J Mol Hist (2012) 43:215–221
DOI 10.1007/s10735-012-9394-2

BRIEF COMMUNICATION

Early specification of GAD67 subventricular derived olfactory interneurons

Céline Plachez · Adam C. Puche

Received: 26 January 2012 / Accepted: 7 February 2012 / Published online: 3 March 2012

Abstract Olfactory bulb interneurons are continuously generated in the subventricular zone (SVZ) and migrate along the rostral migratory stream (RMS) into the olfactory bulb (OB) where the majority becomes local GABAergic interneurons. We previously showed that SVZ-derived progenitor cells expressed glutamic acid decarboxylase 65 kDa (GAD65) very early in the migratory pathway. However, only approximately half of OB GABAergic interneurons use GAD65, an equal number express the 67 kDa GAD enzyme. To investigate the differentiation of these GABAergic interneurons we examined their migration in a transgenic mouse expressing green fluorescent protein (GFP) under the control of the GAD67 promoter. In adult, GFP was expressed by a subpopulation of migratory cells in the SVZ and along the RMS. Using Doublecortin (DCX) as a marker of migrating neuroblasts and bromodeoxyuridine (BrdU) incorporation, we show that these GAD67-GFP neurons co-express DCX and incorporate BrdU indicating they are newly born migratory neuroblasts. This is similar to GAD65 transgene expression, and in contrast to dopaminergic interneuron transgene expression which occurs only after cells reach the olfactory bulb. Although the GAD65/67 transgenes are expressed early in migration, there is minimal protein production in the cells prior to reaching the OB. These results suggest that migrating SVZ-derived neuroblasts acquire GABAergic identity prior to reaching their final location in the olfactory bulb.

C. Plachez · A. C. Puche (✉)
Department of Anatomy and Neurobiology, School of Medicine, University of Maryland, 20 Penn Street, Baltimore, MD 21201, USA
e-mail: apuche@umaryland.edu

Keywords Stem cell · Migration · Rostral migratory stream · Subventricular zone · GABA

Introduction

Over several decades, studies have shown that neurogenesis occurs in the subventricular zone (SVZ) and hippocampus in adult (Altman and Das 1965, 1966; Kaplan and Hinds 1977; Goldman and Nottebohm 1983; Goldman 1998; Richards et al. 1992; Reynolds and Weiss 1992; Lois and Alvarez-Buylla 1993). Neuronal stem cells divide in the SVZ producing neuroblasts that migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB) giving rise to granule and periglomerular GABAergic interneurons (Petreanu and Alvarez-Buylla 2002; Carleton et al. 2003). In these interneurons GABA is synthesized from glutamate in a single enzymatic step by glutamic acid decarboxylase (GAD) from the 65 and 67 kDa isoforms of the enzyme. Although these enzymes have similar kinetics, expression appears to be regulated by cell type. Uniglomerular periglomerular cells preferentially express GAD65 whereas dopaminergic short axon cells preferentially use GAD67 (Parrish-Aungst et al. 2007).

Previously we showed that a subpopulation of the SVZ-derived neuroblasts expresses green fluorescent protein (GFP) transgene under the control of the GAD65 promoter in the SVZ and RMS indicating an early phenotypic differentiation (De Marchis et al. 2004). However, another interneuron population derived from the SVZ, dopaminergic/GAD67, do not exhibit promoter activity for the dopamine synthetic enzyme tyrosine hydroxylase (TH) until the cells are within the bulb (Baker et al. 2001). In both cases protein expression for these enzymes does not

occur until the cells are within the OB (De Marchis et al. 2004; Parrish-Aungst et al. 2007).

In this study, we investigated whether activity of the GAD67 promoter occurred early similar to GAD65 activity, or if GAD67 phenotype was similar to TH and not expressed until the OB. In a transgenic line of mice expressing GFP under the control of the GAD67 promoter we found that a subpopulation of the SVZ-derived progenitors expressed GAD67 transgene within the SVZ/RMS, indicating that the GAD67 neuroblasts have made a lineage specification before they reach their final destination in the OB.

