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Progenitors from the postnatal forebrain subventricular zone differentiate into cerebellar-like interneurons and cerebellar-specific astrocytes upon transplantation

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ABSTRACT

Forebrain subventricular zone (SVZ) progenitor cells give rise to glia and olfactory bulb interneurons during early postnatal life in rats. We investigated the potential of SVZ cells to alter their fate by transplanting them into a heterotypic neurogenic and gliogenic environment—the cerebellum. Transplanted cells were examined 1 to 7 weeks and 6 months post transplantation. Forebrain progenitors populated the cerebellum and differentiated into oligodendrocytes, cerebellar-specific Bergmann glia and velate astrocytes, and neurons. The transplanted cells that differentiated into neurons maintained an interneuronal fate: they were GABA-positive, expressed interneuronal markers, such as calretinin, and exhibited membrane properties that are characteristic of interneurons. However, the transplanted interneurons lost the expression of the olfactory bulb transcription factors Tbr2 and Dlx1, and acquired a cerebellar-like morphology. Forebrain SVZ progenitors thus have the potential to adapt to a new environment and integrate into diverse regions, and may be a useful tool in transplantation strategies.

Introduction

Immature central nervous system (CNS) cells are in general committed to glial vs. neuronal fates early during development by intrinsic, extrinsic and spatial factors (Jessell, 2000; McCarthy et al., 2001; Schuurmans and Guillemot, 2002; Shen et al., 2006). However, the differentiation into specific subtypes may be shaped by environmental signals (Brustle et al., 1995; Fishell, 1995; Magrassi and Graziadei, 1996; Milosevic and Goldman, 2004). The potential for progenitor cells to produce specific cellular subtypes varies across developmental stages and brain regions. For example, embryonic progenitor cells are generally more plastic than progenitor cells from postnatal or adult proliferative areas. Embryonic cells can differentiate into a wide variety of cell types and subtypes upon their transplantation into different brain regions (Brustle et al., 1995; Campbell et al., 1995; Gao et al., 1991; Gao and Hatten, 1994; McConnell and Kaznowski, 1991; Vicario-Abejon et al., 1995). Late embryonic and especially postnatal cells, however, appear to lose the potential to differentiate into region specific cell subtypes (Desai and McConnell, 2000; Frantz and McConnell, 1996).

The postnatal brain contains only a few neurogenic areas, such as the forebrain SVZ (Doetsch and Alvarez-Buylla, 1996; Luskin, 1993; Suzuki and Goldman, 2003), hippocampal dentate gyrus (Kuhn et al., 1996), and cerebellar white matter (Zhang and Goldman, 1996b). Cells from the forebrain SVZ migrate along the anterior–posterior axis of this zone and eventually migrate into the overlying white matter and cerebral cortex, where they differentiate into astrocytes and oligodendrocytes, and also migrate along the rostral migratory stream to the olfactory bulb, where they differentiate into interneurons (Doetsch and Alvarez-Buylla, 1996; Suzuki and Goldman, 2003). During early postnatal life, progenitors from the lateral SVZ are a heterogeneous population, consisting of lineage restricted, as well as bipotential glial progenitors (Levison and Goldman, 1993,1997; Marshall et al., 2005; Zerlin et al., 2004). They express glial markers such as Olig2, Aldolase C (Marshall et al., 2005), and NG2 (Aguirre and Gallo, 2004; Staugaitis et al., 2001), neuronal markers such as Dlx2 (Marshall and Goldman, 2002; Marshall et al., 2005), and doublecortin (Gleeson et al., 1999), and neural precursor markers such as Sox3 (Wang et al., 2006), nestin (Ishii et al., 2008), Mash1 (Kohwi et al., 2005; Parras et al., 2004), and Pax6 (Kohwi et al., 2005) *in vivo*. The anterior part of the SVZ is largely populated by neuronal progenitors that express neuronal markers

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such as Tuj1 and MAP2, and markers of proliferative cells such as PCA-NCAM (Luskin, 1993). Previous studies reported that progenitors from the postnatal and adult anterior SVZ differentiated into neurons after transplantation into the neonatal or adult striatum (Brock et al., 1998; Herrera et al., 1999; Zigova et al., 1998), but the cells did not assume the morphology of region specific neurons upon transplantation into heterotypic or homotypic brain areas. We investigated the plasticity of progenitors from the rat lateral SVZ during the first postnatal week when challenged by a heterotypic environment. We labeled lateral SVZ progenitors *in vivo* with a replication-deficient retrovirus encoding green fluorescent protein (GFP), and then transplanted them into the neonatal cerebellar white matter.

