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NG2-expressing cells in the subventricular zone are type C–like cells and contribute to interneuron generation in the postnatal hippocampus

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The subventricular zone (SVZ) is a source of neural progenitors throughout brain development. The identification and purification of these progenitors and the analysis of their lineage potential are fundamental issues for future brain repair therapies. We demonstrate that early postnatal NG2-expressing ($NG2^+$) progenitor cells located in the SVZ self-renew in vitro and display phenotypic features of transit-amplifier type C–like multipotent cells. $NG2^+$ cells in the SVZ are highly proliferative and express the epidermal growth factor receptor, the transcription factors Dlx, Mash1, and Olig2, and the Lewis X (LeX) antigen. We show that grafted early postnatal $NG2^+$ cells generate hippocampal GABAergic interneurons that propagate action potentials and receive functional glutamatergic synaptic inputs. Our work identifies Dlx^+/$Mash1^+$/LeX^+/$NG2^+$/GFAP-negative cells of the SVZ as a new class of postnatal multipotent progenitor cells that may represent a specific cellular reservoir for renewal of postnatal and adult inhibitory interneurons in the hippocampus.

Introduction

Neurogenesis continues in restricted areas of the postnatal vertebrate brain throughout adulthood (Reynolds and Weiss, 1992; Doetsch et al., 1999a; Alvarez-Buylla and Garcia-Verdugo, 2002). Adult neural stem cells (NSCs) that give rise to neurons are found in specific brain regions, including the subventricular zone (SVZ). This germinal layer is a large reservoir of proliferating NSCs (Doetsch et al., 1999a,b, 2002a; Capela and Temple, 2002), and is therefore a potential source of multipotential precursors for use in cell repair therapies.

Two major multipotent NSC phenotypes have been described in the adult SVZ, based on expression of the intermediate filament protein GFAP (glial fibrillary acidic protein; Doetsch et al., 1999a, 2002a; Capela and Temple, 2002). Type B cells express GFAP and give rise to the rapidly dividing, transit-amplifying type C cells, which are not immunopositive for GFAP (Doetsch et al., 1999a). It has been demonstrated that SVZ type C cells (a) generate neurospheres in the presence of EGF; (b) express the Dlx2 transcription factor and the epidermal growth factor receptor (EGFR); and (c) are the direct precursors of neurons generated in the olfactory bulb (Doetsch et al., 1999a, 2002a).

Adult NSCs express the Lewis X (LeX) carbohydrate antigen, which is detected in both $GFAP^+$ and GFAP-negative cells of the SVZ (Capela and Temple, 2002), indicating that this brain region contains a LeX^+/GFAP-negative cell population with neurogenic potential. Because type C cells are a GFAP-negative and highly proliferative cell population, it is likely that the LeX^+/GFAP-negative stem cells are in fact type C progenitors (Capela and Temple, 2002; Doetsch et al., 2002a).

Cells that express the NG2 chondroitin sulfate proteoglycan represent the largest pool of postnatal/adult proliferative progenitors in the brain (Dawson et al., 2000). NG2-expressing cells were thought to be strictly oligodendrocyte progenitors; however, recent studies have expanded their role by demonstrating that early postnatal $NG2^+$ cells form neurospheres and generate neurons in vitro (Belachew et al., 2003). These properties were established by using a 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP)-EGFP transgenic mouse, in

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Abbreviations used in this paper: CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; DG, dentate gyrus; DIV, days in vitro; EGFR, epidermal growth factor receptor; LeX, Lewis X; NSC, neural stem cell; SVZ, subventricular zone.

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which $NG2^+$ progenitors expressed CNP promoter-driven EGFP (Yuan et al., 2002; Belachew et al., 2003). Consistent with these results, a parallel study demonstrated that $A2B5^+$ precursor cells isolated from the adult human subcortical white matter expressed the CNP gene and generated neurons in vitro and in vivo (Nunes et al., 2003). Given these findings, it is clear that CNP gene-expressing cells that are NG2 and A2B5 immunopositive display properties of multipotent progenitor cells (Belachew et al., 2003; Goldman, 2003; Nunes et al., 2003). These cells could contribute to neurogenesis in the postnatal/adult brain, although the precise characterization and neurogenic potential of $NG2^+$ progenitors in the SVZ and the classification of the neuronal subtypes that they generate remain undefined.

In the present work, we demonstrate that early postnatal $NG2^+/EGFP^+$ cells in the SVZ of CNP-EGFP mice are mitotically active and express markers of multipotent precursor cells of the adult SVZ (Dlx, EGFR, and LeX). Based on these markers, we establish a relationship between $NG2^+$ and LeX^+ precursor cells, and determine a lineage continuum between $NG2^+/Dlx^+$ progenitors and hippocampal GABAergic interneurons. We also show that isochronically transplanted perinatal $NG2^+/EGFP^+$ progenitors migrate to the hippocampus, where they give rise to Dlx^+ GABAergic interneurons. Our findings indicate that early postnatal $NG2^+$ cells are Lex^+/GFAP-negative type C–like cells in the SVZ, and identify such type C–like cells as a source of hippocampal GABAergic interneurons in the postnatal brain.

Results

Early postnatal and adult NG2-expressing cells in the SVZ display a type C–like immunophenotype

Fig. 1 shows that the majority (~75%; Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200311141/DC1) of $EGFP^+$ cells in the SVZ were $NG2^+$ at P8 (Fig. 1, A–G) and at P40 (not depicted). At both ages, all the $NG2^+$ cells were $EGFP^+$ and were found subependymally in the lateral ventricle wall (Fig. 1 A). Staining with anti-Olig2 (Lu et al., 2000) labeled virtually all the $NG2^+/EGFP^+$ cells at P8 (Fig. 1 B) and P40 (not depicted). $Olig2^+/EGFP^+$ cells that did not express NG2 were also found in the SVZ (Fig. 1 B). A significant percentage (26.5 ± 2.4%; mean ± SEM; $n = 356$) of the $NG2^+/EGFP^+$ cells in the SVZ were also labeled with anti-Mash1 (Fig. 1 E and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200311141/DC1).

We determined the proliferative potential of $NG2^+$ cells in the postnatal SVZ. 2 h after a single BrdU injection, 31.9 ± 5.2 and 25.7 ± 4.2% ($n = 360$ and 239) of the $NG2^+/EGFP^+$ cells were $BrdU^+$ at P8 and P40, respectively (Fig. 1 C and Fig. 2 G, inset). Consistent with these findings, a large percentage of $NG2^+/EGFP^+$ cells (P8, 59.0 ± 3.3%, $n = 283$; P40, 37.5 ± 7.3%, $n = 140$; Fig. 1 D and Fig. 2 G, inset) were $Ki67^+$ (Schluter et al., 1993).

To further define the immunophenotype of $NG2^+/EGFP^+$ cells in the SVZ, we immunolabeled tissue sections with anti-NG2 and the MMA monoclonal antibody, which recognizes the LeX antigen (Capela and Temple, 2002). At P8 (Figs. 1 F and 2 G) and P40 (Fig. 2 G and not depicted), 47 and 25% of the $NG2^+/EGFP^+$ cells were LeX^+, respectively. None of the $NG2^+$ cells in the SVZ expressed GFAP or GLAST (Fig. 2, A and B). $NG2^+/EGFP^+$ cells represented 12.9 ± 0.8% of total SVZ cells at P8, and the total LeX^+ SVZ population was divided into a 27.4 ± 1.5% of cells that were $GFAP^+$ and a nonoverlapping subset of 37.9 ± 3.1% of cells that were $NG2^+$ (Fig. S3). Altogether, these results indicate that the $NG2^+/EGFP^+$ cells that express LeX, but do not express GFAP, account for approximately half of total LeX^+/GFAP-negative cells. Thus, $NG2^+/EGFP^+/Lex^+$/GFAP-negative cells likely represent the population of LeX^+/GFAP-negative cells characterized by Capela and Temple (2002), and might correspond to type C–like transit-amplifying cells that are present in the early postnatal SVZ and persist throughout adulthood (Doetsch et al., 2002a).

Adult type C cells express the EGFR and the Dlx2 homeobox-containing transcription factor (Doetsch et al., 2002a). At P8, virtually all (96.0 ± 8.2%, $n = 395$) of the $NG2^+/EGFP^+$ cells also expressed EGFR in the SVZ (Figs. 1 G and 2 G). At P40, more than half of the $NG2^+/EGFP^+$ cells expressed the EGFR (52.0 ± 5.8%, $n = 210$; Fig. 2 G). After immunostaining with a pan anti-Dlx antibody, recognizing both the early (*dlx1/2*) and late (*dlx5/6*) members of the *dlx* homeobox gene family (Panganiban et al., 1997), we found that 45.0 ± 5.2 ($n = 335$) and 37.0 ± 3.3% ($n = 160$) of the $NG2^+/EGFP^+$ cells were also Dlx^+ at P8 and P40, respectively (Fig. 2, C and G). Dlx was also expressed in $LeX^+/EGFP^+$ cells (unpublished data). We confirmed by RT-PCR that the Dlx2 gene was expressed in SVZ $NG2^+$ cells at P8 (Fig. S2). Among the total Dlx^+ cell population of the SVZ, only 7.7 ± 0.9% expressed NG2 at P8 (Fig. S3). Because it has been demonstrated that Dlx proteins are expressed in bipotential oligodendrocyte-GABAergic neuron progenitors (He et al., 2001; Marshall and Goldman, 2002; Panganiban and Rubenstein, 2002), we determined if the pan anti-Dlx antibody also stained $EGFP^+$/NG2-negative oligodendrocytes. $EGFP^+/MBP^+$ (myelin basic protein) oligodendrocytes were consistently Dlx-negative (unpublished data).

In summary, it appears that at the end of the first postnatal week, $NG2^+/LeX^+/Dlx^+/Mash1^+$/GFAP-negative type C–like cells may represent ~2–3% of the total SVZ cell population.

NG2 expression is detected in cells displaying an immature neuronal phenotype in the SVZ

Neuroblasts are Dlx^+/PSA-$NCAM^+$ and can be found migrating through the SVZ into the olfactory bulb (Doetsch et al., 1997, 1999a). In the SVZ, 22.0 ± 2.2 and 11.0 ± 1.8% of the $NG2^+/EGFP^+$ cells expressed PSA-NCAM (Fig. 2, D and G) at P8 and P40, respectively. Confirming the identity of a fraction of $NG2^+/EGFP^+$ cells as immature neuroblasts, we found that 16.0 ± 3.2 and 7.0 ± 1.6% of $NG2^+/EGFP^+$ cells in the SVZ also expressed class III β-tubulin (stained by TUJ1 antibody) at P8 and P40, respectively (Fig. 2, E and G; and not depicted). Similar percentages were also obtained with the immature neuronal marker HuC/D (unpublished data).

These data indicate that ~15% of endogenous $NG2^+/EGFP^+$ cells are postmitotic neuroblasts in the SVZ at P8.

