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Phenotypic and molecular identity of cells in the adult subventricular zone: in vivo and after expansion in vitro

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Abstract

We have studied the molecular identity of adult mouse SVZ cells in situ, and after isolation and expansion as neurospheres in vitro. The gene and protein expression patterns of the adult cells have been compared to that of the cells from the lateral ganglionic eminence (LGE), their putative embryonic counterparts. The LGE gives rise to both striatal projection neurons and olfactory bulb interneurons via spatially and molecularly distinct progenitor populations present in the SVZ of the LGE. These two populations are thought to have a common origin in the GSH2 expressing cells of the embryonic LGE ventricular zone. We found that a significant number of cells in the adult SVZ, and in the in vitro expanded neurospheres, derived from the adult SVZ express GSH2. However, under normal conditions, GSH2-expressing cells in the adult SVZ and in the in vitro expanded neurospheres appear to specify only olfactory bulb progenitors and not striatal progenitors.

Introduction

One of the major neurogenic regions in the adult brain is the subventricular zone (SVZ) of the lateral ventricle (Altman, 1969; Bayer, 1983; Corotto et al., 1993). The neural stem-like cells in the SVZ self renew and give rise to the cells that proliferate and migrate along the rostral migratory stream (RMS) to the olfactory bulb, where they differentiate into interneurons of the granular and glomerular layers. (Luskin, 1993; Lois and Alvarez-Buylla, 1994). The periventricular region (ependymal layer and SVZ) contains at least four different cell types that are defined based on their morphology, ultrastructure, and expression of specific molecular markers (Doetsch et al., 1997, 1999a, 2002): (1) type A cells are neuroblasts that express molecular markers such as PSA-NCAM, TUJI/β-III-tubulin, nestin, and DLX2 (Doetsch et al., 1997, 2002). They have elongated cell bodies with few processes and form tangentially oriented chains in the SVZ and RMS; (2) type C cells are rapidly proliferating, transiently amplifying cells that give rise to type A cells (Doetsch et al., 1999b). These precursors express nestin and DLX2, and represent around 10% of the cells in the SVZ but are rarely found in the intact RMS (Doetsch et al., 1997, 1999b, 2002; Gritti et al., 2002); (3) type B cells are the astrocyte-like cells expressing GFAP and nestin (Doetsch et al., 1997, 1999a), and have been shown to generate the type A neuroblasts via the type C cells in both the intact and depleted SVZ (Doetsch et al., 1999a, 1999b) and in the depleted RMS (Gritti et al., 2002); and (4) type E cells are ependymal cells and they line the ventricle forming an epithelial monolayer between the SVZ and lateral ventricle. These cells express vimentin, nestin, and GFAP can be detected by polyclonal, but not monoclonal, antibodies (Doetsch et al., 1997). Cells from the adult SVZ can be isolated and expanded in vitro as neurosphere cultures under epidermal growth factor (EGF) and/or basic fibroblast growth factor (bFGF) stimulation (Reynolds and

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Weiss, 1992; Richards et al., 1992; Vescovi et al., 1993; Morshead et al., 1994; Gritti et al., 1996). However, it remains to be determined which specific cell type(s) in the adult SVZ that proliferate and form neurospheres in vitro, and which cell types are present in the resulting spheres.

Based on gene expression pattern, morphology, and type of neurons generated, the lateral ganglionic eminence (LGE), located in the floor of the embryonic telencephalon, is the most likely candidate for giving rise to the adult SVZ (Sturrock and Smart, 1980; Chiasson et al., 1999; Stenman et al., 2003). Transplantation studies have shown that both striatal projection neurons and olfactory bulb interneurons are generated from the LGE (Deacon et al., 1994; Olsson et al., 1995, 1997, 1998; Wichterle et al., 1999, 2001). The generation of striatal projection neurons ceases around birth, whereas the production of olfactory bulb interneurons continues in the adult brain (Luskin, 1993; Lois and Alvarez-Buylla, 1994).

During development, spatially and molecularly distinct progenitor populations in the LGE SVZ have been suggested to differentially contribute to the generation of striatal projection neurons and the olfactory bulb interneurons. Both progenitor populations express the transcription factors DLX and MEIS2. In addition, the LIM homeodomain protein Islet1 (ISL1) is expressed in most of the LGE SVZ and marks the progenitors that generate the striatal projection neurons (Toresson et al., 2000; Toresson and Campbell, 2001; Wang and Liu, 2001). Recently, a population of cells that does not express ISL1 has been identified in the dorsal most portion of the LGE. This population expresses the ETS-related transcription factor Er81 and represents the progenitor population that generates the interneurons of the olfactory bulb (Stenman et al., 2003). Genetic studies suggest that both progenitor populations have a common origin in the GSH2-expressing cells residing in the LGE ventricular zone (VZ) (Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001, 2003; Stenman et al., 2003).

In the present study, we have analyzed the expression of a set of LGE characteristic developmental genes and corresponding proteins, with particular emphasis on Gsh2, in the adult SVZ and in neurosphere cultures derived from this region. In addition, we have studied the effect of EGF and bFGF on neurosphere formation and on the phenotypic and molecular composition of cells present in the expanded SVZ neurosphere cultures.

Results

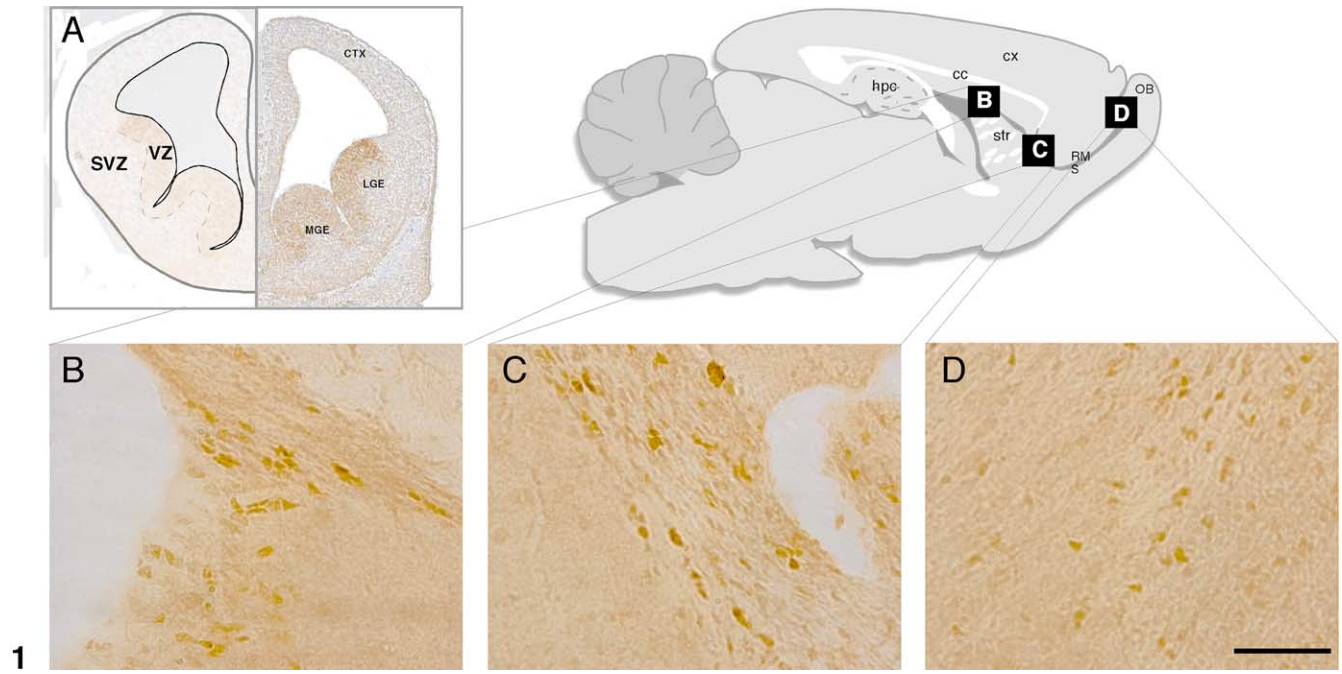
Expression of developmentally regulated transcription factors in the SVZ of adult mice

