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Transcription Factor VAX1 Regulates the Regional Specification of the Subpallium through Repressing *Gsx2*

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Abstract

Specification of the progenitors' regional identity is a pivotal step during development of the cerebral cortex and basal ganglia. The molecular mechanisms underlying progenitor regionalization, however, are poorly understood. Here we showed that the transcription factors *Vax1* was highly expressed in the developing subpallium. In its absence, the RNA-Seq analysis, in situ RNA hybridization, and immunofluorescence staining results showed that the cell proliferation was increased in the subpallium, but the neuronal differentiation was blocked. Moreover, the dLGE region severely expanded ventrally at the expense of the vLGE, MGE, and septum. Finally, overexpressed VAX1 in the LGE progenitors strongly inhibits *Gsx2* expression. Taken together, our findings show that *Vax1* is crucial for subpallium regionalization by repressing *Gsx2*.

1 Introduction

The telencephalon originates from the rostral nerve plate. During development, the telencephalon acquires precise subdivisions both along the dorsal-ventral (DV) and the anteroposterior (AP) axis [1-3]. The telencephalon is subdivided into two different territories: pallium and subpallium. The subpallium consists of the lateral ganglionic eminence (LGE), medical ganglionic eminence (MGE), caudal ganglionic eminence (CGE), preoptic area (POA), and septum. The progenitors located in different regions generate different cell types during telencephalic development. For example, the progenitors in the dLGE give rise to the olfactory bulb (OB) interneurons. In contrast, the progenitors in the vLGE mainly contribute to striatal medium-sized spiny neurons (MSNs) [4-6]. Thus, the maintenance of the dLGE, vLGE, MGE, and POA region in the subpallium is crucial for telencephalon development.

Multiple signaling pathways and transcription factors are involved in regionalization of the telencephalon. In the early embryonic stage, *Fgf8* and *Shh* signaling are required for telencephalon patterning. Deletion of *Fgf8* leads to structural abnormalities of the dorsal and ventral telencephalon [7, 8]. In $Shh^{-/-}$ mouse embryos, all ventral telencephalic progenitors are missing as detected by loss of ventral markers including *Gsx2*, *Nkx2.1*, and *Dlx2* [9, 10]. In the subpallidum development, several genes have been found responsible for regionalization. Transcription factors *Gsx2* and *Pax6* play complementary roles in dorsal-ventral patterning of the telencephalon [11]. A ventral to dorsal transformation leads the pallidal primordium into a striatal-like anlage in the *Nkx2.1* knock out mice [12, 13]. *Otx2* also plays crucial roles in MGE patterning through specifying vMGE fate and repressing POA fate [14]. The mechanisms of activation and mutual repression of the transcription factors in progenitors to establish regional patterning, however, need to be further investigated. For example, the key transcription factor, *Gsx2*, is not only required for the generation of the striatal MSNs and OB interneurons but is also crucial for the regionalization of the PSB (pallial-subpallial boundary) during forebrain development [4, 11, 15, 16]. The exact mechanism of down-regulating its expression in the vLGE/MGE, however, is largely unknown.

The homeobox transcription factor *Vax1* starts to express at approximately embryonic day (E) 8 in mice. *Vax1-mRNA* is detected in the most rostral level of the medial neural plate at E8. As development proceeds, *Vax1* is found in the basal forebrain, optic stalk, optic disk, and medial olfactory placode [17]. Previous studies showed that secreted VAX1 protein regulates retinal axon growth and is required for axon guidance and major tract formation [18]. *Vax1* is also required for the precursor cells proliferating in the SVZ and migrating through the rostral migratory stream (RMS) to the OB [19]. In addition, this generation of cortical interneurons is compromised in the $Vax1^{-/-}$ mice [20]. Recently, studies showed that regulatory interactions between *Vax1* and *Pax6* are crucial for stem cell regionalization during olfactory bulb neurogenesis [21]. Finally, heterozygous deletion of *Vax1* gene causes subfertility in mice and variants in *VAX1* genes are associated with non-syndromic cleft lip in humans [22, 23]. Although *Vax1* plays important roles in subpallium development, the mechanisms of *Vax1* in regulating subpallium regionalization remain largely unknown.

Here, our results show that *Vax1* is crucial for the regionalization of the subpallium. In the *Vax1* mutants, the dLGE expands ventrally, the vLGE and MGE get smaller, and the septum is absent. Furthermore, we found that the expression of *Gsx2* in the vLGE and MGE was significantly increased. Lastly, ectopic overexpression of *Vax1* leads to a significant decrease the expression of *Gsx2* in the LGE. Thus, *Vax1* may regulate subpallium regionalization by repressing *Gsx2*.

2 Materials And Methods

2.1 Mice

All experiments conducted in this research study were in accordance with guidelines from Fudan University. $Vax1^{tm1b(KOMP)MBP}$ ($Vax1^{+/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). $Vax1^{-/-}$ mice were obtained from crossing $Vax1^{+/-}$ with $Vax1^{+/-}$ mice. All mice were maintained in a mixed genetic background of C57BL/6J and CD1. Noon of the day of vaginal plug detection was considered as E0.5 and the day of birth was defined as P0.

Genotyping of $Vax1^{-/-}$ mice by PCR using the following primers:

Vax1-WT-F: CCAAGGGGGCTAACTTCATAC

Vax1-WT-R: CTCCTCCTTCTGCTGTCTGG

Vax1-Mut-F: CGGTCGCTACCATTACCAGT

Vax1-Mut-R: AAGCCCTGGTTTCTCTGACA

2.2 BrdU Labeling

A single intraperitoneal injection of 50 mg/kg BrdU was administered at E16.5. BrdU incorporation was analyzed 30 minutes after BrdU injection.

2.3 Tissue Preparation

Tissue preparation was performed as previously described [24].

2.4 Immunofluorescence

Immunofluorescence staining was performed as previously described [25].

The primary antibodies used in this study were as follows:

(1) Rabbit anti-GSX2 (Millipore Cat#ABN162, RRID: AB_11203296, Dilutions 1:2000)

(2) Goat anti-SP8 (Santa Cruz Biotechnology Cat# sc-104661, RRID: AB_2194626, Dilutions 1:1000).

(3) Rat anti-BrdU (Accurate Chemical and Scientific Corporation Cat# OBT0030S, Dilutions 1:200).

(4) Rabbit anti-FOXP1 (Abcam Cat# ab16645, Dilutions 1:2000).

(5) Rabbit anti-Ki67 (Vector Labs, Cat# VP-K451, Dilutions 1:500).

(6) Chicken anti-GFP (Aves Labs, Cat# GFP-1020, Dilutions 1:2000).

All secondary antibodies used in this study were from Jackson ImmunoResearch Labs.

Secondary Antibodies	Cat Number
Alexa®488-conjugated Affinipure Donkey Anti-Rabbit IgG (H^+L)	711-545-152
Cy^{TM}3-conjugated Affinipure Donkey Anti-Rabbit IgG (H^+L)	711-165-152
Alexa®488-conjugated Affinipure Donkey Anti-Mouse IgG (H^+L)	715-545-151
Cy^{TM}3-conjugated Affinipure Donkey Anti-Mouse IgG (H^+L)	715-165-151
Alexa®488-conjugated Affinipure Donkey Anti-Chicken IgY^{++} (IgG) (H^+L)	703-545-155
Cy™3-AffiniPure Donkey Anti-Chicken IgY (IgG) (H^+L)	703-165-155
Alexa®488-conjugated Affinipure Donkey Anti-Goat $^{++}$ (IgG) (H^+L)	705-545-147
Cy™3-AffiniPure Donkey Anti-Goat IgG (H^+L)	705-505-147
Cy™3-AffiniPure Donkey Anti-Rat IgG (H^+L)	712-165-150
Cy™3-AffiniPure Donkey Anti-Guinea Pig IgG (H^+L)	706-165-148

2.5 In Situ RNA Hybridization

In situ hybridization was performed on 20 µm thick cryo-sections using digoxigenin riboprobes, as previously described [26]. Probes were made from P0 wild type mouse brain cDNA amplified by PCR using the following primers

Vax1	F: TCCTGTCTAGAACAGAGCTGATGGG
	R: CTGTGGTTTTGGTTAAATTGCGAG
Gsx2	F: CCTTTGTTCGAGTCCCAGACACC
	R: AAAGGGACTTCCAGAGACCTGGAT
Etv1	F: TTCATGGCCTCCCACTGAAAATC
	R: CCTTCGTTGTAGGGGTGAGGGTT
Tshz1	F: GAGAAGGTCACGGGCAAGGTCAGC
	R: GAGGCGAGGACACAGCATCTGCCA
Prokr2	F: ATGGGACCCCAGAACAGA
	R: ATAGATACGCTACTTCAAAGATGAG
Isl1	F: ATGGGAGACATGGGCGATCC
	R: CATGCCTCAATAGGACTGGCTACC
Drd1	F: ATGGCTCCTAACACTTCTACCATGG
	R: TCAGGTTGAATGCTGTCCGCTGTG
Drd2	F: CGGGAGCTGGAAGCCTCGA
	R: TGCAGGGTCAAGAGAAGGCCG
Lhx6	F: CAGAGAGGGCGCGCATGGTCACCT
	R: AATTGGGGGGGGGGTCTTTGGCAC
Lhx8	F: GCCTTAGTGTGGCTGAGAGA
	R: AGGATGGTAGGCTTTGTAAACT
Nkx2.1	F: TCCTAGTCAAAGACGGCAAACCCT
	R: TAAAGAGAAAGGAAGGGGGGAAAGA
Gbx1	F: GATGAAGAGAAGCTAGAGCCCCCA
	R: GGTTCAGATCCTGTGACTTCCGGT
Gbx2	F: GTTCGCTATTCGAAGTCAACACCAG
	R: AATTAAACACAACGGAGACGTGCTT
Cdca7	F: AGACTCTCGAGACGTTTGCTA
	R: AGCTGCAGTTGCAAATTCCTC
Ccnd2	F: GGGCTAGCAGATGACGAACACG

	R: TCCCATTCAGCCAAAGGAAGGA
Ascl1	F: CTAACAGGCAGGGCTGGA
	R: TAAGGGGTGGGTGTGAGG
Dlx1	F: ATGACCATGACCACCATGCCAG
	R: TCACATCAGTTGAGGCTGCTGC
Dlx2	F: TTTCCTGTCCCGGGTCAGGAT
	R: AAGTCTCAGACGCTGTCCACTCGA
Dlx5	F: CAGCTTTCAGCTGGCCGCTT
	R: CAAGGCACCATTGATAGTGTCCACA
LacZ	F: CTCTATCGTGCGGTGGTTGAACT
	R: GGGTTGCCGTTTTCATCATATTT

2.6 RNA-Seq

RNA sequencing (RNA-Seq) analysis was performed as previously described [26]. The ganglionic eminence from the E13.5 *Vax1*$^{-/-}$ and littermate *WT*, and lateral ganglionic eminence from the E16.5 *Vax1*$^{-/-}$ and littermate *WT* were dissected (n=3). Gene expression levels were reported in FPKM (fragments per kilobase of exon per million fragments mapped).

