Supplementary Information Titles

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Article Title:	Adult generation of glutamatergic olfactory bulb interneurons
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SUPPLEMENTARY FIGURES

Adult generation of glutamatergic olfactory bulb interneurons

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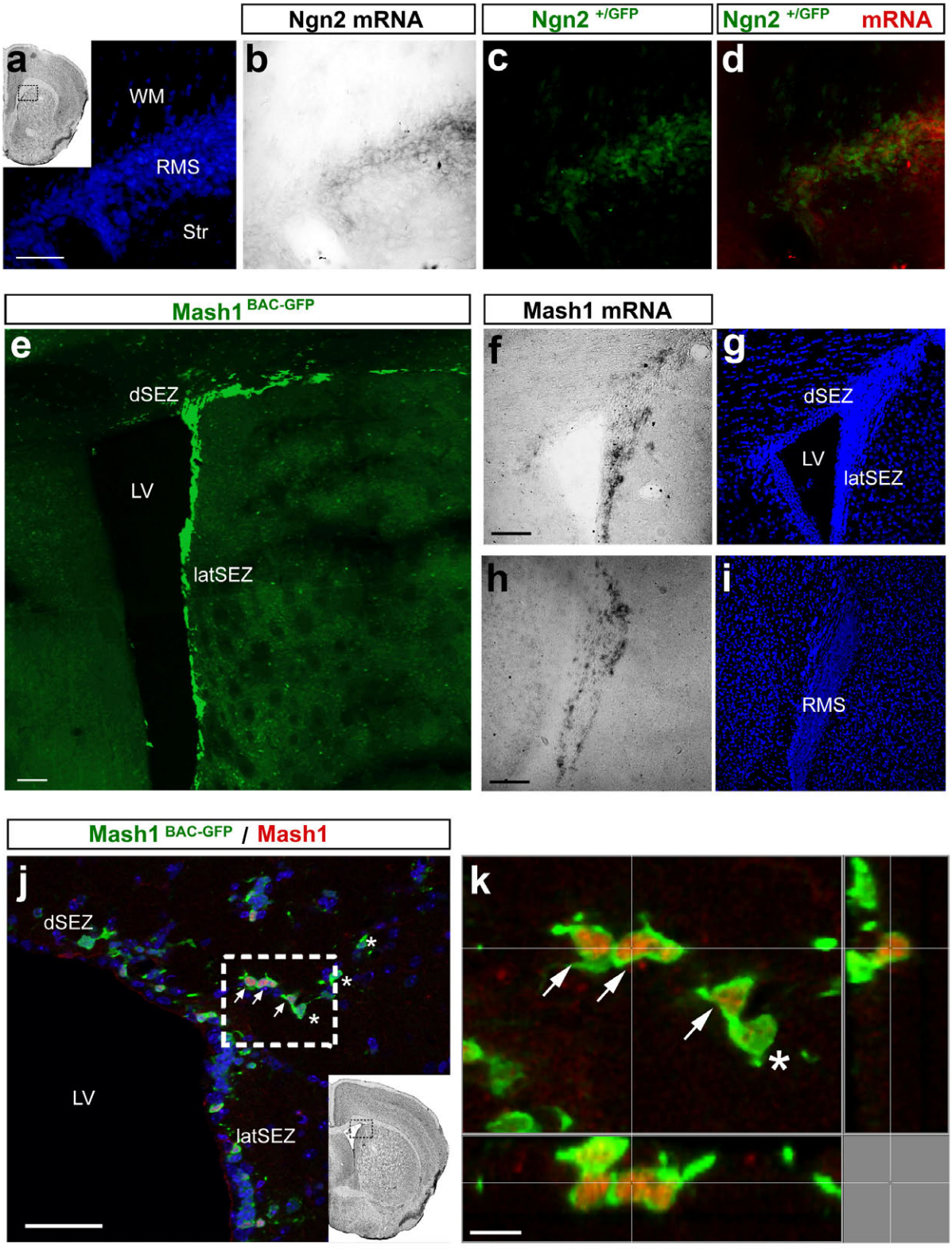
Supplementary Figure 1

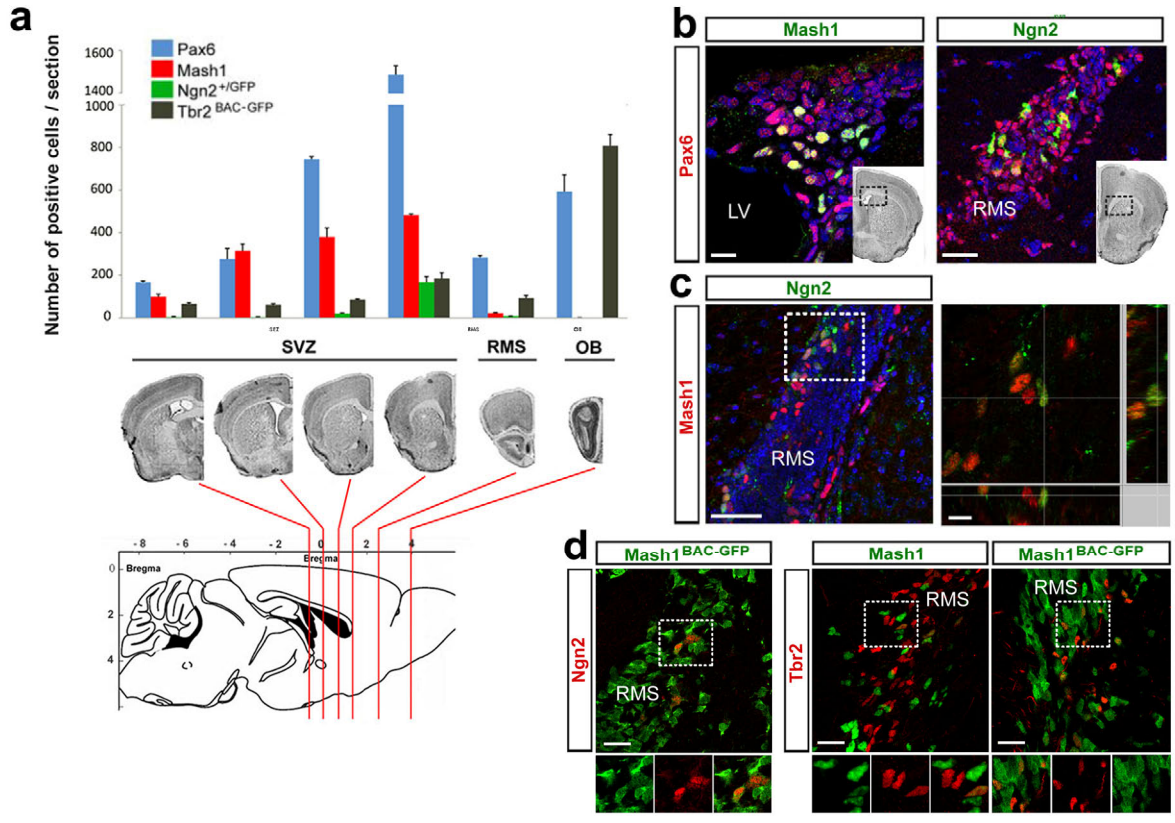


Supplementary Figure 1: Reliability of GFP expression in the adult forebrain of *Neurog2*$^{+/GFP}$ and *Mash1*$^{BAC-GFP}$ mice

(**a-d**) Micrographs depicting *Neurog2* mRNA and GFP protein in the RMS in *Neurog2*$^{+/GFP}$ mice. (**e-k**) Micrographs depicting the expression of GFP in *Mash1*$^{BAC-GFP}$ mice demonstrating the location of GFP mRNA and protein along the lateral wall of the lateral ventricle (**e-g**) and the RMS (**e,h,i**) as shown previously[50] and in (**j,k**) for the endogenous protein, while Neurog2 is restricted dorsally (**a-d**). (**j,k**) Fuorescent micrographs depicting the colocalization of Mash1- and GFP-immunoreactivity in the adult SEZ of *Mash1*$^{BAC-GFP}$ mice. Note that all Mash1-positive cells (red) express GFP (100%, 125 Mash1+ cells counted; arrows). As GFP protein persists longer than Mash1 endogenous protein, only some GFP-positive cells are still Mash1-immunoreactive (15 ± 2%, 1025 GFP+ cells; asterisks). The area framed by dashed white lines in (**j**) is presented at higher magnification in (**k**).

