Cerebellum- and forebrain-derived stem cells possess intrinsic regional character

Corinna Klein[1], Simon J. B. Butt[1], Robert P. Machold[1], Jane E. Johnson[2] and Gord Fishell[1,*]

[1]Developmental Genetics Program and the Department of Cell Biology, The Skirball Institute of Biomolecular Medicine, New York University Medical Center, 540 First Avenue, New York, NY 10016, USA
[2]Center for Basic Neuroscience, UT Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9111, USA
*Author for correspondence (e-mail: fishell@saturn.med.nyu.edu)

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Summary

The existence of stem cells in the adult nervous system is well recognized; however, the potential of these cells is still widely debated. We demonstrate that neural stem cells exist within the embryonic and adult cerebellum. Comparing the potential of neural stem cells derived from the forebrain and cerebellum, we find that progeny derived from each of these brain regions retain regional character in vitro as well as after homotopic transplantation. However, when ectopically transplanted, neurosphere-derived cells from either region are largely unable to generate neurons. With regard specifically to embryonic and adult cerebellar stem cells, we observe that they are able to give rise to neurons that resemble different select classes of cerebellar subclasses when grafted into the perinatal host cerebellum. Most notably, upon transplantation to the perinatal cerebellum, cerebellar stem cells from all ages are able to acquire the position and mature electrophysiological properties of cerebellar granule cells.

Key words: Cerebellum, Neural stem cell, Forebrain, Mouse

Introduction

Traditionally, neurogenesis was considered to end in the period just after birth (Rakic, 1985). Recent work has challenged this idea and it is now well accepted that the generation of neurons continues in the hippocampus and the subventricular zone of the adult central nervous system (CNS) (Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch, 2003b). Furthermore, it has also been argued that neurogenesis at less robust levels also occurs in a number of other sites in the adult CNS, including the spinal cord (Horner et al., 2000), the substantia nigra (Zhao et al., 2003) and the cortex (Gould et al., 1999), although the latter remains controversial (Kornack and Rakic, 2001; Magavi et al., 2000). Moreover, neural stem cells (NSCs) can be isolated from most portions of the embryonic and adult nervous system (reviewed by Klein and Fishell, 2004). These findings have been widely interpreted to suggest that the brain possesses a covert but widespread potential for neurogenesis.

We are just starting to understand the functional role of newborn neurons that are generated from adult NSC populations (Kempermann et al., 2004; Lledo et al., 2004; Schinder and Gage, 2004; Schmidt-Hieber et al., 2004). Despite the prevalence of NSCs in the adult CNS, the range of neuronal subtypes that these cells generate in the adult brain seems to be limited (Alvarez-Buylla et al., 2002; Doetsch, 2003a). However, the developmental potential of these populations appears to be broader when challenged (Auerbach et al., 2000; Eriksson et al., 2003; Parmar et al., 2003; Suhonen et al., 1996). Indeed, when NSCs from the adult brain were introduced into blastocysts they were shown to be capable of contributing to all germ layers, albeit at very low frequency (Clarke and Frisen, 2001; D'Amour and Gage, 2002). Unfortunately, interpretation and generalization of the data as a whole is difficult due to major differences in methodological details used by different labs and the lack of markers to untangle the complexity of neuronal subpopulations (Bithell and Williams, 2005; Gage, 2000; Klein and Fishell, 2004). A direct comparison of the in-vitro and in-vivo potential of NSCs derived from different regions of the CNS has not been systematically examined in a single study.

Here we describe the existence and the potential of NSCs within the embryonic and adult cerebellum, suggesting that despite the lack of evidence for adult neurogenesis in the cerebellum (Altman and Das, 1966), neural stem cells may reside in the postnatal cerebellum. Notably, the cerebellum is the only structure in the brain where the prevalent form of embryonic neural progenitors (Anthony et al., 2004; Gaiano et al., 2000; Malatesta et al., 2000; Noctor et al., 2001), the radial glia cells, persists into adulthood. Having identified a stem cell population in the cerebellum, we compare our findings to NSCs from the ganglionic eminences of the forebrain, both in vitro and after homotopic and heterotopic transplantation. We find that forebrain- and cerebellar-derived neurospheres give rise to progeny in accordance with their region of origin, but require local regional-specific cues to yield neurons with the characteristics of region-specific subtypes.

Materials and methods

Animals

Swiss Webster mice, ICR mice (Taconic) and β-actin^{EGFP} mice (Okabe et al., 1997) used in these studies were maintained according

to protocols approved by the Institutional Animal Care and Use Committee at New York University School of Medicine. Timed-pregnant Swiss Webster mice were obtained from the Skirball Institute transgenic facility. The day on which the sperm plug was identified is referred to as embryonic day (E) 0.5.

The *Math1*^{EGFP} mice were generated in a similar manner, as previously described using the same Math1 enhancer element (Lumpkin et al., 2003). By contrast to Lumpkin and colleagues, the enhanced green fluorescent protein (EGFP) expression matches the endogenous Math1 expression, and we do not see any ectopic EGFP expression in the cortex and hippocampus.

Neurosphere cultures

The cerebellar anlage was identified and dissected by its position and morphology (i.e. the dorsal portion of the metencephalon, superficial to the pons and bordered posteriorly by the lumen of the fourth ventricle and anteriorly by the mesencephalon). Whole cerebelli were prepared from P0 and adult mice (>P42). The medial and lateral ganglionic eminences of the telencephalon were dissected from Swiss Webster mice or β-actin^{EGFP} E14.5 embryos, the meninges were removed and the tissue chopped into smaller pieces and washed twice in cold DMEM. Embryonic tissue was treated with 0.25% trypsin (Worthington) at 37°C for 5 minutes. DNase I (0.1%; Sigma) and ovomucoid inhibitor (2 mg/ml ovomucoid, 0.1 g/ml BSA in PBS) were then added and samples were triturated with a fire polished Pasteur pipette. Postnatal tissue was dissociated using the papain dissociation kit (Worthington) according to the manufacturer's instructions. For the feeder layer, 1×10^5 cells/well from either the cerebellum, the ganglionic eminences or E14 skin (i.e. fibroblasts) were plated onto LabTekII CC2 chamber slides (Nunc) in DMEM/F12 with B27 supplement and 2 mmol/l glutamine (Gibco) 3-5 days before the neurospheres were seeded.

For the neurosphere assay, cells were plated at clonal density (1-2 cells/mm^2) or FACsorted into 96-well plates and cultured in DMEM/F12 with B27 supplement, 2 mmol/l glutamine and 2 μg/ml heparin (Sigma) and EGF and/or FGF (20 ng/ml; Upstate Inc., Waltham, MA). The clonal nature of the neurosphere assays was confirmed under a microscope, which showed that all cells plated were single cell isolates or only one cell was plated per well.

To ascertain that the cells plated at low density gave rise to neurospheres that were clonally derived, we performed the following experiment. We mixed equal numbers of wild-type and GFP-expressing transgenic cerebellar progenitors and plated them at the density used in all our low-density neurosphere cultures (i.e. 1-2 cells/mm^2). We reasoned that if re-aggregation of progenitor and differentiated cells was occurring the resulting neurospheres would be comprised of a mixture of both GFP-positive and -negative cells. Alternatively, if, as we hoped, the spheres were clonally derived, the resulting neurospheres would be either entirely GFP-positive or -negative. In a supplementary figure we show that, as hoped for, the latter proved true and in all cases neurospheres were either entirely GFP-positive or -negative, strongly supporting the clonality of the neurospheres used in our experiments (see Fig. S1B in the supplementary material). Notably this result was observed in all 100 neurospheres we examined.

After 7-10 days in culture, neurospheres were counted using a grid. The neurospheres were then transferred onto an age-matched feeder layer to induce differentiation. Although it did not change the qualitative outcome, neurospheres were generally placed on a feeder layer in DMEM/F12 supplemented with N2 and B27 and 2 mmol/l glutamate (Gibco) to facilitate the survival and differentiation of these cells (see Fig. S1C-F in the supplementary material). Cell cultures were analyzed after 5-10 days. Neurospheres grown at clonal density or clonally in 96-well plates were qualitatively indistinguishable in their developmental potential.

