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REGULAR ARTICLE

A novel classification of quiescent and transit amplifying adult neural stem cells by surface and metabolic markers permits a defined simultaneous isolation

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Abstract Adult neural stem and progenitor cells (NSPCs) are usually defined retrospectively by their ability to proliferate *in vivo* (bromodeoxyuridine uptake) or to form neurospheres and to differentiate into neurons, astrocytes and oligodendrocytes *in vitro*. Additional strategies to identify and to isolate NSPCs are of great importance for the investigation of cell differentiation and fate specification. Using the cell surface molecules Prominin-1 and Lewis X and a metabolic marker, the aldehyde dehydrogenase activity, we isolated and characterized five main populations of NSPCs in the neurogenic subventricular zone (SVZ) and the non-neurogenic spinal cord (SC). We used clonal analysis to assess neurosphere formation and multipotency, BrdU retention to investigate *in vivo* proliferation activity and quantified the expression of NSPC associated genes. Surprisingly, we found many similarities in NSPC subpopulations derived from the SVZ and SC suggesting that subtypes with similar intrinsic potential exist in both regions.

The marker defined classification of NSPCs will help to distinguish subpopulations of NSPCs and allows their prospective isolation using fluorescence activated cell sorting.

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Introduction

Since the discovery of neural stem cells in the adult central nervous system (CNS) and their niches in the subventricular zone (SVZ) and hippocampus, enormous efforts were undertaken to understand the complex mechanisms which control proliferation, migration and differentiation of

endogenous neural stem cells and their progeny. One of the major obstacles the field has to overcome is the lack of specific markers impeding a distinct description of neural stem and progenitor cell subpopulations (NSPCs). Until now NSPCs are usually identified retrospectively by bromodeoxyuridine (BrdU) incorporation assays or by their *in vitro* ability to form neurospheres thereby limiting the search for mechanisms which control NSPC differentiation and fate specification *in vivo*.

Therefore, a prospective isolation of NSPCs defined by surface and intracellular markers would have a big impact on the neural stem cell field. It would allow the genomic and

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proteomic screening of acutely isolated NSPCs from different regions of the CNS and permit the investigation of pathways which may be differentially regulated in NSPCs in neurogenic and non-neurogenic regions.

For the two neurogenic niches in the adult CNS, the SVZ of the lateral ventricle and the dentate gyrus (DG) of the hippocampus, a first characterization of NSPCs by the expression of intracellular markers has been established (Alvarez-Buylla et al., 2002; Kempermann et al., 2004) and shows the diversity of different stem and progenitor cell types. Ultrastructural analysis by light and electron microscopy (Doetsch et al., 1997; Garcia-Verdugo et al., 1998) led to the present model of the stem cell niche identifying six different cell types: migrating neuroblasts (type A cells in the SVZ, type 2b cells in the DG), GFAP positive cells (type B1, B2 in the SVZ, type 1 in the DG), transit amplifying (type C cells in the SVZ, type 2a in the DG), putative precursors (type D cells in the SVZ, type 3 in the DG) and ependymal cells (type E).

However, cells with *in vitro* stem cell properties cannot only be isolated from the two neurogenic regions but also from areas that do not produce new neurons under normal conditions such as the hypothalamus, substantia nigra, subcortical white matter or spinal cord (SC) (Kokoeva et al., 2005; Lie et al., 2002; Nunes et al., 2003; Shihabuddin et al., 2000; Weiss et al., 1996). For NSPCs and possible subpopulations residing in these regions substantially less information is available, although the existence of multipotent stem cells, for example in the SC, was shown almost a decade ago (Shihabuddin et al., 2000; Weiss et al., 1996).

To date the best available method to distinguish between different types of NSPC is based on intracellular markers. Despite their fundamental importance as definition criteria for NSPC subpopulations, intracellular markers are of limited use for the isolation of different NSPC subtypes. Even though the expression of reporter genes under the control of an intracellular marker is a powerful instrument, there are several restrictions like costs, the complexity to generate transgenic animals and low viral transfection rates. Another limitation is given by the number of available fluorescent proteins and the list of markers required to discriminate between NSPC subpopulations. Besides intracellular markers, cell surface antigens offer a unique opportunity to efficiently identify different cell populations and would consequently provide an easy to use ability to isolate different NSPC subpopulations.

Some surface molecules being already used for the isolation of stem cells from different types of tissue including the CNS are Prominin-1 (Prom-1, CD133) a pentaspan membrane glycoprotein (Corti et al., 2007; Uchida et al., 2000), Lewis X (LeX, SSEA-1, FAL) an extracellular matrix-associated carbohydrate (Capela and Temple, 2002), a 473HD-Chondroitinsulfate epitope known to be expressed on radial glia (von Holst et al., 2006), Notch-1 being highly expressed in NSPC (Johansson et al., 1999) and peanut agglutinin (PNA) (Rietze et al., 2001).

In this study we focus on the analysis of the expression of the surface markers Prom-1 and LeX on stem and progenitor cell populations isolated from the adult SVZ and SC. Additionally to the surface markers we investigated a metabolic marker, the aldehyde dehydrogenase (ALDH) activity which is described to be enriched in hematopoietic progenitor (Storms et al., 1999) and neural stem cells (Corti et al., 2006). By combining the three markers we identified five main populations of NSPCs that showed distinct

characteristics with regards to gene expression, *in vitro* multipotency and their proliferation activity *in vivo* in the SVZ and SC.

Materials and Methods

Animals

All experiments were performed with adult male and female C57BL/6 mice (6 to 8 weeks old) and were conducted in compliance with the guidelines of the veterinary department of the canton of Zurich.

Isolation and pre-enrichment of NSPCs

After microdissecting the subventricular zone and isolating the spinal cord the tissue was dissociated as described before (Thallmair et al., 2006). In brief, tissue was dissociated using 2.5 U/ml papain (Worthington, USA), 250 U/ml DNaseI (Worthington) and 1 U/ml dispase (Gibco, Invitrogen, Switzerland). Following dissociation cells were filtered through a 70 µm cell strainer and further purified through Percoll (Amersham Biosciences, Germany) density gradient centrifugation. Afterwards cells were washed with phosphate buffered solution (PBS) and red blood cell lysis was performed by five minutes incubation in ammonium-chloride lysis buffer at room temperature.

Fluorescent activated cell sorting (FACS) of NSPCs

Cells were re-suspended in PBS and immunostained for the different markers. ALDH activity of single cells was measured using Aldefluor[®] (StemCell Technologies, USA) according to the manufacturer's protocol. The principle of the assay is based on Aldefluor, an uncharged substrate of the ALDH, freely diffusing through cell membranes, getting negatively charged after conversion through the ALDH. As a consequence the green fluorescent Aldefluor accumulates faster in cells with increased ALDH activity. After Aldefluor labeling cells were re-suspended in phosphate buffered saline (PBS) containing 2 mM EDTA and 0.5% fetal bovine serum (FBS) and incubated with rat α -Prominin-1-APC (MB9-3G8, Miltenyi, Germany) and mouse α -CD15-PE (VIMC6, Miltenyi) antibody, each at 10 µg/ml for ten minutes. Cells were sorted using a BD-FACS Aria cell sorting system. Dead cells were excluded by using propidium iodide (PI), doublets were eliminated by forward scatter (FSC), side scatter (SSC) area, width and height function. Cells were sorted with 98-100% purity and collected in tubes. For clonal analysis single cells were sorted into 96 well plates (TPP, Switzerland) with 100% purity. CD24 and CD31 positive cells were detected using rat α -CD24 (M1/69, BD) and rat α -CD31 (MEC13.3, BD) antibodies.

