Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb

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SUMMARY (149 words)

We determined the embryonic origins of adult forebrain subventricular zone (SVZ) stem cells by Cre-lox fate mapping in transgenic mice. We found that all parts of the telencephalic neuroepithelium, including the medial and lateral ganglionic eminences (MGE and LGE) and the cerebral cortex, contribute multipotent, self-renewing stem cells to the adult SVZ. Descendants of the embryonic LGE and cortex settle in ventral and dorsal aspects of the dorsolateral SVZ, respectively. Both populations contribute new (BrdU-labeled) Tyrosine Hydroxylase- and Calretinin-positive interneurons to the adult olfactory bulb. However, Calbindin-positive interneurons in the olfactory glomeruli were generated exclusively by LGE-derived stem cells. Thus, different SVZ stem cells have different embryonic origins, colonize different parts of the SVZ and generate different neuronal progeny - suggesting that some aspects of embryonic patterning are preserved in the adult SVZ. This could have important implications for the design of endogenous stem cell-based therapies in the future.

INTRODUCTION

Since the identification of neural stem cells in the adult forebrain subventricular zone (SVZ) (Reynolds and Weiss, 1992;Richards et al., 1992) a lot of work has been done to characterize the phenotype of these stem cells, the SVZ cyto-architecture and factors involved in regulating stem cell maintenance and behaviour (reviewed by Gage, 2000;Doetsch, 2003;Alvarez-Buylla and Lim, 2004;Morshead and van der Kooy, 2004;Young et al., 2007). Adult neural stem cells have been described as a subpopulation of SVZ astrocytes because of their morphology and the fact that they express the astrocyte marker, glial fibrillary acidic protein (GFAP) (Doetsch et al., 1999a;Laywell et al., 2000). It is now well established that the slowly dividing SVZ stem cells generate rapidly dividing neuronal progenitors (transit amplifying cells) that in turn generate post-mitotic neuroblasts. These migrate forwards along the rostral migratory stream (RMS) into the olfactory bulb, where they give rise to various sub-types of olfactory interneurons continuously throughout life (reviewed by Lledo et al., 2006). However, it is still unclear whether the SVZ contains a heterogeneous collection of neural stem cells with different neurogenic potentials or whether cell fate restriction occurs later, within the RMS or the olfactory bulb.

Neuroepithelial precursors in most parts of the embryonic telencephalon have a similar radial morphology and express antigenic markers characteristic of radial glia (Noctor et al., 2002;Malatesta et al., 2003;Anthony et al., 2004). However, there are also regional differences in gene expression,

including regionally-restricted expression of transcription factors (Kriegstein and Götz, 2003). For example, the medial ganglionic eminence (MGE) expresses Nkx2.1, the MGE and the lateral ganglionic eminence (LGE) express Gsh2, the cortico-striatal sulcus expresses Dbx1 and the developing cortex expresses Emx1. As development proceeds, the neuroepithelial germinal zone (ventricular zone, VZ) regresses and a relatively small proportion of neural stem cells persists in the SVZ of the adult forebrain. The relative contributions of different parts of the embryonic VZ to the adult SVZ have not yet been established, although the bulk of evidence suggests that adult stem cells are descendants of the LGE (embryonic striatum). For example, transcription factors that are expressed in the dorsal LGE of the embryo, such as Dlx1/2, Er81, Gsh2 and Pax6 are also present within the adult SVZ and RMS (Parmar et al., 2003;Stenman et al., 2003;Kohwi et al., 2005). In addition, viral lineage tracing of radial glia in the perinatal LGE has demonstrated that some of the progeny of these cells come to reside in the postnatal SVZ and contribute significantly to the adult neurosphere-forming activity (Merkle et al., 2004). Recent evidence supports the contribution of embryonic cortical (*Emx-1* expressing) progenitors to generating the adult SVZ (Tamamaki. 2005;Willaime-Morawek et al., 2006;Ventura and Goldman,2007). However, it is still unknown whether other parts of the embryonic VZ such as the MGE contribute cells to the adult SVZ or, if so, whether adult SVZ cells retain their previously established positional and functional identities into adulthood.

Here in we have examined the origins of adult SVZ stem cells by Cre-*lox* fate mapping of the embryonic telencephalic neuroepithelium, using *Nkx2.1-Cre*, *Gsh2-Cre*, *Dbx1-Cre*, *Emx1-Cre* and *Emx1-CreER*^{T2} transgenic mice (Fogarty et al., 2005;Kessaris et al., 2006). We found that the MGE, LGE and the embryonic cortex all generate cells that populate different parts of the adult SVZ and exhibit the in vitro characteristics of multipotent neural stem cells. Both the LGE- and cortex-derived stem cells generate RMS neuroblasts and are responsible for generating olfactory interneurons throughout life. However, these two stem cell populations make unequal contributions to adult neurogenesis. Cortex-derived stem cells, despite being the minority, generate the majority of Calretinin-positive interneurons but none of the Calbindin-positive interneurons. LGE-derived stem cells generate all of the adult-born Calbindin-positive interneurons for the olfactory glomerulus.

METHODS

Transgenic mice

The generation and genotyping of *Nkx2.1-Cre*, *Gsh2-Cre*, *Emx1-Cre*, *Dbx1-Cre* and *Emx1-CreER^{T2}* mice was described previously (Fogarty et al., 2005;Kessaris et al., 2006). Each transgenic line was crossed with either of two Cre reporter lines: *Rosa26-enhanced Green Fluorescent Protein* (*R26-GFP*) (Mao et al., 2001) (Jackson Laboratories) or *Rosa26-Yellow Fluorescent Protein* (*R26-YFP*) (Srinivas et al., 2001) (kindly provided by S. Srinivas). To activate Cre recombinase activity in *Emx1-CreER^{T2}/ R26-YFP* transgenics (Kessaris et al., 2006), we administered Tamoxifen (Sigma), dissolved by sonication at a concentration of 50 mg/ml in corn oil (Sigma). Induction at embryonic day 10.5 (E10.5) was carried out using a single 4 mg dose of Tamoxifen administered into the stomach of pregnant mothers by gavage. Induction in adult mice was achieved by administering 300 mg/kg Tamoxifen per day by gavage on five consecutive days.

BrdU labeling

To identify cells dividing in vivo, BrdU (Sigma) was dissolved in phosphate buffered saline (PBS) by sonication to a concentration of 20 mg/ml and was administered as four 2 mg intra-peritoneal doses over 24 hours (6pm, midnight, 6am, noon).

Tissue preparation

Tissue was prepared as previously described (Young et al, 2007). Briefly, postnatal and adult mice (> P50) were anaesthetized with pentobarbitone and perfused through the ascending aorta, first with PBS and then with 4% (w/v) paraformaldehyde (PFA, Sigma) in PBS. Brains were removed, immersed in fresh 4% PFA and stored at 4°C overnight. Tissue was cryo-protected in 20% (w/v) sucrose in PBS for 24 hours. All brains were embedded in optimal cutting temperature (OCT) compound and coronal cryo-sections (15 or 30 μm nominal thickness) were collected onto Superfrost Plus slides (BDH) or floated in PBS.

Neurosphere culture

Brains were removed and examined under an inverted fluorescence microscope to confirm GFP expression. Neurosphere cultures were subsequently generated from the SVZ of seven to eight week

old (P50, young adult) mice, by culturing in serum-free medium (Stem Cell Technologies) containing the mitogens EGF (Sigma) and bFGF (Roche), as previously described (Reynolds and Weiss, 1992;Young et al., 2006;Young et al.,2007). Primary cell suspensions were plated at clonal density (one cell per well) or at a density not exceeding 3500 cells/cm^2 and all cultures were maintained at 37°C in a 5% CO_2 atmosphere. Neurospheres were considered mature when they had reached an average diameter of 120 μm.

Expression of GFP in neurospheres was examined using an inverted fluorescence microscope at six to seven days post-plating. The number of GFP-positive neurospheres in each culture was scored and expressed as a percentage of the total. At day seven a portion of the neurosphere culture was taken to test differentiation potential and the remainder was passaged. Neurospheres were differentiated by plating onto glass coverslips pre-coated with poly-D-ornithine (Sigma) and cultured in DMEM/F12 medium (Gibco BRL) with B27 supplement (Gibco BRL) and 2% fetal calf serum (Gibco BRL), for 5 days. Differentiated neurospheres were fixed for 10 minutes in 4% PFA at room temperature, washed three times with PBS, and stored at 4°C for immunostaining. The remainder of the culture was passaged by enzymatic dissociation with trypsin/EDTA (Gibco BRL) to generate a single-cell suspension, re-plated at a density of 10^4 cells/cm^2 and serially passaged seven times or more.

Stem cell colony-forming assays

The proportion of neural stem cells originating from each region of the embryonic neuroepithelium was quantified using the Neurocult Neural Colony-Forming Cell Assay Kit (Stem Cell Technologies) according to the manufacturer's instructions. In brief, a single cell suspension was generated from the SVZ of each Cre-transgenic. The cells were mixed with NeuroCult NCFC Serum-Free Medium (Stem Cell Technologies), containing 20 ng/ml EGF (Sigma), 10 ng/ml bFGF (Roche), 3.5 μg/ml heparin (Sigma) and 30% (v/v) collagen solution (Stem Cell Technologies). The suspension was poured into three 35 mm plates per mouse and incubated for 3 weeks at 37°C in a 5% CO_2 atmosphere. Growth factors were replenished weekly. By the end of three weeks approximately 5% of colonies were ≥ 2mm in diameter.

