
Figures and figure supplements

Stem cell regionalization during olfactory bulb neurogenesis depends on regulatory interactions between *Vax1* and *Pax6*

Nathalie Coré et al

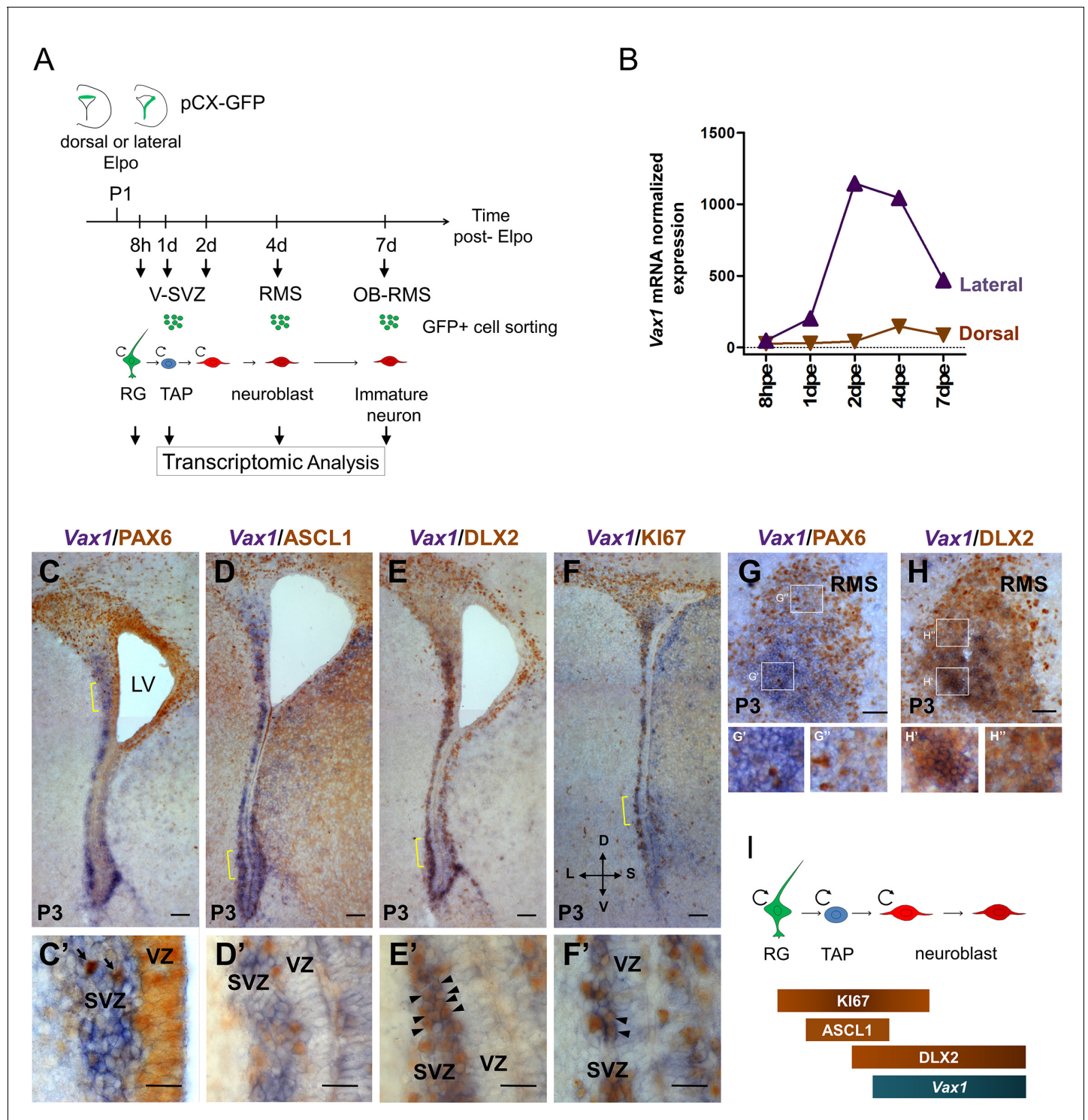


Figure 1. *Vax1* is expressed in the lateral V-SVZ. (A) Representation of the strategy used for transcriptomic analysis in time and space in the dorsal and lateral OB lineages. pCX-GFP plasmid was introduced into neural stem cells (NSCs) residing within the dorsal or lateral V-SVZ and GFP-positive cells were isolated by FACS at different time points after electroporation (Elpo). The mRNA content was analyzed by micro-array (Tiveron et al., 2017). (B) Quantification of *Vax1* mRNA expression detected by micro-array analysis in dorsal (brown) and lateral (purple) progenies during neurogenesis. (C–H) In situ hybridization revealing *Vax1* mRNA (in blue) combined with immuno-histochemistry using antibodies detecting (in brown) PAX6 (C, C', G), ASCL1 (D, D'), DLX2 (E, E', H) or KI67 (F, F') proteins in the V-SVZ (C–F) or RMS (G, H) at postnatal day 3 (P3). (C'–F') High magnification of cellular staining in the V-SVZ (area indicated by the yellow bracket in C–F). Arrows (C'): examples of strong PAX6 only positive cell in the dorso-lateral SVZ; blue staining underneath labels cells from a distinct plane. Arrow heads (E', F'): double positive cells for DLX2 and KI67, respectively. High magnification of the RMS