We chose the cerebellum for transplantation because during the early postnatal period it is both a gliogenic and neurogenic region. Progenitor cells in the cerebellar white matter give rise to oligodendrocytes and astrocytes, including the cerebellar-specific types of astrocytes, Bergmann glia and velate astrocytes, as well as interneurons including Golgi, basket, stellate (Zhang and Goldman, 1996a), Lugaro (Milosevic and Goldman, 2002), interstitial, marginal (Ramon y Cajal, 1995), and granule cells (Altman, 1972). We labeled lateral SVZ progenitors *in vivo* with a replication-deficient retrovirus encoding green fluorescent protein (GFP), and then transplanted them into the neonatal cerebellar white matter. We found that some SVZ cells differentiated into cerebellar-specific glia, assuming the appropriate anatomical position and morphology, and some expressed cell specific markers. The transplanted SVZ neuronal progenitors maintained an interneuron fate in their new environment. They expressed general interneuron markers such as GABA and calretinin, exhibited membrane properties that are typical for interneurons, and assumed the appropriate position and morphology of some cerebellar interneuron subtypes. Transplantation of the SVZ cells into the cerebellum appears to partially shift their fate since they no longer expressed the Tbr2 and Dlx1 markers, that are expressed by olfactory bulb cells did not adopt the expression of cerebellar-specific markers. Thus, given the appropriate environment, glial and to a lesser extent neuronal progenitors from the postnatal SVZ can respond to local signals by differentiating in an environmentally specific manner.

Results and discussion

Transplanted cells from the forebrain SVZ assume proper spatial and morphological characteristics in the cerebellum

The neonatal forebrain SVZ contains neuronal and glial progenitors, both of which can be labeled by replication-deficient retrovirus (Suzuki and Goldman, 2003). To test the potential of progenitors from the forebrain SVZ to develop into cerebellar-specific cell types, we injected a replication-deficient retrovirus encoding GFP into the SVZ at P0/1, isolated GFP-positive cells by fluorescent activated cell sorting 2 days later, and immediately transplanted them into the deep white matter of the cerebellum of postnatal day 3/4 (P3/4) pups. We then examined the cerebella at different times from 10 days post transplantation (dpt), to 6 months post transplantation to determine which types of neurons and glia the forebrain progenitors generated. In this study we transplanted SVZ progenitor cells as a mixed group of GFP-positive cells. While we assume that the SVZ neuroblasts developed into interneurons and the glioblasts into glia in the host cerebellum, our study did not examine this issue. Nevertheless, we asked whether forebrain progenitors could assume any of the characteristics of cerebellar-specific cells in the context of the neonatal cerebellar environment.

We injected cells into the cerebellar white matter and at 10 dpt found that the transplanted cells had migrated away from the injection site and could be detected in all layers of the cerebellum. Many of the transplanted cells closely resembled endogenous cerebellar cells described before (Chan-Palay and Palay, 1972; Douyard et al., 2007; Laine and Axelrad, 2002; Milosevic and Goldman, 2002; Ramon y Cajal, 1995; Rodrigo et al., 2001; Simat et al., 2007). The transplanted cells displayed similar morphologies and also resided at appropriate positions within the cerebellum in comparison to host cells (Figs. 1A–K). Thus, the SVZ cells appeared to have migrated in a manner similar to that of endogenous progenitors in the early postnatal cerebellar white matter (Zhang and Goldman, 1996a,1996c). Some of the transplanted cells acquired the morphologies of cerebellar-specific glia, such as velate astrocytes (Fig. 1A), Bergmann glia (Fig. 1B), and myelinating oligodendrocytes in the white matter (Fig. 1C). Other transplanted cells resembled cerebellar cortical interneurons, such as Lugaro cells (Fig. 1D), basket cells (Fig. 1E), and Golgi cells (Fig. 1F). In addition to cerebellar interneurons found in the cerebellar cortex, two subtypes of interneurons are found in the cerebellar white matter, interstitial and marginal neurons (Ramon y Cajal, 1995). Interstitial neurons have oval or elongated cell bodies with dendrites that run parallel to the white matter axons and eventually reach the granule cell layer; marginal neurons are found on the border between the white matter and the internal granule cell layer, with elongated or spindled-shaped cell bodies and dendrites that run parallel to the white matter axons, but also have a ramified process that invades the granule and molecular layers. Some of the transplanted cells showed morphological and spatial characteristic that are properties of these two interneurons subtypes (Figs. 1G–I). At 6 months post transplantation SVZ cells were found in all cerebellar layers, displaying the same distribution, phenotypes, and antigenic characteristics as cells observed at earlier time points (Fig. 3A).

