Quiescence and Activation of Stem and Precursor Cell Populations in the Subependymal Zone of the Mammalian Brain Are Associated with Distinct Cellular and Extracellular Matrix Signals

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The subependymal zone (SEZ) of the lateral ventricles is one of the areas of the adult brain where new neurons are continuously generated from neural stem cells (NSCs), via rapidly dividing precursors. This neurogenic niche is a complex cellular and extracellular microenvironment, highly vascularized compared to non-neurogenic periventricular areas, within which NSCs and precursors exhibit distinct behavior. Here, we investigate the possible mechanisms by which extracellular matrix molecules and their receptors might regulate this differential behavior. We show that NSCs and precursors proceed through mitosis in the same domains within the SEZ of adult male mice—albeit with NSCs nearer ependymal cells—and that distance from the ventricle is a stronger limiting factor for neurogenic activity than distance from blood vessels. Furthermore, we show that NSCs and precursors are embedded in a laminin-rich extracellular matrix, to which they can both contribute. Importantly, they express differential levels of extracellular matrix receptors, with NSCs expressing low levels of α6β1 integrin, syndecan-1, and lutheran, and in vivo blocking of β1 integrin selectively induced the proliferation and ectopic migration of precursors. Finally, when NSCs are activated to reconstitute the niche after depletion of precursors, expression of laminin receptors is upregulated. These results indicate that the distinct behavior of adult NSCs and precursors is not necessarily regulated via exposure to differential extracellular signals, but rather via intrinsic regulation of their interaction with their microenvironment.

Introduction

Stem cell niches are protective microenvironments constructed by supporting cells and—in vertebrates—blood vessels, that contain stem cells and some of their progeny (Scadden, 2006; Kiel et al., 2007). A neurogenic stem cell niche in the adult rodent brain is the subependymal zone (SEZ) of the lateral walls of the lateral ventricles (LVs). Here, astroglial-like neural stem cells (NSCs, also known as type B cells) generate neuroblasts (NBs, or type A cells) and oligodendrocyte precursors via transit-amplifying precursors (TaPs, or type C cells) (Doetsch et al., 1999). The cell cycle behavior of NSCs and of their downstream more committed progenitors (collectively referred to as “precursors”) within the niche is distinct. In the normal CNS, NSCs are relatively quiescent, while precursors actively proliferate (Morshead et al., 1994). Following ablation of the precursors, however, the NSCs become activated and rapidly generate a new population of TaPs. However, the intrinsic and extrinsic signals that determine these distinct behaviors remain elusive.

A necessary first step in determining the identity of these signals is to establish the cellular and extracellular environment of NSCs and TaPs. Previous studies have shown that proliferating cells in the SEZ reside in close proximity to blood vessels (Shen et al., 2008; Tavao et al., 2008) and to fractones (Kerever et al., 2007), extracellular matrix (ECM) structures proposed to be continuums of the blood vessel basement membrane, and that NSCs...
integrate between ependymal cells creating specific pinwheel cytoarchitectures at the ventricular wall (Mirzadeh et al., 2008). However, these studies did not examine whether NSCs and TaPs differed in their proximity to ependymal cells and blood vessels, critical information in the formulation of hypotheses as to which cell type generates the signals for each stem/precursor population. Nor, in addition, was the role of ECM examined by determining expression of its components and their receptors. Here we have performed this analysis and show that, while stem and precursor cells are equidistant from blood vessels, stem cells are also in close proximity to ependymal cells. Laminins are expressed throughout the niche but only precursors express laminin binding integrins in the normal CNS, and functional studies show that these regulate proliferation and migration. During regeneration NSCs also express integrins, and our study therefore shows for the first time how laminins could regulate proliferation of the different populations in the niche during maintenance and regeneration.