Materials and methods

GAD67-GFP transgenic mice were kindly provided by Dr Yuchio Yanagawa (Gunma University Graduate School of Medicine). The generation of GAD67-GFP (Δneo) mice was previously described by (Tamamaki et al. 2003). GAD67-GFP knock-in mice were bred in-house at the animal facility of the University of Maryland, Baltimore. These facilities are approved by the American Association for Accreditation of Laboratory Animal Care and are under full-time veterinary supervision. All animal procedures were conducted in accordance to the NIH and the Animal Care and Use Committee guidelines and approved by the Institutional Animal Care and Use committee of the University of Maryland.

Adult male GAD67-GFP mice (6–8 week old) received 2 intraperitoneal injections of Bromodeoxyuridine (BrdU, 50 mg/kg, Sigma Chemical Co., St. Louis, MO, USA) 4 h apart BrdU is incorporated into the DNA of dividing cells during the S-phase of the cell cycle (del Rio and Soriano 1989; Soriano et al. 1991). Mice were euthanized 6 days following the injection.

Mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal; 150 mg/kg, Abbott Laboratories, Abbott Park, IL, USA) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). Brains were carefully dissected out from the scalp, embedded in 10% gelatin in 1× phosphate buffer saline (PBS, pH 7.4) and incubated in 30% sucrose and 1% PFA in 1× PBS for 48 h at 4°C. Brain were coronally sectioned (25 μm) using a Leica CM3050 cryostat (Leica, Deerfield, IL, USA).

Free-floating sections were rinsed several times in TBS (0.1 M Tris, 0.9% saline, pH 7.4) and incubated for 30 min in blocking buffer (1% bovine serum albumin, 1% donkey serum, 0.3% Triton X-100 in TBS). Primary antibodies diluted in blocking buffer were incubated overnight at room temperature. Guinea Pig anti-Doublecortin antibody (AB5910, Chemicon, Temecula, CA, USA) was used at 1/5,000 to label the neuroprogenitor cells migrating from the SVZ to the OB. Rat anti-BrdU antibody (AB6326-250 BU 1/75(ICR1), Abcam, Cambridge, MA, USA) was used at 1/2,500 to label the dividing cells at the time of the injections. After several washes, sections were incubated in the appropriate donkey secondary antibodies (1/500, JIR, Jackson ImmunoResearch lab, West Grove, PA, USA) diluted in blocking solution for 2 h at room temperature. Sections were then washes and counterstained with 4′-6-Diamidino-2-Phenyllindole (DAPI, 1:20,000, Sigma) for 15 min. Sections were mounted on gelatin-coated slide (3% gelatin) and then coverslipped with DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma)-based antifade mounting media. Images were collected on a confocal microscope (Fluoview FV5000 Olympus, NY) in 1 μm optical steps.

Results

We previously showed that SVZ-derived progenitors were capable of expressing GAD65 transgene as they migrate along the RMS (De Marchis et al. 2004). Here, we examined the SVZ-RMS-OB pathway in adult transgenic mice expressing GFP under the control of the GAD67 promoter. As expected, GFP expression was prominent within olfactory bulb interneurons (Fig. 1, f–l). GFP positive cells found along the RMS were also present in the subependymal layer (SEL) where newly formed cells are entering the OB.

In order to confirm that GAD67 is expressed in migratory neuroblasts we first performed immunohischemistry for Doublecortin (DCX), a microtubule-associated phosphoprotein expressed in migrating neuroblasts and differentiating neurons (Gleeson et al. 1998; Nacher et al. 2001). Numerous DCX positive cells were present along the RMS (Fig. 2), and a subpopulation of these DCX positive cells were also positive for GAD67-GFP (Fig. 2d, h, l, p). Next, we performed BdrU injections to label newly born cells and examined the RMS 6 days following the BrdU injection, this interval provides enough time to the SVZ newborn cells to distribute throughout the RMS and SEL of the OB. We found that within the SVZ/RMS/OB pathway BrdU positive cells also expressed GAD67-GFP (Fig. 3). Taken together those results show that in adult mice a subpopulation of newly generated progenitors expresses GAD67-GFP, indicating that similar to the GAD65 population of interneurons the GAD67 population also phenotypically differentiate prior to the OB.