2.7 Cloning of the pCAG-*Vax1*-ires-GFP expression plasmid and in vivo electroporation

The *Vax1* CDS was cloned from *WT* P0 cDNA amplified by PCR using the following primers: F-ATGTTCGGGAAACCAGACAAAATGG; R-AAATCAGTCCAGCGCTTTTTTCTCG. It was then inserted into the pCAGGS-ires-EGFP vector, using XbaI and EcoRI restriction sites. DNA-sequencing was performed to make sure no mutation was generated during the cloning. In vivo electroporation was performed at P0. Plasmids pCAG-GFP (Addgene #11150) or pCAG-*Vax1*-Ires-GFP, (final concentration of 1-2 mg/ml, 1µl each embryo), were mixed with 0.05% Fast Green (Sigma), and injected into the lateral ventricle using a beveled pulled glass micropipette. Five electrical pulses (duration: 50 ms) were applied at 130V across the uterine wall with a 950 ms interval between pulses. Electroporation was performed using a pair of 7 mm platinum electrodes (BTX, Tweezertrode 45-0488, Harvard Apparatus) connected to an electroporator (BTX, ECM830). Pups were analyzed at P3.

2.8 Quantification

The numbers of BrdU and *Foxp1* positive cells in the LGE SVZ were quantified in 9 randomly chosen 20 µm sections from each mouse. Three control and *Vax1* mutant mice from each group were analyzed at

E16.5. The integrated density of *Cdca7*, *Ccnd2*, *Ascl1*, *Dlx1*, *Dlx2*, *Dlx5* and *Isl1* measured by Image J in the LGE SVZ were quantified in 9 randomly chosen 20 μm sections from each mouse. Three control and *Vax1* mutant mice from each group were analyzed at E16.5. For quantification of GFP^+ and $GFP^+/GSX2^+$ cells in the mouse lateral ventricular zone at P3. Images were collected with an Olympus VS 120 microscope using a 20X objective. Six 20-μm thick coronal sections from rostral, intermediate, and caudal levels of the striatum were selected (n=5 mice per group). We selected the dorsal-most lateral ventricular VZ/SVZ region (1500 pix X 1500 pix) to quantify the number of GFP^+ and $GFP^+/GSX2^+$ cells in each group.

2.9 Microscopy

Figures were imaged with an Olympus VS 120 microscope. Images were merged, cropped, and optimized in Adobe Photoshop CC without distorting the original information.

3 Results

3.1 Increase of cell proliferation in the *Vax1*$^{-/-}$ subpallium V-SVZ

The Ventral Homeodomain Protein 1 (VAX1) is highly expressed in the subpallium progenitors from embryonic day 12.5 (E12.5) to adult would suggest *Vax1* plays an important role in forebrain development (See Supplementary fig. S1a-f, Additional File 1). To investigate whether *Vax1* regulates cell proliferation, we first performed 30-min BrdU pulse labeling at E16.5 and BrdU immunofluorescence staining. The number of $BrdU^+$ cells in the *Vax1*$^{-/-}$ LGE gradually increased in a rostral to caudal pattern, compared to controls (Fig. 1a-c', j). This means the number of cells in S-phase of the cell cycle was increased after *Vax1* deletion. Since *Cdca7* is a cell division cycle associated gene and *Ccnd2* promotes cell cycle progression from G1 to S phase, we then checked these genes at E16.5. In situ RNA hybridization of *Cdca7* and *Ccnd2* showed that *Cdca7-mRNA*$^+$ and *Ccnd2-mRNA*$^+$ cells were also significantly increased (Fig. 1d-i', k, l). The above results illustrated that there is an increase of cell proliferation in the *Vax1*$^{-/-}$ subpallium. This is consistent with previous *in vitro* neurosphere culture results [19].

3.2 *Vax1* promotes neuronal differentiation in the subpallium V-SVZ

The bHLH transcription factor, *Ascl1*, is heavily expressed in ventral forebrain progenitors, and *Ascl1* is crucial for neurogenesis [27]. At E16.5, the number of *Ascl1*$^+$ cells in the *Vax1*$^{-/-}$ subpallium VZ (ventricular zone) was significantly increased, compared to controls (Fig. 2a-c', m). *Dlx1* and *Dlx2* are mainly expressed in the VZ and SVZ of the subpallium, *Dlx5* appears later than *Dlx1/2*, and is expressed in more differentiated neurons in the SVZ (subventricular zone) and MZ (mantle zone). Our results showed that *Dlx1*$^+$, *Dlx2*$^+$ and *Dlx5*$^+$ cells in the *Vax1*$^{-/-}$ subpallium were increased markedly (Fig. 2d-l', m). Accumulation of *Ascl1*$^+$, *Dlx1*$^+$, *Dlx2*$^+$ and *Dlx5*$^+$ immature neurons in the *Vax1*$^{-/-}$ VZ and SVZ suggests

that neural progenitors in the subpallium could not normally exit the cycle and differentiate. Therefore, *Vax1* promotes neuronal differentiation in the subpallium VZ-SVZ.

3.3 The expansion of the dLGE domain in the *Vax1*$^{-/-}$ mice

The progenitors in the dLGE mainly give rise to olfactory bulb interneurons. Strong expression of *Gsx2* in the dLGE promotes the activation and lineage progression of OB interneuron progenitors. *Sp8* is expressed in migrating neuroblasts in the embryonic dLGE, postnatal SVZ and most OB interneurons, and the OB interneuron defects in conditional inactivation of *Sp8* mice [6]. *Sp8* regulates the expression of *Tshz1* and *Prokr2* [28]. *Prokr2* and *Tshz1* are expressed in the SVZ-RMS-OB, severe tangential, and radial migration defects of neuroblasts in *Prokr2-KO* mice [24, 29]. *Etv1*$^{+}$ cells in the dLGE and postnatal SVZ travel through the RMS into the OB where they mature into OB interneurons. At E16.5, we found that these dLGE markers (*Gsx2*, *Etv1*, *Sp8*, *Tshz1* and *Prokr2*) were significantly increased in the *Vax1*$^{-/-}$ mice (Fig. 3a-o'). *Gsx2* was heavily expressed in progenitors of the dLGE and its expression showed a ventral-low to dorsal-high gradient in the subpallium of the controls whereas, more GSX2^{+} cells were seen in the VZ/SVZ of the vLGE and MGE. The obvious ventral to dorsal gradient in GSX2^{+} cell numbers in the *Vax1*$^{-/-}$ mice disappeared (Fig. 3a-c'). Even more remarkably, *Prokr2*, *Tshz1*, *Etv1*, and *Sp8* showed an increased gradient expression in the dLGE (Fig. 3d-o'). *Gsx2* is expressed in proliferating progenitors, *Sp8* is found in some dividing cells, and *Prokr2* is observed in post-mitotic immature interneurons, suggesting the blockage of neuronal differentiation in the dLGE after *Vax1* gene deletion. The expression of these markers (*Gsx2*, *Sp8* and *Prokr2*) was also significantly increased in the *Vax1*$^{-/-}$ mice at P0 (data not shown). Taken together, the progenitors and immature neurons in the dLGE are significantly increased in the *Vax1*$^{-/-}$ mice.

3.4 The vLGE derived striatal MSNs were reduced in *Vax1* mutant mice

We showed above that the dLGE was expanded, next we wanted to know whether the vLGE and MGE-derived neurons changed or not. *Isl1* was highly expressed in D1-type MSNs [30, 31]. At E16.5, *Isl1*$^{+}$ cells were significantly reduced in the LGE SVZ and MZ of the *Vax1*$^{-/-}$ mice (Fig. 4a-c', m). More than 90% striatal MSNs are expressed *Foxp1*. The number of FOXP1^{+} cells in the *Vax1*$^{-/-}$ mouse SVZ and MZ was greatly reduced at E16.5 (Fig. 4d-f', m). Striatal projection neurons were mainly composed of dopamine D1 receptor (*Drd1*) expressing MSNs and dopamine D2 receptor (*Drd2*) expressing MSNs. In situ hybridization of *Drd1-mRNA* and *Drd2-mRNA* showed that *Drd1*$^{+}$ and *Drd2*$^{+}$ MSNs were also reduced in *Vax1* mutant mice (Fig. 4g-l'). These results suggest striatal MSNs are decreased in *Vax1*$^{-/-}$ mice at E16.5.

At P0, we can see a thicker SVZ in *Vax1*$^{-/-}$ mice from the nuclear staining of DAPI (See Supplementary Fig. S2a, b, Additional File 1). The partial sequences of exon 2 and exon 3 of the *Vax1* gene were replaced by a *LacZ* reporter gene in the *Vax1*$^{-/-}$ mice. In situ hybridization of *LacZ-mRNA* showed a marked increase of LacZ^{+} cells, suggesting that most *Vax1* mutant cells were still there (See Supplementary Fig. S2 c, d, Additional File 1). *Ki67* is expressed in dividing cells and the KI67^{+} region was the thicker LacZ^{+} SVZ

region in *Vax1*$^{-/-}$ mice (See Supplementary Fig. S2e, f, Additional File 1), whereas, FOXP1^{+} cells were rarely detected (See Supplementary Fig. S2g-h', Additional File 1). Altogether, these results indicated the blockage of striatal MSN differentiation in the *Vax1*$^{-/-}$ mice.