A large majority of the transplanted cells, 78%, showed cerebellar-like morphology. Cells that resembled cerebellar interneurons and expressed typical interneuronal markers, such as GABA and calretinin (CR) accounted for 30% of these cells. The proportion of glial cells was 48%, of which oligodendrocytes accounted for 27%, and astrocytes for 13%, while the proportion of the cerebellar-specific astrocytes, Bergmann glia and velate astrocytes, was 4% each. The proportion of transplanted cells that had progenitor-like or unspecific morphology was 22%.

Previous transplantation studies have demonstrated that both the donor cells and the host environment are critical variables in determining the developmental outcome of transplanted cells. Thus, for example, neurospheres generated from E14.5 forebrain generate mostly astrocytes and no cerebellar-type neurons when transplanted into P4 cerebellum (Klein et al., 2005). In contrast, cerebellar neurospheres generated neurons with characteristics of granule cells and GABAergic interneurons after transplantation into P4 cerebellum. Klein et al. concluded that neural stem cells retain characteristics of their source tissue.

Cerebellar cells transplanted into embryonic telencephalic ventricles differentiate into neurons with cerebellar characteristics (Carletti et al., 2002). However, cerebellar cells isolated at E12 generated both projection neurons and interneurons, while postnatal cerebellar cells only generated interneurons, thus following the normal developmental program, in which Purkinje and deep cerebellar projection neurons are generated before interneurons. This study suggests that by E12, immature cells in the cerebellum have been specified to a cerebellar fate, which local environmental influences, in this case, do not overcome. However, the specification of cerebellar cells to different cerebellar neurons changes over time in concordance with the normal developmental schedule, early-generated cells producing projection neurons and late-generated cells producing interneurons from the multipotent progenitor that adopts specific morphology in response to the local environment (Carletti et al., 2002; Leto et al., 2006). Similarly, cerebellar cells transplanted back into the embryonic cerebellum assume fates consistent with the age of the donor cells, i.e. postnatal derived cells produce only interneurons (Jankovski et al., 1996).

Our observations suggest a capacity for developmental plasticity in SVZ cells such that interneuron progenitor cells destined to

