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GABAergic phenotypic differentiation of a subpopulation of subventricular derived migrating progenitors

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Abstract

Olfactory bulb interneurons are continuously generated throughout development and in adulthood. These neurons are born in the subventricular zone (SVZ) and migrate along the rostral migratory stream into the olfactory bulb where they differentiate into local interneurons. To investigate the differentiation of GABAergic interneurons of the olfactory bulb we used a transgenic mouse which expresses green fluorescent protein (GFP) under the control of the glutamic acid decarboxylase 65 kDa (GAD65) promoter. During development and in adulthood GFP was expressed by cells in the SVZ and along the entire length of its rostral extension including the distal portion within the olfactory bulb. The occurrence of GAD65 mRNA in these zones was confirmed by PCR analysis on microdissected regions along the pathway. Polysialic acid neural cell adhesion molecule, a marker of migrating neuroblasts in adults, was coexpressed by the majority of the GFP-positive SVZ-derived progenitor cells. Cell tracer injections into the SVZ indicated that $\approx 26\%$ of migrating progenitor cells expressed GFP. These data show the early differentiation of migrating SVZ-derived progenitors into a GAD65–GFP-positive phenotype. These cells could represent a restricted lineage giving rise to GAD65-positive GABAergic olfactory bulb interneurons.

Introduction

The forebrain of both neonatal and adult rodents is characterized by persistent neurogenesis in the olfactory bulb (OB; Hinds, 1968a; Hinds, 1968b; Altman, 1969; Bayer, 1983) and hippocampal dentate gyrus (Altman & Das, 1965; Kaplan & Hinds, 1977; Kuhn et al., 1996), and cells with a neurogenic potential have been isolated from many other brain regions (Palmer et al., 1995; Palmer et al., 1999; Lie et al., 2002). The large numbers of newly generated neurons in the adult OB originate from a multipotent stem cell compartment located in the subventricular zone (SVZ; Luskin, 1993; Lois & Alvarez-Buylla, 1994; Weiss et al., 1996). Cells generated in the SVZ migrate tangentially along the rostral extension (RE) of the SVZ up to its distal portion into the olfactory bulb, following a well-defined pathway, the ‘rostral migratory stream’ (RMS). Upon reaching the core of the OB, these cells migrate radially towards the granule and glomerular layers where most assume the morphology of granule cells and juxtglomerular cells, respectively (Luskin, 1993; Lois & Alvarez-Buylla, 1994; De Marchis et al., 2001).

Neuronal progenitors, in vitro, form cell aggregations (‘neurospheres’) containing self-renewing multipotent stem cells, whose differentiation fate can be manipulated by single factors added to the culture. These factors can induce the generation of all three major CNS cell types: astrocytes, oligodendrocytes and neurons. For example, epidermal growth factor and leukaemia inhibitory factor both increase the formation of astrocytes from stem cells (Kuhn et al., 1997; Koblar et al., 1998), whereas several of the fibroblast growth factors (FGF-1 and FGF-2) and insulin-like growth factor-1 increase the formation of neurons (Kuhn et al., 1997; Brooker et al., 2000). Thus,

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combinations of factors applied to SVZ-derived progenitor cells in vitro can shift the proportions of differentiated cells and cell lineages. As stated above, the SVZ-derived cells that reach the OB in vivo give rise exclusively to cells of the neuronal lineage. The main targets of these migrating cells, the OB granule and glomerular layers, consist each of several morphologically and chemically defined subpopulations (Shipley et al., 2004). However, it is unclear whether the SVZ-derived newly generated cells contribute to all these different subpopulations or whether only specific phenotypes are renewed throughout life. Moreover, it is unclear when SVZ progenitor cells en route to the olfactory bulb make lineage decisions in vivo.

In adult mice, SVZ-derived migrating neuroblasts are easily identifiable by the expression of PSA-NCAM and doublecortin (Bonfanti & Theodosis, 1994; Gleeson et al., 1999). Baker and colleagues reported that some migrating neurons begin to express a dopaminergic phenotype (mRNA) in the deep bulb layers prior to reaching their final glomerular layer target location (Baker et al., 2001). Also, a few cells in the pathway express calretinin (Jankovski & Sotelo, 1996a), a marker expressed by a discrete population of mature olfactory bulb interneurons. To investigate other neurochemical lineages we examined the SVZ–RMS–OB pathway in a transgenic line of mice expressing green fluorescent protein (GFP) under the control of the glutamic acid decarboxylase (GAD)65 promoter. GAD65 is one of the two isoforms (the other being GAD67) of the enzyme responsible for the conversion of l-glutamic acid to GABA, both expressed by the GABAergic OB interneurons (Mugnaini et al., 1984), although GAD65 accounts for $\approx 80\%$ of all GAD protein in the OB (Sheikh et al., 1999). In these transgenic mice, cells along the entire length of the RMS and in the OB expressed GAD65-GFP. Immunostaining with PSA-NCAM, and injections of cell migration tracers, showed that there are both GAD65-GFP-positive and GAD65-GFP-negative cells migrating to the olfactory bulb. These data demonstrate that a subpopulation of SVZ progenitors en route to the OB acquire GABAergic phenotype before reaching their final target.

Materials and methods

Tissue preparation

Newborn and adult mice expressing GFP under the control of the glutamic acid decarboxylase 65 kDa promoter (GAD65-GFP) were deeply anaesthetized with halothane inhalation or intraperitoneal injection of sodium pentobarbital (6.5 mg/100 g body weight). All animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed and postfixed for a further 4 h, washed in 0.1 M PB for 15 min and placed in 30% sucrose solution in 0.1 M PB for 48 h. The brains were embedded in Tissue-Tek optimal cutting compound (OCT; Sakura Finetek, Torrance, CA, USA), frozen on dry ice and stored at -70°C until sectioned. Cryostat serial parasagittal and coronal sections (25 μm) were cut and collected as floating sections. At least three animals were examined for each immunohistochemical stain and cell counting.

Stereotaxic surgery and tracer injections

Stereotaxic injections were performed on postnatal day 0 mice (P0; the day of birth was designated as P0) and adults as previously described (De Marchis et al., 2001). Briefly, newborn mice were deeply anaesthetized by hypothermia and either 100–200 or 300–400 nL of cholera toxin β or Cell Tracker Orange (Molecular Probes, Eugene, OR, USA) injected at stereotaxic coordinates of 0 mm bregma, 0.8 mm lateral to sagittal sinus and 1.5 mm depth, by means of a glass micropipette and a pneumatic pressure injection apparatus (Picospritzer II, General Valve Corp, Fairfield, IL, USA). The pups were revitalized under a heat lamp and returned to the dam.