Figure 1 continued on next page

Figure 1 continued

highlights the differential expression of *Vax1* and *Pax6* along the dorso-ventral axis (**G',G''**) and the co-localization with *Dlx2* (**H',H''**). (I) Schematic representation of gene expression profile in different cell types of the neurogenic sequence. Circular arrow indicates proliferating cells. LV: lateral ventricle, RG: radial glia, TAP: transit amplified precursor, VZ: ventricular zone, SVZ: sub-ventricular zone. D: dorsal, L: lateral, S: septal, V: ventral. Scale bars: 100 μm (**C–F**), 20 μm (**C'–F'**), 50 μm (**G–H**).

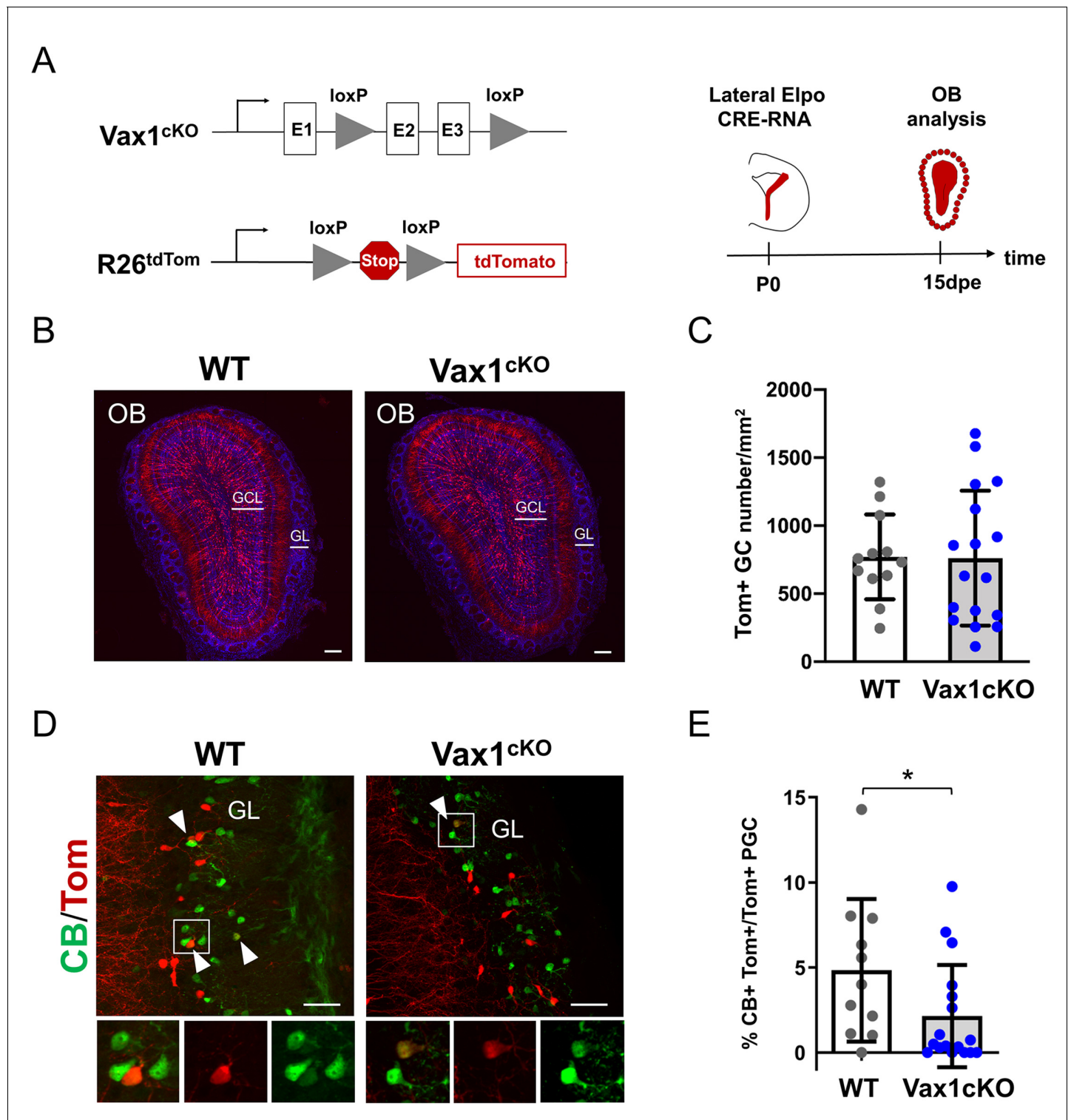


Figure 2. *Vax1* is necessary for the production of Calbindin-positive interneurons in the olfactory bulb. (A) Representation of the *Vax1* conditional allele (*Vax1*^{cKO}) and the inducible reporter *tdTomato* allele in the *Rosa26* locus (*R26*^{tdTom}). Right panel: strategy used to recombine the *Vax1* mutant allele in the V-SVZ cells in the lateral wall at postnatal day 0 (P0). *TdTomato* (Tom)-positive cells were analyzed 15 days post-electroporation (dpe) in the olfactory bulb (OB). (B) Images showing the distribution of Tom+ cells (in red) in the OB at 15dpe in control and mutant brains. Nuclei (in blue) are stained by