For the intracellular labeling of cells we followed a protocol described previously (Sergent-Tanguy et al., 2003). In brief, cells were fixed with 2% paraformaldehyde (PFA) for 15 minutes on ice, washed and incubated for 20 minutes in PBS containing 0.5% saponin. Subsequently, cells were washed three times with 0.1% saponin PBS and incubated for 30 minutes on ice with primary antibodies for mouse α -NeuN (AB5320, Chemicon, Germany), mouse α -NF160 (RM0270,

Zymed, USA), mouse α -GFAP (6F2, Dako, Denmark), and mouse α -CC1 (OP80, Calbiochem, Switzerland). After another three washes cells were incubated with secondary antibodies α -mouse Alexa 488 and α -rabbit Alexa 488 (Molecular Probes) for 30 minutes on ice. Cells were washed and re-suspended in PBS containing 2 mM EDTA and 0.5% FBS and analyzed on a Cytomics FC 500 system (Beckman Coulter).

Cell culture

Isolated NSPCs were cultured in DMEM:F12 media (Invitrogen) supplemented with B27 (Invitrogen) and N2 (Invitrogen) and 20 ng/ml FGF2 (Peprotech, Germany) and EGF (Peprotech). Every second day one third of the media was exchanged with fresh media supplemented with growth factors. To determine multipotency, freshly isolated single cells were FAC sorted in 96 well plates (TPP). Ten plates per population were filled with single cells, and regularly checked for the presence of spheres (n=3) for four weeks. Neurospheres derived from single cells were dissociated by using Accutase (Omnilab, Switzerland) and expanded for two passages. Differentiation was induced by withdrawal of growth factors and cultivation with 0.5% FBS on poly-L-lysine coated coverslips.

Immunocytochemistry

After ten days under differentiation conditions, isolated cells were fixed with 4% PFA and immunostained for the presence of RIP (1:100, DSHB, USA), GFAP (1:2000, 6F2, Dako) and β III Tubulin (1:1200, 5G8, Promega), secondary antibodies α -mouse Alexa 488 (1:2500, Molecular Probes), Alexa 546 (1:2500, Molecular Probes).

In vivo proliferation assay

The proliferation activity of the cell populations we identified through FACS was investigated using *in vivo* BrdU labeling. We applied BrdU via drinking water (1 mg/ml) for 14 days or one day to adult mice (Fig. 6E). The BrdU solution was light protected and changed daily to avoid degradation of BrdU. One day and one week after the last BrdU application we isolated LeX⁺, Prom-1⁺, ALDH^{high} and triple negative cells from the SVZ and SC using a pre-enrichment by percoll gradient followed by FACS and quantified the percentage of BrdU positive cells in the respective population. For each experiment the tissue of ten animals was pooled, dissociated to a single cell suspension and sorted as described above (n=pooled tissue of 10 animals, n=4-6 for animals with 14 days of BrdU application, n=3 for animals with 1 day of BrdU application). Cells were plated on poly-L-lysine coated chamber slides, allowed to adhere for 3-4 hours and then fixed with 4% PFA. BrdU staining was performed according to the protocol described before (Thallmair et al., 2006). Briefly, cells were incubated for 30 minutes in 2 N HCL at 37 °C, rinsed with 0.1 M borate buffer for 10 minutes, washed and incubated with rat α -BrdU (Biozol, Germany) over night at 4 °C. After three washing steps in PBS, cells were labeled with goat α -rat Alexa 488 antibody (Molecular probes) for two hours and counterstained with Hoechst.

Immunohistochemistry

Transcardial perfusion with 4% PFA in 0.1 M phosphate buffer (PB) at pH 7.4 was performed after mice were anesthetized. Isolated brains and spinal cords were postfixed overnight at 4 °C and cryopreserved in 30% sucrose in PB. Brain and spinal cord tissue was cut on a cryostat into 25 μ m thick cross sections. The BrdU and Prom-1 immunostainings were performed using the same protocols as described previously (Thallmair et al., 2006). The primary antibodies used were: rat α -BrdU (Biozol, Germany) and mouse α -Prominin-1 (1:100, MAB4310, Chemicon). LewisX stainings were performed with mouse α -CD15 (1:50, VIMC6, Miltenyi) and mouse α -CD15 (1:250, MMA, BD) according to a previously published protocol (Reifenberger et al., 1992). Cell nuclei were identified using Hoechst bisbenzidine 33342 (Axonlab, Switzerland).

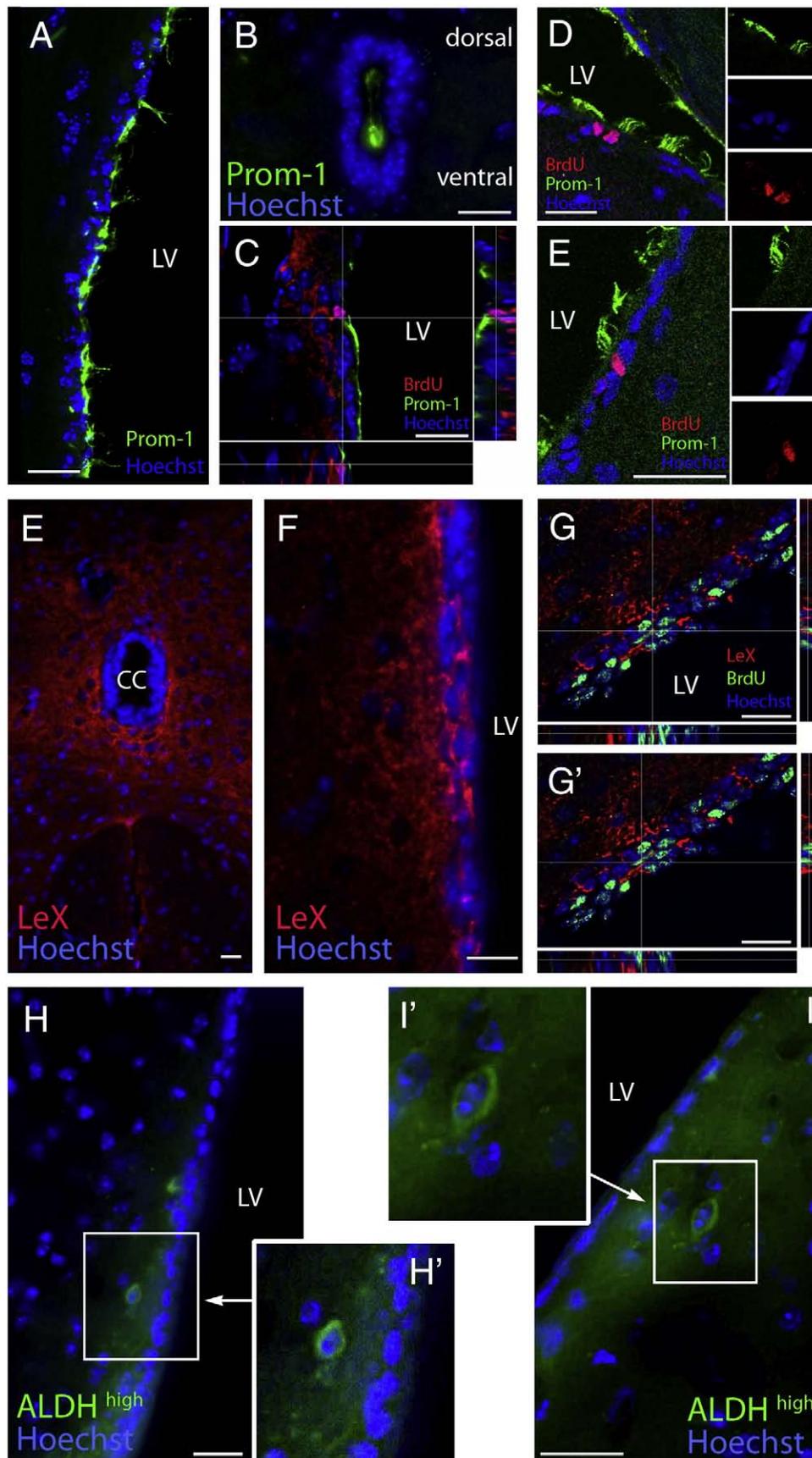
Images were acquired using a fluorescent microscope (Axioscope 2, 2 plus; AxioVision, software, Zeiss, Switzerland) and a confocal microscope (TCS SP2 AOBs, Leica Microsystems AG, Germany).