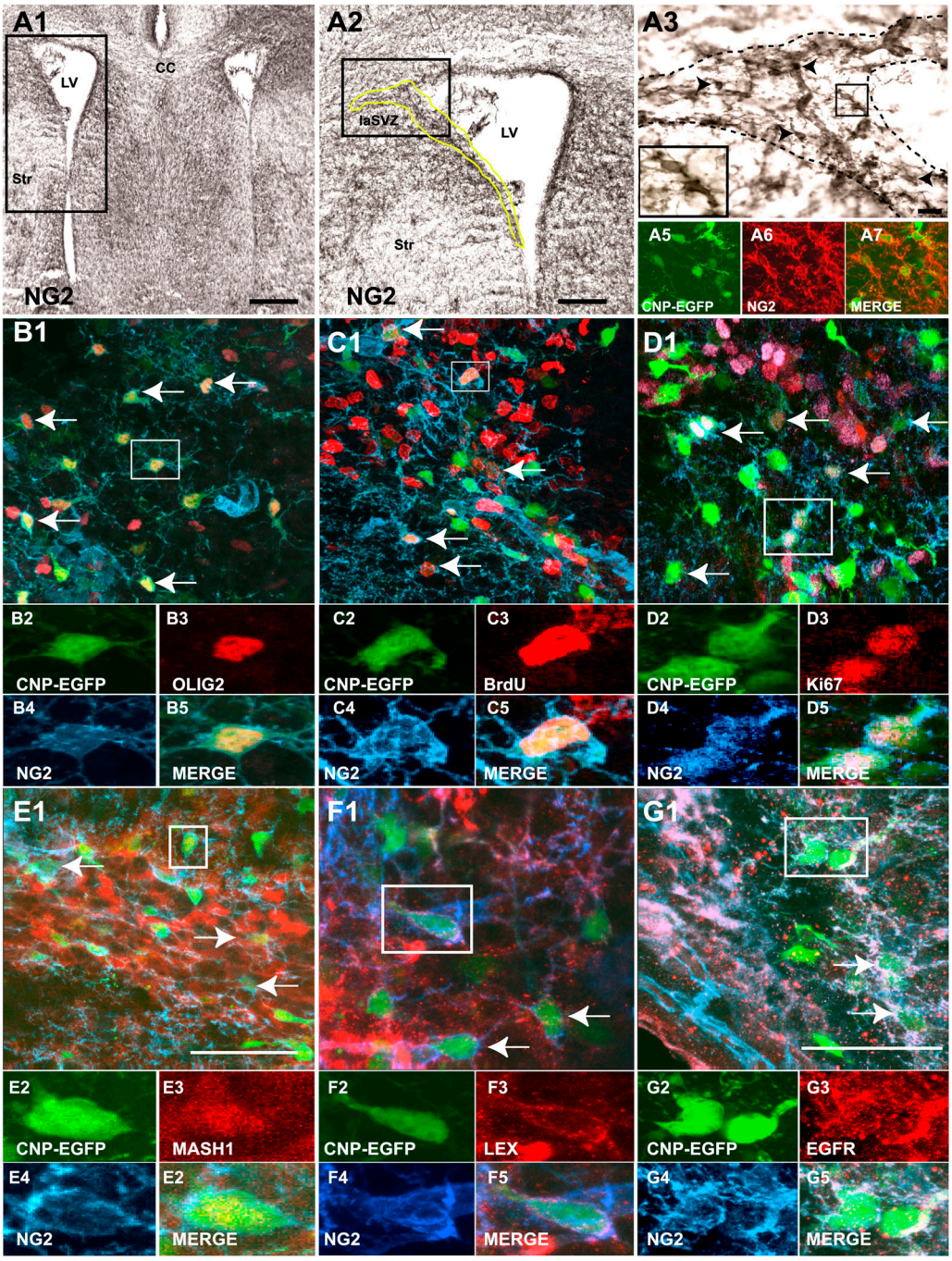

Figure 1. **NG2$^+$/EGFP$^+$ cells express NSCs markers in the SVZ.** Coronal sections of the SVZ at P8. (A1–A3) Anti-NG2 staining (DAB reaction, brown) shows that NG2 cells are found lining the wall of the lateral ventricle (yellow line) and throughout the entire lateral SVZ. The dotted line (A3) indicates the area analyzed in this paper. (A5–A7) Most of the EGFP$^+$ cells (A5, green) were labeled with NG2 antibodies (A6, red), and all NG2$^+$ cells were EGFP$^+$ (A7). (B–G) All micrographs were obtained from the lateral SVZ (laSVZ). (B) All NG2$^+$/EGFP$^+$ cells (blue/green, respectively) express Olig2 (red). (C and D) NG2$^+$/EGFP$^+$ cells proliferate in the SVZ, as shown by BrdU incorporation (C, red) and by Ki67 immunolabeling (D, red). (E and G) A large percentage of NG2$^+$/EGFP$^+$ cells (blue/green, respectively) express the transcription factor Mash1 (E, red), the adult NSC markers LeX antigen (F, red), and EGFR (G, red). Arrows indicate NG2$^+$/EGFP$^+$ cells double-labeled with any of the markers used. NG2$^+$/EGFP$^+$ cells in boxed areas are shown at higher magnification. LV, lateral ventricle; Str, striatum; CC, corpus callosum. Bars: (A1) 500 μm; (A2) 300 μm; (A3) 50 μm; (B–E) 50 μm; (F and G) 100 μm.

Consistently, we found that the majority of SVZ NG2$^+$/EGFP$^+$ cells that expressed class III β-tubulin were immunoreactive for Dlx at P8 (Fig. 2 F and Fig. S3). Finally, we could also detect LeX$^+$ cells that were TUJ1$^+$ (Capela and Temple, 2002; unpublished data).

The LeX-expressing subset of early postnatal NG2$^+$/EGFP$^+$ cells generates neurospheres and is self-renewing

To demonstrate self-renewal of early postnatal NG2$^+$/EGFP$^+$ cells, we determined whether or not single cells derived from the dissociation of primary neurospheres

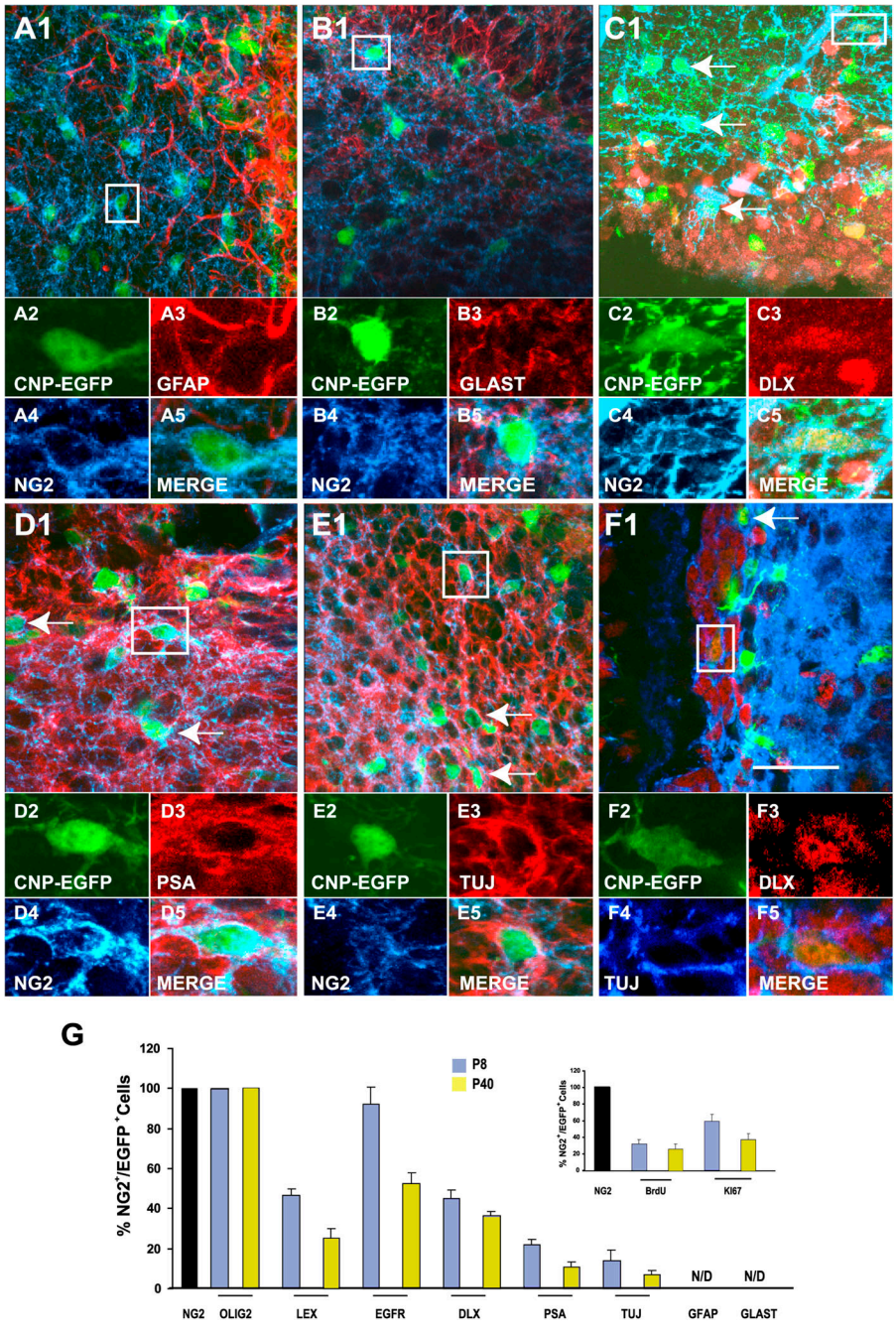

Figure 2. **A subpopulation of NG2$^+$/EGFP$^+$ cells displays an immature neuronal phenotype in the SVZ.** P8 coronal sections. (A and B) NG2$^+$/EGFP$^+$ cells (blue/green, respectively) in the SVZ are not labeled for the astroglial markers GFAP (A, red) or GLAST (B, red). (C) A large percentage of the NG2$^+$/EGFP$^+$ cells (blue/green, respectively) in the SVZ are labeled with anti-Dlx antibodies (red) for neuronal progenitor cells. (D) A significant percentage of the NG2$^+$/EGFP$^+$ cells (blue/green, respectively) in the SVZ express the early neuronal markers PSA-NCAM (D, red) and βIII-tubulin (E, TUJ1; red). (F) The majority of the EGFP$^+$/TUJ1$^+$ cells (green/blue, respectively) are Dlx$^+$ (red). Arrows indicate double-labeled NG2$^+$/EGFP$^+$ cells. NG2$^+$/EGFP$^+$ cells in boxed areas are shown at higher magnification. Bar, 50 μm. (G) NG2$^+$/EGFP$^+$ cells in the lateral ventricle of the SVZ. Virtually all NG2$^+$/EGFP$^+$ cells expressed Olig2 at P8 and P40, and EGFRs at P8. At P40 the percentage of EGFR$^+$/NG2$^+$ cells decreases by 50%. A similar decrease is also observed for Lex, Dlx, PSA-NCAM, and TUJ1. None of the NG2$^+$/EGFP$^+$ cells are labeled with anti-GFAP or anti-GLAST antibodies. (inset) Percentage of NG2$^+$/EGFP$^+$ cells that incorporated BrdU after 2 h of pulse labeling. No significant differences are detected between P8 and P40. This result was also confirmed by anti-Ki67 immunostaining. Percentages were obtained by counting NG2$^+$/EGFP$^+$ cells located into the SVZ region. Data are means ± SEM (total NG2$^+$/EGFP$^+$ cells counted equals 850 at P8 and 400 at P40; for each age, four to six brain sections from four different brains were used).