Hoechst. (C) Quantification of granule cells (GC) number in the OB GCL in both conditions. Data are shown as means ± SD, dots represent individual animals. WT: n = 12, *Vax1*^{cKO}: n = 17. (D) Images showing Calbindin+ (in green) and Tom+ cells in the GL at 15dpe. Arrow heads indicate double stained neurons. High magnification of representative double positive cells is shown below. (E) Quantification of the percentage of Calbindin+ cells in the GL. *** indicates statistical significance (p < 0.05).

Figure 2 continued on next page

Figure 2 continued

neurons among the Tom+ PGC population (WT: n = 11, Vax1cKO: n = 17) showing reduction of CB-N in the mutant. GCL: granule cell layer, GL: glomerular layer. * $p \leq 0.05$. Scale bars: 200 μm (B), 50 μm (D).

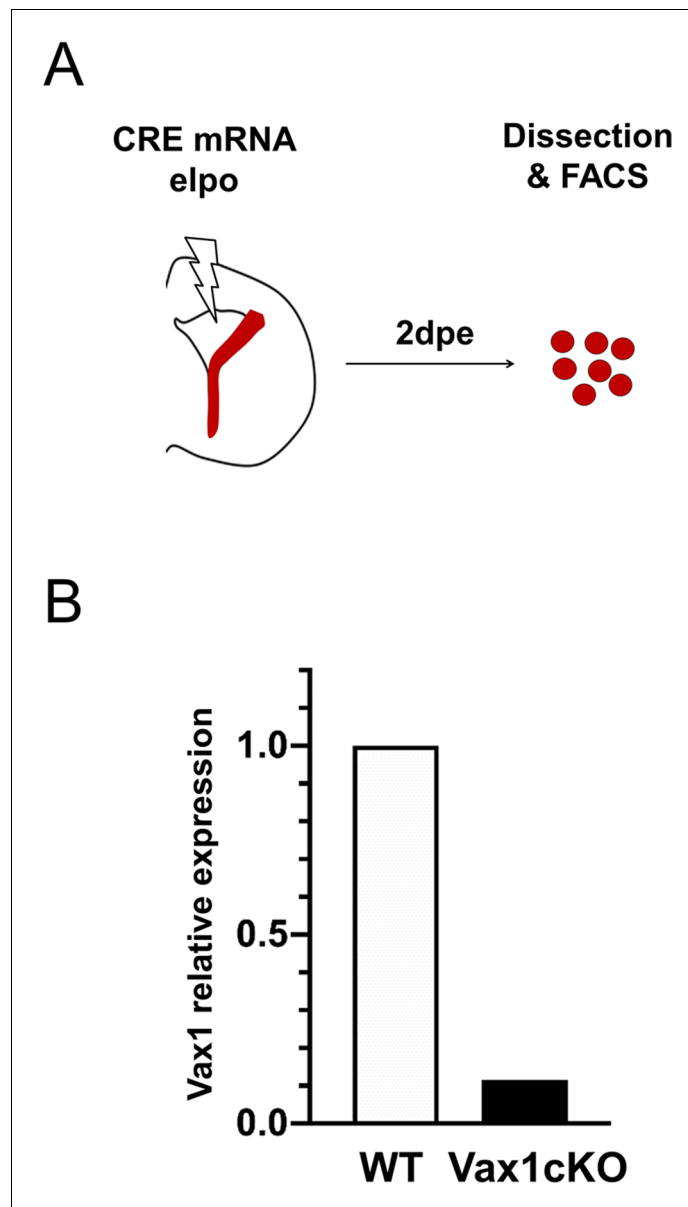


Figure 2—figure supplement 1. CRE-recombination of the *Vax1*^{fl^{ox}} allele in progenitors induce a substantial reduction of *Vax1* mRNA expression in the homozygote mutant (*Vax1*cKO) compared to WT animal. (A) Strategy used to target lateral V-SVZ NSCs with *Cre*-mRNA. Tomato+ recombined cells were isolated 2 days after electroporation. (B) *Vax1* mRNA level quantified by RT-PCR was normalized to beta-actin and reported in *Vax1*cKO condition as relative level to control (WT).