Adult animals were anaesthetized with intraperitoneal injection of Nembutal (4–5 mg/100 mg body weight in 0.9% saline solution) and positioned in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The mice were maintained on a warm platform at 35°C to maintain the body temperature constant during anaesthesia. Injections of either 100–200 or 300–400 nL of cholera toxin β (CTb; 1% in 0.9% sodium chloride; List Biologicals, Campbell, CA, USA) or Cell Tracker Orange

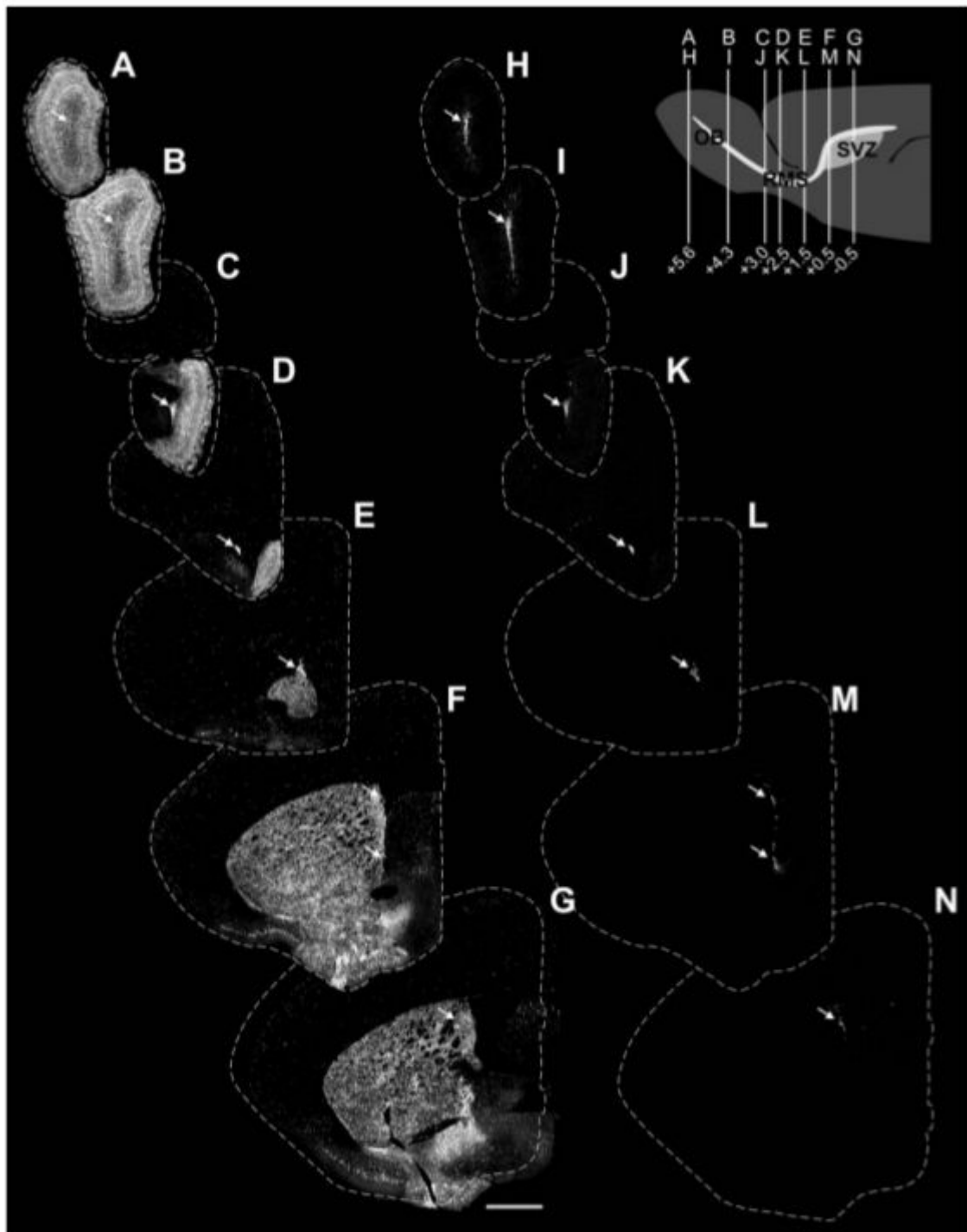
CMTMR (CTO; 5-(and-6)-(((4-chloromethyl)benzoyl)amino)-tetramethylrhodamine; 10 mm in dimethylsulfoxide; Molecular Probes, Eugene, OR, USA) were made at stereotaxic coordinates of 0.5 mm anterior to bregma, 1 mm lateral to the sagittal sinus and a depth of 2 mm. Following surgery, the animals were left under a heat lamp and constantly monitored until recovery. All the surgical procedures were carried out in accordance with protocols approved by our institutional animal care and utilization committee (IACUC).

Immunohistochemistry

Immunohistochemical procedures were performed on floating sections using antibodies directed against cholera toxin β (goat anti-Ctb; 1 : 5000; List Biological Laboratories), polysialic acid neural cell adhesion molecule (mouse monoclonal IgM anti-PSA-N-CAM; 1 : 1000; courtesy of Dr Rougon, Marseille, France), glial fibrillary acidic protein (rabbit anti-GFAP; 1 : 5000; Boehringer-Mannheim, Indianapolis, IN, USA), neuronal nuclear marker (mouse monoclonal IgG anti-NeuN; 1 : 5000; Chemicon, Temecula, CA, USA) or GAD65 or 67 kDa (mouse monoclonal IgG anti-GAD65 or 67; 1 : 5000; Chemicon). Sections were rinsed twice in PBST (0.1 M phosphate buffer, 0.9% sodium chloride and 0.3% Triton X-100), then incubated for 30 min in 1% bovine serum albumin (BSA; Sigma Chemical Corporation) in PBST for 30 min. Sections were sequentially incubated with primary antibody diluted in 1% BSA in PBST for 16 h, washed in PBST (three washes of 5 min duration), incubated in the appropriate secondary antibodies conjugated with either Cy3 or Cy2 (1 : 500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h, and washed in PBST (three washes of 5 min duration). All incubations were performed at 22 °C on a rotating platform. Some sections were counterstained by incubation in 5 nm TOTO3 Nucleic Acid Stain (Molecular Probes) for 30 min at 22 °C following immunohistochemical labelling. Sections were then coverslipped with a DABCO (Sigma Chemical Corporation)-based antifade mounting medium.