In vivo aldehyde dehydrogenase activity staining

After drilling a 4 mm² hole in the skull of deeply anesthetized 8 week old mice 5 μ l of Aldefluor stock solution was injected into the lateral ventricle using a Micro4 syringe controller (World Precision Instruments, USA) in combination with a Hamilton syringe (Hamilton, Switzerland). The injection flow rate was 80 nl/min. During the injection and drilling process mice were fixed in a mouse stereotactic frame (Kopf, Germany). After 4 hour incubation, animals were perfused and the tissue was processed as described above.

Quantitative Real-Time polymerase chain reaction (qRT-PCR)

Immediately after separating the cells by FACS, mRNA was isolated with RNeasy Micro kit (Qiagen). For cDNA synthesis we used SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed with Applied Biosystems 7500 Real-Time PCR Systems, in combination with SYBR master mix (Applied Biosystems). Gene expression of the target genes was normalized to three housekeeping genes: Elongation factor 1A1 (NM_010106.2; FWD: TCCACTTGGTCGCTTTGCT, REV: CTCTTGTCCACAGCTTTGATGA), TATA box binding protein (NM_013684.3; FWD: TTGACCTAAAGACCATTGCACTTC, REV: TTCTCATGATGACTGCAGCAAA) and Tubulin β (NM_001080971.1; FWD: GCAGTGGCAACCAGAT, REV: AGTGGGATCAATGCCATGCT). Primers for Notch-1 (NM_008714.2) and Pax6 (NM_013627.4) were achieved from Qiagen (QT00156982, QT01052786), for Sox2 (NM_011443.3) and Musashi-1 (Msi1, NM_008629.1) from SuperArray (PPM04762A, PPM25245A). Primer sequences for: Mash-1 (NM_008553; FWD: GACTTTGGAAGCAGGATGG, REV: GCTGTCTGTTTGTGTTTGTTC), CyclinD1 (CycD1, NM_007631.2; FWD: CTGGATGCTGGAGGTCTGTGAGG, REV: CTGACGGCGCTCTTCTTCAAG), ABCG2 (NM_011920.3; FWD: CTAACCTGCCATTCAAATGCT, REV: GTTGAAGTCGAAGAGCTGCTGAGA), MCM2 (NM_008564.1; FWD: ACCAGGACAGGACCAGCATCCA, REV: TGGGGTTGGCAGCAGCTATGA), Nestin



(Nes, NM_016701.3; FWD: TACAGGACTCTGCTGGAGGCTGAGA, REV: CTGGTATCCCAAG GAAATGCAGCTT), GFAP (NM_010277.3; FWD: GCAGAAGCTCCAAGATGAAAC, REV: CCTTTCTCTCAAATCCACAC). Every sample was analyzed in quadruplicates, graphs show the gene expression fold change related to the sample with the lowest detectable expression. The specificity of the primers was controlled by amplicon melt curve analysis.

Statistics

Graphs show the mean values of independent experiments \pm standard deviation. Statistical significance was evaluated using unpaired Student's *t* test ($p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$).

Results

Localization of cells positive for Prominin-1, LewisX and increased ALDH activity in the SVZ and SC

To assess the *in vivo* localization of cells expressing the putative stem/progenitor markers Prominin-1 (Prom-1), LewisX (LeX) or showing increased ALDH activity we performed immunohistochemistry. Prom-1 was detected along the lateral ventricle walls and was highly expressed on ependymal cells or cells close to the ependymal layer (Fig. 1A). Additionally, Prom-1 immunoreactivity was detected in close proximity of BrdU positive nuclei suggesting that Prom-1 cells underwent mitosis (Figs. 1C, D). In the spinal cord ependymal cells forming the central canal (CC) express Prom-1 in the thoracic, cervical and lumbar area (Fig. 1B). Cells located ventrally in the central canal show an increased expression of Prom-1 compared to cells in the dorsal region and few Prom-1⁺ cells detected lateral to the CC.

The immunostaining for LeX, which is also found in the extracellular matrix (Capela and Temple, 2002; Gocht et al., 1996), showed LeX immunoreactivity around the CC of the spinal cord (Fig. 1E) and in subependymal layers of the SVZ (Figs. 1F, G) - regions known for the presence of NSPCs (Reynolds and Weiss, 1992; Sakakibara et al., 2007).

To identify cells with an increased ALDH activity in the SVZ and SC *in vivo*, we injected Aldefluor into the lateral ventricle as we hypothesized that Aldefluor (the substrate of ALDH) is able to freely diffuse through the tissue as it is uncharged. Four hours after injection we detected single cells in the SVZ with increased levels of Aldefluor in the cytosol marking them as cells with high ALDH activity (Figs. 1H, I).

Undifferentiated neural cells are enriched through density gradient centrifugation

To assess whether density gradient centrifugation reliably and exclusively enriches undifferentiated neural cells we per-

formed intracellular stainings for mature neural cell markers after the Percoll gradient and analyzed the cells using FACS. We detected ~15% GFAP-labeled cells after density gradient centrifugation of isolates from the SVZ (Fig. 2A) and SC (Fig. 2G), suggesting the presence of GFAP positive NSPCs and/or astrocytes after this enrichment step. However, we did not detect any NeuN, NF160 (neurons) or CC1 (oligodendrocytes) in cells isolated from the SVZ (2B, E, D) or SC (2H, K, J) after purification. To quantify the amount of ependymal cells present after Percoll gradient we assessed cells for the co-expression of CD24, an ependymal and neuroblast marker (Coskun et al., 2008), and Prom-1 which is found also on ependymal cells. CD24⁺ cells were almost absent and we only detected about 0.1% Prom-1⁺/CD24⁺ cells in the SVZ (Fig. 2C) and the SC (Fig. 2I). Positive controls for all markers shown in Fig. 2 are shown in supplementary Fig. 2.

The percentage of endothelial cells which are known to express LeX (Majuri et al., 1999) was assessed by labeling cells with CD31 and LeX after Percoll. About 1.1% of cells isolated from the SVZ (Fig. 2F) and 0.8% of cells derived from the spinal cord (Fig. 2L) were positive for CD31 and LeX identifying them as endothelial cells. Thus, the majority of LeX⁺ cells (more than 90%) did not express CD31 after pre-enrichment identifying them as non-endothelial. Taken together purification by density gradient centrifugation enriches for undifferentiated cells and yields a high amount of NSPCs.

Identification of different NSPC populations characterized by the expression of Prom-1, LeX and ALDH activity using FACS

After pre-enrichment of NSPC with density gradient centrifugation (Palmer et al., 1999) cells were gated for living and dead cells (Fig. 3A) and cell doublets were excluded. Only living cells were analyzed for the surface expression of Prom-1, LeX and for an increased ALDH activity. For the identification of cells with an increased ALDH activity, we performed the Aldefluor assay as described by the manufacturer. The specificity for the labeling of ALDH^{high} cells and ideal incubation periods were investigated through time series experiments (supplementary Fig. 1). A well defined separation of ALDH^{high} and ALDH^{low} cells was detected after 30 minutes incubation at 37 °C with Aldefluor.

