Development/Plasticity/Repair

Postnatal Generation of Neurons in the Ventrobasal Nucleus of the Rat Thalamus

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Most CNS systems, including the trigeminal–somatosensory system, develop via a hierarchical order (from the periphery and up the neuraxis). We tested the hypothesis that development of the trigeminal system can proceed via a nonhierarchical mechanism (i.e., that neuronogenesis can occur postnatally). Preweanling rats were perfused, and brain sections were stained with cresyl violet or immunolabeled with NeuN (for neuronal counts), or processed for acetylcholinesterase (AChE) activity or p75 immunoreactivity [to identify boundaries of the ventrobasal nucleus (VB)]. Neuronal number decreased during the first postnatal week but increased 2.5-fold over the next 3 weeks. To determine whether this remarkable rise resulted from the generation of new neurons, preweanlings were given injections of bromodeoxyuridine (BrdU) on postnatal day 6 (P6) or P21. BrdU-positive VB cells were apparent on both days. Cumulative BrdU labeling showed that the cell cycle was 17.3 h on P6. Moreover, Ki-67, a protein elaborated throughout the cell cycle, was expressed by 25.8–29.3% of all VB cells on P6–P15, falling to 7.7% by P21. BrdU-positive VB cells coexpressed neuronal markers: NeuN, HuC/D, microtubule-associated protein 2, and a dextran placed in the somatosensory cortex. Note that postnatal neuronal generation was also evident in other thalamic nuclei (e.g., the lateral geniculate nucleus). Thus, the developing VB experiences two periods of neuronal generation. Prenatal neuronogenesis is part of hierarchical trigeminal–somatosensory development. Postnatal nonhierarchical neuronogenesis is intrathalamic and matches changes in neuromodulatory systems (exemplified by AChE activity and p75) and the arrival of corticothalamic afferents.

Key words: **bromodeoxyuridine; lateral geniculate nucleus; neural stem cells; neurogenesis; proliferation; acetylcholinesterase (AchE)**

Introduction

Development of components of the trigeminal–somatosensory system proceeds in a hierarchical order (i.e., up the neuraxis). This order is evident in the sequence of neuronogenesis. In the rat, most second-order neurons in the principal sensory nucleus of the trigeminal nerve (PSN) and spinal trigeminal nuclei are generated between gestational day 12 (G12) and G14 (Nornes and Morita, 1979; Altman and Bayer, 1980; Miller and Muller, 1989). Third-order neurons in the ventrobasal nucleus of the thalamus (VB) are generated on G14 and G15 (Altman and Bayer, 1989), and fourth-order neurons in layer IV of the somatosensory cortex, the primary target of afferents from the VB, are born on G17 and G18 (Miller, 1988). Like neuronogenesis, death of postmigratory neurons proceeds hierarchically. It peaks in the PSN, VB, and cortex neonatally (Ashwell and Waite, 1991; Miller and Al-Ghoul, 1993; Miller, 1999), during the middle of the first postnatal week (Waite et al., 1992; Mai et al., 1998), and at the end of the first postnatal week (Finlay and Slattery, 1983; Ferrer et al., 1990; Miller, 1995; Miller and Kuhn, 1997), respectively.

Overlap in the timing within each stage of development of trigeminal–somatosensory components allows for feedforward passage of information (e.g., the thalamus affects cortical development via ingrowth of thalamocortical axons). One advantage of this hierarchical pattern is the matching of neuronal numbers among components of the system (Wetts and Herrup, 1983). That is, the number of VB neurons is matched to the number in the brainstem, and cortical neuronal numbers are matched to the number of VB neurons.

Interestingly, some systems (e.g., olfactory and hippocampal) have a nonhierarchical aspect to their development. In these structures, proliferating neural stem cells (NSCs) produce neurons that are continually integrated into the adult system (Schlessinger et al., 1975, 1978; Hinds and McNelly, 1977, 1981; Miller, 1995; Kempermann et al., 1996; Luskin, 1998; Kornack and Rakic, 1999, 2001a). Apparently, these new neurons are critical for maintaining neural plasticity. The development of the visual pathway also exhibits a nonhierarchical component (Fitzgibbon, 2006). The perigeniculate nucleus matures before its afferent sources and its target.

Neuromodulatory systems are expressed during postnatal VB

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development. These include serotonin (Fujimiya et al., 1986; Bennett-Clarke et al., 1991; Bruning and Liangos, 1997), glutamate (Spreafico et al., 1994), acetylcholine (Kristt, 1983; Broide et al., 1995, 1996), and neurotrophins (Crockett et al., 2000; Vitalis et al., 2002). The latter two are of particular interest because they are important for the development of NSCs in the adult (Li et al., 2001; Hosomi et al., 2003; Cooper-Kuhn et al., 2004; Giuliani et al., 2004). They keep NSCs cycling and promote lineage definition. Potentially, such neuromodulatory systems support cycling or newly differentiating NSCs in the postnatal VB.

Table 1. Antibodies used in immunohistochemical studies

Cell type	Marker	Dilution	Source	Host
Proliferating cells	BrdU	1:30	347580; BD Biosciences (Franklin Lakes, NJ)	Mouse
	Ki-67	1:150	RM9106-50; Lab Vision (Fremont, CA)	Rabbit
NSCs	Nestin	1:200	MAB 353; Millipore (Bedford, MA)	Mouse
	Vimentin	1:500	V6630; Sigma	Mouse
Immature neurons	Tuj1	1:1000	MMS-435-P; CRP (Berkeley, CA)	Mouse
	Doublecortin	1:400	AB 5910; Millipore	Guinea pig
Mature neurons	NeuN	1:50	MAB 377; Millipore	Mouse
	HuC/D	1:150	A21271; Invitrogen	Mouse
	MAP2	1:500	M4403; Sigma	Mouse
Glia	GFAP	1:1000	G3893; Sigma	Mouse
	Iba1	1:300	100369-764; VWR Scientific (West Chester, PA)	Rabbit
VB subnuclear borders	p75	1:200	G323A; Promega (Madison, WI)	Mouse
	pan–trk	1:1000	sc-139; Santa Cruz Biotechnology (Santa Cruz, CA)	Rabbit

The present study tested the hypotheses that the trigeminal–somatosensory system uses a nonhierarchical manner of development and, in contrast to the contemporary understanding of thalamic development, that the VB experiences postnatal neuronogenesis.

Materials and Methods

Animals

Pregnant Long–Evans rats were obtained from Harlan Sprague Dawley (Indianapolis, IN) on G4. The day that a sperm-positive plug was first seen was defined as G1. Animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility at the Syracuse Veterans Affairs Medical Center (VAMC). All procedures were approved by the Institutional Animal Care and Use Committee of the VAMC and the Committee on the Humane Animal Use at Upstate Medical University.