Materials and Methods

**Tissue preparation and immunostaining.** Experiments were performed in accordance with the Animals (Scientific Procedures) Act (1986). Adult (4–6 months) C57BL/6 and 129sv male mice were fixed in 4% paraformaldehyde in PBS. Cryostat (6μm) or vibratome (70μm) brain sections were cut from the area extending from bregma up to 2.0 mm posterior. For immunostaining, sections were treated with 0.1% Triton X-100, 10% normal goat serum (Sigma) in PBS and subsequently incubated overnight with the appropriate primary antibodies at 4°C (antibodies used are listed in supplemental Table 1, available at www.jneurosci.org as supplemental material). For laminin immunostaining, sections were also post-fixed with acetone (10 min) and the blocking buffer contained 3% BSA (Sigma). Alexa Fluor 350, 488, 568, and 647 (Invitrogen) were used as secondary antibodies. To identify slowly dividing, BrdU-retaining cells, mice received 2 BrdU injections/d (Sigma; 50 mg/kg of body weight) for 3 d and were killed 40 d later. Brains were processed and analyzed as described above with the addition of a pretreatment in 2N HCl for 30 min at 37°C. HCl treatment resulted in significant alterations of the immunostaining for most laminins and laminin receptors; therefore, only the BrdU/1 integrin double staining results have been incorporated to the analysis. Images were acquired using an Olympus IX50 or a Zeiss fluorescence microscope and with a Leica SP5 confocal microscope and were processed using MagnaFire and Photoshop (Adobe) software. Triple labelings were analyzed using DeltaVision imaging as previously reported (Lathia et al., 2007). Briefly, an Olympus IX60 inverted microscope was used as the platform for image acquisition (Applied Precision). The interface for stack building was done with a SoftWorx acquisition module (Resolve3D). The center of an image stack was determined by the auto-focus function of Resolve3D and images were automatically compiled (Resolve3D). The cell types in a multichannel image were enumerated as described below. First, all the DAPI-stained nuclei in an image were counted using FARSIGHT. In the case of nuclear markers, all the nuclei above a certain intensity threshold in the corresponding channels were counted. For membrane markers, boundary pixels of the DAPI-stained nuclei were inspected and the nuclei were deemed positive or negative if the pixels were above or below an intensity threshold. The fraction of a cell type in the SEZ was defined as the ratio of the total number of nuclei counted from various images to the total number of DAPI-stained nuclei in the corresponding images. The cell types were cataloged from a pool of 2200 cells in the SEZ. To measure the distance of the various cell types to the blood vessels, the nuclei were segmented as described above, the vessels were then segmented, and the distances from the centroid of the nuclei to the surface of the vessels were measured using FARSIGHT. The density of vessels was measured using a custom program written in MATLAB, and vessel diameters as well as the proximity of vessels to the ventricle were measured using LSI image browser from 60 z-stacks that were 10–45 μm thick. The size of each image that was analyzed was 143 μm (parallel to the lateral ventricles) × 20 μm (distance from the ventricle). The measurements involving GFAP immunostaining were made using Imaris 5.0.3.

**AraC treatment and β1 integrin-blocking experiments.** Adult mice were anesthetized and a cannula (BIK-II, Alzet) was fixed on the skull (1 mm lateral to bregma) connected to a subcutaneously implanted miniosmotic pump (1007D, Alzet). For AraC treatment, 4% AraC (Sigma) or saline alone was infused for 4 d onto the surface of the brain and animals were killed at different time points after the end of the infusion (Kazanis et al., 2007). For β1 integrin-blocking experiments, isotype-control or β1 integrin antibodies (clone Ha2/5, BD Biosciences PharMingen) were infused for 3 d in one ventricle and mice were killed 1 or 4 d after treatment.

**Fluorescence-activated cell sorting and neurosphere assays.** The SEZ from 10- to 12-week-old heterozygous Sox2;EGFP (Ellis et al., 2004) and CD1 male mice was microdissected into DMEM/F12 (Sigma). Samples were digested with papain (Worthington) for 30–45 min with occasional resuspension (Panchision et al., 2007; Coskun et al., 2008). The sample was passed through a 40μm filter to exclude undigested tissue and large debris and the cell concentration adjusted to 10^6 cells/100 μl for staining. Cell suspensions were incubated with antibodies against β1 integrin conjugated to Alexa Fluor 700 (Biolegend) at a concentration of 1:100 on ice for 20 min. Propidium iodide was added (1:100), at least 10 min before sorting, to exclude dead or dying cells. Experiments were performed with a BD FACSAria machine. Samples from wild-type mice were used as negative controls to set up voltages and to establish positive and negative gates (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Data were collected using FACSDiva software and analyzed using Flowjo software. Sorted cells were plated at clonal density (50 cells/cm^2) (Coskun et al., 2008) in neurosphere medium (containing FGF2 and EGF). Fresh medium was added after 3 d to maintain growth factor concentrations and the sphere forming potential was assessed at 7 d. Single primary spheres were placed in individual wells of a 96-well plate and dissociated into single cells to assess formation of secondary spheres.
Results

Structural characteristics of the neurogenic niche

The SEZ neurogenic niche is a narrow periventricular area extending a few micrometers into the mature brain tissue at the lateral side of the LV wall, with the middle and ventral parts being thin and compact areas and the dorsal part being wider and dominated by large clusters of migrating NBs (Fig. 1 and supplemental Fig. 2, available at www.jneurosci.org as supplemental material). We compared the structure of the SEZ on the lateral side of the LV with the non-neurogenic area situated at the medial side (middle and dorsal parts) of the SEZ (Fig. 1), focusing on the ependymal cells, astrocytes, and vasculature. Ependymal cells form a monolayer that lines the ventricle, and at the medial side below the ependymal layer multiple astrocytes form a layer of processes running in parallel to the ventricular wall (Fig. 1, inset 1). This ependymal-astrocyte architecture was not observed in the SEZ, where astroglial processes run radially, or randomly (Fig. 1, inset 2). The SEZ niche was also characterized by an extensive network of blood vessels, many positioned in parallel to the ventricular wall (Fig. 1, inset 2). The SEZ niche was also characterized by an extensive network of blood vessels, many positioned in parallel to the ventricular wall (Fig. 1, inset 2). The SEZ niche was also characterized by an extensive network of blood vessels, many positioned in parallel to the ventricular wall (Fig. 1, inset 2).