Representative FACS density plots in Fig. 3 show the major cell populations identified in the SVZ (Figs. 3C-E) and in the SC (Figs. 3I-K) and unstained controls (Figs. 3F-H, L-N). The main cell types detected were Prom-1⁺, LeX⁺, ALDH^{high}, LeX⁺/ALDH^{high} and triple negative cells. The percental distribution of marker defined populations isolated from SVZ and SC are shown in Fig. 3B. ALDH^{high} and LeX⁺ cells showed a similar percental distribution in the SVZ and SC. Differences were observed for the LeX⁺/ALDH^{high} and Prom-1⁺ populations. While the percentage of LeX⁺/ALDH^{high} cells was twice as high in the SC compared to the SVZ (SC

Figure 1 Cells expressing Prominin-1, LewisX and with an increased ALDH activity in the adult SVZ and SC. Confocal images of Prom-1 expression in the SVZ are shown in A and in combination with BrdU in C, D and E. Cell expressing high levels of Prom-1 in the SC are mainly detected in the ventral part of the central canal (CC; arrows) and a few Prom-1 positive cells were found in the periventricular region (B). LeX enriched areas were detected in the SC around the CC (E) and in the SVZ in subependymal layers (F, G). BrdU/LeX double labeling is illustrated in confocal images in H. ALDH^{high} cells are shown in I-J. Scale bars indicate 20 μ m. LV, lateral ventricle; CV, cresyl violet; CC, central canal.

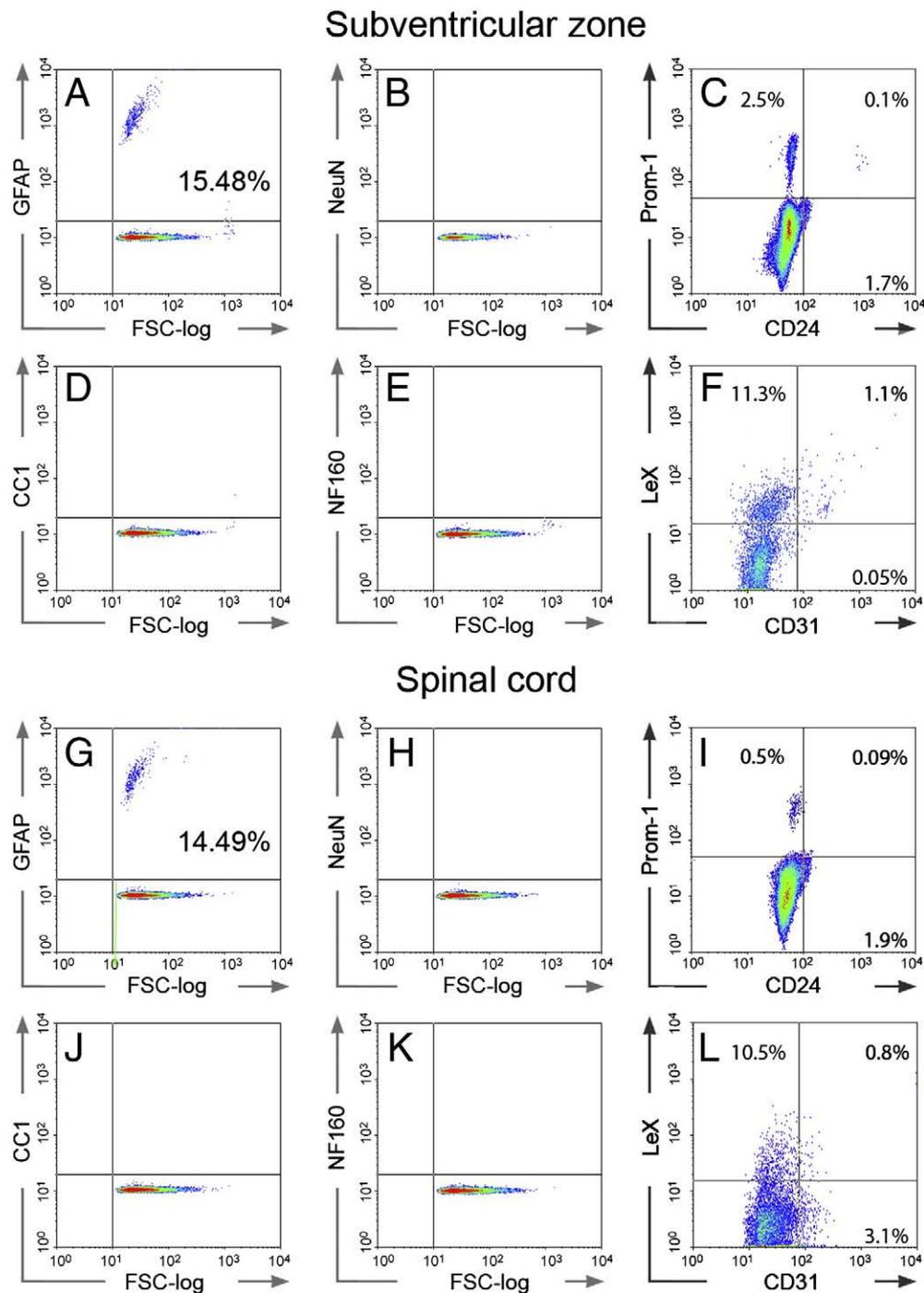


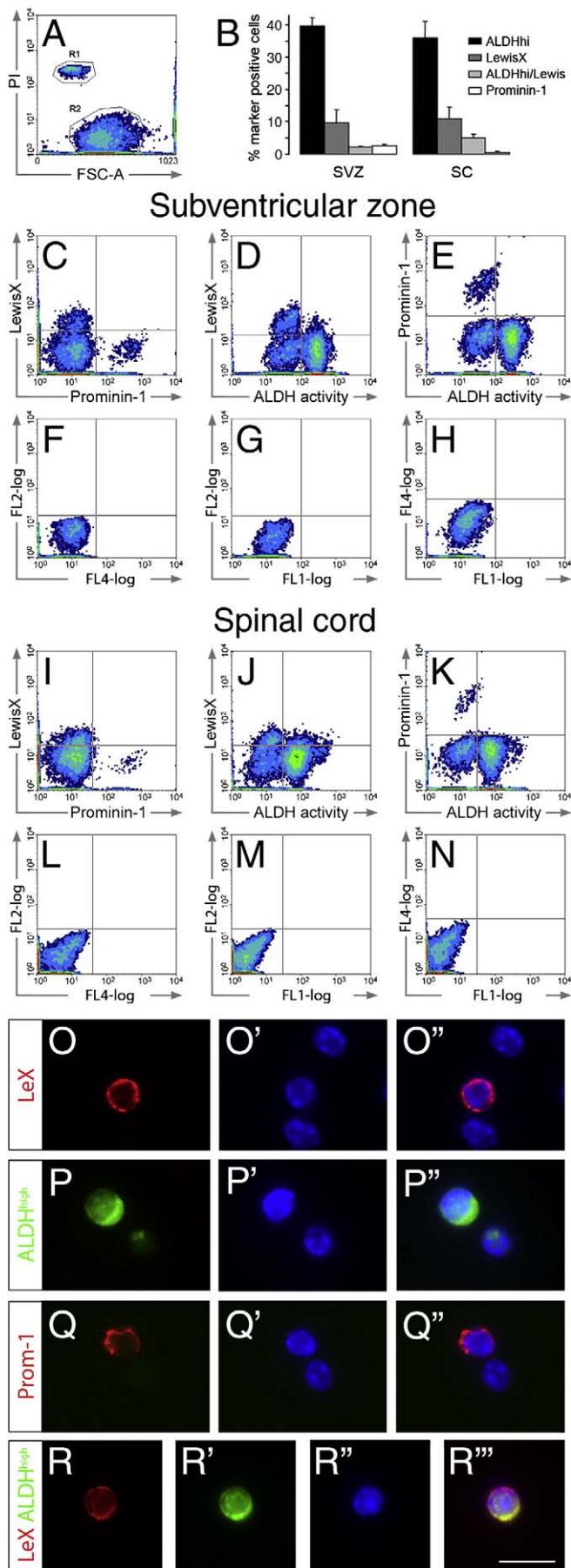
Figure 2 Cell type analysis using FACS after density gradient centrifugation. To verify whether density gradient centrifugation enriches specifically for undifferentiated cells (NSPCs) we intracellularly immunostained cells isolated from the SVZ and SC after Percoll for mature cell lineage markers. The neuronal markers NeuN, NF160 and the oligodendrocyte marker CC1 were not detected in cell isolates from the SVZ (B, E, D) or the SC (H, K, J). However, GFAP (astrocytic or stem cell marker) positive and a few CD24 (ependymal marker) and CD31 (endothelial marker) positive cells were present after the Percoll gradient (SVZ A, C, F and SC G, I, L). Positive controls before Percoll density gradient centrifugation are shown in supporting Fig. 2. The numbers in the FACS blots correspond to the percentage of positive cells in the respective quadrant.