Only about 6% *Vax1*$^{-/-}$ mice can survive to P20, and we were fortunate to have collected two P20 *Vax1*$^{-/-}$ mouse brains. We then performed DAPI, and in situ hybridization staining of *Drd1* and *Drd2*. We observed the absence of the septum and preoptic area in the *Vax1* mutant mice, the change in the shape of the striatum, and that *Vax1* mutants had enlarged lateral ventricles (See Supplementary Fig. S3a, a', Additional File 1). The *Vax1* mutants had a low density of *Drd1-mRNA*$^{+}$ and *Drd2-mRNA*$^{+}$ in the medial striatum (See Supplementary Fig. S3b-c', Additional File 1). Taken together, our results showed the number of vLGE derived striatal MSNs was reduced in the *Vax1*$^{-/-}$ mice.

3.5 The MGE domain had defects in the *Vax1*$^{-/-}$ mice

Since the dLGE domain is enlarged and the vLGE domain is atrophic in the *Vax1*$^{-/-}$ mice, we wanted to further investigate the change of the MGE domain in the *Vax1* mutants. We then performed in situ hybridization staining of *Nkx2.1*, *Lhx6* and *Lhx8* at E13.5. The induction and regional pattern formation of MGE are dependent on the transcription factor *Nkx2.1* [13]. *Nkx2.1* plays an important role in maintaining the specificity and differentiation of MGE progenitors [12, 32]. The *Nkx2.1*$^{+}$ MGE domain in the *Vax1*$^{-/-}$ mice was significantly smaller than that in the controls (Fig. 5a-c'). LIM-homeodomain transcription factors *Lhx6*, and *Lhx8*, are expressed in the MGE SVZ, and are crucial for the migration and differentiation of MGE-derived neurons. In *Vax1*$^{-/-}$ mice, the expression of *Lhx6* and *Lhx8* was greatly reduced (Fig. 5d-i'). Thus, the interneurons in the neocortex were reduced by 30%-44% after *Vax1* gene deletion [20], possibly due to the smaller MGE domain in the *Vax1*$^{-/-}$ mice. The progenitors in the MGE not only give rise to cortical interneurons, but also striatal interneurons and globus pallidus neurons. Many transcription factors have been found to be expressed in the globus pallidus, such as: *Nkx2.1*, *Gbx1*, *Gbx2*, *Arx*, *Dlx1*, *Etv1*, *Lhx6*, and *Lhx8* [33]. In order to know clearly the changes in the globus pallidus, we performed in situ hybridization staining of *Nkx2.1*, *Gbx1*, and *Gbx2* at E12.5 in the *Vax1*$^{-/-}$ and control mice. We also detected *Etv1*, *Nkx2.1*, *Lhx6* and *Lhx8* at E18.5. There was a reduction in globus pallidus neurons after *Vax1* deletion (See Supplementary Fig. S4, Fig.S5, Additional File 1). In summary, the MGE domain gets smaller and MGE-derived neurons are decreased in the *Vax1*$^{-/-}$ mice.

3.6 RNA-Seq analysis provided further molecular evidence for defects in *Vax1* mutant mice

To characterize the molecular changes in *Vax1*$^{-/-}$ mice, we performed RNA-Seq analysis. Gene expression profiles from the embryonic day (E) 13.5 and E16.5 ganglionic eminence (GE) were analyzed. Changed expression of genes revealed by the RNA-Seq was consistent with our staining results, above. At E13.5, there was a remarkable increased expression of *Gsx2* and *Sp8* in the GE, with significantly decreased *Isl1*, *Ebf1*, *Foxp1*, *Tac1*, *Otx2*, *Lhx8*, *Gbx2*, and *Gbx1* (Table1). At E16.5, the dLGE markers (*Sp8*, *Etv1*, *Tshz1*) were up-regulated and the vLGE- derived neuronal markers (*Foxp1*, *Foxo1*, *Ikzf1*, *Ebf1*, *Gpr88*, *Tac1*, *Sox8*,

Zfp503, *Drd1*, *Ppp1r1b*, *Gpr6*, *Isl1*, *Zcchc12*, *Adora2a*) were down-regulated, with an upregulation of genes that promote cell cycle and maintenance of the progenitor state (Table2). The combined with the above staining results, compared with controls in the *Vax1*$^{-/-}$ mice, showed that the dLGE cells invaded ventrally into the vLGE, causing the dLGE region to get larger while the vLGE and MGE regions get smaller.

3.7 Increased expression of *Gsx2* in the vLGE and MGE in *Vax1* mutant mice

Previous studies showed that *Vax1* is a fundamental regulator of ventral identity for retinal ganglion cells and *Gsx2* is a key regulator of dLGE identity for OB interneurons [4, 34-37]. In an attempt to understand the relationship between *Vax1* and *Gsx2*, we performed immunofluorescence or in situ RNA hybridization staining of *Gsx2* or *Vax1* from immediate adjacent 20 μm sections in *WT* mice at E11.5 and E16.5. We observed *Gsx2* expression as a ventral-low to dorsal-high gradient in the subpallium (Fig. 6a-c). In contrast, *Vax1* was expressed in a ventral-high to dorsal-low gradient along the subpallium (Fig. 6b-d). The expression pattern of *Vax1* is largely complementary to that of *Gsx2*, supporting our hypothesis that *Vax1* may be crucial for subpallium regionalization. To test this, we performed immunofluorescence or in situ RNA hybridization of *Gsx2* at early stages, and our results showed that the expression pattern of *Gsx2* (ventral-low to dorsal-high) was disrupted in the *Vax1*$^{-/-}$ mice at E11.5 and E13.5 (Fig. 7a-d). *Gsx2* was highly expressed in the whole subpallium VZ and SVZ in the *Vax1*$^{-/-}$ mice suggesting that *Vax1* may be as an inhibitor to suppress the expression of *Gsx2* in the subpallium VZ and SVZ. Altogether, these results suggest that *Vax1* may regulate subpallium regionalization by repressing *Gsx2*.

3.8 *Vax1*inhibits the expression of *Gsx2* in the progenitor cells.

To further investigate whether *Vax1* inhibits the expression of *Gsx2* during forebrain development, we over-expressed *Vax1* in the lateral neural progenitors by electroporation and analyzed the impact on *Gsx2* expression. A *Vax1* expression plasmid (pCAG-*Vax1*-GFP) or a control vector (pCAG-GFP) was electroporated into the lateral ventricular wall at P0 in the *WT* mice. Three days later, animals were sacrificed and the number of total GFP^{+} or GFP/GSX2 double positive cells was measured in the dorsal-lateral region (Fig. 8a-b). Our results showed no difference in the total number of GFP^{+} cells between control and pCAG-*Vax1*-GFP mice (Fig. 8c). However, the number of GFP/GSX2 double positive cells showed a significant decrease (5-fold) in the pCAG-*Vax1*-GFP mice compared to control mice (Fig. 8a'-b' and d). Thus, we conclude that in progenitor cells, *Vax1* has the capacity to act as a negative regulator of *Gsx2* expression.

4 Discussion

In this study, we investigated the function of the *Vax1* gene in subpallium regionalization using *Vax1*$^{-/-}$ mice. Our results showed an increase in cell proliferation and the blockage of neuronal differentiation in the subpallium of the *Vax1*$^{-/-}$ mice. More importantly, the dLGE expanded to the whole subpallium VZ and SVZ in the *Vax1*$^{-/-}$ mice (Fig. 9a). We found that the *Gsx2* was highly expressed in the whole subpallium VZ and SVZ caused by *Vax1* gene deletion (Fig. 9b). Overexpression of *Vax1* in the LGE progenitors

strongly inhibits the expression of *Gsx2*. Thus, we proposed that *Vax1* may regulate the subpallium regionalization by suppressing *Gsx2* expression.

4.1 The dLGE, vLGE and MGE domain in the *Vax1-KO* mice

The full name of the *Vax1* gene is ventral anterior homeobox 1. The *Vax1* gene also is a member of the *Emx* and *Not* gene families [17]. The genes of the *Emx* and the *Not* gene families play an important role in the patterning development of the regions where they are expressed. In the $Vax1^{-/-}$ mice, the progenitors in the dLGE/dSVZ domain are significantly increased, vLGE-derived striatal projection neurons are significantly decreased, MGE progenitors are decreased and the septal area is absent. This suggests that the *Vax1* gene regulates the regionalization of the subpallium. It seems that the dLGE region was significantly expanded ventrally in $Vax1^{-/-}$ mice and the dLGE was enlarged at the expense of the vLGE, MGE, and septum.

The dLGE progenitors mainly give rise to OB interneurons. *Prokr2*, *Tshz1*, *Etv1*, and *Sp8* showed increased gradient expression in the dLGE/dSVZ at E16.5 and P0. Since *Gsx2* is expressed in proliferating progenitors, *Sp8* is found in some dividing cells, and *Prokr2* is observed in post-mitotic immature interneurons, this would suggest the blockage of neuronal differentiation in the dLGE after *Vax1* gene deletion. Despite the significantly increased of dLGE/dSVZ progenitors, the blockage of neuronal differentiation and defects of migration, results in $Vax1^{-/-}$ mice with a severely hypoplastic OB [38].

There is a reduction of $Ebf1^{+}$, $Isl1^{+}$, $Foxp1^{+}$, $Drd1^{+}$, and $Drd2^{+}$ striatal projection neurons in the striatum of *Vax1* mutant mice. Despite the increase of cell proliferation in the $Vax1^{-/-}$ subpallium V-SVZ, the vLGE domain gets smaller and there is blockage of striatal MSN differentiation in the $Vax1^{-/-}$ mice, this thereby, led to the developmental defects of the striatum in the $Vax1^{-/-}$ mice.

MGE contributes approximately 70% of cortical interneurons and most globus pallidal projection neurons [39, 40]. The MGE domain got smaller and MGE progenitors were decreased in the $Vax1^{-/-}$ mice, leading to a reduction of cortical interneurons [20] and globus pallidal neurons.