Nuclei are visualized with DAPI (**a,g,i,j**). dSEZ = dorsal subependymal zone, latSEZ = lateral subependymal zone, LV = lateral ventricle, RMS = rostral migratory stream, Str = Striatum, WM = white matter, scale bars: (**e**) 100 µm; (**a-d**,**f-j**) 50 µm, (**k**)10 µm.

Supplementary Figure 2

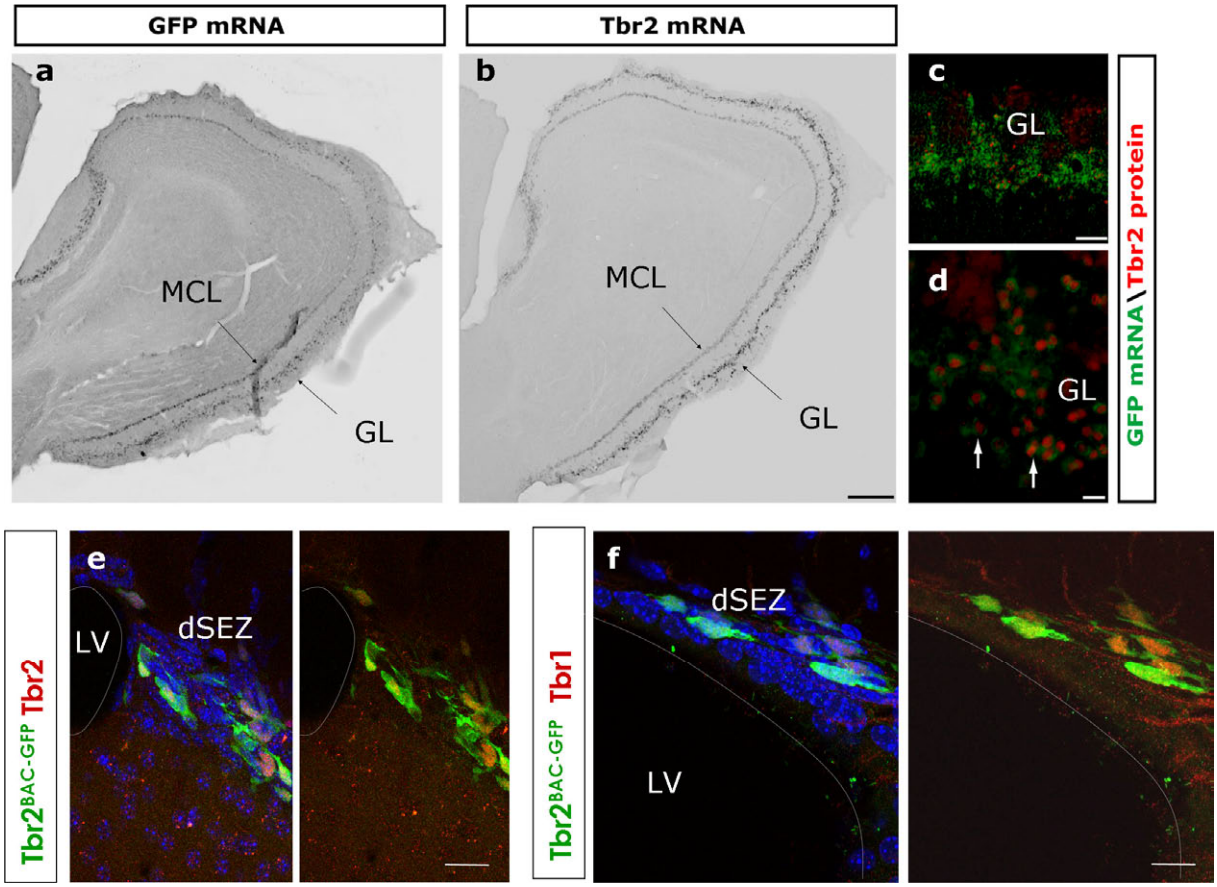


Supplementary Figure 2: Location of Mash1-, Pax6-, *Tbr2*$^{BAC\text{-}GFP}$-, and *Neurog2*$^{+/GFP}$-expressing cells along the rostro-caudal axis of the adult forebrain

(**a**) Histogram depicts the number of Pax6- (blue), Mash1- (red), *Neurog2*$^{+/GFP}$-(green), and *Tbr2*$^{BAC\text{-}GFP}$-(dark grey) immunoreactive cells at different rostro-caudal levels of the subependymal zone (SEZ), rostral migratrory stream (RMS) and olfactory bulb (OB) as indicated in the examples depicted below the x-axis. Counts were obtained from coronal sections taken at different coordinates illustrated by the bottom panel. While Pax6 and Mash1 protein are expressed throughout the entire axis of the SEZ, *Neurog2*$^{+/GFP}$ and *Tbr2*$^{BAC\text{-}GFP}$ expression is restricted largely to the dorsal SEZ and proximal RMS. Both Pax6 and *Tbr2*$^{BAC\text{-}GFP}$ expressions rise again in some mature neurons in the olfactory bulb. (**b-d**) Fluorescent micrographs depicting the colocalization of these transcription factors. Micrographs in (**b**) show that *Neurog2*$^{+/GFP}$- and Mash1-positive progenitors are immunoreactive for Pax6 (red) in the dorsal SEZ. Inserts indicate the location of the migrographs. Micrographs in (**c**) demonstrate colocalization of Mash1- and Neurog2-immunoreactivity in some cells (49 ± 4% of 69 Neurog2-positive cells are Mash1-positive; boxed area is presented at higher magnification) and in (**d**) a low degree of coexpression of Tbr2 (middle panel in **(d);** 10.5 ± 2% of 375 Tbr2+ cells are Mash1-positive; n = 5 animals), while no colocalization Mash1 with Tbr1 was observed (data not shown, 2.6 ± 1%, 321 Tbr2+ cells; n = 3 animals). Note that short-term fate mapping due to the longer persistence of GFP in *Mash1*$^{BAC\text{-}GFP}$ mice reveals many more GFP/Tbr2-double-positive cells (right hand panel in (**d**)) reaching 74% ± 5% GFP-positive cells amongst 728 Tbr2+ cells. A similarly high colocalization of GFP driven by the *Mash1* promoter was also observed for Neurog2-immunopositive cells (84 ± 4%, 173 Neurog2+ cells analyzed, left panel in (**d**)) and Tbr1-positive cells (72% ± 3%, 414 Tbr1+ cells analyzed, data not shown), indicating that Neurog2, Tbr2 and Tbr1 are expressed downstream of Mash1 in differentiating progenitors of the dorsal SEZ. Note the low level of GFP expression in those cells, confirming the down-regulation of Mash1 protein in these progenitor populations as suggested by protein immunostaining (see above).

LV= lateral ventricle, RMS = rostral migratory stream, SEZ = subependymal zone, OB = olfactory bulb, scale bars: 20 μm.