At birth, litters were culled to 10. Offspring were weaned on postnatal day 21 (P21). To ensure a common scale for the developmental timeline, G22 was designated as P0 regardless of the actual day of birth. One animal from each of five litters was perfused transcardially with 4.0% paraformaldehyde in 0.10 M phosphate buffer (PB), pH 7.4, at various times between P0 and P21. Each brain was removed, postfixed in buffered paraformaldehyde for 4 h, cryoprotected, and stored in 30% sucrose in PB.

Cell proliferation studies

Prenatal neurogenesis. Four dams were given injections of [^{3}H]thymidine ([^{3}H]dT) on each day during the period between G13 and G17 inclusive. [^{3}H]dT was used for these studies because it is ideal for use in birthdating studies wherein it is necessary to discriminate first-generation neurons from succeeding generations (Miller, 1985, 1988). An offspring from each litter was killed on P21. These animals were used to verify prenatal times of generation of VB neurons.

Postnatal neurogenesis. One animal from each of three litters was given an injection of the thymidine analog bromodeoxyuridine (BrdU; 25 mg/kg; Sigma, St. Louis, MO) on P6 or P21. BrdU was appropriate for these studies because it lent itself to short-term studies of cell proliferation and for fluorescence labeling studies (Miller and Nowakowski, 1988). These animals were perfused at various times (2–6 h or 3 or 15 d) after the injection. Sections were collected as described below. Immunohistochemistry was used to localize dual expression of BrdU and one of various cell type-dependent and developmental state-dependent markers (Table 1).

One animal from each of three litters that received an injection of BrdU on P6 also received an intracerebral injection of biotinylated dextran (0.020 μl of 1.0 mg/100 μl saline; D1956; Invitrogen, San Diego, CA) on P24. Animals were perfused 1 week after dextran injection, and brains were processed for double immunofluorescence.

Three animals from two different litters were used to assess cell cycle kinetics. These animals received injections of BrdU (25 mg/kg) on P6. Thirty minutes later, one animal from each litter was perfused, and the brain was collected. Two hours after the first injection, the remaining four siblings received a second injection of BrdU. Once again, 30 min after the second injection, one animal per litter was killed and the brain was harvested. The remaining two rats received a third injection of BrdU 2 h after the second and were perfused 30 min later. Brains from all six animals were processed for BrdU immunolabeling.

Tissue preparation

Using a Leica (Nussloch, Germany) CM1900 cryostat, sets of 12-μm-thick sections of the forebrain were collected from each brain. The periodicity of the series varied among the different ages: at P0 and P3, every 10th section was taken; at P6, every 15th section was taken; and at P12 and later, every 20th section was collected. One set was stained with cresyl violet for counting neurons. A second set was stained to localize acetylcholinesterase (AChE) activity for delineation of the boundaries of the developing VB. Third, fourth, fifth, and sixth sets of sections were immunolabeled for NeuN or HuC/D (markers for mature neurons), Ki-67 (a marker for cells in all phases of the cell cycle), p75 (the low-affinity neurotrophin receptor), and pan–trk (the high-affinity neurotrophin receptors). Other sets were used in the double-labeling studies (see below).

AChE histochemistry

Sections were incubated in the stain solution containing 0.50 mg/ml acetylthiocholine iodide, 0.10 M sodium acetate, 0.10 M acetic acid, 0.10 M sodium citrate, 30 mM copper sulfate, 4.0 mM tetraisopropylpyrophosoamide, and 5.0 mM potassium ferricyanide, pH 5.5 (El-Badawi and Schenk, 1967; Mooney and Miller, 1999). This incubation was performed in the dark at 37°C for 3 h. Subsequently, tissue was dehydrated through graded alcohols and cleared before coverslipping. Differential thalamic AChE staining was used to delineate the borders of the VB, specifically the medial and lateral segments of the ventral posterior nucleus (VPm and VPl, respectively) (Kristt, 1983). The VB was identified using the criteria described by Paxinos and Watson (1982).

Immunohistochemistry

Sections were immunolabeled for markers identifying stage-specific cells. Antigen retrieval was performed by heating sections in 0.01 M citrate buffer, pH 6.0. Nonspecific antibody binding was quenched by a wash in PB containing 0.10% Triton X-100 (TPB), 4.0% goat serum, and 1.0% bovine serum albumin. Sections were incubated for 1 h with a primary antibody directed against p75, pan–trk, Ki-67, BrdU, NeuN, or HuC/D (Table 1). Unbound antibody was removed by washing in 5.0% nonfat milk in PB (mPB). Biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) was applied for 1 h. Sections were rinsed in mPB and incubated with a Vectastain Elite kit (Vector Laboratories). Immunoreactivity was visualized by incubating the sections in 3,3′-diaminobenzidine (Vector Laboratories) in the presence of hydrogen peroxide. Sections were counterstained with cresyl violet. All steps were performed at room temperature. Nonspecific labeling was examined by omitting either the primary or the secondary antibody.

To determine the identity of the dividing cells, dual immunofluorescence was used to colocalize BrdU with markers for immature and ma-

ture neural cells. Nonspecific antibody labeling was blocked by incubation in TPB containing 4.0% goat serum and 1.0% bovine serum albumin. All sections were treated with 0.0070N NaOH to produce single-stranded DNA and incubated in an antibody to BrdU on single-stranded DNA. The BrdU immunolabel was visualized with a secondary antibody conjugated with fluorescein. Subsequently, the sections were processed with a second primary antibody for a cell type-specific marker (Table 1) and a rhodamine-tagged secondary antibody. Sections were also labeled with bis-benzamide (1:400 in PB; Hoescht 33341, B2261; Sigma), a DNA marker, to label all nuclei and thus allow determination of a cytoarchitectonic area. For the double-labeling studies with the dextran, the dextran was conjugated with fluorescein isothiocyanate (1:200 in PB; 016-070-084; Jackson ImmunoResearch, West Grove PA) and the BrdU with a primary antibody and a secondary antibody conjugated to Texas Red strepavidin (1:200 in PB; Fl6000; Vector Laboratories). Fluorescent immunolabeling was visualized with a Zeiss (Thornwood, NY) Axioplan 2E Imaging fitted with appropriate filters and structured illumination (Zeiss Apotome) or with a Leica microscope outfitted with a confocal laser (Bio-Rad, Hercules, CA).

Tritiated-thymidine autoradiography

The brains of the offspring of rats given injections of [^{3}H]dT were removed, embedded in paraffin, and cut into a series of 10 μm sections. The sections were deparaffinized, dehydrated, cleared, rehydrated, and dipped in NTB2 Nuclear Track Emulsion (Kodak, Rochester, NY). The autoradiographs were developed for 25–28 d, fixed, cleared, dehydrated, counterstained with cresyl violet, and coverslipped.