GAD65-GFP is expressed early during neuroblast migration, however protein expression does not turn on within the cells until they reach the bulb (De Marchis et al. 2004). The similarity in early expression of the GAD67 transgene leads us to conjecture that GAD67 protein would also be expressed only within the olfactory bulb and not

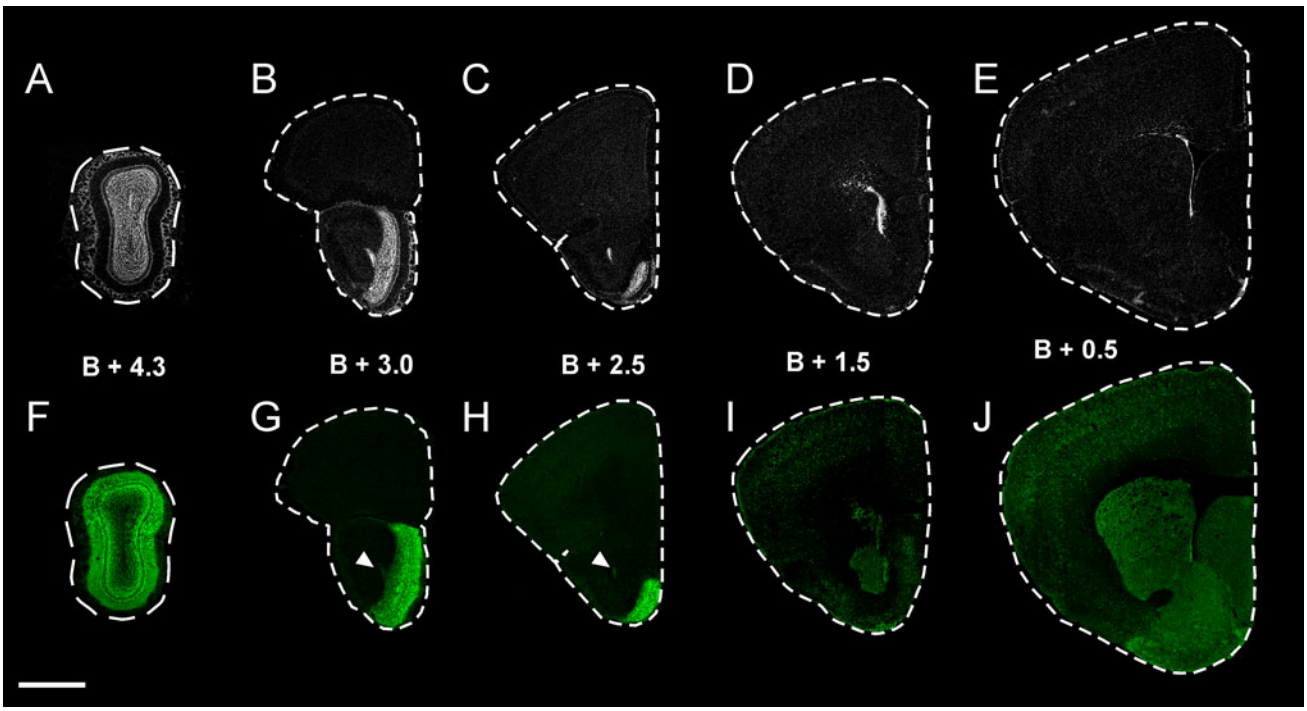

Fig. 1 GAD67-GFP expression in adult mouse brain. Bregma (B) coordinates are indicated going from +4.3 to +0.5 Bregma. DAPI staining is shown on the top panels (**a**–**e**) and GAD67-GFP expression is shown on the bottom panels (**f**–**j**), going from rostral to caudal. Only half of the adult mouse brain (coronal sections) is showed in each panel. *Dotted line* is outlining the brain section. GAD67-GFP is strongly expressed in the OB (**f** and **g**). *Arrowheads* in **g** and **h** point out SEL layer where Figs. 2a–d and Fig. 3a–d were taken from, respectively. *Scale bar* is 1 mm

within migratory neuroblasts. In both the GAD67-GFP and GAD65-GFP mice, immunohistochemistry for the respective GAD67 and GAD65 proteins showed protein expression only within the mature granule cell layer of the OB (Fig. 4b, d). GFP positive cells in the SVZ, RMS, and SEL did not express GAD67 or GAD65 proteins (Fig. 4c, f). Thus, both GABAergic interneurons subtypes begin exhibiting GAD promoter activity early in their lineage/migration, but only express the protein only after exiting the migratory pathway.