Each plate was fixed with 4% PFA for 45 minutes at room temperature, and then washed 3 times with PBS. The collagen matrix was removed from the 35 mm dishes and placed into a 100 mm Petri dish (three into one) containing 10 ml of blocking solution (PBS containing 0.1% (v/v) Triton-X100, 10% (v/v) normal sheep serum (NSS, Sigma) and placed on an orbital shaker at room temperature for three

hours. The blocking solution was replaced with 10 ml of blocking solution containing rabbit anti-GFP (1:6000, Abcam) and placed on a shaking platform overnight at 4°C. Matrices were washed in PBS during the day and stained with AlexaFluor 488-conjugated goat anti-rabbit IgG (1:1000, Invitrogen) overnight at 4°C. Matrices were again washed in PBS for four hours at room temperature before colony size was measured using a gridded Petri dish (Stem Cell Technologies). The neural stem cell colonies were characterized as ≥ 2mm in diameter (Brent Reynolds unpublished data; Bull and Bartlett, 2005) and scored as GFP-positive or -negative under a fluorescence dissecting microscope.

Immunocytochemistry

For GFP immunolabeling, sections were washed with PBS and permeabilized with blocking solution containing Triton-X100 and NGS (see previous paragraph) before incubating overnight with rabbit anti-GFP (1:6000) or rat anti-GFP IgG2a (1:1000, Nacalai Tesque Inc.). Sections were washed three times with PBS before adding AlexaFluor 488-conjugated anti-rabbit IgG or AlexaFluor 488-conjugated anti-rat IgG (1:1000; Invitrogen) in blocking solution. Sections were treated with Hoescht 33258 ($1:10^4$; Sigma) to detect cell nuclei and washed with PBS. Floating sections (30 μm) were transferred onto glass slides and mounted using Fluoromount (DAKO). Sections were co-stained with either CY3-conjugated mouse anti-GFAP (1:3000; Sigma); rabbit anti-GFAP (1:500; Dako); mouse anti-NeuN (1:500, Chemicon); rabbit anti-Calbindin (1:1000, Swant); rabbit anti-Calretinin (1:1000, Swant), rabbit anti-Parvalbumin (1:1000, Chemicon), rabbit anti-Tyrosine Hydroxylase (1:1000, Chemicon); guinea-pig anti-doublecortin (1:3000, Chemicon); mouse anti-PSA-NCAM IgM (1:1000, Chemicon); rabbit anti-Gsh2 polyclonal (1:2000, kind gift from Kenneth Campbell, University of Cincinnati) and detected with AlexaFluor 568-conjugated anti-mouse IgG1; AlexaFluor 568-conjugated anti-rabbit IgG; AlexaFluor 568-conjugated anti-mouse IgM (Invitrogen, all used at 1:1000;) or CY3-conjugated anti-guinea-pig (1:500, Pierce).

For BrdU labeling to determine the identity of newly born cells, 30 μm floating sections were first stained as above to detect GFP expression (or other listed antigens). The sections were subsequently collected onto glass slides and fixed with 70% ethanol/ 20% glacial acetic acid and treated for a further 20 minutes with 70% ethanol at -20°C. Slides were then washed with PBS / 1% Triton-X100 (v/v) and acid treated with 6M HCl / 1% Triton X-100 in PBS for 15 minutes before washing in PBS and staining with anti-BrdU (1:15, ATCC mouse hybridoma), detected with AlexaFluor 568-conjugated anti-mouse IgG1 or AlexaFluor 647-conjugated anti-mouse IgG1 (1:1000, Invitrogen).

Protein co-localization (multiple fluorochromes) was assessed in the confocal microscope. Three mice were examined for each transgenic genotype. From each mouse, three 30 μm coronal brain sections (i.e. six olfactory bulb sections) were immunolabeled to count Calretinin/ BrdU/ GFP triple-positive cells or Tyrosine Hydroxylase/ BrdU/ GFP-positive cells, or ten 30 μm coronal sections (20 olfactory bulb sections) for Calbindin/ BrdU/ GFP. A minimum of 20 newly born (BrdU+) neurons of each sub-type (Tyrosine Hydroxylase, Calretinin or Calbindin-positive) were scored for GFP expression (Fig. 7I). The proportions of BrdU- or Calbindin-positive cells in the olfactory bulb were also examined solely for GFP co-expression (minimum of 300 positive cells per mouse) (Fig. 5H, Fig. 7J, K). Statistical comparisons were made by ANOVA (for multiple variables) or t-test (for single variables).

To determine whether GFP-positive neurospheres could generate multipotent progeny, neurospheres were cultured on coverslips under differentiating conditions and were double- labeled (Young et al., 2006;Young et al., 2007) with anti-rabbit GFP and one of the following: mouse anti-β-III-tubulin (1:2000, Promega); rabbit anti-GFAP-CY3 (1:2500, Sigma); mouse anti-GFAP (1:1500, Sigma); monoclonal O4 IgM (1:5, ATTC mouse hybridoma). Sections were post-stained with Hoescht 33258 nuclear stain (1:10^4; Sigma). Fixed coverslips were treated with blocking solution (as above) for 30 minutes at 37°C, before the addition of the primary antibodies. The coverslips were washed three times with PBS prior to the addition of the appropriate secondary antibodies for three hours at room temperature. Secondary antibodies included Alexa 568-conjugated goat anti-mouse IgG1 (1:1000, Invitrogen), Alexa 680-conjugated goat anti-mouse IgM (1:1000, Invitrogen), Alexa 488-conjugated goat anti-rabbit IgG (1:1000, Invitrogen). Coverslips were washed and mounted as above.

RESULTS

The embryonic LGE and cortex both contribute cells to the adult SVZ

To map the contributions of different regions of the embryonic telencephalic VZ to the adult forebrain SVZ, we made use of a battery of transgenic lines that express Cre-recombinase in defined neuroepithelial domains: *Nkx2.1*-Cre (to label the MGE), *Gsh2*-Cre (LGE and MGE), *Emx1-Cre* (cerebral cortex) and *Dbx1-Cre* (cortico-striatal sulcus) (Fogarty et al., 2005;Kessaris et al., 2006) (**Fig. 1O**; E12). Each individual line (or combined *Emx1/Dbx1*) was crossed to the Cre-dependent *R26-GFP* reporter line to label specific subsets of neuropithelial cells and all of their progeny in the embryonic and postnatal brain. Immunolabeling for GFP identified a significant contribution of cells from all parts of the embryonic neuroepithelium to the adult brain (**Fig. 1A-H**). The *Nkx2.1* domain (MGE) made a minor contribution to the adult SVZ, with the greater number of GFP-positive cells

being located near the ventral extremity of the lateral ventricle (**Fig. 1E, J**). In occasional sections cells were also observed at the ventral edge of the dorso-lateral corner of the SVZ (**Fig. 1I**). The majority of cells lining the lateral wall of the lateral ventricle, including the dorso-lateral SVZ, were derived from the *Gsh2* domain (**Fig. 1F, K, L**). Together with the previous result, this indicates that most of the lateral wall cells are descendants of the LGE, as previously thought (see Introduction). However, we also observed a significant population of GFP-labeled SVZ cells in the *Emx1-Cre/R26-GFP* mice. These were found in the roof of the lateral ventricle and in the dorso-lateral corner of the SVZ (**Fig. 1G, M**)

The *Emx1*-derived SVZ cells were found mainly in the dorsal half of the dorso-lateral corner of the SVZ (**Fig. 1M**), suggesting that they were genuine descendants of the embryonic cortical VZ, not *Gsh2*-derived cells that had up-regulated *Emx1* postnatally and consequently recombined de novo in the adult. We tested this directly by inducing transient recombination in *Emx1-CreER^{T2}/R26-YFP* mice with a single dose of Tamoxifen at E10.5 and following the subsequent fates of labeled cells in the adult brain. The distribution of GFP-positive cells within the SVZ of pulse-labeled *Emx1-CreER^{T2}/ R26-YFP* mice (**Fig. 1N**) was similar to the constitutive *Emx1-Cre/R26-YFP* mice (**Fig. 1M**). These data demonstrate that cells from the embryonic cortex do indeed give rise to the dorsal and dorso-lateral edge of the adult SVZ. Therefore we conclude that the adult SVZ is comprised of cells derived predominantly from the VZ of the embryonic LGE and cortex (**Fig. 1O**), and that the location of these cells potentially defines germinal domains in the SVZ similar to those known to exist developmentally.

Multipotent, self-renewing SVZ stem cells are descended from both the LGE and cortex

To determine whether SVZ cells derived from both *Gsh2*- and *Emx1*-expressing embryonic territories continue to divide in the adult SVZ, as would be expected for stem or progenitor cells, we asked whether they can incorporate BrdU in vivo. Adult *Cre/ R26-GFP* mice were given a dose of BrdU every 6 hours for 24 hours and analyzed three hours after the final dose. Double immunolabeling for GFP and BrdU detected small numbers of *Nkx2.1* (MGE)-derived cells in the lateral wall of the SVZ that were BrdU-positive (**Fig. 2A, B**). The majority of BrdU-positive cells in the SVZ were derived from *Gsh2*-expressing territories (**Fig. 2C, D**), so most proliferative SVZ cells have their origins within the LGE. A significant number of BrdU-labeled cells in the dorso-lateral corner of the SVZ were *Emx1*-derived (**Fig. 2E, F**), confirming that the embryonic cortex also contributes proliferative cells to the adult SVZ. These could be either multipotent stem cells or neural progenitor cells with more restricted potential.