Reverse transcription–polymerase chain reaction (RT-PCR)

Microdissections of small regions of the SVZ-RMS-OB pathway were performed on fresh 250- μ m coronal vibratome slices harvested from the forebrain of P5 and adult mice (the day of birth designated P0). Under a high-magnification dissecting microscope each region was carefully dissected away from surrounding brain structures (\approx 25 μ m accuracy). Microdissected tissue regions were collected in Trizol (Invitrogen) and stored at -80° until use in RNA extraction following the manufacturer's protocols. The yield of total RNA was determined by measuring the absorbance at 260/280 nm (Eppendorf Biophotometer). In each region the same amount of total RNA was used for the RT-PCR reaction. Aliquots containing 500 ng of RNA were denatured for 5 min at 65 °C and incubated in reverse transcription reaction mix (containing 200 U of Superscript II reverse transcriptase, Invitrogen; 2.5 μ M random hexamers, Amersham-Pharmacia Biotech; 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM MgCl₂; 2 U/ μ L RNasin, Amersham-Pharmacia Biotech; 10 mM DTT; and 1 mM deoxynucleotide triphosphates, Amersham-Pharmacia Biotech) for 10 min at room temperature, followed by 42 °C for 50 min and 70 °C for 15 min. Following reverse transcription RNA the cDNA product was stored at -80°C.



GAD65 and G3PDH were amplified from reverse-transcribed cDNA with REDTaq DNA Polymerase (Sigma-Aldrich) in an MJ Programmable Thermal Cycler. The amplification mixture
 Fig. 1. Expression of (A–G) GAD65-GFP and (H–N) PSA-NCAM at different rostrocaudal levels in the adult mouse. (A–G) Low magnification coronal sections through the brain of a GAD65-GFP mouse showing the (A–D) olfactory bulb and (E–G) rostral forebrain. The insert shows a diagram of the bregma co-ordinates of each section. Arrows indicate the position of the subventricular zone (SVZ), rostral migratory stream (RMS) and its rostral extension in the olfactory bulb (OB). (H–N) The same sections shown in (A–G) immunostained for PSA-NCAM showing the presence of PSA-NCAM staining in the same locations as GAD65-GFP-positive cells. Scale bar, 1 mm.

contained cDNA, 0.5 μ m primers, 200 μ m dNTPs, 1.5 mm $MgCl_2$, 10 mm Tris-HCl, pH 8.3, 50 mm KCl, 0.01% gelatin and 0.025 units/mL of REDTaq Polymerase in 30 μ L volume. These mixtures were amplified for 25 cycles (G3PDH) or 32 cycles (GAD65). Specific primers designed to amplify mouse G3PDH (forward, 5'-TTTGCCGTGAGTGGAGTCATACTG-3'; reverse, 5'-

CGTCCCGTAGACAAAATG GTGAAG-3'; accession number M32599), were used to monitor the quality and quantity of RNA that had been reverse transcribed into cDNA. GAD65-specific primers (forward, 5'-GGCTCTGGCTTTTGG TCCTTC-3'; reverse, 5'-TGCCAATTCCCAATTATACTCTTGA-3') were from Szabo et al. (2000). Amplification products were separated by agarose gel electrophoreses and the DNA bands visualized by ethidium bromide staining of the gels. Negative controls for PCR were performed using templates derived from reverse transcription reactions lacking either reverse transcriptase or total RNA, and positive controls from diluted whole-bulb RNA.

Photography and cell counting

All images were captured on a FluoView X confocal microscope (Olympus Instruments, CA, USA) fitted with standard excitation and emission filters for the visualization of Cy2/GFP, Cy3/CTO and Cy5/TOTO3. Confocal images were captured through the full 25 μ m thickness of the slice at 1 μ m optical steps resulting in an image stack of \approx 25 images. These image stacks were used for cell counting (below) or assembled into extended focus photographs, brightness-, colour- and contrast-balanced, and assembled into panels with CorelDraw 11 (Core Corporation, USA).

Sections were randomly selected at three rostrocaudal levels from the SVZ to the OB. Image stacks were analysed using the optical dissector counting methodology (Russ & Dehoff, 1999). Briefly, the number and position of each cell in the section or confocal image stack that contained CTO was marked using the neuroLucida stereology software (MicroBrightfield). To ensure unbiased estimates of cell numbers in each region the following standard exclusion criteria were used. Any cell contacting the top of the section (i.e. present in the first image of the confocal image stack) was excluded and any cell contacting the bottom of the slice (i.e. present in the last image of the confocal image stack) was included. Cells contacting a line on the upper or right edge of a counting grid were excluded and cells contacting the lower or left edge of the grid were included in the counts. These reconstructions yield the number and laminar position of cells present in the 25 μ m thickness of each section. Double-labelled (CTO and GFP) cells were also demarcated to determine the proportion of migratory CTO-positive cells that express GFP.

Results

The majority of olfactory bulb interneurons are generated postnatally (Altman, 1969; Bayer, 1983) and derive from SVZ progenitor migration along the RMS into the OB. Although the majority of OB interneurons are generated during postnatal development, cells continue to be born in the SVZ, migrate along the RMS and differentiate into OB interneurons throughout life. Most of these SVZ-derived cells assume the morphology of granule cells or juxtglomerular interneurons (Luskin, 1993; Lois & Alvarez-Buylla, 1994; Reid et al., 1999). However, it is unclear whether all the cells migrating into the OB are undifferentiated multipotent cells or whether they are lineage-restricted and begun to differentiate into specific phenotypes. To investigate when SVZ progenitors differentiate into a GABAergic phenotype during migration we examined the SVZ-RMS-OB pathway in adult transgenic mice that express GFP under the control of the glutamic acid decarboxylase 65 kDa promoter (GAD65-GFP). In these mice there was robust GFP expression in the GABAergic granule and periglomerular cells in the olfactory bulb and GABAergic cells in other regions of the nervous system (Fig. 1A–G).