Mitotic activity of NSCs and precursors occurs in the same microdomains of the SEZ

Having defined the position of ependymal cells and blood vessels within the SEZ, we next asked whether the location of NSCs and TaPs relative to these structural components suggested a dominant role for one or the other in providing a cellular environment for neurogenesis. First, we established the number of the different cell types within the SEZ using an automated object recognition software (FARSIGHT): ependymal cells (33%), TaPs (12%), NBs (23%; commonly found in clusters), astrocytes including NSCs (16%), neurons (11%), and unidentified cells (5%) (for cell-type-specific markers, see supplemental Fig. 2, available at www.jneurosci.org as supplemental material)—figures that agree closely with previous studies (Doetsch et al., 1997).

Our analysis then focused primarily on mitotic cells (i.e., the “real-time” neurogenic activity as opposed to cells that might be resting in G1 phase of the cell cycle), marked by the expression of phosphohistone 3 (PH3). We used expression of GFAP to distinguish the combined population of TaPs and NBs (that are GFAP negative) from the NSCs (that are GFAP positive), so enabling the necessary triple labeling experiments for the structural analysis using antibodies against PH3 and laminins (to identify blood vessels). The addition of antibodies against further markers that distinguish between TaPs and NBs resulted in a level of signal inadequate for analysis. One hundred fifty-seven mitotic cells from seven mice were identified and separated into potential NSCs (coexpressing GFAP) or more committed precursors (GFAP negative). For the analysis of the positional characteristics of different cell types, the shortest distance from the center of the nucleus of each PH3+ cell to the ventricle and to the nearest blood vessel was measured (supplemental Fig. 4, available at www.jneurosci.org as supplemental material), using FARSIGHT. As expected, the vast majority of mitotic cells were GFAP negative (91 ± 2%) and they were observed at all rostral and dorsal-ventral levels, although with lower average density at the middle of the niche, while no GFAP+ mitotic cells were observed in the ventral SEZ (supplemental Fig. 4, available at www.jneurosci.org as supplemental material).
All mitotic cells were located within a 20-μm-wide zone adjacent to the ventricle. Few mitotic nuclei were positioned among ependymal cells (<5 μm from the ventricular surface; 7% of all mitotic cells), and these included almost half of dividing astrocytes (40% of GFAP+/PH3+ cells) and a small fraction of the mitotic GFAP− cells (6% of GFAP−/PH3+ cells). More mitotic cells (43% of all mitotic cells) were observed in the second cell layer (5–10 μm from the ventricular surface), belonging both to GFAP+ (60% of GFAP+/PH3+ cells) and GFAP− (42% of GFAP−/PH3+ cells). Reflecting this, the average distances from the ventricle were 7.2 ± 1.5 μm for the mitotic GFAP+ cells (NSCs) and 10.7 ± 4.3 μm for the mitotic GFAP− precursors (p = 0.316 using the nonparametric Friedman test). The third and fourth cell layers (10–20 μm) contained numerous GFAP− mitotic nuclei (35% and 17% of GFAP−/PH3+ cells, respectively) but no mitotic astrocytes, although these layers were rich in nondividing astrocytes (containing 25% of all SEZ astrocytes, with another 9% positioned farther than 20 μm). These data therefore demonstrate that neurogenic activity (either of NSCs or downstream precursors) occurs in the peryventricular area, with the GFAP+ mitotic cells (NSCs) restricted to the 10 μm adjacent to the ventricle while the GFAP− cells (precursors) are dispersed throughout the 20 μm depth of the SEZ.

Having established above that the neurogenic regions of the SEZ are more vascularized than non-neurogenic regions, we next examined the position of the mitotic cells relative to blood vessels. The average distance of mitotic NSCs, mitotic precursors, and nonmitotic astrocytes from the nearest blood vessel was not statistically different (NSCs: 16.6 ± 15.4 μm, precursors: 13.7 ± 10.6 μm, nonmitotic astrocytes: 16.8 ± 10.7 μm; p = 0.12 using the nonparametric Friedman test), and we observed significant numbers of mitotic NSCs and precursors at distances >20 μm from the nearest blood vessel (NSCs: 20% 0–5 μm, 30% 5–10 μm, and 50% >20 μm; precursors: 29% 0–5 μm, 19% 5–10 μm, 6% 10–15 μm, 23% 15–20 μm, and 23% >20 μm). Indeed, when the distance from the ventricle and from the nearest blood vessel was compared for each mitotic cell, using the nonparametric paired Wilcoxon test, it was found that both mitotic NSCs and precursors were positioned significantly nearer to the ventricle rather than to blood vessels (p = 0.007 for NSCs and p = 0.013 for precursors), even though many of the latter population are located 10–20 μm away from the ventricle.