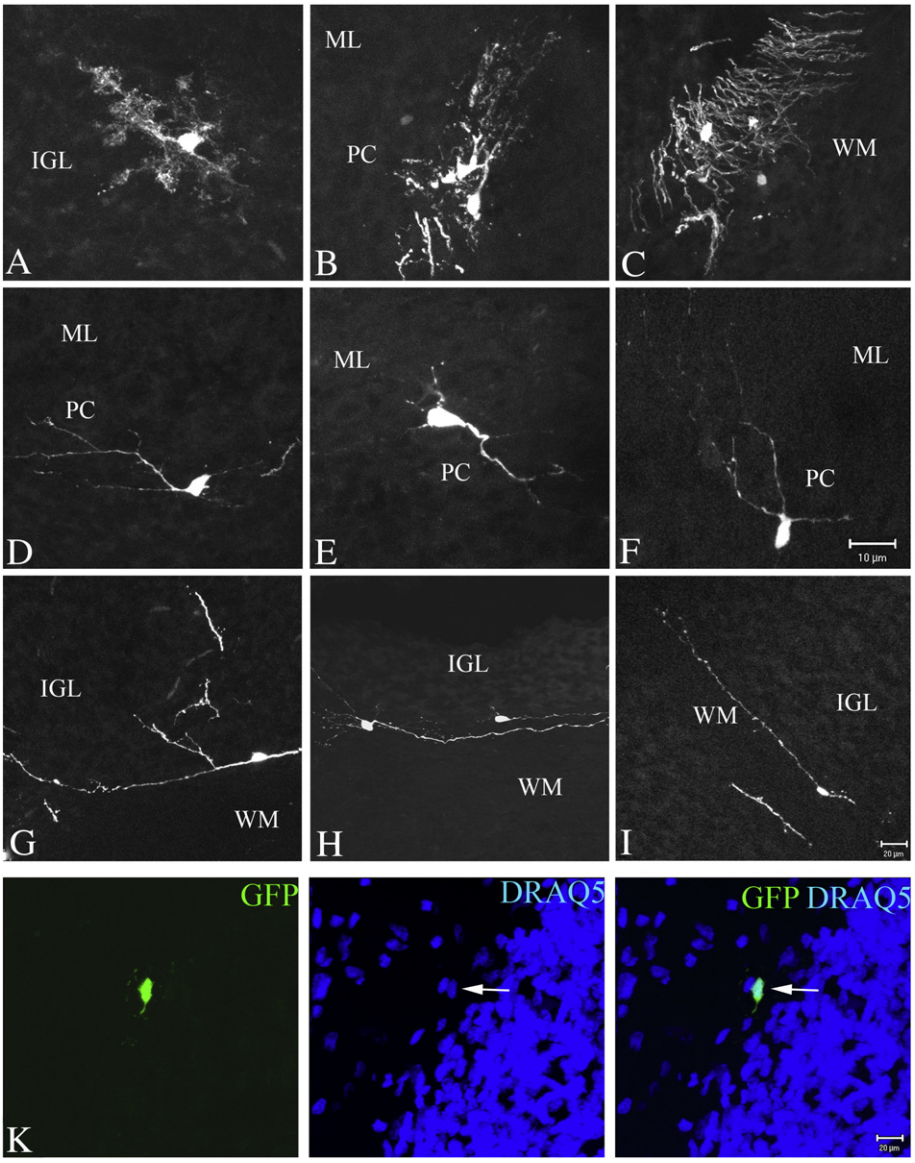

Fig. 1. Morphology and distribution of GFP-positive cells transplanted from forebrain SVZ into the cerebellar white matter, 10–21 days post transplantation. (A) Velate astrocyte-like in the IGL; 10 dpt. (B) Bergmann glia-like cells with the cell bodies in the PC layer and processes with branches extended toward the pia; 14 dpt. (C) Myelinating oligodendrocytes in white matter; 10 dpt. (D) Lugaro-like cell in the IGL, with cell body below the PC layer and dendrites that run perpendicular to the PC layer; 14 dpt. (E) Basket-like cell in ML with cell body just above the Purkinje cells and dendrites that branch in ML; 21 dpt. (F) Golgi-like cell with cell body just below the PC layer and dendrites that protrude to the ML, and axon pointing into the IGL; 10 dpt. (G–I) Transplanted cells that reside in the white matter, with long dendrites parallel to white matter fibers. Cell in G also has a dendritic branch that spans into the IGL towards the PC and ML; 14 dpt. (K) Transplanted cell residing in the white matter is labeled with the nuclear stain DRAQ5. Scale bar for A–F is 10 µm. Scale bar for G–K is 20 µm.

become olfactory bulb cells can acquire cerebellar-like characteristics and astrocyte precursors destined to become cortical or white matter glia acquire characteristics of Bergmann glia and velate astrocytes, forms that are not present in the forebrain.

Transplantation of neonatal or adult SVZ cells into other regions, such as striatum, dentate gyrus or cerebral cortex results in their differentiation into neurons, but not necessarily the neuronal population specific to the host location (Herrera et al., 1999; Zigova et al., 1998). Why would neonatal SVZ cells transplanted into the early postnatal cerebellum acquire cerebellar characteristics? It might be that the cerebellum, a neurogenic and gliogenic area during early postnatal life, provides positional and differentiation cues for the development of these progenitors, whereas the striatum or cerebral cortex does not. Thus, the local host environment plays a key role in determining regional specificity of cellular differentiation.