We analyzed the expression of transcription factors, known to be active during the development of the LGE, in the SVZ of adult mice by immunohistochemistry and by RT-PCR. Interestingly, we found a strong correlation between GSH2 expression and the areas involved in adult olfactory bulb neurogenesis. GSH2 was found to be expressed at high levels in many of the cells in the adult SVZ (Fig. 1B), and in the RMS (Fig. 1C), and at a lower level also in the subependymal layer of the olfactory bulb (Fig. 1D). The only other site in the adult brain that we detected GSH2 expression was in a few scattered cells in, or just underlying, the corpus callosum (data not shown).

GSH2 is expressed in the dividing cells in the VZ of the embryonic LGE (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001, 2003), and is implicated in the generation of both striatal projection neurons and olfactory bulb interneurons (see discussion for details). Thus, the expression of Gsh2 (Figs. 1A–D, 2A, and 3A) may indicate the presence of an immature precursor population with the potential to specify formation of both striatal and olfactory bulb progenitors/neurons. We looked for the presence of these different progenitors in the adult SVZ using immunohistochemistry (Fig. 2A–D), and RT-PCR (Fig. 3A). Meis2 is expressed in both these progenitor populations during development, and we found that its expression persisted in the adult SVZ (Figs. 2D and 3A). As a marker of the olfactory bulb progenitor population, we found Er81 to be expressed in the adult SVZ (Figs. 2B and 3A) and RMS (Stenman et al., 2003). Some cells in the striatum were also positive for Er81. The majority of the Er81 positive cells in the striatal parenchyma expressing cells are parvalbumin-positive interneurons (Z.K., unpublished observation), and they have been shown to coexpress NKX2.1, thus are likely to be interneurons derived from the medial ganglionic eminence (MGE) (Stenman et al., 2003). In contrast, no ISL1-immunopositive striatal progenitor cells were found in the adult SVZ (Fig. 2C). Low levels of *Isl1* mRNA were detected in RNA isolated from adult SVZ (Fig. 3A). Since Isl1 is expressed by differentiating striatal projection neurons and by differentiating and mature striatal cholinergic inteneurons (derived from the MGE) (Toresson et al., 2000; Toresson and Campbell, 2001; Wang and Liu, 2001), it is likely that the PCR signal was due to the contamination by striatal interneurons.

The GSH2 expressing precursors are expandable as neurospheres in vitro

In vitro, SVZ cells can be expanded as neurospheres in the presence of EGF and/or bFGF (Reynolds and Weiss, 1992). To determine whether the molecular phenotypes are maintained in the in vitro expanded SVZ cells, and if EGF and bFGF differentially affect the molecular identity of the in vitro expanded cells, we analyzed the gene expression in undifferentiated neurospheres. Independent of growth factor condition, the neurospheres formed after the first passage are similar to their in vivo counterparts expressing *Gsh2, Meis2,* and *Er81,* but not *Isl1* (Fig. 3B). At passage 5, the EGF and EGF + bFGF cultures (we did not expand cells in bFGF alone up to passage 5) maintained this molecular identity (Fig. 3C). The extent of



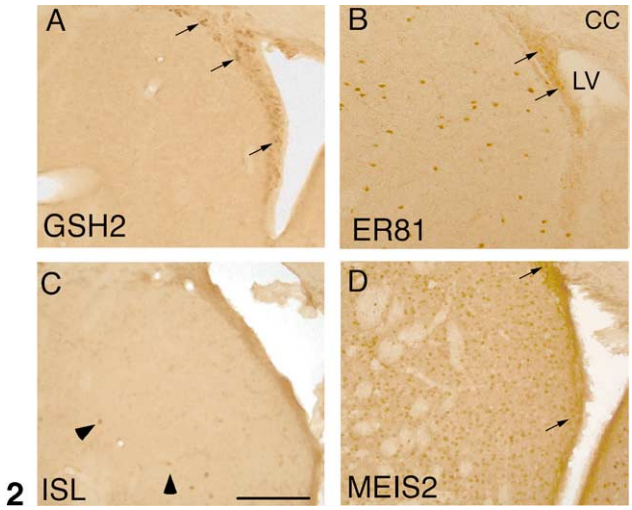


Fig. 1. Expression of GSH2 in the adult brain. (A) Schematic drawing (left) and GSH2 staining (right) illustrating the VZ restricted expression of GSH2 in the developing brain. (B–D) schematic drawing indicates the level of the sagittal sections at which the images have been captured. GSH2 is expressed at high levels in the SVZ (B), RMS (C), and at lower levels in the olfactory bulb (D). VZ = ventricular zone; SVZ = subventricular zone; LGE = lateral ganglionic eminence; MGE = medial ganglionic eminence; CTX = cortical primordia; hpc = hippocampus; cc = corpus callosum; cx = cortex; str = striatum; RMS = rostral migratory stream; OB = olfactory bulb. Scale bar = 50 μm.