The neurogenic microenvironment is rich in laminins

Having established that NSCs and precursors differ in their distribution within the SEZ, we next asked whether this resulted in differences in the extracellular matrix environment of the two populations. We focused on laminins, as these molecules are found in other niches and have previously been shown to be present in fractones within the SEZ. Laminins are trimers consisting of one α, one β, and one γ chain, and we examined the expression of these chains using available chain-specific antibodies as detailed in Table 1. Blood vessels of the niche were immunopositive for numerous laminin chains as well as for agrin, collagen IV, and fibronectin (Table 1). No difference was detected in this ECM profile among blood vessels within the SEZ and those within the adjacent striatal area (data not shown). Examination of blood vessels revealed that laminin expression was confined at the outer surface of vessels, and GFAP+ cells were often found to be in close contact with the laminin-rich zone of vessels (supplemental Fig. 3A,B, available at www.jneurosci.org as supplemental material). The parenchyma of the ependymal and subependymal regions was also immunopositive for the same laminins (Table 1). In contrast, in the adjacent striatal area laminin immunoreactivity was associated mainly with vessels. The immunoreactivity was diffuse within the SEZ but notably higher at the ependymal layer and around clusters of TaPs and NBs (supplemental Fig. 5, available at www.jneurosci.org as supplemental material and data not shown). In addition, immunopositive fractone-like structures were observed (Table 1; supplemental Fig. 5, available at www.jneurosci.org as supplemental material). Importantly, no differences in the chains expressed were seen between any of these regions. Overall, therefore, these data indicate that the neurogenic niche is characterized by high concentrations of laminins, as compared to adjacent non-neurogenic areas, with NSCs and precursors equally exposed to this ECM and with no differences in trimer composition as judged by the expression of specific chains.

### Table 1. Expression profile of ECM molecules in the normal and regenerating SEZ

<table>
<thead>
<tr>
<th></th>
<th>Normal Ependymal cell layer</th>
<th>SEZ</th>
<th>b.v.</th>
<th>Fr.</th>
<th>Post-AraC Ependymal cell layer</th>
<th>SEZ</th>
<th>b.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin α1</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Laminin α2</td>
<td>Diffuse</td>
<td>Diffuse</td>
<td>High</td>
<td>p</td>
<td>Diffuse</td>
<td>n</td>
<td>High</td>
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<tr>
<td>Laminin α3</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Laminin α4</td>
<td>Diffuse</td>
<td>Diffuse</td>
<td>High</td>
<td>p</td>
<td>Diffuse</td>
<td>n</td>
<td>High</td>
</tr>
<tr>
<td>Laminin α5</td>
<td>Diffuse</td>
<td>Diffuse</td>
<td>High</td>
<td>p</td>
<td>Diffuse</td>
<td>n</td>
<td>High</td>
</tr>
<tr>
<td>Laminin β1</td>
<td>Diffuse</td>
<td>Diffuse</td>
<td>High</td>
<td>p</td>
<td>Diffuse</td>
<td>Astrocyte related</td>
<td>High</td>
</tr>
<tr>
<td>Laminin β2</td>
<td>n</td>
<td>Low</td>
<td>High</td>
<td>p</td>
<td>Diffuse</td>
<td>n</td>
<td>High</td>
</tr>
<tr>
<td>Laminin γ1</td>
<td>Diffuse</td>
<td>Diffuse</td>
<td>High</td>
<td>p</td>
<td>Diffuse</td>
<td>n</td>
<td>High</td>
</tr>
<tr>
<td>Laminin γ2</td>
<td>Diffuse</td>
<td>Diffuse</td>
<td>High</td>
<td>p</td>
<td>Diffuse</td>
<td>n</td>
<td>High</td>
</tr>
<tr>
<td>Laminin γ3</td>
<td>Diffuse</td>
<td>Diffuse</td>
<td>High (~5%)</td>
<td>p</td>
<td>Diffuse</td>
<td>n</td>
<td>High (~5%)</td>
</tr>
<tr>
<td>Agrin</td>
<td>n</td>
<td>n</td>
<td>High</td>
<td>n</td>
<td>n</td>
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<td>High</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>n</td>
<td>n</td>
<td>High</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>High</td>
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<tr>
<td>Fibronectin</td>
<td>n</td>
<td>n</td>
<td>High</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>High</td>
</tr>
<tr>
<td>CSPG</td>
<td>Diffuse</td>
<td>n</td>
<td>High</td>
<td>n</td>
<td>n</td>
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<td>High</td>
</tr>
<tr>
<td>Kalanin</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
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<tr>
<td>Nidogen-1</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Perlecán</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
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</tbody>
</table>

Note the decrease in laminin immunoreactivity in the SEZ after treatment with AraC. b.v., Blood vessels; Fr., fractones; n, negative; p, positive.