Discussion

The rodent OB contains a large number of GABAergic interneurons, concentrated in the glomerular, mitral and granule cell layers (Kosaka et al. 1987; Mugnaini et al. 1984). In adult brain, GABA is synthesized by either GAD65 or GAD67. These isoforms are preferentially expressed by different interneurons subpopulations (Kiyokage et al. 2010; Parrish-Aungst et al. 2007). Newly generated interneurons contributing to both GAD65 and GAD67 expressing interneuron subpopulations, originate in the SVZ and migrate along the RMS to the OB. Neuroblasts express GAD65 transgene in the SVZ and along the RMS (De Marchis et al. 2004) and here we showed that GAD67 transgene is also expressed by a subpopulation of migrating neuroblasts. However, neither GAD population expresses GAD proteins until reaching the OB. Taken together, our studies demonstrate that both major GABAergic neuroprogenitor cell populations in the OB undergo an early phenotypic specification as soon as they leave the SVZ.

In adult, the SVZ is a stem cells niche, generating self-renewing and multipotent progenitors (Miller and Gauthier-Fisher 2009). Those neuroblasts migrate long distance along the RMS to the OB, where they differentiate into periglomerular cells and granule interneurons. SVZ derived progenitors are different from other immature neurons since they have the capacity to undergo mitosis as they migrate (Menezes et al. 1995), whereas other immature neurons become postmitotic before they migrate (Coskun and Luskin 2001). Adult neurogenesis differs from developmental neurogenesis since newly born neurons have to integrate into an established, functioning network.

Early differentiation of migratory neuroblasts in this pathway has been suggested by expression of TuJ1 (a marker for neuron-specific class III β-tubulin) (Menezes et al. 1995), MAP2 (microtubule-associated protein 2)