Cells expressing GFP were present in the SVZ lining the lateral ventricle and along the entire length of the RMS up to the core of the olfactory bulb (Fig. 1A–G). Immunohistochemistry for PSA-NCAM, a specific marker for migrating neuroblasts in adult labelled GFP-positive cells along the SVZ-RMS-OB pathway (Figs 1H–N and 2). The GFP-positive–PSA-NCAM-positive cells were restricted to the migratory pathway, although other differentiated GFP-positive–PSA-NCAM-negative cells were present in adjacent brain regions. Double-labelled cells were present in the SVZ of the lateral wall of the ventricle (Fig. 2A–C; bregma co-ordinate -0.5 mm), along the horizontal limb of the RMS (Fig. 2D–F and G–I; bregma co-ordinates +1.5 mm and +2.5 mm, respectively),

and in the olfactory bulb (Fig. 2J–L; bregma co-ordinate +4.3 mm). In the olfactory bulb PSA-NCAM-positive cells were localized mainly in the SVZ rostral extension and deep granule cell layers (Fig. 2K), with many of these cells coexpressing GFP (Fig. 2L). Infrequent GFP-positive–PSA-NCAM-positive cells were also present in the outer bulb layers (not shown). Double-labelled cells along the length of the RMS and OB had morphology typical of migrating neuroblasts, with an elongated cell body, a trailing process and a leading process terminating in a growth cone orientated in the direction of migration (Fig. 2M). GFP-positive–PSA-NCAM-positive cells were often arranged into ‘chains’ along the RMS (Fig. 2N), typical of the SVZ progenitor chain migration previously reported (Lois et al., 1996; Peretto et al., 1997). Interestingly, not all PSA-NCAM-positive cells in the pathway expressed GFP, suggesting that there are different subpopulations of SVZ progenitors.

Expression of a GAD65-GFP transgene may not represent expression of GAD65 mRNA per se. To test for the presence of GAD65 mRNA along the SVZ-RMS-OB system we microdissected small regions of the pathway and tested for GAD65 expression with PCR. In both postnatal and adult mice, GAD65 mRNA was present at different rostrocaudal positions along the pathway with expression levels equivalent to striatum (Fig. 3A and B). These data strongly support the possibility that expression of GAD65-GFP transgene in the neuroblasts of the SVZ-RMS-OB system is a reliable sign of GAD65 mRNA expression. Immunohistochemistry for GAD showed that only low levels of protein were expressed in the RMS (not shown) and the inner layer of the bulb (Fig. 3C–E). Robust GAD protein expression was not present in GFP-positive cells until they were in the olfactory bulb granule cell layer.

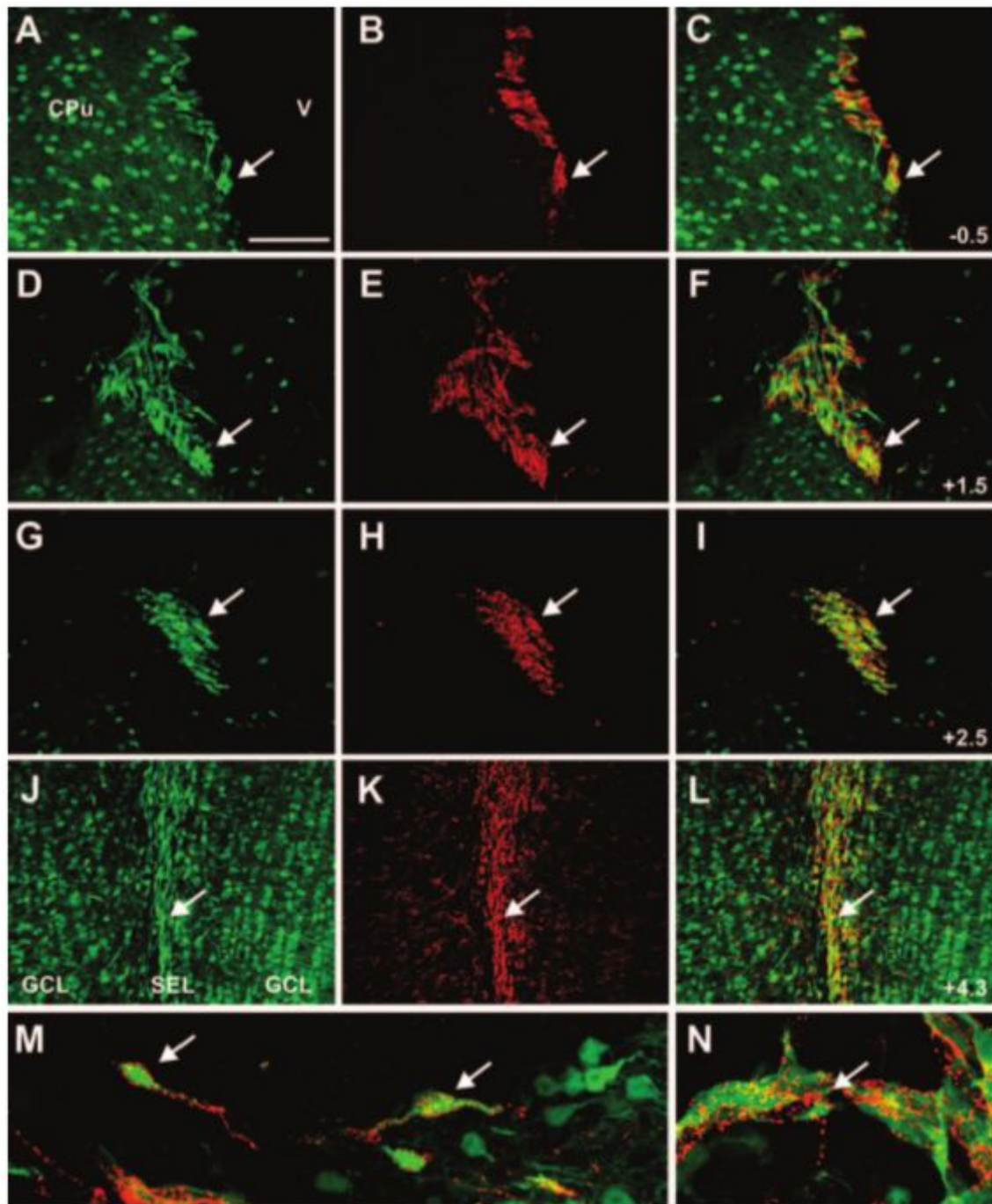


Fig. 2. GAD65-GFP-positive cells in the SVZ-RMS-OB pathway coexpress PSA-NCAM. (A–C) Cells within the SVZ (arrows, bregma co-ordinate -0.5 mm) express GAD65-GFP (green, panel A) and PSA-NCAM (red, panel B). Double-labelled cells show as yellow in the overlay (panel C). Differentiated GABAergic interneurons are visible in the caudate-putamen (striatum) underlying the SVZ. V, ventricle. (D–E) SVZ-derived progenitors in the posterior RMS (arrows, bregma co-ordinate +1.5 mm) coexpress GAD65-GFP (green, panel D) and PSA-NCAM (red, panel E) and in the overlay (yellow, panel F). (G–I) SVZ-derived progenitors in the anterior RMS (arrows, bregma co-ordinate +2.5 mm) coexpress GAD65-GFP (green, panel G) and PSA-NCAM (red, panel H), and in the overlay (yellow, panel I). (J–L) GAD65-GFP (green, panel J) is expressed in the RMS of the olfactory bulb (arrows, bregma co-ordinate +4.3 mm) and in granule cells (green, panel J). PSA-NCAM densely labels the subependymal layer (SEL) and individual PSA-NCAM-positive cells are present in the surrounding granule cell layers (red, panel K). Double-labelled cells show as yellow in the overlay (panel L). (M) Individual GAD65-GFP cells (green) entering the caudal olfactory bulb. Punctate PSA-NCAM staining (red) is present on the soma and processes of these cells (arrows). (N) GAD65-GFP (green)- and PSA-NCAM (red)-positive cells along the RMS are organized into chains. Scale bar in A, 100 μ m (A–L), 10 μ m (M,N).