Immunocytochemistry, immunohistochemistry and quantification

Standard immunostaining procedures were used to stain the neurosphere cultures and the histological sections. The following antibodies were used: rabbit α-AN2 (1:1000, gift from J. Trotter); rabbit α-calbindin (1:5000; Swant), rabbit α-DARPP-32 (1:500; Chemicon), rabbit α-GABA (1:500; Sigma), rabbit α-GFAP (1:1500; Accurate, Westbury, NY), chicken α-GFP (1:2000; Chemicon), rabbit α-glutamate (1:500; Sigma), rabbit α-Math1 (1:100, provided by J. Johnson), mouse α-parvalbumin (1:500; Swant), mouse α-synaptophysin (1:200; Sigma), mouse α-TAG1 (4D7; Developmental Studies Hybridoma Bank), mouse α-Tuj1/βIIITubulin (1:1000; Covance). Secondary antibodies were obtained from Jackson ImmunoResearch and were used at a dilution of 1:200. Fluorescent images were obtained using an Axioscope (Zeiss), a cooled-CCD camera (Princeton Scientific Instruments) and Metamorph software (Universal Imaging). Confocal imaging was done on an LSM 510 Axioplan (Zeiss). Optical sections were taken every 1 μm. Most of the presented confocal pictures are single sections, but for some pictures two or three consecutive confocal sections were combined after confirming the double labeling to better illustrate the elaborate morphology of the transplanted cell.

For the quantification of the in-vitro double-labeled cells, pictures of five to ten visual fields of each condition were taken, and the total number of EGFP-expressing cells was counted to determine the percentage of these cells expressing specific markers.

For the quantification of the transplantation results in the forebrain, five regions (olfactory bulb, striatum, cortex, corpus callosum and hippocampus) per forebrain sections were chosen. The number of EGFP per region was determined and correlated with the total number of EGFP cells. For the quantification of the transplantation results in the cerebellum, the total number of EGFP-expressing cells was counted and correlated with the number of cells expressing specific markers.

Transplantations of neural stem cells

Neurospheres derived from EGFP-expressing transgenic mice were collected, pooled and partially dissociated by triturating them at 37°C in Leibowitz's L-15 medium (Gibco Invitrogene) with DNase I (0.1%; Sigma) and collected by centrifugation. The cells were taken up in Leibowitz's L-medium at a concentration of ~10^5 cells/μl. The perinatal mouse pups (P4) were cryo-anesthetized for 2 minutes. A small incision was made into the skin overlaying the midbrain and the cerebellum for cerebellar injection or the forebrain for forebrain injections using a surgical blade. The mouse pup was placed in a self-made mold for stabilization and 1 μl of the cell suspension was unilaterally injected into one cerebellar hemisphere or into the subventricular zone (SVZ) with the help of a Hamilton syringe mounted vertically into a stereotactic holder. Although the cell suspension was injected slowly into the host brain, some leakage of some of the cell suspension could not be avoided. The incision was sealed with Vetbond (World Precision Instruments, Sarasota, FL), and the animals were then warmed to 36°C and returned to the litter. Twelve to 17 days after transplantation the animals were either used for electrophysiology or perfused for histological analyses.

Fluorescent activated cell sorting

Cerebellar anlagen of E14.5 Math1^{EGFP} were dissected and dissociated as described previously. The cell suspension was fluorescence activated cell sorted (FACS) using a DakoCytomation MoFlo cell sorter. The purity of sorted cells was determined by immunostaining for GFP 2 hours after sorting. Math1^{EGFP}-positive, -negative and unsorted fractions were analyzed using the neurosphere assay.

Electrophysiology

GFP-positive and control GFP-negative profiles were recorded in the

granule cell layer in acute cerebellar slices obtained from neurosphere transplanted mice (P15-P18) similar to that previously described (D'Angelo et al., 1995; D'Angelo et al., 1997). In brief, mice were anesthetized, killed by decapitation and the brain dissected out and immediately immersed in ice cold Ringer's solution consisting of (mmol/l): 125 NaCl, 2.5 KCl, 20 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$ and 2 $CaCl_2$ (pH 7.4 when bubbled with 95% O_2, 5% CO_2). Two hundred micrometer slices were cut using Leica VT1000 vibratome and transferred to an incubation chamber for a minimum of 1 hour prior to recording. Cells were visualized using an infrared contrast system (Stuart et al., 1993). All recordings were performed at room temperature. Intracellular electrodes were pulled from borosilicate glass capillaries and filled with a solution consisting of (mmol/l): 128 K-gluconate, 10 HEPES, 0.0001 $CaCl_2$, 4 NaCl, 0.3 GTP, 5 ATP, 1 glucose (pH adjusted to pH 7.4 with KOH). Current clamp recordings were performed using an Axoclamp 2B amplifier (Axon Instruments, USA). Electrode capacitance was cancelled as best as possible prior to obtaining electrical access to the cell. To ascertain if the cells exhibited a current-voltage relationship consistent with them being cerebellar granule cells, they were stimulated at 0.25 Hz with 300 or 500 millisecond hyperpolarizing and depolarizing current pulses.

Results

Multipotent progenitor cells persist postnatally in the cerebellum

To test for the existence of NSCs in the cerebellum, we examined cells derived from the E14.5, perinatal (P0) and adult (>P42) cerebellum, using the neurosphere assay (Reynolds and Weiss, 1992). Although the neurosphere assay is an in-vitro assay and much has been argued about its physiological relevance (Gage, 2000), it is a very useful tool to analyze the proliferation, self-renewal capacity and potency of neural progenitors.

We compared the cerebellar cells with those derived from a combination of the medial and lateral ganglionic eminences of E14.5 forebrain. Although E14.5 cells from both the forebrain and cerebellum can generate neurospheres, twice the rate of neurosphere formation was observed in forebrain cultures (4.6±1.1%) than that of those derived from the cerebellum (2.1±0.2%). Furthermore, P0 and adult cerebellum maintained their ability to form neurospheres, albeit at reduced frequencies (1.3±0.1% and 0.3±0%: see Fig. S1A in the supplementary material). When dissociated and replated, neurospheres from each of these ages formed secondary neurospheres at the same rate as the primary neurospheres that gave rise to them (data not shown). Furthermore, when individual cerebellar cells were sorted into 96-well plates, neurospheres formed at higher frequency, as observed in low-density cultures (for adult tissue: 0.8±0.3% for 96-well plates and 0.3±0% for low-density cultures). Regardless of the culture conditions that led to their generation, the numbers and range of differentiated cell types generated from either embryonic or adult cerebellar-derived neurospheres was indistinguishable in vitro (see Fig. 1G-I in the supplementary material).

Embryonic and adult multipotent progenitor cells give rise to cerebellar-specific neuronal cell fates in vitro

Two of the advantages of studying the cerebellum are its relatively simple cellular composition and the fact that the specific subclasses within this structure are readily identifiable (Altman and Bayer, 1996). Excluding the deep cerebellar nuclei, the cerebellum is comprised of eight neuronal classes that can be distinguished by morphology and immunocytochemistry as well as their location within the cerebellar cortex.

The cerebellar neurons can be divided into the five 'classic' neuronal subtypes (granule cells, Golgi, stellate, basket neurons and Purkinje cells) and into the less common neuronal subtypes (Lugaro, brush and candelabrum neurons) (Flace et al., 2004; Laine and Axelrad, 1994).

The only excitatory neurons residing in the cerebellum are the granule cells [except for the brush neurons, which are primarily located in the granule cell layer of the flocculonodular node (Dino et al., 1999)]. Granule cells reside in the granule cell layer, have very small cell bodies, protrude three to five dendrites and a long axon projecting into the molecular layer, forming the parallel fibers. They can be identified with antibodies against the excitatory neurotransmitter glutamate, and when in an immature state by their expression of the cell surface protein TAG1 (Kuhar et al., 1993; Pickford et al., 1989).