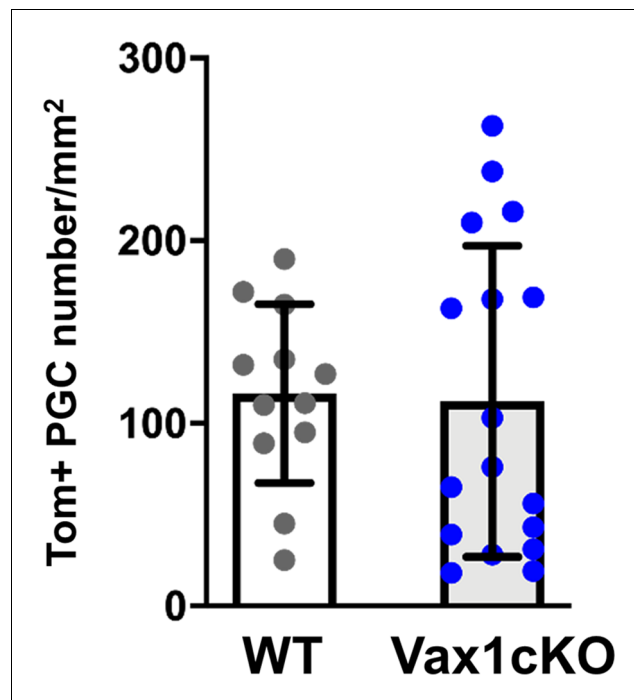


Figure 2—figure supplement 2. Quantification of tdTomato-positive periglomerular cell (PGC) number in the OB of *Vax1* mutant brains. Cells were analyzed 15 days after electroporation of lateral V-SVZ progenitors by *Cre* mRNA in WT or *Vax1*cKO mice. Data are shown as means \pm SD, dots represent individual animals. WT: n = 12, *Vax1*cKO: n = 17.