Supplementary Figure 3

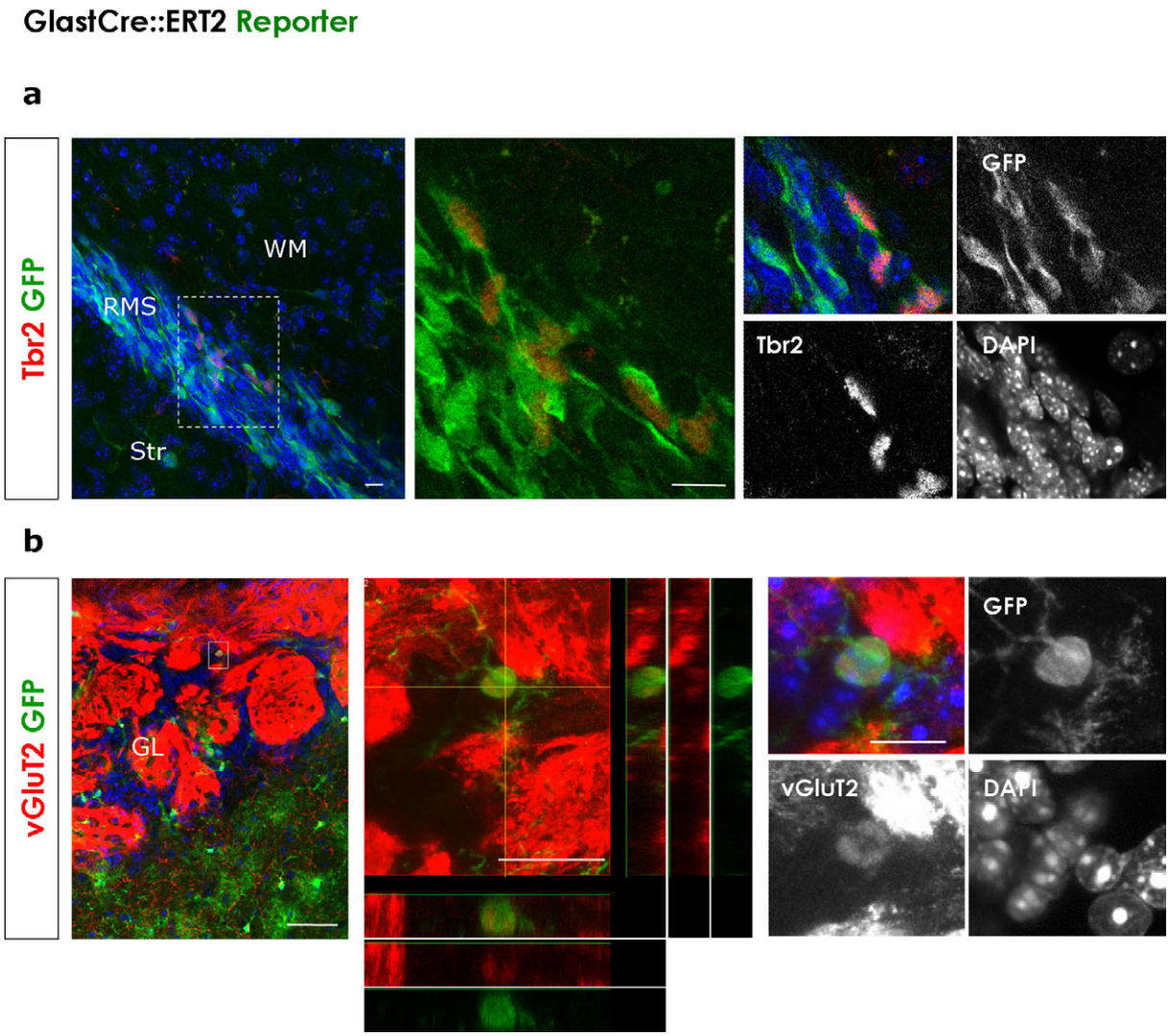


Supplementary Figure 3: Reliability of GFP expression in *Tbr2*$^{BAC\text{-}GFP}$ mice

(**a,b**) Micrographs show comparable localization of *GFP* (**a**) and *Tbr2* (**b**) mRNA in the olfactory bulb (OB). (**c,d**) Fluorescence micrographs showing the colocalization of GFP mRNA and Tbr2 protein in the glomerular layer (GL) demonstrating the faithful expression of GFP mRNA in Tbr2-immunoreactive cells. (**e,f**) Micrographs depicting the colocalization of Tbr2 (**e**) or Tbr1 (**f**) protein (red) with *Tbr2*-driven GFP. Note that virtually all Tbr2-immunoreactive cells are GFP-positive (88 ± 5% of Tbr2 protein positive cells are also GFP-positive (n = 3 animals, 352 cells), supporting the reliability of the *Tbr2*$^{BAC\text{-}GFP}$ mouse line. Conversely, only 44 ± 5% of *Tbr2*$^{BAC\text{-}GFP}$ cells are Tbr2 protein positive (n = 3 animals, 710 cells) consistent with the longer perdurance of the GFP protein compared to the endogenous native protein. This allows using this mouse line for short term fate mapping. Nuclei are visualized with DAPI in the left panels of (**e,f**).

dSEZ = dorsal subependymal zone, LV = lateral ventricle, MCL = mitral cell layer, GL = glomerular layer, LV = lateral ventricle, scale bars: 20 µm.