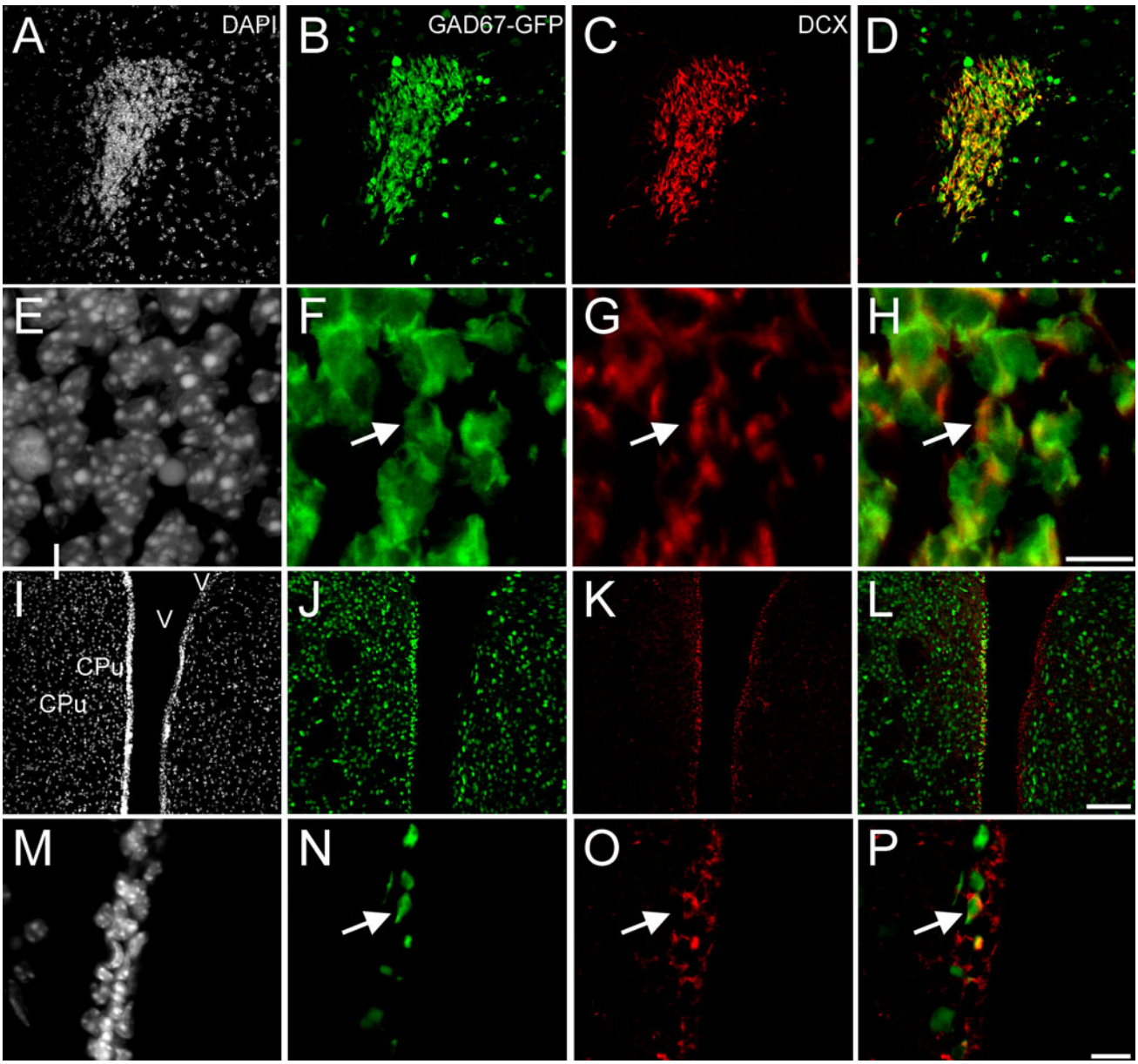

Fig. 2 GAD67-GFP expression in migrating neuroblast. DCX immunostain (in *red*, **c**, **g**, **k** and **o**) on GAD67-GFP animals (in *green*, **b**, **f**, **j**, **n**) shows that a subpopulation of migrating neuroblasts expresses GAD67-GFP (merge, **d**, **h**, **l**, **p**) in adult olfactory bulb (**a**–**h**, coronal sections) and SVZ (**i**–**p**, coronal sections; CPu, caudate Putamen, V, ventricle). Low magnification of panel b is panel j in Fig. 1. *Arrows* are pointing out cells expressing both DCX and GAD67-GFP. **e**, **f** are high magnifications of **a**–**d**, and **m**–**p** are high magnifications of **i**–**j**, respectively. Panels **a**, **e**, **i**, and **m** show DAPI staining. *Scale bar* in **d** is 50 μm for **a**–**d**; in **h** is 10 μm for **e**, **f**; in **l** is 100 μm for **i**, **l**; in **p** is 10 μm for **m**–**p**. (Color figure online)

(Pencea et al. 2001; Faiz et al. 2008), DCX (a microtubule-associated phosphoprotein expressed in migrating neuroblasts and differentiating neurons) (Nacher et al. 2001), and PSA-NCAM (polysialylated form of neural cell adhesion molecule) (Pencea et al. 2001). Those studies showed that migrating neuroblasts express general neuronal markers indicative of neural lineage selection, and here our results indicate that the major GAD lineages are also specify early. In contrast, the dopaminergic subtype of GABAergic interneurons begins to express tyrosine hydroxylase only after reaching the olfactory bulb (Baker et al. 2001). This suggests that general neuronal and GABAergic lineage selection occurs immediately upon exiting the SVZ, whereas interneuron subtype specification may be a later event upon entering the bulb.