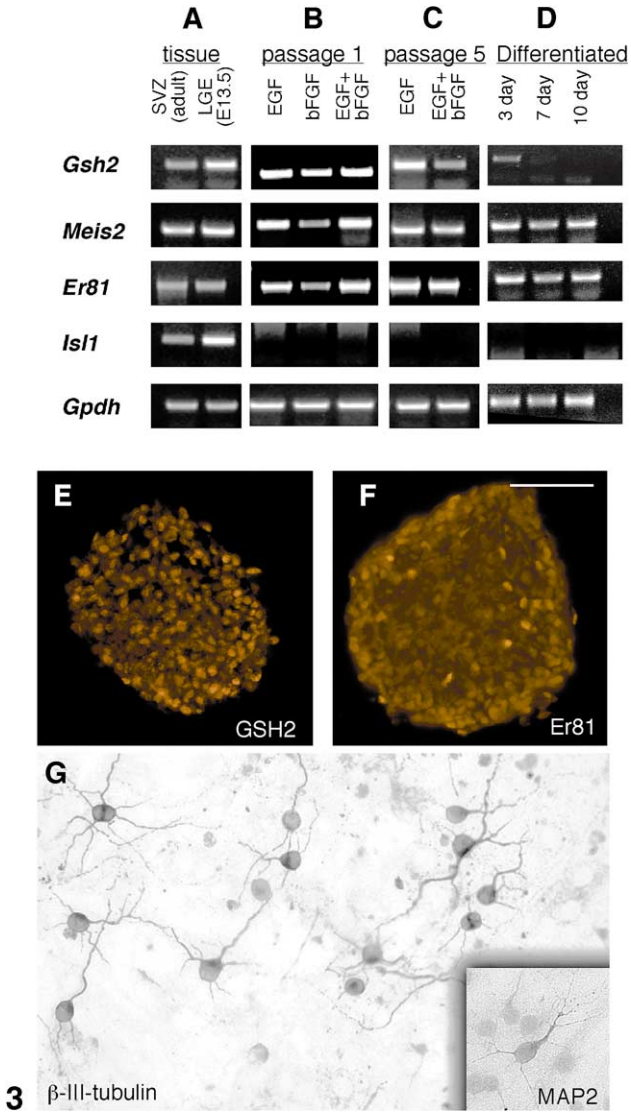
Fig. 2. Expression of developmentally important transcription factors in the SVZ of adult mice. (A) GSH2, (B) ER81, (C) ISL1, and (D) MEIS2. Note that GSH2 is expressed exclusively in the SVZ (arrow in A), ER81 and MEIS2 in the SVZ (arrows in B and D) and striatum, and ISL1 only in interneurons in the striatum (arrowhead in C). CC = corpus callosum; LV = lateral ventricle. Scale bar = 100 μm.

GSH2 and Er81 protein expression was further analyzed by immunohistochemistry in sectioned spheres. Virtually all analyzed spheres contained GSH2-expressing cells (Fig. 3E). We counted the total number of cells and the number of GSH2-expressing cells in acutely dissociated and fixed spheres from three separate dissections at passage 2 and passage 5/6. In all quantified groups, between 6% and 7% of the total number of cells were found to express GSH2, and this number was not different between passages.

Unlike GSH2, that was expressed in virtually all spheres analyzed, only a subset of the spheres also expressed Er81 (Fig. 3F). No ISL1-positive cells, however, were found in any of the analyzed spheres (data not shown).

Differentiation potency of in vitro expanded adult SVZ cells

The majority of neurospheres (83.6 ± 3.7%, n = 26) expanded in EGF and bFGF for five passages in vitro,



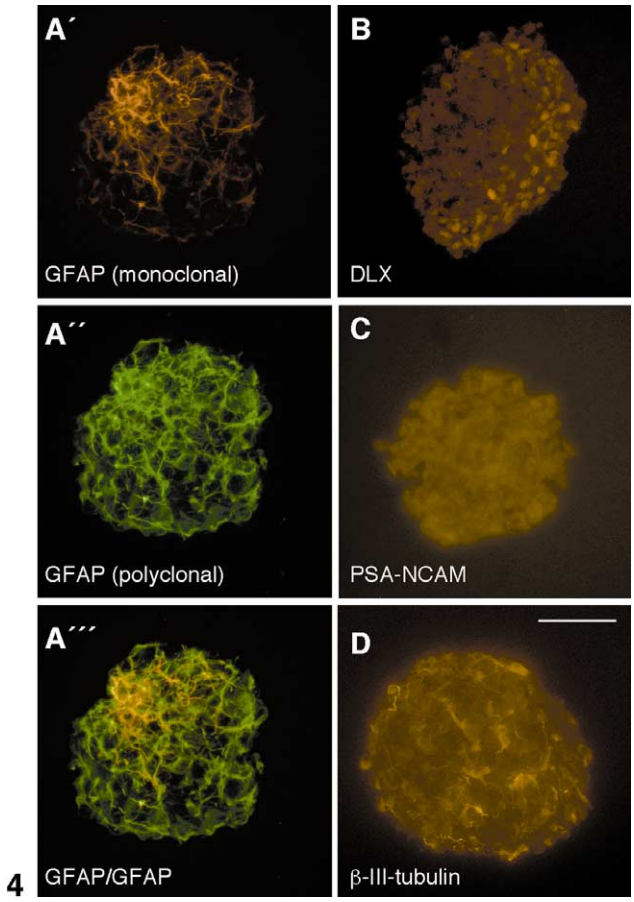


Fig. 3. Gene and protein expression in neurosphere cultures isolated from the adult SVZ. Expression of *Gsh2, Meis2, Er81,* and *Isl1* in (A) acutely dissected adult SVZ and E13.5 LGE, (B) passage 1 neurospheres isolated from adult SVZ in EGF, bFGF or EGF + bFGF, (C) passage 5 neurospheres expanded in EGF alone, or in combination with bFGF, and (D) spheres expanded in EGF + bFGF and allowed to differentiate for 3, 7, and 10 days. Immunocytochemistry on sectioned spheres show that a significant number of cells in the spheres express GSH2 (E) and Er81 (F). After differentiation, around 10% of the cells differentiate into neurons, as detected by β-III-tubulin (G). Many of the neurons also express MAP2 (G, inset). Scale bar in E and F = 100 μm.

Fig. 4. Heterogenous populations of cells within the neurospheres. Immunocytochemistry performed on cryostat sectioned proliferating neurospheres show that the spheres are composed of cells with different phenotypes. Cells immunopositive for GFAP was detected by a polyclonal (A″, A‴, green) and monoclonal (A′, C‴, red) antibody. A subpopulation of spheres stained positive for DLX (B), and few cells expressed β-III-tubulin (D), but no spheres contained cells expressing PSA-NCAM (C). Scale bar = 100 μm.

followed by 7 days of differentiation, were multipotent, generating neurons (Fig. 3G), astrocytes, and oligodendrocytes (data not shown). The number of neurons generated, as detected by expression of β-III-tubulin, represented approximately 10% of the total cell number. The cells had a neuronal morphology (Fig. 3G), and also expressed MAP-2 (Fig. 3D, insert). We isolated RNA from neurospheres that had been allowed to differentiate for 3, 7, or 10 days and looked at the gene expression changes during differentiation. We found that expression of *Gsh2* was significantly downregulated early in the differentiation process. After 3 days of differentiation, only a week expression of *Gsh2* was detected, and the expression was completely abolished after 7 days of differentiation (Fig. 3D). In contrast, the expression of *Meis2* and *Er81* were maintained throughout the time course of the in vitro differentiation (Fig. 3D). We did not detect *Isl1* mRNA at any stage of differentiation (Fig. 3D).