Data analyses

Labeling frequencies. The labeling frequency for VB cells expressing BrdU or Ki-67 was determined in brains collected 6 h after injection of BrdU on P6 or P21 using the Bioquant Image Analysis system (R&M Biometrics, Nashville, TN). Counts were made of the total number of neurons within a square box (100 × 100 μm) and the number of cells immunopositive for BrdU or Ki-67. The labeling frequency was calculated as the number of immunopositive cells divided by the total number of neurons counted.

Autoradiographic analyses. Cells were identified as being heavily or lightly labeled depending on the relative number of grains overlying them (Miller 1985, 1988). Heavily labeled cells had greater than half the maximal number of grains over their nuclei. These cells were presumed to have been in their final mitosis when the [^{3}H]dT was injected. Lightly labeled neurons had less than half the number of grains as that over the most heavily labeled neurons and more than double background. Conservatively, background was two grains per nucleus (i.e., two grains per 50 μm^2). Cells were lightly labeled (1) if they underwent more than one mitotic division subsequent to the [^{3}H]dT injection or (2) the somata of a heavily labeled neuron was too deep in the section to be detected as heavily labeled (e.g., the crown of the nucleus only broached the upper 2 μm of the section, the zone that affects the overlying emulsion).

Stereological methods. The boundaries of the VB were determined in the AChE- and cresyl violet-stained sections using the Bioquant Image Analysis system. Each image was projected to a monitor, and the borders of the VB and its subnuclei (VPm and VPl) were traced. Cavalieri's estimator of volume was used to calculate the volume (V_T) by the following formula:

$$V_T = \Sigma a_i \times s_f,$$

where a_i is the cross-sectional area of the thalamic nucleus on the ith profile and s_f is the mean distance between sections (section thickness multiplied by the inverse of the periodicity of sections in the series).

A corrected number of neurons (N_{corr}) was estimated using Smolen's correction to Floderus's formula (Smolen et al., 1983; Miller and Muller, 1989; Mooney and Miller, 1999). The formula was as follows:

$$N_{corr} = (n \times t)/(t + \bar{D} - 2k),$$

where n is the number of neurons counted in sampled areas (the counting boxes), t is the section thickness, $\bar{D}$ is the mean maximal diameter, and k is the diameter of the smallest recognizable cap of a cut nucleus.

The density of neurons (N_V; also called a cell packing density) was determined from the following formula:

$$N_V = N_{corr}/(\#_{boxes} \times V_{box}),$$

where V_{box} is the volume of one counting box (area of counting frame by section thickness).

The estimated total number of neurons in the VB, VPm, or VPl (N_T) was calculated as the product of the total volume of the VB (or a subnucleus) and the cell packing density:

$$N_T = N_V \times V_T.$$

Cell cycle kinetics. The total length of the cell cycle (T_C) was determined using a cumulative BrdU labeling technique (Nowakowski et al., 1989; Siegenthaler and Miller, 2005). BrdU incorporates into DNA of cells passing through the S phase, and over time, all cycling cells take up the label. The timing of the incorporation (i.e., the temporal change in the number of cells that take up BrdU) depends on the cell cycle kinetics. The data for the numbers of cells that incorporated the BrdU shortly after one, two, or three injections of BrdU were used to calculate the T_C. Counts did not endeavor to differentiate among cell types (e.g., NSCs, neurons, or glia); however, endothelial cells were eliminated from the tallies. The labeling index (LI) was determined for BrdU-immunolabeled sections from animals treated with multiple BrdU injections. The total number of cells and the number of BrdU-positive cells in a 100 × 100 μm counting box were counted. The LI was calculated as the number of BrdU-immunolabeled cells divided by the sum of the numbers of labeled and unlabeled cells.

Conventionally, the proportion of cells that are actively cycling, the GF, is determined by the cumulative labeling method that relies on the saturation of the cycling population with BrdU via a series of periodic injections (Nowakowski et al., 1989). To use fewer animals and to examine the change in the growth fraction (GF) over time, in the present study the GF was determined using Ki-67 labeling. Empirical data show that the number of cells expressing Ki-67 is the same as the number of cells that are cumulative labeled with BrdU (Siegenthaler and Miller, 2005). Accordingly, the LI for Ki-67 was determined at four ages: P6, P12, P15, and P21.

Statistical analyses. Only one rat per litter was used in generating any particular datum. The means (SEMs) were calculated for V_T, N_v, N_T, T_C, and GF and for the proportion of neurons generated on a particular gestational day. A one-way ANOVA was used to examine the effect of age. In situations of statistical significance, specific *post hoc* comparisons were performed using *t* tests.

Results

Appearance of the developing VB

The VB was discernible in cresyl violet-stained sections of the preweanling brains (Fig. 1). Throughout the early postnatal period, the VB was nestled in the crook of the internal capsule and the zona incerta. During the first postnatal week, the two subnuclei, the VPm and VPl, were difficult to distinguish in the cresyl violet; however, by the second postnatal week, the two subnuclei had distinctive appearances. The neuronal packing density was not significantly different between the subnuclei; however, between P6 and P21, the cross-sectional area of profiles of both the nuclei and somata of neurons in the VPm was ~30% greater than those of neurons in the VPl.

The pattern of AChE staining changed over time. In the neonate, the VB was richly AChE positive. In fact, at this time, the VB was more intensely stained than any of the adjacent thalamic nuclei. Moreover, as early as P0, the VPl was darker that the VPm. This differential was detectable during the first postnatal week. By age P12, the VPm had noticeably lost its staining intensity, and by P21, the staining pattern was characteristic of the mature VB (i.e., both subnuclei were paler than surrounding thalamic nuclei) (cf. Kristt, 1983; Mooney and Miller, 1999).

The temporal change in the distribution of p75 immunolabeling was similar to that of AChE activity (Fig. 1). In the neonate, p75 was richly expressed in the VPm. Staining intensity decreased with age, and by P21 there was no detectable p75 immunoreactivity in the VB. This spatiotemporal pattern of staining agrees with that described previously (Crockett et al., 2000). In contrast to the pattern of AChE staining, p75 immunoreactivity in the young pup was rich in the VPm and virtually absent in the VPl. Labeling with the pan–trk antibody, which labels all three high-affinity neurotrophin receptors (trkA, trkB, and trkC), showed the same spatiotemporal pattern as seen with p75. Trk expression was high in the young animal and attenuated by the third postnatal week (data not shown).