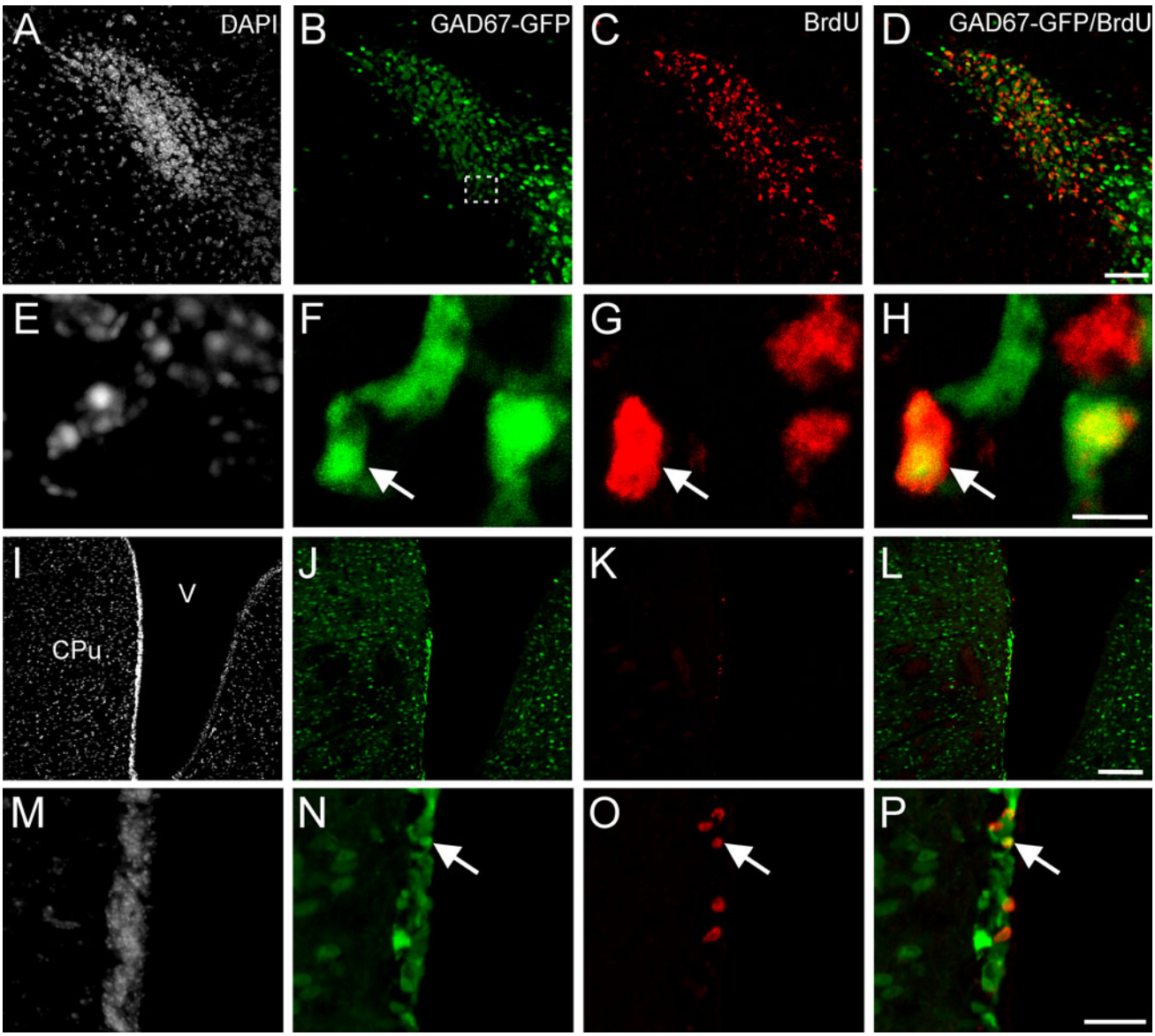

Fig. 3 GAD67-GFP expression in dividing neuroblasts. BrdU immunostain (in *red*, **c**, **g**, **k** and **o**) on GAD67-GFP animals (in *green*, **b**, **f**, **j**, and **n**) shows that a subpopulation of dividing neuroblasts expresses GAD67-GFP (merge, **d**, **h**, **l** and **p**) in adult olfactory bulb (**a**–**h**, coronal sections) and SVZ (**i**–**p**, coronal section; CPu, caudate Putamen, V, ventricle). *Arrows* in **f**–**h** and **n**–**p** are pointing out co-label cells with BrdU and GAD67-GFP. **e**, **f** are high magnifications of **a**–**d** (from square box in **b**), and **m**–**p** are high magnifications of **i**, **j**, respectively. Low magnification of panel b is panel i in Fig. 1. Panels **a**, **e**, **i**, and **m** show DAPI staining. *Scale bar* in **d** is 50 μm for **a**–**d**; in **h** is 5 μm for **e**, **f**; in **l** is 100 μm for **i**–**l**; in **p** is 20 μm for **m**–**p**. (Color figure online)