Neurospheres from adult SVZ contain a heterogenous population of cells, including both GFAP- and DLX-positive cells

Immunostainings of sectioned neurospheres originating from the adult SVZ revealed that they contain several of the

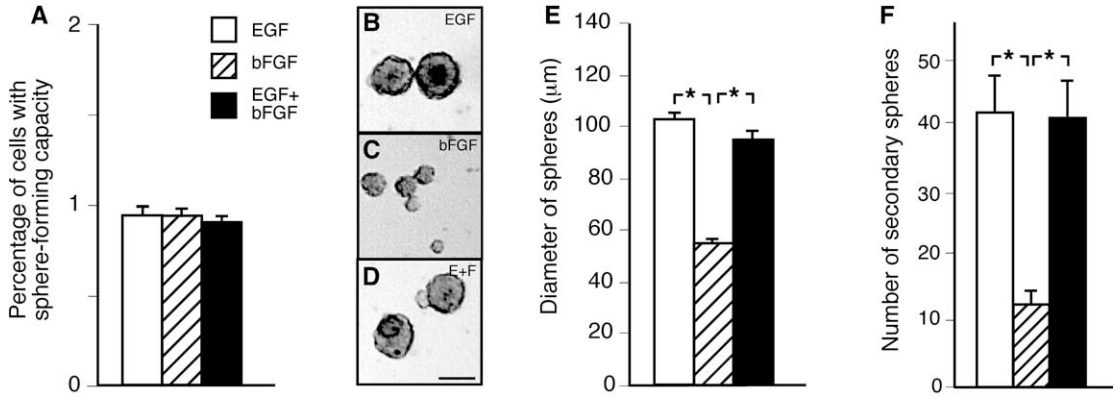


Fig. 5. Growth factor effect on initiation, proliferation, and self-renewal of neurosphere cultures. (A) Bar graphs illustrating the percentage of primary cells that form neurospheres 1 week after isolation in either or both mitogens ($n = 30$ for each mitogen). Microscopic images of neurospheres formed in (B) EGF alone, (C) bFGF alone, and (D) EGF + bFGF combined. (E) Bar graphs illustrating the diameter (μm) of individual spheres 1 week after isolation in either or both mitogens ($n = 279$ for EGF, $n = 271$ for bFGF, $n = 255$ for EGF + bFGF). (F) Bar garphs illustrate total number of secondary spheres formed after dissociation of individual primary spheres ($n = 26$ for EGF, $n = 30$ for bF, $n = 29$ for E + F). Scale bar = 100 μm.

cell types present in situ in the SVZ. Many of the cells in the neurospheres expressed GFAP, as detected with a polyclonal antibody (Fig. 4A′ and A″′, green). A subset of these cells were also stained with a monoclonal anti-GFAP antibody (Fig. 4A″, A″′, red); thus, it is possible that they represent astrocytes (type B cells) and not ependymal (type E) cells. Recently, DLX2 has been reported as a marker of the transiently and rapidly proliferating progenitor population (type C cells) (Doetsch et al., 2002). We detected DLX expression in a subset of the analyzed spheres (Fig. 4B). PSA-NCAM is expressed by immature neurons, including the migrating neuroblasts (type A cells) in the RMS (Bonfanti and Theodosis, 1994; Rousselot et al., 1995), but we did not detect any PSA-NCAM-positive cells in the spheres (Fig. 4C) (using two different antibodies, that resulted in positive staining in brain sections processed in parallel). Thus, the undifferentiated spheres do not seem to contain cells that are as differentiated as the migrating neuroblasts (type A cells) under proliferating conditions. In a few neurospheres under expansion conditions, we detected some spontaneous neuronal differentiation as marked by the presence of cells expressing the neuron-specific protein β-III-tubulin (Fig. 4D).

The same population of neurosphere-forming cells respond to both EGF and bFGF

It is unclear which cells types in the adult SVZ possess neurosphere forming potential, and whether the same or different cells respond to EGF and bFGF. To address this issue, we isolated cells from adult SVZ in EGF alone, bFGF alone, or in both mitogens combined. We found that cells isolated at clonal density (10,000 cells/ml) gave rise to the same number of neurospheres under each growth factor condition. Independent of whether the cells had been isolated in EGF or bFGF, approximately 1% (0.90 ± 0.05% for EGF and 0.96 ± 0.05% for bFGF, $n = 30$ separate wells) of the cells plated formed neurospheres (Fig. 5A). To verify that the same population of cells proliferate in response to EGF and bFGF, as opposed to two separate populations of cells (one EGF responsive and one bFGF responsive) we isolated cells in medium containing both EGF and bFGF. Under these conditions, neurospheres were formed at a similar frequency (0.96 ± 0.06%, $n = 30$ separate wells) as the cells isolated in EGF alone or bFGF alone (Fig. 5A). The absence of additive effect on neurosphere forming potential of EGF and bFGF is in agreement with the theory that the same population of cells in the lateral ventricular wall of the adult forebrain proliferate in response to EGF and bFGF in vitro (Gritti et al., 1999).

Cell counting during passages of the cultures revealed that the cultures that had been exposed to EGF contained more cells. In addition, we noticed that the neurospheres cultured in bFGF alone were significantly smaller (Fig. 5C) than the neurospheres cultured in the presence of EGF (Fig. 5B) alone or in combination with bFGF (Fig. 5D). We therefore measured the diameter of individual spheres 1 week after isolation in the different mitogen conditions. We observed no difference in average size between the EGF alone (102 ± 2 μm, $n = 279$), or the EGF + bFGF combined spheres (103 ± 2 μm, $n = 255$) (Fig. 5E), while the spheres isolated in bFGF alone were approximately 50% smaller (average diameter of 54 ± 1 μm, $n = 271$), than the spheres grown in EGF or EGF + bFGF combined ($P < 0.05$; one-way ANOVA followed by Bonferoni-Dunn's post hoc test) (Fig. 5E). This difference in sphere size was maintained after several passages in vitro and the diameter of individual cells within the spheres from all different conditions was similar, approximately 12 μm (data not shown).