$4.98 \pm 1.32\%$, SVZ $2.14 \pm 0.23\%$), the Prom-1⁺ population was five-fold lower in the SC (SC $0.53 \pm 0.31\%$, SVZ $2.55 \pm 0.39\%$).

To validate the cell populations identified through FACS, we analyzed unsorted cells for the presence of the three markers using immunofluorescence. We observed the expected cell types and were able to verify the specificity

of the stainings (Figs. 3O-R). As expected LeX (Figs. 3O, R) and Prom-1 (Fig. 3Q) were detected on the cell surface while the Aldefluor accumulation, identifying ALDH^{high} cells, was observed in the cytosol (Figs. 3P, R).

Very few cells in the SVZ and SC (Figs. 3E, K) ($\sim 0.01\%$) were Prom-1⁺/ALDH^{high}, however, due to the limited



number of cells they were not further characterized in this study. We were not able to identify a cell population in the adult SVZ or in the SC being double positive for Prom-1 and LeX, confirming an earlier finding (Coskun et al., 2008).

Expression of stem and progenitor cell associated genes

The current classification of subpopulations of neural stem and progenitor cells is mainly based on intracellular markers, which are used to discriminate between quiescent or rarely dividing stem and rapidly dividing progenitor cells. Thus, we examined the expression of commonly used stem cell genes, in the sorted cell populations. ABCG2, Musashi-1, Nestin and Notch-1 (Ernst and Christie, 2005; Hitoshi et al., 2002; Islam et al., 2005; Sakakibara et al., 1996), all associated with stem cells in the SVZ, were highly expressed in the Prom-1⁺ population of the SVZ and SC. The gene expression pattern of the Prom-1⁺ population which is in general similar in the SVZ and SC (Fig. 4, Table 1) could be an indication for a preserved NSPC subtype in the two regions. Comparable results in the SVZ and SC were also observed for cells being LeX⁺ and/or ALDH^{high}. ABCG2, Musashi-1, Nestin, Sox2, GFAP and Notch-1 are expressed in these populations but at a lower level compared to Prom-1⁺ cells. The main difference between the populations derived from the neurogenic SVZ and the non-neurogenic SC concerned the expression of Pax6 which is known to be a key factor in neuronal differentiation (Hack et al., 2005; Osumi et al., 2008), and which was absent in SC populations. Besides its expression in the Prom-1⁺, LeX⁺ and LeX⁺/ALDH^{high} fractions Pax6 showed a high expression in Prom-1⁻, LeX⁻ and ALDH^{low} cells. Additionally to the high Pax6 expression this triple negative cell type showed high levels of CyclinD1 and Mcm2 mRNA indicating a high proliferation rate. Together with the increased level of Mash-1 transcript the gene expression pattern of the triple negative population from the SVZ suggests that this subpopulation is representative for transit amplifying neural progenitor cells. Interestingly, we detected high Mash-1 levels in the triple negative population of the SC likely attributable to oligodendrocyte progenitors that were shown

Figure 3 Identification of different NSPC populations characterized by the expression of Prom-1, LeX and ALDH activity using FACS. After NSPC pre-enrichment through density gradient centrifugation cells were analyzed for the expression of LeX, Prom-1 and ALDH activity through FACS. Dead cells were identified using PI (gate R1 in A) and separated from living cells (gate R2 in A). Only living cells were further investigated for the presence of markers. FACS plots identifying different cell populations derived from the SVZ and from the spinal cord are shown in C-E, and unstained controls are shown in F-H (SVZ) and L-N (SC). We observed no co-expression of LeX and Prom-1 in the SVZ (C) or in the SC (I). Populations expressing LeX and/or ALDH are shown in D for the SVZ and J for the SC. Very few cells being Prom-1⁺/ALDH^{high} in both regions are shown in E and K respectively. The percentage of the populations found after pre-enrichment and FACS is shown in B. Representative pictures of immunolabeled cells before the separation through FACS are depicted in O-R. Hoechst nuclear staining is shown in blue. Scale bar represents 20 μ m.