The remaining neuronal cell types are immunoreactive for GABA, the inhibitory neurotransmitter used by these cells. Furthermore, all classical interneuron classes within the cerebellum (Golgi, stellate and basket cells) express the calcium-binding protein parvalbumin at high levels (Bastianelli, 2003), whereas Purkinje cells also express the calcium-binding protein calbindin (Altman and Bayer, 1996; Celio, 1990; Rogers, 1989). Basket and stellate cells reside in the molecular layer, whereas Golgi cells are situated in the granule cell layer. Most prominent are the Purkinje cells, which form a discrete row of cells between the molecular and internal granule cell layers. The Purkinje cells extend large dendritic arborizations into the molecular layer and send their axon to the white matter track (Altman and Bayer, 1996). Finally, the recently described candelabrum neurons (Laine and Axelrad, 1994) and Lugaro neurons are located directly underneath the Purkinje cell layer. Lugaro cells project their dendrites horizontally and remain within the molecular layer (Laine and Axelrad, 2002).

Using immunocytochemical markers, we analyzed the potential of cells within cerebellar neurospheres, which upon removal of the growth factors and presentation of an adhesive substrate undergo differentiation. The use of neurospheres derived from a mouse line that ubiquitously expresses EGFP under the β-actin promoter (Okabe et al., 1997) allowed neurosphere-derived cells (expressing EGFP) to be distinguished from feeder-layer-derived cells.

Regardless of the age of the donor tissue, cerebellar neurospheres can give rise to populations characteristic of each of the major cell subtypes observed within the cerebellum. In all instances, a subset of cells derived from embryonic (Fig. 1A-E) or adult (Fig. 1F-K) cerebellar neurospheres differentiated into GABA-ergic (Fig. 1A,F; E14.5: 30.2±1.2%; adult: 26.3±2.6%) and parvalbumin-expressing neurons (Fig. 1B,G; E14.5: 27±2.2%; adult: 17.3±2%). We also observed calbindin-expressing neurons with the morphology of Purkinje cells, as indicated by their elaborated dendritic arborization and the presence of a single long axon (Fig. 1C,H; E14.5: 4.9±1.3%; adult 4.3±0.7%) (Baptista et al., 1994). Surprisingly, we were also able to generate a population of

cells with the morphology and the immunocytochemical profile of granule cells. These cells possessed a small rounded cell body, had thin projections and expressed glutamate (Fig. 1D,I; E14.5: 29.4±0.3%; adult: 29.4±3.2%) or TAG1 (Fig. 1E,J; E14.5: 29.4±1.3%; adult: 27.4±3.3%). The presence of this population was unexpected, as the cerebellar GABA-ergic progenitor population is thought to be segregated early in development (~E10.5) from the granule precursor cells (Goldowitz and Hamre, 1998; Wingate and Hatten, 1999). In addition to the various cerebellar neuronal populations, we also observed oligodendrocytes expressing the oligodendroglial precursor marker AN2/NG2 and astrocytes, expressing the intermediate filament GFAP (data not shown). Neurosphere-derived neurons intermingle with feeder-layer-derived cells and express the synaptic vesicle marker synaptophysin (Fig. 1K), suggesting that these neurons have the ability to generate synaptic vesicles in vitro. All neurospheres contained cells with the immunological characteristics of granule cells, interneurons and astrocytes, suggesting that the cerebellar stem cell population we are studying may be uniform in their potency, at least under in-vitro conditions. However, only a subpopulation of neurospheres contained cells with the characteristics of Purkinje neurons. While this may suggest that only a subpopulation of cerebellar neural stem cells can generate this cell type, it is also possible that this is simply the result of the low frequency at which this cell type is generated.

Math1^{EGFP}-negative cerebellar progenitor cells give rise to Math1^{EGFP}-expressing granule precursor cells

While our immunological findings supported the idea that cerebellar neurospheres can give rise to granule cells, given the unexpectedness of this observation, we sought to confirm this result. Given that cerebellar granule cells require Math1 for their normal generation, we reasoned that it is likely that Math1 would be required to generate granule cells from neurospheres. We therefore made use of a transgenic mouse expressing EGFP under an enhancer element found to be sufficient to drive Math1 expression in its normal in-vivo pattern (Helms et al., 2000; Lumpkin et al., 2003).

At E14.5, EGFP is expressed in the external granule layer of the cerebellum (Fig. 2A). We dissected the cerebellar

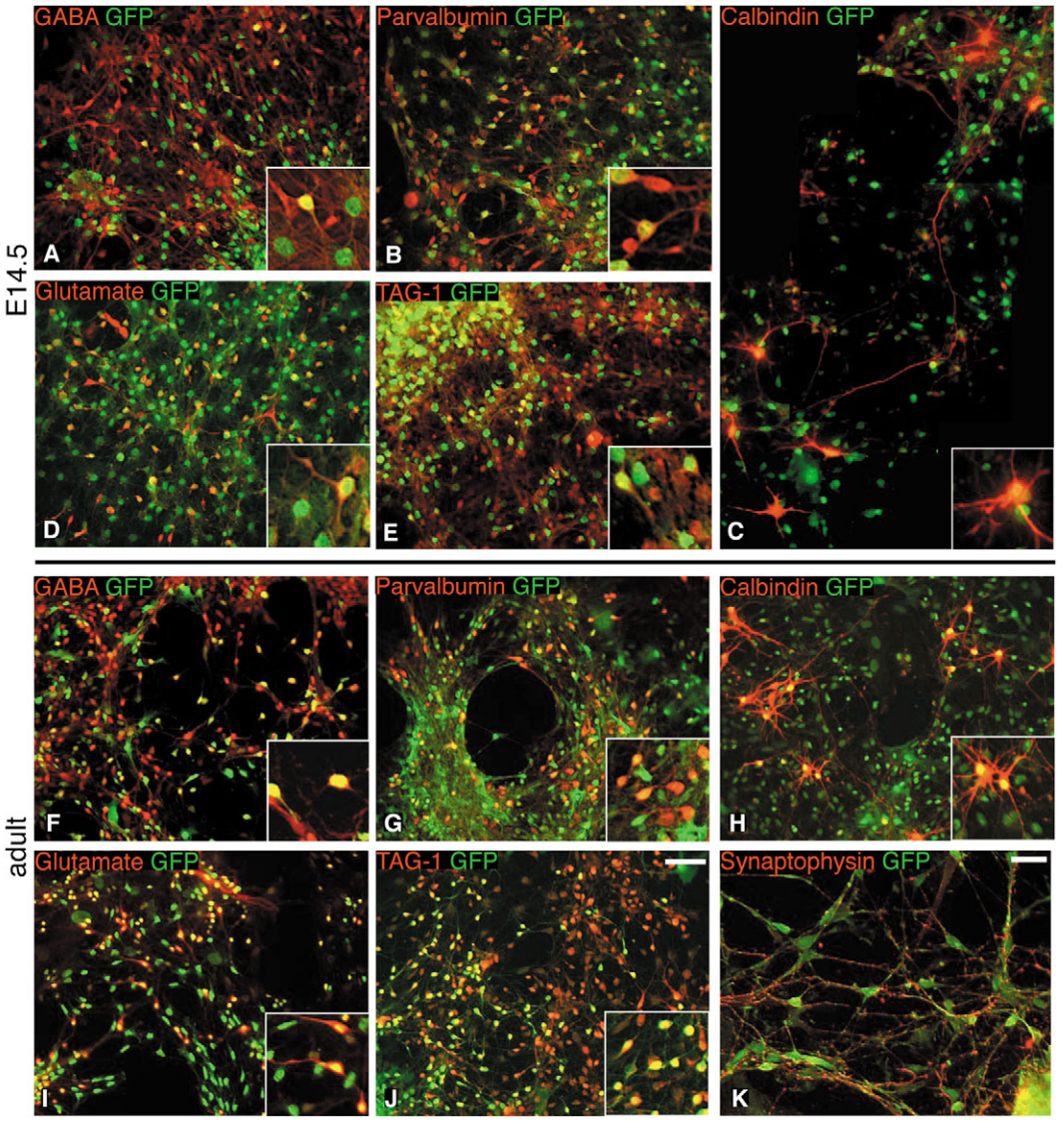

Fig. 1. Cerebellar neurospheres give rise to neurons that resemble distinct cerebellar cell types in vitro. E14.5 (A-E) or adult (F-K) cerebellar neurospheres expressing EGFP were plated on a cerebellar feeder layer and allowed to differentiate. Cells derived from the neurospheres that express the molecular marker indicated are double-labeled and appear yellow, whereas neurosphere-derived cells not expressing the marker examined appear green, while feeder-layer derived cells expressing the analyzed marker appear red. A subpopulation of neurosphere-derived cells gave rise to GABA-ergic neurons (A,F) and can be subdivided into the parvalbumin-expressing interneurons (B,G) and the calbindin-positive Purkinje-like cells (C,H). Neurosphere-derived cells also gave rise to neurons that resemble granule cells, as indicated by their expression of glutamate (D,I) and TAG1 (E,J). The expression of synaptophysin implies that the cells can generate synaptic vesicles in vitro (K).