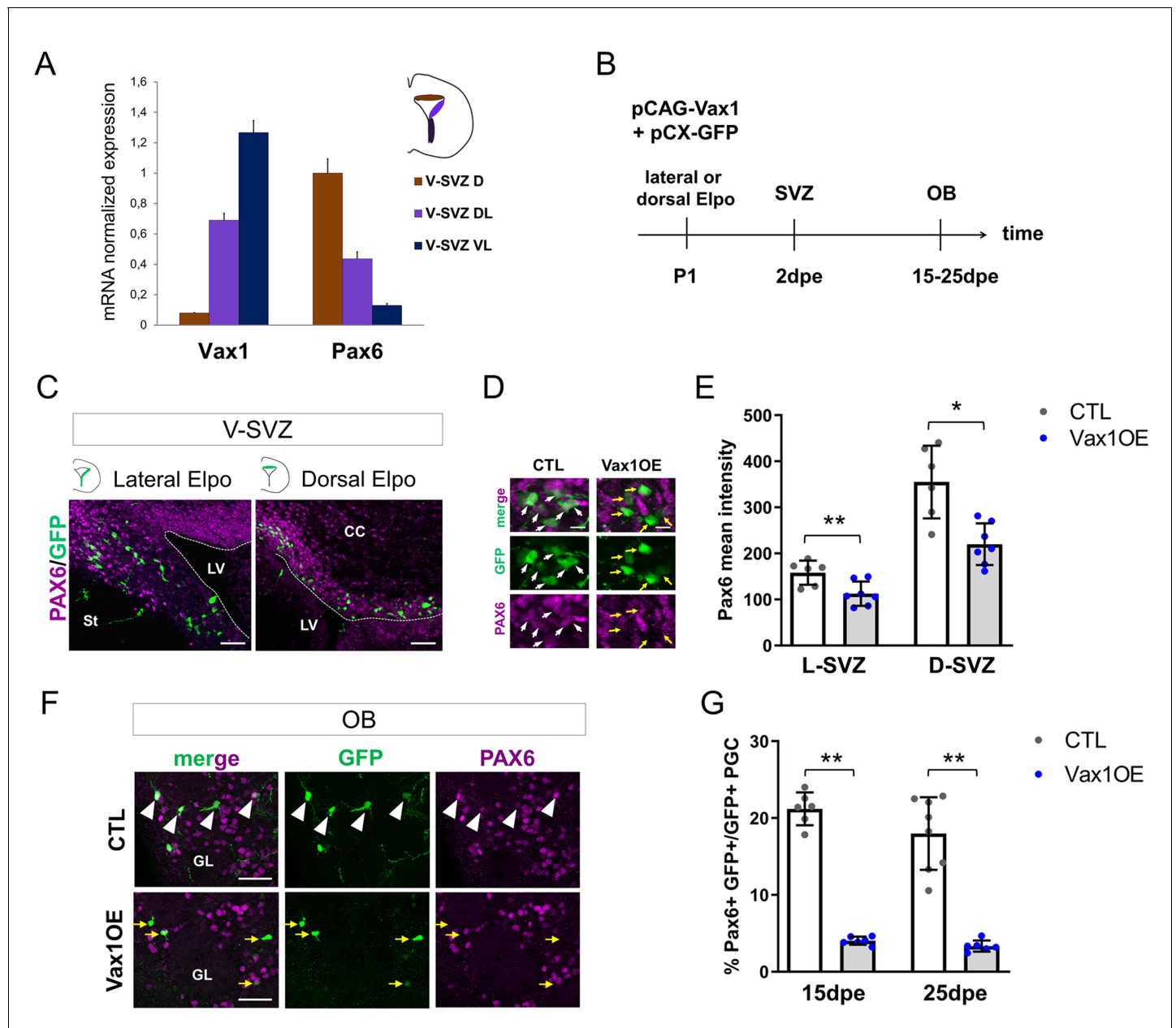


Figure 3. *Vax1* inhibits PAX6 expression in the V-SVZ and the OB. (A) Quantitative RT-PCR revealing *Vax1* and *Pax6* gene expression in tissue micro-dissected from three distinct areas of the V-SVZ. D: dorsal, DL: dorso-lateral, VL: ventro-lateral. (B) Strategy design for the *Vax1* gain-of-function experiment. The *Vax1* expressing plasmid (pCAG-Vax1) was introduced into lateral or dorsal progenitors in combination with pCX-GFP by electroporation at P1. Brains were analyzed at different time points in the V-SVZ or the OB. (C) Representative images showing simultaneous expression of PAX6 and GFP proteins in dorsal or lateral lineage in the V-SVZ. (D) High-magnification images illustrating the downregulation of Pax6 in GFP+ cells after electroporation of lateral V-SVZ by *Vax1*. White arrows: GFP/Pax6 double positive cells, yellow arrows point to cells with reduced or absent Pax6 expression. (E) Quantification of PAX6 mean intensity in control or *Vax1*-overexpressing (OE) V-SVZ GFP+ cells from dorsal (D, $n = 6$ for the control, $n = 7$ for *Vax1* condition) or lateral (L, $n = 6$ for the CTL, $n = 7$ for *Vax1* condition) walls. (F) Images showing simultaneous expression of PAX6 and GFP in the OB glomerular layer of control or *Vax1*OE brains. Arrow head: double GFP/PAX6-positive cells; yellow arrow: GFP only cells. (G) Quantification of GFP+PAX6+ neurons in the OB GL at 15dpe ($n = 6$ for the CTL, $n = 6$ for *Vax1*OE) and 25dpe ($n = 8$ for the CTL, $n = 6$ for *Vax1*OE). PGC: periglomerular cell. $*p \leq 0.05$, $**p \leq 0.01$. Scale bars: 50 μm (C,F), 10 μm (D).

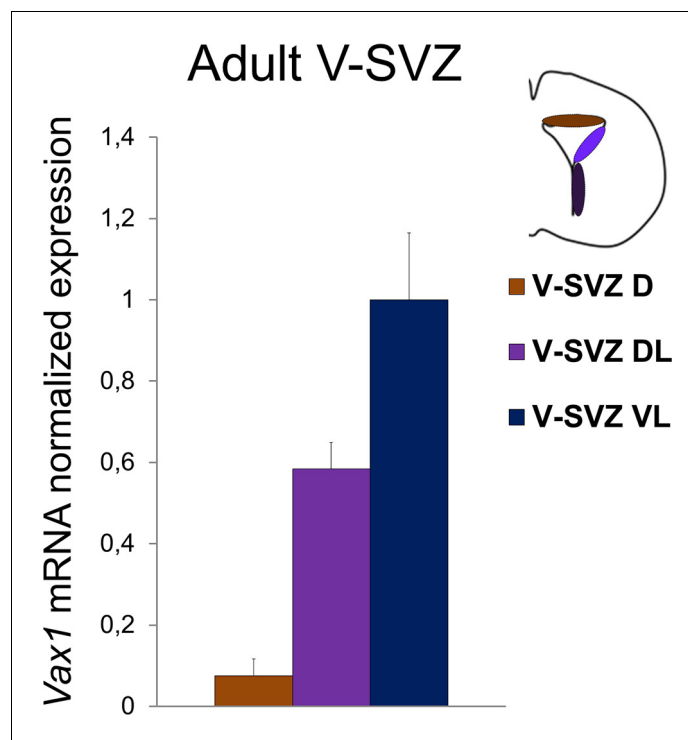


Figure 3—figure supplement 1. Quantitative RT-PCR revealing *Vax1* gene expression in adult brain. Cells were dissected out from three distinct areas of the V-SVZ. Error bars represent technical triplicate. D: dorsal, DL: dorso-lateral, VL: ventro-lateral.