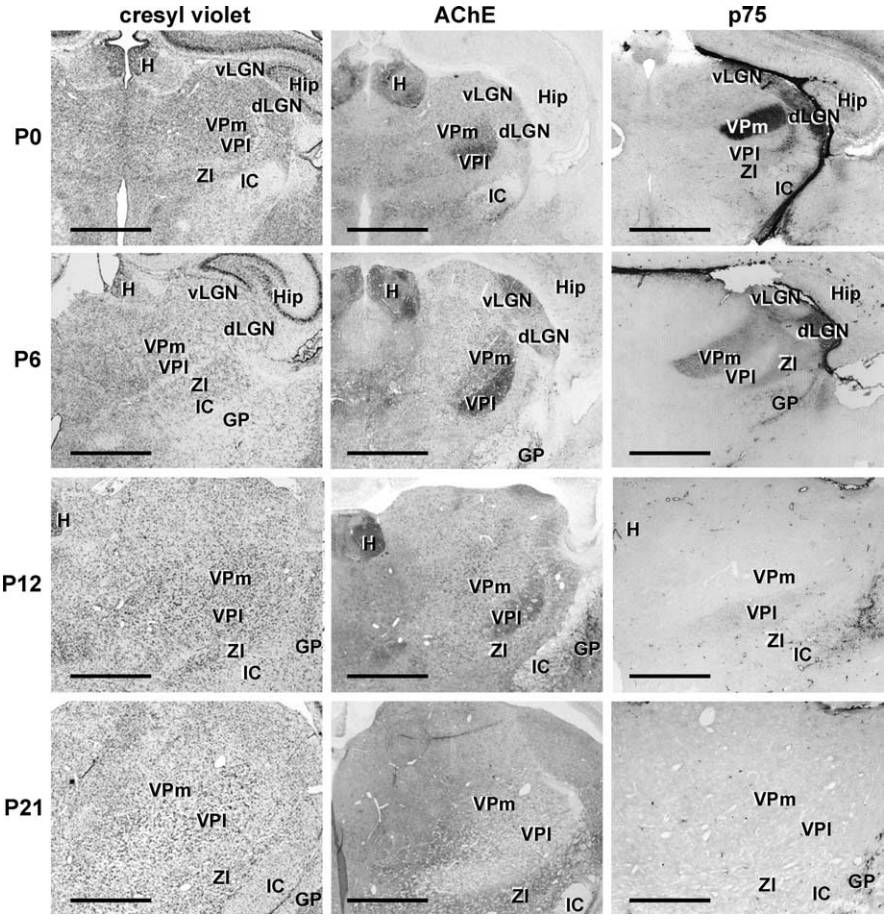

Figure 1. Appearance of the VB. The VB was identifiable by a distinctive pattern of AChE activity and p75 immunolabeling. Both AChE and p75 were highly expressed in the VPl and the VPm, respectively, during the first postnatal week. Labeling waned during the second postnatal week and was absent by P21. p75 expression was also evident in the border neurons of the globus pallidus (GP) and the LGN, particularly the dorsal subnucleus (dLGN). The latter labeling fell temporally, as did the labeling in the VB. Cresyl violet-stained sections were used for stereological analysis. H, Habenula; Hip, hippocampal formation; IC, internal capsule; vLGN, ventral LGN; ZI, zona incerta. Scale bars, 1.0 mm.

[^{3}H]dT labeling of prenatally generated neurons

Neurons in the two subnuclei of the VB were heavily labeled by injections of [^{3}H]dT on either G14 or G15 (Fig. 2). Heavily labeled neurons were seen in both the VPm and VPl after [^{3}H]dT injection on G14 or G15. Similar numbers of neurons were born on each day. No heavily labeled neurons were evident in the VB after an injection on G13, G16, or G17 (data not shown).

Numbers of neurons in the VB

The total number of neurons in the VB was calculated as the product of the volume of the VB and the mean cell packing density. The total volume of the VB increased 10-fold during the first postnatal month ($F_{(5,20)} = 80.427$; $p < 0.001$) (Fig. 3). The volume of each subnucleus also increased over this time. Moreover, during the first three postnatal weeks, the VPm was consistently (1.41- to 1.72-fold) and significantly ($p < 0.05$) larger than the VPl.

Concomitant with the increase in the VB volume, there was a progressive decrease in the neuronal packing density with age ($F_{(5,20)} = 147.22$; $p < 0.001$). The biggest decrease occurred during the first postnatal week, and the density evident on P12 was not significantly different from that in 21- and 30-d-old rats. No differences between the neuronal packing density in the VPm and VPl were detected at any age examined.

The number of neurons in the VB was determined by calculating the product of the volume and neuronal packing density. Accordingly, there was a significant effect of age ($F_{(3,12)} = 12.410$; $p < 0.001$) on the total number of neurons in the VB. *Post hoc* analyses showed that the total number of neurons significantly ($p < 0.001$) fell between P0 and P3. Surprisingly, the number of neurons increased significantly ($p < 0.001$) between P3 and P12 and then again between P12 and P21. The number of neurons fell significantly ($p < 0.05$) between P21 and P30 [the latter data were described in a previous study (Mooney and Miller, 1999)].

To verify that only neurons were included in the analyses of the cresyl violet-stained sections, sections immunolabeled with an anti-NeuN antibody were examined. The numbers of VB cells that were NeuN positive on P12 and on P21 were not significantly different from numbers determined using sections stained with cresyl violet.

The change in the number of neurons in the two subnuclei was determined. As with the overall numbers of VB neurons, the numbers of neurons in the VPm and VPl rose during the first three postnatal weeks. Furthermore, over the times examined, the VPm comprised 1.61-fold more neurons than the VPl.

Postnatal generation of neurons in the VB

BrdU labeling

To address the possibility that neuronal generation occurred in the VB during the postnatal period, animals received injections of BrdU on P6 or P21. Six hours after injecting the BrdU, 12.5% of cells in the VB were labeled by an injection on P6 (Fig. 4). In contrast, only 1.5% of VB cells incorporated BrdU that was administered on P21. Animals given injections on P6 exhibited BrdU-positive cells in the VB as early as 30 min after injection, indicating that the cell proliferation occurred within the VB and that the cells did not migrate from the ventricular zone (VZ) of the third ventricle.