Supplementary Figure 4

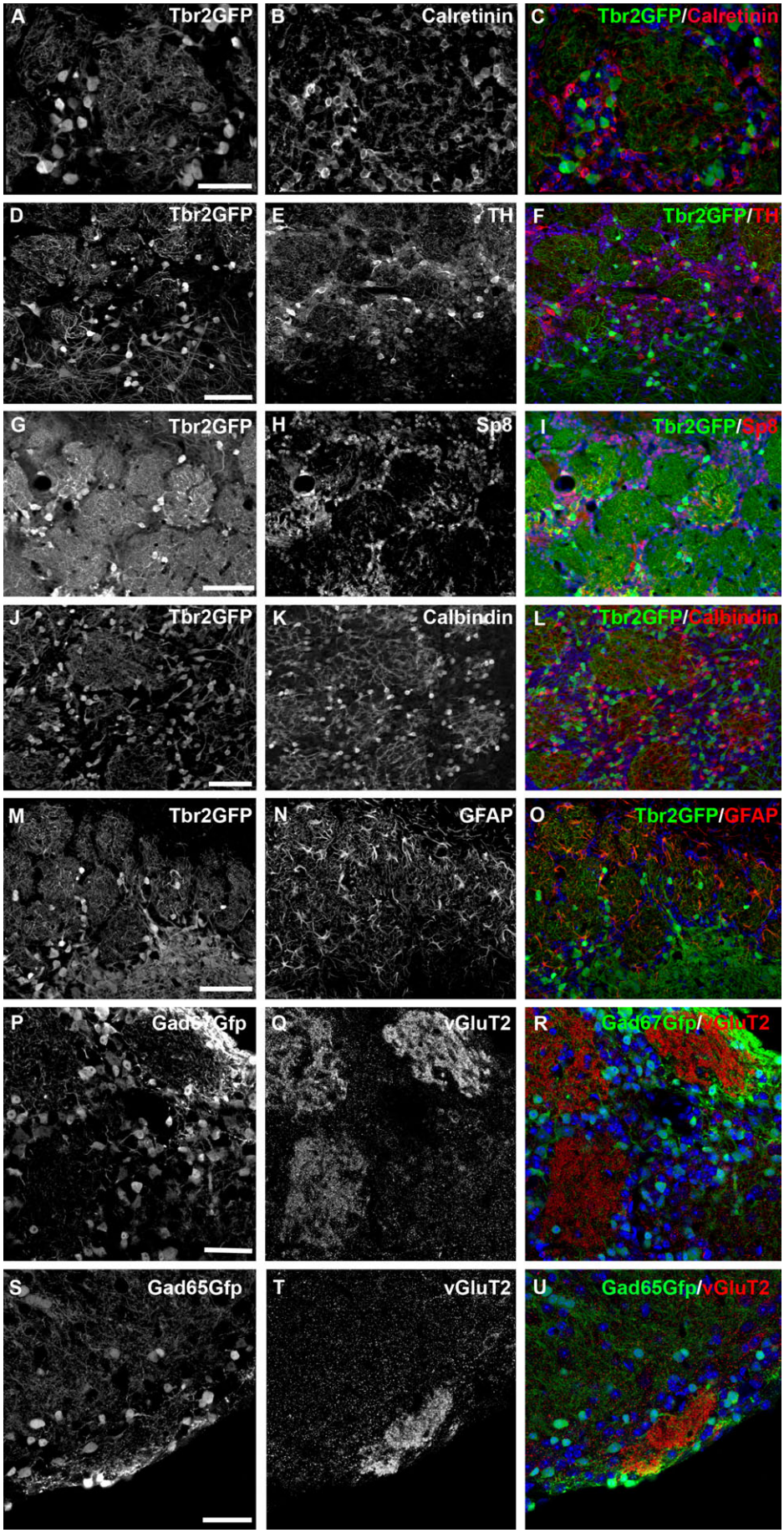


Supplementary Figure 4: Genetic fate mapping of adult neural stem cell derived cells reveals vGluT2 immunoreactive juxtaglomerular neurons as their progeny

Fate mapping of the progeny of *GLAST* expressing astrocytes in *GLAST::CreERT2* mice crossed with the *R26R-CFP* reporter line by Tamoxifen-induced recombination at adult ages reveals in (**a**) Tbr2-positive cells (red) expressing the GFP reporter demonstrating that the majority of Tbr2-expressing cells are derived from adult neural stem cells labelled 4 weeks earlier by Tamoxifen-induced recombination (Tbr2+/GFP+ of Tbr2+ 64%, 104 cells; Tbr2+/GFP+ of GFP+ 5% in RMS, 779 cells; n = 3 animals). The micrograph in (**b**) shows reporter-positive juxtaglomerular cells with somatic vGluT2-immunoreactivity (red), indicating that these neurons are derived from adult neural stem cells labelled 4 weeks earlier by Tamoxifen-induced recombination. Nuclei are visualized with DAPI (**a,b**).

WM = white matter, RMS = rostral migratory stream, Str = Striatum, GL = glomerular layer, scale bars: 20 µm (**a,b**).

Supplementary Figure 5

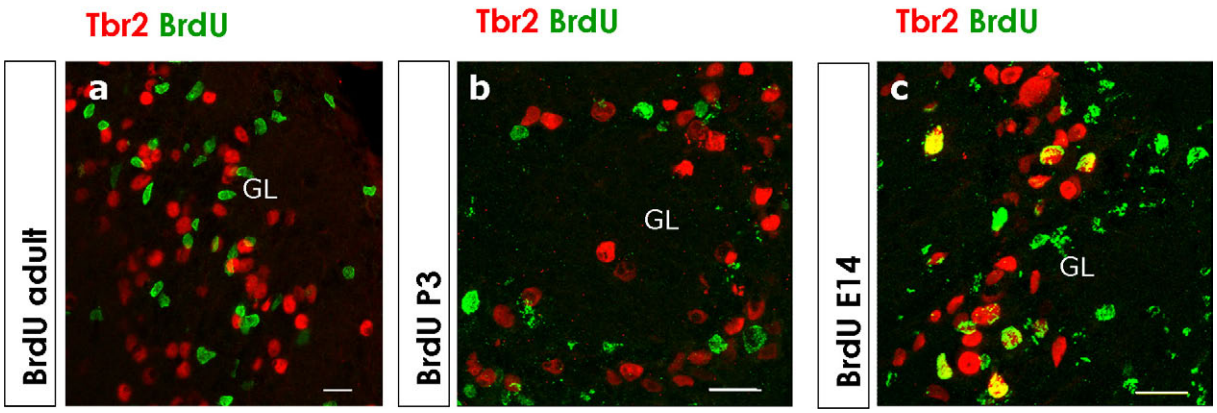


Supplementary Figure 5: *Tbr2*-driven GFP-positive juxtaglomerular neurons are not labelled by markers for periglomerular neurons and are *GAD*-negative

(**a-l**) *Tbr2*-driven GFP-positive neurons do not colocalize with markers specific for periglomerular neurons such as calretinin (**a-c**), tyrosine hydroxylase (TH; **d-f**), Sp8 (**g-i**), or calbindin (**j-l**). (**m-o**) *Tbr2*-driven GFP-positive cells are also not expressing Gfap consistent with their neuronal nature. (**p-u**) vGluT2 immunoreactive cells are not expressing *GAD65*- or *GAD67*-driven GFP in the glomerular layer of the olfactory bulb.

Nuclei are visualized with DAPI, scale bar: 50 µm.

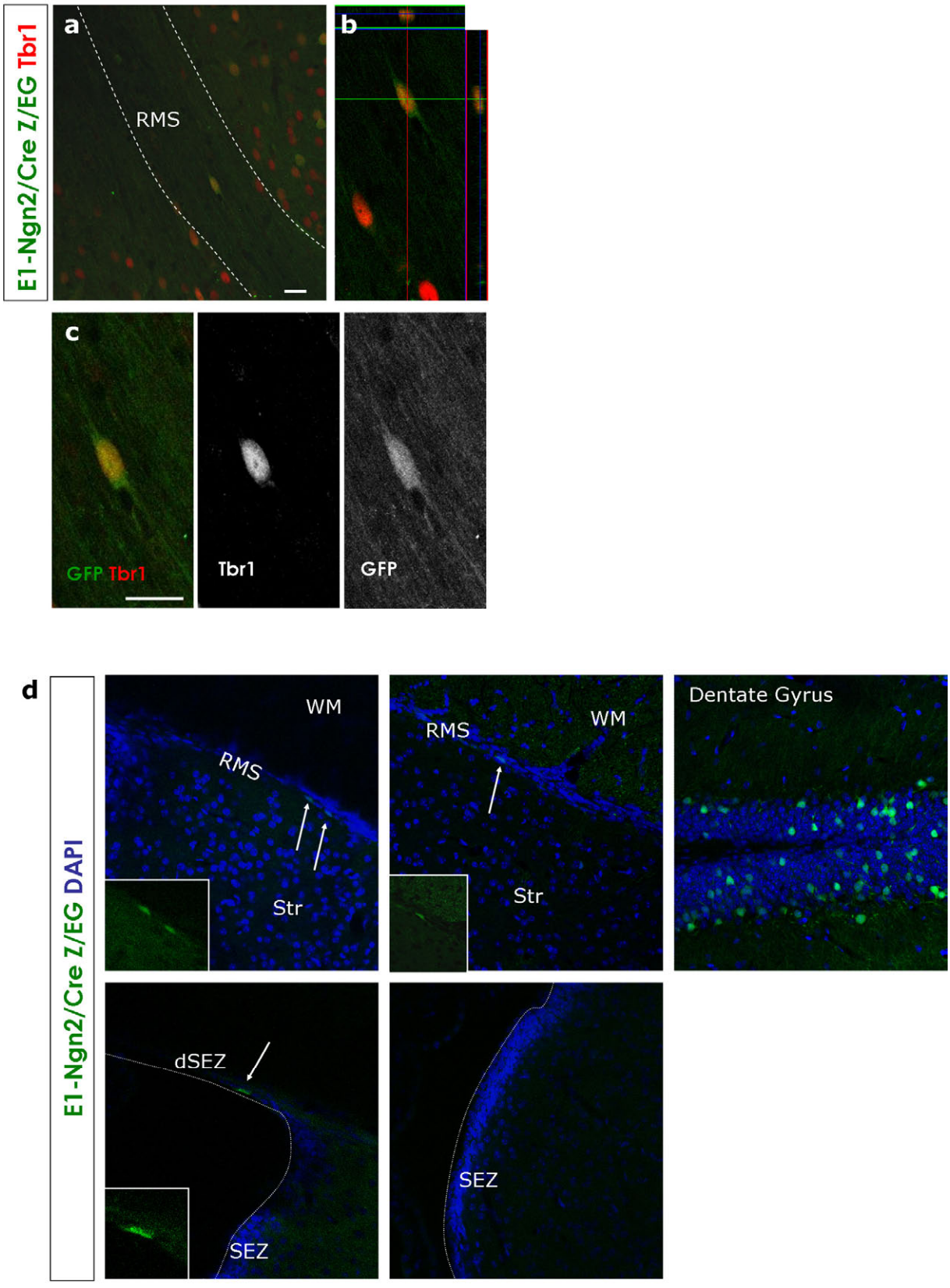
Supplementary Figure 6



Supplementary Figure 6: Tbr2-immunoreactive neurons in the adult olfactory bulb are generated exclusively during embryonic development