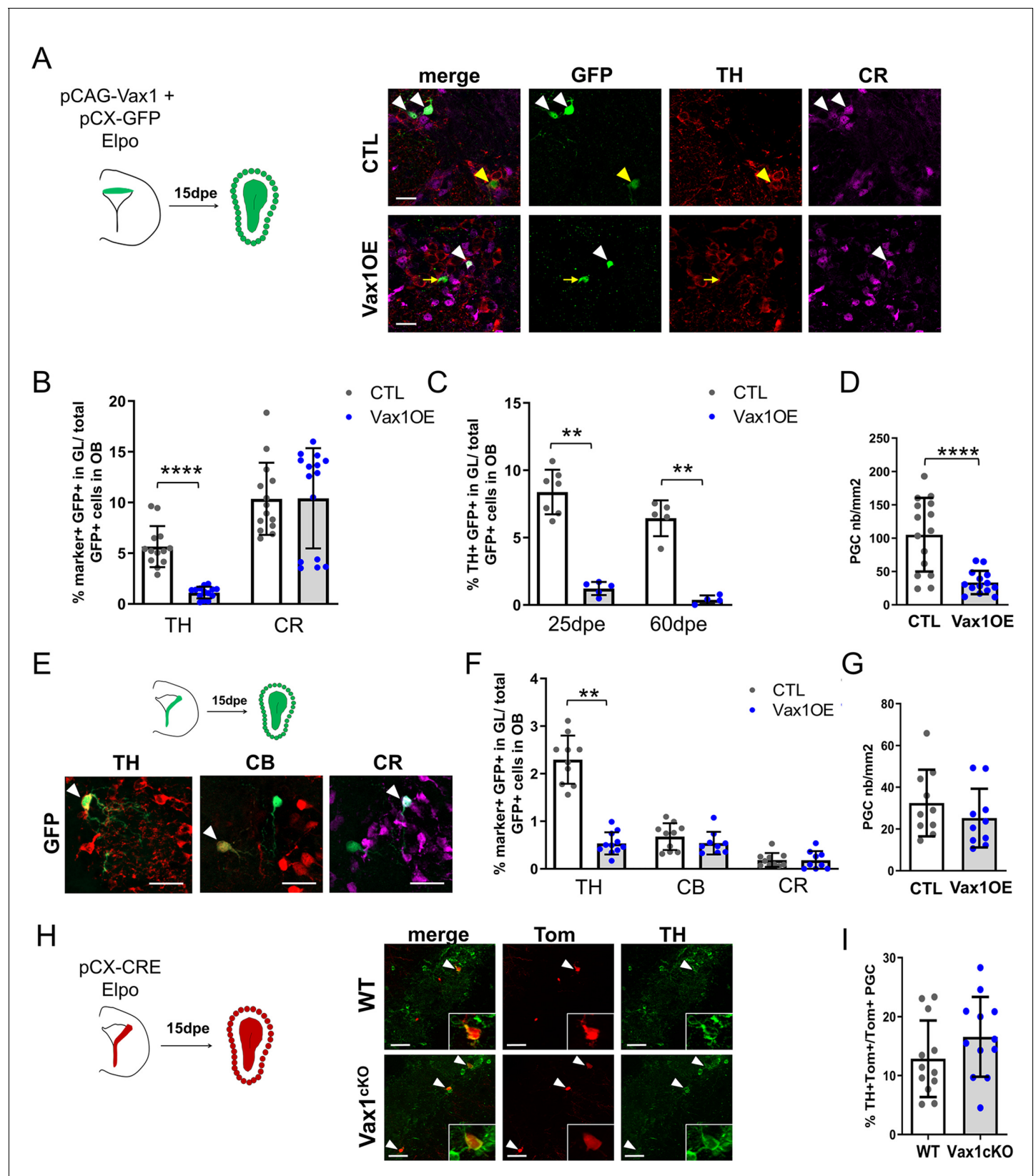


Figure 4. Overexpression of Vax1 in V-SVZ neural stem cells inhibits dopaminergic phenotype. (A) Experimental design (left) for the electroporation of NSCs in the dorsal wall with pGAC-Vax1 + pCX-GFP. Images (right) showing expression of Tyrosine Hydroxylase (TH) and Calretinin (CR) in the OB
Figure 4 continued on next page