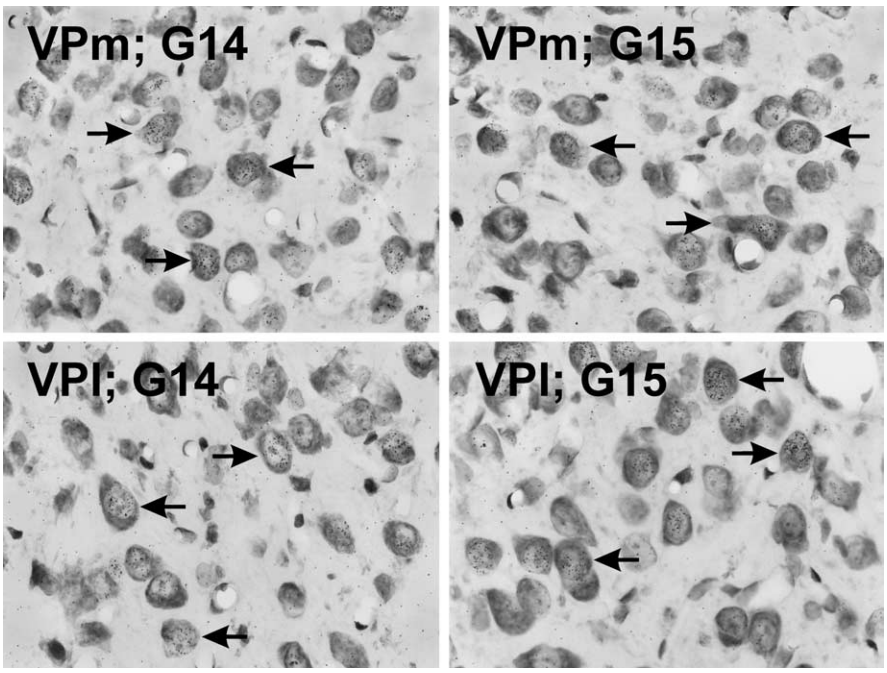

Figure 2. Prenatal neuronogenesis. Mature offspring of pregnant dams that received injections of [^{3}H]dT on G14 (left) or G15 (right) had many heavily labeled neurons (arrows) in the VB. Top, VPm; bottom, VPl. Scale bars, 50 μm.

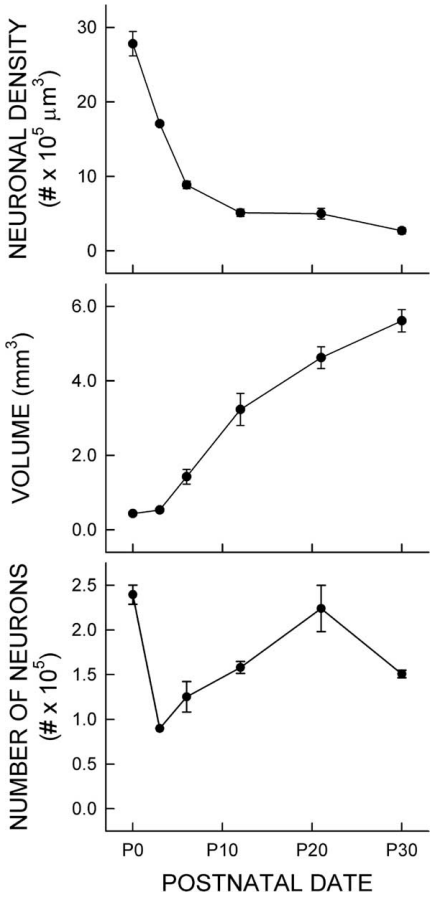

Figure 3. Morphometry of the VB. Three parameters pertaining to the development of the VB were quantified. Top, The neuronal packing density was determined stereologically. Middle, Cavalieri's estimator of volume was used to determine the total volume of the VB. Bottom, The product of these values was the total number of neurons. Means are based on five animals per time point. Error bars indicate SEM.

Cell cycle kinetics

The combination of cumulative BrdU and Ki-67 labeling allowed for determination of the cell cycle kinetics (Fig. 5). During the 6.5 h after the first injection of BrdU, the number of BrdU-positive cells increased. By fitting these data to a linear regression ($r = 0.9936$; $p = 0.0064$), the T_C was estimated to be 17.3 $\pm$ 0.9 h. This was similar to that determined for cells in the cortical VZ on G21 (Miller and Nowakowski, 1991). The LI for Ki-67 (i.e., the GF) was stable between P6 and P12. The mean GF was 27.4 $\pm$ 0.9%. By P21, the LI dropped significantly ($p < 0.01$) to 7.7%, indicating a reduction in the proliferative population.

Identity of proliferating cells

To determine the identity/fate of BrdU-positive cells, brains were examined 2, 6, or 15 d after injection (i.e., on P8, P12, or P21). On P8, BrdU mostly colocalized with markers for NSCs (vimentin or nestin) or immature neurons (Tuj1) (Fig. 6). By P12, BrdU colocalized with mature neuron-specific markers NeuN, HuC/D (Fig. 7 and supplemental Figs. 1, 2, available at www.jneurosci.org as supplemental material), or MAP2 (microtubule-associated protein 2; data not shown). It is noteworthy that the NeuN and HuC/D immunostaining were expressed in the nuclei and perikarya (Mullen et al., 1992; Wolf et al., 1996). (n.b. The balance of nuclear/perikaryal NeuN immunolabeling was tipped by the pH of the antigen-retrieval buffer.) Double labeling was also evident in pups receiving injections of BrdU on P6 and with a biotinylated dextran placed in somatosensory cortex on P24 (Fig. 6 and supplemental Fig. 3, available at www.jneurosci.org as supplemental material). The VB of these animals exhibited a subset of BrdU-positive cells that were also dextran positive. This indicated that the neurons generated postnatally (1) projected to the cortex and (2) these projection neurons survived for at least 3 weeks. Some cells exhibited BrdU and GFAP immunolabeling or BrdU and Iba1 immunolabeling.

Cerebral cortex and hippocampus were examined for comparative purposes. BrdU-positive cells in neocortex did not coexpress NeuN, but BrdU-positive/GFAP-positive cells were common (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). The implication is that early postnatal neurogenesis in cortex leads to the production of glia and not neurons. In contrast, double NeuN–BrdU labeling was evident in the hippocampal formation.

Lateral geniculate nucleus

It is noteworthy that the VB was not unique among thalamic nuclei insofar as its cytoarchitectural and chemoarchitectural development. For example, the lateral geniculate nucleus (LGN) followed a parallel developmental course to that described for the VB. The spatiotemporal pattern of both AChE activity and p75 immunoreactivity in the dorsal and ventral LGN was identical to that for the VPm and VPl, respectively (Fig. 1). Moreover, BrdU- and Ki-67-positive cells were common in the LGN throughout the period from P3 to P21 (data not shown). Double labeling with

BrdU and a neuronal marker was also identified in the LGN 6 d after the BrdU was administered on P6 (data not shown).

Discussion

Periods of VB neuronogenesis

VB neurons are generated during two periods. The first period occurs prenatally, on G14 and G15 (Altman and Bayer, 1989). The second period occurs during the preweaning period, between P3 and P21. Over this time, neuronal number increases 2.5-fold.