To investigate the stem and/or progenitor cell nature of the *Emx1*- and *Gsh2*-derived SVZ cells we turned to cell culture assays. The adult SVZ was dissected from *Cre/R26-GFP* mice and dissociated to generate neurosphere cultures (see Methods). Since each neurosphere was derived from a single proliferating cell in the starting culture, each sphere was either entirely positive or entirely negative for GFP (**Fig. 3A**). The proportions of GFP-positive and GFP-negative neurospheres were determined for each of our Cre lines. The majority of neurosphere-forming cells in the SVZ were of striatal origin; ~70% of all neurospheres were GFP-positive in cultures from *Gsh2-Cre/R26-GFP* mice (**Fig. 3B**). By comparison, *Emx1*-derived SVZ cells accounted for ~20% of the neurosphere-forming activity (**Fig. 3B**). Some *Nkx2.1*-derived neurospheres were detected but their contribution was small, in keeping with the low contribution of the *Nkx2.1*-expressing VZ to the adult SVZ (see above). All SVZ neurosphere-forming activity could be accounted for by the combination of *Gsh2*-derived (LGE plus MGE) and *Emx1/Dbx1*-derived (cortex plus cortico-striatal sulcus) SVZ cells (**Fig. 3B**)**.** These data provide clear evidence that the neurosphere-forming SVZ cells are heterogeneous in origin.

Neurospheres derived from *Gsh2-Cre*, *Emx1-Cre* and *Emx1/Dbx1-Cre* mice could be serially passaged more than seven times while remaining GFP-positive, confirming the presence of self-renewing stem cells (data not shown). Moreover, cultures from each of these Cre mice contained GFP-positive neurospheres that were able to generate GFAP-positive astrocytes, O4-positive oligodendrocytes and βIII-tubulin-positive neurons when transferred to differentiation conditions, confirming their multipotency in vitro (see Methods) (**Fig. 3C**). However, the *Nkx2.1*-derived neurospheres were less neurogenic than the others, because a smaller proportion of GFP-positive neurospheres generated βIII-tubulin-positive neurons (**Fig. 3D**).

To quantify the proportions of true SVZ stem cells descended from different regions of the embryonic VZ, we dissociated SVZ cells from *R26-GFP* reporter mice carrying *Nkx2.1-Cre*, *Gsh2-Cre*, *Emx1-Cre* or *Emx1/Dbx1-Cre* and plated them in a mitogen-containing collagen matrix. Neural progenitor cells with limited self-renewing capacity formed small colonies and stopped proliferating after 1-2 weeks, whereas self-renewing stem cells continued to proliferate for more than 3 weeks, forming colonies ≥ 2mm in diameter. Stem cell colonies that were GFP-positive (**Fig. 3E, F**) and others that were GFP-negative (**Fig. 3G, H**) were identified in all cultures after three weeks (**Fig. 3I**). The proportions of self-renewing SVZ stem cells contributed from each embryonic territory, as judged by this collagen matrix assay, were not the same as the proportions of neurosphere-forming cells measured previously. The embryonic LGE (*Gsh2-Cre*) provided around 70% of the neurosphere-forming cells but only ~40% of the self-renewing stem cells, whereas the cortex (including cortico-striatal sulcus, *Emx1/Dbx1-Cre*) provided only ~25% of the neurosphere-forming cells but ~50% of the self-renewing stem cells

(**Fig. 3B, I**). This suggests that LGE-derived adult SVZ cells might contribute a greater proportion of the transit amplifying cells and RMS neuroblasts in vivo.

LGE- and cortex-derived stem cells make neurons in the adult olfactory bulb

Adult SVZ stem cells have been identified as a subtype of subependymal astrocytes ("B cells") because they express GFAP in vivo. We confirmed that a subset of GFP-labeled adult SVZ cells were also GFAP-positive in *R26-GFP* reporter mice carrying either *Gsh2-Cre* or *Emx1-Cre* (**Fig. 4A, B**). There were also small numbers of cells co-labeled for GFP and GFAP in *Nkx2.1-Cre/ R26-GFP* mice (not shown). To visualize migratory neuroblasts, we immunolabeled each transgenic to detect GFP expression and either Doublecortin (Dcx) or PSA-NCAM. Dcx and PSA-NCAM-positive neuroblasts in the SVZ and RMS were detected only in the *Gsh2-Cre* and *Emx1-Cre* transgenics, indicating that the LGE- and cortex-derived stem cells are responsible for most or all olfactory neurogenesis in the adult (**Fig. 4D, E;** PSA-NCAM shown). Equivalent immunohistochemical analysis of the SVZ of adult *Emx1-creER^{T2}/R26-YFP* mice (**Fig. 4C, F**), induced at E10.5, produced a similar staining pattern to that of the adult *Emx1-cre/R26-GFP*, confirming that stem cells originating in the embryonic cortex and retained in the adult SVZ generate neuroblasts throughout life.

To determine directly the separate contributions of *Nkx2.1*-, *Gsh2*-, Dbx1 and *Emx1*-derived stem cells to olfactory neurogenesis we mapped GFP-labeled cells within the adult olfactory bulbs of our *Cre/R26-GFP* mice. GFP-positive cells were detected in the granule cell layer (gcl), mitral cell layer (mcl), external plexiform layer (epl) and glomerular layer (gl) of *R26-GFP* reporters carrying the *Nkx2.1-Cre*, *Gsh2-Cre* or *Emx1-Cre* transgenes (**Fig. 5A-C**) as well as the *Emx1/Dbx1-Cre/R26-GFP* triple-transgenic (not shown). Most of the GFP-labeled cells represent olfactory bulb cells that were generated during embryonic or early postnatal development, as opposed to adult-born cells. To identify SVZ-derived cells that migrated into the olfactory bulb during adulthood we administered BrdU at P50 and examined the olfactory bulbs four weeks later. Only ~1% of all BrdU-positive cells were *Nkx2.1*-derived (**Fig. 5D, H**). The majority (~70%) were *Gsh2*-derived (**Fig. 5E, H**), indicating that most adult-born cells in the olfactory bulb are generated from LGE-derived stem cells. Around ~30% of BrdU-labeled olfactory bulb neurons were *Emx-1* derived (**Fig. 5F, H**). No additional contribution was observed in *Emx1/Dbx1-Cre/R26-GFP* mice (**Fig. 5G, H**), indicating that *Dbx1*-derived cells do not generate significant numbers of adult olfactory cells.

LGE-and cortex-derived adult SVZ stem cells have different properties

Gsh2- and *Emx1*-derived cells were found in all layers of the olfactory bulb, although there was a relatively higher contribution of *Emx1*-derived cells to the glomerular layer than the granule cell layer (**Fig. 5K**). To investigate whether this laminar bias might result from different cell fate specificities of *Gsh2*- and *Emx1*-derived stem cells, we categorized their adult-born (BrdU-positive) neuronal progeny by immunolabeling for Parvalbumin (PV), Tyrosine Hydroxylase (TH), Calretinin (Crt) or Calbindin (Cb). The locations and connections of olfactory bulb neurons that express these markers are illustrated in **Fig. 6**.

BrdU was administered at seven weeks of age and the olfactory bulbs analyzed four weeks later (P80) as before. Parvalbumin-positive neurons were detected in the external plexiform layer but were never labeled with BrdU (more than 100 Parvalbumin-labeled cells examined in 3 mice of both *Emx1-Cre* and *Gsh2-Cre* backgrounds) suggesting that this population is not turned over significantly in the adult. Parvalbumin-positive neurons had dual developmental origins, with some cells co-expressing YFP in both the *Emx1-Cre* and *Gsh2-Cre* transgenics (**Fig. 7A, B**).

Tyrosine Hydroxylase-positive, BrdU-positive neurons were detected in the glomerular layer. These adult-born Tyrosine Hydroxylase-positive interneurons were generated from both LGE- and cortex-derived SVZ stem cells (**Fig. 7C, D, I**) in numbers proportional to the overall contributions of these stem cell pools to the olfactory bulb (~70% and ~30% respectively) (**Figs. 5H, 7I**) and similar to their neurosphere-forming activities in vitro (**Fig. 3B**). These data suggest that LGE- and cortex-derived neuroblasts are equally likely to generate Tyrosine Hydroxylase-positive (dopaminergic) interneurons.

The situation was different for Calretinin-positive, adult-born neurons, the majority of which (~60%) were generated from cortex-derived (*Emx1*) stem cells (**Fig. 7E, F, I**), despite the fact that cortex-derived SVZ stem cells generate only ~30% of the total (**Fig. 5H**). This was the first indication that LGE-derived and cortex-derived stem cell progeny might have differential cell fates. This conclusion was strikingly reinforced when we immunolabeled for Calbindin. We found that while there were far fewer new adult-born Calbindin-expressing interneurons generated in the olfactory glomeruli relative to Calretinin (~10-15%), they were all produced by LGE-derived SVZ stem cells, and none from their cortex-derived counterparts (**Fig. 7G, H, I**). Interestingly 88% $\pm$ 6% of all Calbindin-positive olfactory interneurons present at P80 were generated from striatum (*Gsh2*)-derived progeny (Calbindin and GFP double-positive; **Fig. 7J**). By comparison, only 1.5% $\pm$ 0.8% of Calbindin-positive interneurons appeared to be GFP-positive in *Emx1-Cre /R26-GFP* mice (**Fig. 7K**) (see Methods - Immunocytochemistry for experimental details). For further confirmation that embryonic cortex-derived cells fail to generate Calbindin-positive olfactory interneurons we again turned to the *Emx1-*

CreER^{T2}/ R26-YFP transgenic line. Tamoxifen was administered at E10.5 and the olfactory bulbs examined at P50. This experiment confirmed that descendants of embryonic cortical precursors included significant numbers of Tyrosine Hydroxylase- and Calretinin-positive interneurons but very few Calbindin-positive interneurons (**Fig. 7L-N)**. Collectively, these data strongly support the idea that cortex-derived and striatum-derived precursor/ stem cells remain functionally distinct throughout life, generating different subsets of olfactory neurons during development and even in the adult.

