells because they also expressed β-gal (F and J).

(K–R) Sp8 marks cells in the dLGE (K) and many of these are observed to coexpress β-gal (L), indicating that the *Emx1*^{cre} fate-mapped cells normally give rise to dLGE cells. Sp8 expression is severely reduced in the dLGE of *Gsx2* cKO embryos (M). Moreover, few, if any, fate-mapped (i.e., β-gal) cells colocalize Sp8 expression (N). The Isl1 expression domain is relatively unchanged in *Gsx2* cKO LGE (Q) compared to the control LGE (O). Interestingly, *Emx1*^{cre} fate-mapped cells were observed to colocalize Isl1 in both the control (P) and cKO LGE (R).

2001; Yun et al., 2001, 2003; Stenman et al., 2003a; Waclaw et al., 2004, 2006). Accordingly, dLGE identity as labeled by Sp8 expression (Figures 6K and 6L and S4C and S4I) is severely disrupted in the *Gsx2* conditional mutants (Figures 6M and 6N and S4F and S4L). Unlike in the control embryos where Sp8 and β-gal (i.e., fate-mapped cells) are broadly coexpressed (Figure 6L), little overlap is detected in *Gsx2* conditional mutants (Figure 6N). In contrast to the dLGE, vLGE identity as labeled by Isl1 expression is not severely affected in the *Gsx2* conditional mutant (compare Figures S4E and S4K with S4B and S4H). In fact, we were able to identify β-gal-positive *Emx1* fate-mapped cells that also express Isl1 in the *Gsx2* conditional mutant LGE (Figure 6R). These findings suggest that the vLGE may be specified by Gsx2 in LGE progenitors earlier than E10.5, since *Emx1*^{cre}-mediated recombination starts in the LGE around this time point. Taken together, these results suggest that *Emx1*^{cre}-induced mosaic recombination of *Gsx2* in the LGE severely affects the establishment of dLGE identity while leaving vLGE specification largely intact.

To examine dLGE and vLGE derivatives in *Gsx2* conditional mutants, we analyzed markers of olfactory bulb interneurons and striatal projection neurons in E18.5 embryos. Germline *Gsx2* mutants are known to have severe defects in striatal development, notably a greater than 50% reduction in striatal size as observed by Isl1 and FoxP1 expression (Toresson and Campbell, 2001; Waclaw et al., 2004). Consistent with this result, germline deletion of our *Gsx2* conditional allele *Gsx2*$^{RA/RA}$ (i.e., null allele) results in a 57% reduction in striatal volume, as marked by FoxP1 expression, compared to that of control embryos ($p < 0.01$; $n = 3$) (compare Figure 7B to