### Differential expression of laminin receptors on NSCs and progenitors

A uniform expression of laminins could still generate distinct signals in stem and precursor populations if the expression of laminin receptors differed on the two populations. We therefore performed an immunohistochemical analysis of the expression...
Table 2. Expression profile of laminin receptors in the normal and regenerating SEZ

<table>
<thead>
<tr>
<th></th>
<th>E</th>
<th>B</th>
<th>TaP</th>
<th>NB</th>
<th>b.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3 Integrin</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>α4 Integrin</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>α5 Integrin</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>α6 Integrin</td>
<td>High</td>
<td>n</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>β1 Integrin</td>
<td>High</td>
<td>n</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Syndecan-1</td>
<td>n</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Lutheran</td>
<td>High</td>
<td>n</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

Dystroglycan n n n n High
Lutheran High n High High High

Note that type B cells express low or nondetectable levels of laminin receptors in the normal niche. E, Ependymal cells; B, type B astrocytes; b.v., blood vessels; n, negative.

Figure 2. Cell-type-specific expression of β1 integrin in the SEZ. Expression of β1 integrin by different cell types of the SEZ was investigated with double immunostainings on coronal sections. Sox2 marks numerous cells within the niche (A), most of which are immunopositive for β1 integrin, although a few Sox2+/β1 integrin negative cells are also observed (A, arrows). Transit-amplifying precursors identified by Mash1 expression (B, arrow indicates 1 example) and PSA-NCAM-positive neuroblasts (C) coexpress β1 integrin. Astrocytes do not express detectable levels of β1 integrin (D, arrows indicate 2 β1 integrin-negative astrocytes). Ependymal cells (indicated by arrowheads in all panels) and blood vessels (“bv” at C and D) also express high levels of β1 integrin. The lateral ventricle is to the right in all images. Scale bar, 30 μm.

of the major receptors for laminins: integrins (consisting of one α and one β chain), syndecans, dystroglycans, and lutheran—which is a laminin α5 chain-specific ligand. The laminin receptors with the widest distribution of expression were α6 and β1 integrins, expressed by blood vessels, ependymal cells, and progenitors (Table 2). Double immunostaining showed that these subunits were highly colocalized (data not shown), consistent with the expression of the laminin α6β1-binding integrin. Double immunostaining for β1 integrin and the transcription factor Sox2 (expressed on NSCs, precursors, and ependymal cells) revealed that most, but not all, of Sox2-positive cells expressed β1 integrin (Fig. 2A). To identify the integrin-negative population, we used cell-type-specific markers. These confirmed that ependymal cells, TaPs, and neuroblasts were β1 integrin positive (Fig. 2) and that all actively dividing cells in the SEZ expressed high levels of β1 integrin (Fig. 3). Interestingly, however, the cell bodies and processes of astrocytes did not express β1 integrin (Fig. 2), suggesting that the integrin-negative, Sox2+ cells might include the NSC population.

The finding that NSCs did not express β1 integrin was surprising, as this integrin has been reported to be highly expressed on other stem cells. We therefore verified this conclusion in two further ways. First, we performed label-retaining studies, in which mice were given a cumulative dose of BrdU and killed 40 d later. This identifies the slowly dividing cell population within which NSCs will be found, while rapidly dividing precursors dilute out BrdU and so are unlabeled. In keeping with our initial results, we found that 90% of BrdU-retaining cells were β1 integrin negative (Fig. 3). As a second way to confirm this conclusion, we used a neurosphere assay. Neurospheres are three-dimensional (3D) aggregates containing a mixture of stem, precursor, and more differentiated cells that grow from a single stem cell and can be passaged to form secondary neurospheres, and therefore provide a semi-quantitative method for assessing stem cell numbers within dissociated cell populations. We used fluorescence-activated cell sorting (FACS) to collect subpopulations of cells, isolated from microdissected SEZs of adult Sox2-EGFP mice and immunostained with β1 integrin-conjugated antibodies (n = 4). In these mice, EGFP reporter has been shown to represent accurately endogenous Sox2 expression (Ellis et al., 2004), and we validated this within the adult SEZ by comparing EGFP expression with Sox2 immunoreactivity (Fig. 4A). Four populations were sorted in the FACS analysis based on high or low expression of EGFP and β1 integrin, with the EGFP+ β1 integrin− population then containing the putative GFAP+ NSCs (Figs. 2A, D, 4B), and each was collected and seeded into growth medium to assess neurosphere formation (Fig. 4C). Over 60,000 cells were assayed, and the results revealed that virtually all cells with neurosphere-forming ability were Sox2+ but β1 integrin negative, with 830 of 833 spheres formed from the Sox2+/β1 integrin− population. Secondary spheres were formed by 83% (75/90) of primary spheres from this population. These results therefore confirmed that the Sox2+/β1 integrin− population of the SEZ is highly enriched for NSCs, and thus we conclude that the great majority of NSCs in the normal SEZ do not express detectable β1 integrin.