That VB neurons are generated postnatally is supported by two independent sets of data: (1) stereological determination of total neuronal number traces a longitudinal increase in the number of neurons during the second and third postnatal weeks; and (2) VB neurons incorporate BrdU administered postnatally. It is important to note that the BrdU is incorporated into cycling cells (as opposed to cells repairing their DNA) as evidenced by colabeling of BrdU with nestin and Ki-67 (Gerdes et al., 1983, 1984; Scholzen and Gerdes, 2000; Siegenthaler and Miller, 2005).

Discovering a second period of neuronogenesis is surprising. Although dividing cells in the postnatal VB have been demonstrated (Altman and Das, 1966), the identity of these cells has remained unknown and presumed to be glia. The present study shows that many cells cycling in the postnatal VB become neurons. Indeed, colocalization of a dextran transported retrogradely from neocortex with BrdU in thalamic neurons shows that postnatally generated thalamic neurons not only survive but they integrate into the system.

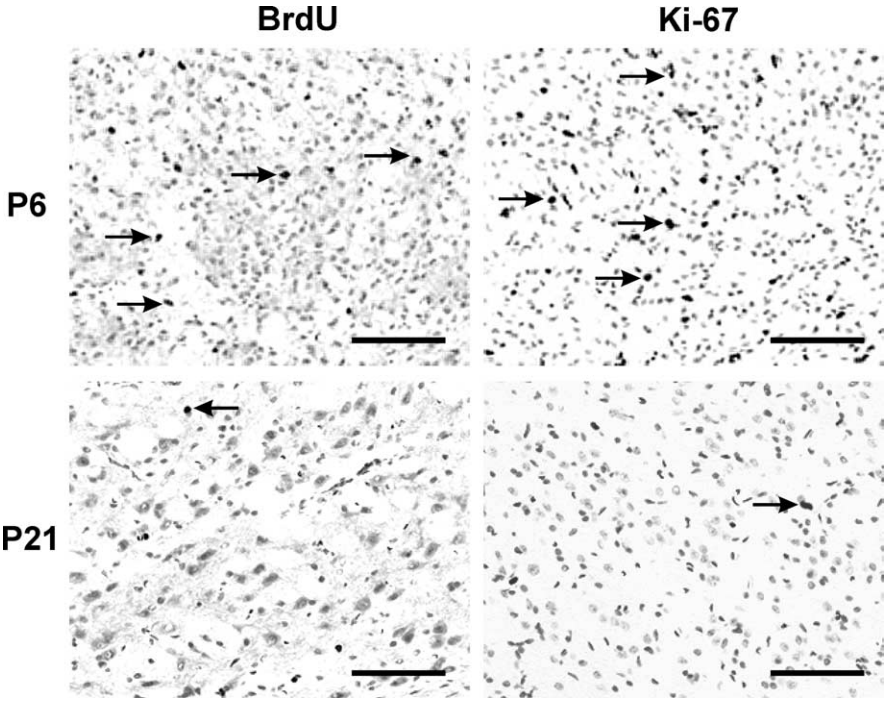

Figure 4. Evidence that cells in the VB are cycling. BrdU-positive cells are evident in the VB of an animal given an injection of BrdU on P6 and killed 6 h later. In contrast, animals receiving injections of BrdU on P21 have few labeled cells. Likewise, the expression of Ki-67 immunoreactivity parallels the pattern of BrdU labeling. Arrows identify labeled cells. Scale bars, 50 μm.

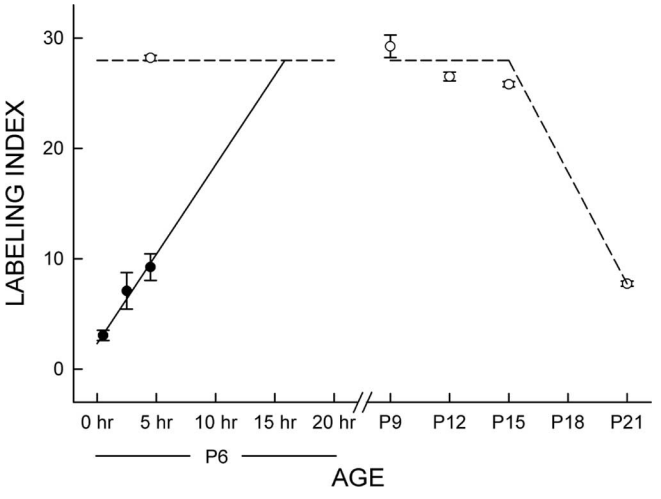

Figure 5. Cell cycle kinetics for postnatal proliferation in the VB. BrdU incorporation among cycling cells increases after a series of injections on P6 (solid symbols and line). This is evident by an increase in the mean LI (SEMs). A regression line was fitted to the primary data, the slope (m) of which was the dividend of the GF and the total length of the cell cycle (T_C): $m = \mathrm{GF}/T_C$. The GF was determined as the percentage of cells that was Ki-67 immunoreactive (open circles and dashed lines). This LI was measured at five times: P6, P9, P12, P15, and P21. It remained at a steady amount until the end of the third postnatal week when it fell significantly ($p < 0.01$).

Source of postnatal neuronogenesis

In contrast to the prenatal generation of VB neurons that occurs in the diencephalic VZ, postnatal neuronogenesis occurs in the VB per se. Evidence of BrdU-positive cells in the VB 30 min after injection shows that cells divide *in situ* rather than being generated elsewhere (e.g., the VZ) and migrating into the VB. After all, the cells that incorporate the BrdU are in S phase, and they must complete the cycle (i.e., pass through G_2, M, and the beginning of G_1) before they can migrate. Furthermore, radial migration of prosencephalic neurons in the rat occurs at a rate of 5–6 μm per hour (Miller, 1999; Siegenthaler and Miller, 2004), and if the cells are born in the diencephalic VZ, they must travel a distance of 600 μm or more. Neither the passage of cells through the remaining cell cycle nor their migration can be accomplished in 30 min, much less the sum of both processes. Therefore, the postnatally generated neurons must have originated in the VB.

Using the cell cycle kinetic data (Fig. 5) and the change in cell number (Fig. 3), the number of cells produced daily can be estimated. Based on a mathematical model (Miller, 2003), it is estimated that 51,300 cells (i.e., neurons and glia) per day are produced in the VB. Even allowing for gliogenesis and the death of a fraction of these cells, this production more than explains the documented addition of 7500 neurons per day.

Postnatal neuronal generation beyond the VB

Postnatal neural generation is not confined to the VB. BrdU- and Ki-67-positive cells are present in other structures, notably the hippocampus, olfactory system, neocortex, and lateral geniculate nuclei. In essence, the hippocampal formation serves as a positive control, and the neocortex serves as a negative control.