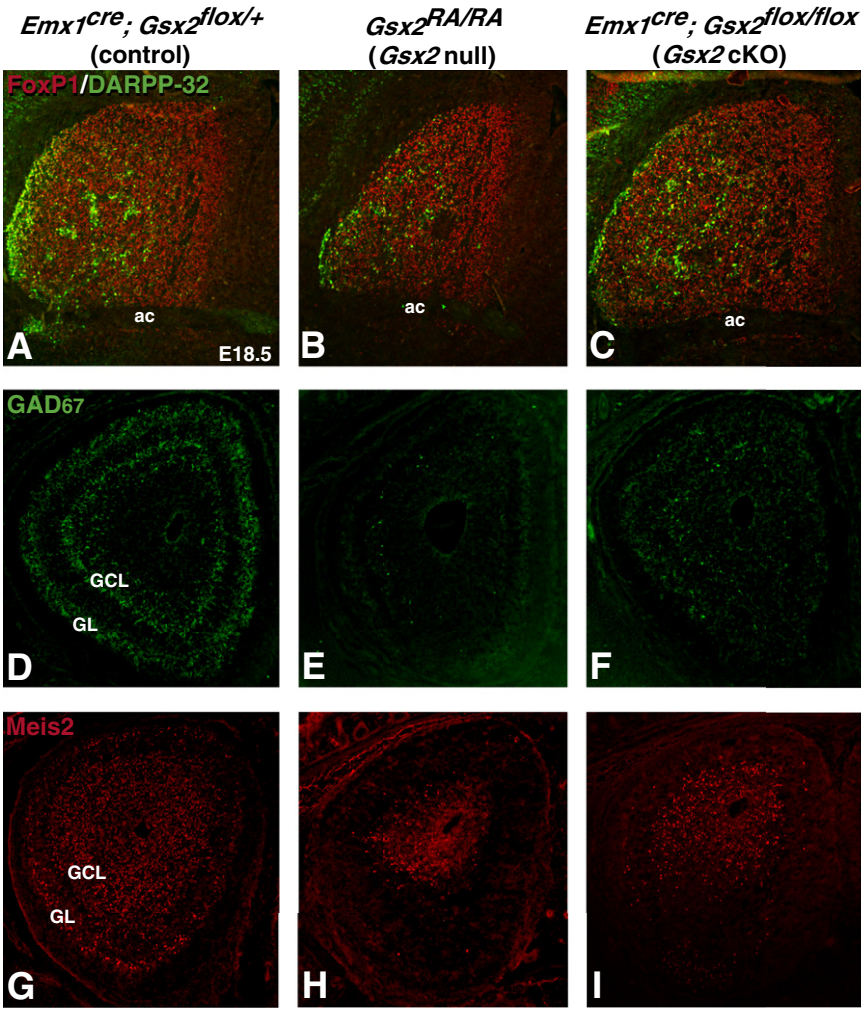

Figure 7. *Gsx2* Conditional Mutant Embryos Exhibit More Severe Defects in dLGE Cell Fate Compared to vLGE Cell Fate

Representative coronal sections of E18.5 control (A, D, and G), *Gsx2* null (B, E, and H), and *Gsx2* cKO (C, F, and I) embryos. Striatal development as marked by the expression of FoxP1 and DARPP-32 in *Gsx2* cKO embryos (C) is dramatically improved compared to the *Gsx2* null embryo striatum (B) and, in fact, is very similar to control striatum (A). Conversely, *Gsx2* cKO embryos exhibit severe reductions in the olfactory bulb interneuron markers GAD_{67} (F) and Meis2 (I) when compared to control embryos (D and G). Indeed, the expression of GAD_{67} and Meis2 in *Gsx2* cKO olfactory bulbs (F and I) is more similar to that in the *Gsx2* null olfactory bulb (E and H). ac, anterior commissure; GCL, granule cell layer; GL, glomerular layer.

7A). On the contrary, striatal volume in the $Emx1^{cre}$;$Gsx2^{flox/flox}$ mutants was not significantly different from that in controls (compare Figure 7C to 7A). In fact, the striatal volume of $Gsx2^{RA/RA}$ mutants was 53% reduced from that in the $Emx1^{cre}$ conditional mutants ($p < 0.01$; $n = 3$) (compare Figures 7B and 7C). At birth, most striatal projection neurons have been born but are still undergoing maturation, and thus most mature markers of these cells are not yet expressed. DARPP-32, which is ultimately expressed by all striatal projection neurons, only marks the early-born neurons at birth, which largely belong to the patch compartment (Foster et al., 1987). The germline *Gsx2* mutants show a disproportionate reduction in DARPP-32 neurons (Corbin et al., 2000; Toresson and Campbell, 2001; Waclaw et al., 2004). $Emx1^{cre}$;*Gsx2* conditional mutants (Figure 7C) exhibited considerably more DARPP-32-positive cells than the $Gsx2^{RA/RA}$ mutants (Figure 7B), the numbers of which seem to be only slightly reduced from that seen in controls (compare Figures 7A and 7C). Together these data support the notion that the delayed recombination of *Gsx2* that is induced by $Emx1^{cre}$ results in minimal alterations of vLGE specification or striatal development, at least with respect to what can be examined at perinatal stages.