The septum is an important structure that connects many important brain structures. It begins to develop in the early embryonic stage, from E10.5 to E14.5 when septal neurons are mainly produced [41]. Progenitors in the septum not only contribute to septal neurons but also OB interneurons [37, 42]. The septum is absent in the $Vax1^{-/-}$ mice suggesting that *Vax1* plays an important role in its development.

4.2 The relationship between *Vax1* and *Gsx2*

There was increased expression of *Gsx2* in the vLGE and MGE in *Vax1* mutant mice. The transcription factor *Gsx2* plays a powerful role in the development of the telencephalon [35] and is crucial for the dorsal-ventral patterning [16]. *Gsx2* and *Pax6* play important roles in the maintenance of the pallium and subpallium boundary [11, 43]. In *Gsx2* mutant mice, dLGE progenitor cells express markers of the ventral pallium (*Pax6*, *Ngn2*, *Tbr2* and *Dbx1*) during early telencephalic development. The dLGE is respecified into

a ventral pallium like structure [35, 44, 45]. *Vax1* (ventral-high, dorsal-low) and *Gsx2* (ventral-low, dorsal-high) display largely complementary patterns of expression in the developing subpallium, suggesting that *Vax1* may regulate ventral telencephalic development by repressing *Gsx2*. We performed in vivo electroporation to overexpress *Vax1* in the LGE progenitors, our results showed that *Vax1* can strongly inhibit the expression of *Gsx2*. These results suggested that *Gsx2* was down-regulated in the progenitors due to *Vax1* expression. We also performed EMSA (electrophoretic mobility shift assay) to verify whether VAX1 directly inhibits the expression of *Gsx2*. This may be due to the real motif which is not in the predicted motifs, however, we did not get a positive result.

4.3 *Gsx2* cooperated with *Pax6* and *Vax1* which are essential for telencephalic regionalization

The telencephalon is subdivided into molecularly and functionally distinct progenitor regions along the dorsal-ventral (D-V) axis that generate different subclasses of neurons. A number of developmental control genes are specifically expressed by progenitors in either the pallium or subpallium. For instance, progenitor cells in the pallium express *Emx1*, *Pax6*, and *Ngn2* [11, 46]. In the pallium, *Pax6* is critical for the progenitors' identity. The dLGE expands to the pallium in the *Pax6*$^{-/-}$ mice as detected by dLGE markers such as *Dlx1/2*, *Sp8* and *Etv1* [47]. Progenitors in the dLGE express *Gsx2*, *Sp8*, *Etv1*, *Tshz1* and *Prokr2*. In this region, *Gsx2* is a core regulator for dLGE identity. *Gsx2* function was found to be essential to maintain the molecular identity of early striatal progenitors and in its absence the pallium regulatory genes *Pax6* and *Ngn2* are ectopically expressed in the LGE [16]. Progenitors in the MGE or POA express *Nkx2.1*, *Lhx6*, *Lhx8*, and *Otx2*. In the *Nkx2.1* mutants, the MGE gets smaller and LGE progenitors invade into the MGE primordium [13, 33]. *Otx2* was also reported to be essential for promoting vMGE identity and repressing POA identity [14]. In this study, we found that *Vax1* was highly expressed in the subpallium (dorsal-low, ventral-high). *Vax1* functions as a repressor to inhibit *Gsx2* expression for subpallium regionalization. Overall, we speculated that *Vax1*, *Gsx2*, and *Pax6* are the key regulators of the progenitors' regionalization during telencephalic development.

4.4 The increase of cell proliferation and the blockage of neuronal differentiation in the *Vax1*$^{-/-}$ mice

In the *Vax1*$^{-/-}$ mouse subpallium, the expression of *Gsx1* is down-regulated. *Gsx2* maintains LGE progenitors in an undifferentiated state, whereas *Gsx1* promotes progenitor maturation and the acquisition of neuronal phenotypes [48]Therefore, that is the reason for the increase of cell proliferation and the blockage of neuronal differentiation in the *Vax1*$^{-/-}$ subpallium.

Declarations

Acknowledgements

Not applicable.

Ethics approval and consent to participate

All experiments conducted in this research study were in accordance with guidelines from Fudan University.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest statement

The authors declare that they have no competing interests.

Author contributions

All authors had full access to the data and take responsibility for the integrity of the results and the accuracy of the data analysis. Y.W., Z.Y., and Z.Z. designed the research and Y.W., Z.S., and Z.W. performed experiments and analysis. L.Y., G.L., Z.S., Y.D., H.D., Z.L., Y.Y., and X.L. helped conduct experiments. Y.W., Z.Y., and Z.Z. drafted the manuscript.

Consent for publication References

Not applicable.

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Tables

Table1: The significantly changed gene at E13.5

Gene Name	*Vax1*$^{-/-}$(FPKM)	*WT*(FPKM)	P-value	Gene Name	*Vax1*$^{-/-}$(FPKM)	*WT*(FPKM)	P-value
Gsx2	23.14	13.38	1.14E-05	*Sp8*	14.54	9.97	0.000109
Bmp2	0.33	0.15	0.019487	*Gsx1*	1.35	3.34	1.60E-07
Wnt8b	0.26	0.01	0.000507	*Olig2*	24.76	33.79	0.009027
Col2a1	9.47	3.43	1.03E-08	*Lhx8*	7.22	18.93	7.45E-08
Rpl21	9.39	3.54	9.74E-06	*Gbx2*	1.54	3.56	2.27E-05
Pcdhga9	9.16	1.67	2.00E-06	*Gbx1*	4.07	8.90	5.27E-08
Zfp125	6.61	2.51	0.018824	*Isl1*	13.07	19.52	0.000176
Pcdhgb5	5.09	1.22	0.003204	*Ebf1*	9.35	13.38	0.000134
Zbtb9	5.05	0.75	0.000337	*Pcdhgb8*	0.60	14.38	3.11E-07

Table2: The significantly changed gene at E16.5

Gene Name	*Vax1*$^{-/-}$(FPKM)	*WT*(FPKM)	P-value	Gene Name	*Vax1*$^{-/-}$(FPKM)	*WT*(FPKM)	P-value
Gsx2	48.57	21.93	6.51E-20	*Ikzf1*	5.13	95.76	4.1E-194
Sp8	129.05	40.62	3.97E-64	*Ebf1*	19.71	279.67	3.4E-186
Etv1	121.40	68.67	1.12E-11	*Gpr88*	40.54	157.96	1.2E-128
Tshz1	218.54	124.77	1.77E-34	*Foxp1*	136.78	509.22	1.6E-128
Id2	8.60	2.79	1.02E-13	*Foxo1*	11.09	72.42	1.1E-110
Btg2	71.76	53.39	1.31E-06	*Tac1*	1.56	30.79	9.2E-119
Cdk1	88.43	59.89	3.04E-08	*Sox8*	65.38	169.49	1.21E-97
Cdk6	103.61	63.07	0.000564	*Aldh1a3*	0.71	24.35	1.05E-83
Cdk17	183.13	114.80	4.78E-17	*Zfp503*	51.52	165.63	8.52E-66
Cdk2	63.56	45.21	9.82E-07	*Drd1*	2.29	16.78	7.88E-44
Cdk2ap1	184.03	118.16	2.69E-32	*Ppp1r1b*	4.07	19.33	4.32E-39
Cdca7	442.29	233.64	7.27E-32	*Gpr6*	0.51	2.58	5.01E-09
Hes5	43.41	29.83	2.17E-05	*Isl1*	13.88	212.04	1.8E-150
E2f1	59.81	31.77	9.53E-20	*Zcchc12*	23.66	154.32	1.2E-140
Rnd3	355.58	234.16	1.07E-18	*Adra2a*	1.06	6.39	1.47E-24
Ube2ql1	193.94	117.69	4.57E-35	*Pdyn*	0.55	4.13	5.49E-18
Smo	108.05	63.65	1.41E-27	*Zfp521*	18.90	90.34	1.7E-125
Gli3	117.85	55.41	1.62E-27	*Cadm1*	64.43	193.31	9.8E-122
Gli2	59.45	38.33	7.8E-08	*Syt4*	13.74	177.66	8.5E-302
Fgf3	9.48	4.97	6.73E-07	*Plk2*	39.49	228.74	2.1E-268
Wnt5a	52.20	14.95	1.87E-43	*Rarb*	8.12	109.08	2.4E-236
Tgif2	86.59	36.18	5.74E-48	*Nrxn1*	35.01	165.06	2.5E-222
Grik3	492.09	340.76	2.28E-08	*Cdh13*	7.57	43.06	2.49E-73
Slit1	208.78	126.45	9.4E-37	*Cdh8*	14.27	115.01	6.2E-174
Erbb4	184.81	142.53	4.59E-06	*Rph3a*	18.23	120.03	1.9E-165
Tcf4	771.87	374.27	2.59E-22	*Tgfa*	9.71	52.16	9.81E-55
Atp5e	63.64	1.61	1.7E-230	*Gsx1*	1.68	6.11	5.46E-15
Ppp1r14b	189.02	103.54	1.35E-47	*Nkx2-1*	14.10	63.19	5.18E-14
Sox5	78.28	36.11	3.3E-34	*Gbx2*	0.03	0.81	1.1E-07
Ascl1	211.46	142.65	4.75E-12	*Gbx1*	0.23	1.70	1.82E-05
Dlx1	1405.97	735.12	1.02E-09	*Olig1*	7.37	16.12	5.66E-07
Sp9	457.87	279.05	4.37E-16	*Sox10*	0.76	3.08	8.77E-06
Nr2f2	38.23	12.90	2.32E-33	*Calb1*	4.39	24.55	4.76E-33
Arx	399.99	310.76	3.42E-06	*Npy*	4.79	20.62	3.69E-39
Pbx1	582.92	418.62	4.01E-06	*Sst*	2.66	15.65	2.86E-28
Cux1	321.52	260.59	1.66E-05	*Cxcl14*	2.76	6.37	1.38E-07
Htr3a	17.76	3.92	4.92E-28	*Robo1*	136.64	295.71	7.08E-60
Prox1	115.39	41.09	1.22E-48	*Reln*	11.53	108.95	5E-178