To investigate if the increased number of cells in each neurosphere when EGF had been present during expansion was also reflected by an increase in cells with self-renewing potential, we performed a secondary neurosphere-forming

assay. We found that all primary neurospheres, independent of which growth factor they were isolated in, gave rise to more than one secondary sphere. The number of secondary spheres formed in each growth factor condition varied greatly: from 11 to 71 for EGF ($n = 26$), from 2 to 41 for bFGF ($n = 30$), and from 3 to 87 for both growth factors combined ($n = 29$) (Fig. 5F). We found no significant difference in secondary sphere-forming potential between the EGF and EGF + bFGF cultures, whereas the number of secondary spheres formed from the neurospheres isolated in bFGF alone were significantly less compared to EGF and EGF + bFGF cultures ($P < 0.05$; one-way ANOVA followed by Bonferoni-Dunn's post hoc test) (Fig. 5F). No difference in the size of cells from different neurospheres, and smaller size of bFGF spheres, means that these spheres contain fewer cells. Since each of the primary spheres subcloned derives from a single cell, the lower number of secondary spheres indicates that the neurosphere-forming cell made fewer self-renewing divisions when exposed to bFGF alone. However, if one takes into account the smaller sphere size, the proportion of sphere-forming cells in all conditions was the same (approximately 7–8%, assuming all cells in the spheres had a diameter of 12 μm).

EGF and bFGF expands the same cell type, but at different stages of maturation

We set up a model using the combined expression of *Gsh2*, *Dlx2*, and *Dlx5* as an indication of the maturation state of the cells (see Discussion). Using RT-PCR, with the amount of cDNA normalized with *Gpdh* expression, we found that *Gsh2* is expressed at approximately equal levels in bulk neurosphere cultures generated in EGF alone, bFGF alone, and in EGF and bFGF combined (Fig. 3B), but is rapidly downregulated upon differentiation (Fig. 3D). We also investigated the presence of *Dlx2* and *Dlx5* by RT-PCR in bulk preparations of neurosphere cultures isolated and propagated in EGF alone, bFGF alone, or in EGF and bFGF combined, during expansion and differentiation (Fig. 6A). We found that when cells had been propagated in bFGF alone, *Dlx2* was expressed at a lower level than if EGF had been present during the expansion. However, when the neurospheres were exposed to differentiation conditions, the bFGF neurospheres started to express both *Dlx2* and *Dlx5* to the same extent as the neurospheres cultured in the presence of EGF. The PCR analysis was performed on bulk neurosphere cultures from three separate dissections. In each case, the results were similar.

To further study the effect of EGF and bFGF on the differentiation state of the cells, we performed RT-PCR analysis for *Gsh2, Er81, Isl1,* and *Dlx2* expression in individually picked neurospheres. A total of 75 spheres from three separate dissections were analyzed ($n = 25$ for EGF, bFGF, and EGF-bFGF conditions, respectively). Except for a few samples (most likely containing to low cDNA amounts since *Gpdh* expression was very weak, and therefore excluded from the analysis), all of the neurospheres expressed *Gsh2,* and none expressed *Isl1*. When analyzing the gene expression in individual spheres, we found that the *Gsh2*-expressing neurospheres showed three different genetic profiles: one population expressed only *Gsh2;* a few spheres were found to express both *Gsh2* and *Dlx2;* and a third group expressed all three markers *Gsh2, Dlx2,* and *Er81*. The proportion of each subpopulation of neurospheres was similar in the EGF and EGF + bFGF cultures: 17% of the neurospheres grown in EGF and 21% of neurospheres grown in EGF and bFGF combined expressed only *Gsh2* ($\chi^2 = 0.14$, $P > 0.05$). Few of the neurospheres in the EGF and EGF + bFGF cultures expressed *Gsh2* and *Dlx2,* and the majority (66% for EGF and 65% for EGF + bFGF; $\chi^2 = 0.09$, $P > 0.05$) expressed *Gsh2,* together with *Dlx2,* and *Er81*. In contrast, the bFGF-only cultures had many more (55%) *Gsh2*-only expressing spheres ($\chi^2 = 6.77$; $P < 0.001$, and $\chi^2 = 5.15$; $P < 0.025$ compared to EGF and EGF + bFGF cultures, respectively), and only 22% expressed all three markers ($\chi^2 = 8.22$; $P < 0.001$, and $\chi^2 = 6.71$; $P < 0.001$ compared to EGF and EGF + bFGF cultures, respectively) (Fig. 6B). We therefore conclude that the EGF and bFGF expanded spheres represent the same population of cells, but at different stages of maturation.

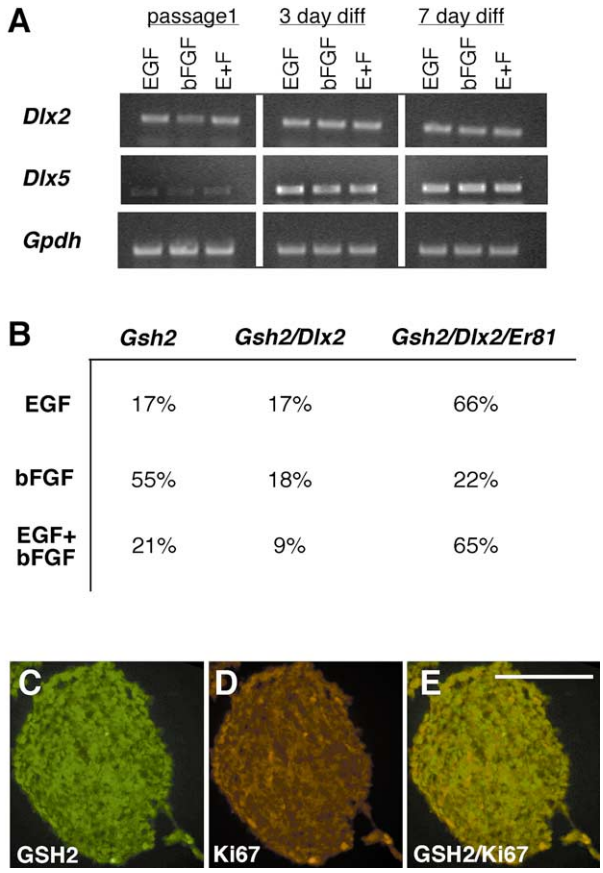
Furthermore, since all spheres analyzed expressed Gsh2 both at the mRNA and protein level, and the number of GSH2-expressing cells is similar (6–7%) to the number of secondary spheres formed (7–8%), we propose that the Gsh2 is expressed by neurosphere-forming cell itself, or by its direct descendent.

In the VZ of the LGE, all GSH2-expressing cells are actively proliferating (Yun et al., 2003). Double-immunolabeling with antibodies against GSH2 (Fig. 6C) and the cell cycle marker Ki67 (Fig. 6D) showed that also in neurospheres isolated from adult SVZ, the majority of the GSH2-positive cells were also labeled with Ki67, thus being mitotically active (Fig. 6E).