(**a-c**) Fluorescent micrographs depicting Tbr2-positive cells in the adult olfactory bulb and BrdU-immunoreactive cells that incorporated BrdU after application in the adult (3 weeks drinking water followed by 3 weeks BrdU-free water) (**a**), at postnatal day P3 (**b**) and at embryonic day E14 ((**c**), analyzed at postnatal day P16). Note that Tbr2-positive cells are only labelled by BrdU-application during embryonic development indicating that they are not generated in adulthood. GL = glomerular layer, scale bars: 20 µm

Supplementary Figure 7

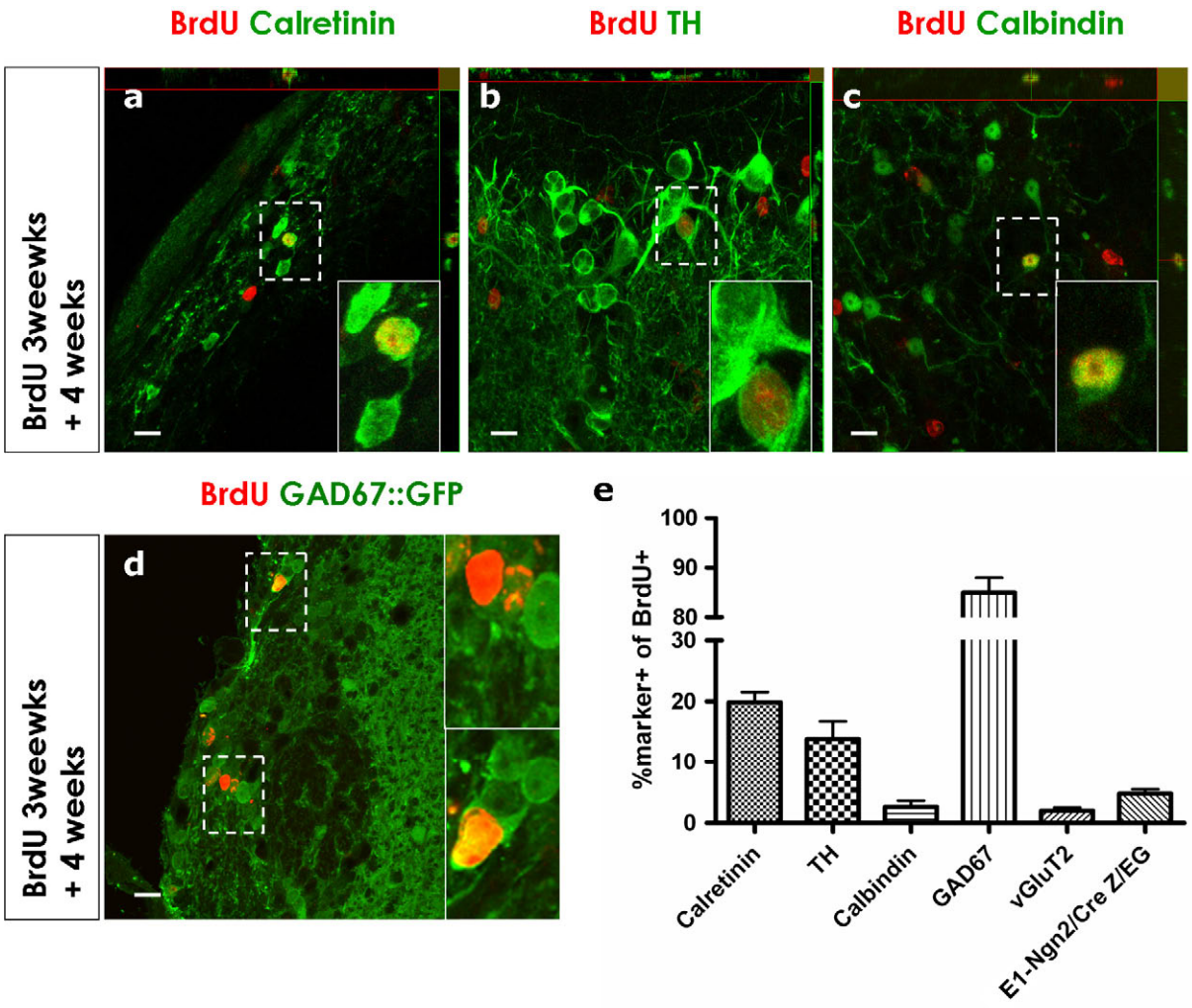


Supplementary Figure 7: Tbr1 expressing cells in the RMS are derived from Neurog2-expressing progenitors as revealed by *E1-Neurog2 driven Cre*-mediated recombination

(**a-c**) Fluorescent micrographs demonstrating GFP-reporter-positive cells with Tbr1-immuno-reactivity (red) in the RMS close to the olfactory bulb upon *E1-Neurog2 driven Cre*-mediated recombination. Location of the RMS in (**a**) is outlined by dotted white lines and panels (**b,c**) show higher magnifications. (**b**) is shown as Z-projection and (**c**) as maximum intensity projection. (**d**) Fluorescent micrographs showing GFP-reporter-positive cells in the subependymal zone (SEZ), rostral migratory stream (RMS), and dentate gyrus. Note, that there are no *Neurog2*-derived GFP-positive cells in the lateral SEZ. Nuclei are visualized with DAPI (**d**).

dSEZ = dorsal subependymal zone, RMS = rostral migratory stream, SEZ = subependymal zone, Str = Striatum WM = white matter, scale bars: 20 µm.