Figures

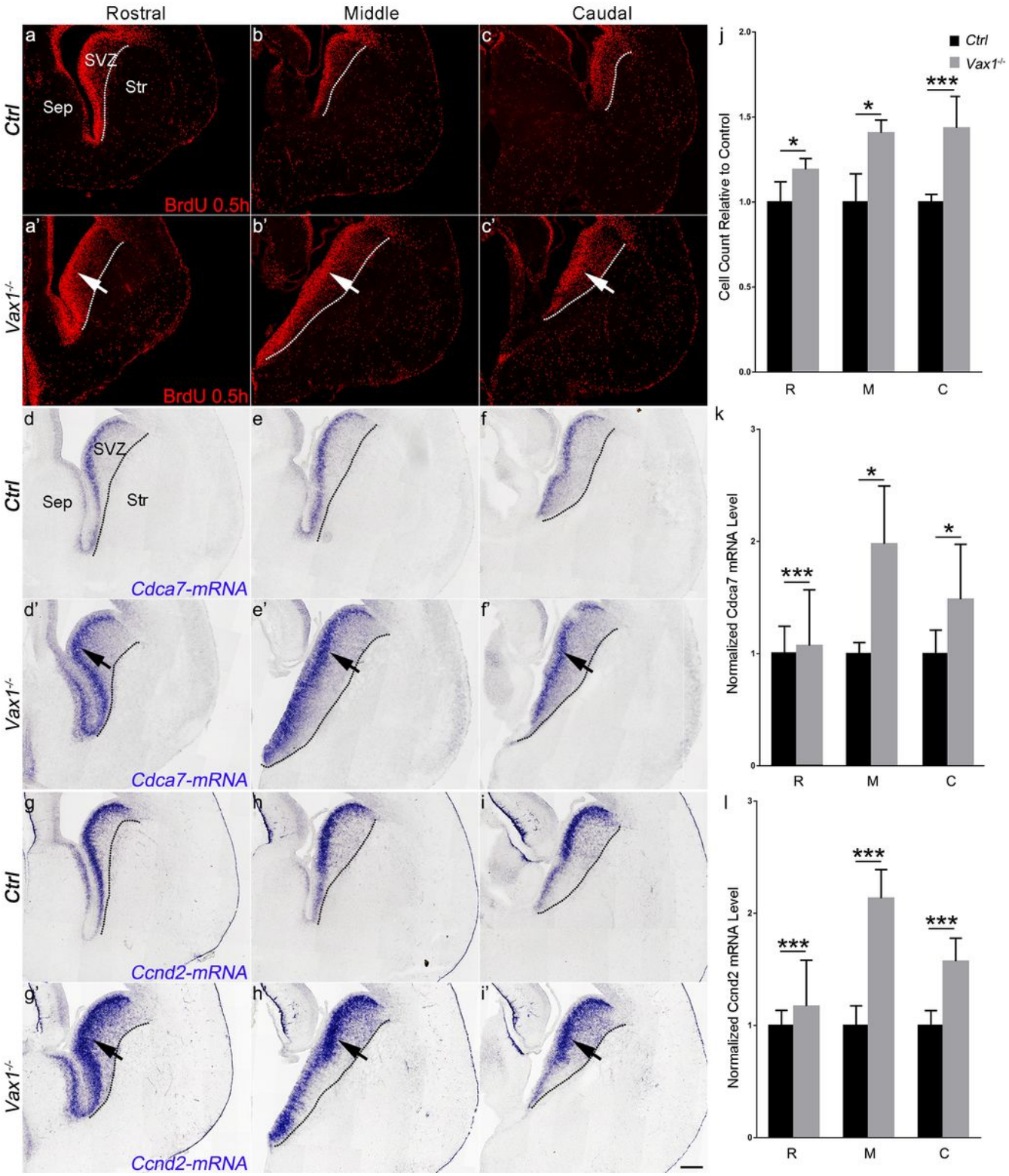

Figure 1

Increase of cell proliferation in the Vax1-/- subpallium V-SVZ at E16.5. (a-c') A single injection of BrdU was administered into mice at E16.5 and BrdU immunostaining was analyzed 30-minutes later. Immunofluorescence staining of BrdU showed that BrdU+ cells were significantly increased in the Vax1-/- subpallium V-SVZ. (d-i') In situ hybridization showed that Cdca7-mRNA+ and Ccnd2-mRNA+ cells were also significantly increased in the Vax1-/- subpallium. (j-l) Histograms show quantification data of BrdU,

Cdca7 and Ccnd2. (Student's t-test, *P < .05, ***P < .001, n=3 mice per group, mean ± SEM). Arrows indicate the number of BrdU, Cdca7 and Ccnd2 is increased. Abbreviations: Sep (septum), SVZ (subventricular zone), Str (Striatum), R (rostral), M(middle) and C (Caudal). Scale bars: 200 μm in i' for a-i'.

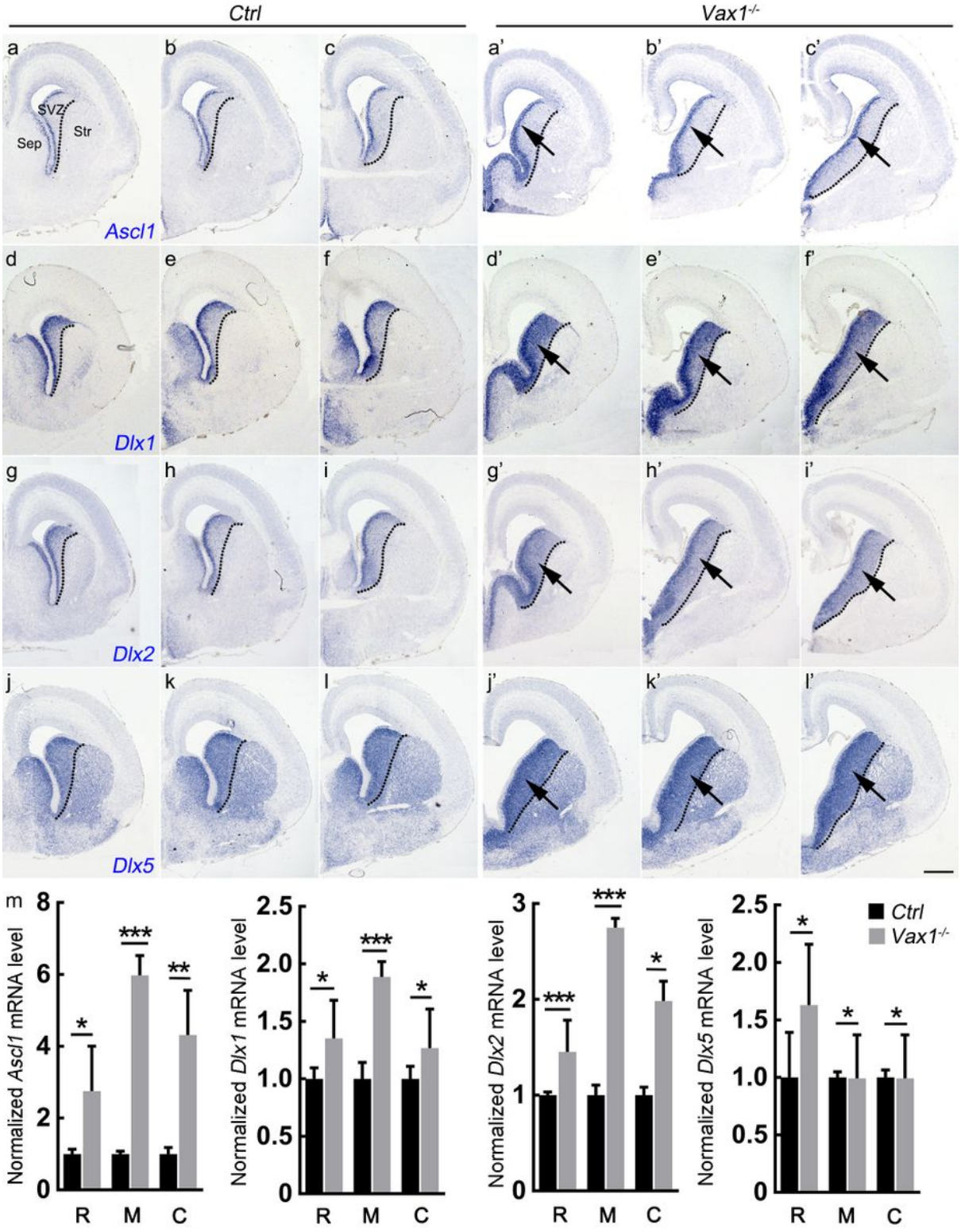

Figure 2

Neural progenitor cells accumulate in the subpallium V-SVZ of Vax1-/- mice. (a-l') In situ hybridization of Ascl1, Dlx1, Dlx2 and Dlx5 in the E16.5 Vax1-/- subpallium. Ascl1+, Dlx1+, Dlx2+, and Dlx5+ cells in the Vax1-KO subpallium were markedly increased. (m) The quantification data showed that the expression of the Ascl1, Dlx1, Dlx2 and Dlx5 was increased in the LGE SVZ of Vax1-KO mice. (Student's t-test, *P < .05, **P < .01, ***P < .001, n=3 mice per group, mean ± SEM). Scale bars: 500 μm in l' for a-l'.