anlagen of E14.5 Math1^{EGFP}-expressing mice and used FACS to separate EGFP-expressing cells (i.e. granule cell precursors, 8.8±0.1% of the whole fraction) from the cells that do not express EGFP (78.4±3.9% of the remaining cerebellar cells; Fig. 2B). Both fractions, as well as an unsorted control population, were examined using the neurosphere assay. Whereas the unsorted and the Math1/EGFP-negative cells formed neurospheres at the previously observed frequency (unsorted: 2.3±0.2%, Math1/EGFP-negative: 2.3±0.1%), the Math1/EGFP-positive cells failed to form neurospheres (0.04±0.01%; Fig. 2C). This latter result could perhaps have been anticipated, as previous work has demonstrated that granule cell proliferation is maintained by sonic hedgehog (Dahmane and Ruiz-i-Altaba, 1999; Wechsler-Reya and Scott, 1999).

The neurospheres derived from the Math1/EGFP-negative fraction were then placed onto a cerebellar feeder-cell layer and allowed to initiate differentiation. After 2-3 days we observed the upregulation of the Math1^{EGFP} expression within the cultures (Fig. 2D,E). This is consistent with the presence of granule cell precursors within these cultures, although based on this experiment we cannot rule out the possibility of the generation of other dorsally derived Math1-expressing cell types. FACS analysis of the whole culture (feeder-layer and differentiated neurospheres) confirmed that the cells were viable and expressed EGFP (Fig. 2D). Notably all EGFP expression was lost in these cultures after 1 week, suggesting that the granule cell precursors progressed to a Math1-negative differentiated state.

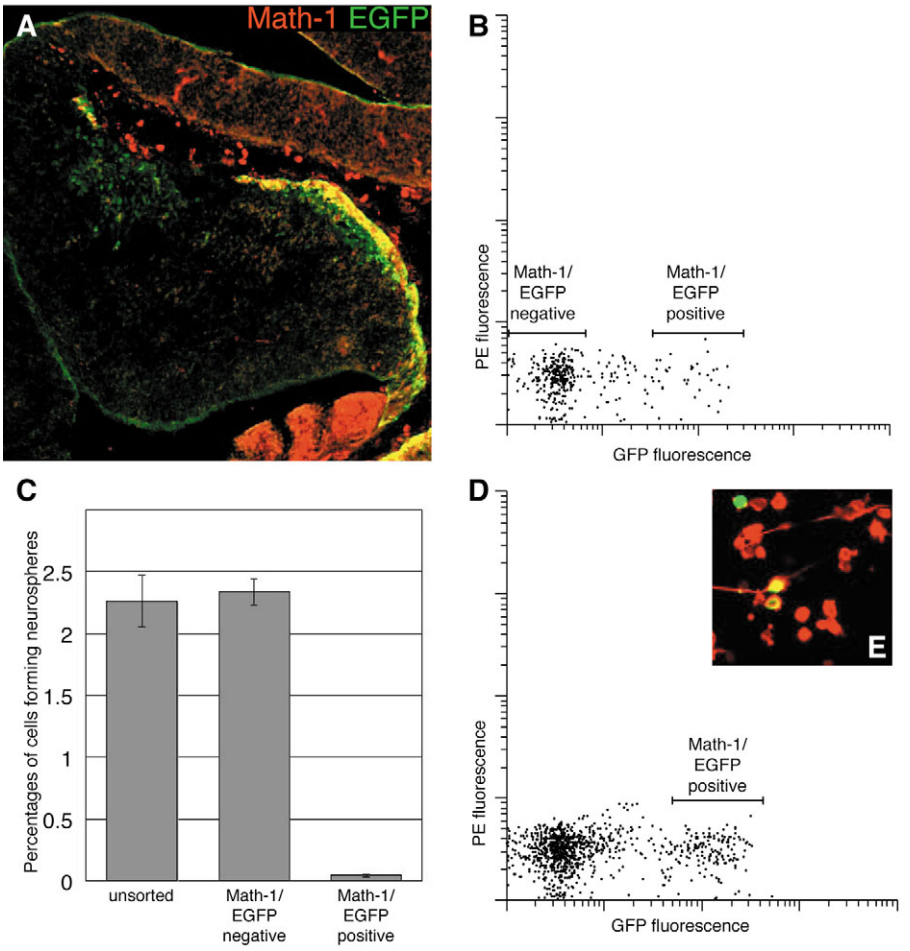

Fig. 2. Math1-negative progenitors give rise to Math1-expressing cells. (A) Math1^{EGFP} expression is restricted to the external granule layer of the cerebellum at E14.5 and overlaps with endogenous Math1 expression. (B) FACS subdivides the Math1^{EGFP}-negative fraction from the Math1^{EGFP}-positive cells. (C) The unsorted cells, as well as the Math1^{EGFP}-negative cells, give rise to neurospheres, whereas the Math1^{EGFP}-positive cells do not form neurospheres. (D,E) FACS of neurospheres derived from Math1^{EGFP}-negative cells plated on a feeder layer reveals the upregulation of Math1^{EGFP} after 2 days in culture. The perdurance of the EGFP allows the double-labeling of Math1^{EGFP}-positive neurons expressing Tuj-1 (E).

Cerebellar NSCs and forebrain-derived NSCs give rise to distinct cellular phenotypes in vitro

Our results suggest that cerebellar progenitors possess region-specific character that governs the mature cell types they generate. To examine this in more detail, we compared the cell types generated by cerebellar versus forebrain neurospheres, using cellular markers expressed exclusively in either one or other of these brain regions (DARPP-32 in the forebrain, TAG1 in the cerebellum). In addition, we also examined calbindin, which although expressed in both regions appears in morphologically distinct cell types (e.g. forebrain interneurons versus Purkinje cells). Consistent with the idea that stem cells from the forebrain and cerebellum possess regional character, cerebellar neurosphere cultures expressed TAG1 upon differentiation (29.4±1.3%) while forebrain-derived neurosphere cultures did not (0.1±0.1). Conversely, while no DARPP-32 staining was apparent in cerebellar-derived cultures (0±0%), DARPP-32 staining was expressed in a subpopulation of forebrain-derived cells (3.6±0.5%) (Jensen et al., 2004). Similarly, while the calbindin-immunopositive cells in cerebellar cultures exhibited the characteristic morphology of Purkinje neurons (4.9±1.3%), forebrain-derived calbindin-expressing cells had a bipolar morphology characteristic of the interneuron populations in this region (5.2±0.5%). We also examined the differentiation of neurospheres from both regions on a primary fibroblast feeder layer, to provide an equivalent neutral environment for differentiation. Cerebellar-derived neurospheres gave rise to Tag1-expressing (27.4±3.2%) and calbindin-expressing (2.7±0.7%) cells, whereas no DARPP-32 expression was observed (0±0%). Conversely, forebrain-derived neurospheres gave rise to DARPP-32-expressing (3.2±0.8%) and calbindin-expressing (4.9±1.3%) cells, but no TAG1 staining was apparent (0±0%). Hence, our in-vitro

results support the idea that the in-vitro cell fate of the cerebellar-derived neurospheres differs from telencephalic-derived neurospheres in a manner that reflects the normal cell fate of neural progenitors in these regions (Hitoshi et al., 2002; Horiguchi et al., 2004; Jensen et al., 2004; Ostenfeld et al., 2002).