As mentioned above, germline *Gsx2* mutants exhibit defects in dLGE specification and an attendant reduction in the generation of embryonic olfactory bulb interneurons (Corbin et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001, 2003; Stenman et al., 2003a; Waclaw et al., 2006). To determine if $Emx1^{cre}$;$Gsx2^{flox/flox}$ mutants displayed defects in embryonic olfactory bulb neurogenesis, we analyzed the expression of the GABA synthesizing enzyme GAD_{67}, an established marker of many embryonic olfactory bulb interneurons (Kohwi et al., 2007; Kosaka and Kosaka, 2007; Parrish-Aungst et al., 2007). *Gsx2* conditional mutants exhibited an 81% reduction in GAD_{67}-positive cells in the E18.5 olfactory bulb compared to levels in controls (Figures 7F and 7D) ($p < 0.01$; $n = 3$). This reduction was similar to, but not as severe as, that observed in $Gsx2^{RA/RA}$ mutants that show a 97% reduction compared to levels in controls (Figures 7E and 7D) ($p < 0.01$; $n = 3$). The difference between the $Gsx2^{RA/RA}$ and $Emx1^{cre}$ conditional mutants is likely due to the remaining Gsx2 expression in the dLGE (approximately 20% of control) that is not recombined by $Emx1^{cre/+}$ during development (see Figure 5). In addition to expression of GAD_{67}, we analyzed expression of Meis2, which is a broad marker of olfactory bulb interneuron subtypes (Allen et al., 2007). The amount of Meis2 is also clearly reduced in the *Gsx2* conditional mutant (Figure 7I) and $Gsx2^{RA/RA}$ mutant (Figure 7H) compared to that in controls (Figure 7G). Note that there is almost a complete loss of Meis2 in the forming glomerular layer of both *Gsx2* conditional mutants (Figure 7I) and germline *Gsx2* mutants (Figure 7H) compared to controls (Figure 7G). These results, combined with the analysis of dLGE identity (i.e., Sp8), indicate that the delayed inactivation of *Gsx2* by $Emx1^{cre}$ significantly affects dLGE development and the generation of olfactory bulb interneurons in a manner similar to that observed in the $Gsx2^{RA/RA}$ mutants.

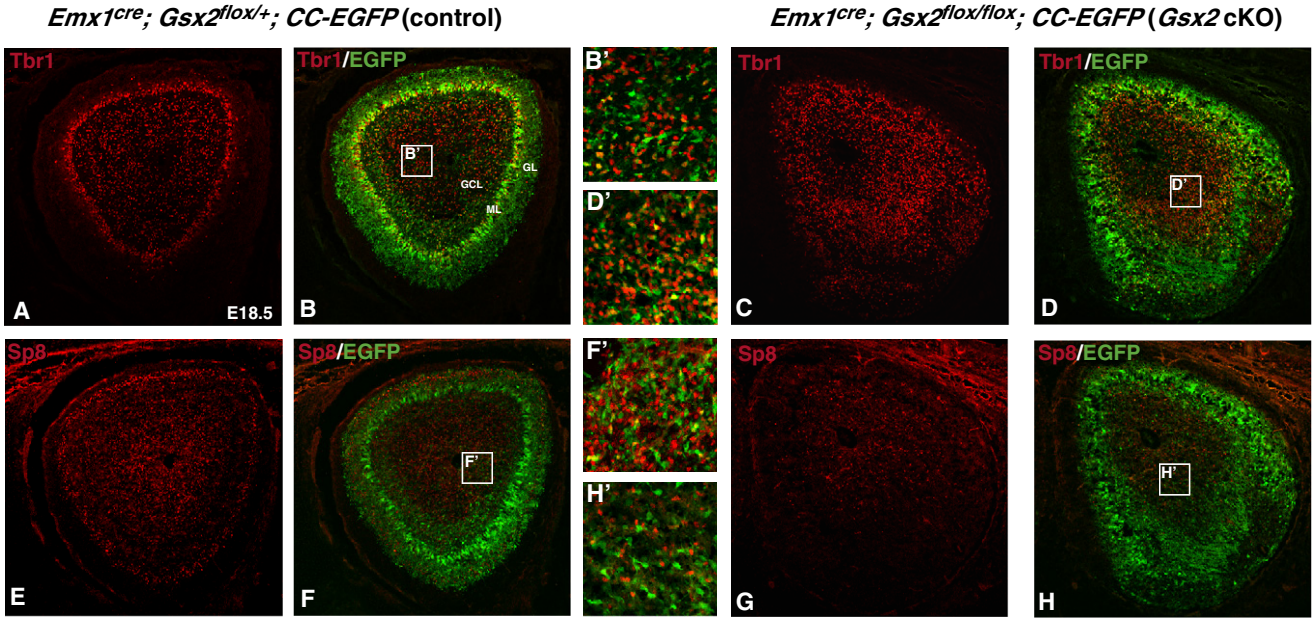

Figure 8. Respecification of Olfactory Bulb Interneurons toward Olfactory Bulb Projection Neuron Cell Fate