Supplementary Figure 8

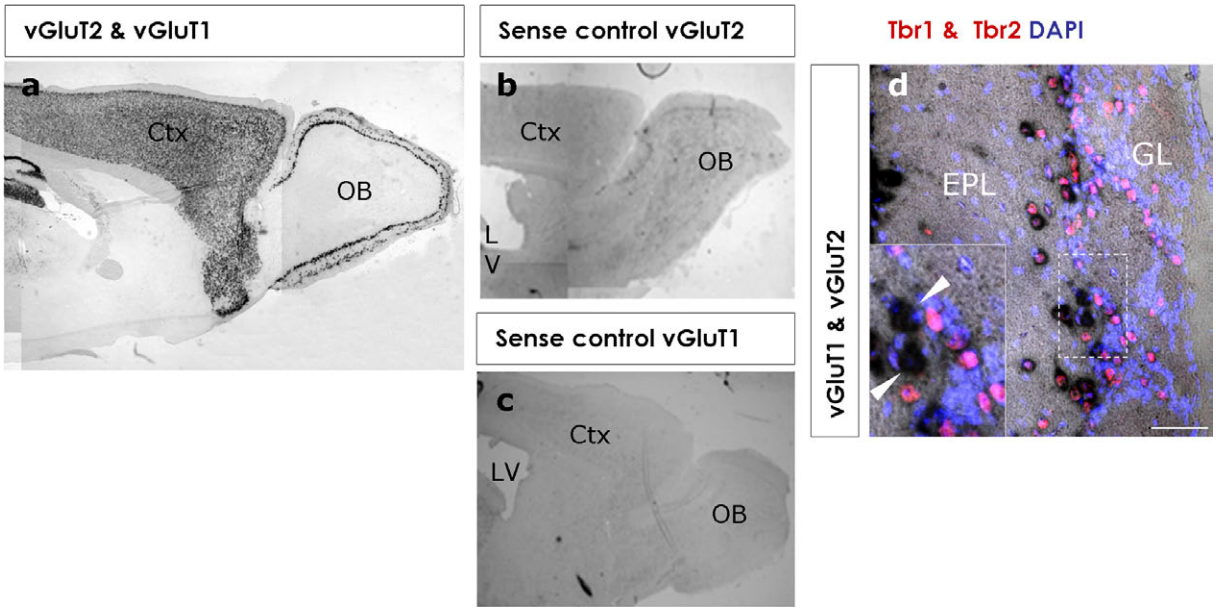


Supplementary Figure 8: Identity of adult generated neurons in the glomerular layer of the olfactory bulb

To label adult generated cells BrdU was given to adult mice for three weeks in the drinking water, followed by three weeks BrdU-free water. Fluorescent micrographs in (**a-d**) depict BrdU-labelled cells in the glomerular layer in red double-stained for (**a**) calretinin, (**b**) tyrosine hydroxylase (TH), (**c**) calbindin and (**d**) GFP driven from the endogenous *GAD67* locus in green. Boxed areas in (**a-d**) (white) are shown at higher magnifications on the right side of the panels.

Quantification of the colocalization of BrdU-labelled cells in the glomerular layer with these and other markers for neuronal subtypes depicted in (**e**) reveals that the majority of the BrdU labelled cells are of GABAergic nature, while a small, but notable population of the BrdU incorporating cells are positive for *vGluT2* mRNA or are derived from *Neurog2* expressing progenitors (*E1- Neurog2/Cre Z/EG* mice). (Calretinin+ cells = 568, 20 ± 2%; Calbindin+ cells = 429, 3 ± 1%; TH+ cells = 512, 14 ± 3%; *GAD67::GFP*+ cells = 534, 85 ± 3%; *vGluT2* mRNA: BrdU+cells = 1632, 2 ± 0.5%; *E1- Neurog2/Cre Z/EG*+ cells = 228); scale bars: 20 µm.

Supplementary Figure 9

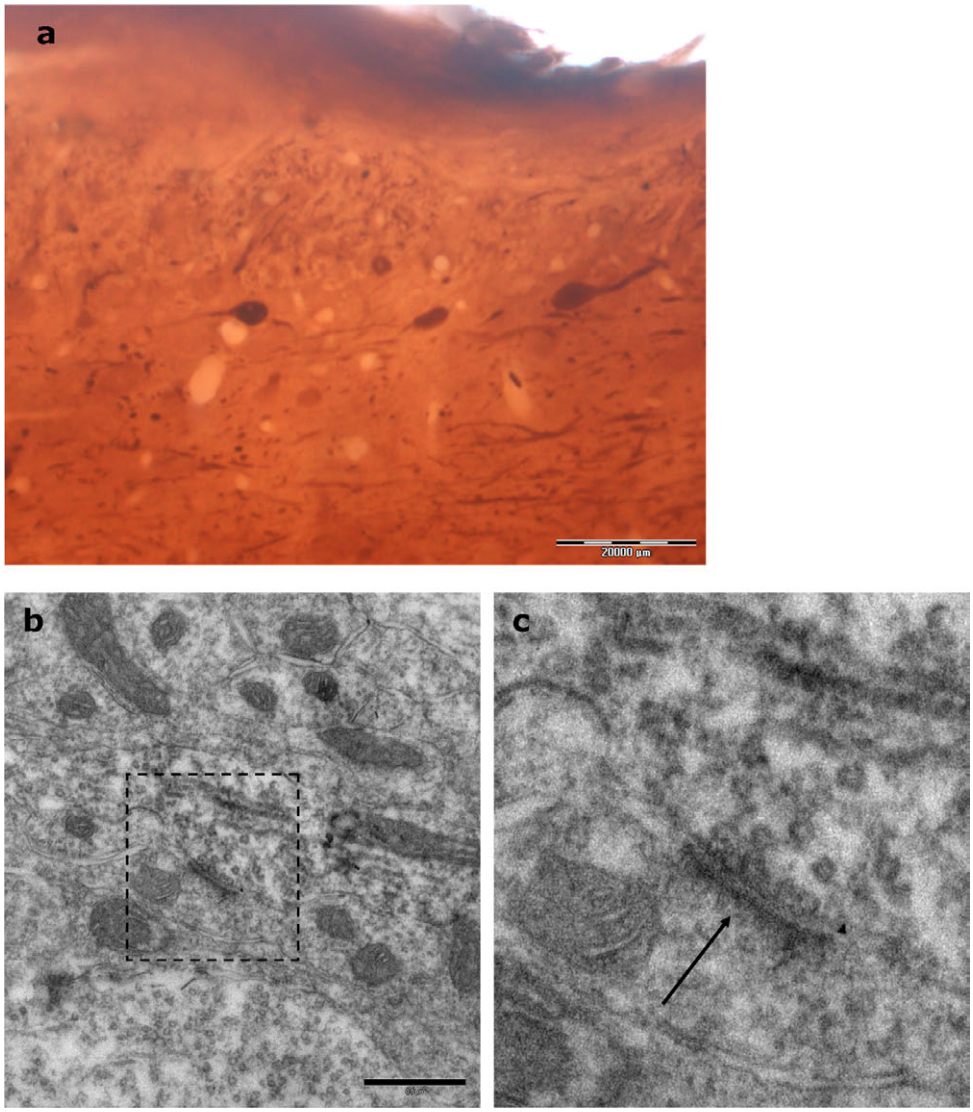


Supplementary Figure 9: Only a subset of glutamatergic neurons in the olfactory bulb express Tbr1 and Tbr2

(**a-c**) Micrographs depicting *in-situ* hybridization for both *vGluT1* and *vGluT2* mRNA (**a**) and the respective sense controls in (**b,c**). The micrograph in (**d**) depicts a high power view of the glomerular layer of the olfactory bulb for *vGluT1* mRNA and *vGluT2* mRNA counterstained for Tbr1 and Tbr2 (red), showing that some *vGluT* expressing cells are negative for Tbr protein (indicated by arrowheads in the insert in the lower left corner). Boxed area (white) is shown at higher magnification in the lower left corner. Thus, at least two populations of glutamatergic interneurons exist in the glomerular layer, and only one stains for Tbr transcription factors. To reveal the glomeruli nuclei are visualized with DAPI (**d**).

Ctx = Cortex, LV = lateral ventricle, OB = olfactory bulb, GL = glomerular layer, EPL = external plexiform layer, scale bar: 50 µm (**d**).