Discussion

We report here the expression of the homeodomain transcription factor GSH2 in the adult SVZ, and its maintenance in neurosphere cultures originating from this region. This observation is of significance in two respects.

First, it is known that during development the LGE consists of two proliferative zones, the VZ and the SVZ (Smart, 1976), but the lineage relationship between cells in these two zones during development and the proliferative cells in the adult SVZ has not yet been fully clarified. Morphologically, the LGE is the most likely source, as the postnatal/adult SVZ comes to reside close to the ventricular wall of the striatum (Sturrock and Smart, 1980). Recent studies have shown that some proteins known to be expressed in the LGE SVZ, such as DLX and Er81, are also present in the adults SVZ (Liu et al., 1997; Eisenstat et al.,



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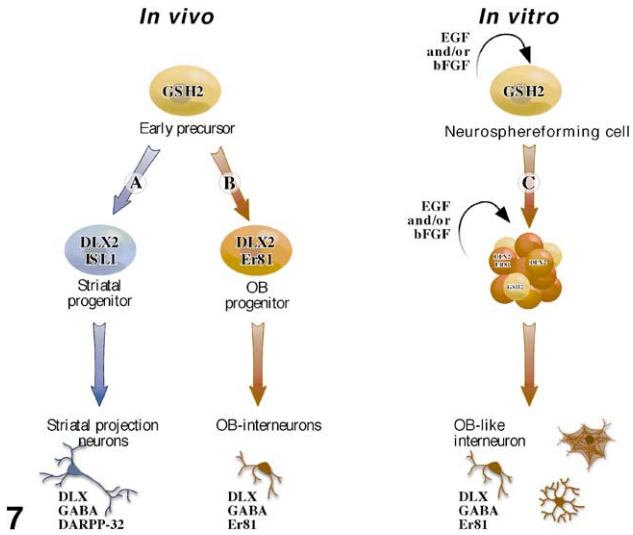

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Fig. 6. Growth factor effect on state of differentiation in neurosphere cultures. (A) Expression of *Dlx2* and *Dlx5* in undifferentiated and differentiated neurosphere cultures, propagated in EGF, bFGF, or in EGF and bFGF combined. (B) Proportion of subtypes of neurospheres based on gene expression in individual spheres ($n = 25$ for each condition). (C–E) Expression of GSH2 (green) and Ki67 (red) in a sectioned neurosphere.

Fig. 7. Schematic diagram showing the potency of the GSH2 expressing cell in vivo and in vitro. In vivo, the GSH2-expressing cells reside in the ventricular zone of the LGE. During development, its expression is necessary to specify both the striatal projection neurons via the ISL1 expressing progenitors (pathway A) and the olfactory bulb interneurons via the Er81-expressing progenitors (pathway B). The GSH2-expressing cells is maintained in the adult SVZ, but in the intact adult brain it generates only the olfactory bulb progenitors (pathway B). In vitro, we suggest that the GSH2-expressing cell is the neurosphere-forming cell, that in turn generates the DLX2 and Er81 expressing progenitors (pathway C). Upon differentiation, the cells in the neurospheres differentiate into neurons, astrocytes, and oligodendrocytes. The neurons formed are olfactory bulb-like, expressing DLX2, DLX5, GABA, and Er81.

1999; Doetsch et al., 2002; Andrews et al., 2003; Stenman et al., 2003). We now report the expression of GSH2 in the adult SVZ, a homeodomain transcription factor that is developmentally expressed in the cells of the VZ of the LGE, but not in the SVZ (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001, 2003). This is the first report that a region-specific protein expressed only in the LGE VZ is also expressed in the adult SVZ, which may suggest that at least some of the adult SVZ cells derive, directly or indirectly, from the VZ of the embryonic LGE.

Second, Gsh1 and 2 homeodomain transcription factors are expressed in the ventral telencephalon at early stages of development (Szucsik et al., 1997; Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001, 2003). Because GSH2 is expressed early in mitotically active VZ cells, it is implicated as a key regulator in defining the identity of LGE progenitor cell populations (Yun et al., 2003). Genetic studies show that Gsh2 regulates the development of the LGE and plays a fundamental role in specifying the striatal and olfactory bulb progenitors. In fact, both the Er81 and Isl1 populations are severely depleted in the gsh2 and gsh1/2 mutants. Subsequently, there is a significant reduction in Er81 positive cells in the olfactory bulb and ISL1 and in DARPP-32 positive cells in the developing striatum (Toresson and Campbell, 2001; Stenman et al., 2003; Yun et al., 2003). Therefore, it is interesting to observe that some cells in the adult SVZ maintain the expression of GSH2, and that these cells are expandable in vitro under EGF and/or bFGF stimulation. The presence of GSH2-positive cells implies that the adult SVZ, and neurospheres derived from this region, contain a cell population with the potential to specify the identity of both striatal and olfactory bulb progenitors. However, in contrast to the developing brain, where the GSH2 expression is needed for the generation of progenitors for both olfactory bulb interneurons and striatal neurons (see Fig. 7, pathways A and B), the GSH2-expressing cells in the adult SVZ (see Fig. 7, pathway B), as well in the neurosphere cultures (see Fig. 7,

pathway C) derived from the adult SVZ (present study) and embryonic LGE (Parmar et al., 2002), seem to only generate neurons that exhibit characteristics of olfactory bulb interneurons. We are currently investigating if this restriction in developmental potential is due to factors missing in the in vitro differentiation environment, or if the GSH2-expressing cells in vitro, have lost their potential to direct the generation of striatal projection neurons.

EGF and bFGF act on the same cells, but at different stages of differentiation

The germinal zone in the adult forebrain is made up of a complex mixture of both proliferating and differentiated cells (Alvarez-Buylla and Garcia-Verdugo, 2002), and both EGF and bFGF receptors are present (Wanaka et al., 1991; Morshead et al., 1994; Gonzalez et al., 1995; Weickert and Blum, 1995; Craig et al., 1996). As previously reported (Gritti et al., 1999), we found that it is the same cells that proliferate and form neurospheres in EGF as in bFGF. However, the individual spheres were significantly larger, containing more cells, when EGF was present during the expansion phase.