Tamoxifen was also administered to adult *Emx1-CreER^{T2}/R26-YFP* transgenic mice (~P50), allowing us to label and trace the descendants of any stem or progenitor cells that continue to express *Emx1* into adulthood. Animals were analyzed at different times after Tamoxifen induction to follow the progress of YFP-labeled cells from the SVZ to the olfactory bulb. At one week post-induction, the shortest time lag examined, YFP-positive cells were observed in the dorsal part of the adult SVZ and the proximal end of the RMS. The location of these cells within the SVZ was consistent with the restricted location of cortex-derived progeny reported previously (**Fig 1M, N**). The GFP-positive cells identified were also GFAP-positive (**Fig. 8A, B**). By four weeks post-induction (YFP, GFAP) double-positive cells were still present in the SVZ (**Fig. 8C**) and, in addition, there were clusters of GFP-positive cells that were GFAP-negative (**Fig. 8D**). Some of these cells were Dcx-positive neuroblasts. Other YFP-positive, Dcx-negative cells were observed on the dorsal edge of the SVZ and in the corpus callosum (not shown).

The olfactory bulb was devoid of YFP-positive cells at short times after Tamoxifen induction, as expected if they migrate into the bulb from the SVZ via the RMS (**Fig. 8E**). After four weeks, both (YFP, PSA-NCAM) double-positive neuroblasts and (YFP, NeuN) double-positive neurons could be found in the granule cell layer (**Fig. 8F, G**) and the glomerular layer (not shown). The number of YFP-labeled cells in the olfactory bulb remained small because of the inefficiency of Tamoxifen induction in adulthood. However, there were some cells that co-labeled for YFP and Calretinin in the granule cell layer (**Fig. 8H**) and the glomerular layer (not shown). None of >250 YFP-positive cells that we examined co-expressed Calbindin, reinforcing the results obtained with constitutive *Emx1-Cre/ R26-YFP* mice and BrdU (**Fig. 7H, K**). The fact that the *Emx1-CreER^{T2}* transgene remains active in the adult implies that cortex-derived SVZ stem cells continue to express *Emx1* into adulthood. In addition, the YFP-positive cells detected in the SVZ of *Emx1-Cre/R26-YFP* mice never immunolabeled for Gsh2 (supplementary **Fig. S1**). Therefore, we conclude that the different SVZ stem cell pools not only have different embryonic origins but also different molecular properties and different neurogenic fates in the adult olfactory bulb.

DISCUSSION

The main findings of this study are as follows: 1) the adult SVZ contains stem cells derived from multiple regions of the embryonic neuroepithelium - MGE, LGE and cortex 2) stem cells from both the LGE and cortex contribute to adult neurogenesis in the olfactory bulb and 3) cortical and LGE-derived stem cells are distinct populations with regard to their distribution within the SVZ, their molecular properties and their behaviour, since they make non-proportional contributions to the genesis of olfactory interneuron subtypes.

SVZ stem cells are heterogeneous in origin and behaviour

Previous evidence had suggested a purely striatal origin for adult SVZ stem cells (Stenman et al., 2003). However, we show here that the LGE contributes most but not all neurosphere-forming cells and less than half of the self-renewing stem cells, the remainder originating mainly from the embryonic cortex (*Emx1*- plus *Dbx1*-domains). Our findings confirm the recent report from Willaime-Morawek et al. (2006) that cells from the embryonic cortex contribute to the neurosphere-forming cells of the adult SVZ. Our findings are also consistent with the report from Ventura and Goldman (2007) that dorsal (cortical) radial glia are stem cells that contribute interneurons to the adult olfactory bulb, in addition to astrocytes and oligodendrocytes.

Since neurosphere cultures derived from our *Cre/ R26-GFP* transgenic mice contained neurospheres that were entirely GFP-positive and others that were entirely GFP-negative, it is clear that the patterns of gene expression established early in the embryo remain stable in the adult, even when the cells are dissociated and cultured at low density - that is, cells from the embryonic cortex do not activate Gsh2 expression in culture. Stability of gene expression is also supported by the fact that SVZ cells that express YFP in the *Emx1-Cre/R26-YFP* transgenic do not exhibit Gsh2 immunoreactivity (supplementary **Fig. S1**) and that our *Emx1-CreER*^{T2} transgene made a similar contribution to the adult SVZ whether Tamoxifen was administered in the embryo (E10.5) or in the adult (P50). We therefore presume that the adult SVZ contains distinct populations of Emx1-expressing and Gsh2-expressing stem cells (and possibly others), which have distinct and overlapping neurogenic properties.

It is known that at least three subtypes of GABAergic interneurons expressing the GABA-synthesizing enzyme Glutamate Decarboxylase can be identified in the periglomerular layer of the olfactory bulb. These three largely non-overlapping populations can be identified by their expression of Calretinin, Calbindin-D28K (two members of the EF-hand family of calcium-binding proteins) or Tyrosine

Hydroxylase (dopaminergic sub-population) and are all generated or regenerated continuously during adulthood (Kosaka et al., 1995;Dellovade et al., 1998;Hack et al., 2005;Kohwi et al., 2005). Adult-born Calretinin-positive interneurons are also found in the granule neuron layer (see **Fig. 6**). Our data confirm that these neuronal subtypes continue to be generated during adulthood. However when we examined the turnover of interneurons expressing Parvalbumin, a third calcium-binding protein of the EF-hand family, we found that these neurons, located in the external plexiform layer (EPL), are not turned over significantly during adulthood. In this sense they resemble projection neurons such as mitral cells but differ from most olfactory interneurons. Parvalbumin-positive neurons are also present in the human olfactory bulb and these were reported to be generated continuously in the adult (Bedard and Parent, 2004). However, human Parvalbumin neurons are found in the glomerular layer, not the EPL, suggesting that Parvalbumin marks different populations of neurons in humans and mice. Otherwise, adult-born neurons in the human olfactory bulb include GAD65-, Tyrosine Hydroxlyase- and Calretinin-positive interneurons, similar to those of the mouse (Kosaka et al., 1995;Bedard and Parent, 2004).

There are stem cells that reside in the RMS throughout life, in addition to those in the SVZ (Gritti et al., 2002). Hack et al. (2005) found that the RMS stem cells generate the majority of Tyrosine Hydroxylase-positive interneurons in the adult olfactory bulb, the remainder coming from the SVZ. Ventura and Goldman (2007) labeled dorsal radial glia directly using an adenovirus applied to the cortical surface and showed that they generate Tyrosine Hydroxylase-positive periglomerular neurons as well as astrocytes and oligodendrocytes. The RMS is contiguous with the SVZ and the cortical (*Emx1*-expressing) neuroepithelium contributes to the development of both structures, so our own fate-mapping experiments with *Emx1-Cre* cannot distinguish adult stem cells that reside in the RMS from those of the dorsal SVZ. Nevertheless, we too have confirmed that cortex-derived stem cells – presumably a combination of RMS and SVZ stem cells - generate Tyrosine Hydroxylase-positive (and Calretinin-positive) olfactory interneurons in the adult.

Olfactory bulbectomy has provided evidence that interneuron cell type identity is established prior to neuroblasts reaching the olfactory bulb in vivo (Jankovski et al., 1998). Our data support this conclusion by demonstrating that the neurogenic properties of adult SVZ stem cells are determined very early, according to their sites of origin in the embryonic neuroepithelium. We found that LGE- and cortex-derived stem cells (whether from RMS or SVZ) generated all of the adult-born Tyrosine Hydroxylase interneurons in the olfactory bulb (Fig 7I) and did so in proportion to the contributions of these stem cell populations to olfactory neurogenesis overall. However, this was not true for adult-born Calretinin interneurons, most of which were generated from cortex-derived SVZ stem cells,

despite their being less neurogenic overall than those from the LGE. Strikingly, all of the adult-born Calbindin-D28K-positive interneurons originated from LGE-derived SVZ stem cells and none from cortex-derived stem cells. These data indicate that the fates of adult SVZ stem cells are already determined within the SVZ (or RMS). Together with the finding of Hack et al. (2005) that the neurogenic properties of the RMS and SVZ are distinguishable, our studies raise the possibility that some aspects of embryonic patterning along both dorsal-ventral and anterior-posterior axes are preserved in the adult SVZ.

Different SVZ stem cells for different purposes?