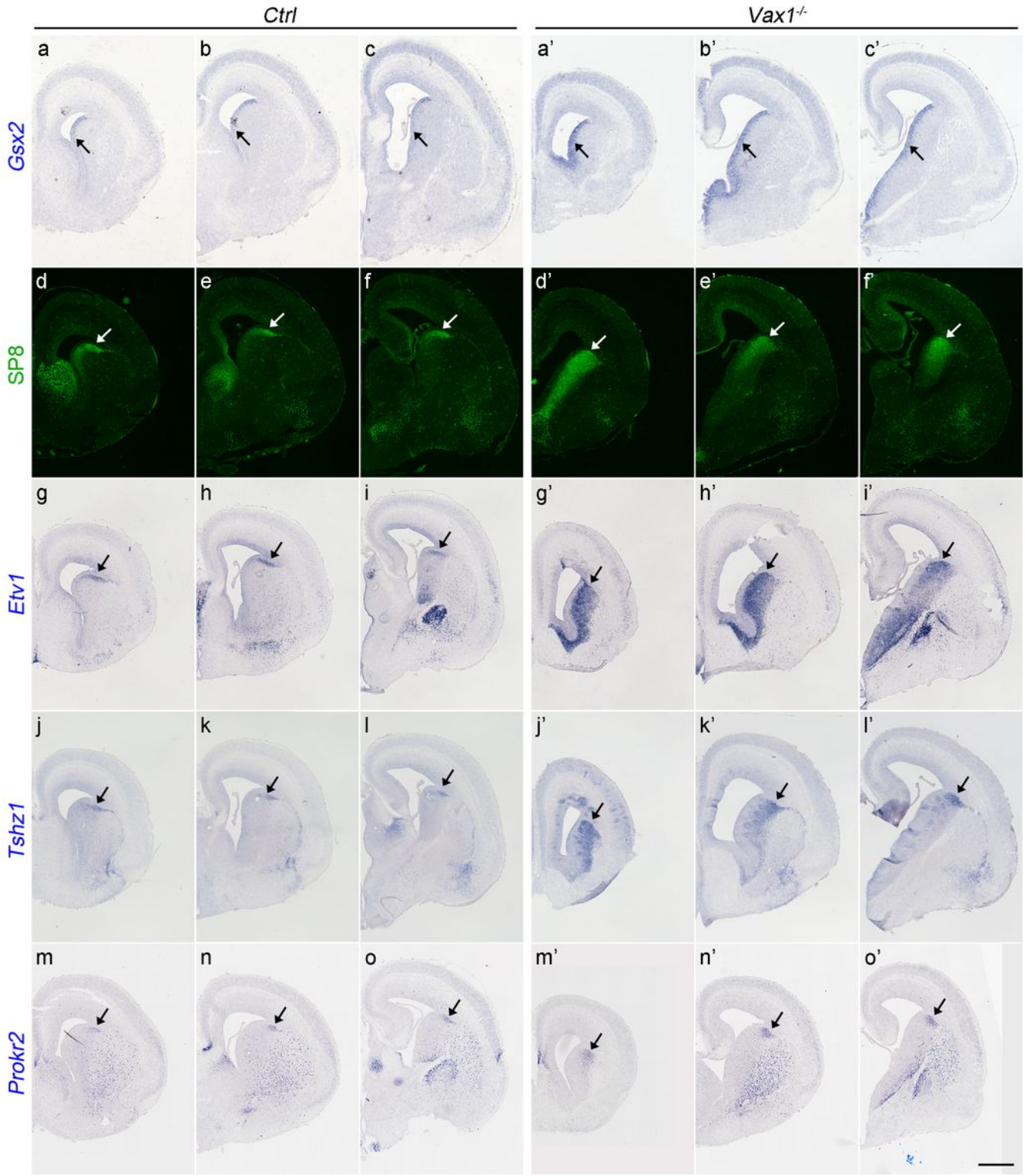

Figure 3

Increased expression of Gsx2, Sp8, Etv1, Tshz1 and Prokr2 in the E16.5 Vax1-/- mice. (a-c') In situ hybridization of Gsx2 showed increased expression of Gsx2 in the vLGE in Vax1 mutant mice. (d-f') SP8 immunostaining showed that the number of SP8+ cells in the Vax1-/- mouse dLGE was significantly increased at E16.5. (g-o') Etv1+, Tshz1+, and Prokr2+ cells in the Vax1-/- mouse dLGE were also increased. N = 3 mice per group. Scale bars: 500 μm in o' for a-o'.

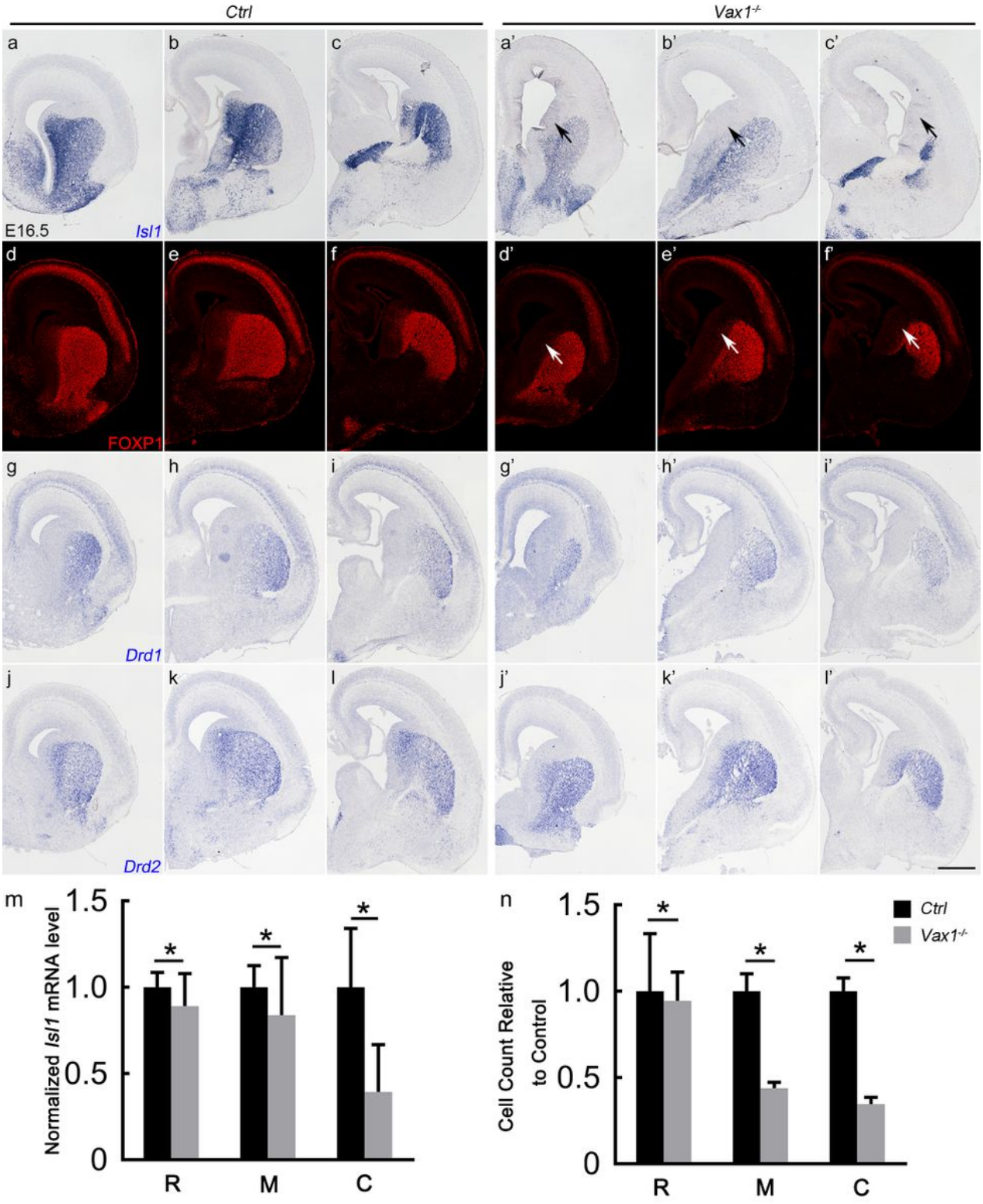

Figure 4

The vLGE-derived striatal MSNs were disrupted in Vax1-KO mice at E16.5. (a-c') In situ hybridization of Isl1 at E16.5 showed Isl1-mRNA+ cells in the vLGE SVZ were significantly decreased (arrows). (d-f') Immunofluorescence staining showed that the FOXP1+ cells in LGE SVZ were greatly reduced (arrows) at E16.5. (g-l') In situ hybridization of Drd1 and Drd2 showed Drd1+ and Drd2+ MSNs were reduced in Vax1 mutant mice. (m-n) Quantification data showed that the expression of Isl1 and Foxp1 was reduced in the LGE SVZ from rostral to caudal. (Student's t-test, *P < .05, n=3 mice per group, mean ± SEM). Scale bars: 500 μm in l' for a-l'.