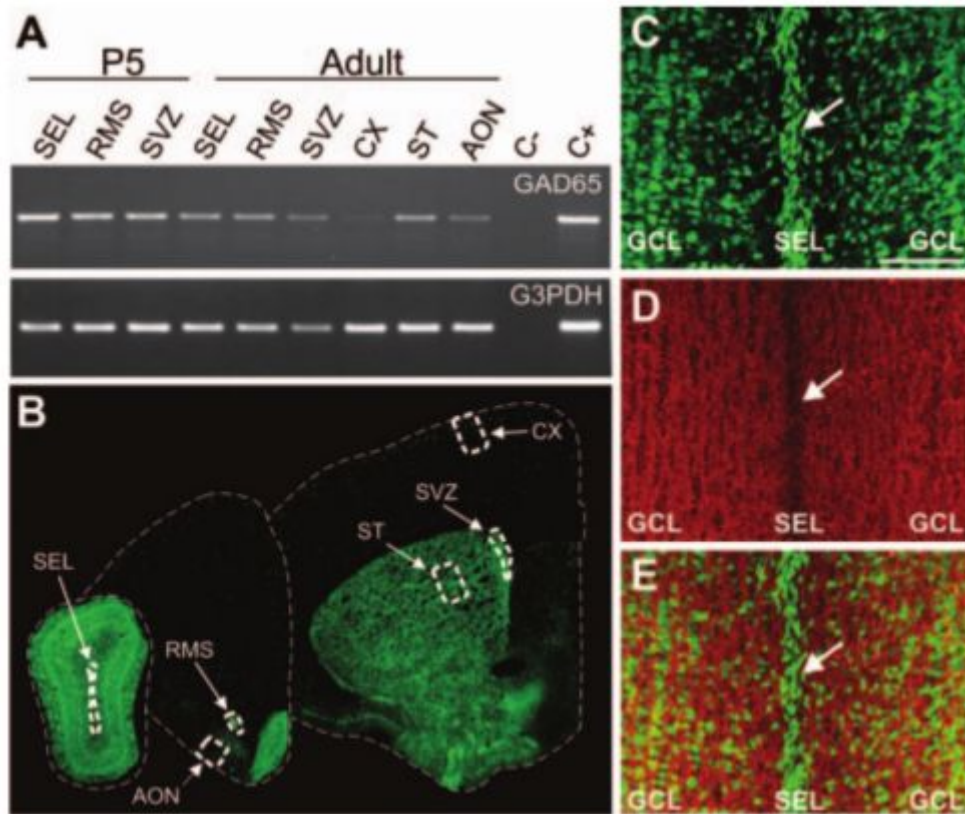


Fig. 3. GAD65 expression in the rostral migratory stream . (A) Gel showing expression of GAD65 mRNA in the olfactory bulb subependymal layer (OB, SEL), the rostral migratory stream (RMS) and the subventricular zone of the lateral ventricle (SVZ) in P5 and adult. Expression in the SVZ-RMS-OB pathway is higher than in the cortex (CX) and anterior olfactory nucleus (AON), and equivalent to expression levels in the striatum (ST). (B) Diagram showing the microdissected regions used in A. (C) GAD65-GFP expression in cells of the SEL (arrows) and granule cell layer (GCL). (D) Immunohistochemistry for GAD65/67 showing very low protein expression in the SVZ-derived neuroblasts migrating into the core of the OB (arrow). (E) Overlay of (C) and (D). Scale bar in C, 100 μ m (C–E).

In the adult SVZ, progenitors have been shown to migrate along specialized astrocyte arrangements, the glial tubes (Lois et al., 1996; Peretto et al., 1997). To show that the GFP-positive cells in these transgenic mice were present within the glial tubes, sections were immunostained for glial fibrillary acidic protein (GFAP). As previously described, astrocyte processes along the length of the RMS form a tube-like arrangement. GFP-positive cells were present within these glial tubes (Fig. 4; bregma co-ordinate +2.0 mm). There were also blank spaces within these tubes where GFP-negative cells were present. In the SVZ rostral extension of the olfactory bulb glial tubes progressively disappeared and longitudinal astrocyte processes were present. GFP-positive cells were also observed within this glial arrangement in the OB (Fig. 4C and D). GFP-positive cells were never observed to express GFAP. These data show that GFP-positive SVZ progenitors were present within the SVZ-RMS-OB pathway.

Expression of GAD-65-GFP by SVZ progenitors suggests that these cells have begun to select a GABAergic lineage as early as the SVZ and could represent a more differentiated population of cells migrating along this pathway. Sections were stained with NeuN, a marker for differentiated neurons which is also present on some migratory neuron populations in early brain development (Shu et al., 2003). Numerous NeuN-positive–GFP-positive neurons were present in surrounding brain structures and throughout the olfactory bulb. Cells within the SVZ at the level of the lateral ventricle and rostral extension (Fig. 4E and F) up to the OB (Fig. 4G and H) did not express NeuN, suggesting that GFP-positive SVZ progenitors are not fully differentiated neurons despite early expression of GAD65-GFP.