(Belachew et al., 2003) generated secondary neurospheres. FACS®-purified NG2$^+$/EGFP$^+$ cells from P2 brains represented 4.1 ± 0.2% of the total cells (12 separate sortings). After 7 d in EGF, these cells seeded at clonal dilution formed neurospheres, displaying the typical morphology and growth properties of NSCs (Reynolds and Weiss, 1992; Belachew et al., 2003). NG2$^+$/EGFP$^+$ cells dissociated from primary neurospheres generated secondary neurospheres, thus indicating self-renewal (Fig. 3 A1, inset).

NG2$^+$ cell–derived clonal neurospheres plated on coverslips comprised TUJ$^+$ neurons, GFAP$^+$ astrocytes, O4$^+$ preoligodendrocytes, and nestin$^+$ progenitors (unpublished data). Each clone contained ∼15–20% of neurons. The vast majority of the neurons in each clone were GABAergic, based on GAD-67 coexpression (93 ± 0.6% of total TUJ1-expressing cells; Fig. 3, A–C; and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200311141/DC1). All of the GAD-67$^+$/EGFP$^+$ neurons were Dlx$^+$ (Fig. 3 B and Fig. S1), and a small subset coexpressed the Ca^{2+}-binding protein PV (parvalbumin; Fig. 3 C). In contrast with the mixed neuronal fate potential of typical E14.5 forebrain NSCs, ∼5% of neurons derived from early postnatal NG2$^+$/EGFP$^+$ cells were immunoreactive for markers of dopaminergic and glutamatergic neurons (Fig. S1). These results indicate that NG2$^+$/EGFP$^+$ cells predominantly generate inhibitory GABAergic interneurons in culture, as demonstrated by their expression of GAD-67, Dlx, and PV (Ma et al., 1994; Jinno and Kosaka, 2002; Panganiban and Rubenstein, 2002; Liu et al., 2003).

By proposing that early postnatal SVZ NG2$^+$/EGFP$^+$ cells are type C–like cells (Doetsch et al., 2002a) and that the

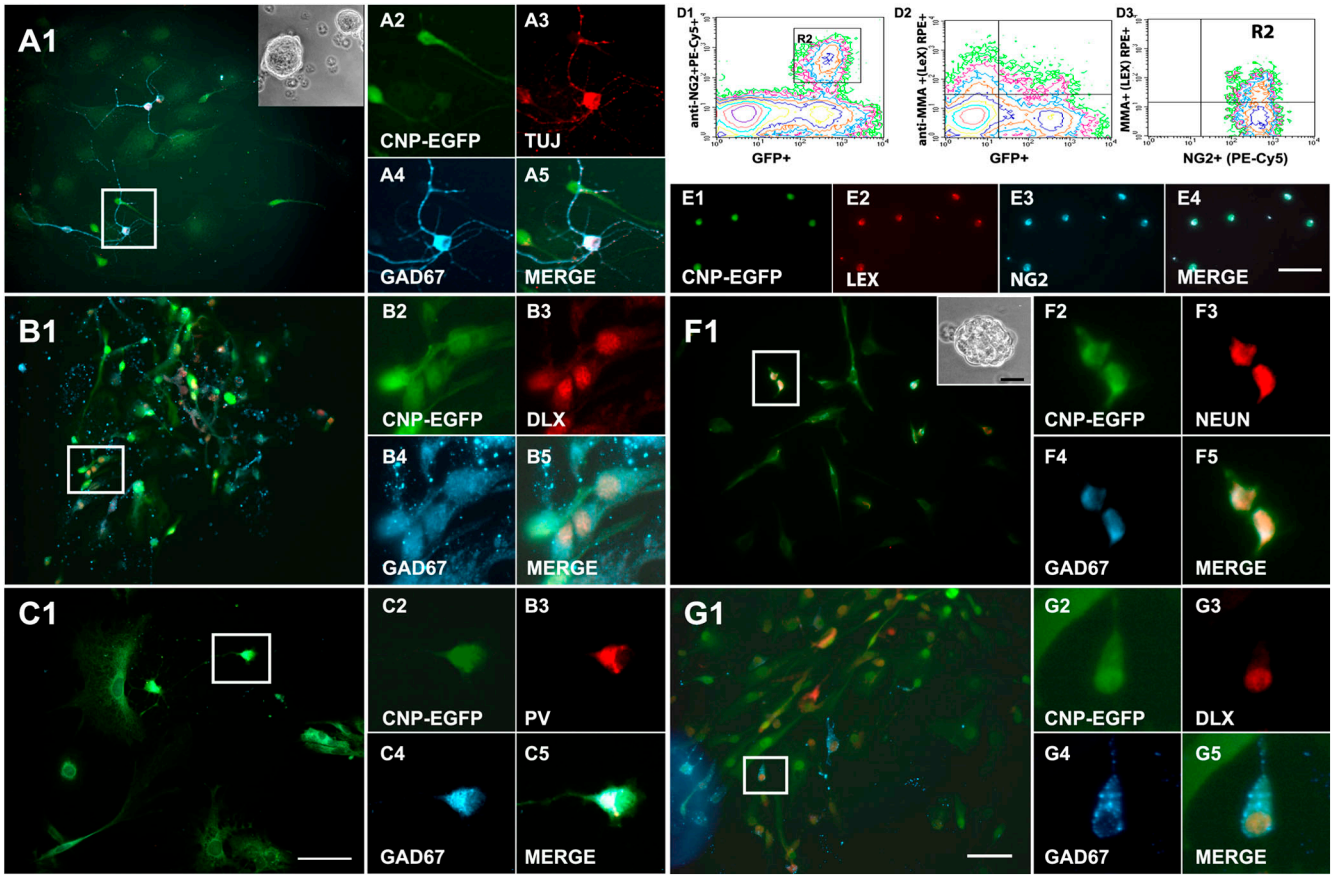

Figure 3. **FACS®-purified NG2$^+$/EGFP$^+$ and LeX$^+$/NG2$^+$/EGFP$^+$ cells self-renew and generate GABAergic interneurons in vitro.** FACS®-purified NG2$^+$/EGFP$^+$ (A–C) and LeX$^+$/NG2$^+$/EGFP$^+$ (D–G) cells generate neurospheres. After primary neurosphere formation, cell dissociation to single cell suspension and replating in SCM at clonal dilution generated secondary neurospheres (A1 and F1, insets). (A) A single secondary neurosphere was plated and processed for immunocytochemistry 6 d after plating. All the TUJ1$^+$ cells (red) are labeled with anti–GAD-67 (blue) antibodies. (B and C) The phenotype of the neurons derived from NG2$^+$/EGFP$^+$ cells is GABAergic. Neurons are double labeled with anti-Dlx (red) and anti–GAD-67 (blue) antibodies (B), and anti-parvalbumin (PV, red) and anti–GAD-67 (C, blue). (D) Sorting profile for NG2$^+$/EGFP$^+$ cells (D1; NG2 immunolabeling was scattered with PE-Cy5, R2 box) or MMA$^+$/EGFP$^+$ cells (D2; LeX antigen was scattered with R-phycoerythrin, RPE). To scatter the triple positive (LeX$^+$/NG2$^+$/EGFP$^+$) cells, the NG2$^+$/EGFP$^+$ fraction (R2 box in D1) was double sorted using PE-Cy5 for NG2 and RPE for LeX (D3). Note that a significant percentage of NG2$^+$/EGFP$^+$ cells (D1, R2 window) express the LeX antigen (D3). (E) A typical sample of LeX$^+$/NG2$^+$/EGFP$^+$ cells after FACS® shows high purity. (F) LeX$^+$/NG2$^+$/EGFP$^+$ FACS®-purified cells were assayed for neurosphere formation. LeX$^+$/NG2$^+$/EGFP$^+$ neurospheres displayed similar properties to those derived from NG2$^+$/EGFP$^+$ cells and generated differentiated GABAergic neurons, as shown by immunocytochemistry with anti-NeuN (red) and anti–GAD-67 (blue) antibodies. (G) The GABAergic neuronal fate is confirmed by anti-Dlx (red) and anti–GAD-67 (blue) coexpression. EGFP$^+$ cells (green) in boxed areas are shown at higher magnification. Bars, 50 μm.

LeX$^+$/GFAP-negative cells described by Capela and Temple (2002) are NG2$^+$, we are suggesting that NG2$^+$/EGFP$^+$ cells, which also express LeX, are multipotent and have the potential to self-renew. To demonstrate this property, we purified P2 LeX$^+$/NG2$^+$/EGFP$^+$ cells by triple FACS® (Fig. 3, D1–D3). All FACS®-purified Lex$^+$ cells were consistently NG2$^+$/EGFP$^+$ (Fig. 3, E1–E4), whereas only 26% of the NG2$^+$/EGFP$^+$ cells were also Lex$^+$ (Table I).

FACS®-purified LeX$^+$/NG2$^+$/EGFP$^+$ cells formed primary and secondary neurospheres (Fig. 3 F1, inset). Also, these neurospheres generated GFAP$^+$, O4$^+$, and nestin$^+$ cells in each clone (unpublished data). All clones contained

Table I. **Antigenic characterization of FACS®-purified perinatal and adult NG2$^+$/EGFP$^+$ cells**

	NG2	Ki67	LEX	DLX	EGFR	PSA	TUJ	GFAP
Postnatal (P2)	100	65.99 ± 0.42	26.0 ± 1.8	13.13 ± 2.2	97.52 ± 5.3	26.26 ± 0.30	10.17 ± 0.50	N/D
Adult (P40)	100	26.43 ± 2.2	14.58 ± 1.8	9.62 ± 1.2	42.20 ± 3.4	16.23 ± 0.4	6.2 ± 1.0	N/D

Postnatal day 2 (P2) and 40 (P40) brains from CNP-EGFP transgenic mice were dissociated and processed for NG2/EGFP FACS® purification, as described in Materials and methods. After FACS®, cells were plated on polyornithine-coated coverslips (20,000 cells per 35-mm coverslip) and processed for immunocytochemistry after 2 h. Percentages of NG2$^+$/EGFP$^+$ cells labeled with each marker are shown. Cells were not detected at P2 or P40 with the astrocyte marker GFAP (N/D). Values represent means ± SEM obtained from a total of five to six separate FACS® experiments and 10 microscopic fields per experiment. Total number of cells counted for each marker ranged between 2,787 and 3,251 at P2 and 2,020 and 2,485 at P40.