Examination of other laminin receptors revealed that lutheran was colocalized with β1 integrin (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). Syndecan-1 was expressed on TaPs and NBs (supplemental Fig. 7, available at www.jneurosci.org as supplemental material), but, in contrast to β1 integrin, only low levels of expression were observed in the ependymal cell layer. Additionally, and again in contrast to β1 integrin and lutheran, a fraction of astrocytic cell bodies and
processes (10 ± 1.5%, n = 3 mice) expressed syndecan-1. Finally, blood vessels were also immunopositive for α7 integrin and dystroglycan (Table 2).

There are two possible interpretations of the result that NSCs do not express detectable levels of β1 integrin. First, β1 integrin may be expressed only on dividing cells, and as the great majority of NSCs are quiescent, they are β1 integrin negative. Second, NSCs and precursors may differ in integrin expression such that NSCs are always β1 integrin negative whatever their cell cycle status. These two possibilities could in theory be distinguished in the normal niche by examining dividing NSCs, but these are very rare cells. To distinguish these possibilities, we therefore took advantage of the regenerative properties of the SEZ, where significant numbers of NSCs become activated and reenter the cell cycle to reconstitute the TaP and NB populations after their ablation with the cytotoxic drug AraC. AraC was infused on the surface of the brain for 4 d, and mice were killed immediately after the end of treatment (day 0, n = 5) and 2 or 4 d later (n = 5 per time point). One hundred eighty-four PH3 cells were identified. As expected, in these initial phases of regeneration the majority were astrocytes with their percentage declining later on (data not shown). Notably, a few mitotic astrocytes were now observed farther than 10 μm from the ventricle (in contrast to the normal SEZ), but never beyond 20 μm from the ventricle (the distribution was as follows: 11% 0–5 μm, 63% 5–10 μm, 22% 10–15 μm, 4% 15–20 μm). As in the normal SEZ, these cells were closer to the ventricle than they were to blood vessels (8.6 ± 3.4 μm from the ventricle and 15.8 ± 10.5 μm from the vessels; p < 0.05 using the non-parametric Wilcoxon test). Mitotic GFAP− cells were very sparse immediately after the end of AraC treatment, but their numbers increased thereafter and their distribution was similar to those of the normal niche (20% 0–5 μm, 36% 5–10 μm, 34% 10–15 μm, 10% 15–20 μm from the ventricle). The expression of lutheran and syndecan-1 was upregulated on SEZ astrocytes immediately after the end of AraC infusion (Fig. 5), while expression of α6β1 integrin remained undetectable. However 2 d later, when neuroblasts were still absent and the TaP pool had only partially been reconstituted, β1 integrin expression was upregulated on GFAP-positive cells. Triple immunostaining revealed that these β1 integrin+GFAP+ astrocytes were also Sox2+, confirming that

Figure 3. β1 integrin expression and cell cycle. Actively dividing cells immunolabeled with anti-phospho histone 3 (A, 1 PH3+ cell is indicated by the arrow), or anti-Ki67 antibodies (B, 2 Ki67+ cells are indicated by arrows) express β1 integrin. In A1, A2, B1, and B2, single-channel immunolabeling is shown. In A3 and B3, these are merged with PH3/Ki67 in green and integrin in red. Blue shows DAPI in A3. In contrast, slowly dividing cells, that retain BrdU 40 d after the last injection, are negative for β1 integrin (arrows in C and D). Note the presence of multiple β1 integrin+ cells in close proximity to BrdU-retaining cells. In D, the BrdU-retaining cells are GFAP+, compatible with a NSC identity. C3 and D4 show merged images with BrdU in green, integrin in red, and in D4, GFAP in blue. Scale bars: A, B, D, 10 μm; C, 5 μm.
NSCs in the SEZ niche upregulate β1 integrin at the time of their mitotic activation (Fig. 5E, F). We conclude that integrin expression correlates with mitotic status rather than being a difference between stem and precursor cells.

Interestingly, although blood vessel- and ependymal cell-related laminin immunoreactivity, as well as the occurrence of fractone-like dots, was unaffected by AraC treatment, there was a significant reduction in the levels of diffuse parenchymal laminin expression (Fig. 6). This indicates that TaPs and NBs (that constitute ~35% of the total number of cells in the niche) contribute laminins to this compartment of the niche microenvironment. No other change in laminin expression was seen except for high laminins to this compartment of the niche microenvironment.

Percentage of cells that form neurospheres from each of the four populations shown in B. Note that only Sox2+ cells generate neurospheres and predominantly those that are Sox2+/β1 integrin negative. Data in the graph represent means and SEM.