Cerebellar- and forebrain-derived NSCs give rise to region-specific cellular phenotypes in vivo

To further analyze the differentiation potential of forebrain- and cerebellar-derived neurospheres, we transplanted them in to the SVZ of developing forebrain (postnatal day 4). As with our in-vitro experiments, the transplanted cells could be unambiguously identified by their constitutive expression of EGFP (Okabe et al., 1997). Two weeks post-transplantation, the distribution, morphology and expression of neural marker genes of transplanted cells were examined. Cells from forebrain-derived neurospheres integrated into the host tissue and migrated away from the injection site in a manner resembling the normal migration of SVZ-derived neurons (Fig. 3). Most EGFP-labeled cells were found in either the rostral migratory stream (RMS) and olfactory bulb (46.4±6.9%) or the cortex (36.6±5.6%). Fewer numbers of cells were located in the striatum (6.5±3.5%), corpus callosum (3.1±1.3%) and hippocampus (10.2±4.4%). Cells in the olfactory bulb were mainly found in the inner granule cell layer of the olfactory bulb, a subpopulation of which was immunopositive for GABA (Fig. 3B1,B2). Cells within the olfactory stream appeared to be migrating in chains and expressed the neuronal progenitor marker Tuj-1 (Fig. 3C1,C2) (Doetsch and Alvarez-Buylla, 1996). Forebrain-derived cells were found scattered in most layers of the cortex and were immunopositive for GABA, suggesting that they are interneurons (Fig. 3D1,D2). Most cells that remained in the injection site expressed the astroglial marker GFAP (Fig. 3E1,E2).

By comparison, the behavior of both grafted embryonic and adult cerebellar-derived neurospheres was markedly different from that of forebrain-derived neurospheres (as embryonic and adult transplants were comparable, for simplicity only the quantitation of adult cerebellar grafts are shown below). Two weeks after transplantation to the forebrain they showed limited dispersion (Fig. 4). Most cells were found along the corpus callosum (73.9±6.9%) and in the hippocampus (15.6±5.9%). Few cells were located in the cortex (8.2±5.8%) and striatum (2.3±1.3%), and these were mostly scattered along the needle track. No cells were found within the RMS or in the olfactory bulb (0±0%). In all analyzed areas the majority of cells expressed GFAP, indicating that they became astroglial cells (Fig. 4B1-D2). Some cells, mainly close to the graft site, expressed TAG1, implying that they maintained cerebellar identity (Fig. 4D3,D4).

We also did the converse experiment of transplanting both forebrain- and cerebellar-derived neurospheres into the cerebellum. Forebrain-derived cells integrated into the host

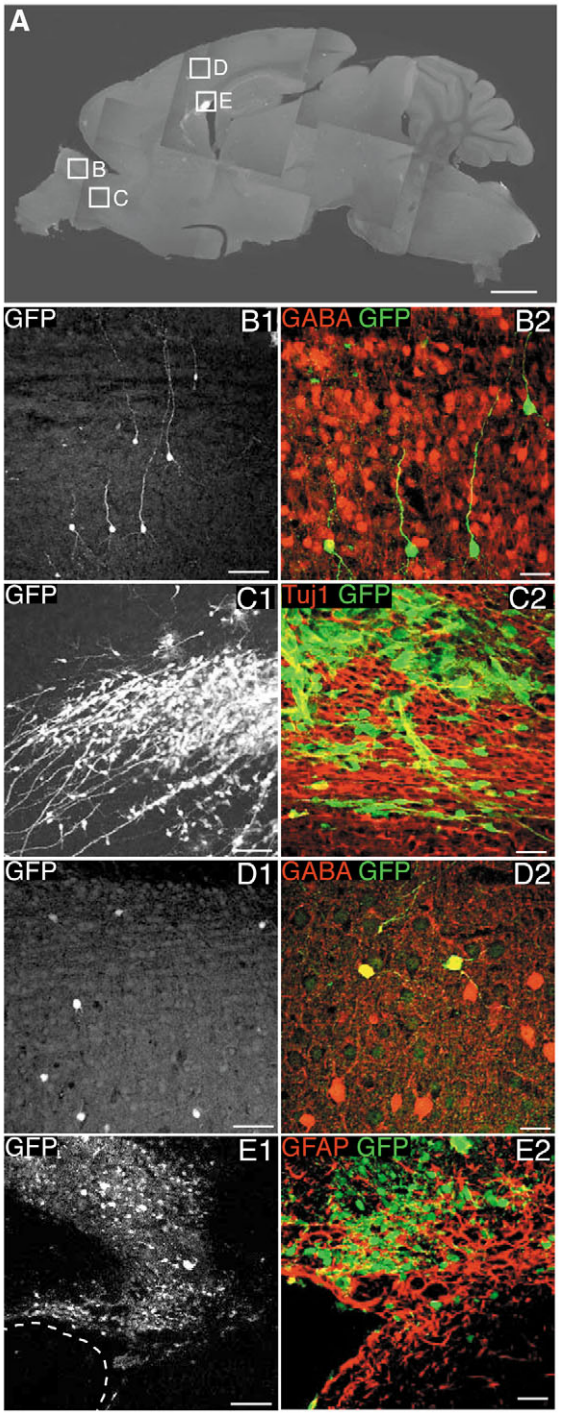

Fig. 3. Forebrain-derived neurospheres use normal migratory routes and give rise to neurons with the characteristics of olfactory bulb granule cells and cortical interneurons when transplanted to the forebrain. Constitutive EGFP-expressing forebrain-derived neurospheres were transplanted into the neonatal forebrain (P4) and analyzed at P21. Neurosphere-derived cells were using normal migratory routes and were found in various regions of the brain (A). GABA-immunopositive cells with the morphology of granule neurons were found in the granule cell layer of the olfactory bulb (B), and neuroblasts utilizing chain-migration were observed within the olfactory stream and expressed Tuj1 (C). GABA-positive cells were found distributed over most layers of the cortex (D). Some cells stayed close to the graft site and were expressing GFAP (E). Scale bars: 1 mm in A; 50 μm in B1,C1,D1,E1; 20 μm in B2,C2,D2,E2. Note that regions shown in boxes B1-E2 represent higher power taken from the approximate regions shown in A and that the two higher power pictures in C1 versus C2, as well as D1 versus D2, are different examples.

tissue and could be found in all cerebellar layers, including the white matter. Virtually all forebrain-derived cells observed after a 2-week survival period appeared to be glial cells that expressed GFAP. Consistent with this neurosphere–derived forebrain, cells generally adopted the morphology of either astrocytes or Bergman radial glia (Fig. 5A-F) (only one neurofilament-expressing cell was found in the analysis of 16 brains; Fig. 5G-I). In no cases did we observe forebrain cells that acquired the morphologies or markers characteristic of cerebellar neurons (data not shown).

We also transplanted E14.5- or adult-derived cerebellar neurospheres into P4 cerebellum. Two weeks post-grafting, EGFP-expressing cells were found distributed throughout all the layers of the cerebellum, as well as in the white matter tracks. GABA-expressing neurosphere-derived cells were found located in both the molecular and the inner granule cell layer (E14.5: 20.4±5.4%; adult: 20.7±3.7%; Fig. 6A–F). A few calbindin-expressing cells with the morphology of Purkinje cells were observed within the Purkinje cell layer (Fig. 6G-I), albeit only when derived from embryonic neurospheres and then with the same low frequency observed in vitro (0.5±0.4%). By contrast, transplantations of adult neurospheres, although they did not produce cells resembling Purkinje cells, gave rise to a population of cells that assumed the position and characteristic morphology of Lugaro cells (Fig. 6J-L). Notably, after either transplantation of embryonic or adult-derived cerebellar neurospheres the most abundant cell population observed possessed small cell bodies and were found in the inner granule cell layer (E14.5, 45.8±5.4%; adult, 58.9±5.3%). Consistent with this population being granule cells, they often both protruded 3-5 dendrites and were immunopositive for glutamate (Fig. 6M-R). In addition, subpopulations of cells after embryonic or adult neurosphere transplantation gave rise to glial cells. Notably, three-dimensional reconstructions of cells of each of these classes indicated that they were not multinuclear, suggesting that they do not result from cell fusion events.