Supplementary Figure 10

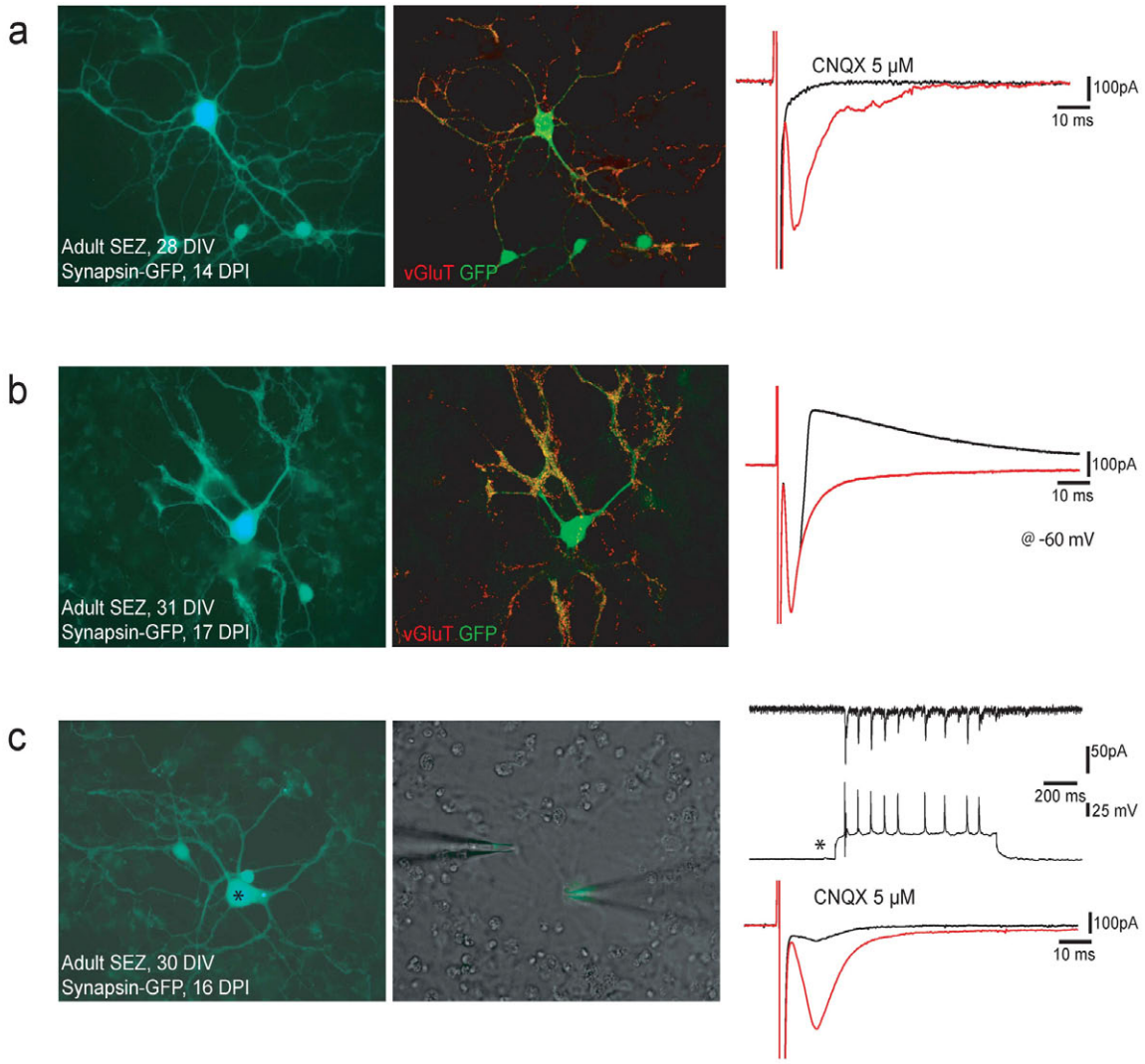


Supplementary Figure 10: Immunoelectron analysis of neuronal contacts in the glomerular layer of the olfactory bulb labelled by GFP reporter in *E1-Neurog2/Cre Z/EG* mice

Immunoelectron microscopy in the glomerular layer of *E1-Neurog2/Cre Z/EG* mice in which GFP expression was labelled by DAB-staining (**a**). (**b**) A DAB-labelled axon forms an asymmetric contact demonstrating the glutamatergic nature of *Neurog2*-derived progeny in the adult olfactory bulb. Boxed area (black) in (**b**) is represented in (**c**) at higher magnification. The black arrow in (**c**) indicates the postsynaptic density of an asymmetric contact.

Scale bar: (**a**) 20.000µm, (**b,c**) 0.5 µm.

Supplementary Figure 11



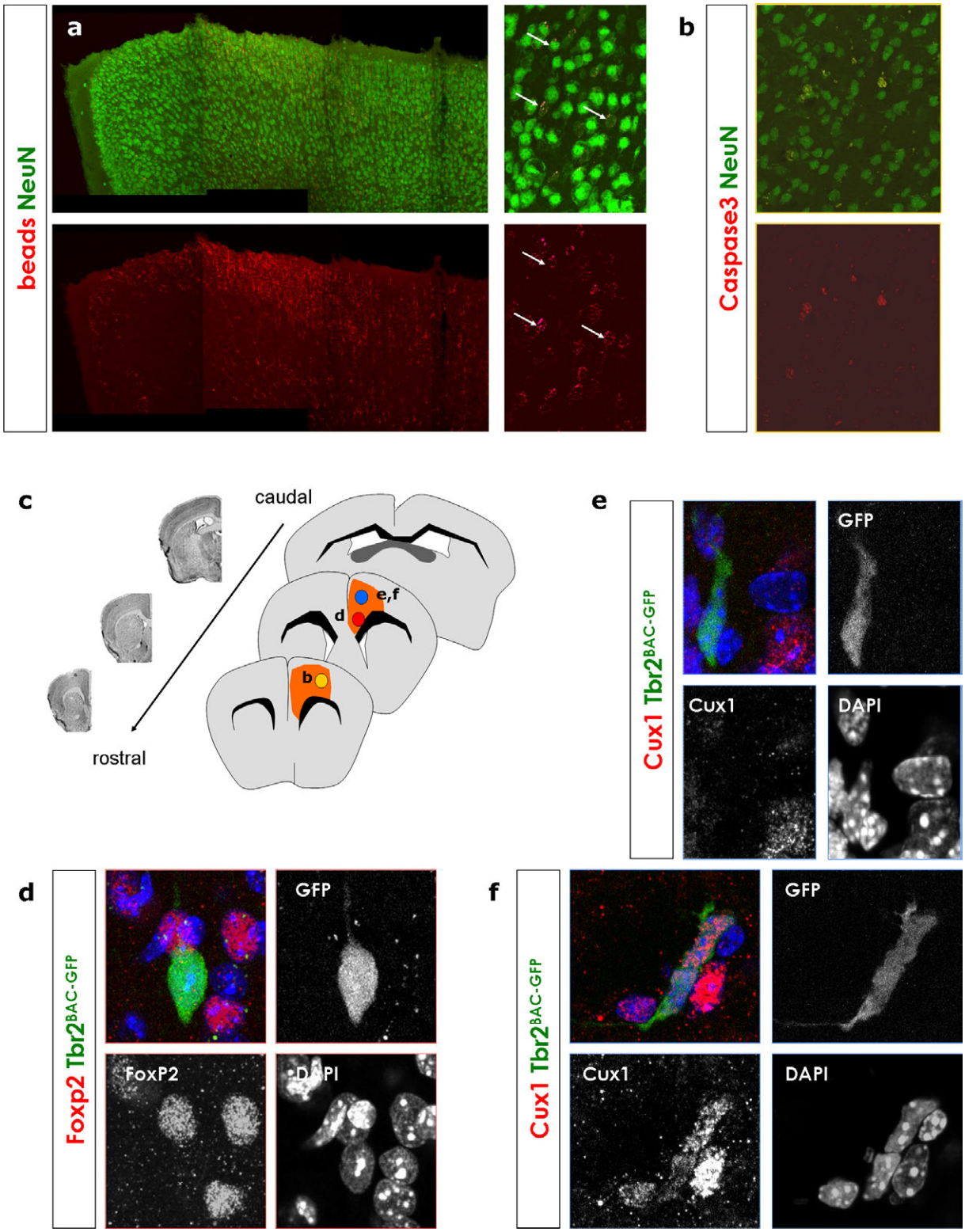
Supplementary Figure 11: A subpopulation of adult SEZ stem and progenitor cells form glutamatergic autapses

(**a**) Example of a glutamatergic neuron transduced with a lentiviral vector expressing GFP under the synapsin promoter. Left panel shows live GFP fluorescence after 28 days in vitro (div), 14 days post infection (dpi); middle panel shows post-recording immunocytochemistry of the same neurons for GFP and vGluT; note the dense puncta of vGluT on the dendrites of the large GFP-positive neuron consistent with the presence of autaptic connections as shown in the right panel. The autaptic response was entirely blocked by 5 μM CNQX demonstrating its glutamatergic nature.