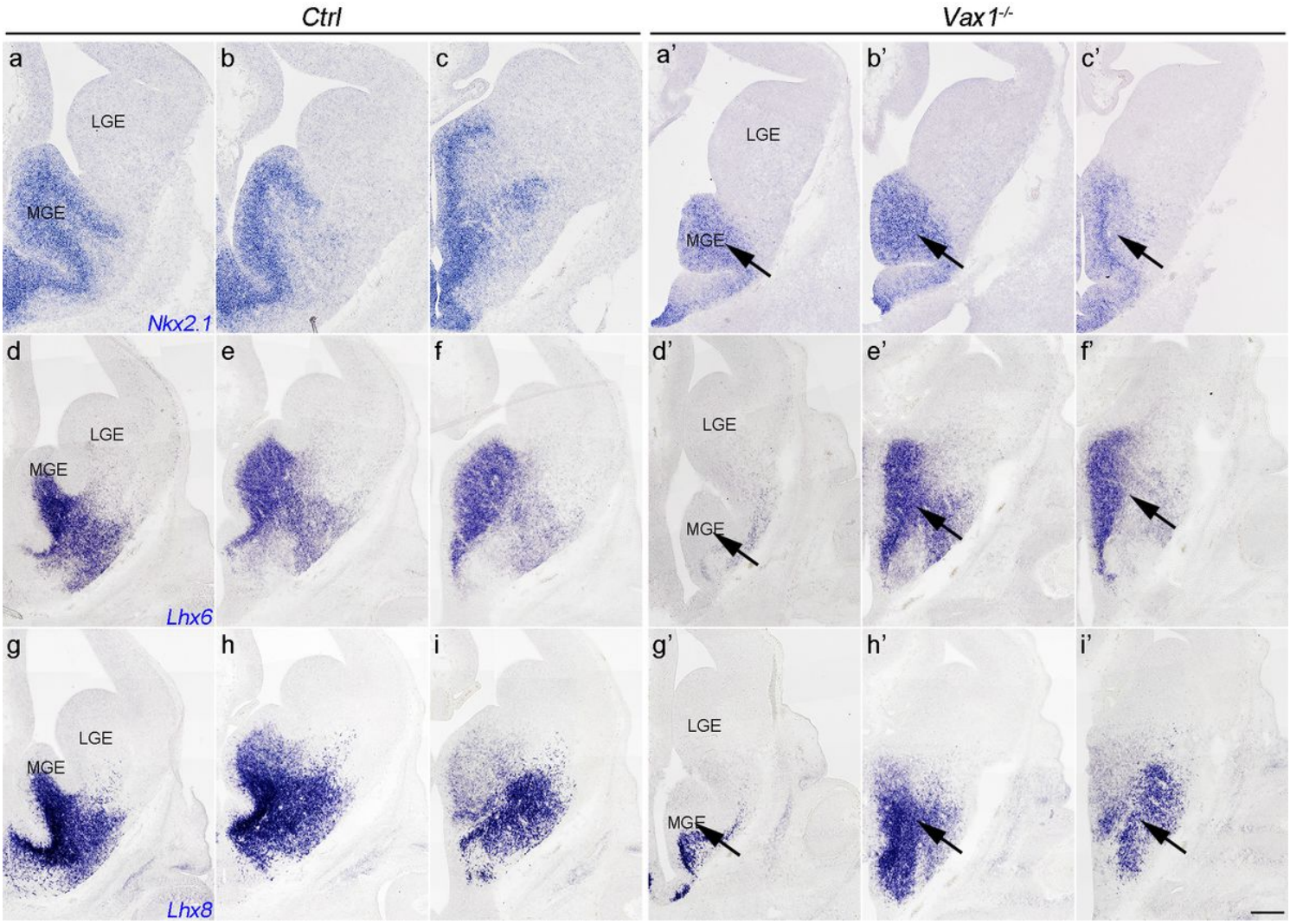

Figure 5

The MGE domain gets smaller in the Vax1-KO mice at E13.5. (a-i') In situ RNA hybridization staining for Nkx2.1, Lhx6 and Lhx8 at E13.5. The Nkx2.1+ domain in the Vax1-/- mice was smaller than that in the controls and the expression of Lhx6 and Lhx8 was greatly reduced in the MGE of Vax1 mutants. Arrows indicate the expression of Nkx2.1, Lhx6 and Lhx8 is decreased. N = 3 mice per group. Scale bars: 200 μm in i' for a-i'.

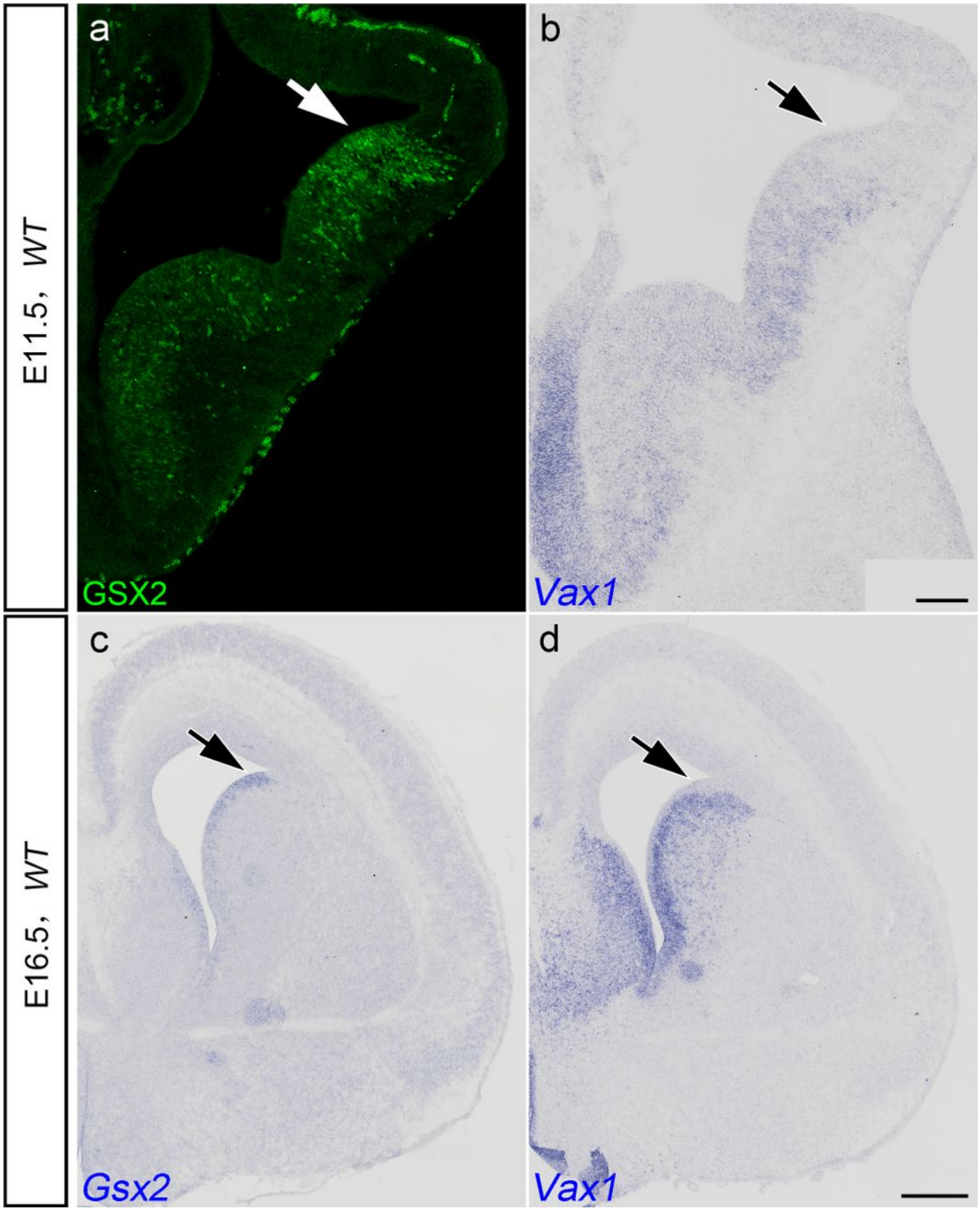

Figure 6

Complementary expression of Vax1 and Gsx2 in the subpallium. (a, b) Immunofluorescence staining of Gsx2 and in situ RNA hybridization staining of Vax1 from immediately adjacent 20-μm sections in WT mice at E11.5. Gsx2 was strongly expressed in the dLGE and the expression of Vax1 in this region was weak. (c, d) In situ RNA hybridization staining of Gsx2 and Vax1 from immediately adjacent 20-μm

sections in WT mice at E16.5; Vax1 and Gsx2 display largely complementary patterns of expression in the subpallium. N = 3 mice per group. Scale bars: 200 μm in b for a-b, 500μm in d for c-d.

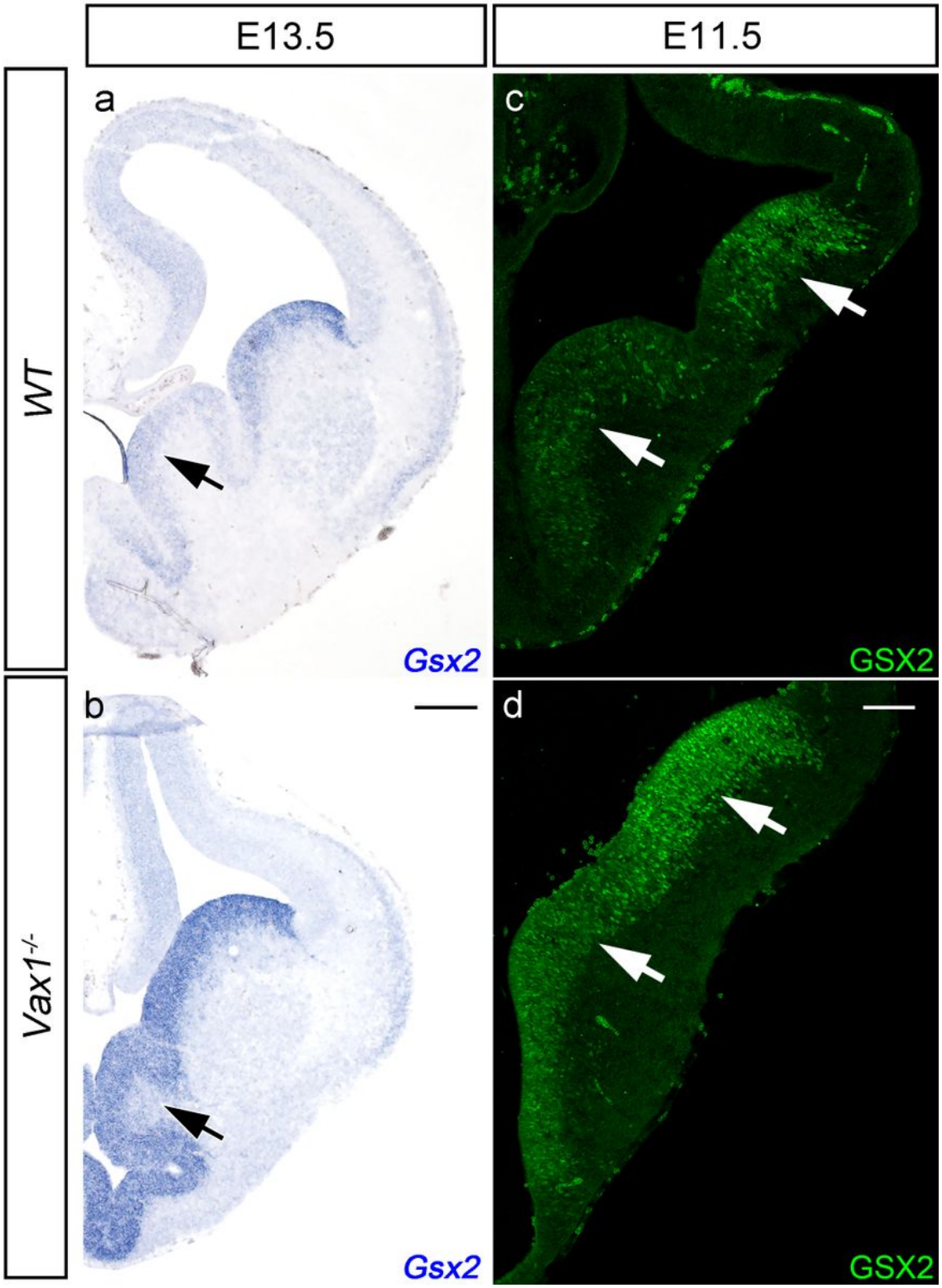

Figure 7

Increased expression of Gsx2 in the vLGE and MGE in Vax1 mutant mice. (a, b) The Gsx2+ cells were seen in the VZ of the vLGE and MGE and there was no longer an obvious ventral to dorsal gradient of the Gsx2 expression pattern in the Vax1-/- mice. (c, d) Immunofluorescence staining of GSX2 at E11.5. There

was increased expression of Gsx2 in the vLGE and MGE in the Vax1 mutant mice. N = 3 mice per group. Scale bars: 500 μm in b for a-b, 200 μm in d for c-d.