To further characterize the cerebellar-derived population resembling granule cells, we undertook an analysis of their electrophysiological properties. From animals receiving neurosphere transplants, we recorded 36 of the cells found in the internal granule cell layer of the cerebellum, using whole cell patch clamp recording (EGFP-positive: 12 E14.5 NSCs, nine adult NSCs and 15 EGFP-negative). The current-voltage response of the recorded cells was ascertained from holding potentials of approximately –70 mV and three classes were defined, non-spiking, immature and mature (see Table 1), in both the EGFP-positive and negative populations similar to that previously described in young rats (D'Angelo et al., 1997). Immature cells exhibited long-duration, non-repetitive intermediate- and high-threshold calcium spikes (Fig. 6S,U) (D'Angelo et al., 1997). Mature granule cells of all populations exhibited characteristic inward rectification at subthreshold voltage steps and repetitive fast spikes (spike half width ⩽2 mseconds) (Fig. 6T,V). The ability of the EGFP-positive cells to generate action potentials and their overall passive membrane properties, size and position are strongly indicative of a cerebellar granule cell phenotype. In addition, we observed spontaneous excitatory synaptic potentials when recording from grafted cells [sensitive to the glutamatergic antagonist CNQX (10-20 μmol/l; n=3)], demonstrating that they receive afferent input.

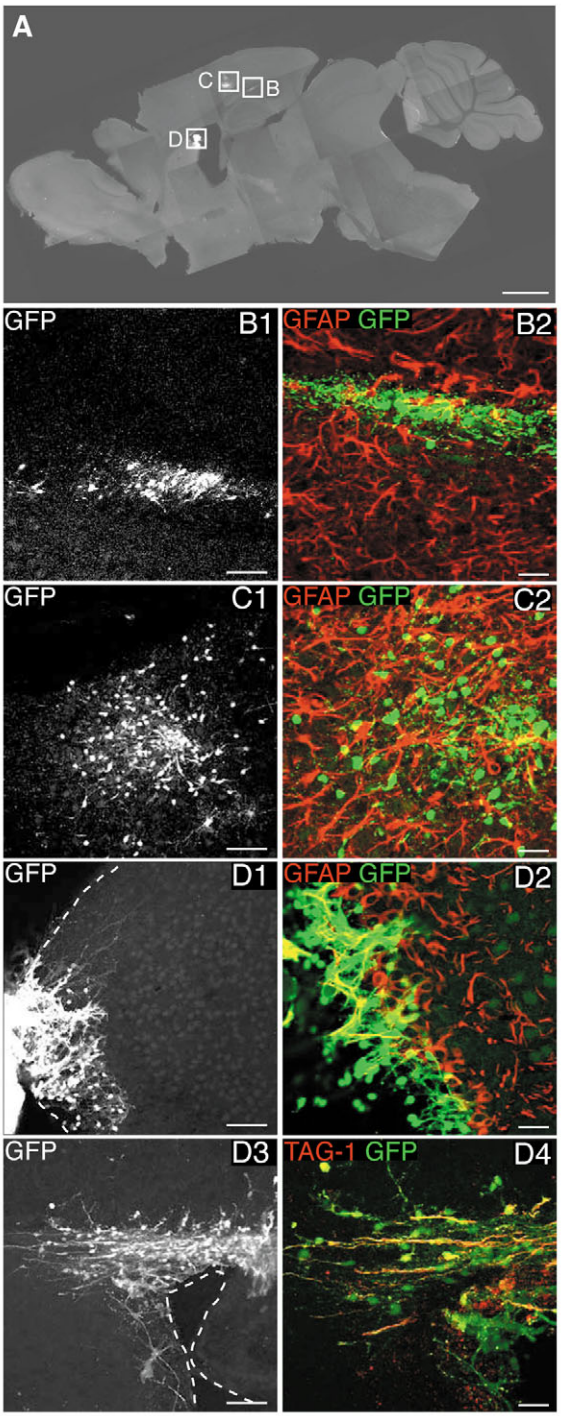

Fig. 4. Cerebellar-derived neurospheres mainly give rise to astroglia when transplanted to the forebrain. Constitutive EGFP-expressing adult cerebellar-derived neurospheres were transplanted into the neonatal forebrain (P4) and analyzed at P21. The grafted cells mainly remained in close proximity to the injection site (A). GFAP-expressing cells were found in the corpus callosum (B), in the cortex along the needle tract (C) and close to the ventricle (D). TAG1-expressing cells were found close to the graft site (D). Scale bars: 1 mm in A; 50 μm in B1,C1,D1,E1; 20 μm in B2,C2,D2,E2. Although it appears that many of the GFP cells in B2-D2 are not expressing GFAP, this is a result of the different localization of GFP and GFAP in these cells. Reconstruction of optical stacks demonstrates that the GFP cells shown in these figures are largely GFAP positive.

Discussion

In this paper we present data demonstrating that in-vitro expanded NSCs give rise to progeny that retain the character of their region of origin. Specifically we show that in vitro and after homotopic transplantation, forebrain and cerebellar-derived neurospheres give rise to cells that resemble those found endogenously in the brain. Furthermore, while forebrain-derived neurospheres grafted to the perinatal forebrain migrate both along the RMS and tangentially into the cortex, cerebellar-derived neurospheres are able to generate cells that resemble multiple cerebellar neuronal cell types, including granule cells, interneurons, Purkinje cells and Lugaro cells. Moreover, both embryonic and adult cerebellar-derived neurosphere cells preferentially adopt granule cell character with electrophysiological properties that are indistinguishable from the endogenous population. By contrast, forebrain- and cerebellar-derived neurosphere cells largely give rise to glial populations when transplanted heterotopically. Hence, these data suggest that NSCs possess regional character that is bestowed on their progeny and that stem cell-derived neurons require environmental regional cues for proper differentiation.

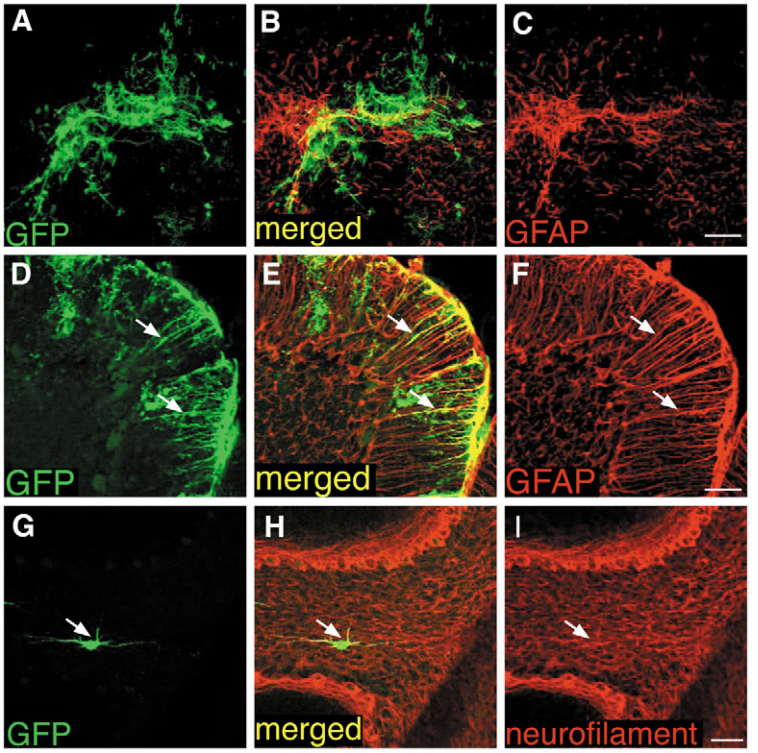

Fig. 5. Forebrain-derived neurospheres mainly give rise to astroglia when transplanted to the cerebellum. Constitutive EGFP-expressing E14.5 forebrain-derived neurospheres were transplanted into the neonatal cerebellum (P4) and analyzed at P21. The cells integrated into the host tissue and expressed GFAP, displaying morphology of either astrocytes (A-C) or Bergman radial glial cells (D,E, arrows). Only one neurosphere-derived neurofilament-expressing cell was observed (G-I).