Confocal images of E18.5 control (A, B, E, and F) and *Gsx2* cKO (C, D, G, and H) olfactory bulbs showing *Emx1*^{cre} fate-mapped cells using the CC-EGFP mice. (A) Tbr1 is strongly expressed in the mitral cell layer (ML) of the control olfactory bulb with scattered cells in the forming granule cell layer (GCL). *Emx1*^{cre} fate-mapped (i.e., EGFP -expressing) cells strongly colocalize Tbr1 in the ML as well as in the GCL (B). In fact, just over half of all fate-mapped cells in the GCL were observed to be Tbr1 expressing (B′). (C and D) The *Gsx2* cKO olfactory bulbs showed increased numbers of Tbr1-positive cells and a loss of the normal laminar organization. (D) A similar pattern was observed for the fate-mapped EGFP cells in which the proportion of fate-mapped cells that expressed Tbr1 increased dramatically (D′). (E) Sp8 marks olfactory bulb interneurons in the GCL and GL of control embryos, a significant number of which are observed to be fate mapped by the *Emx1*^{cre} mice (F and F′). (G) In *Gsx2* cKO embryos, Sp8 is severely depleted in all regions of the olfactory bulb (compare G to E). Contrary to the results with Tbr1, the fate-mapped mutant olfactory bulb cells were only rarely seen to express Sp8 (H and H′).

Previous studies have indicated that the dLGE contributes to the generation of olfactory bulb interneurons (Stenman et al., 2003a; Yun et al., 2003; Waclaw et al., 2006). However, recent studies have suggested that multiple telencephalic regions, including the pallium, may also produce these interneurons (Kohwi et al., 2007; Merkle et al., 2007; Ventura and Goldman, 2007; Young et al., 2007). Some of the supporting data for the pallial contribution has come from fate-mapping *Emx1*^{cre} cells in the olfactory bulb. As would be expected, many of the *Emx1*^{cre} fate-mapped cells comprise projection neurons (i.e., mitral or tufted cells) (Gorski et al., 2002); however, a significant number of recombined cells possess interneuron phenotypes (Kohwi et al., 2007; Young et al., 2007). To determine the effect of *Gsx2* conditional loss of function in dLGE cells destined for the olfactory bulb, we crossed the CC-EGFP recombination reporter mice (Nakamura et al., 2006) onto the *Emx1*^{cre};*Gsx2*$^{flox/flox}$ mutant background. Tbr1 can be used to mark projection neurons (mitral and tufted cells) as well as their progenitors in the olfactory bulb at perinatal time points (Bulfone et al., 1998). We found that 56.2% ± 6.1% of the *Emx1*^{cre} fate-mapped cells located near the progenitor regions of the olfactory bulb were Tbr1 expressing (i.e., projection neuron identity) (Figures 8B and 8B′). In contrast, Sp8 can be used to mark many olfactory bulb interneurons, particularly those expressing calretinin (Waclaw et al., 2006), which have also been shown to be *Emx1*^{cre} derivatives (Kohwi et al., 2007; Young et al., 2007). Germline *Gsx2* mutants show severe reductions in Sp8-positive cells within the olfactory bulb (Waclaw et al., 2006). We observed that 34.9% ± 3.1% of the *Emx1*^{cre} fate-mapped cells in the control olfactory bulbs were Sp8 positive (Figures 8F and 8F′). In the *Emx1*^{cre};*Gsx2*$^{flox/flox}$ mutant bulb, we noticed a dramatic increase in Tbr1 staining at E18.5 together with a disorganization in the typical staining pattern for Tbr1 (Figure 8C). Since this result has not been reported in the germline *Gsx2* mutants, we examined the *Gsx2*$^{RA/RA}$ mutant olfactory bulbs at E18.5 and found that they also show increased numbers of Tbr1-positive cells (Figure S5B). Quantification of the *Emx1*^{cre} fate-mapped cells in the conditional mutant olfactory bulb showed that 77.6% ± 1.2% of the EGFP cells expressed Tbr1, which was a 38% increase from that seen in controls ($p < 0.05$; $n = 3$) (Figures 8D and 8D′). Conversely, Sp8 showed a dramatic reduction in the *Gsx2* conditional mutant olfactory bulb (Figure 8G) and only 6.0% ± 0.2% of the fate-mapped mutant cells contained Sp8 staining, which was an 82% reduction from that observed in controls ($p < 0.001$; $n = 3$) (Figures 8H and 8H′). Therefore, it seems that the increase of Tbr1-positive cells in the *Emx1*^{cre};*Gsx2*$^{flox/flox}$ mutant olfactory bulb occurs at the expense of the normal generation of Sp8-positive interneurons from *Gsx2*-dependent dLGE cells. Moreover, these findings suggest that the respecified (i.e., ectopic Tbr1 and Tbr2) cells in the conditional mutant dLGE retain the ability to migrate rostrally and populate the olfactory bulb. Indeed, using *Gsx2*^{EGFP} mice