SVZ neural stem cells have been defined in vitro by their ability to proliferate, self-renew and generate multipotent progeny (Richards et al., 1992;Reynolds and Weiss, 1992;Young et al, 2006;Young et al, 2007). In vivo, the slowly dividing neural stem cells generate more rapidly-dividing neural progenitor cells ("transit amplifying cells"), which in turn generate post-mitotic neuroblasts destined for the olfactory bulb (Doetsch et al., 1999a;Doetsch et al., 1999b). Transit amplifying cells and neural stem cells are both capable of generating neurospheres (Doetsch et al., 2002) so, in addition to neurosphere assays, we cultured SVZ cells in a semi-solid collagen matrix to identify the true stem cells, which proliferate longer and form larger colonies than transit-amplifying cells. We found that the numbers of stem cells and neurosphere-forming cells were not in proportion to one another. For example, the LGE contributed a greater proportion of neurosphere-forming cells than stem cells (~70% versus ~40% of the total), whereas for the cortex (*Emx1* plus *Dbx1* domains) the opposite was true (~30% versus ~50%). These data suggest that LGE-derived stem cells might be more active in generating transit-amplifying cells for the RMS than their cortex-derived counterparts. It is also worth noting that whereas the embryonic *Dbx1*-expressing domain at the lateral edges of the cortex appears to contribute ~20% of the in vitro stem cell activity of the SVZ (**Fig. 3I**), this domain did not contribute significant numbers of adult born olfactory cells in vivo (**Fig. 5H**). Therefore, the *Dbx1*-derived stem cells might serve some function other than olfactory neurogenesis, perhaps contributing preferentially to adult gliogenesis, another known function of the postnatal SVZ (Levison and Goldman, 1993;Luskin and McDermott, 1994;Menn et al., 2006). Alternatively, they might lie dormant until activated following an ischemic insult or other CNS damage (Magavi et al., 2000;Picard-Riera et al., 2002;Sundholm-Peters et al., 2005) or in response to a physiological stimulus (Shingo et al., 2003).

Cell fate determinants in the SVZ and RMS

We have taken advantage of the spatially restricted embryonic expression of several transcription factors to show that embryonic origin is important for the future properties of adult stem cells and their progeny. This was highlighted by our examining the origin of Calbindin-positive olfactory interneurons. The great majority of these cells are generated during early postnatal development (DeMarchis et al., 2007) from striatum-derived precursor cells (this study). Smaller numbers of Calbindin-positive interneurons are generated continuously during adult life and these are all derived from striatum-derived SVZ stem cells. In contrast, the cortex made an insignificant contribution to the genesis of Calbindin-positive interneurons at any stage of development or in the adult. It therefore seems likely that the transcription factors we used to demarcate the embryonic stem cell domains (Gsh2 and Emx1) - and possibly others - remain active in adult stem cells and play a role in restricting their subsequent cell fates. It is known that Emx1 has an important function in olfactory behaviour (Cao and Li, 2002). Other transcription factors that have been detected in the SVZ and/or RMS include Pax6 (Hack et al., 2005; Kohwi et al., 2005), Mash1 (Kohwi et al., 2005), Dlx2 (Doetsch et al., 2002), Olig2 (Parras et al., 2004;Hack et al., 2005), Er81 (Stenman et al., 2003) and Sp8 (Waclaw et al., 2006). Sp8 is retained in Calretinin-expressing and non-dopaminergic GABAergic interneurons of the glomerular layer. It will be interesting to determine whether any of these transcription factors or specific combinations of them are expressed in subsets of neuroblasts that can be traced back specifically to either LGE- or cortex-derived SVZ stem cells.

Our demonstration that striatum-derived and cortex-derived stem cells generate distinct subsets of olfactory interneurons in the adult, taken together with evidence that they are spatially and developmentally distinct populations, indicates that adult SVZ stem cells are non-uniform with respect to their cellular properties and behavior. This is potentially an important consideration when attempting to harness endogenous neural stem cells for therapy or repair.

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FIGURE LEGENDS

Figure 1 All regions of the telencephalic neuroepithelium contribute to the adult SVZ. Coronal sections (30 μm) of adult (P50) mouse brains were stained with rabbit anti-GFP (green) and Hoescht 33258 to visualize cell nuclei (blue). GFP-positive cells were detected by immuno-labeling in sections from *R26-GFP* reporters carrying *Nkx2.1-Cre* (**A, E**), *Gsh2-Cre* (**B, F**), *Emx1-Cre* (**C, G**) or *Emx1/Dbx1-Cre* (**D, H**) transgenes. Each region of the embryonic neuroepithelium contributed preferentially to distinct regions of the adult SVZ. Images (I-M) are higher-magnification confocal images of the areas boxed in panels (E-H). (**I, J**) *Nkx2.1-Cre/R26-GFP* brain. Groups of GFP-positive cells were occasionally detected at the ventral edge of the dorso-lateral corner of the SVZ (**I**), as well as the ventral region of the lateral wall (**J**). (**K, L**) *Gsh2-Cre/R26-GFP* brain. Many GFP-positive cells were found in the lateral wall (**K**) and the dorso-lateral corner (**L**) of the SVZ. (**M**) *Emx1-Cre/R26-GFP* brain. Significant numbers of GFP-labeled cells are present, mainly in the dorsal half of the dorso-lateral corner of the SVZ. (**N**) Adult (P50) *Emx1-CreER^{T2}/ R26-YFP* brain, following Tamoxifen induction at E10.5. The distribution of GFP-labeled cells was found to be similar to the *Emx1-*

Cre/R26-GFP animals – i.e. in the dorsal part of the dorso-lateral corner of the SVZ. (**O**) Schematic depicting the different embryonic neuroepithelial domains targeted by our Cre mice and their relative contribution to generating the adult SVZ. Scale bars: (A-D) 1 mm, (E-H) 0.5 mm, (I-N) 25 µm.

Figure 2 The embryonic striatum and cortex both contribute proliferative cells to the adult SVZ. BrdU was administered to adult *R26-GFP* reporter mice carrying *Nkx2.1-Cre* (**A, B**), *Gsh2-Cre* (**C, D**) or *Emx1-Cre* (**E, F**) transgenes and the brains were analyzed 24 hours later by immuno-labeling with anti-BrdU (red) and anti-GFP (green) and confocal fluorescence microscopy. BrdU/GFP double-positive cells (yellow) were present in all animals, the greatest proportion being found in *Gsh2-Cre/R26-GFP* mice. The inset high-magnification images are of the cells indicated by arrows (**B, D, F**)**.** All images are single confocal scans. Scale bars: (A-C) 0.5 mm, (D-F) 50 µm.

Figure 3 All regions of the embryonic neuroepithelium give rise to multipotent, self-renewing stem cells in the adult SVZ. (**A**) Neurosphere cultures were generated from the SVZ of adult *R26-GFP* reporter mice carrying *Nkx2.1-Cre*, *Gsh2-Cre* or *Emx1-Cre* transgenes, or both *Emx1-* and *Dbx1-Cre* together. Individual neurospheres were either uniformly GFP-positive or –negative, consistent with each being derived from a single cell in the starting culture. (**B**) The proportions of GFP-positive neurospheres are shown graphically (mean ± standard deviation, three independent cultures from three adult mice of each genotype). (**C**) Neurospheres were re-plated under differentiation-inducing conditions for five days before being fixed and triple-immuno-labeled with anti-GFP (green), anti-βIII-tubulin (red, to label neurons) and monoclonal O4 (magenta, to label oligodendrocyte lineage cells), then counter-stained with DAPI nuclear stain (blue). The example shown in (**C**) depicts a GFP-positive and a GFP-negative neurosphere from an *Emx1-Cre/R26-GFP* culture differentiating side by side, illustrating the generation of neurons and oligodendrocytes from stem cells of differing embryonic origins. (**D**) The proportions of GFP-positive neurospheres of each genotype that generated βIII-tubulin-positive neurons are presented graphically. *Nkx2.1*-derived neurospheres were significantly less neurogenic than the others ($P<0.05$). All neurospheres regardless of genotype generated GFAP-positive astrocytes (not shown). (**E**) SVZ cells were dissected free of surrounding tissue, dissociated and cultured in a semi-solid matrix with mitogens for three weeks (see Methods). Long term self-renewing stem cells gave rise to large colonies that distinguished them from transit-amplifying precursors which have less proliferative potential. Individual colonies were either GFP-positive (**E, F**) or GFP–negative (**G, H**) depending on the origin of the founder stem cell. The examples shown are from an adult *Emx1-Cre/R26-GFP* culture. (**I**) The proportions of GFP-positive stem cell colonies of each genotype are shown graphically. Scale bars: (A) 100 µm, (C) 25 µm, (E-H) 0.5 mm.

Figure 4 Cortex- and LGE-derived SVZ stem cells generate new neuroblasts in vivo.
Coronal sections through the forebrain of *R26-GFP* reporters carrying either the *Gsh2-Cre* (**A, D**), *Emx1-Cre* (**B, E**) or *Emx1-CreER*^{T2} (**C, F**) transgene were double-immuno-labeled for GFP (green) together with either GFAP (red; to identify multipotent stem cells) (**A-C**) or PSA-NCAM (red; to identify neuroblasts) (**D-F**). All sections were counterstained with Hoechst nuclear dye (blue). Images show the dorso-lateral corner of the SVZ (compressed confocal series with orthogonal views taken at the level indicated by the dashed line). GFAP-positive stem cells and PSA-NCAM-positive neuroblasts descend from both the embryonic LGE and cortex (single confocal scan inset). Scale bar: 30 µm.