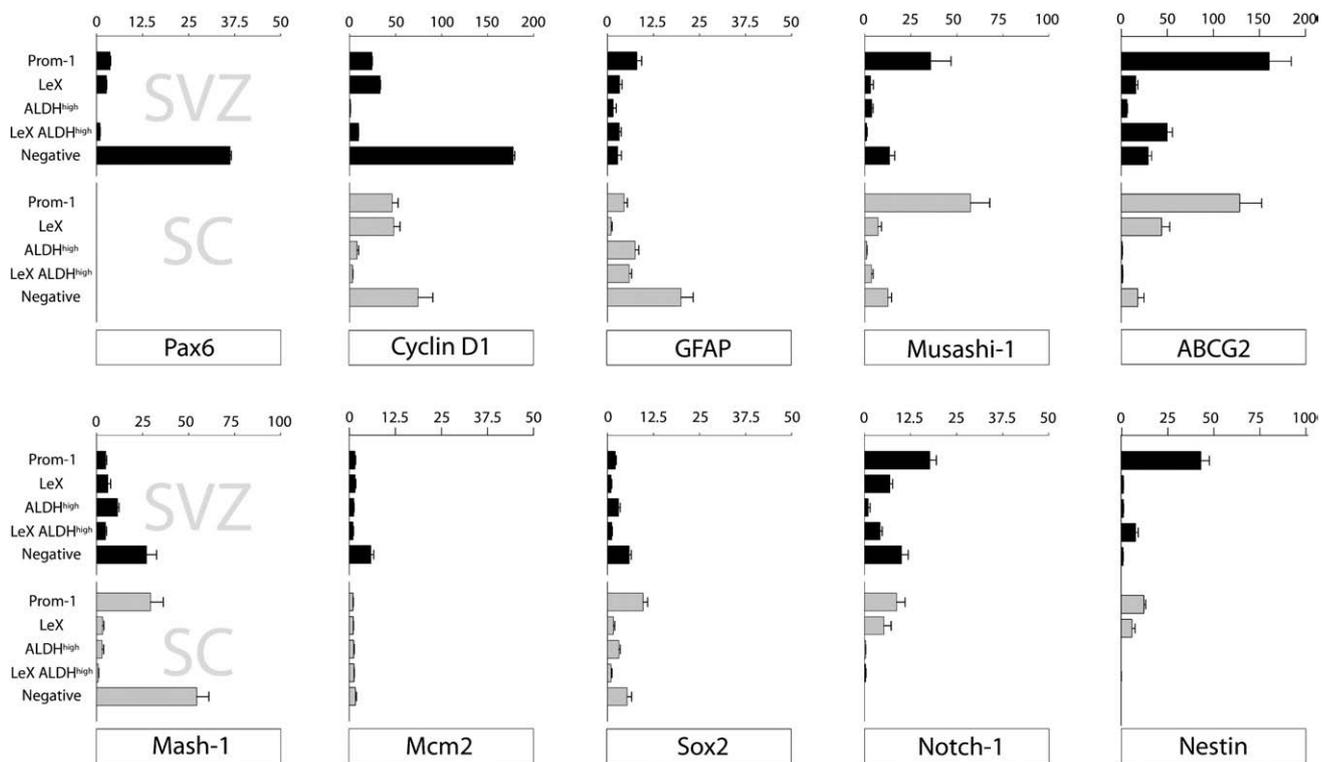


Figure 4 qRT-PCR analysis of NSPC associated genes. Prom-1⁺, LeX⁺, ALDH^{high} and LeX⁺/ALDH^{high} cells were analyzed for the expression of NSPC associated genes. The graphs show the normalized expression of genes relative to the cell population with the lowest expression. Black represents SVZ, gray SC derived cells.

to express Mash-1 in the SC (Ohori et al., 2006). The absence of a rapidly dividing population in the intact SC was supported by reduced expression of CyclinD1 and Mcm2 compared to the SVZ in the triple negative population.

Neurosphere formation and multipotency

The stem cell potential of the isolated populations was tested by a single cell clonal analysis using the sphere

forming assay. Neurospheres were only generated from LeX⁺, ALDH^{high} and LeX⁺/ALDH^{high} cells derived from the SVZ, and from LeX⁺ and ALDH^{high} populations of the SC. The frequency of spheres formed by single cells isolated from the SVZ was one out of 221 ± 80 of the LeX⁺ cell population, one out of 240 ± 64 from ALDH^{high} cells and one out of 411 ± 92 of LeX⁺/ALDH^{high} cells. In the SC one in 320 ± 122 LeX⁺ cells and one in 720 ± 277 ALDH^{high} gave rise to neurospheres. The remaining populations did not form neurospheres under the culture conditions used. Single cell derived neurospheres formed

Table 1 Data summary

		Proliferation		Multipotency			Gene expression							
		<i>in vivo</i>	<i>in vitro</i> *	clonal analysis	ABCG2	Msi1	Nestin	GFAP	Sox2	Notch-1	CycD1	Mcm2	Pax6	Mash-1
Subventricular zone	Prom-1 ⁺	slow	no	-	++++	+++	++++	++	+	++++	+	+	+	+
	LeX ⁺	rare	yes	yes	+	+	+	+	+	++	+	+	+	+
	ALDH ^{high}	rare	yes	yes	+	+	+	+	++	+	+	+	-	+
	LeX ⁺ ALDH ^{high}	rare	yes	yes	++	+	+	+	+	+	+	+	+	+
	negative	rapid	no	-	+	+	+	+	+++	++	++++	++++	++++	++
Spinal cord	Prom-1 ⁺	rare	no	-	++++	++++	++	+	++++	++	+	+	-	++
	LeX ⁺	rare	yes	yes	+	+	+	+	+	++	+	+	-	+
	ALDH ^{high}	rare	yes	yes	+	+	-	++	++	+	+	+	-	+
	LeX ⁺ ALDH ^{high}	rare	no	-	+	+	+	+	+	+	+	+	-	+
	negative	rare	no	-	+	+	-	++++	+++	-	++	+	-	++++

Table 1 illustrates an overview of the results obtained for proliferation, multipotency and gene expression of the different subpopulations derived from SVZ and SC. More detailed data about the gene expression is shown in Fig. 4. *single cell derived neurospheres.

secondary and tertiary spheres, which were dissociated and then cultured under differentiation conditions. Every single cell derived neurosphere contained cells of the neuronal (β III Tubulin), astroglial (GFAP) and oligodendroglial (RIP) lineage (Fig. 5), identifying them as multipotent. Even though single Prom-1⁺ cells did not form neurospheres, we cultivated them in differentiation medium to assess whether they give rise to cells from the neuronal and glial lineage. After ten days we observed β III Tubulin, GFAP and RIP positive cells (data not shown).

Proliferation *in vivo*

To investigate the proliferation of the identified cell populations *in vivo*, animals received BrdU over a period of 14 days. One day or one week after the last BrdU application NSPCs were isolated and sorted for Prom-1, LeX and ALDH activity (Fig. 6A) after pre-enrichment by a Percoll gradient. We then assessed the sorted cell population for BrdU retention (Fig. 6B-G). A significant decline in the percentage of BrdU⁺ cells one week after the last BrdU application - indicating a dilution of the BrdU signal by several rounds of proliferation - was only detected in the Prom-1⁺ ($5.27\% \pm 0.41$ to $2.22\% \pm 0.48$, $p < 0.01$, $n = 5$) and triple negative population of the SVZ ($10.4\% \pm 1.03$ to $2.25\% \pm 0.49\%$, $p < 0.001$, $n = 5$; Fig. 6D). In contrast, the ALDH^{high} and LeX⁺ cells from the SVZ did not show a significant reduction of BrdU⁺ cells and therefore represent a more quiescent NSPC population. To exclude the possibility that the decline of BrdU⁺ cells is due to migration of progenitor cells toward the olfactory bulb rather than a reduction in proliferation, we applied BrdU only over one day and analyzed the percentage of BrdU⁺ cells in each population. Again the triple negative cell fraction of the SVZ showed the highest percentage of BrdU⁺ cells (Fig. 6E), a clear indication that this cell population includes frequently dividing cells.

In the SC BrdU⁺ cells were detected in all four populations, but the percentage of BrdU⁺ cells was lower than in cells derived from the SVZ (Fig. 6F). After one day of BrdU application, very few BrdU labeled cells were detected only in the triple negative cell population (Fig. 6G) suggesting a low proliferation rate of SC NSPCs.

Discussion

We show here that distinct populations of NSPCs that differ in gene expression, self renewal and potency can be isolated from neurogenic and non-neurogenic regions of the adult CNS using surface markers that allow the prospective separation of discrete populations by FACS.