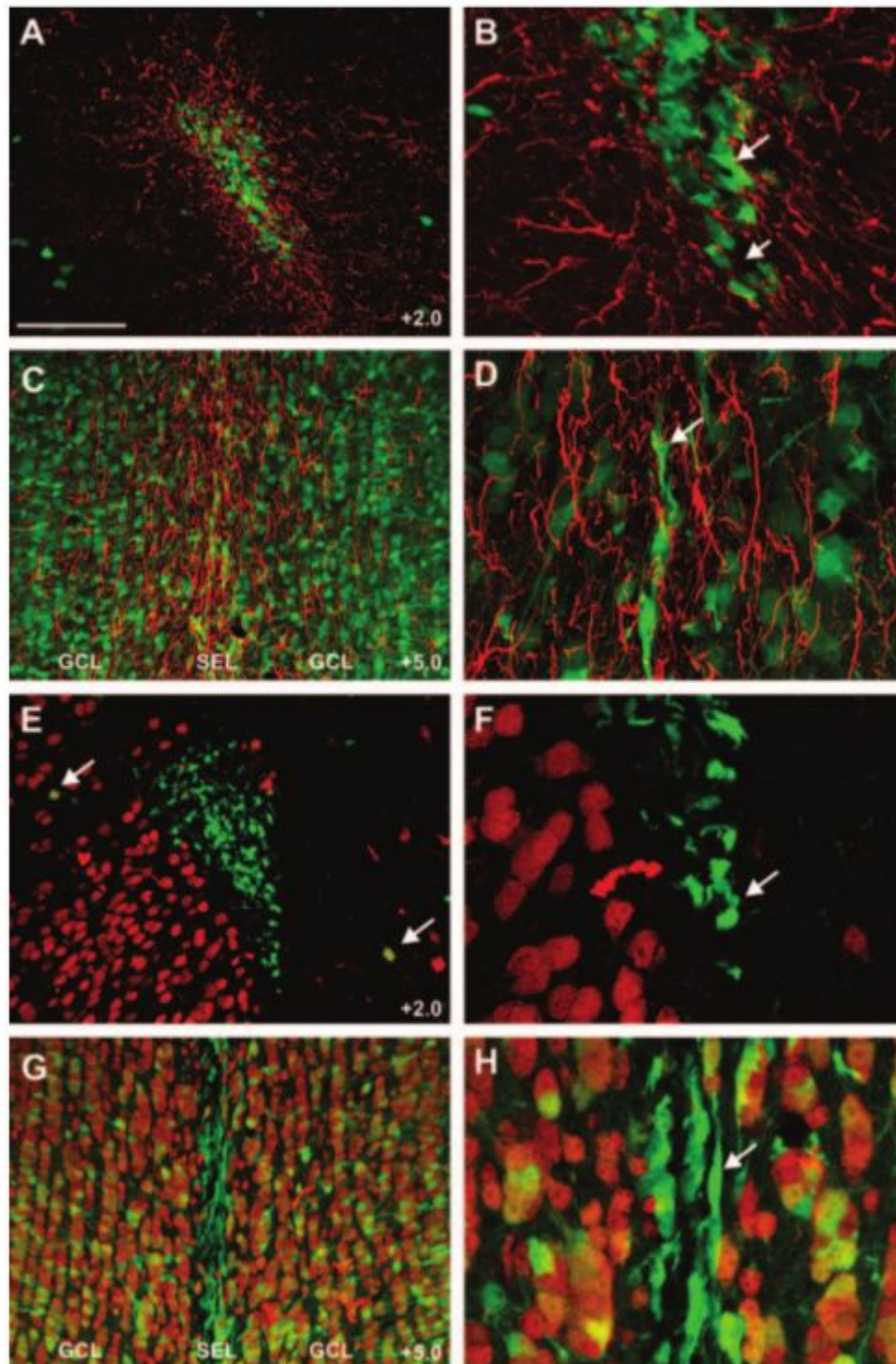


Fig. 4. GAD65-GFP cells migrate within the glial tubes of the RMS and do not express NeuN. (A) Low magnification coronal section showing the arrangement of GFAP-positive astrocytes (red) and GAD65-GFP-positive cells (green) at bregma co-ordinate +2.0 mm. (B) GAD65-GFP-positive (green, arrow) and GAD65-GFP-negative (arrowhead) cells are present within the GFAP-positive astrocyte glial tubes. (C) GFAP-positive astrocyte organization in a coronal section of the olfactory bulb from a GAD65-GFP mouse (bregma co-ordinate +5.0 mm). (D) High magnification showing individual GAD65-GFP cells (green) and GFAP-positive astrocytes (red) in the subependymal layer of the olfactory bulb. (E) At the level of the RMS (bregma co-ordinate +2.0 mm) GAD65-GFP cells are not colocalized with NeuN immunostaining. Several differentiated GABAergic interneurons in surrounding tissues coexpress GFP and NeuN (arrows). (F) High magnification of part of the RMS in panel D. (G) In the subependymal layer of the OB GAD65-GFP cells did not express NeuN; however, GAD65-GFP cells in the surrounding granule cell layers did coexpress NeuN

(yellow). (H) High magnification of part of the SEL in panel G. Scale bar in A, 100 μ m (A,C,E,G), 20 μ m (B,D,F,H).

Expression of PSA-NCAM and the presence of GFP-positive cells in glial tubes strongly suggest that the GFP-positive subsets of SVZ progenitor cells are migrating to the olfactory bulb. In order to identify the proportion of migrating cells that express GFP, we injected the dye CTO into the SVZ. Indeed, due to the 'chain' arrangement of the migrating neuroblasts and the punctate immunohistochemical staining for PSA-NCAM it is difficult to count the total number of migrating cells by PSA-NCAM staining. Cell Tracker dyes are a robust, reliable method for labelling migrating SVZ progenitors (De Marchis et al., 2001). CTO-labelled progenitor cells were present along the SVZ-RMS-OB pathway following a 6-day survival (Fig. 5A–D). There were also CTO-labelled cells which colocalized with GFP throughout the pathway (Fig. 5B–D). If the GFP-positive cells are a stable predetermined population of SVZ progenitors we predict that the proportion of CTO-labelled–GFP-positive cells along the length of the pathway and in the bulb would be similar. The proportion of CTO-labelled cells expressing GFP was counted at three rostrocaudal levels from the SVZ to the OB. While there was a tendency for a lower proportion of cells closer to the SVZ the trend was not significant and all the counts were pooled. The proportion of CTO-labelled progenitors expressing GFP was $\approx 26 \pm 5\%$ (mean \pm SEM) and this proportion was similar along the length of the SVZ-RMS and in the OB. The presence of CTO-labelled–GFP-positive and CTO-labelled–GFP-negative migrating progenitor cells show unambiguously that the GFP-positive progenitor cells are a subpopulation of the total migratory population. One month following labelling with Ctb, which is stable in SVZ-derived progenitors for extended periods of time (De Marchis et al., 2001), CTb-positive–GFP-positive neurons were present in the glomerular (Fig. 6A) and granule cell (Fig. 6B) layers. These data show that some SVZ progenitors that have integrated into the bulb express GAD65-GFP.