Discussion
The exact position of stem cells within their niche will be critical for the regulation of cell behavior, as it will determine to which extracellular cues the stem cell is exposed. In the case of the SEZ neurogenic niche, both the ventricle (CSF, ependymal layer) and the vasculature have been implicated in the regulation of neurogenesis (Sawamoto et al., 2006; Mirzadeh et al., 2008; Shen et al., 2008; Tavazoie et al., 2008). In keeping with this, our analysis revealed that the periventricular area where neurogenesis occurs is characterized by significantly higher vascularization compared to non-neurogenic areas, a result of the penetration of the blood vessel network nearer to the ventricle. This specialization of the vasculature of the niche might be very important not only for homeostatic neurogenesis (i.e., the turnover seen in the undamaged CNS) but also for the regenerative responses after injury, since inflammation mediated by the innate microglia and the blood vessel-derived infiltrating T-cells has been shown to modulate NSC and precursor behavior (Pluchino et al., 2008; Ekdahl et al., 2009). Interestingly, however, within the 3D SEZ microenvironment the distribution of mitotic cells within both the normal and regenerating tissue was not tightly linked to blood vessels, with either NSCs or precursors going through mitosis at a diversity of distances from <5 µm to >30 µm from the vessels. This is not to say that NSCs do not receive signals from vessels, as previous work has shown that fine processes from astrocytes can extend significant distances to contact blood vessels (Mirzadeh et al., 2008). However, as the distance from the ventricular wall emerged as a strong limiting factor, with all mitotic activity being reduced to ≤5 µm from the vessels.

normal niche, and increased to 4.8 ± 2% after 4 d with β1 integrin-blocking antibody (p < 0.05 using t test). In contrast, the percentage of Sox2-low/Ki67+ cells did not change significantly: 11.1 ± 2.4% of total cells in the normal niche and 13.7 ± 2.7 at 4 d (p = 0.54 using t test). As ~85% of dividing cells in the normal SEZ are neuroblasts expressing Dcx and low levels of Sox2 (supplemental Fig. 8, available at www.jneurosci.org as supplemental material), while the other 15% that express high levels of Sox2 are predominately TaPs (NSCs and ependymal cells that are also Sox2 high-expressing cells divide only occasionally or are quiescent, respectively) (supplemental Fig. 8, available at www.jneurosci.org as supplemental material), we conclude that the majority of the increased proliferation is present in the TaP cells. As we would have predicted given their lack of β1 integrin expression, there was no increase in the numbers of proliferating astrocytes (that remained <0.5% of total cells both in the normal and the infused niche). These results therefore confirm a role for β1 integrin in the regulation of TaP proliferation in the SEZ. They also revealed a migration phenotype, with an increase in the appearance of Dcx-positive and proliferating cells on the medial side of the ventricle (Fig. 7), where normally only very few migrating cells are seen.
The nature of these signals remains to be determined, as only a few possible ependyma-derived regulators of neurogenesis have been identified previously, such as pigment epithelium-derived factor (Ramírez-Castillejo et al., 2006) and molecules of the bone morphogenetic protein pathway (Lim et al., 2000; Colak et al., 2008). Interestingly, and in support of the hypothesis that ependymal cells provide critical signals for neurogenesis, in non-neurogenic periventricular regions an additional layer of astrocytic processes running parallel to ependymal cells is observed. Thus, it is only within the neurogenic area that the subependymal region is separated from the ventricle only by the ependymal cell monolayer. Moreover, specific subtypes of ependymal cells (e.g., E2 bicilia cells) are observed exclusively in the neurogenic region of the ventricular wall (Mirzadeh et al., 2008). Nevertheless, the possible role of ependymal cells in the regulation of adult NSCs remains controversial since hippocampal neurogenesis occurs in a niche devoid of ependymal cells (Riquelme et al., 2008), albeit one producing different types of neurons via intermediate progenitors distinct from those seen in the SEZ (Seaberg and van der Kooy, 2002; Seri et al., 2004). In the future it will therefore be important to extend this type of structural investigation to other CNS niches and also to include in the analysis other cell types,
such as microglia, that have been shown to regulate the behavior of neuronal progenitors (Walton et al., 2006).

Previous experimental work has shown that the SEZ is rich in ECM molecules, such as fibronectin and laminins β1 and γ1 (Mercier et al., 2002), chondroitin sulfate proteoglycans (Thomas et al., 1996; Akita et al., 2008), and tenascin-C (Peretto et al., 2005; de Chevigny et al., 2006; Kazanis et al., 2007). Here, we showed that NSCs and precursors are exposed to high levels of many additional laminin chains within the niche, including laminin α5. Laminins are important for proliferation and survival of neuronal progenitors (Barnabé-Heider et al., 2005; Hall et al., 2008) and for hippocampal regeneration (Grimpe et al., 2002) and regulate growth factor concentrations in the SEZ (Kerever et al., 2007). Moreover, recent in vivo studies have revealed a role of laminins and especially of laminin 511 (the α5β1γ1 trimer), produced both by vessel endothelial cells and other sources, in the regulation of adult stem cells in the skin (Paquet-Fifield et al., 2009) and pancreas (Otonkoski et al., 2008). In vitro work has also demonstrated a role for α5 laminins in the survival and proliferation of embryonic stem cells (Yoshihara et al., 2007; Domogatskaya et al., 2008; Evseenko et al., 2009; Vuoristo et al., 2009). This laminin may therefore have a significant role in the regulation of adult NSCs within the SEZ.