(Wang et al., 2009) as a short-term fate map of cells derived from *Gsx2*-expressing cells, we observed many EGFP and Tbr1 coexpressing cells in the olfactory bulb of $Gsx2^{EGFP/RA}$ (i.e., null) mutants (Figure S5D), unlike the case in $Gsx2^{EGFP/+}$ embryos (Figure S5C). Finally, our data question the assumption that $Emx1^{cre}$ recombination indicates pallial ancestry since we find that this cre driver significantly recombines *Gsx2* in the LGE in a temporally progressive manner. This leads to a loss of dLGE specification and ultimately a fate shift in dLGE cells (normally destined to become olfactory bulb interneurons) toward pallial fates.

DISCUSSION

Our results show that Gsx2 is capable of directly ventralizing pallial progenitors and promoting LGE neuronal cell fate specification. Interestingly, this homeobox transcription factor appears to function within a temporal framework to specify predominantly striatal projection neuron (i.e., vLGE) fates at early stages of LGE neurogenesis, and subsequently, dLGE fates (i.e., olfactory bulb interneurons) at later stages of development. Accordingly, our conditional loss-of-function studies demonstrate that at late stages of development, Gsx2 is required for the specification of dLGE fates. Indeed, in the conditional *Gsx2* mutants we found that many of the cells normally fated to become olfactory bulb interneurons appeared to be respecified toward pallial fates, as marked by Tbr1 expression. Taken together, our findings indicate that the dorsal-ventral patterning activities of Gsx2 function within an independently controlled temporal framework.

Gsx2 and Dorsal-Ventral Patterning

Gsx2 is known to be required for correct dorsal-ventral patterning in the telencephalon (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001); however, it has been thought to play an indirect role in this process. In fact, viral misexpression of Gsx2, which was used to examine Gsx2's ability to impart ventral telencephalic identity within early pallial progenitors, did not support a ventralizing role (Corbin et al., 2000). These authors reported that neither *Ascl1* nor *Dlx* genes were induced, nor was *Pax6* expression in the dorsal telencephalon reduced by ectopic Gsx2. The authors did, however, find that the LGE-specific radial glial marker retinol binding protein (RBP-I) (Toresson et al., 1999) was moderately upregulated in some of the infected pallial progenitors. These findings are in stark contrast to those presented here. It could be that widespread ectopic Gsx2 (as shown here) is required to ventralize pallial progenitors, essentially representing a "community effect." However, on rare occasions, we obtained $Foxg1^{tTA}$*;tet-O-Gsx2-IE* embryos that exhibited mosaic expression of Gsx2 that appeared similar to the pattern observed after viral transductions. The effects of Gsx2 misexpression in small clones of EGFP-expressing cells were identical to those observed in the embryos showing widespread misexpression, thus indicating a cell autonomous function for Gsx2 in repressing dorsal identity and promoting LGE specification. The discrepancy between the present results and those of Corbin et al. (2000) could be due to downregulation of virally misexpressed Gsx2 protein prior to the assay points. If this was the case, the induced RBP-I that Corbin et al. (2000) observed may represent a very sensitive readout of transient Gsx2 activity, while the regulation of *Pax6*, *Ascl1*, and *Dlx* genes may require sustained Gsx2 expression.

Although viral misexpression of Gsx2 did not drastically alter the development of cortical progenitors (Corbin et al., 2000), it was found to repress Nkx2.1 in the MGE when delivered at E9.5 (Corbin et al., 2003). This was similar to our results of early misexpression of Gsx2, where Nkx2.1 was reduced or missing in the MGE. Interestingly, delayed misexpression of Gsx2 was not efficient at repressing Nkx2.1 in the MGE, indicating that by E13.5 MGE progenitors are resistant to Gsx2 influence. Despite this, the present findings clearly show, using the binary transgenic system described here, that ectopic Gsx2 robustly and reproducibly promotes LGE specification in pallial progenitors at both early and late time points.