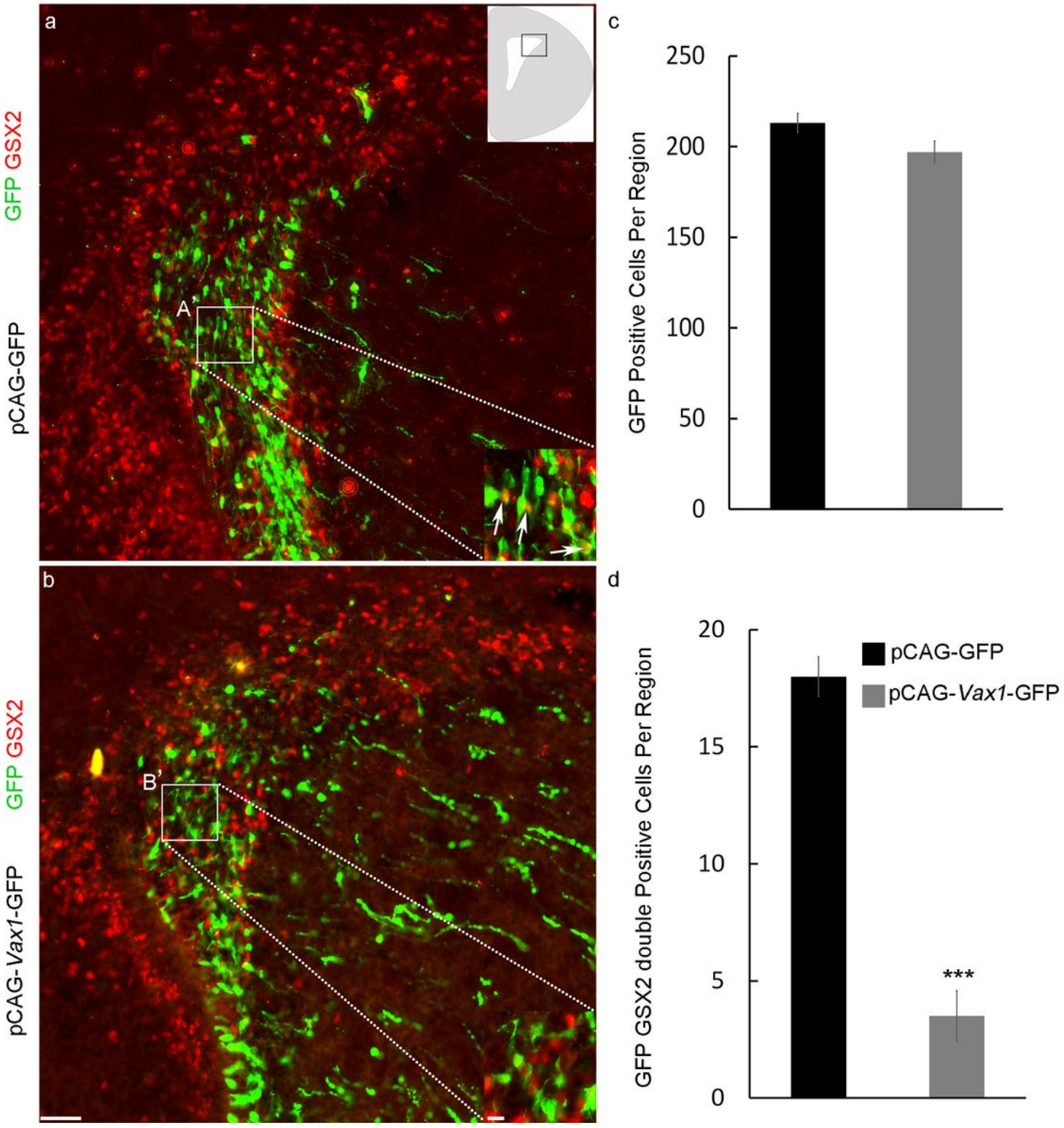

Figure 8

The expression of Gsx2 was suppressed by Vax1 in progenitor cells. (a) Many GSX2+ cells were co-labelled with GFP+ cells in control mice. (b) Very few GSX2 /GFP double positive cells were observed in Vax1 over-expressing mice. (c) Quantification of GFP+ cells in control or Vax1 over-expressing mice. (d)

Quantification GFP /GSX2 double positive cells in control or Vax1 over-expressing mice. (a', b') High-magnification images illustrating the downregulation of GSX2 in GFP+ cells after electroporation of the lateral V-SVZ by Vax1. White arrows: GFP / GSX2 double positive cells. Student's t-test, ***P < .0001, n=5 mice per group, mean ± SEM. N = 5 mice per group. Scale bars: 50µm in b for a-b, 20 µm in b' for a'-b'.

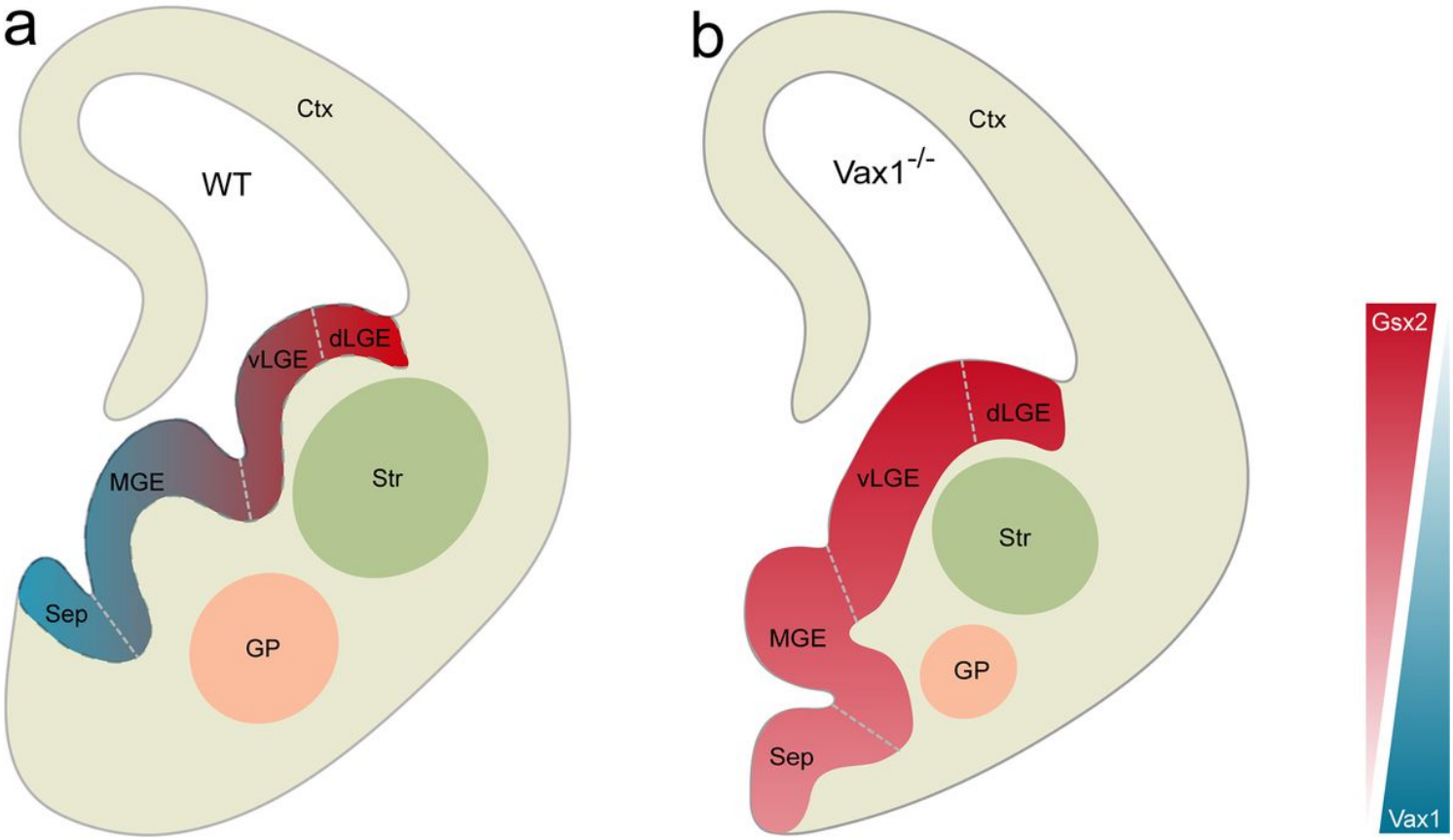

Figure 9

A model of Vax1 regulates subpallium regionalization. (a) The expression patterns of Vax1 and Gsx2 are largely complementary. Gsx2 expressed in a ventral-low to dorsal-high gradient while the Vax1 was expressed in a ventral-high to dorsal-low gradient along the subpallium. (b) Increased expression of Gsx2 in the vLGE and MGE leads to the abnormal cell proliferation and differentiation of the subpallium neurons in Vax1 mutant mice.

Supplementary Files

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- AdditionalFile1.pdf