Neuroscience 227 (2012) 180–190

CHARACTERIZATION OF IMMATURE AND MATURE 5-HYDROXYTRYPTAMINE 3A RECEPTOR-EXPRESSING CELLS WITHIN THE ADULT SVZ–RMS–OB SYSTEM

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Abstract—Neural stem cells in the subventricular zone (SVZ) generate progenitors which in turn give rise to neuroblasts. These neuroblasts then migrate along the rostral migratory stream (RMS) in chains and reach the olfactory bulb (OB), where they mature into local interneurons. Interneurons in the OB are heterogeneous, which are mainly located in granular cell layer (GCL), external plexiform layer (EPL) and glomerular layer (GL). In this study, we show that green fluorescent protein-expressing (GFP+) cells in the SVZ and RMS of the 5HT3aR-BAC^{EGFP} transgenic mouse exclusively express transcription factor *Sp8* which is expressed in lateral ganglionic embryonic eminence (LGE) and postnatal SVZ.

These GFP+ neuroblasts of the 5HT3aR-BAC^{EGFP} transgenic mouse migrate along the RMS to the OB where they differentiate into calretinin+ (CR+), parvabumin+ (PV+), vasoactive intestinal peptide+ (VIP+), somatostatin+ (Som+) and tyrosine hydroxylase+ (TH+), but not calbindin+ (CB+) interneurons. These GFP+ interneurons continuously express *Sp8* in the OB. Furthermore, these results suggest that GFP-expressing cells are derived from LGE, and this transgenic mouse line will be a useful tool for studying the development and function of interneurons in both neocortex and OB.

Key words: subventricular zone, rostral migratory stream, olfactory bulb, 5-HT3AR, interneuron, *Sp8*.

INTRODUCTION

Adult neurogenesis occurs in the subventricular zone (SVZ) of the lateral ventricular wall and the dentate gyrus of the hippocampus (Ming and Song, 2005). Neural stem cells that function as primary precursors in the SVZ correspond to type B cells, a subpopulation of slowly dividing cells that has the morphology, ultrastructure and markers of astrocytes (Doetsch et al., 1999). Type B cells produce the transient-amplifying progenitors (termed C cells), which are present in clusters scattering throughout the SVZ. Type C cells divide rapidly to produce young neurons, also known as neuroblasts or type A cells. Neuroblasts are present in SVZ and migrate anteriorly through the rostral migratory stream (RMS) to the olfactory bulb (OB), where they mature into interneurons located in the granule cell layer (GCL), external plexiform layer (EPL) and glomerular layers (GL) (Lledo et al., 2008).

Neuromodulator systems play major roles in odor processing through the activation of inhibitory interneurons in the OB (Chameau and Van Hooft, 2006). 5-Hydroxytryptamine (serotonin) receptor 3 (5-HT$_3$R) is the only known murine serotonergic ionotropic receptor. And of the two subunits 5-HT$_{3A}$ and 5-HT$_{3B}$, only the former is expressed in GABAergic interneurons in the brain (Morales and Bloom, 1997). Previous studies have described 5-HT$_{3A}$R was present exclusively in GABAergic interneurons in the mature neocortex (Morales and Bloom, 1997; Ferezou et al., 2002), including vasoactive intestinal peptide (VIP) and/or cholecystokinin (VIP+ and/or CCK+) cells and neuropeptide Y (NPY+) cells (Inta et al., 2008; Varga et al., 2009; Vucurovic et al., 2010). In the neonatal mice, the majority of 5-HT$_{3A}$R+ cells born in the SVZ migrate to the OB along the RMS. Some of these cells exit RMS and migrate to the corpus callosum and neocortex, where they express markers of GABAergic interneurons (Inta et al., 2008; Le Magueresse et al., 2011; von Engelhardt et al., 2011).

Sp8, a zinc finger transcription factor, is continuously expressed in the neurogenic regions of the embryonic (i.e. dorsal–lateral ganglionic eminence [dLGE] and caudal ganglionic eminence [CGE]) and postnatal (i.e. SVZ and RMS) brain (Waclaw et al., 2006; Liu et al., 2009; Ma et al., 2012; Wei et al., 2011). In the adult OB, *Sp8* is expressed and required for the production of CR+ and PV+ interneurons (Waclaw et al., 2006; Li et al., 2011).