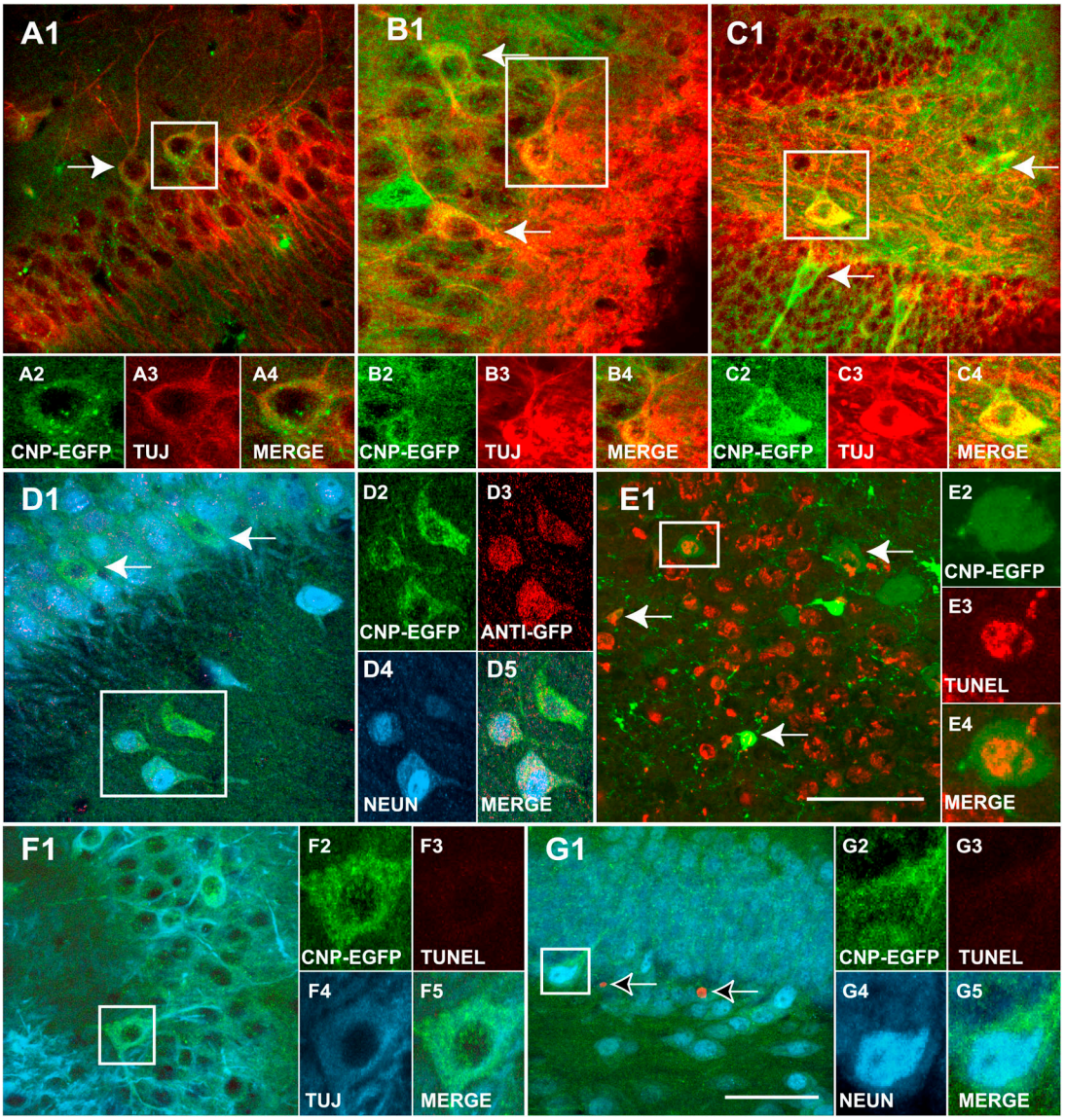

Figure 4. **Grafted NG2$^+$/EGFP$^+$ cells differentiate to neurons in the hippocampus.** (A–C) 3 wk after transplantation, all the grafted EGFP$^+$ cells (green) in the hippocampus are labeled with TUJ1 (red) antibodies. EGFP$^+$/TUJ1$^+$ cells (green/red, respectively) are found in the pyramidal layer of the CA1 (A1) and CA3 (B1). Grafted cells are also found in the hilar region of the DG (C1). (D) A large percentage of transplanted EGFP$^+$ cells (green) are also labeled with anti-NeuN (blue) antibodies in the CA3 area and hilar region of the DG (not depicted), confirming the neural fate of the grafted NG2$^+$/EGFP$^+$ cells. The tissue was also stained with anti-GFP antibodies (D3, red), confirming that these neurons are derived from the transplanted NG2$^+$/EGFP$^+$ fraction. (E–G) Neurons derived from grafted NG2$^+$/EGFP$^+$ cells are viable, as determined by TUNEL assay. (E) Positive control (Dnase I-treated tissue). EGFP$^+$/TUJ1$^+$ (F, green/blue, respectively) and EGFP$^+$/NeuN$^+$ (G, green/blue, respectively) grafted cells are TUNEL-negative (red) 6 wk after transplantation. Arrows in A1–D1 indicate EGFP$^+$ neurons derived from grafted cells. Arrows in E1 indicate EGFP$^+$/TUNEL$^+$ cells after DNAse treatment of the tissue (positive control). Arrows in G1 indicate endogenous EGFP-negative TUNEL$^+$ cells in DG. EGFP$^+$ cells in boxed areas are shown at higher magnification. Bars: (A–D , F, and G) 50 μm; (E) 100 μm.

~10% of NeuN$^+$/GAD-67$^+$/Dlx$^+$ neurons (Fig. 3, F and G). FACS®-purified Lex-negative/NG2$^+$/EGFP$^+$ cells did not generate neurospheres, indicating that, under our culture conditions, LeX$^+$ cells represent the multipotent neurosphere-generating cells among the NG2$^+$/EGFP$^+$ lineage.

NG2-expressing progenitors transplanted into the lateral ventricle migrate to the hippocampus and differentiate into GABAergic interneurons

To determine their developmental fate in vivo, we transplanted perinatal NG2$^+$/EGFP$^+$ cells FACS®-purified from P2 CNP-EGFP brains into wild-type mouse brains. Table I shows that, in agreement with the in vivo results (Figs. 1 and 2), a high percentage of the FACS®-purified NG2$^+$/EGFP$^+$ cells were Ki67$^+$, i.e., they displayed a proliferative phenotype. None of the cells were stained with anti-GFAP (Table I) or with anti-NeuN antibodies (Belachew et al., 2003). Both at P2 and P40, a significant percentage of the cells were stained for all the markers expressed by NG2$^+$/EGFP$^+$ cells in the SVZ in vivo, i.e., LeX, Dlx, EGFR, PSA-NCAM, and TUJ1 (Table I). These percentages were very similar to those found in the SVZ

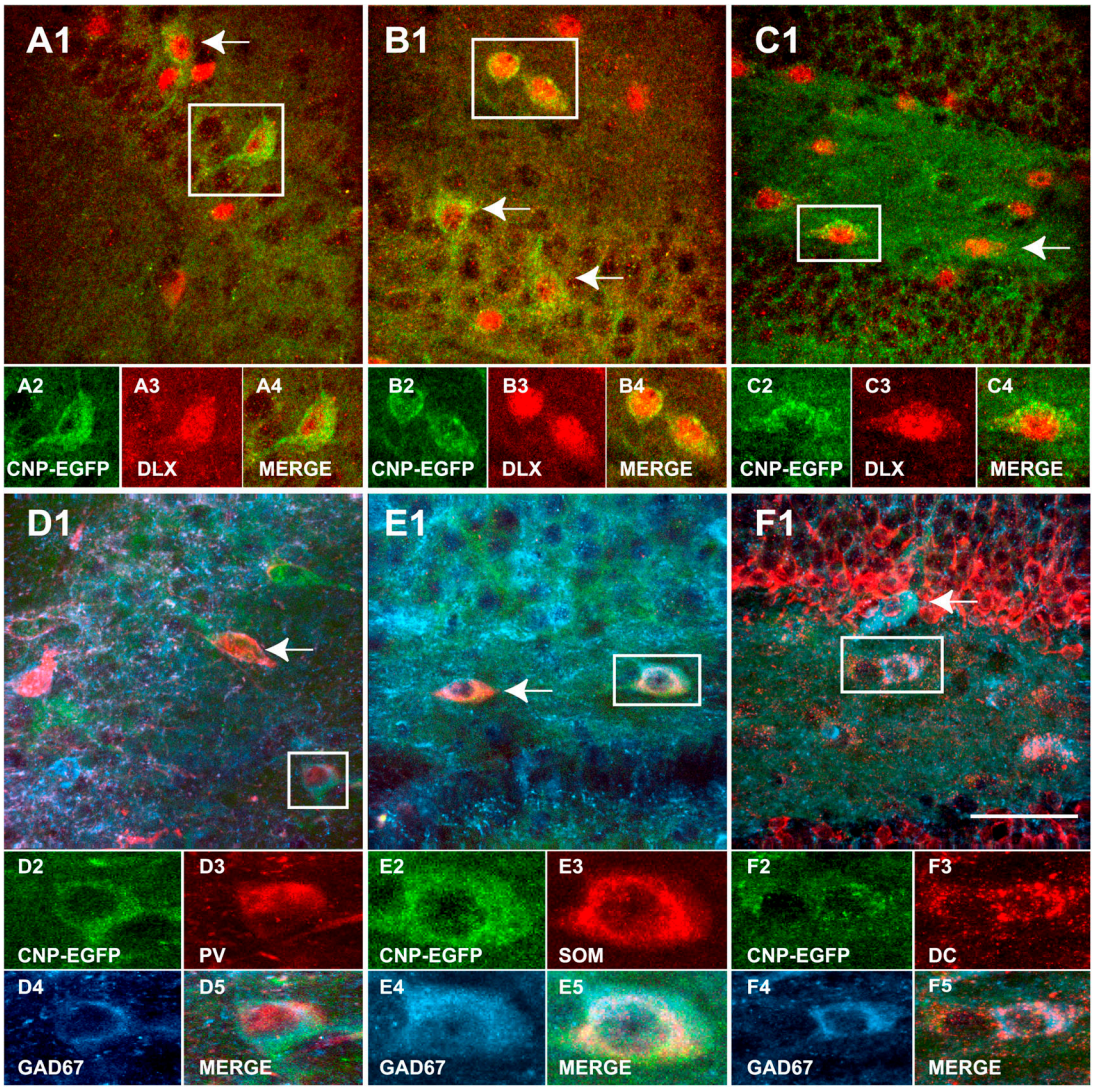

Figure 5. **Grafted NG2$^+$/EGFP$^+$ cells give rise to GABAergic interneurons in the hippocampus.** (A–C) 3 wk after transplantation, EGFP$^+$/Dlx$^+$ cells were found in the stratum radiatum, stratum oriens, and stratum pyramidale of the CA1 (A) and CA3 (B) and in the hilar region of the DG (C). (D–F) Immunostaining with anti–GAD-67 antibodies 3 wk after transplantation. Note that at this time the vast majority of the grafted EGFP$^+$ cells (green) were labeled with anti–GAD-67 (blue). Immunoreactivity of the graft-derived GAD-67$^+$ neurons for calcium-binding proteins and neuropeptides confirmed the interneuron fate of the grafted EGFP$^+$ cells. (D and E) A proportion of the graft-derived neurons are double labeled with anti–GAD-67 (blue) and parvalbumin (D, PV; red), somatostatin (E, SOM; red), or doublecortin (F, DC; red). Arrows in A1–F1 indicate EGFP$^+$ neurons derived from grafted cells. EGFP$^+$ cells in boxed areas are shown at higher magnification. Bar, 50 μm.

(compare Table I with Fig. 2 G and Fig. S3), indicating that the majority of FACS®-purified NG2$^+$/EGFP$^+$ cells display an SVZ-like immunophenotype.

We injected FACS®-purified NG2$^+$/EGFP$^+$ cells into the LV of P2 wild-type mice and analyzed the grafted cells over the following 3–4 wk. In some experiments, cells were also labeled with the fluorescent dye PKH26 before transplantation (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200311141/DC1). Labeled migrating cells were seen within the wall of the lateral ventricle and striatum as soon as 48 h after injection (Fig. S4 A). Some of the cells had migrated tangentially through the subcortical white matter (Fig. S4 A, arrow and inset). As negative controls, we transplanted NG2$^+$/CNP-EGFP$^+$ cells, which underwent six freeze–thaw cycles. These cells did not migrate, but were found as clumps surrounding the area of the lateral ventricle (unpublished data). EGFP fluorescence intensity was sufficient to detect the grafted cells up to 5 wk after transplantation. Within the first 2–3 wk, a large number of the transplanted cells were found dispersed throughout the olfactory bulb and striatum (not depicted), hippocampus (Figs. 4 and 5), and cortex and white matter (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200311141/DC1).