Our experiments indicate at least three different sources of laminins. The marked reduction of the laminin content of the niche after ablation of precursors revealed that cells of the NSC lineage are one major source of these molecules. In contrast, laminin immunostaining around ependymal cells and blood vessels remained stable after AraC treatment, showing that both these two additional sources—containing cells that are not affected by anti-mitotic treatment (Doetsch et al., 1999)—are producing laminins independently of the presence of precursors. Finally, the preservation of laminin β1 expression in the parenchyma of the SEZ after AraC treatment, and its colocalization with astrocytic cell bodies and processes, suggests that SEZ astrocytes may represent a fourth source exhibiting a cell-type-specific expression pattern of laminins seen only following injury.

How do NSCs and precursors interact with the ECM derived from all of the cellular components of the niche? We found that NSCs and precursors express different levels of laminin receptors, with the latter expressing high levels of α6β1 integrin, syndecan-1, and lutheran while the former did not express detectable levels of these same receptors. Specifically for β1 integrin, this result was confirmed with

neurosphere generation assays after FACS separation of SEZ cells by expression levels of Sox2 and β1 integrin. β1 Integrin has been previously identified as a marker of actively dividing neuronal progenitors (Campos et al., 2004; Nagato et al., 2005; Hall et al., 2006). Therefore, we propose that expression of ECM receptors such as α6β1 integrin could be a mechanism that NSCs use to
regulate their activity (Fig. 8). Although NSCs and precursors reside in similar niche domains (Mirzadeh et al., 2008) and are thus exposed to similar extracellular signals from the ECM, during normal conditions NSCs will have limited interaction with their microenvironment (as a result of low receptor expression) and so remain relatively quiescent. This conclusion is supported by the lack of any effect on NSC behavior in the β1 integrin-blocking experiments. When NSCs are stimulated to become active, to replenish the depleted niche after AraC infusions, their position relative to the ventricle and the vasculature remains unaltered, but they upregulate expression of α6β1 integrin, laminin-511/H9251, and syndecan-1 and thus increase their interaction with their cellular and extracellular environment (Fig. 8). β1 Integrin has been shown to mediate interactions with laminin 511 (Otonkoski et al., 2008; Evseenko et al., 2009; Vuoristo et al., 2009), while laminin is a laminin α5-specific receptor (Rahuel et al., 2008). Syndecan-1 has been previously shown to promote proliferation of breast cancer cells (Blaess et al., 2004) and to enhance FGF2 activity (Filla et al., 1998) via paracrine activity, in some cases signaling in cooperation with integrins (Banerjee et al., 2006). The correlation between the level of activity of adult NSCs and alterations in laminin–integrin interactions is reminiscent of recent findings in the follicle stem cell niche of the Drosophila ovary (O’Reilly et al., 2008) where follicle stem cells cell autonomously control their self-renewing behavior in a two-step process: first by producing laminin and second by up-regulating integrin expression.

What is the role of β1 integrin on activated NSCs and precursors? The increased proliferation of precursors after antibody blocking of β1 integrin activity, similar to results obtained previously after antibody blocking of α6 integrin (Shen et al., 2008), suggests that an important function of integrins might be to control levels of proliferation in actively dividing cells. Here, the observation that increased proliferation was seen in cells expressing high levels of Sox2 suggests that the affected population is the TaPs rather than NBs, but further work using specific TaP markers such as Mash1 and Olig2 (Hack et al., 2005) and cell surface markers of activated NSCs and TaPs such as the EGF receptor (Pastrana et al., 2009) will be required to confirm this. In the intestine adult stem cell niche, perturbation of β1 integrin also resulted in increased proliferation of progenitors, partially due to defective Shh signaling (Willaime-Morawek et al., 2008). Another aspect of integrin function is the control of cell migration, and it has been shown that genetic deletion or blocking of β1 integrin results in disrupted architecture of the rostral migratory stream (Blaess et al., 2004) and altered migration of NBs (Emsley and Hagg, 2003). In agreement with this, we showed that blocking β1 integrin resulted in increased ectopic migration of NBs at the medial side of the LVs.

In conclusion, our descriptive and functional study highlights three important properties of the cellular and extracellular architecture of the SEZ in the regulation of stem cell behavior: first, the greater proximity of mitotic cells to the ependymal cells than to blood vessels; second, the lack of change in this architecture during NSC activation and niche regeneration; and third, the generation by neural precursor cells of their own ECM microenvironment, the interaction to which depends on the state of activation and variation in the level of receptor expression. The recognition that ependymal cells are likely to be an important source of signals in addition to blood vessels for the regulation of NSC behavior, and that NSCs alter their interactions with their microenvironment during successful regeneration not by exposure to a different ECM but by altering their receptor expression, has significant implications in regenerative medicine and in any attempt to transplant (into areas of degeneration) neural stem and precursor cells while providing them with the signals required to retain their self-renewing capacity and to promote repair.

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