During postnatal development many OB interneurons derive from SVZ progenitor migration into the olfactory bulb. Our mRNA expression studies demonstrated that microdissected regions of the SVZ-RMS-OB pathway of newborn mice express GAD65 at levels equivalent to that observed in adult (Fig. 3A). To determine if migrating neurons in the developing OB also express GFP, transgenic mice were injected with CTO on P0 and the SVZ-RMS-OB pathway examined 2–3 days later. The SVZ-RMS-OB pathway of these neonatal mice contained numerous GFP-positive cells (Fig. 7A). CTO-labelled progenitor cells were present along the entire SVZ-RMS-OB pathway with $\approx 35\%$ of CTO-labelled cells also GFP-positive (Fig. 7B–D). The early expression of GFP in the SVZ-RMS-OB pathway in GAD65-GFP transgenic mice throughout development suggest that a subpopulation of SVZ progenitors are specified through a GABAergic phenotype immediately upon exiting the stem cell proliferation stage and beginning migration.

Discussion

Continual neurogenesis in the SVZ of postnatal and adult forebrain has been well documented, but the mechanisms underlying cell migration and differentiation from this region are poorly understood. Here we show that a subpopulation of migratory cells derived from the SVZ express GFP under the control of the GAD65 promoter during migration from the SVZ to the olfactory bulb. The expression of GAD65-GFP occurs prior to the expression of GAD protein, GABA, or the differentiated neuronal marker NeuN, suggesting that while early phenotypic differentiation is occurring during migration it is only a partially mature phenotype. It is always possible with transgenic animals that the promoter drives ectopic expression in this population of cells and that GAD expression does not occur until the cells reach the olfactory bulb. However, consistent proportions of transgene-positive cells along the length of the SVZ-RMS-OB pathway suggest the same population of cells is differentiating into GAD65-GFP-positive interneurons in the olfactory bulb. Moreover, RT-PCR analysis of microdissected portion of tissue along the SVZ-RMS-OB pathway confirms the occurrence of significant levels of GAD65 mRNA. Previous studies have shown the presence of GAD protein and receptors in migrating cells along the RMS (Pencea &

Luskin, 2003; Wang et al., 2003). Regardless, this transgenic mouse provides a useful model in which to investigate the mechanisms specifying subpopulations of migrating SVZ cells and fate determination.

Dissociated stem cells harvested from the SVZ can be induced to form several different phenotypes *in vitro*, including different classes of neurons and glia (Kuhn et al., 1997; Koblar et al., 1998; Brooker et al., 2000). However, it is not known whether progenitor cells that have left the SVZ and are migrating into the OB remain multipotent or have a restricted cell lineage. In other words, at what point in its migration from the SVZ to the OB is a cell's fate determined? There are several hypotheses: (i) all nonstem cells are lineage-restricted before as they leave SVZ and environmental factors can no longer alter cell fate; (ii) cells become restricted during migration in the RMS and are lineage-restricted by the time they reach the bulb; (iii) migrating cells remain multipotent until they reach their ultimate target location. Calretinin, a marker expressed by a subpopulation of mature olfactory bulb interneurons, has been shown to be expressed along the RMS by a very low number of migrating neuroblasts (Jankovski & Sotelo, 1996b). Moreover, Baker and colleagues recently showed that cells within the OB begin to express a tyrosine hydroxylase (TH) β -galactosidase transgene and TH mRNA prior to reaching the glomerular layer where TH protein is expressed (Baker et al., 2001). In this study, the presence of GAD65-GFP cells in the SVZ-RMS-OB pathway suggests that partial phenotypic specification is also taking place in the GAD65-GFP population of cells prior to the neurons reaching their final locations in the olfactory bulb.

One of the underlying principles of neural development is that once neurons initiate differentiation they become permanently postmitotic. Progenitor cells of the embryonic ventricular zone become postmitotic following phenotypic differentiation during early development. Expression of the cyclin-dependent kinase inhibitors (CKIs) is thought to regulate exit from the cell cycle and the decision for a cell to become postmitotic. In telencephalon the CKI p19^{INK4d} is expressed as cells permanently exit the mitotic region and expression persists in the cell as it differentiates (Coskun & Luskin, 2002). In contrast, it has been suggested that progenitor cells along the RMS progress through successive rounds of differentiation and dedifferentiation during migration. Progenitor cells in the RMS down-regulate and up-regulate p19^{INK4d} in order to re-enter the cell cycle as they migrate to the olfactory bulb (Coskun & Luskin, 2002). The progressive increase in the number of cells expressing p19^{INK4d} along the RMS suggests more cells permanently exiting the cell cycle as they approach the bulb (Coskun & Luskin, 2002). The expression of GAD65-GFP along the entire RMS may represent cells that have permanently chosen to exit the cell cycle or that maintain transgene expression through rounds of mitosis. Unlike the expression of p19^{INK4d} the numbers of GAD65-GFP-positive cells remains relatively constant along the length of the RMS suggesting that expression of the transgene is not directly related to the cell cycle marker p19^{INK4d}. Potentially the selection of cell fate, GAD65-GFP-positive, is independent of cell cycle.

The phenotypic maturation of migrating neurons is not restricted to early expression of GAD65-GFP during migration. SVZ progenitors also express neurotransmitter receptors and voltage-dependent ion channels during migration. Migrating cells in the RMS respond to GABA via a GABA A receptor and to glutamate via AMPA receptors without the presence of NMDA receptors (Carleton et al., 2003; Wang et al., 2003). The early expression of AMPA-mediated responses prior to NMDA receptor responses is in contrast to other developing neuroblasts which show low AMPA-receptor expression until up-regulation by NMDA receptor activation as the cell differentiates (Durand et al., 1996). Taken together with GAD65-GFP expression it suggests that migrating SVZ progenitors are not undifferentiated cells but have a partially differentiated neuronal phenotype and potentially preselected interneuron class lineages. Experiments in the cerebral cortex also suggest that GAD65-GFP-positive neuroblasts have specific birthdates and laminar specificity (Lopez-Bendito et al., 2004). Taken together, these data suggest neuroblasts in the developing and adult brain have a high degree of precommitment *in vivo*.

In summary, our results, together with previous data (Jankovski & Sotelo, 1996a; Baker et al., 2001), suggest that the SVZ-derived migrating neuroblasts are represented by a mixed population of

phenotypically determined cell types. Our data show that migrating SVZ progenitors acquire a GAD65-GFP phenotype very early in the SVZ-RMS-OB pathway. This suggests that this population of SVZ progenitors phenotypically differentiates prior to reaching their final location in the olfactory bulb. However, the cells do not acquire a mature phenotype (GAD protein and NeuN expression) until they enter the olfactory bulb cellular layers.