Postnatal neuronogenesis occurs in nonthalamic parts of the CNS. Granule neurons of the dentate gyrus are generated in the subgranular (intrahilar) zone in the early postnatal period and

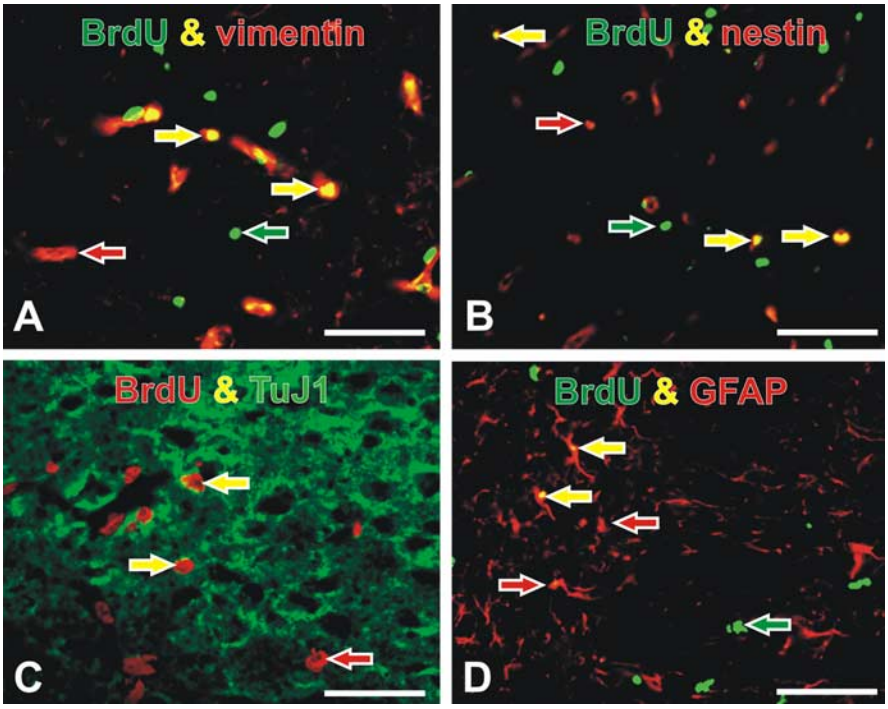

Figure 6. Phenotype of immature cells. Animals were given injections of BrdU on P6 and killed on P8. ***A***, ***B***, Cells that were singly labeled with BrdU or vimentin/nestin are indicated by red and green arrows, respectively. Some BrdU-labeled cells were vimentin or nestin positive (yellow arrows). ***C***, A subset of BrdU-positive cells were TuJ1 positive (yellow arrows). ***D***. Some BrdU-positive cells expressed the glia-specific marker GFAP (yellow arrows). Scale bars, 50 μm.

throughout life (Schlessinger et al., 1975, 1978; Miller 1995; Kempermann et al., 1996; Kornack and Rakic, 1999). These neurons are generated in the region proximal to the granule cell layer, but they must migrate to the positions that they ultimately occupy.

There is consensus that new olfactory bulb neurons are generated postnatally (Hinds and McNelly, 1977, 1981; Luskin, 1998; Kornack and Rakic, 2001a). These neurons are generated in a site of origin (the anterior subventricular zone) distant from their eventual residence, and the young neurons must traverse a great distance via the rostral migratory stream to get to the bulb.

Neurogenesis occurs in neocortex postnatally. It is agreed that glia are generated (Levison, 2006). Although controversial, it has also been argued that neocortical neurons are also produced postnatally. Some studies show that adult cortical cells incorporate a DNA precursor ([^{3}H]dT or BrdU) and that the ultrastructure (Kaplan and Hinds, 1977, 1980) or protein profile (Gould et al., 2001) indicate that some of these cells become neurons. This situation is further complicated because some evidence shows that cortical neuronogenesis occurs *in situ* (Kaplan and Hinds, 1977, 1980), whereas other data show that these new neurons are born in the neocortical proliferative zones and migrate to neocortex (Gould et al., 2001). Others disagree with the interpretation of these data arguing that incorporation of [^{3}H]dT or BrdU is evidence of DNA synthesis that is not associated with cell division (Kornak and Rakic, 2001b; Rakic, 2002). In the case of the VB, this criticism is addressed by finding BrdU and Ki-67 colabeling in VB cells.

Factors influencing postnatal neuronogenesis

Perhaps the most interesting question to ponder is why the number of neurons drops neonatally and then rises again. The initial drop concurs with the transient appearance of pyknotic cells (Waite et al., 1992; Mai et al., 1998) and a change in the balance between expression of the anti-apoptotic bcl-2 and the pro-apoptotic bax (Mooney and Miller, 2000). Therefore, this drop can be attributed to naturally occurring neuronal death (NOND). This death is consistent with a hierarchical sequence in that it follows the death occurring in the PSN (Ashwell and Waite, 1991; Miller and Al-Ghoul, 1993; Miller, 1999) and precedes the NOND in the cortex (Finlay and Slattery, 1983; Ferrer et al., 1990; Miller, 1995; Miller and Kuhn, 1997).

Immature neurons compete for trophic substances that are in limited supply; some fail to find sufficient support and die (Oppenheim, 1991). For VB cells, trophic substances likely come from somatosensory cortex. Survival of neonatal thalamic neurons *in vitro* is enhanced by cortically conditioned medium (Lotto et al., 1997; Asavaritikrai et al., 2003). One vital trophic substance in CNS-conditioned medium responsible for the maintenance of thalamic neurons is brain-derived neurotrophic factor (BDNF). Four independent sets of data imply that transported cortex-derived BDNF plays a role in the survival of VB neurons: (1) BDNF mRNA is undetectable in the VB between G15.5 and P15, and VB neurons are BDNF responsive (Baquet et al., 2004); (2) the thalamus of the preweanling expresses p75 and trk during the period of postnatal neuronogenesis, and this expression wanes by P21 when this production is virtually complete; (3) inactivation of trkB, the high-affinity receptor for BDNF, results in increased cell death in VB (Vitalis et al., 2002); and (4) conversely, injection of BDNF into cortex on P2 enhances the survival of thalamic neurons (Lotto et al., 2001).

As discussed above, the rise in the number of VB neurons during the second and third postnatal weeks results from postnatal neuronogenesis. Growth factors such as BDNF appear to be involved in this generation. Infusion of BDNF into the lateral ventricle of adult rats induces cell generation in numerous brain areas including thalamus (Pencea et al., 2001). This is consistent with evidence of a population of BDNF-responsive quiescent stem cells in mature thalamus. Conceivably, the transiently high expression of BDNF in cortex in the second and third postnatal weeks (Climent et al., 2002) triggers the second wave of proliferation seen in the postnatal VB. It is unclear whether the BDNF is transported through corticothalamic or thalamocortical pathways or both, although the data cited above strongly implicate corticothalamic projections. After all, neurotrophins can act through anterograde (Pitts and Miller, 1995, 2000; Wahle et al., 2003) and retrograde (DiStefano et al., 1992; Crockett et al., 2000; Wahle et al., 2003) mechanisms. The end of the second wave of neuronogenesis is punctuated by the loss of the neurotrophin receptors, both p75 and trk, during the third postnatal week.