Temporal Control of vLGE versus dLGE Fates by Gsx2

Gsx2 is expressed in a temporally dynamic fashion within the VZ progenitors of the LGE. At early stages, it is expressed at high levels in progenitors throughout the presumptive LGE, while at later stages high-level expression is confined to VZ cells in the dLGE. The early (E9–E11) high-level expression of Gsx2 correlates with the appearance of Isl1 cells throughout most of the LGE with limited numbers of Sp8-expressing cells in the dorsal-most region. This is not the case at later stages (E12 onward), when only Sp8 cells are found in close association with high-level Gsx2-expressing LGE (i.e., dLGE) cells. This suggests that Gsx2 can specify both vLGE and dLGE at early stages of LGE neurogenesis; however, vLGE specification appears to be favored. Our early misexpression data support this notion. In contrast, high levels of Gsx2 at later stages (i.e., E12 onward) may exclusively promote dLGE fates. Indeed, when Gsx2 misexpression is delayed by Dox treatment to around E13, only dLGE specification is observed. Moreover, in the ventral telencephalon, this appears to be at the expense of the vLGE (at least its most dorsal portion). These results correspond well with the fact that striatal projection neurons commence neurogenesis at an earlier time point than the olfactory bulb interneurons (Bayer and Altman, 2004).

Previous studies using germline *Gsx2* mutants have shown that this factor is required for the correct development of both the striatal projection neurons and olfactory bulb interneurons (Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001, 2003). The fact that delayed inactivation of *Gsx2* using $Emx1^{cre}$ preserves striatal specification indicates that correct striatal development depends largely on early (i.e., pre-E10.5) Gsx2 expression. Conversely, dLGE and olfactory bulb interneuron development requires *Gsx2* function at least until birth. Taken together, our gain-of-function and loss-of-function results suggest that vLGE fate is specified earlier than dLGE fate and that this specification occurs in a sequential manner. This does not appear to be the case in the developing spinal cord, where Gsx1/2 are required for the normal production of both early- and late-generated excitatory interneurons that share similar transcription factor profiles (Mizuguchi et al., 2006). Thus, our results reveal a specific role for Gsx2 in the temporal production of molecularly distinct neuronal subtypes from LGE progenitors.

Given the protracted period of neurogenesis in the developing telencephalon, it is not surprising that temporally regulated mechanisms would also be involved in the generation of neuronal diversity within this brain region. In fact, previous transplantation studies have demonstrated a progressive restriction in the fate potential of cortical progenitors. Those transplanted from early stages are capable of generating neurons that populate both the deep and superficial cortical layers (McConnell, 1988), while the later stage progenitors are limited to populating the superficial layers, even if back-transplanted to an earlier cortical environment (Frantz and McConnell, 1996; Desai and McConnell, 2000). A likely explanation for this finding is that the molecular nature of telencephalic progenitors changes over time and that this constrains fate specification accordingly. In this respect, Hanashima et al. (2004) previously investigated the role of Foxg1 in the temporal production of different cortical neuron subtypes. They found that Foxg1 suppresses the generation of Cajal-Retzius neurons, the earliest cell type generated in the cerebral cortex. Moreover, these authors demonstrated that conditional inactivation of Foxg1 in progenitors that normally produce deep layer cortical neurons results in the production of Cajal-Retzius neurons. A subsequent study by Muzio and Mallamaci (2005) suggested that Foxg1 may regulate areal patterning and, through suppression of Cajal-Retzius neurogenesis in most of the pallium, restrict the production of these early-born neurons to the dorsal medial telencephalon. Cortical progenitors isolated in vitro have recently been shown to follow the same schedule of neuron generation as their in vivo counterparts, indicating that the factors responsible for progressive restriction in developmental potential are cell intrinsic (Shen et al., 2006). Furthermore, these authors demonstrated that knockdown of Foxg1 in midgestation (e.g., E12) cortical progenitors actually appeared to reset the timing of neuron generation such that early fates (i.e., Cajal-Retzius neurons) were first generated followed by later cortical fates. Interestingly, late-stage cortical progenitors (e.g., E15) were not reverted in their developmental potential by the knockdown of Foxg1 (Shen et al., 2006). Thus Foxg1 plays an important role in restricting the developmental potential of cortical progenitors, specifically by repressing the earliest cortical neuron fate. However, factors that restrict the fate of cortical progenitors at later embryonic stages have yet to be identified.