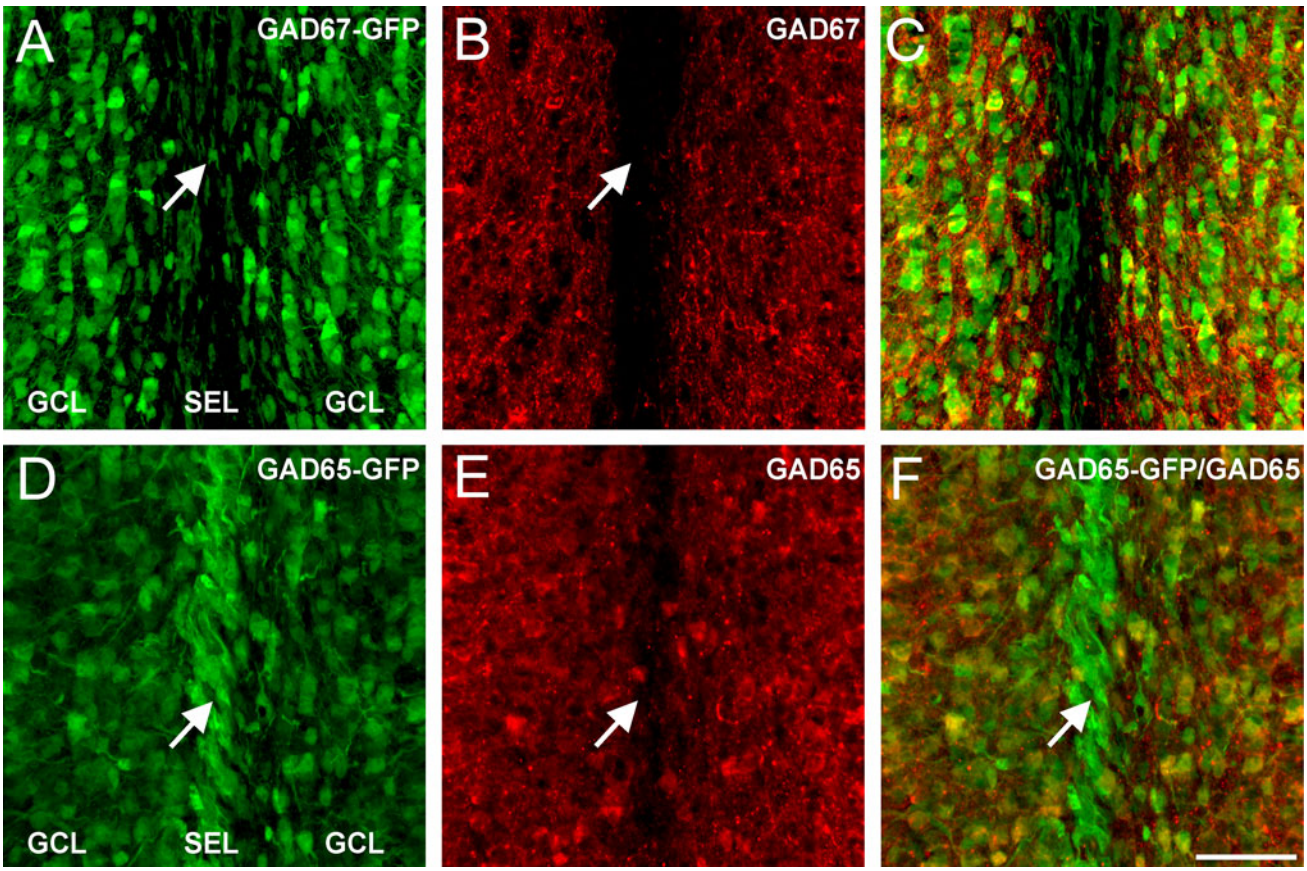

Fig. 4 Transgene expression versus protein expression. GAD67 immunostain (in *red*, **b**) on GAD67-GFP animals (in **green**, **a**) shows that GAD67 protein is not present in migrating neuroblasts (merge, **c**, *arrows* pointing out cells expressing only GAD67-GFP) in adult olfactory bulb (coronal section). GAD65 immunostain (in *red*, **e**) on GAD65-GFP animals (in *green*, **d**) shows that GAD65 protein is not present in migrating neuroblasts (merge, **f**, *arrows* pointing out cells expressing only GAD65-GFP) in adult olfactory bulb (coronal section). *Scale bar* is 50 μm in **c** and **f**. (Color figure online)

Acknowledgments This work was supported by grants from Maryland Stem cell research foundation (MSCRFE-0239 to ACP, 100728-002 to CP).