4 d after transplantation, EGFP$^+$ migrating neuroblasts were encountered in the stratum oriens of the hippocampus and in striatum (Fig. S4, B1 and B2, respectively). The majority of the migrating cells were PSA-NCAM$^+$ (Fig. S4 C) and TUJ1$^+$ (Fig. S4 D). We were not able to identify a single EGFP$^+$/TUJ1$^+$ cell in the hippocampus or surrounding the area of injection at 48 h after transplantation (unpublished data). These findings demonstrate that neuronal differentiation of NG2$^+$/EGFP$^+$ cells observed after 3 wk does

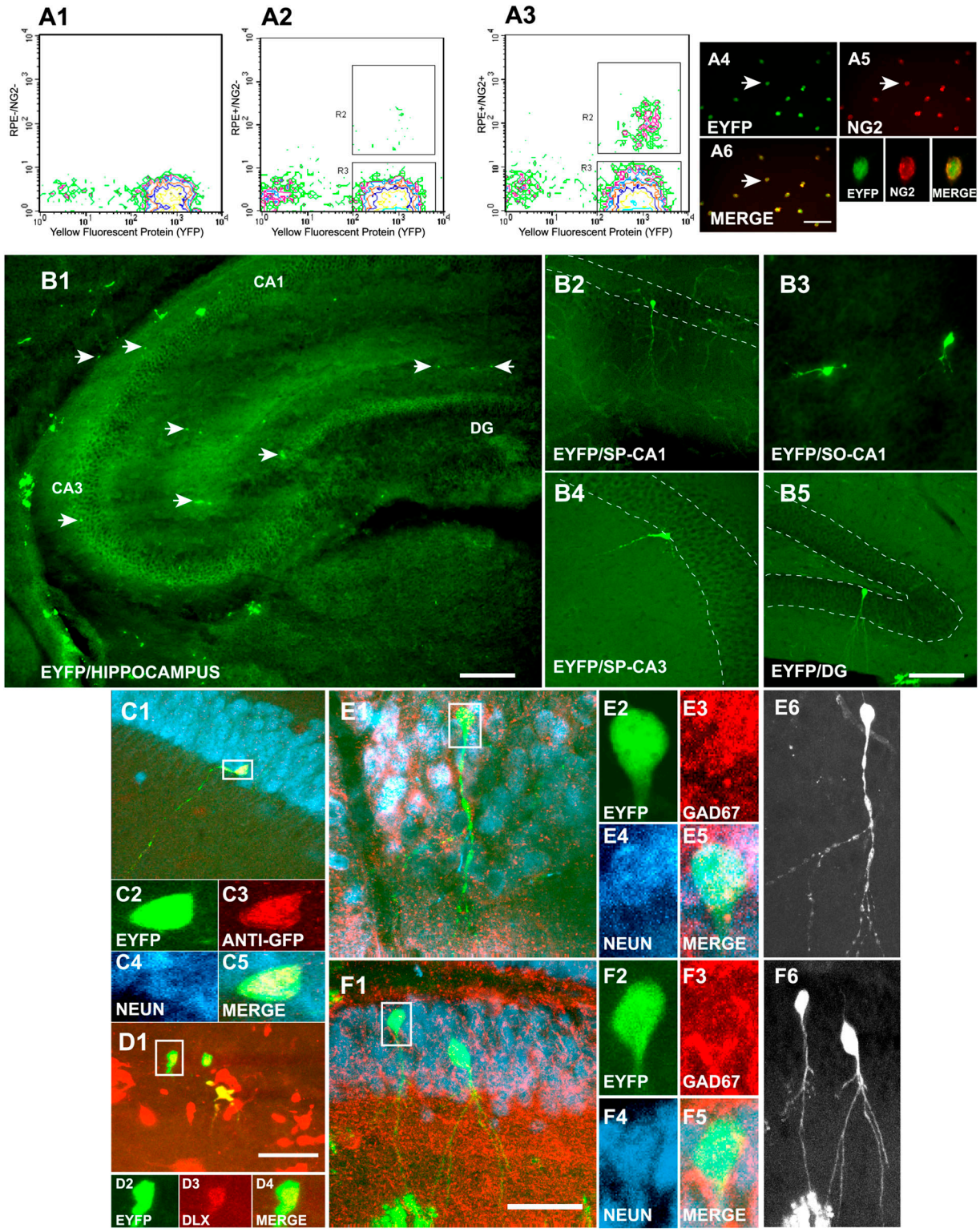

Figure 6. **Grafted NG2$^+$/EYFP$^+$ cells give rise to GABAergic interneurons in the hippocampus.** (A1–A3) Typical sorting profile for NG2$^+$/EYFP$^+$ cells (NG2 immunolabeling was scattered with RPE-Cy5 [R2 box]). (A4–A6) A typical sample of NG2$^+$/EYFP$^+$ cells after FACS® shows high purity. (B) 1 wk after grafting, EYFP$^+$ cells (green) were found in the hippocampus (arrows) displaying a typical neuronal morphology in the stratum oriens of the CA1 (B3) and CA3 (not depicted), in the stratum pyramidale of the CA1 and CA3 (B2 and B4, respectively), and in the DG (B5). The tissue was also stained with anti-GFP antibodies (C, red), confirming that these neurons are derived from the transplanted NG2$^+$/EYFP$^+$ fraction. Migratory EYFP$^+$ cells acquired immature neuronal markers 1 wk after grafting, as shown by Dlx (D, red) and TUJ1 (not depicted). (E and F) 3 wk after transplantation, all the grafted EYFP$^+$ cells (green) found in the hippocampus are labeled with anti-NeuN antibodies (blue). EYFP$^+$/NeuN$^+$ cells (green/blue) are found in the pyramidal layer of the CA1 (not depicted) and CA3 (E) and in the subgranular layer of the DG (F). EYFP$^+$ cells are also labeled with anti–GAD-67 (red) antibodies in the CA3 area (E) and DG (F), confirming their neural fate. In E6 and F6, EYFP was converted to grayscale. Anti-GFP antibodies recognize all the GFP variants, including EGFP, EYFP, and ECFP. EYFP$^+$ cells are shown at higher magnification in boxed areas. Bars: (A, C, E, and F) 50 μm; (B1) 300 μm; (B2–B5) 100 μm.

not arise from the small fraction of NG2$^+$/EGFP$^+$ cells that may already express markers of early neuronal commitment (Table I). Finally, no EGFP$^+$ cells were found outside the area of injection, including the hippocampus, within the first 24–48 h after grafting. These results indicate that the grafted NG2$^+$/EGFP$^+$ cells acquire a neuronal fate during the first week after transplantation.

3 wk after transplantation, grafted NG2$^+$/EGFP$^+$ cells were found scattered in the hippocampus (Fig. S4, E–G). EGFP$^+$ cells were seen in the stratum radiatum (Fig. S4, E and F), stratum lucidum (Fig. S4 E), stratum oriens (Fig. S4 F), and stratum pyramidale (Fig. S4, E and F) of the CA1 and CA3 regions. Cells were also found in the hilar region of the dentate gyrus (DG; Fig. S4 G). Complete overlap between green fluorescence and EGFP was confirmed in all grafted cells found in the hippocampus by using anti-GFP antibodies (Fig. 4 D). None of the EGFP$^+$ cells found in the hippocampus was labeled for astrocyte (GFAP) or oligodendrocyte (MBP) markers (unpublished data). However, EGFP$^+$/MBP$^+$ cells with a typical oligodendrocyte morphology were found both in subcortical white matter and in cerebral cortex 2–3 wk after grafting (Fig. S5).

3 wk after transplantation, the majority of the grafted EGFP$^+$ cells in the hippocampus were immunolabeled with TUJ1 (Fig. 4, A–C) and were found in the stratum oriens, stratum radiatum, and stratum pyramidale of the CA1 and CA3 regions (Fig. 4, A and B). Cells with the same phenotype were also found in the hilar region of the DG (Fig. 4 C). In the same hippocampal regions, a large percentage of the EGFP$^+$ cells also expressed NeuN (Fig. 4 D), indicating that grafted cells had differentiated into mature neurons. TUNEL assays demonstrated that graft-derived TUJ1$^+$ and NeuN$^+$ neurons in the hippocampus were still viable 6 wk after transplantation (Fig. 4, F and G).

Transcription factors of the Dlx homeobox gene family are implicated in GABAergic interneuron identity and differentiation (Panganiban and Rubenstein, 2002). The majority of the hippocampal graft-derived EGFP$^+$ cells were labeled with anti-Dlx antibody (Fig. 5, A–C) and anti–GAD-67 (Fig. 5, D–F), indicating that NG2$^+$/EGFP$^+$ cells generated GABAergic interneurons in vivo. Many EGFP$^+$ grafted cells found in CA1, CA3, and DG were GAD-67$^+$/PV$^+$ (Fig. 5 D), GAD-67$^+$/SOM$^+$ (somatostatin; Fig. 5 E), or GAD-67$^+$/DC$^+$ (doublecortin; Fig. 5 F), confirming their interneuron identity (Jinno and Kosaka, 2002).

We repeated the aforementioned grafting experiments by using NG2$^+$/EYFP$^+$ (enhanced YFP$^+$) cells FACS® purified from P2 β-actin-EYFP mouse brain (Fig. 6 A). NG2$^+$/EYFP$^+$ cells transplanted into the lateral ventricle migrated to different regions of the hippocampus (Fig. 6 B) and differentiated into NeuN$^+$/Dlx$^+$/GAD-67$^+$ GABAergic interneurons displaying typically arborized morphologies (Fig. 6, C–F).

GABAergic hippocampal interneurons derived from transplanted NG2-expressing progenitor cells are functionally integrated

We investigated whether or not EGFP$^+$ cells in the hippocampus that could be identified after transplantation elicited action potentials and received synaptic innervation. We performed whole cell current and voltage clamp from nine graft-derived EGFP$^+$ hippocampal cells (Fig. 7 A). Examples of two of such cells are shown in Fig. 7 (H and I) for hilar region and stratum oriens of the CA3, respectively. Cells displayed a morphologically differentiated neuronal phenotype (Fig. 7, H and I). In all cases, depolarizing current pulses (30–180 pA for 1,000 ms; step = 30 pA) from a membrane potential of ~−70 mV resulted in the generation of action potentials (Fig. 7 B, step size 120 pA) at a range of frequencies (Fig. 7 C). At the maximum current injection used, the mean firing frequency was 50 ± 10 Hz (Fig. 7 C; $n = 9$; range 35–106 Hz). Interestingly, minimal spike frequency adaptation was noted within the duration of the depolarizing current injections (Fig. 7 D). In all cells tested ($n = 6$), spontaneous inward currents were present (single example in Fig. 7, E and F), which were consistently abolished by the inclusion of 10 μM NBQX (Fig. 7 E, arrow). The average inward current (267 events) from the single example trace shown in Fig. 7 E displayed a decay with $\tau = 2.2$ ms when fitted to a single exponential (Fig. 7 G).