Although evidence for the temporal restriction of developmental potential has been demonstrated for dorsal telencephalic progenitors, our genetic data indicate that similar restrictions may also occur in the ventral telencephalon. Importantly, the response of telencephalic progenitors to misexpressed Gsx2, at early versus late developmental stages, was similar regardless of their location in the pallium or LGE. This suggests that similar temporal patterning mechanisms may regulate the progressive restriction of developmental potential in both pallial and LGE progenitors. It may be that in order to specify vLGE fates, Gsx2 needs to cooperate with a factor or factors that are restricted in their telencephalic expression to early time points. In this way, downregulation of such a factor or factors at later stages would limit Gsx2 to specifying dLGE fates exclusively. At present, the identity of such a factor or factors are unknown. Therefore, it seems important to perform molecular profiling studies on telencephalic progenitors, over time, in order to identify candidate factors involved in regulating this process. Another mechanism that could regulate progressive restriction in developmental potential within pallial and LGE progenitors is cell cycle length, as suggested previously (Shen et al., 2006). In fact, it seems that the cell cycle length of dorsal telencephalic progenitors increases as corticogenesis proceeds, and that this increase is rather specific to G_1 (Caviness et al., 2003; Calegari et al., 2005). Thus it could be that the duration of Gsx2 activity during G_1 is fundamental to its role in specifying dLGE versus vLGE at different stages of development. Finally, it is possible that progressive restriction in developmental potential is also regulated in SVZ progenitors downstream of Gsx2 such that the induction of Sp8 leads to a repression of Isl1 and thus vLGE identity, or vice versa. We are currently performing gain-of-function studies to address this possibility.

Ventral Recombination by *Emx1^{cre}*

The conditional mutant analysis shown here was facilitated by the *Emx1^{cre}* mice (Gorski et al., 2002). These mice have been widely used to recombine floxed alleles within pallial progenitors; however, in their initial characterization the authors noted recombination also within scattered cells of the LGE (Gorski et al., 2002). Furthermore, they found recombined cells within interneuron layers of the olfactory bulb as well as dispersed cells in the striatum. The *Emx1^{cre}*-derived cells in the striatum expressed the calcium binding protein calbindin (which marks the projection neurons of the matrix; Gerfen et al., 1987), but not markers of striatal interneuron subtypes. The existence of *Emx1^{cre}* recombined cells in the striatum and interneuron layers of the olfactory bulb has recently been used to argue for a pallial contribution to ventral telencephalic neuronal diversity, particularly within the striatal projection neuron and olfactory bulb interneuron populations (Willaime-Morawek et al., 2006; Kohwi et al., 2007; Young et al., 2007; Willaime-Morawek and van der Kooy, 2008). While our data do not exclude this possibility, they support a simpler explanation whereby *Emx1^{cre}* is expressed in LGE progenitors and, by virtue of this, labels significant numbers of striatal projection neurons and olfactory bulb interneurons. Indeed, we show recombination in the presumptive LGE beginning as early as E10.5, commensurate with a slight reduction of Gsx2. By E12.5, large portions of the Gsx2 expression domain in the LGE are recombined, and surprisingly, at late stages, nearly 80% of the Gsx2 cells are gone from the LGE. The kinetics of this recombination were well suited to our questions concerning temporal constraints on Gsx2 in LGE specification. Indeed, the delayed recombination of *Gsx2* that was facilitated by the *Emx1^{cre}* mice revealed that vLGE development was largely preserved, but that the dLGE specification depends on late stage Gsx2 expression. Indeed, no significant changes were detected in the striatum of the *Emx1^{cre}* conditional mutants at birth, while the reductions in olfactory bulb interneuron markers in these mutants were reduced to levels similar to that seen in the germline *Gsx2* mutants. The dorsal septum has also been suggested to give rise to olfactory bulb interneurons at perinatal stages (Merkle et al., 2007). Interestingly, Gsx2 is expressed in the dorsal septum and was observed to be recombined in the *Emx1^{cre}* conditional mutants (Figure 6B), suggesting that Gsx2

may also be required for septal-derived olfactory bulb interneuron generation.

To trace the mutant cells in the LGE, striatum, and olfactory bulb, we used the recombination reporters R26R (Mao et al., 1999) or CC-EGFP (Nakamura et al., 2006) in combination with the *Emx1^{cre};Gsx2$^{flox/flox}$* alleles. We consistently found that the fate-mapped cells grossly underrepresent the number of recombined LGE cells (as indicated by the loss of Gsx2 expression in the mutants). The mutant cells in the dLGE lost the expression of Sp8 and at the same time upregulated the pallial markers Pax6, Tbr2, and Tbr1. Surprisingly, *Emx1^{cre}* fate-mapped, mutant cells in the vLGE did not appear to change fate, since they retained expression of Isl1, suggesting that vLGE fate was specified in these cells prior to cre activity (i.e., before E10.5). In the conditional mutant olfactory bulb, a dramatic decrease in Sp8-positive, *Emx1^{cre}* fate-mapped cells was observed coincident with a near doubling of the number of fate-mapped mutant cells that express the pallial marker Tbr1. The expansion of pallial markers into the *Gsx2* mutant LGE has previously been suggested to indicate a respecification toward ventral pallial fates (Yun et al., 2001, 2003; Stenman et al., 2003b). While ventral pallial progenitors have been proposed to give rise to projection neurons of the amygdala and pyramidal cortex (Fernandez et al., 1998; Puelles et al., 1999, 2000; Stenman et al., 2003c; Hirata et al., 2009), no reports to date suggest that these progenitors migrate rostrally to populate the olfactory bulb. Thus, it may be that the respecified (i.e., Tbr1-positive) cells in the dLGE of *Gsx2* conditional mutants retain the ability to migrate rostrally as they would normally do despite their apparent pallial respecification.