Figure 4 continued

glomerular layer 15 days after electroporation in *Vax1* over-expression (OE) and control brains. White arrow head: GFP/CR- double positive neuron, yellow arrow head: GFP/TH double positive neuron, yellow arrow: GFP-only cell. (B) Histogram showing the reduction of the density of GFP+ periglomerular cells (PGC) in the *Vax1*OE OB (CTL *n* = 15, *Vax1* *n* = 14). (C) The quantification of TH+ and CR+/GFP-positive cells shows a large decrease of the proportion of dopaminergic neurons among the total GFP+ cells in the OB of *Vax1* condition (TH *n* = 14, CR *n* = 15) compared to control (*n* = 13/14). (D) The reduction of the TH+ population is sustained with time as it is still observed at 25- (CTL *n* = 7, *Vax1* *n* = 5) and 60- (CTL *n* = 5, *Vax1* *n* = 4) days post electroporation. (E) Experimental design (left) for the electroporation of NSCs in the lateral wall with pGAC-*Vax1* + pCX-GFP. Representative images (right) of immunostaining with TH, Calbindin (CB), and CR antibodies in the OB GL. Arrow head: example of double positive staining with GFP for each marker. (F) Histogram presenting the quantification of the three different neuronal populations among the GFP+ neurons in the OB of control (*n* = 10 for each marker) or *Vax1*OE (TH *n* = 11, CB and CR *n* = 9) conditions. (G) Histogram showing the density of GFP + PGC in both conditions (CTL *n* = 10, *Vax1* *n* = 10). (H) Lateral NSCs of *Vax1*cKO: *rosa26tdTom* brains were electroporated at birth with pCX-CRE and neuronal phenotype was analyzed in OB at 15 dpe. Representative images of TH staining in the GL of control or *Vax1* deficient OB. Arrow head: GFP+ cells co-labelled with TH. Insert: high magnification of a double positive neuron. (I) Histograms presenting the percentage of TH+ neurons among Tom + PGC (CTL: *n* = 12, three independent litters; *Vax1*: *n* = 12, three independent litters). A slight increase of the TH+ population was observed in absence of *Vax1* compared to control but statistical test (Mann Whitney U test) failed to give significant *p* values (*p*=0.16). ***p*≤0.01, *****p*≤0.0001. All scale bars: 20 μm except in H (50 μm).

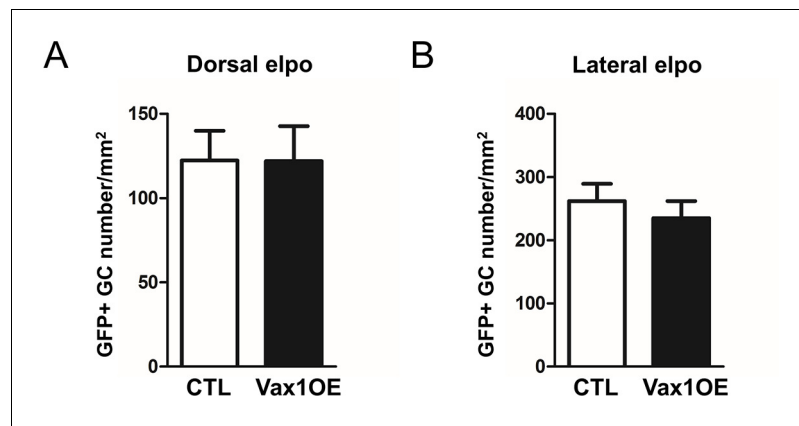


Figure 4—figure supplement 1. Forced expression of *Vax1* has no effect on cell density in the OB granule cell (GC) layer, 15 days after electroporation of dorsal (CTL $n = 15$, *Vax1* $n = 14$) or (B) lateral ($n = 10$ for both conditions) V-SVZ progenitors. Data are represented by mean \pm SEM.