Cerebellar stem cells exist in both the embryonic and adult cerebellum

In this study we extend previous analysis of CNS stem cells by characterizing their existence in the embryonic and adult cerebellum. While the existence of such a population in the embryonic and adult telencephalon is well recognized (Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch, 2003b), the presence of these populations in other parts of the nervous system is still a matter of debate (Gould et al., 1999; Horner et al., 2000; Kornack and Rakic, 2001; Magavi et al., 2000; Zhao et al., 2003). Similarly, the majority of NSC data suggest that they generically give rise to all cells in the CNS. Such claims are compromised by the lack of analysis of the regional character of these cells by markers other than ubiquitous markers of neurons and glia. The very existence of this population in the cerebellum raises the question of whether some basal neurogenesis may occur in the adult cerebellum.

Cerebellar stem cells recapitulate normal steps in cell differentiation to give rise to granule cells

The progenitors giving rise to the granule cell layer are known to occupy the rhombic lip and are thought to be segregated

Table 1. Active and passive membrane properties of EGFP-positive and control, EGFP-negative cerebellar granule cells

	Non-spiking (*n*=2)	E14.5 EGFP non-spiking (*n*=1)	Adult EGFP non-spiking (*n*=3)	Immature (*n*=5)	E14.5 EGFP immature (*n*=5)	Adult EGFP immature (*n*=2)	Mature (*n*=8)	E14.5 EGFP mature (*n*=6)	Adult EGFP mature (*n*=4)
RMP (mV)	–59	**–64**	**–61±7**	–57±9	**–55±13**	**–61**	–68±14	**–69±13**	**–76±16**
Rin (GΩ)*	2.8	**3.6**	**6.2±3.2**	6.1±3.5	**2.8±1.2**	**2.3**	2.6±1.5	**2.0±0.6**	**1.9±0.8**
Spike threshold (mV)†	–	–	–	–45±7	**–47±8**	**–50**	–45±3	**–42±11**	**–43±7**
Spike height (mV)†	–	–	–	12±7	**19±16**	**44**	54±7	**47±7**	**55±10**
Spike width (mseconds)†	–	–	–	55±50	**44±28**	**12**	0.9±0.2	**1.5±0.6**	**1.3±0.4**
Maximum firing freqency (Hz)	–	–	–	–	–	–	55±18	**40±18**	**62±13**
Tau (mseconds)*	57	**88**	**110±50**	77±30	**33±21**	**51**	23±10	**23±8**	**20±6**
Cm (pF)*	20	**25**	**18±5**	13±5	**11±4**	**16**	10±3	**12±3**	**11±2**

All values quoted±s.d.
RMP, resting membrane potential; Rin, input resistance.
*Calculated from 10 mV, 500 msecond hyperpolarizing pulses.
†Derived from the first spike at threshold.
Student's *t*-test between control and GFP populations.
EGFP-positive values are in bold.

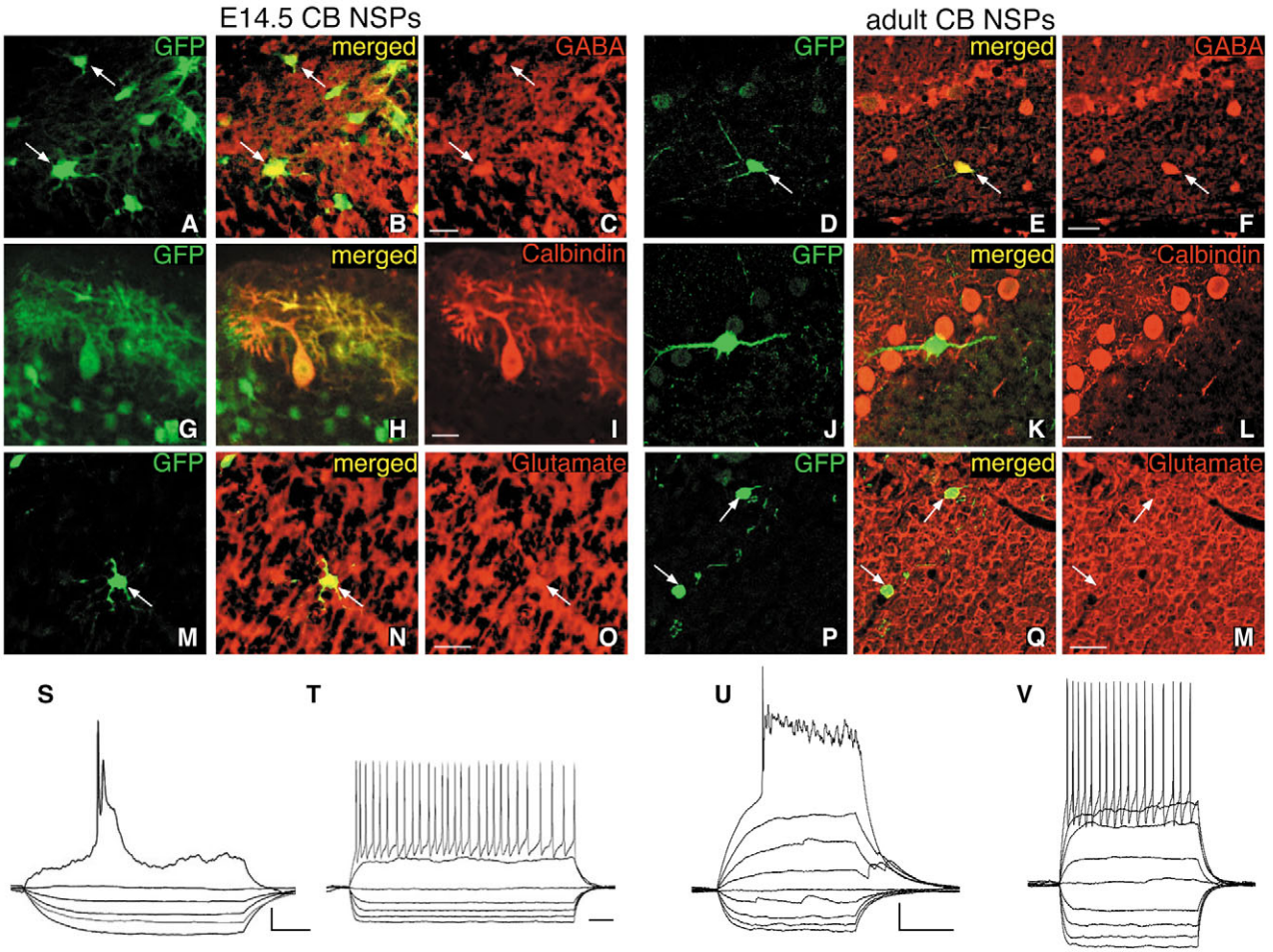

Fig. 6. Embryonic and adult-derived cerebellar neurospheres give rise to neurons with the characteristics of GABA-ergic interneurons and granule cells in vivo. Constitutive EGFP-expressing E14.5- and adult-derived cerebellar neurospheres were transplanted into the neonatal cerebellum (P4). A subpopulation of cells was observed to integrate into the host cerebellum and express GABA (A-F), indicating that they are interneurons. EGFP-positive calbindin-expressing cells with the morphology of Purkinje cells were detected in the Purkinje cell layer of E14.5-derived neurospheres, albeit at low frequency (G-I). In adult-derived NSC transplantations, large fusiform cells within the Purkinje cell layer that did not express calbindin were observed (J-L). Most commonly observed were EGFP-expressing cells with the characteristics of granule cells. These possessed a small cell body with three to five protruding dendrites and were immunoreactive for glutamate (M-R). Current-voltage responses of immature (S,U) and mature (T,V) GFP-positive cerebellar granule cells. Immature cells exhibited long-duration, non-repetitive intermediate- and high-threshold calcium spikes, whereas mature granule cells exhibit pronounced inward rectification and fast, repetitive spiking. Synaptic input was not blocked. Step protocol from initial step of –20pA, with 5pA increments. In T, the +10pA step is not shown for clarity. Scale bars: 30 μm in C,F,R; 20 μm in I,F,L. Horizontal bar, 50 milliseconds; vertical bar 10 mV in S,T; horizontal bar, 200 mseconds; vertical bar, 10 mV in U,V.

from progenitors giving rise to other cerebellar lineages from a time prior to the onset of cerebellar neurogenesis (Alder et al., 1996) (reviewed by Wingate, 2001). We were therefore very surprised to find that NSCs can give rise to neurons that resembled both granule neurons, as well as other cerebellar cell types including Purkinje cells, interneurons and presumably Lugaro cells. Given that granule cell lineage is considered to arise from the more restricted rhombic lip progenitor population, it seemed unlikely that the cerebellar stem cell population derived from this population. Consistent with this expectation, FAC-sorted Math1/EGFP cells did not give rise to neurospheres in our culture system.