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Abbreviations: 5-HT$_{3A}$R, 5-hydroxytryptamine receptor 3A; CB, calbindin; CGE, caudal ganglionic eminence; CR, calretinin; DAPI, 4′, 6-diamidino-2-phenylindole; Dcx, doublecortin; dLGE, dorsal–lateral ganglionic eminence; EPL, external plexiform layer; GCL, granular cell layer; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; GL, glomerular layer; M/T, mitral and tufted; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; OB, olfactory bulb; POA, preoptic area; PV, parvabumin; RMS, rostral migratory stream; Som, somatostatin; SVZ, subventricular zone; TBS, Tris–buffered saline; TH, tyrosine hydroxylase; VIP, vasoactive intestinal peptide.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.09.062

In the present study we found that the majority of GFP+ cells in the SVZ, RMS and OB express *Sp8* in the young adult 5HT3aR-BAC^{EGFP} transgenic mouse. In OB, GFP+ cells mature into CR+, PV+, Som+, VIP+, and TH+, but not CB+ interneurons, and continuously express *Sp8*.

EXPERIMENTAL PROCEDURE

Animals

5HT3aR-BAC^{EGFP} transgenic mouse line was obtained from GENSAT project at the Rockefeller University. The transgenic mice were generated by homologous recombination with a bacterial artificial chromosome (BAC) containing the promoter of the 5-HT$_{3A}$ receptor with the 5-HT$_{3A}$ receptor-coding sequence being replaced with a sequence encoding the EGFP reporter gene. The experiments were carried out on mice at 30 days (young adult mice) and150 days (adult mice) old. All experiments using animals were carried out in accordance with institutional guidelines, and the study was approved by the Fudan University Animal Care and Use Committee. All efforts were made to minimize the number of animals used.

Immunohistochemistry

5HT3aR-BAC^{EGFP} transgenic mice (either sex) were deeply anesthetized before intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were post-fixed with 4% paraformaldehyde overnight and then cryoprotected at 4 °C for at least 24 h in 30% sucrose. The brain samples were frozen in embedding medium (O.C.T., Sakura Finetek, Torrance, CA, USA). Free-floating sections of the brain and OB were collected in 30-μm thickness in 24-well plates and were sampled 180 μm apart. Immunohistochemistry staining was performed on 30-μm free-floating sections in 24-well tissue culture plates. Sections were blocked for 1 h in Tris–buffered saline (TBS) with 0.5% Triton X-100 and 10% donkey serum. Primary antibodies were incubated for 48 h at 4 °C. The following primary antibodies were used: rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, 1:500, Z0334, Dako, Carpinteria, CA, USA), rabbit polyclonal anti-Ki67 (1: 500, VP-K451, Vector Laboratories, CA, USA), chicken anti-green fluorescent protein (GFP, 1: 2000, GFP-1020, Aves Labs, Tigard, OR, USA), rabbit anti-doublecortin (Dcx, 1: 600, Ab-18723, Abcam, Cambridge, MA, USA), goat anti-*Sp8* (1: 400, sc-104661, Santa Cruz Biotechnology, CA, USA), rabbit anti-calretinin (CR, 1: 3000, AB5054, Chemicon, MA, USA), rabbit anti-neuropeptide Y (NPY, 1: 500, 22940, Incstar, MN, USA), mouse anti-parvalbumin (PV, 1:400, MAB1572, Chemicon), rabbit anti-somatostatin (Som, 1:100, sc-13099, Santa Cruz), rabbit anti-vasoactive intestinal peptide (VIP, 1:500, 20077, Incstar), goat anti-neuronal nitric oxide synthase (nNOS, 1: 300, ab1376, Abcam), mouse-anti TH(1: 500, MAB318, Chemicon), rabbit anti-CB (1: 2,500, CB-38a, Chemicon), rabbit anti-Tbr1(1: 200, ab31940, Abcam), rabbit anti-Tbr2 (1: 300, ab23345, Abcam). Secondary antibodies against the appropriate species were incubated for 2 h at RT (all from The Jackson Laboratory, 1: 200). Omission of primary antibodies eliminated staining. All sections were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma, 400 ng/mL, 5 min).