We used the expression of two different *Dlx* genes to address the differentiation state of the cells. Cycling precursor cells in the LGE VZ express GSH2 (Yun et al., 2003). A subset of these cells also express DLx1 and DLx2 and these cells are likely to represent a slightly more specified cell population (Liu et al., 1997; Eisenstat et al., 1999). As differentiation proceeds, GSH2 is downregulated, whereas the DLX1 and DLX2 expression is maintained. As the cells mature, they also start to express DLX5 and DLX6, both in the SVZ and the striatal mantle region (Liu et al., 1997; Eisenstat et al., 1999), as well as in the mature olfactory bulb inteneurons (Long et al., 2003). When analyzing the gene expression in single neurospheres derived from the adult SVZ, we found two major subtypes of neurospheres: one immature subtype expressing only *Gsh2* and one slightly more differentiated subtype of neurosphere, containing cells that expresses both *Gsh2* and *Dlx2*. These more mature neurospheres most often also contained cells expressing *Er81,* but never *Isl-1,* suggesting that they are specified to generate olfactory bulb interneurons, but not striatal projection neurons. A larger proportion of the neurospheres expanded in bFGF alone expressed *Gsh2,* but not *Dlx2* or *Er81,* reflecting that they are less mature than the majority of the neurospheres cultured in the presence of EGF, where most of the neurospheres expressed *Gsh2, Dlx2,* and *Er81*. We therefore conclude that EGF and bFGF stimulates proliferation of the same population of cells from the adult SVZ, but that the cells proliferate faster and the neurospheres contain a higher number of more mature cells when EGF has been present during expansion.

Identity and specification of the neurosphere forming cell

We show that Gsh2 is expressed in all neurospheres analyzed, and the number of GSH2-expressing cells (6–7%) in an sphere is similar to the number of secondary neurospheres formed (around 7–8%). Therefore, we suggest that the Gsh2 expressing cell may represent the sphere-forming cell in the adult SVZ, that in turn generates the Dlx2 and *Er81* expressing progenitor population. However, both in vivo and in vitro, there are only correlative evidence that GSH2 is expressed in stem cells. In vivo, a subpopulation of the LGE VZ cells are though to be stem cells. Since almost all cells in the LGE VZ express GSH2 (Yun et al., 2003), it seems reasonable to conclude that some of the GSH2 expressing cells are stem cells. In vitro, long-term maintenance of GSH2 expression in clonally derived neurospheres suggests that GSH2 is expressed in the neurosphere forming cell, i.e., the stem cells themselves, or in their direct descendents. However, by no means does GHS2 have to be restricted to stem cells. Likewise, we do not suggest that GSH2 is a marker for stem cells. Rather, GSH2 expression is an indication of maintained regional specification in neurosphere cultures, as reported previously by us and other groups (Zappone et al., 2000; Hitoshi et al., 2002; Parmar et al., 2002).

Neurospheres originating from the adult SVZ contain cells that express putative markers for both type B cells (GFAP, detected by both polyclonal and monoclonal antibodies), and type C cells (detected by DLX2 expression), but lack PSA-NCAM (typically expressed by the migrating neuroblasts, the type A cells in the RMS). Our data do not allow us to conclude which of the cell types (A, B, and/or C cells) in the adult SVZ/RMS, or in neurospheres generated from this region that express GSH2. Since very few, if any, type C cells are present in the RMS under normal conditions, and the number of GSH2-expressing cells in the neurospheres are larger than the number of DLX-expressing cells, it is fair to conclude that GSH2 is not exclusively expressed in type C cells. However, it is possible that GSH2 is expressed in both type C and type B cells.

A number of studies (Zappone et al., 2000; Yamamoto et al., 2001; Hitoshi et al., 2002; Ostenfeld et al., 2002; Parmar et al., 2002) have assessed maintenance of regional specification in neurosphere cultures derived from distinct regions of the embryonic and adult brain and spinal cord. It is clear that cells propagated as neurospheres from the embryonic LGE maintain many aspects of their regional identity and molecular specification in vitro, even after long-term expansion (Zappone et al., 2000; Hitoshi et al., 2002; Parmar et al., 2002). In the present study we show that adult SVZ cells, just like their embryonic LGE counterparts, maintain aspects of their molecular identity after growth factor stimulated expansion in vitro. Cells expanded from the adult SVZ in neurosphere culture maintained the expression of *Gsh2, Er81,* and *Meis2* for at least five passages in vitro, independent of the growth factor condition, and upon

differentiation generate cells expressing *Dlx5, Meis2,* and *Er81,* all of which are expressed by olfactory bulb interneurons.

Implication for increased differentiation potential of adult SVZ cells

We have shown the presence of GSH2-positive cells in adult SVZ and neurosphere cultures derived from the adult SVZ. However, in contrast to the developing LGE, the adult GSH2-expressing cells seem to generate only olfactory bulb progenitors/neurons. This raises the question of whether there is something in the environment of the adult SVZ that blocks the generation of the ISL1-positive progenitor pool. If an inhibitory factor was present, one would expect that removing the cells from their normal environment, and culturing them in vitro, may remove this inhibition. However, when expanded in neurosphere culture, the adult SVZ cells maintain their expression of *Gsh2, Er81, Dlx2,* and *Meis2,* but *Is11* is never expressed. This is also the case for neurosphere cultures of embryonic LGE cells (Parmar et al., 2002). We therefore suggest that a positive regulator(s) is necessary to allow the GSH2-positive cells to generate ISL1-positive progenitors, and in turn, striatal projection neurons. This factor seems to be present in LGE during embryonic development, but absent in SVZ of the adult brain as well as in the neurosphere culturing system. In line with the notion that the cells in the adult SVZ may have maintained a latent potential for specifying striatal projection neurons, striatal neurogenesis under experimental conditions have been reported from several groups. Virus-mediated expression of BDNF in the adult ependymal layer resulted in the genesis of medium-sized spiny projection neurons expressing DARPP-32 (Benraiss et al., 2001). Other studies showed that after stroke, the damaged DARPP-32-expressing striatal projection neurons, but not interneurons, can at least partly be replaced from resident progenitors that are most likely located in the SVZ (Arvidsson et al., 2002; Parent et al., 2002). Thus, the Gsh2-expressing cells present in the adult SVZ may not be restricted to the specification of the Er81-expressing progenitors. Given the right conditions, they may have the capacity to specify the generation of striatal projection neurons as well.

Experimental methods

Animals

Cultures were generated from 4–6-week-old female NMRI mice. All animal-related procedures were conducted in accordance with local ethics guidelines and approved animal care protocols.