Conversely, given the absolute requirement for Math1 for the generation of granule cells (Ben-Arie et al., 1997), if a non-Math1 progenitor was able to give rise to granule cells, one would predict that at some intermediate stage this progenitor must express Math1/EGFP. Consistent with this, a subpopulation of cells within the neurospheres transiently expresses Math1/EGFP when they are induced to differentiate. Generalizing from this it seems that in giving rise to the different cerebellar lineages the normal programs by which subsets of cerebellar neurons are generated are recapitulated in the differentiation steps utilized by cerebellar NSCs.

Forebrain and cerebellar NSCs maintain their regional character

Having demonstrated the existence of cerebellar stem cells, we sought to compare their differentiation potential to those stem cell populations known to exist in the forebrain. Indications that NSCs retain discrete regional character in vitro have come

from a number of recent studies (Hitoshi et al., 2002; Horiguchi et al., 2004; Jensen et al., 2004; Parmar et al., 2002). These studies suggest that the NSCs from distinct regions of the brain maintain some aspects of their molecular profile and can differentiate into some but not all neuronal classes in accordance with their regions of origin (Hitoshi et al., 2002; Horiguchi et al., 2004; Parmar et al., 2002). However, extrinsic factors appear to influence the developmental potential of NSCs by promoting their intrinsic cellular program when the NSCs are placed in a matching environment (Hitoshi et al., 2002; Jensen et al., 2004). Here we present the first systematic comparison of NSCs derived from two different regions of the CNS. Our in-vitro results support this trend by showing that after differentiation both forebrain and cerebellar NSCs give rise to neuronal cell types appropriate to their region of origin.

Furthermore, as shown by other researchers (Auerbach et al., 2000; Gage et al., 1995; Parmar et al., 2003; Sabate et al., 1995; Suhonen et al., 1996), our transplantation studies show that forebrain-derived NSCs integrate when grafted into the neonatal SVZ, make use of normal migratory routes and differentiate appropriately. Moreover, forebrain-derived NSCs only give rise to astrocytes when transplanted to the cerebellum as previously described (Suhonen et al., 1996). By contrast, cerebellar derived-NSCs can integrate and differentiate into cerebellar neurons when transplanted into the developing cerebellum (see below). Conversely, when cerebellar-derived NSCs are grafted into the SVZ they fail to migrate along the RMS and largely differentiate into astrocytes.

These observations suggest that NSCs, as well as the populations they give rise to, retain regional character that reflects their place of origin. Upon passaging, numerous examples suggest that to varying degrees region characteristics are lost (Gabay et al., 2003; Hack et al., 2004; Hitoshi et al., 2002; Jensen et al., 2004; Ostenfeld et al., 2002; Parmar et al., 2002; Santa-Olalla et al., 2003). In this regard, our studies only examine neural progenitors expanded in culture without passaging, and even so full differentiation to normal fates could only be achieved in vivo upon homotopic transplantation.

Considerable data support the notion that NSCs in the CNS are maintained by specialized niche environments (Alvarez-Buylla and Lim, 2004; Doetsch, 2003b; Doetsch et al., 1999; Doetsch et al., 1997; Lai et al., 2003; Lim et al., 2000; Machold et al., 2003). The progressive loss of regional character of NSCs when expanded through multiple passages in vivo probably reflects the requirement of these specialized niches in reinforcing the regional character of NSCs. However, our observation that NSCs expanded in vitro without passaging fail to attain appropriate regional character when transplanted heterotopically supports the argument that epigenetic cues in their normal postmitotic environment are required for NSCs to express proper regional character in vivo.

Studies examining the fate of heterotopically transplanted NSCs have generated mixed results. Work by the Gage laboratory has shown that hippocampal NSCs transplanted into the olfactory bulb can adopt seemingly normal olfactory granule cell identity (Auerbach et al., 2000; Suhonen et al., 1996). By contrast, heterotopic transplantation of NSCs from the spinal cord to the forebrain has at best led to NSCs adopting phenotypes only partially appropriate to the host region (Shihabuddin et al., 2000; Yang et al., 2000). These results are generally consistent with our findings that both forebrain and cerebellar NSCs require homotypic environments to differentiate with appropriate regional character. The exception to this rule appears to be when the transplants are restricted to the same region of the neuraxis (Auerbach et al., 2000; Suhonen et al., 1996).

Cerebellar stem cells can functionally integrate into the perinatal cerebellum

When transplanted into the perinatal cerebellum, embryonic as well as adult cerebellar-derived neurospheres generated cells with characteristics of multiple neuronal and glial cerebellar cell types. Both embryonic and adult NSCs were observed to give rise to neurons that resemble GABA-ergic interneurons, granule cells, oligodendrocytes and astroglia. In addition, at low frequencies transplanted embryonic cerebellar NSCs gave rise to Purkinje cells, while adult cerebellar NSCs generated cells that resemble Lugaro neurons, indicating that the developmental potential of neurosphere-derived NSCs might get restricted over time, as has been shown for acutely dissociated cells grafted into the developing cerebellum (Carletti et al., 2002). These findings are consistent with recent evidence that demonstrates that the potential of neural crest stem cells changes during maturation (White et al., 2001). Alternatively, the populations of NSCs we isolated from E14.5 cerebellum versus the adult cerebellum may represent distinct populations. Given that these two populations were indistinguishable based on their in-vitro differentiation potential and only differed in their generation after in-vivo grafting of neurons with Purkinje (E14.5) versus Lugaro characteristics (adult), we think it likely that they represent the same population, the potential of which changes somewhat over time. This is consistent with data suggesting that the embryonic radial glial progenitor population in the forebrain ultimately transforms into the adult NSC ‘B cell’ population (Merkle et al., 2004).

Patch-clamp recordings of NSC-derived neurons revealed that those resembling granule cells acquired electrophysiological properties that were indistinguishable from the host cerebellar granule cell population. The functional integration of these cells into the host environment was indicated by the frequent occurrences of spontaneous excitatory postsynaptic potentials, demonstrating that the grafted cells received afferent input.

There is the notion that the in-vivo acquisition of neural identities can be attained through cell fusion (Alvarez-Dolado et al., 2003). If this phenomenon explained our findings, it is surprising that cerebellar-derived NSCs could give rise to the neurons resembling the different normal cerebellar populations, while forebrain cells do not. Similarly, reports of in-vivo cell fusion suggest that this phenomenon apparently occurs at low frequencies. It is implausible that the large number of integrated cells in the cerebellum of animals receiving neurosphere grafts could be accounted for by this phenomenon. Taken together, our data support the idea that NSCs persist in the cerebellum into adulthood. Furthermore, our analysis suggests that NSCs derived from different brain regions possess intrinsic developmental character.

The fact that grafted cerebellar stem cells can assume appropriate cytoarchitecture in the perinatal cerebellum suggests that cues permitting the proper integration of nascent cells persist at least during this phase of development. These

cues are likely to be permissive rather than instructive, as forebrain-derived stem cells do not adopt cerebellar neuronal character when transplanted into the neonatal cerebellum. It would be interesting to see whether cerebellar NSCs cells can also integrate into more mature cerebellum. Similarly, it would be interesting to ascertain how precisely various cerebellar neuronal populations can be generated from cerebellar neurospheres. In this regard, recent work has unexpectedly demonstrated that by E13.5 three distinct sublineages of granule cells exist (Zong et al., 2005). As more understanding of the lineages and diversity of cerebellar cells is garnered, it will be intriguing to explore more fully the differentiation capacity of both embryonic and adult cerebellar NSCs.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/20/4497/DC1

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