Endogenous CNP-EGFP$^+$ neurons in the hippocampus are GABAergic interneurons

Similar to the SVZ, a significant percentage (18.4 ± 4.0%; $n = 342$) of NG2$^+$/EGFP$^+$ cells in the postnatal and adult DG of the hippocampus coexpressed NG2 and PSA-NCAM (Fig. 8 A). Hippocampal TUJ1$^+$/EGFP$^+$ cells were also observed and expressed Dlx (Fig. 8 B). The majority of the Dlx$^+$ cells were stained with anti–GAD-67 antibodies both at P8 and P40 (Fig. 8 C and Table II), confirming their GABAergic identity. Finally, a large percentage of Dlx$^+$/EGFP$^+$ cells were NeuN$^+$ (Fig. 8 D and Table II).

To define the phenotype of the EGFP$^+$ GABAergic neurons, we performed immunolabeling for the interneuron markers PV (Fig. 8 E), DC (Fig. 8 F), SOM (Fig. 8 G), and CAL (calretinin; Fig. 8 H). A large percentage of the hippocampal EGFP$^+$ neurons were double-labeled with these markers and anti–GAD-67 (Fig. 8, E–H; and Table II). The majority of GAD-67$^+$/EGFP$^+$ neurons were found in CA3 and DG regions (Belachew et al., 2003). Table II shows that the percentage of GAD-67$^+$/EGFP$^+$ cells expressing PV increased by 2–5-fold in all regions between P8 and P40. The percentage of GAD-67$^+$/EGFP$^+$ cells that were DC$^+$ also increased to a similar extent (Table II). In contrast, the percentage of GAD-67$^+$/EGFP$^+$ cells that were SOM$^+$ remained constant (Table II). No EGFP$^+$/CAL$^+$ cells were found (Fig. 8 H). In all hippocampal regions, we observed that the absolute density of GAD-67$^+$/EGFP$^+$ cells and the total number of EGFP$^+$ cells decreased between P8 and P40 (unpublished data).

Together, these results show that endogenous hippocampal GAD-67$^+$/EGFP$^+$ interneurons display a cellular phenotype similar to that of interneurons derived from transplanted NG2$^+$/EGFP$^+$ progenitors or neurons generated from NG2$^+$/EGFP$^+$ neurospheres.

Discussion

Recent works demonstrated that the majority of adult CNS multipotent progenitor cells are rapidly dividing in the SVZ

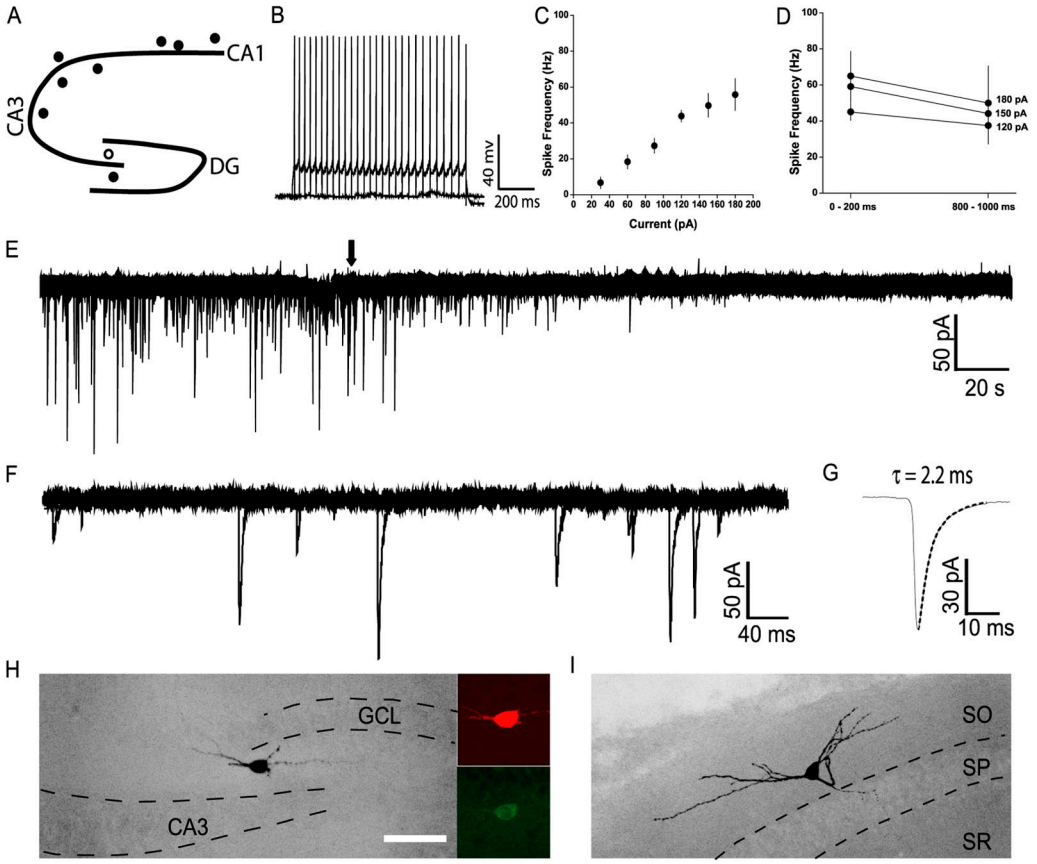

Figure 7. **Grafted NG2$^+$/EGFP$^+$ cells in the hippocampus possess physiological properties of mature inhibitory interneurons.** (A) Schematic of hippocampus illustrating the positions of the grafted EGFP$^+$ cells that physiological recordings were attained from. The open circle indicates the cell from which the single example current (B) and voltage clamp (E and F) traces were obtained. (B) Action potential generation in response to a depolarizing current injection (120 pA for 1,000 ms) from a membrane potential of −70 mV. (C) Pooled data of injection current (30–180 pA; step = 30 pA) versus spike frequency during the 1,000-ms depolarizing pulse. (D) Pooled data illustrating spike frequency during the first and last 200-ms epochs (0–200 and 800–1,000 ms) of depolarizing current injections (120, 150, and 180 ms pA). (E) Continuous voltage clamp trace (holding potential −70 mV; 3 min) illustrating the presence of spontaneous inward currents that were abolished by inclusion of 10 μM NBQX (arrow, 2 min; NBQX is present for the remainder of trace). (F) Magnification of part of the trace in E, illustrating the time course of individual spontaneous inward currents (four superimposed consecutive traces of 700 ms each). (G) Average time course (267 events; average amplitude equals 73 pA) of the inward currents, as determined by single exponential fitting of the decay (dotted line). (H and I) Biocytin (brown) images of two cells from which recordings were taken. GCL, granule cell layer; SO, stratum orien; SP, stratum pyramidale; SR, stratum radiatum. Images were converted to grayscale and inverted for clarity. Insets in H illustrate a high magnification of the biocytin-conjugated fluorescence (red; top inset) and the corresponding EGFP$^+$ fluorescence (green; bottom inset). Bar, 50 μm.

(Capela and Temple, 2002; Doetsch et al., 2002a). In the present work, we show that a large percentage of NG2$^+$/EGFP$^+$ cells in the SVZ incorporated BrdU after a 2-h pulse at P8 and P40. Furthermore, a similar percentage of the cells were immunopositive for Ki67 antibodies (Schluter et al., 1993). From our data, it can be concluded that NG2$^+$ progenitors represent a major population of rapidly cycling cells in the postnatal and adult SVZ. To further characterize the properties of NG2$^+$ cells in the SVZ, we analyzed expression of several NSC markers (Capela and Temple, 2002; Doetsch et al., 2002a). Based on LeX expression, Capela and Temple (2002) have previously identified two main subpopulations of multipotent NSCs in the adult SVZ. Lex$^+$/GFAP$^+$ cells represent only a small percentage (6%) of the entire Lex$^+$ stem cell population, whereas LeX$^+$/GFAP-negative cells are the majority and represent a large percentage of cells (Capela and Temple, 2002). Importantly, only a small percentage of the LeX$^+$/GFAP$^+$ cells proliferate in vivo, whereas the majority of LeX$^+$/GFAP-negative cells incorporate BrdU (Capela and Temple, 2002). Based on these observations, it was proposed that the SVZ contains a stem cell population with a novel identity, neither astrocytic nor ependymal, but Lex$^+$/GFAP-negative (Capela and Temple, 2002). Our experiments demonstrate that this population corresponds to NG2 proteoglycan-expressing cells, based on the findings that half of Lex$^+$/GFAP-negative cells are NG2$^+$ in the early postnatal SVZ and, vice versa, that a large fraction of NG2$^+$ cells in the postnatal and adult SVZ express LeX, do not express GFAP, and incorporate BrdU.

A separate study by Doetsch et al. (2002a) demonstrated that the Lex$^+$/GFAP-negative cells belong to the transit-amplifying, multipotent type C cells in the adult SVZ. These cells are also GFAP negative and display intrinsic properties of multipotent NSCs. Furthermore, type C cells express Dlx and the EGFR (Doetsch et al., 2002a). In the present work, we show that a high percentage of NG2$^+$ cells in the early