Neurotrophins are not the only important molecules regulating VB development. For example, interactions between the VB and somatosensory cortex depend on transient serotonin transporter and AChE-expressing systems (Bennett-Clarke et al., 1991, 1996; Schlaggar et al., 1993; Schlaggar and O'Leary, 1994). The spatiotemporal change in the pattern of AChE activity (in the VPl) dovetails with the changes in the neurotrophin system (in

the VPm). Thus, whereas BDNF may be important for the VPm, other systems (e.g., serotonin transporter systems, AChE-expressing systems, other growth factors) may be key for the VPl.

The second wave of neuronal generation is nonhierarchical, at least in the sense of development following an ascending sequence (periphery → brainstem → thalamus → cortex). On the other hand, this generation coincides with the establishment of the descending corticothalamic projection (Jones, 1997; Uziel et al., 2006). This notion is supported by evidence that (1) layer VI, the source of most corticothalamic projections, has a considerable number of neurotrophin-expressing neurons (Pitts and Miller, 1995, 2000) and (2) the thalamus does not have neurons capable of manufacturing a neurotrophin(s) (Baquet et al., 2004), but it does have cells that express neurotrophin receptors (Vitalis et al., 2002; present study). Thus, cortex apparently plays a major role in establishing thalamic structure via a nonhierarchical mechanism.

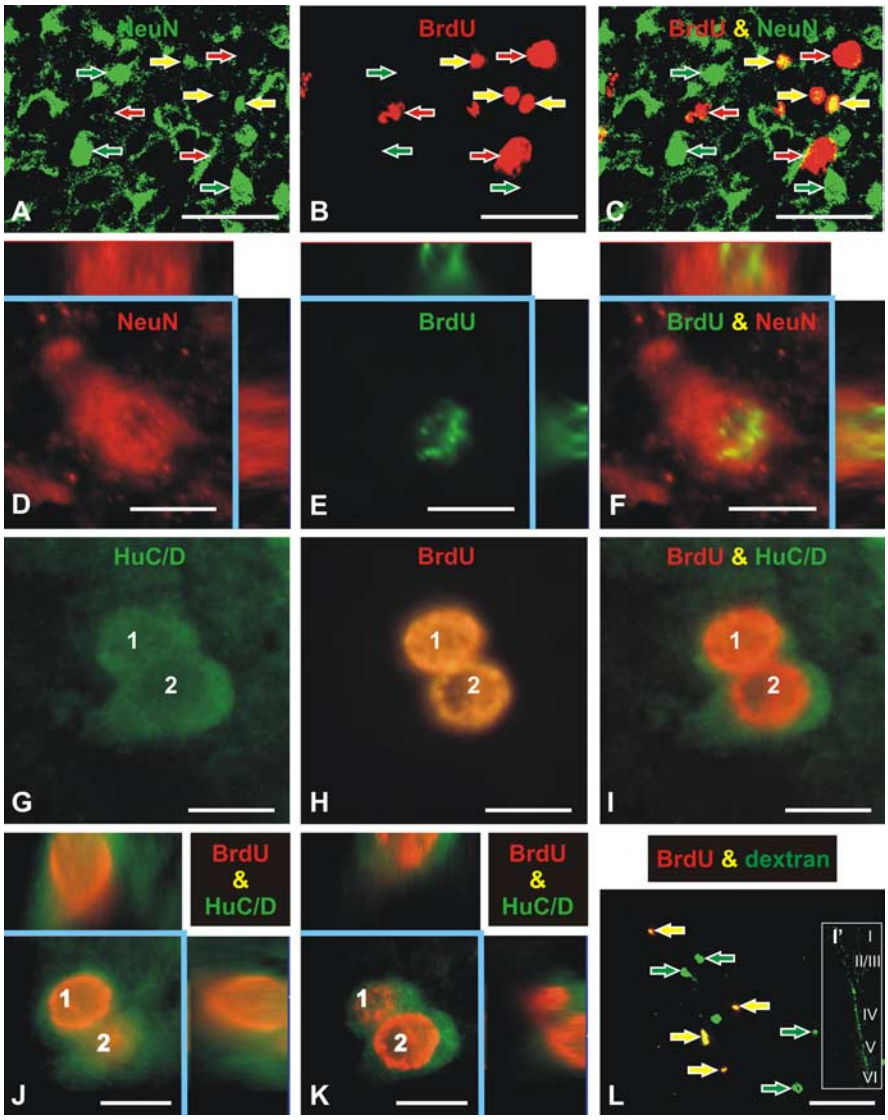

Figure 7. Phenotype of cycling cells. Double immunofluorescent labeling studies were used to determine that some of the cycling cells (labeled by an injection of BrdU on P6) expressed neuron-specific markers ≥6 d after injection. ***A–C***, Confocal images were obtained with optical section thickness of 1.0 μm. Sections processed for NeuN (***A***; green arrows) and BrdU (***B***; red arrows) immunohistochemistry are shown. Many BrdU-positive cells were NeuN positive (***C***; yellow arrows). ***D–F***, Images of double labeling with anti-NeuN (***D***) and anti-BrdU (***E***) antibodies. ***F*** shows colocalization of the labels in a merged image flanked by images at orthogonal planes through the cell. The orthogonal images were compiled from stacks of 1.0 μm optical images. ***G–K***, Images of labeling with anti-HuC/D antibody (***G***) and anti-BrdU antibody (***H***). The merged images show colocalization of the labels. ***I***, ***J***, A z-series of images was taken through the section shown in ***G***. Individual images were taken at planes that highlighted a nuclear profile of cell 1 (***I***) and cell 2 (***J***). Orthogonal views show that for each cell, both antigens were coexpressed. Images for each antibody reaction are shown in supplemental Figure 2 (available at www.jneurosci.org as supplemental material). ***L***, Animals given injections of BrdU on P6 were given an intracerebral injection of biotinylated dextran on P24 (box inset, ***I'***). One week later (on P31), cells in the VB that colocalized BrdU and dextran were identified (yellow arrows). Images representing the individual channels are shown in supplemental Figure 3 (available at www.jneurosci.org as supplemental material). Scale bars: ***A–C***, ***L***, 50 μm; ***D–K***, 10 μm.

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