Transplanted forebrain progenitors differentiate into cerebellar-specific glia

At 21 dpt we found that transplanted cells had differentiated into cerebellar-type glia. We characterized these cells with detailed analysis of morphology, marker expression and electrophysiology. Some of the transplanted cells were labeled with an antibody to glial fibrilary acidic protein (GFAP, Fig. 2A). In the internal granule layer some of the GFAP-positive cells displayed the morphology of the velate astrocytes that are characteristic of this layer (data not shown). Velate astrocytes have large cell bodies and thick branches with many extensions, giving the cell a bushy appearance, which is different from that of fibrous astrocytes that have fewer ramified branches (Zhang and Goldman, 1996b). The velate-like astrocytes also made contact with blood vessels. This is a typical feature of astrocytes, but is not specific to the cerebellum (Milosevic and

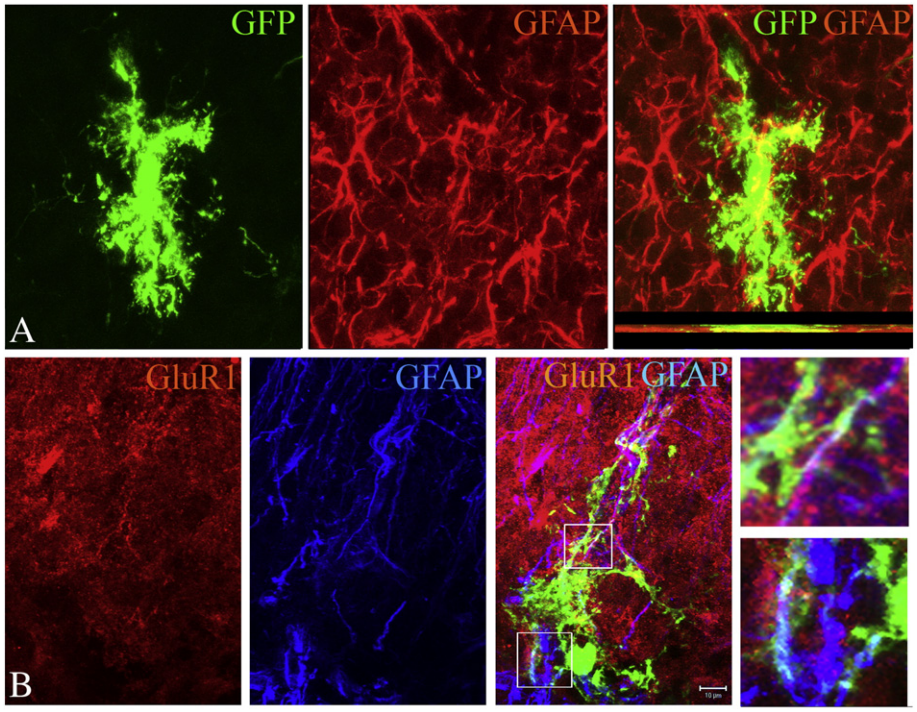

Fig. 2. Immunolabeling of astrocyte-like transplanted cells in the cerebellar cortex; 10–21 dpt. (A) Transplanted astrocyte in IGL labeled with antibody to GFAP (red); the inset in merged panel represents the image rotated 180° along the *x*-axis, showing the processes of the astrocyte labeled with GFAP antibody; 21 dpt. (B) Bergmann glia labeled with antibodies to GluR1 (red) and GFAP (blue); the section is 30 μm thick, and penetration of the antibodies was insufficient, therefore only the superficial portion of the cell was labeled. The insets are magnified parts of the cell on the left to show the expression in the processes; 10 dpt. Scale bar for A and B is 10 μm.

Goldman, 2002; Zerlin and Goldman, 1997; Zerlin et al., 1995; Zhang and Goldman, 1996d).

Transplanted cells also differentiated into Bergmann glia-like astrocytes. These cells exhibited the proper morphology and position of Bergmann glia, with cell bodies in the Purkinje cell layer and processes oriented radially toward the pial surface terminating in end feet at the pia. In addition, these cells were labeled with brain lipid-binding protein (Blbp, data not shown), GFAP, and glutamate receptor 1 (GluR1) antibodies that labeled Bergmann glia (Douyard et al., 2007) (Fig. 2B, insets).