(**b**) Another example as shown in (**a**). The right panel shows autaptic responses following step depolarisation (red trace). Note that in some cases (black trace), the autaptic excitatory postsynaptic current was followed by an inhibitory postsynaptic current (outward current at a holding membrane potential of -60 mV). Both the delay and the high failure rate (> 80 %) of the outward current indicate its polysynaptic nature. Each trace is an average of 10 single responses. The high density of vGluT puncta in the middle panel are consistent with the presence of the glutamatergic autaptic connection revealed by the recording.

(**c**) Example of a glutamatergic neuron (asterisk) synapsing on a nearby neuron. The left panel shows live fluorescence; the middle panel depicts the configuration of the two patch electrodes; the upper right panel shows the postsynaptic response in the smaller neuron following action potential firing in the large neuron marked by the asterisk in the left panel. The lower trace shows the autaptic connection of the neuron marked by the asterisk demonstrating its glutamatergic nature.

Supplementary Figure 12



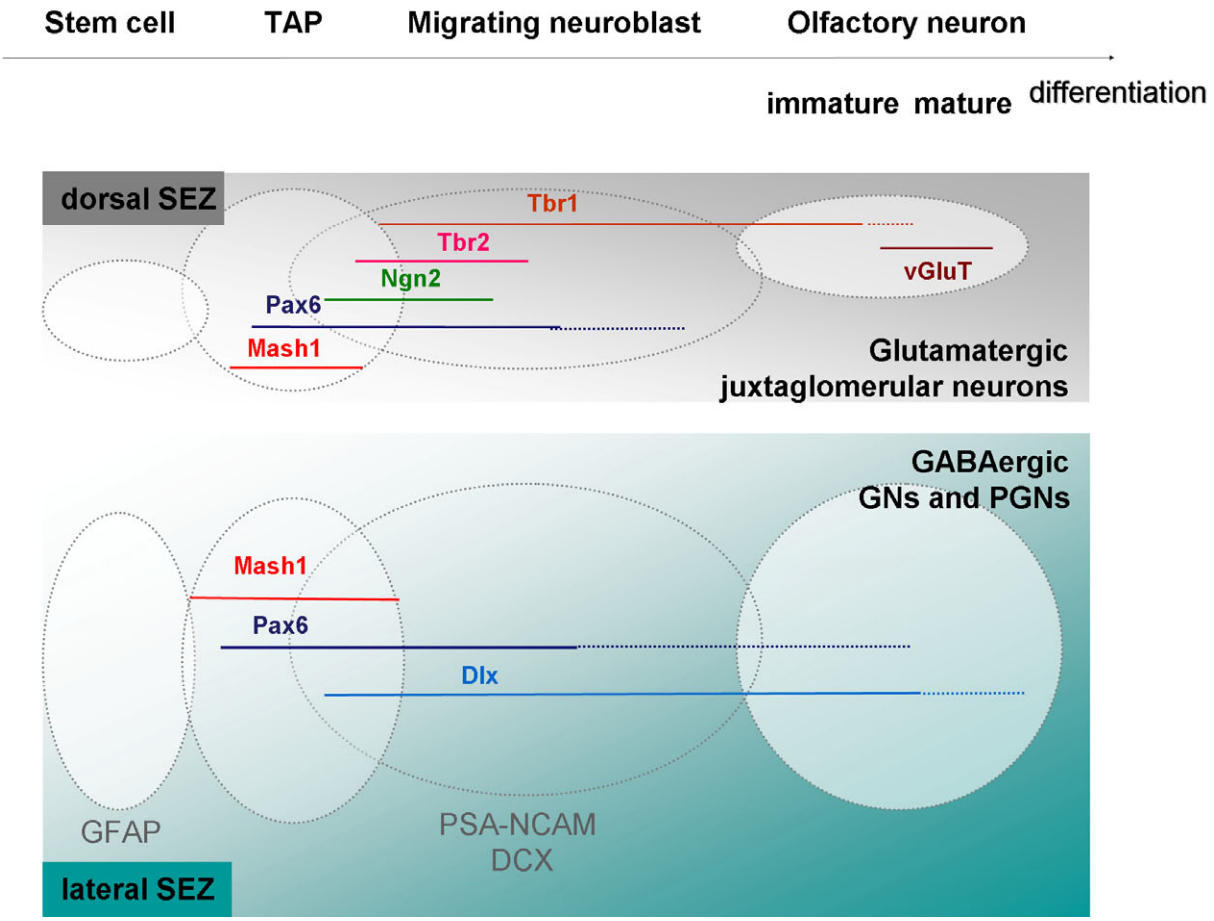
Supplementary Figure 12: Some *Tbr2*$^{BAC\text{-}GFP}$-positive cells recruited towards the lesioned cortex express Cux1

(**a**) Chlorine e_6 coupled red fluorescent latex beads were injected into the cerebral cortex. Immunostaining for NeuN (green) shows that 7 days after injection callosal projection neurons in layers 2/3 and 5 of the cortex accumulate fluorescent beads (red) in their soma. Higher magnification is shown on the right side of panel (**a**). Latex beads incorporating neurons are highlighted by white arrows. (**b**) Dying neurons (NeuN, green) were detected 3 days after laser illumination of the contralateral hemisphere as shown by immunohistochemistry for active Capase 3 (red). The location of panel (**b**) is colour coded in yellow in the schematic overview in (**c**).

(**c**) Schematic overview depicts the location of panels (**d**), and (**e,f**) and the majority of the *Tbr2*$^{BAC\text{-}GFP}$-positive cells in the lesioned cortex in orange. (**d-f**) Fluorescent micrographs immunostained for GFP and the respective layer-specific antigen (Foxp2 in panel (**d**); GFP cells are negative; colour coded in red) and Cux1 (panels (**e,f**), with (**f**) showing one GFP-positive cell (upper cell) that is clearly double-positive for Cux1-immunoreactivity in one of the two DAPI-labelled nuclei, while the GFP labelled cells in (**e**) is not Cux1-immunoreactive; colour coded in blue).

Nuclei are visualized by DAPI (**d-f**). Scale bars: 20 μm.

Supplementary Figure 13



Supplementary Figure 13: Schematic summary of transcription factor expression and lineage progression

The drawing depicts the cell types indicated on top by dotted circles with lineage progressing from Gfap-positive stem cells to transit-amplifying progenitors (TAP) and PSA-NCAM or Dcx expressing migrating neuroblasts.

As detailed in the manuscript we observed a lineage progression from Mash1 and Pax6 expressing tansit-amplifying progenitors to Pax6/Neurog2/Tbr2-positive neuroblasts in the dorsal SEZ that then generate vGluT2-expressing neurons in the olfactory bulb. We have further shown that Neurog2- or Tbr-expression does not colocalize with Dlx transcription factors or any other marker of the GABAergic lineage, thereby proposing two distinct sets of progenitors, one residing in the dorsal SEZ giving rise to the glutamatergic lineage, and a second one in the lateral SEZ giving rise to the majority of olfactory neurons, namely granule neurons (GN) and periglomerular neurons (PGN).

PGN = periglomerular neuron, TAP = transit-amplifying progenitor, GN = granule neuron, PGN = periglomerular neuron.