Confocal imaging and cell counting

All images presented in the study were acquired using the Olympus FV1000 confocal microscope system. Images were cropped, adjusted, and optimized in Adobe Photoshop CS2.

GFP+/Dcx+ and GFP+/*Sp8*+ cells in the adult SVZ were quantified with confocal microscopy (n = 3 mice). We selected six sections in each brain. Confocal Z sectioning was performed at 1-μm intervals in each double-labeling section (GFP/Dcx and GFP/*Sp8*) using a 20× objective. Cells were counted from individual optical sections, not collapsed projection images. About 300–700 cells were analyzed in each SVZ.

For cell counting of GFP/CR/*Sp8* triple labeling in the OB, at least 10 non-overlapping fields from each 30-μm section were analyzed using an Olympus BX 51 microscope with a 40× objective; from each OB, three to eight coronal sections were quantified (n = 3). For quantification of other interneurons in the adult OB, cell counting was performed under an Olympus BX 51 microscope. Six to eight sections at 180-μm intervals of each OB (n = 3) were selected. All data were presented as means ± standard error of the mean (SEM).

All statistical analysis was conducted in STATISTICA (StatSoft, USA). Data were analyzed using Student's t-test. A probability level of less than 0.05 was accepted as statistical significance. Degrees of statistical significance are presented as $^{*}P < 0.05$.

RESULTS

GFP+ neuroblasts in the SVZ express the transcription factor *Sp8*

Previous studies have shown 5-HT$_{3A}$R is accurately reported by GFP in the 5HT3aR-BAC^{EGFP} transgenic mouse (Lee et al., 2010; Vucurovic et al., 2010). 5-HT$_{3A}$R mRNA and GFP are detected in the juvenile SVZ by using *in situ* hybridization and immunohistochemistry (Inta et al., 2008). By double immunostaining for GFP and GFAP, we did not find double labeled (GFP+/GFAP+) cells in the SVZ as shown previously (Inta et al., 2008; Kreuzberg et al., 2010). We only found some GFP+/Ki67+ cells in the SVZ (data not shown), this suggests that some of the GFP+ cells in the SVZ are dividing cells (Uda et al., 2007; Cheng et al., 2009).

As shown in Fig. 1A, B1, B2, GFP+/Dcx+ cells located in the anterior SVZ of young adult 5HT3aR-BAC^{EGFP} transgenic mice, and we quantified the double-labeled cells and found that virtually all of the GFP+ cells are GFP+/Dcx+ in the SVZ as shown previously (Inta et al., 2008; Kreuzberg et al., 2010).

Previous studies have shown that *Sp8* is not expressed by transient-amplifying progenitor cells, but by most neuroblasts in the SVZ of mice (Waclaw et al., 2006; Wei et al., 2011) and rats (Liu et al., 2009). To investigate whether GFP+/Dcx+ cells in the SVZ express *Sp8*, we performed GFP/Dcx/*Sp8* triple immunostaining on brain sections of young adult 5HT3aR-BAC^{EGFP} transgenic mice. The *Sp8* antibody utilized in this study is specific in immunohistochemistry, because no *Sp8* immunoreactivity was detected in the OB section of *Sp8* conditional knockout (Dlx5/6-CIE; Sp8$^{flox/flox}$) mice (Li et al., 2011). Interestingly, nearly all the GFP+ neuroblasts (GFP+/Dcx+ cells) express *Sp8* in the anterior SVZ of young adult 5HT3aR-BAC^{EGFP} transgenic mice (Fig. 1A).

In juvenile mice, the expression of 5-HT$_{3A}$R was detected in the neocortex (Inta et al., 2008; Le Magueresse et al., 2011) and corpus callosum (von Engelhardt et al., 2011). Immature and mature