Prom-1⁺ cells represent a slowly dividing NSPC subtype

Prom-1⁺ cells were detected in the ependymal layer of the SVZ and around the central canal in the SC being consistent with an earlier report (Coskun et al., 2008). Gene expression analysis revealed that these cells show elevated levels of Nestin, Musashi-1, ABCG2 and Notch-1 but do also express GFAP and Sox2, genes which are associated with NSPCs (Doetsch et al., 1999; Islam et al., 2005; Lendahl et al., 1990; Mizutani et al., 2007; Sakakibara et al., 1996). We demonstrate that Prom-1⁺ cells divide *in vivo* and that more quiescent (LeX⁺ and/or

ALDH^{high} cells) and more rapidly dividing populations (triple negative cells) are present in the SVZ. Contradictory to some studies published earlier (Corti et al., 2007; Coskun et al., 2008), but in accordance with another report (Pfenninger et al., 2007), isolated Prom-1⁺ cells did not form neurospheres after single cell isolation under the culture conditions used, thus, we were not able to perform a clonal analysis of multipotency. To see if the absence of neurospheres in the Prom-1⁺ population could be due to the lack of CD133⁺/CD24⁺ ependymal cells, we isolated this population and performed a single cell neurosphere assay. As reported previously, these cells were not able to produce any neurospheres (Capela and Temple, 2002; Corti et al., 2007; Coskun et al., 2008). A reason for the conflicting data found in the literature on neurosphere formation ability of Prom-1⁺ cells might be different isolation and separation protocols. One of the main differences between these and our study concerns our isolation process by a combination of a density gradient centrifugation followed by FACS, which is a rather harsh treatment for the cells and as a result could lead to a decreased number of neurospheres. A second important difference is the cell density we used in this study. Unlike other reports we did not cultivate the isolated cells in clonal density, but carried out single cell assays, which conceivably may lead to a lack of factors or cell cell interactions that would otherwise enhance neurosphere formation.

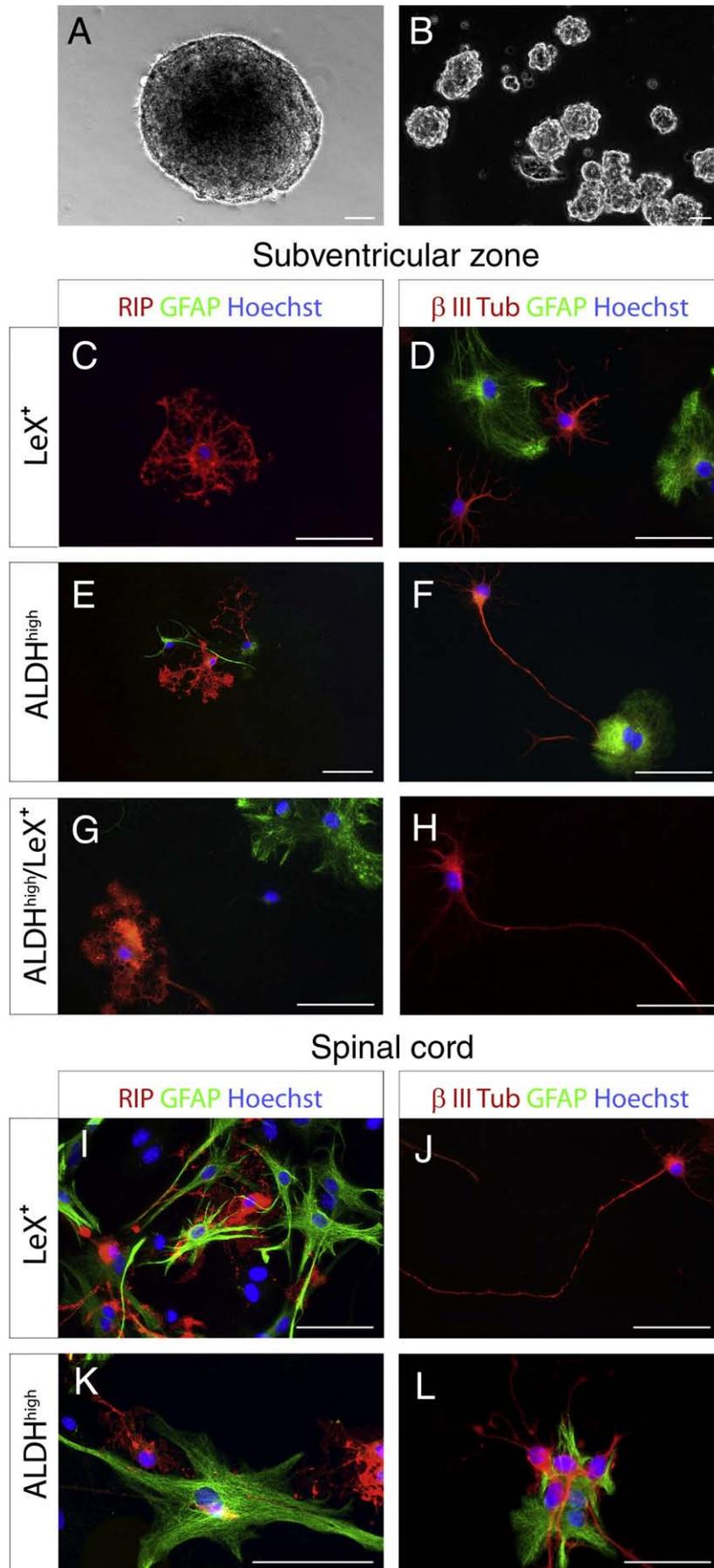
Whether the inability of cells to form neurospheres *in vitro* excludes neural stem cells identity *in vivo* has been doubted in different reports (Marshall et al., 2007; Morshead and van der Kooy, 2004; Reynolds and Rietze, 2005). In summary, we conclude that Prom-1⁺ NSPCs reside in the SVZ as well as in the SC and, interestingly, show very similar characteristics in terms of gene expression despite their origin from a neurogenic and non-neurogenic environment. Therefore, we suggest that Prom-1⁺ cells represent a conserved NSPCs subtype in the SVZ and SC.

Rarely dividing stem or progenitor cells are LeX⁺, ALDH^{high} and LeX⁺/ALDH^{high}

We observed single cells with accumulated Aldefluor in the SVZ indicating their presence in this neurogenic region. Compared to Prom-1⁺ cells, the ALDH^{high} cells were located a few cell layers away from the lateral ventricle and were never found in the ependymal layer. The unequal diffusion of Aldefluor, the ALDH substrate, in the CNS parenchyma and the complexity to intracellularly label enzyme activity in the tissue probably impeded a broad identification of cells with increased ALDH activity.

The presence of LeX in the extracellular matrix (Capela and Temple, 2002; Gocht et al., 1996) leads to rather diffuse immunostainings in the tissue and exacerbates the identification of individual LeX⁺ cells. An enrichment of LeX was found in the SVZ and around the central canal of the SC. Because of the high LeX immunosignal in these areas we suggest that LeX⁺ and LeX secreting cells reside in these regions, in close proximity to Prom-1⁺ cells in the SVZ and SC. Specificity of the immunostainings for LeX⁺ and ALDH^{high} was shown by immunofluorescent labeling of dissociated cells isolated from the SVZ and SC.

The ability of cells in these three populations - ALDH^{high}, LeX⁺, LeX⁺/ALDH^{high} - to form GFAP, beta III Tubulin and RIP positive cells from single cell derived neurospheres identifies