Figure 5 LGE- and cortex-derived stem cells give rise to new cells in the adult olfactory bulb.
30 µm coronal sections through the olfactory bulb of adult *R26R-GFP* reporter mice carrying *Nkx2.1-Cre*, *Gsh2-Cre* or *Emx1-Cre* transgenes were immunolabeled for GFP (**A-C**). To identify newly-generated cells, we injected BrdU at P50 and analyzed the olfactory bulb 30 days later (P80) for the presence of (GFP, BrdU) double-positive cells. Co-labeled cells were rarely detected in *Nkx2.1-Cre/R26-GFP* olfactory bulbs (**D, H**). However, many (GFP, BrdU) double-positive cells were present in *Gsh2-Cre/R26-GFP*, *Emx1-Cre/R26-GFP* and *Emx1/Dbx1-Cre/R26-GFP* olfactory bulbs (**E-G,** arrows). The proportions of newly-born adult cells were quantified according to genotype for the entire olfactory bulb (OB) and separately for the granule cell layer (gcl), mitral cell layer (mcl), external plexiform layer (epl) and glomerular layer (gl) (see panel **A**). There is laminar variation in the relative proportions of *Gsh2-* and *Emx1-*derived adult-born cells. See Methods (Immunocytochemistry) for experimental details. Scale bars: (**A-C**) 200 µm, (**D-G**) 20 µm.

Figure 6: Locations and connections of interneurons in the olfactory bulb. The diagram represents a coronal section through an adult mouse olfactory bulb. New neuroblasts (Dcx-positive) enter the bulb from the rostral migratory stream (RMS), migrate radially and differentiate as interneurons in 1) the granule cell layer (GCL), 2) the mitral cell layer (MCL), 3) the external plexiform layer (EPL) and 4) the glomerular layer (GL). Immunolabeling for Calretinin (Crt), Calbindin (Cb), Tyrosine Hydroxylase (TH) or Parvalbumin (PV) identifies sub-populations of olfactory interneurons distributed throughout the bulb as shown. The majority of granule neurons (G) are not identified by any of these markers. The MCL also contains mitral projection neurons (M), which are not replaced during adulthood from RMS neuroblasts. Olfactory sensory neurons (O) in the olfactory epithelium in the nasal cavity are also not replaced by RMS neuroblasts. Adapted from Lledo et al (2006).

Figure 7: LGE- and cortex-derived SVZ stem cells generate different sub-populations of olfactory interneurons in the adult. BrdU was administered to adult (seven week old) *R26-GFP* reporter mice carrying either *Gsh2-Cre* or *Emx1-Cre* transgenes. Four weeks later (~P80), coronal sections of olfactory bulbs were triple-immunolabeled for BrdU (blue), GFP (green) and either Parvalbumin (**A, B**), Tyrosine Hydroxylase (TH) (**C, D**), Calretinin (Crt) (**E, F**) or Calbindin (Cb) (**G, H**) (red). Cells indicated by arrows in the compressed confocal series are shown at high magnification in the insets (single confocal scans). The proportions of adult-born interneurons of each genotype are presented graphically in (**I**). *Gsh2*- and *Emx1*-derived stem cells generate different proportions of TH-, Crt- and Cb-positive neurons - the most striking example being Cb-positive neurons which were exclusively *Gsh2* (LGE)-derived. The vast majority of Cb-positive neurons (red) co-express GFP (green) in olfactory bulb sections from *Gsh2-Cre/ R26-GFP* (**J**) but not *Emx1-Cre/ R26-GFP* mice (**K**), demonstrating that production of Cb-positive interneurons is the preserve of striatum-derived precursor/stem cells throughout development as well as in the adult. (**L-N**) Tamoxifen was administered to *Emx1-CreER^{T2}/ R26-GFP* transgenic mice at E10.5 and the progeny of these cells were traced into adulthood (P50). The olfactory bulbs were immuno-labeled to detect GFP (green) and either TH (**L**), Crt (**M**) or Cb (**N**) (red). All nuclei were counterstained with Hoescht 33258 (blue). These data demonstrate that cells derived from the embryonic cortex make a significant contribution to the genesis of TH-positive and Crt-positive interneurons, but fail to generate Cb-positive interneurons. Compressed confocal Z-series are shown with orthogonal views taken at the levels indicated by the dashed lines. See Methods (Immuncytochemistry) for experimental details. Scale bars: (**A-H**) 20 µm, (**J-K**) 40 µm, (**L-N**) 50 µm.

Figure 8: A population of adult SVZ stem cells expresses *Emx1* into adulthood. Tamoxifen was administered to young adult (seven week old) *Emx1-CreER^{T2}/R26-YFP* transgenic mice. After one week (**A**, **B**) or 6 weeks (**C**, **D**) brain sections were double-immuno-labeled for YFP (green) together with GFAP (red) to label stem cells in the SVZ (**A, C, D**) or proximal RMS (**B**). Sections were counter-labeled with Hoechst nuclear stain (blue). (YFP, GFAP) double-positive stem cells were present at both one week and six weeks post-Tamoxifen. YFP-single-positive presumptive neuroblasts were also present at six weeks (**D**). No YFP-labeled neurons (NeuN-positive) were present in the olfactory bulb at one week post-Tamoxifen (**E**) but small numbers were found at six weeks (**F**). At six weeks post-Tamoxifen, (YFP, PSA-NCAM) double-positive neuroblasts and (YFP, Calretinin) double-positive interneurons were also present (**G, H**). High magnification images of representative cells are shown as insets (single confocal scans). These data are consistent with the interpretation that Emx1-positive

stem cells in the SVZ generate neuroblasts that migrate via the RMS into the olfactory bulb and differentiate as olfactory interneurons. Scale bars: 50 µm.

Supplementary Figure S1: Emx1-derived cells in the adult SVZ do not express Gsh2. Coronal sections through the SVZ of an adult *Emx1-Cre/R26-YFP* transgenic mouse were immuno-labeled with anti-Gsh2 (red), anti-GFP (green) and counter-stained with Hoechst 33258 (blue). (**A, B**) Scattered Gsh2-positive nuclei (arrow) were detected, but never in YFP-positive cells (**C**), suggesting that there are distinct Emx1-expressing and Gsh2-expressing cell populations in the adult SVZ. A single confocal scan (~1 µm) is shown. Scale bar: 20 µm.

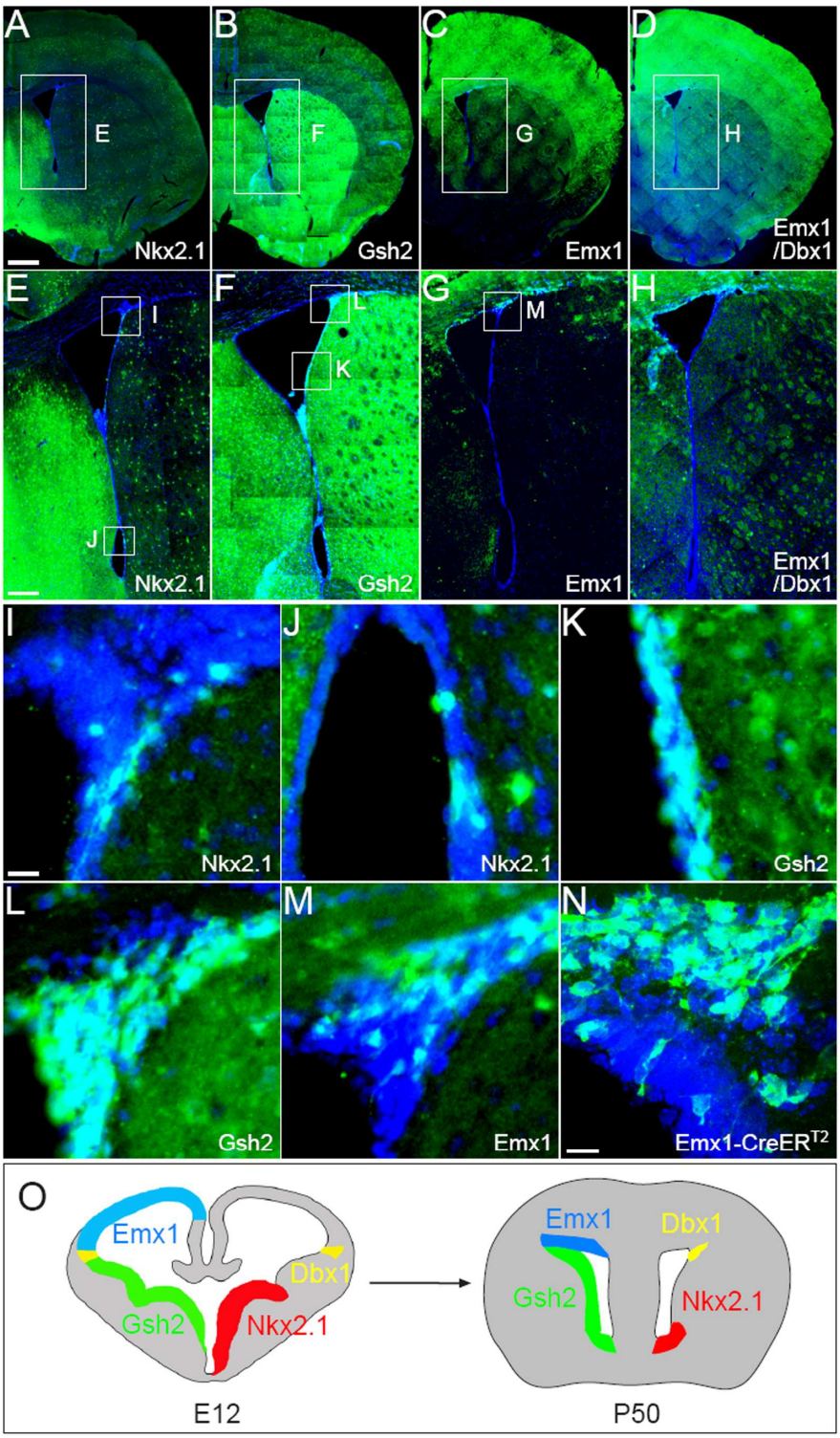

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Nkx2.1
Gsh2
Emx1
Emx1
/Dbx1
Emx1-CreER^{T2}
Dbx1
Nkx2.1
E12
P50