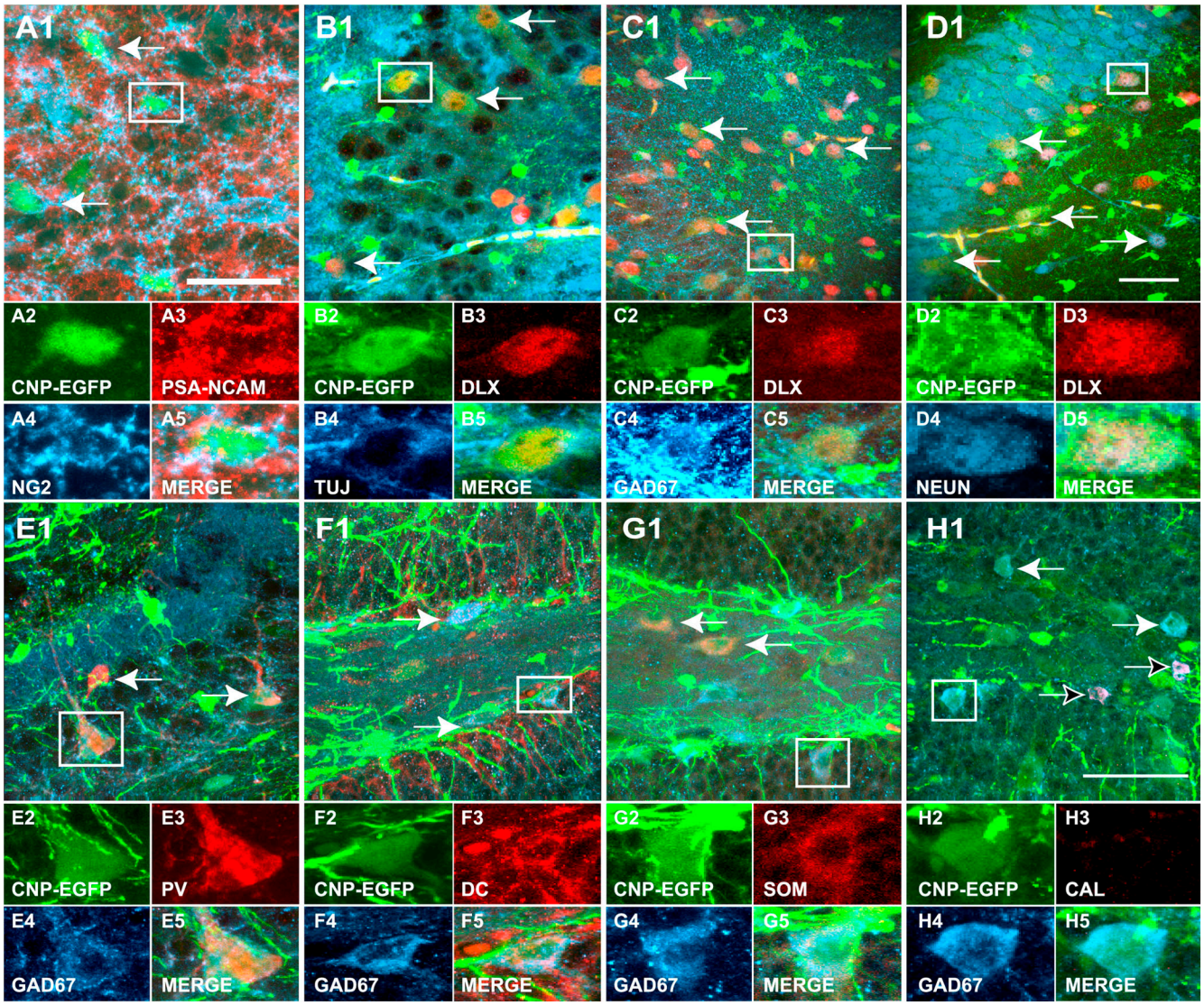

Figure 8. **Endogenous EGFP$^+$ cells in the postnatal hippocampus display a GABAergic interneuron phenotype.** P8 sagittal sections and CA3 and DG regions of the hippocampus. (A) A proportion of the NG2$^+$/EGFP$^+$ cells (blue/green, respectively) in the DG express PSA-NCAM (red). (B) A proportion of the EGFP$^+$ cells coexpress Dlx (red) and class III β-tubulin (blue). (C) The majority of the EGFP$^+$ cells (green) that express Dlx (red) also express GAD-67 (blue). (D) All the EGFP$^+$/NeuN$^+$ cells (green/blue, respectively) also express Dlx (red). (E and F) A proportion of the EGFP$^+$ cells in CA3 (E) and DG (F) express parvalbumin (E, PV; red), doublecortin (F, DC; red), and GAD-67 (blue). (G) In the hilar region of the DG and in CA3 (not depicted), a significant proportion of the EGFP$^+$ cells express the neuropeptide somatostatin (SOM, red) and GAD-67 (blue). (H) EGFP$^+$ cells that are positive for GAD-67 (blue) are not labeled with antibodies against calretinin (CAL, red). White arrows in A1–H1 indicate EGFP$^+$ cells that are also labeled for all the markers analyzed. In H1, black arrows indicate EGFP-negative CAL$^+$/GAD-67$^+$ cells. Cells in boxed areas are shown at higher magnification. Quantitative analysis of these data is shown in Table II. Bars, 50 μm.

postnatal and adult SVZ expresses the EGFR, and that a significant proportion expresses Dlx. These results, combined with the proliferative properties of NG2$^+$ cells and LeX expression in these progenitors, indicate that NG2$^+$ cells in the perinatal SVZ represent a transit-amplifying type C–like cell phenotype, as described by Doetsch et al. (2002a). This notion is further supported by previous studies (Casaccia-Bonnefil et al., 1997; Doetsch et al., 2002b) showing that the absence of p27Kip1 significantly increased the proliferation of both NG2$^+$ cells and of the transit-amplifying type C cells in the mouse SVZ. This evidence not only corroborates that NG2$^+$ and type C cells are overlapping populations, but also that their highly proliferative rate is essential to maintain a reservoir of multipotent progenitor cells in the SVZ. The unambiguous identification of NG2$^+$/Lex$^+$ cells as type C cells would necessitate the demonstration of ultrastructural features that were first described for this phenotype in the adult SVZ (Doetsch et al., 1999a, 2002a). However, our findings indicate that perinatal NG2$^+$/Lex$^+$ cells fulfil all the functional and antigenic criteria to be considered as type C–like progenitor cells of the SVZ.

NG2$^+$/EGFP$^+$ cells can be reliably purified by FACS®, form primary neurospheres, and generate secondary neurospheres after cell dissociation, i.e., they display self-renewal. Cells in secondary neurospheres maintain their multipotentiality and generate GABAergic neurons that express Dlx

Table II. **Immunocytochemical characterization of GAD-67$^+$/EGFP$^+$ neurons in the perinatal and adult hippocampus**

	PV		DC		SOM		DIx		NeuN	
	P8	P40	P8	P40	P8	P40	P8	P40	P8	P40
DG										
GL	23 ± 4	52 ± 5	0	67 ± 7	71 ± 7	58 ± 8	94 ± 2	86 ± 2	70 ± 8	65 ± 8
HL	14 ± 4	30 ± 4	9 ± 3	78 ± 5	68 ± 5	74 ± 6	92 ± 1	92 ± 2	86 ± 5	83 ± 7
CA3										
SR	11 ± 3	45 ± 8	12 ± 3	76 ± 5	67 ± 6	66 ± 3	92 ± 4	83 ± 6	84 ± 6	83 ± 5
SP	11 ± 2	53 ± 6	15 ± 3	67 ± 4	76 ± 2	72 ± 7	90 ± 4	86 ± 4	86 ± 2	92 ± 4
CA1										
SR	0	28 ± 2	0	0	0	58 ± 5	95 ± 2	89 ± 1	90 ± 8	92 ± 8
SP	0	69 ± 2	0	25 ± 2	62 ± 7	63 ± 2	95 ± 2	85 ± 2	88 ± 2	94 ± 6

Sagittal sections from postnatal day 8 (P8) and 40 (P40) brains of CNP-EGFP transgenic mice were stained with different antibody combinations, as described in Materials and methods. Cells were analyzed in dentate gyrus (DG) granule layer (GL) and hylus (HL), and in CA3 and CA1 stratum radiatum (SR) and stratum pyramidale (SP). Percentages of GAD-67$^+$/EGFP$^+$ cells labeled with each marker are shown. Values represent mean ± SEM obtained from four different brains (six tissue sections per brain). Total cells counted were 1,080 for P8 and 770 for P40. PV, parvalbumin; DC, doublecortin; SOM, somatostatin.

and GAD-67. Regarding the self-renewal and multipotential properties of the NG2$^+$ cell population, our results suggest that the high percentage of NG2$^+$/EGFP$^+$ cells in the early postnatal SVZ that also express LeX correspond to the cell population that generate neurospheres and displays multipotentiality. The NG2$^+$/EGFP$^+$/Lex-negative cells that did not form neurospheres could either require different growth conditions or could be progenitors committed to an oligodendroglial fate. These results indicate that, under our experimental conditions, only the LeX$^+$ subpopulation of NG2$^+$/EGFP$^+$ cells is capable of self-renewal and is multipotential. Our analysis establishes a link between NG2$^+$/Lex$^+$/EGFP$^+$ cells in the early postnatal SVZ and a GABAergic neuronal progeny expressing Dlx and GAD-67.

To demonstrate that NG2$^+$/EGFP$^+$ cells are intrinsically neurogenic in vivo, and to define the neuronal subtypes they can generate, we isochronically grafted early postnatal NG2$^+$/EGFP$^+$ cells into wild-type host brains. Our results clearly show that transplanted NG2$^+$/EGFP$^+$ progenitors generate differentiated and synaptically integrated neurons. The antigenic properties of these grafted NG2$^+$ cell–derived hippocampal neurons indicate that they are interneurons based on expression of GAD-67 and markers such as PV, SOM, and DC. The hippocampal graft-derived neurons display the ability to respond to depolarizing current pulses with spikes at high frequencies of discharge that display minimal spike frequency accommodation. Both of these features are indicative of an inhibitory interneuron phenotype and are distinct to the physiological properties commonly seen in principal cells of the hippocampus (McBain and Fisahn, 2001). In addition, these cells also receive functional AMPA receptor-mediated inputs. Together, these data illustrate the neuronal maturation and functional integration of the early postnatal NG2$^+$/EGFP$^+$-derived cells after transplantation into a wild-type host.

Several studies that analyzed development of transplanted neural progenitors concluded that these precursors were unable to generate the appropriate neuronal cell types in various brain regions (Olsson et al., 1997; Desai and McConnell, 2000). In contrast, our results indicate that NG2$^+$/EGFP$^+$ cells express molecular cues, such as Dlx, that are necessary for cellular and regional identity. We also show that temporal and regional information is maintained after transplantation because both grafted NG2$^+$/EGFP$^+$ precursors and hippocampal interneurons derived from these cells still express Dlx. It has been previously shown that Dlx expression identifies a bipotential neural progenitor population capable of generating GABAergic interneurons and oligodendrocytes during embryonic CNS maturation (He et al., 2001; Yung et al., 2002). Therefore, postnatal Dlx$^+$/NG2$^+$/EGFP$^+$ cells are likely to be developmentally related to such bipotential embryonic Dlx$^+$ progenitors. Dlx expression in neural progenitors is also associated with a GABAergic fate and plays a crucial role in terminal differentiation of these neurons (Panganiban and Rubenstein, 2002; Stuhmer et al., 2002). Because we transplanted acutely purified NG2$^+$/EGFP$^+$ cells immediately after FACS®, we suggest that NG2$^+$/EGFP$^+$ cells possess intrinsic properties to generate GABAergic interneurons (Belachew et al., 2003).

The grafted NG2$^+$ cell–derived interneurons express the same antigenic profile and similar physiological properties of endogenous EGFP$^+$/GAD-67$^+$ interneurons found in CA1, CA3, and DG. This finding indicates that the grafted NG2$^+$ cells follow an existing endogenous developmental pathway that results in interneuron generation in the postnatal hippocampus, and recruitment of GABAergic interneurons in the hippocampus significantly contributes to early postnatal and possibly adult neurogenesis. In agreement with our results, a recent study demonstrated that A2B5$^+$, CNP gene-expressing progenitors isolated from the adult human white matter, generate GABAergic hippocampal neurons after transplantation into fetal rat brain (Nunes et al., 2003). These findings contribute to the changing view that a significant percentage of newly generated neurons in the postnatal and adult hippocampus are inhibitory interneurons, as demonstrated by Liu et al. (2003) for PV$^+$ GABAergic basket cells in the adult DG.

Our work suggests that postnatal newly-formed GABAergic interneurons derive from exogenous NG2$^+$ progenitors that migrate out of the SVZ into the hippocampus and most likely to other brain regions. It remains to be determined to what extent our data suggest that endogenous newborn post-

natal interneurons in the hippocampus may derive from NG2$^+$ SVZ progenitor cells, likely located in the posterior SVZ. This would raise the possibility that a subpopulation of type C–like progenitor cells from the SVZ are able to migrate backward and radially (Marshall and Goldman, 2002; Suzuki and Goldman, 2003), instead of following their classical route of tangential forward migration through the rostral migratory stream toward the olfactory bulb.

In conclusion, we have taken advantage of a transgenic mouse to define the cellular properties of CNP gene-expressing NG2$^+$ progenitors in the early postnatal and adult SVZ, and to demonstrate that they are a subpopulation of type C–like cells. Based on EGFP expression, we were also able to graft NG2$^+$ cells into the early postnatal brain and follow their developmental fate. We established that neurons generated from early postnatal NG2$^+$/EGFP$^+$ cells in vivo have the same cellular and functional phenotype of endogenous populations of hippocampal inhibitory interneurons. The possibility of obtaining a viable and highly purified population of endogenous neural progenitors and to direct the generation of GABAergic neurons from these precursors could have very important implications for future therapeutic approaches to epilepsy, stroke, and degenerative damage of the brain.