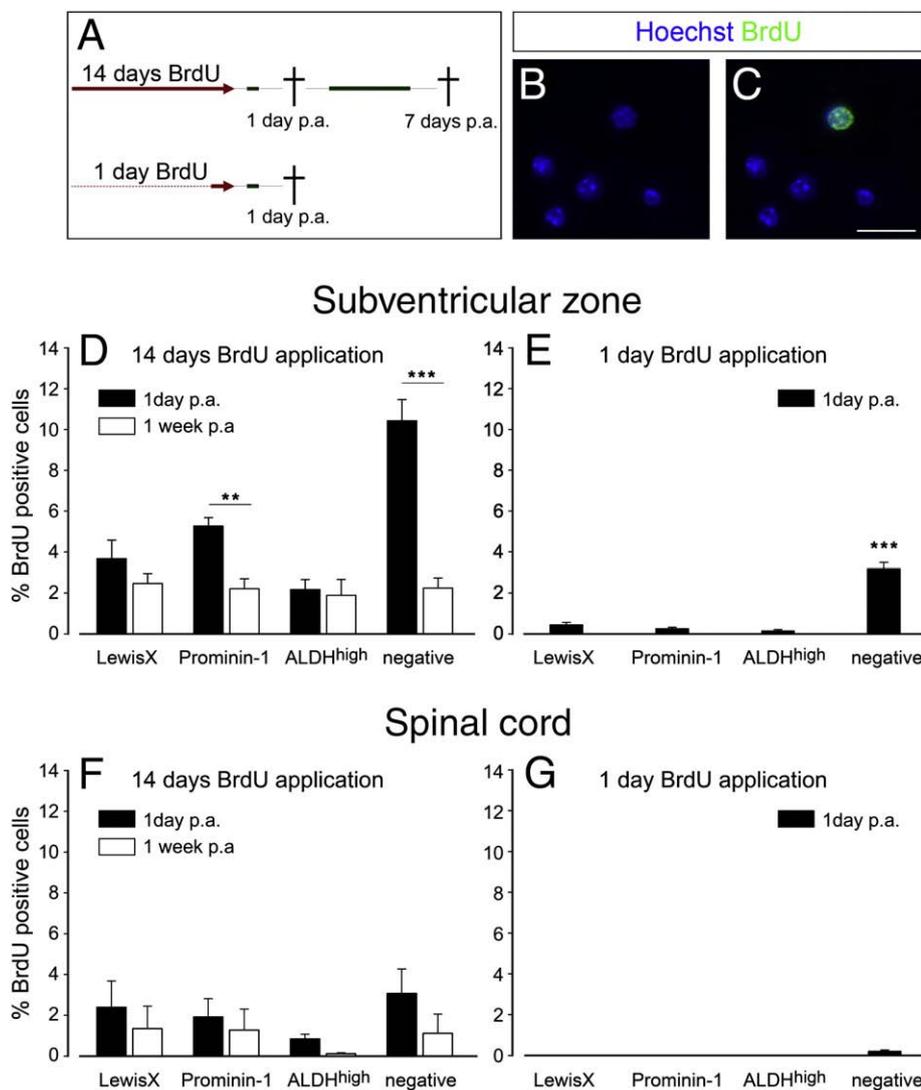


Figure 6 Proliferation activity of isolated NSPC populations *in vivo*. (A) Scheme of experimental procedure. To investigate *in vivo* the proliferation activity of the isolated cell populations we applied BrdU via drinking water for 14 days or one day to adult mice. One day after the last BrdU application (1 day p.a.) we isolated LeX⁺, Prom-1⁺, ALDH^{high} and triple negative cells from the SVZ and SC and quantified the percentage of BrdU positive cells found in the respective population. (B, C) Representative pictures of a BrdU⁺ cell found in the triple negative population. (D-G) Quantification of the percentage of BrdU⁺ cells found in the different populations of the SVZ (D) or SC (F) one day post BrdU application (1 day p.a., black bars), or 7 days p.a. (white bars). A significant decrease in the percentage of BrdU⁺ cells was only observed in the Prom-1 ($p \leq 0.01$) and negative population ($p \leq 0.001$) of the SVZ (D). The strong decrease of BrdU⁺ cells in the SVZ negative population is a clear indication for rapidly dividing cells. To exclude the possibility that the reduction of BrdU⁺ cells was due to a migration of BrdU⁺ cells towards the olfactory bulb, we applied BrdU for one day and quantified the percentage of BrdU⁺ cells 1 day p.a. (E, G). A significant increase of BrdU⁺ cells ($p \leq 0.001$) was observed only for triple negative cells of the SVZ (E). Scale bar represents 20 μm .

them as multipotent. Additionally, ALDH^{high}, LeX⁺, LeX⁺/ALDH^{high} cells divide very rarely or are almost quiescent as shown by the BrdU retention assay, and by the low expression of Cyclin D1 and MCM2. The expression of Nestin, Musashi-1, ABCG2, Notch-1 and GFAP in these populations is decreased compared to Prom-1⁺ cells.

To sum up, we suggest that NSPC included in the LeX⁺, ALDH^{high} and LeX⁺/ALDH^{high} cell populations are representative for quiescent neural stem cells in the SVZ and the SC. As shown for Prom-1⁺ cells, LeX⁺ and/or ALDH^{high} cells exhibit similar properties in both regions, the neurogenic SVZ and the non-neurogenic SC, in terms of gene expression, *in vivo*

Figure 5 Multipotency of adult NSPCs derived from single cell clones. Only LeX⁺, ALDH^{high}, LeX⁺/ALDH^{high} cells from the SVZ and LeX⁺, ALDH^{high} cells from the SC formed neurospheres. A shows a representative picture of a neurosphere derived from a single LeX⁺ cell from the SVZ and B secondary spheres. After transferring cells into differentiation conditions, the expression of RIP, GFAP and β III Tub was detected in cells derived from single cell clones of the SVZ (C-H) and spinal cord (I-L). Nuclei are stained with Hoechst (blue). Scale bars represent 50 μm .

proliferation, their ability to form neurospheres and multipotency.

Rapidly dividing cells in the SVZ are LeX⁻, Prom-1⁻ and ALDH^{low}

Within the SVZ cell population expressing none of the three markers (LeX⁻, Prom-1⁻, ALDH^{low}), we observed a high proliferation activity *in vivo* which we did not find in any other population. After 14 days of BrdU application about 10% of the triple negative population was BrdU positive. One week later without BrdU application the number of BrdU⁺ cells declined to about 2% indicating that either this population includes rapidly dividing cells and/or BrdU⁺ cells migrating away from the SVZ towards the olfactory bulb. To test the two possibilities we applied BrdU for only one day. Again, we observed a statistically significant decline of BrdU⁺ cells in the triple negative fraction clearly indicating the presence of rapidly dividing transit-amplifying progenitors. This conclusion is supported by a 20 fold higher expression of Cyclin D1 and 10 fold higher expression of Mcm2 in triple negative cells compared to the other populations. Additionally to the increased proliferation activity found in this cell population, we detected a very high expression of Pax6 and Mash-1, known to be upregulated in rapidly dividing neuronal progenitor cells in the SVZ (Osumi et al., 2008; Parras et al., 2004; Sakaguchi et al., 2006). Moreover, we found a decreased expression of GFAP which was shown to be downregulated in rapidly dividing cells (Aguirre et al., 2004). The increased dividing rate along with Pax6 and Mash-1 expression of these cells suggests that LeX⁻, Prom-1⁻, ALDH^{low} cells belong to a rapidly dividing cell population described earlier (Doetsch et al., 1997). We assume that the transit amplifying population is downstream of LeX⁺, ALDH^{high} and Prom-1⁺ cells and its presence depends on differentially expressed environmental cues in the SVZ and SC.

Conclusion

The ongoing search for definitive markers of neural stem and progenitor cells needs distinct positive and negative selection criteria for the different subpopulations, comparable to the hematopoietic system. We show here that Prominin-1, Lewis X and an increased ALDH activity can be used as selection marker to define quiescent, slowly dividing and rapidly dividing NSPC populations. Prom-1⁺, LeX⁺ and/or ALDH^{high} cells share similar characteristics in the SVZ and SC in terms of gene expression patterns, their ability to form neurospheres and multipotency *in vitro*. Thus, we describe an approach to prospectively isolate different NSPC populations by a combination of density gradient centrifugation and FACS allowing direct investigations of these cells by genomic and proteomic approaches. Our prospective isolation method contributes to the definition of different stem and progenitor cell populations by surface and intracellular markers and will help to circumvent the identity crisis of adult neural stem cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.scr.2010.05.001](https://doi.org/10.1016/j.scr.2010.05.001).

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