FIGURE 1

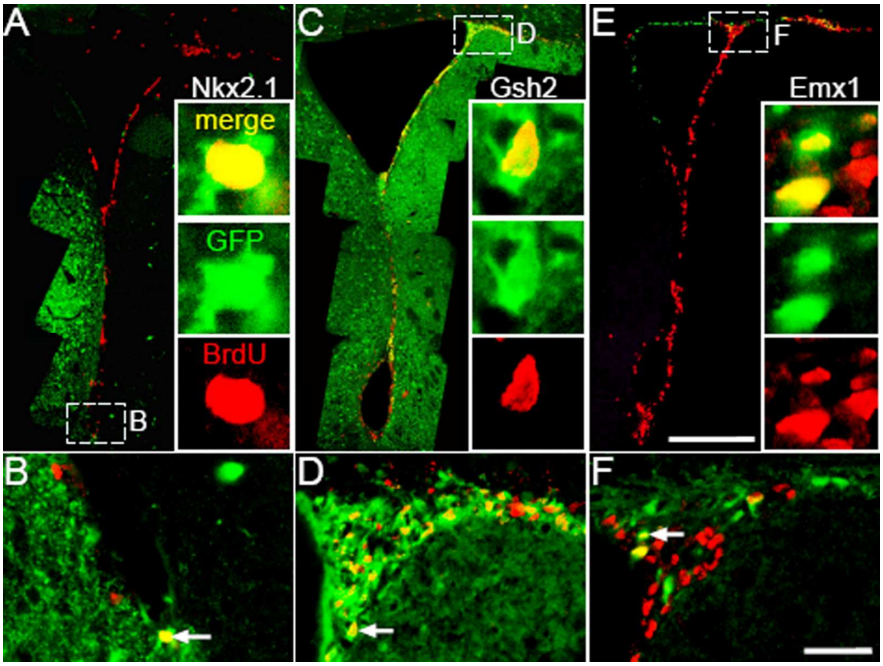


FIGURE 2

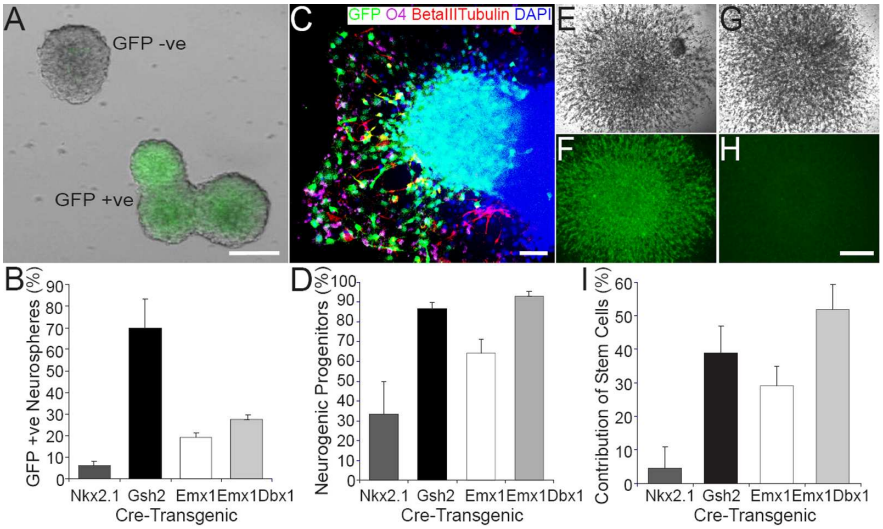


FIGURE 3

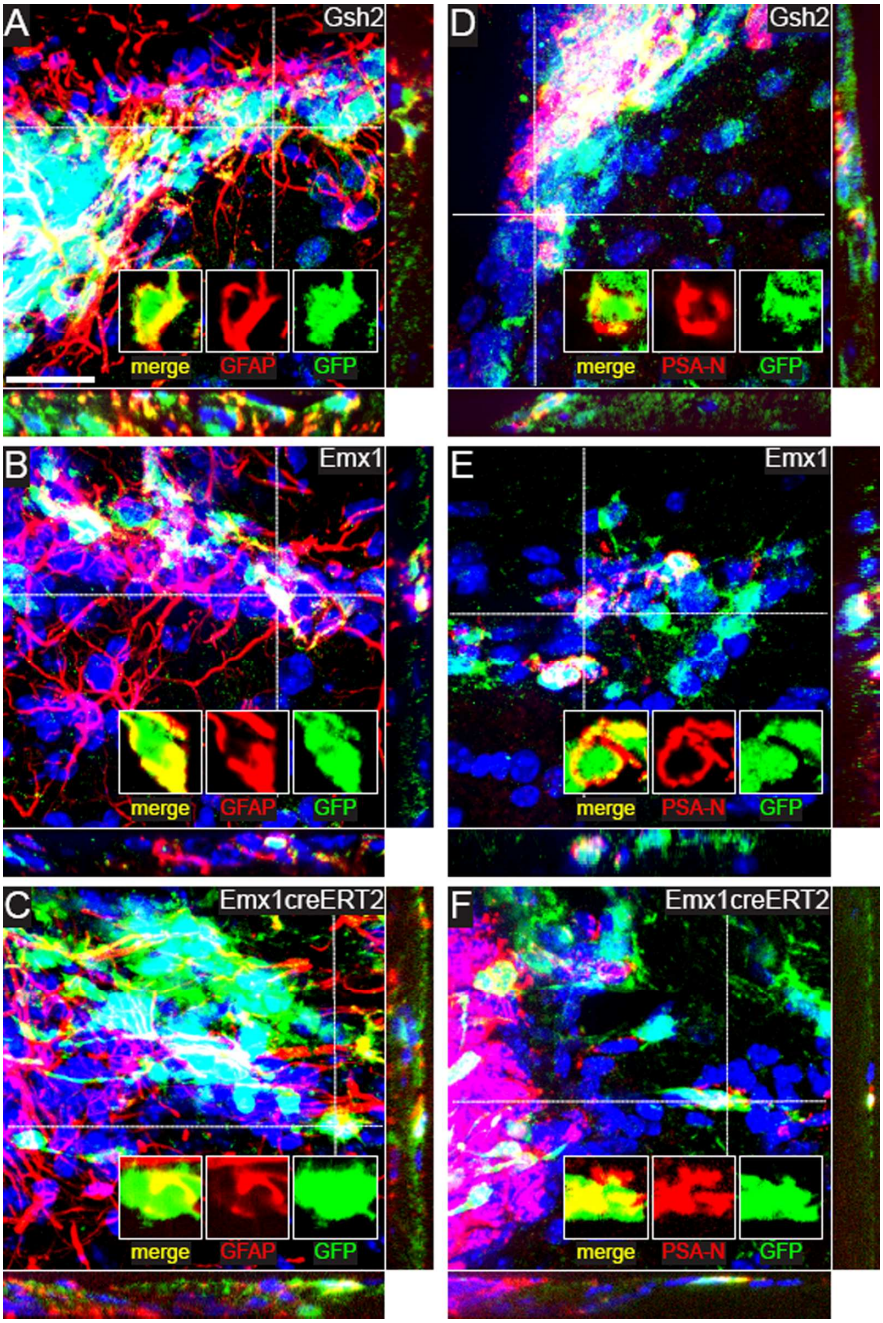

A
Gsh2
merge
GFAP
GFP
D
Gsh2
merge
PSA-N
GFP
B
Emx1
merge
GFAP
GFP
E
Emx1
merge
PSA-N
GFP
C
Emx1creERT2
merge
GFAP
GFP
F
Emx1creERT2
merge
PSA-N
GFP