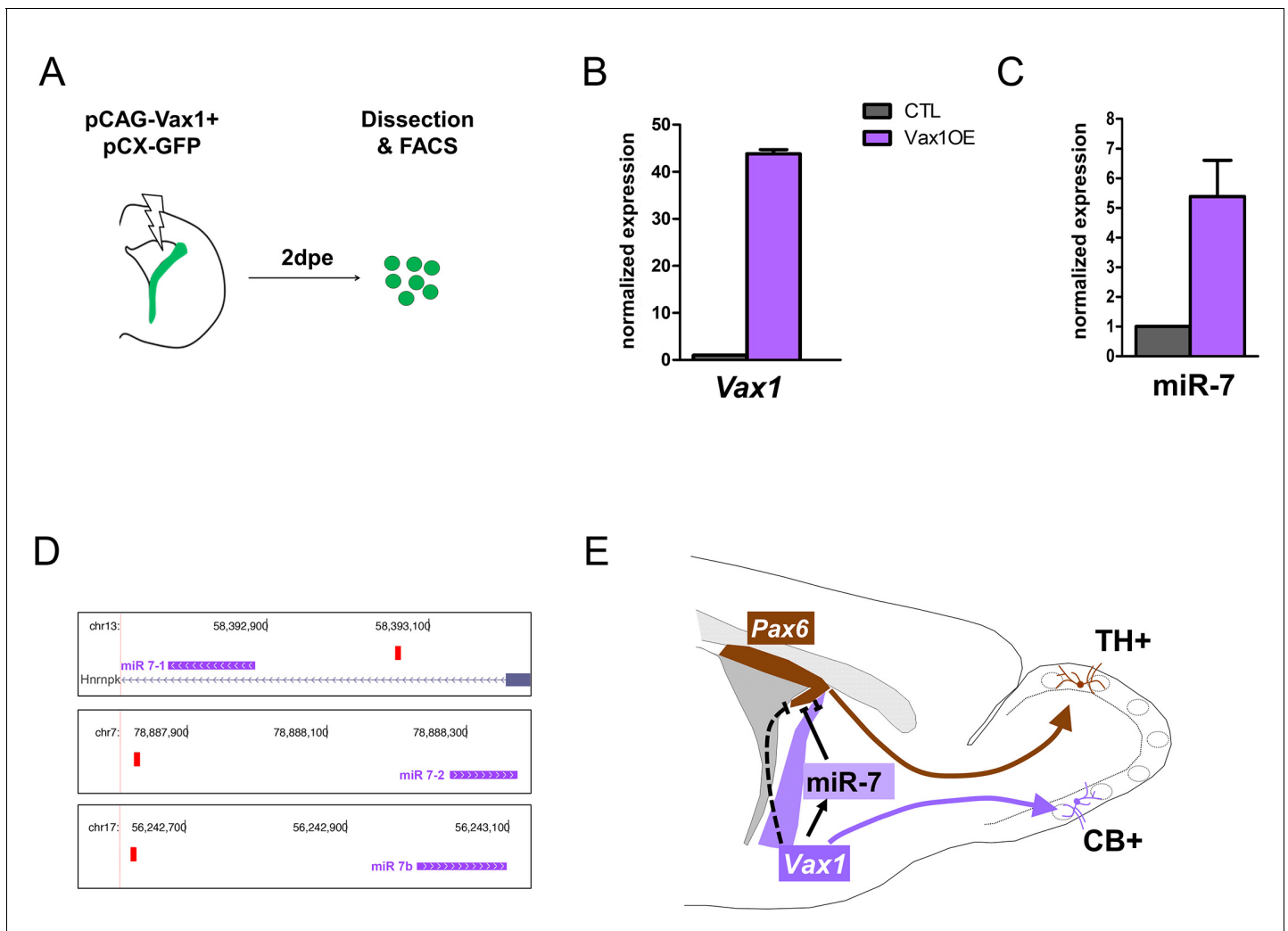


Figure 5. *Vax1* induces the expression of *miR-7* in the lateral V-SVZ. (A) Strategy used to determine the expression of microRNAs in *Vax1*-overexpressing progenitors. PCAG-*Vax1* and pCX-GFP were simultaneously introduced into NSCs by electroporation of the lateral wall of postnatal P1 brains. Lateral V-SVZ was dissected out 2 days after electroporation and GFP+ cells were isolated by flow cytometry (FACS) to perform quantitative RT-PCR analysis. (B) Quantification of *Vax1* mRNA level by qRT-PCR in control and *Vax1*OE conditions, normalized to beta-actin and reported in *Vax1* condition as relative level to control, validating the overexpression of *Vax1* after electroporation. (C) Quantification of *miR-7* expression in both conditions. Expression level of *miR-7* was normalized by invariant expression of microRNA let-7a and reported in *Vax1* condition as relative level to control. Experiments in B and C were performed in triplicate, and data were obtained from (B) two independent biological replications or (C) three technical replications. (D) Genome browser images representing the chromosomal portions encoding the three *Mir-7* loci (depicted in pink). *Mir-7-1* lies within an intronic sequence of the *Hnrnpk* gene whereas *Mir-7-2* and *Mir-7b* reside within intergenic sequences. *Vax1*-binding sites found in the upstream regulatory region of the three *Mir-7* are represented by red boxes. (E) Model of cross-regulatory interaction between *Vax1*, *miR-7*, and *Pax6* in the lateral V-SVZ to control the number of dopaminergic neurons generated by the neural stem cells regionalized in this aspect. This model is supported by our present data and previous work (de Chevigny et al., 2012a) where it was shown that *miR-7* was required to inhibit *PAX6* expression in lateral NSCs to produce the correct number of dopaminergic neurons in the postnatal OB. Here, we propose that *Vax1* acts upstream of *miR-7* by positively regulating its expression and consequently inhibiting *PAX6*. However, it is also possible that *Vax1* directly represses the expression of *Pax6* mRNA (dashed line) by acting on its promoter (Mui et al., 2005). Additionally, *Vax1* is required to generate Calbindin neurons from the ventral aspect of the lateral V-SVZ.