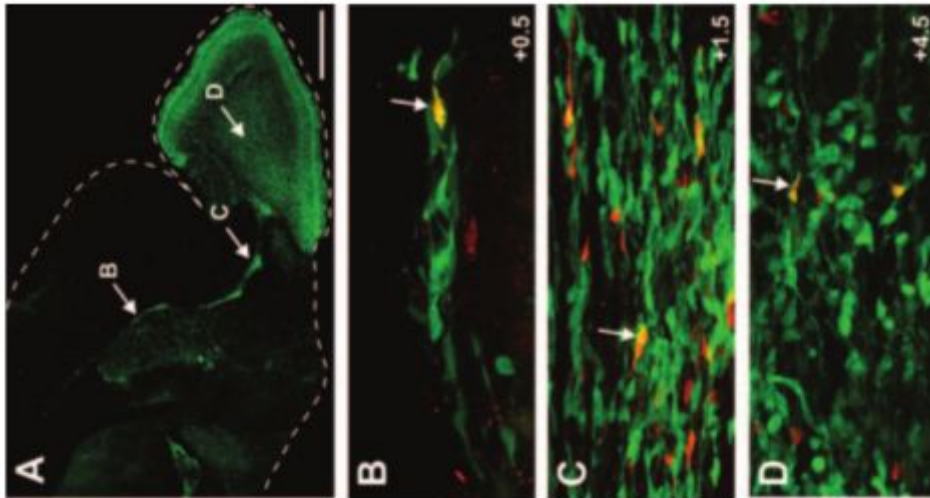


Fig. 7

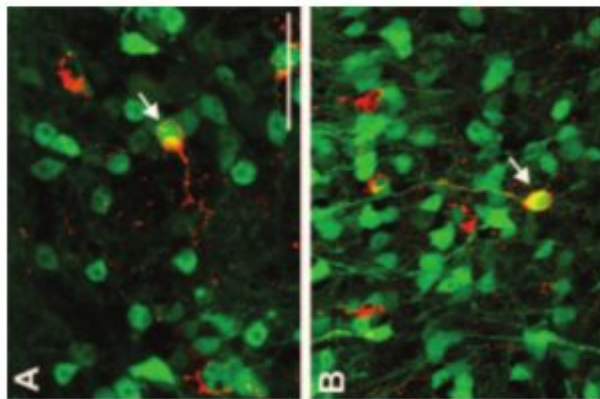


Fig. 6

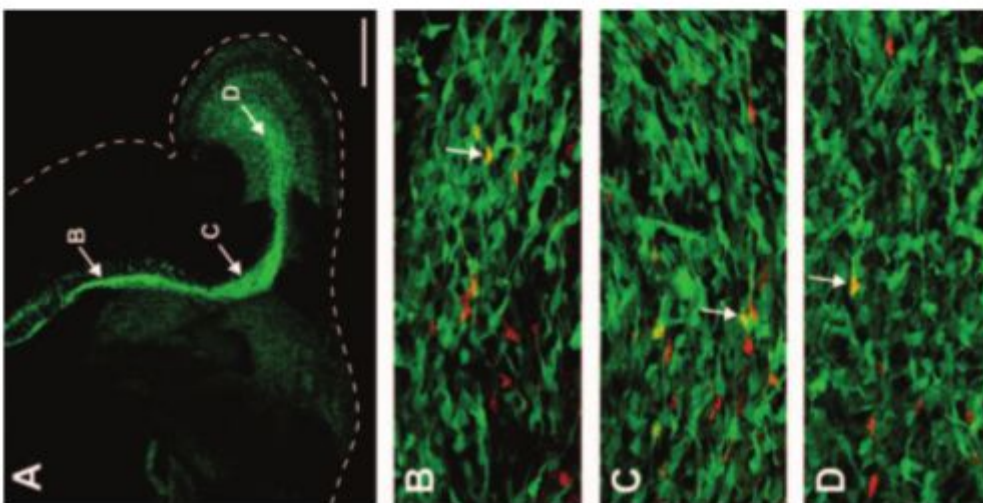


Fig. 5

Fig. 5. A subpopulation of migrating SVZ progenitor cells express GAD65-GFP. (A) Sagittal section through an adult GAD65-GFP mouse showing the RMS and olfactory bulb (arrows indicate the positions of panels B–D). (B) CTO-labelled (red) and GAD65-GFP (green) cells migrating in the SVZ–RMS. GAD65-GFP-positive–CTO-positive (yellow, arrow) and GAD65-GFP-negative–CTO-positive cells (red) are present in the pathway. (C) Double- and single-labelled cells are also present in the horizontal limb of the RMS. (D) At 6 days post injection only a few cells have reached the

centre of the olfactory bulb, but these cells are also a mixed population of CTO–GAD65-GFP-positive and CTO–GAD65-GFP-negative cells. Scale bar in A, 1 mm (A), 25 μ m (B–D).

Fig. 6. GAD65-GFP-positive cells derived from SVZ cell migration became resident in the olfactory bulb. (A) Cells present in the glomerular layer labelled by cholera toxin b (CTb) injection in the SVZ 1 month previously. The arrow highlights a CTb-positive–GAD65-GFP-positive periglomerular cell. (B) In the granule cell layer CTb-positive–GAD65-GFP-positive cells are also present 1 month following injection (arrow). Scale bar, 25 μ m.

Fig. 7. SVZ progenitor cells express GAD65-GFP during postnatal development. (A) Saggital section through P1 GAD65-GFP mouse forebrain showing the RMS and olfactory bulb (arrows indicate the position of panels B–D). (B) CTO-labelled (red) and GAD65-GFP (green) cells in the SVZ–RMS. A subpopulation of SVZ progenitor cells labelled by the CTO injection express GAD65-GFP as well as CTO-positive–GAD65-GFP-negative cells. (C) Double- and single-labelled cells are also present at the ‘elbow’ of the RMS. (D) In the olfactory bulb cells there is also a mixed population of CTO–GAD65-GFP-positive and CTO–GAD65-GFP-negative cells. Scale bar in A, 500 μ m (A), 25 μ m (B–D).

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Abbreviations

CTb, cholera toxin b; CTO, Cell Tracker Orange CMTMR; FGF-1 and FGF-2, fibroblast growth factors; GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; OB, olfactory bulb; P0, postnatal day 0; PB, phosphate buffer; RE, rostral extension; RMS, rostral migratory stream; RT-PCR, reverse transcription–polymerase chain reaction; SVZ, subventricular zone; TH, tyrosine hydroxylase.

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