Dissection and culturing

Animals were deeply anesthetized with halothane (Astra Zeneca) and euthanized by cervical dislocation. Whole brains were removed and placed in ice-cold Leibovitz L-15 medium (GIBCO). Each brain was cut with microtome blades (Feather) in a brain matrix into three 1.0-mm-thick slices. The lateral aspects of the ependymal zone and SVZ were precisely removed under a Leica dissection microscope. The tissue pieces were incubated at 37°C for 15 min in 10-ml of dissociation solution [HBSS without Ca^{2+} and Mg^{2+} (GIBCO) supplemented with 0.015 M HEPES (GIBCO), 5.4 mg/ml D-glucose, 1.33 mg/ml trypsin, 80 U/ml DNase, 0.7 mg/ml hyaluronidase, and 2 mg/ml kynerenic acid (all from Sigma)], followed by gentle trituration, and incubation in the same solution at 37°C for another 10 min. Excess solution, except 2 ml, was removed, and the tissue further mechanically dissociated to a single cell suspension. The cells were spun down and resuspended at a concentration of 10 cells/μl (clonal density) or 50 cells/μl in expansion medium: DMEM/F12 supplemented with B27 (GIBCO), 2 mM L-glutamine, 15 mM HEPES, 0.6% glucose, 0.1 % $NaHCO_2$ (all from Sigma), penicillin/streptomycin (GIBCO). EGF and bFGF (R&D Systems) was added to the medium alone or in combination as appropriate, at concentrations of 20 and 10 ng/ml, respectively. The total number of viable cells was assessed at isolation and at each passage by the trypan blue exclusion method. Cells were passaged by trypzination once a week.

Secondary neurosphere forming assay

Seven days after isolation in EGF only, bFGF only, or EGF and bFGF combined, the neurospheres that had formed were individually picked, put into wells of 96-well plates, and mechanically dissociated into single cells. To ensure that only one sphere was dissociated per well, the wells were microscopically screened before dissociation and the wells that contained more than one sphere were excluded from the experiment. Seven days after dissociation, the number of secondary neurospheres originating from each primary sphere were counted.

Sphere size measurement

Photos of the neurospheres formed after 7 days in vitro (DIV) were taken. Calibration of the microscope was performed with a 1-mm scale of 10-μm resolution. Photos were then printed and individual sphere diameters determined.

Differentiation

Seven days after passage, neurospheres were either individually picked and plated one-by-one in PLL-coated wells of 16-well chamber slides, or differentiated as bulk cultures in PLL-coated T25 flasks. EGF and bFGF were

removed from the expansion medium and 1% serum was added (differentiation medium). The neurospheres were maintained under differentiation conditions for 3, 7, and 10 days before fixation and/or RNA isolation.

Immunocytochemistry

Whole spheres and differentiated cultures were fixed in ice-cold 4% PFA for 15 min at room temperature, followed by three rinses in PBS. Cultures were then processed for immunocytochemistry directly, while spheres were put in 20% sucrose overnight before cryostat sectioning. For in situ analysis of the adult SVZ, 4–6-week-old female NMRI mice were perfused with 0.9% NaCl solution followed by 4% PFA. Brains were removed and post-fixed in 4% PFA at 4°C overnight followed by incubation in 20% sucrose. Brains were sectioned on a microtome to a thickness of 30 μm. Before immunostaining the sections were quenched for 20 min in 3% H_2O_2 and 10% methanol in KPBS. Cultures and sections were rinsed in KPBS, followed by preincubation in 5% normal serum and 0.025% Triton X-100 for 1 h at room temperature. Primary antibodies used were: rabbit-anti-β-III-tubulin (COVANCE/biosite, 1:250), rabbit anti-GFAP (DAKO, 1:1000), Cy3-monoclonal-anti-GFAP (Sigma, 1:20 000), mouse anti-CNPase (Sigma, 1:100), rabbit anti-Er81 (provided by T. Jessell and S. Morten, 1:1000), rabbit anti-Gsh2 (provided by K. Campbell, 1:3000), rabbit anti-Isl1/2 (provided by H. Edlund, 1:400), rabbit anti-Meis2 (provided by A. Buchberg, 1:5000), rabbit anti-distalless (i.e., DLX, provided by G. Panganiban, 1:500), mouse anti-PSA-NCAM (Chemicon, 1:200), rabbit anti-PSA-NCAM (Seki and Arai, 1991) (provided by T. Seki, 1:10000), mouse anti-Ki67 (Novo Castra, Sweden, 1:200), mouse anti-MAP2 (Chemicon, 1:200). Incubations in primary antisera were carried out overnight at room temperature. Detection with secondary antibodies was carried out using standard protocols. Both fluorescent (1:400) and biotin-conjugated (1:200) antibodies were diluted in the preincubation solution and incubated for 2 h at room temperature. The biotinylated antibodies were detected using the ABC method (Vector Labs) with diaminobenzidine (DAB) as the final chromogen.

RT-PCR

RNA was isolated from passage 1 and passage 5 neurosphere cultures, differentiated cultures, freshly dissected adult SVZ, and E13.5 LGE. Total RNA was prepared from cells or tissue using RNAqueous-4PCR (Ambion). RNA isolation was followed by two rounds of DNaseI treatment; 500 ng DNA-free total RNA was used to synthesize cDNA with oligo-(dT)30 primers and superscript-II-RT (GIBCO, Life Technologies). For single sphere RT-PCR, cDNA was obtained from individually picked neurospheres by using Cells-to-cDNAII (Ambion). In all cases, an RT-negative control was always included for each sample. This control never resulted in any amplification product. cDNA was amplified in a thermal cycler using the following conditions: all programs were initiated by a 4-min denaturation step at 95°C followed by 35 amplification cycles. All extension steps were carried out at 72°C but annealing temperature was primer specific. Mg^{2+} concentration was 1.5 mM in all reactions. The primers and annealing temperatures used were: *Gpdh:* sense 5′-ACC ACA GTC CAT GCC-ATC, antisense 5′-TCC ACC ACC CTG TTG CTG TA, annealing 56°C 1 min. *Er81:* sense 5′-CAG AGA TCT GGC TCA TGA TTC AG, antisense 5′-CAC ATG CAG CCT TCT GTT CTG C, annealing 54°C 30 s. *Gsh2:* sense 5′-CAG CTT TCC GGA CAG TGC TC, antisense 5′-GGT AGC AGA AG GAG CCT CTG, annealing 60°C 1 min. *Isl-1:* sense 5′-GCA GCA TAG GCT TCA GCA AG, antisense 5′-GTA GCA GGT CCG CAA GGT, annealing 56°C 1 min. *Meis2:* sense 5′-AAG ACT CCG AGA GTT AT, antisense 5′-GTT TGA AAC TAA AGG ACA, annealing 56°C 1 min. *Dlx2:* sense 5′-AGG ATG ACT GGA GTC TTT GAC, antisense 5′-TCG GAT TTC AGG CTC AAG GTC, annealing 52°C 50 s. *Dlx5:* sense 5′-ATG ACA GGA GTG TTT GAC AG, antisense 5′-CTA ATA AAG CGT CCC GGA GG, annealing 56°C 1 min.

Statistical analysis

Evaluation of differences in number or size of neurospheres between different culture conditions was performed using one-way analysis of variance (ANOVA) followed by Bonferroni-Dunn post hoc test. The differences between gene expression pattern by neurospheres isolated under different conditions was assessed using χ^2 test. Significance for all statistical analysis was set at $P < 0.05$.

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