References

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124(3):319–335

Altman J, Das GD (1966) Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. J Comp Neurol 126(3):337–389

Baker H, Liu N, Chun HS, Saino S, Berlin R, Volpe B, Son JH (2001) Phenotypic differentiation during migration of dopaminergic progenitor cells to the olfactory bulb. J Neurosci 21(21):8505–8513

Carleton A, Petreanu LT, Lansford R, Alvarez-Buylla A, Lledo PM (2003) Becoming a new neuron in the adult olfactory bulb. Nat Neurosci 6(5):507–518

Coskun V, Luskin MB (2001) The expression pattern of the cell cycle inhibitor p19(INK4d) by progenitor cells of the rat embryonic telencephalon and neonatal anterior subventricular zone. J Neurosci 21(9):3092–3103

De Marchis S, Temoney S, Erdelyi F, Bovetti S, Bovolin P, Szabo G, Puche AC (2004) GABAergic phenotypic differentiation of a subpopulation of subventricular derived migrating progenitors. Eur J Neurosci 20(5):1307–1317

del Rio JA, Soriano E (1989) Immunocytochemical detection of 5′-bromodeoxyuridine incorporation in the central nervous system of the mouse. Brain Res Dev Brain Res 49(2):311–317

Faiz M, Acarin L, Villapol S, Schulz S, Castellano B, Gonzalez B (2008) Substantial migration of SVZ cells to the cortex results in the generation of new neurons in the excitotoxically damaged immature rat brain. Mol Cell Neurosci 38(2):170–182

Gleeson JG, Allen KM, Fox JW, Lamperti ED, Berkovic S, Scheffer I, Cooper EC, Dobyns WB, Minnerath SR, Ross ME, Walsh CA (1998) Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. Cell 92(1):63–72

Goldman SA (1998) Adult neurogenesis: from canaries to the clinic. J Neurobiol 36(2):267–286

Goldman SA, Nottebohm F (1983) Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci USA 80(8):2390–2394

Kaplan MS, Hinds JW (1977) Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197(4308):1092–1094

Kiyokage E, Pan YZ, Shao Z, Kobayashi K, Szabo G, Yanagawa Y, Obata K, Okano H, Toida K, Puche AC, Shipley MT (2010) Molecular identity of periglomerular and short axon cells. J Neurosci 30(3):1185–1196

Kosaka T, Kosaka K, Heizmann CW, Nagatsu I, Wu JY, Yanaihara N, Hama K (1987) An aspect of the organization of the GABAergic system in the rat main olfactory bulb: laminar distribution of immunohistochemically defined subpopulations of GABAergic neurons. Brain Res 411(2):373–378

Lois C, Alvarez-Buylla A (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci USA 90(5):2074–2077

Menezes JR, Smith CM, Nelson KC, Luskin MB (1995) The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain. Mol Cell Neurosci 6(6):496–508

Miller FD, Gauthier-Fisher A (2009) Home at last: neural stem cell niches defined. Cell Stem Cell 4(6):507–510

Mugnaini E, Wouterlood FG, Dahl AL, Oertel WH (1984) Immunocytochemical identification of GABAergic neurons in the main olfactory bulb of the rat. Arch Ital Biol 122(2):83–113

Nacher J, Crespo C, McEwen BS (2001) Doublecortin expression in the adult rat telencephalon. Eur J Neurosci 14(4):629–644

Parrish-Aungst S, Shipley MT, Erdelyi F, Szabo G, Puche AC (2007) Quantitative analysis of neuronal diversity in the mouse olfactory bulb. J Comp Neurol 501(6):825–836

Pencea V, Bingaman KD, Freedman LJ, Luskin MB (2001) Neurogenesis in the subventricular zone and rostral migratory stream of the neonatal and adult primate forebrain. Exp Neurol 172(1):1–16

Petreanu L, Alvarez-Buylla A (2002) Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. J Neurosci 22(14):6106–6113

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255(5052):1707–1710

Richards LJ, Kilpatrick TJ, Bartlett PF (1992) De novo generation of neuronal cells from the adult mouse brain. Proc Natl Acad Sci USA 89(18):8591–8595

Soriano E, Del Rio JA, Martinez-Guijarro FJ, Ferrer I, Lopez C (1991) Immunocytochemical detection of 5′-bromodeoxyuridine in fluoro-gold-labeled neurons: a simple technique to combine retrograde axonal tracing and neurogenetic characterization of neurons. J Histochem Cytochem 39(11):1565–1570

Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J, Obata K, Kaneko T (2003) Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. J Comp Neurol 467(1):60–79