In summary, we show here that Gsx2 is sufficient to specify LGE fates in pallial progenitors. Additionally, our results indicate that Gsx2 functions within an independently regulated temporal framework to specify vLGE and dLGE neuronal fates at distinct time points in telencephalic development.

EXPERIMENTAL PROCEDURES

Generation of *tetO-Gsx2-IE* Mice

tetO-Gsx2-IE mice were generated by pronuclear injection and characterized as described in the Supplemental Data. Dox was used to repress transgene expression in *Foxg1$^{tTA/+}$;tetO-Gsx2-IE* embryos. Specifically, doxycycline hyclate (Sigma) was given to pregnant mice in the drinking water at 0.02 mg/ml beginning at E7 (morning of vaginal plug was defined as E0.5) and removed approximately 48 hr later (i.e., E9). This Dox dose was the lowest that we found to completely repress transgene expression in *Foxg1$^{tTA/+}$;tetO-Gsx2-IE* embryos.

Generation of the *Gsx2* Conditional Allele

A conditional allele of *Gsx2* was generated by homologous recombination in W4 ES cells (kindly provided by A. Joyner). Briefly, the floxed allele was made by flanking the second exon of *Gsx2* with *loxP* sites as described in the Supplemental Data (Figures 5A and S3). Chimeric mice were generated from two correctly targeted clones (7-B11 and 7-F3) by the transgenic core at CCHMC. All experiments were performed on the 7-B11 line.

Animals

β-actin-FLPe (enhanced Flpase) mice, *R26R* mice, *EIIA-cre*, and *Emx^{cre}* mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and genotyped following protocols from the Jackson Laboratory. *CAG-CAT(CC)-EGFP* were genotyped as described in Nakamura et al., (2006). See Supplemental Data for genotyping details concerning the *Foxg1$^{tTA/+}$*, *tetO-Gsx2-IE*, and *Gsx2^{flox}* mice and embryos. For staging of embryos, the morning of vaginal plug detection was designated E0.5. Embryos and postnatal brains were fixed overnight in 4% paraformaldehyde, rinsed thoroughly in PBS, and cryoprotected in 30% sucrose in PBS before sectioning at 12 μM on a cryostat.

Immunohistochemistry

Immunohistochemical staining was done as previously described in Waclaw et al. (2006). For details concerning the antibodies used, see Supplemental Data.

Quantification

Quantification for each experiment was done on three different embryos of each genotype. GAD_{67}-positive cells in the granule cell layer (GCL) were counted in three olfactory bulb sections at E18.5 in control embryos, *Gsx2* null embryos, and *Gsx2* conditional mutant embryos. Striatal size of control embryos, *Gsx2* null embryos, and *Gsx2* conditional mutants was determined by quantifying the FoxP1 expression area using the NIH ImageJ program. Statistics were performed between control embryos, *Gsx2* null embryos, and *Gsx2* conditional mutants using a one-way ANOVA with a Tukey post hoc test. Gsx2-positive cells in the LGE were counted at 400x magnification. Each genotype was analyzed at identical rostral and caudal levels. For double staining quantification of *Emx1$^{cre/+}$* fate-mapped cells (EGFP) and either Sp8-positive or Tbr1-positive cells, double- and single-labeled cells in the GCL were counted in two separate areas (400x magnification) of two olfactory bulb sections for each genotype (at least 100 EGFP-positive cells per embryo). Statistics were performed between control and *Gsx2* conditional mutants using a Student's unpaired t test.

SUPPLEMENTAL DATA

Supplemental data for this article include Supplemental Experimental Procedures and five figures and can be found at http://www.cell.com/neuron/supplemental/S0896-6273(09)00544-3.

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