Astrocytes display widely varying morphologies in different regions of the CNS (Ramon y Cajal, 1995). How much of this variation is locally determined, and how much is determined early in the stages of astrocyte development? *In vitro* clonal studies suggest that some astrocyte precursors or astrocytes generate morphological copies of themselves during proliferation (Miller and Szigeti, 1991), but this does not indicate when a decision to become a specific subtype is reached. The observation that progenitors from the forebrain SVZ will differentiate into cerebellar-specific astrocytes indicates that the local environment must play an important role in cell subtype specification. For example, Purkinje cells may influence astrocyte precursors to differentiate into Bergmann glia, whereas internal granule cells may influence the same astrocyte precursors to differentiate into velate astrocytes. Consistent with this model of local influences is the finding that Sonic hedgehog, secreted by Purkinje cells, can induce differentiation of immature astrocytes from embryonic day 10–12 chick cerebellar cortex explants and astroglia isolated from P1–3 mouse cerebellum (roughly equivalent to E18 rats) into Bergmann glia *in vitro*, as judged by the expression of Blbp (Dahmane and Ruiz i Altaba, 1999). The same study showed that the number of Bergmann glial cells in chick embryos is reduced significantly if the secreted Sonic hedgehog is blocked.

Notch signaling is also involved in radial glia development. Activated Notch1 promotes radial glia and eventually astrocyte identity in the embryonic brain (Gaiano et al., 2000) and also glial formation in the cerebellum (Lutolf et al., 2002). The transcription of Blbp in radial glia cells is directly induced by Notch effector CBF1 (Anthony et al., 2005). The components of the Notch signaling system are indeed present both in Bergmann glia and SVZ cells. During postnatal development co-localization of Notch1 and its ligands Jagged1 or 2 were found in Bergmann glia (Stump et al., 2002; Tanaka and Marunouchi, 2003) and Delta-like 3 was detected in the Purkinje cell layer (Stump et al., 2002). Notch1 and Jagged1 were also detected in SVZ cells, whereas Jagged2 and Delta-like 1 and 3 were not expressed (Stump et al., 2002). Thus, it is possible that Purkinje cells induce the differentiation of transplanted SVZ cells into Bergmann glia by activating either the Sonic hedgehog or the Notch1 pathway, or both.

We found that 27% of the transplanted cells differentiated into oligodendrocytes that were labeled with oligodendrocyte markers CNPase and MBP (data not shown). These oligodendrocytes were found mainly in the white matter, frequently in small clusters consisting of 3–4 cells with small cell bodies and smooth processes that ran parallel to the fibers in white matter (Fig. 1C). It is not known whether cerebellar oligodendrocytes have different characteristics from their forebrain counterparts, so we could not conclude whether oligodendrocyte precursors had differentiated in a host-specific manner in the cerebellum. We did not observe NG2-positive transplanted cells, but this is most likely because NG2-positive glia appear later in cerebellar development (Zhang and Goldman, 1996b).

Transplanted cells acquire the general antigenic characteristics of interneurons

We investigated further the characteristics of the transplanted cells that had developed neuron-like morphologies. We found that these cells were all labeled with an antibody to the pan-neuronal marker, MAP2 (Fig. 3A). To determine the specific subtype of neurons we used interneuron specific markers, such as antibodies directed against GABA, which labels cerebellar interneurons (Eccles