Materials and methods

CNP-EGFP transgenic mouse

Line C1 of the CNP-GFP transgenic mouse was used this work (Yuan et al., 2002). The same results were obtained with line D2. All animal procedures were performed according to Children's National Medical Center, Institutional Animal Care and Use Committee, and National Institutes of Health guidelines.

Isolation of NG2$^+$/EGFP$^+$ and NG2$^+$/EYFP$^+$ progenitor cells by FACS® and cell culture

Brains were dissected out from P2 and P40 CNP-EGFP mice or P2 β-actin-EYFP mice (Tg ActbEYFP; JAX Mice). Cells were dissociated (Belachew et al., 2003) and analyzed for light forward and side scatter using a FACStar plus instrument (Becton Dickinson). For double NG2/EGFP and LeX/EGFP FACS® analysis, cell suspensions were incubated with appropriate primary antibodies (see Immunocytochemistry), and then with R-PE– and PECy5-conjugated secondary antibodies (Caltag).

SVZ microdissection

SVZ areas were microdissected from 300-μm-thick coronal sections of P8 CNP-EGFP brains. Cells were dissociated (Belachew et al., 2003), seeded (20,000 cells per well) on coverslips, and processed for immunocytochemistry 2 h after plating.

Neurosphere preparation and differentiation

FACS®-purified NG2$^+$/EGFP$^+$ cells (P2) or E14.5 forebrain cells were seeded (10 cells/μl) on uncoated 24-well plates (BD Biosciences) and grown in SCM (StemCell Technologies Inc.) for 6 d in vitro (DIV) with daily addition of 20 ng/ml EGF. For E14.5 cultures, 10 ng/ml bFGF was also added. Primary neurosphere colonies (Reynolds and Weiss, 1992) were subcloned by mechanical dissociation and replating at 10 cells/μl in 24-well plates. Stem cell self-renewal was assessed after a further 6 DIV. For differentiation experiments, single neurosphere colonies were transferred to individual wells of a 24-well culture plate precoated with 0.1mg/ml polyornithine in serum-free medium + 1% FBS (GIBCO). Cells were processed by immunocytochemistry after 6 DIV (Belachew et al., 2003).

Immunocytochemistry

For cell sorting, cell suspensions were incubated with anti-NG2 antibody (1:1,000; Chemicon) or with antibody from the hybridoma clone MMA (for LeX antigen; 1:50; Becton Dickinson). For immunocytochemistry, cell cultures were processed (Yuan et al., 2002) with the following primary antibodies: anti-NeuN, antityrosine hydroxylase, anti–PSA-NCAM (all from Chemicon), anti-Ki67 (Novocastra), and anti-panDlx (obtained from G. Panganiban, University of Wisconsin Medical School, Madison, WI), all at 1:250. At a dilution of 1:500, TUJ1 (BabCo), anti-NG2, anti-GFAP (Sigma-Aldrich), anti–GAD-67, and anti–choline acetyltransferase (both from Chemicon). Anti-MMA mouse monoclonal (for LeX antigen; Becton Dickinson), antivesicular glutamate transporter 1 (Chemicon), and anti-EGFR (Santa Cruz Biotechnology, Inc.) were used at 1:50, 1:10,000, and 1:100, respectively.

RT-PCR

RNA was isolated from P8 FACS®-purified NG2$^+$/EGFP$^+$ cells and from neurospheres after 4 d in differentiation medium using Trizol® (Invitrogen). 1 μg RNA from each sample was reverse-transcribed using the SuperScript™ First-Strand cDNA Synthesis kit (Invitrogen). The mouse gene-specific primers used were obtained from Integrated DNA Technologies, Inc. Sequences were as follows: Mash1, sense 5′ AGC AGC TGC TGG ACG AGC A 3′, antisense 5′ CCT GCT TCC AAA GTC CAT TC 3′; Dlx2, sense 5′ GGC ACC AGT TCG TCT CCG GTC AA 3′, antisense 5′ CGC CGA AGT CCC AGG ATG CTG 3′; GAD-67, sense 5′ AAG GCA TGG CGG CTG TGC CCA AAC 3′, antisense 5′ ACC ACC CCA GGC AGC ATC CAC ATG 3′; GAD-65, sense 5′ CCA TTA CCC CAA TGA GCT TCT 3′, antisense 5′ CCC CAA GCA GCA TCC ACG T 3′; Actin, sense 5′ CGT GGG CCG CCC TAG GCA CCA 3′, antisense 5′ TTG GCC TTA GGG TTC AGG GGG 3′. Genes were amplified by denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 35 cycles. PCR products were resolved by 1.2% agarose gel electrophoresis and visualized under UV light.

Immunohistochemistry

Frozen 50-μm tissue sections were prepared as described previously (Yuan et al., 2002). Primary antibody dilutions were as follows: 1:500 for anti-NG2 antibody, anti-Ki67, anti-CNP (Sternberger Monoclonal Inc.), anti-MBP (Sternberger Monoclonal Inc.), anti-NeuN, anti-HuC/D (Molecular Probes), and anti-GFAP; 1:1,000 for anti–Mash-1 (from D. Anderson, California Institute of Technology, Pasadena, CA), anti-PV (Sigma-Aldrich), anti–GAD-67, anti–PSA-NCAM, antisomatostatin, antidoublecortin, and anticalretinin (all from Chemicon). Anti-GLAST (Chemicon) and anti-Olig2 (obtained from D. Rowitch, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA) were both used at a 1:5,000 dilution. Anti-BrdU and MMA antibodies (1:100) were purchased from Becton Dickinson.

BrdU administration and detection

Mice were injected intraperitoneally with a single dose of 50 μg/g of BrdU (Sigma-Aldrich) in sterile 0.9% NaCl solution and killed 2 h after the injection. Sections were incubated in 2 N HCl for 30 min at 37°C, immersed in 0.1 M Na$^+$-borate at pH 8.5, and stained with anti-BrdU and anti-NG2.

Microscopy and cell counting

A confocal laser-scanning microscope (model MRC 1024; Bio-Rad Laboratories) equipped with a krypton-argon laser and an inverted microscope (model IX-70; Olympus) was used to image localization of FITC (488-nm laser line excitation; 522/35 emission filter), Texas red (568-nm excitation; 605/32 emission filter) of Cy5 (647 excitation; 680/32 emission filter). Optical sections (z = 0.5 μm) of confocal epifluorescence images were sequentially acquired using a 40× (NA = 1.35), a 60× (NA = 1.40), or 100× oil objective (NA = 1.35) with LaserSharp v3.2 software (Bio-Rad Laboratories). Confocal Assistant 4.02 software was subsequently used to merge images. Merged images were processed in Photoshop 7.0 with minimal manipulations of contrast. For cell counting, NG2$^+$/EGFP$^+$ cells were counted in the SVZ and hippocampus. We consistently found that 100% of the NG2$^+$ cells were EGFP$^+$. The analysis of the SVZ was performed at different rostrocaudal levels of the lateral ventricle. An average of 15–20 sections were counted for the SVZ and hippocampus to obtain an estimate of the total number of NG2$^+$ cells. Percentages of cells expressing different antigens were estimated by scoring the number of cells double-labeled with the marker in question. In cultured cells, 2 coverslips/culture and 8–10 microscopic fields/coverslip were counted from three separate cultures. Statistical analysis was performed by paired t test.

Transplantation and analysis of grafted cells

FACS®-purified NG2$^+$/EGFP$^+$ or NG2$^+$/EYFP$^+$ donor cells were prepared from P2 CNP-EGFP or β-actin-EYFP transgenic mice. Immediately after FACS®, cells were stained with PKH26 (Sigma-Aldrich) and incubated with the diluent C (5:1,000) for 5 min at RT. Cells were washed with DME + 10% FCS and resuspended in DME at 20,000 cells/μl. As a negative control, NG2$^+$ cells were exposed to six freeze–thaw cycles before grafting.

Microinjection was performed under deep hypothermia, and 1–2 μl of the cell suspension was injected directly into the ventricles of wild-type *P2* FVB/NxCB6 pups. In some experiments, trypan blue (0.1%) was added to the cell suspension to confirm targeting of injections. The injection was performed with a glass capillary pipet (100–150 μm outer diameter with beveled tip) driven by a micromanipulator (Harvard Instruments). For the studies of migration and proliferation, mice were reared 24–72 h after injection and processed for immunohistochemistry. For anatomical and electrophysiological studies of the grafted cells, mice were killed 1–4 wk after the injection. Grafted $NG2^+$ cells were readily visible under confocal microscopy using a 488-nm laser-line excitation. An anti-GFP rabbit polyclonal antibody (IgG; 1:250; BD Biosciences) was used to confirm $NG2^+$ cell differentiation by immunohistochemistry. In three experiments, we quantified the percentage of transplanted $NG2^+/EYFP^+$ cells found in the hippocampus after 1 wk as $NeuN^+$ neurons. After injecting 20,000 $NG2^+/EYFP^+$ cells, we detected an average of 215.0 ± 9.1 $NeuN^+$ cells per hippocampus (total four brains counted), i.e., 1.07 ± 0.05% of the total injected cells, and 0.04 ± 0.002% NeuN-negative cells.

TUNEL assay

Sagittal sections (50 μm, $n = 6$ sections per animal) were prepared 6 wk after transplantation and were processed for TUNEL by using an apoptosis detection kit (Roche Bioscience) following the protocol from the kit.

Electrophysiology

Acutely isolated hippocampal slices (sagittal; 150–200 μM; Belachew et al., 2003) were prepared from P10–P15 mice that had received a ventricular injection of FACS®-sorted $NG2^+/EGFP^+$ cells. After a 1-h recovery, slices were perfused in a recording chamber with extracellular solution (Belachew et al., 2003). $EGFP^+$ cells in the stratum oriens and stratum radiatum of CA1 and CA3 were identified as described previously (Belachew et al., 2003). Due to the low levels of EGFP fluorescence, the InvestiGater integrating box (DAGE-MTI) was used to amplify the fluorescent signal detected by the CCD camera (model CCD-100; DAGE-MTI). Patch electrodes and intracellular solution were as described previously (Belachew et al., 2003). Action potentials and spontaneous AMPA-receptor currents were analyzed under current-clamp (current injection 30–180 pA steps; 1,000 ms duration) and voltage-clamp, respectively, as described previously (Belachew et al., 2003). All data were filtered at 2 kHz and digitized at 10–20 KhZ. Offline analysis was performed using Clampfit 8 (Axon Instruments, Inc.) and MiniAnalysis (Synaptosoft Inc.).

Online supplemental material

Fig. S1 shows that neurons derived from P2 $NG2^+/EGFP^+$ spheres give rise primarily to GABAergic neurons. Fig. S2 shows the presence of *Dlx2* and *Mash1* mRNA in FACS®-purified $NG2^+/EGFP^+$ cells. Fig. S3 shows the antigenic characterization of acutely dissociated P8 striatal-SVZ. Fig. S4 shows the cellular distribution and migration into the hippocampus of transplanted $NG2^+/EGFP^+$ cells at different times after transplantation. Fig. S5 shows the progenies derived from transplanted $NG2^+/EGFP^+$ cells in the subcortical white matter 3 wk after transplantation. Online supplemental material is available at http:www.jcb.org/cgi/content/full/jcb.200311141/DC1.

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