FIGURE 4

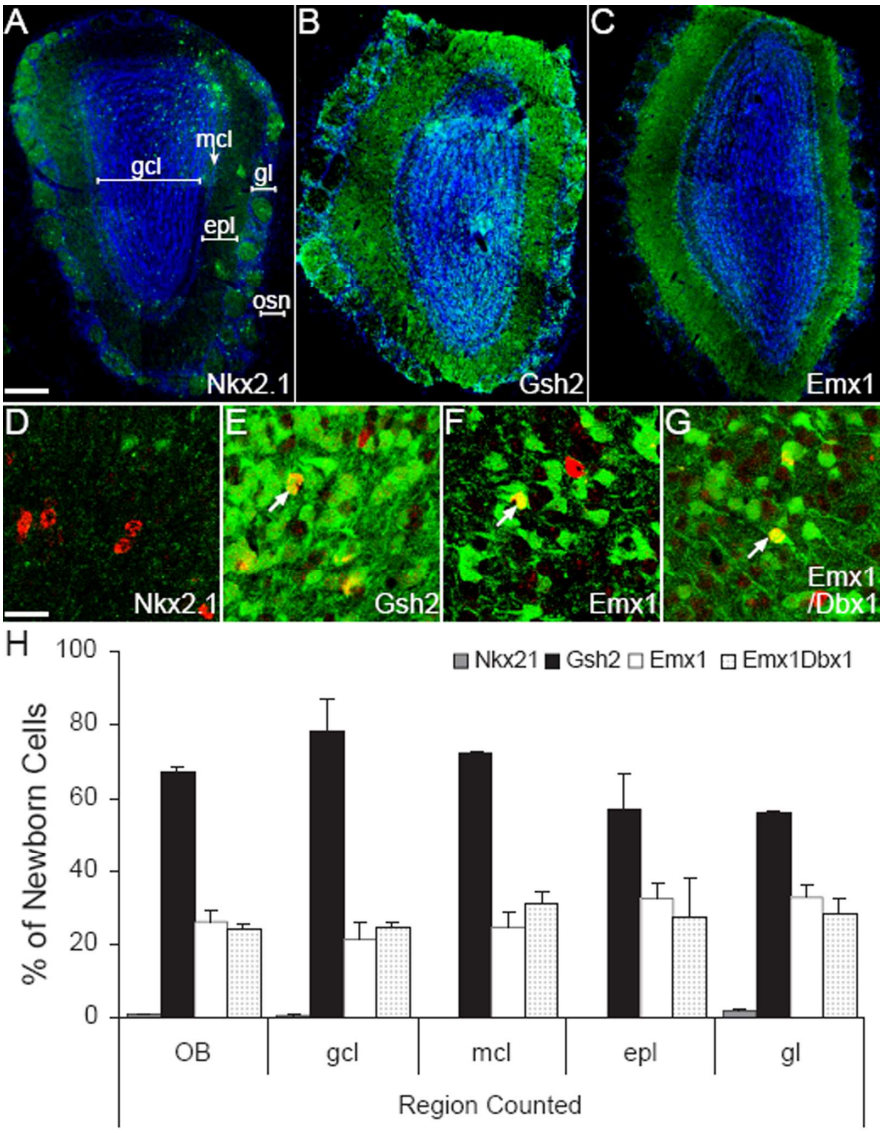

A
B
C
mcl
gcl
gl
epl
osn
Nkx2.1
Gsh2
Emx1
D
E
F
G
Nkx2.1
Gsh2
Emx1
Emx1
/Dbx1
H
Nkx21
Gsh2
Emx1
Emx1Dbx1
% of Newborn Cells
100
80
60
40
20
0
OB
gcl
mcl
epl
gl
Region Counted

FIGURE 5

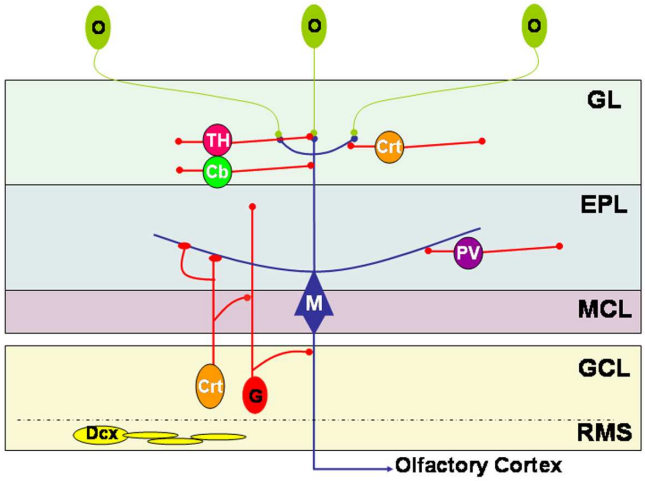


FIGURE 6

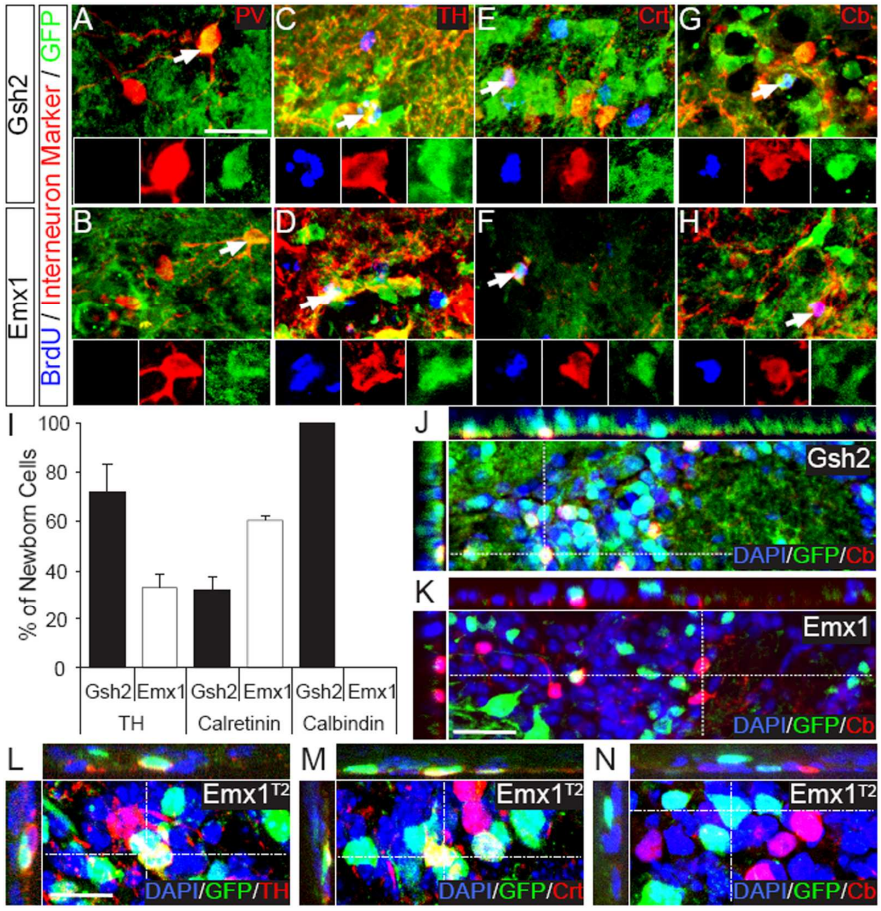


FIGURE 7

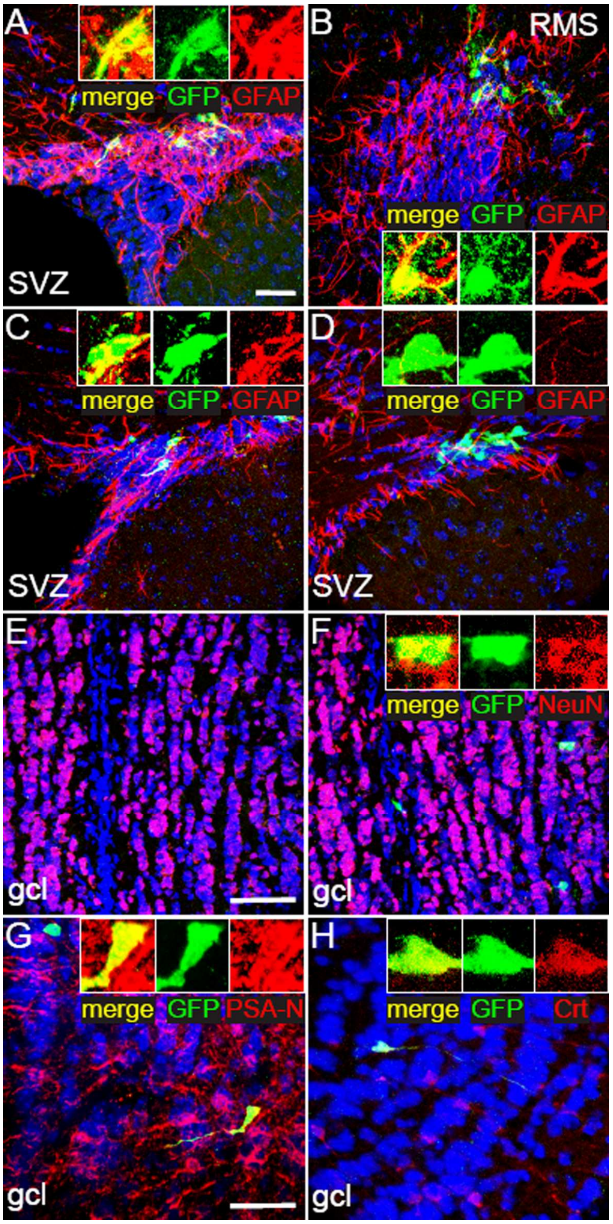
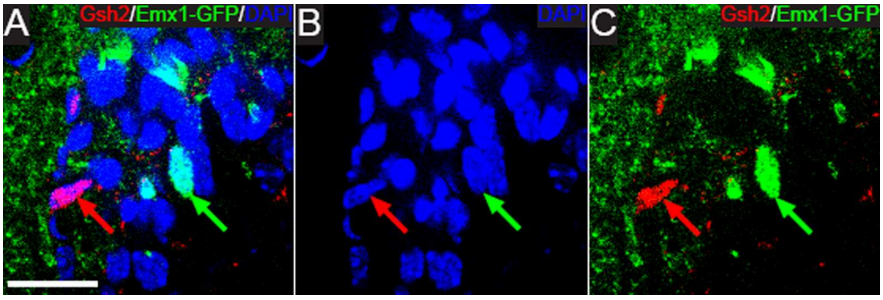


FIGURE 8



Supplementary FIGURE 1