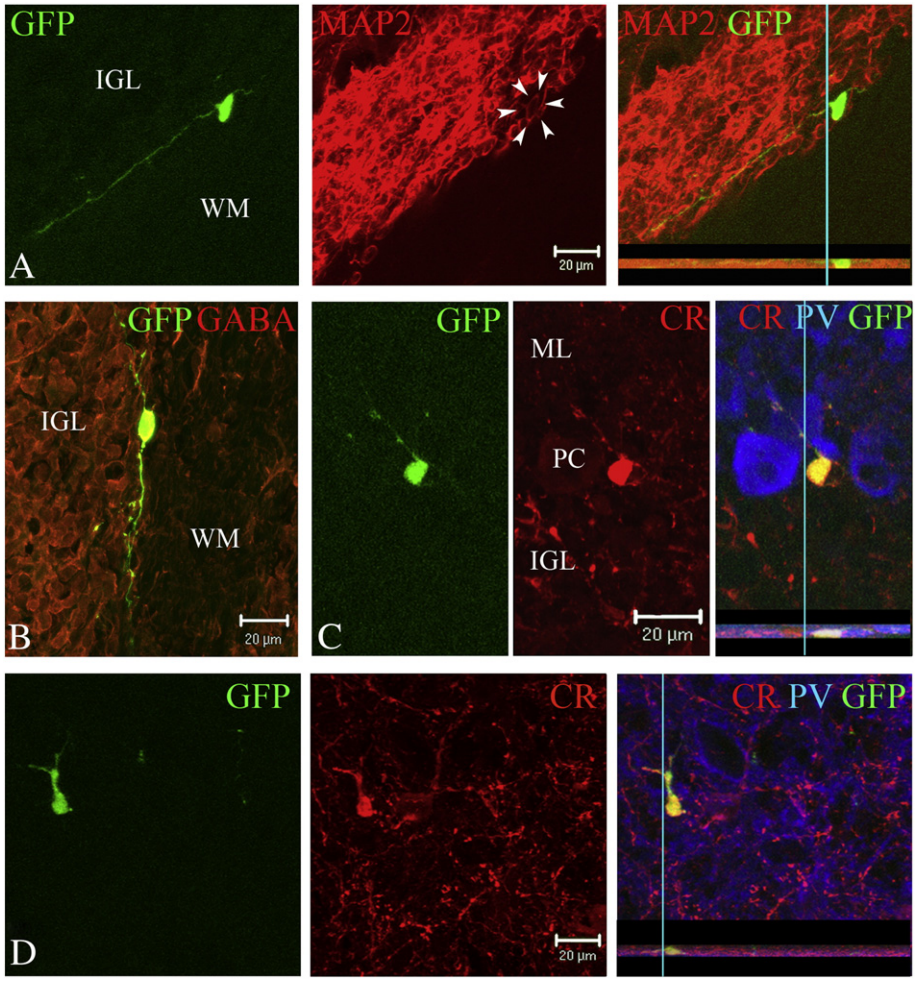

Fig. 3. Immunolabeling of transplanted cells differentiated into cerebellar-like interneurons; 14–21 dpt and 6 mpt. (A–B) Cells on the border between white matter and IGL labeled with MAP2 (red) in A and GABA (red) in B; arrowheads in A are pointing to the cytoplasm labeled with MAP2 antibody; A) 6 mpt, B) 21 dpt. (C) Cell in the IGL, just below the PC layer, labeled with antibody to CR (red); 21 dpt. (D) Small calretinin-positive (red) transplanted cell in cerebellar dentate nucleus. Parvalbumin (blue) labels Purkinje cells axons, wrapped around the large projection neurons of the dentate nucleus. Insets in the panels A, C and D are 3-D representation of z-stack image rotated at 180° along the *x*-axis to show that immunolabeling is indeed in the transplanted cell. Scale bars are 20 μm in A–C, and 10 μm in D.

et al., 1966; Gabbott et al., 1986), or the calcium-binding proteins calretinin and parvalbumin, which are expressed by a subpopulation of interneurons in forebrain cortex (Kubota et al., 1994; Xu et al., 2006) and cerebellum (Bastianelli, 2003; Celio, 1989). The transplanted cells with neuronal morphology were labeled with the anti-GABA antibody (Fig. 3B), and few were labeled with anti-CR antibody (Fig. 3C). We also detected a few CR-positive transplanted cells in the deep cerebellar nuclei (Fig. 3D). Interneurons in the cerebellar deep nuclei are born at the end of gestation and in the early postnatal period (Leto et al., 2006). The transplanted SVZ cells in the deep nuclei had rather small cell bodies that did not morphologically resemble host projection neurons of the deep nuclei.

The transplanted cerebellar-like neurons that were distributed in the cerebellar folia were not labeled with anti-parvalbumin antibody, which labels basket cells (Kosaka et al., 1993), or with an antibody directed against LIM homeobox gene 5 (Lhx5), which is expressed exclusively by stellate and basket cells in the postnatal cerebellum, but not by olfactory bulb interneurons or their progenitors in the postnatal SVZ (Gong et al., 2003); Gensat database, www.gensat.org). We also found that transplanted cells were not labeled with an antibody directed against the transcription factor paired box gene 2 (Pax2), which is expressed specifically by the GABAergic interneurons in the developing cerebellum (Maricich and Herrup, 1999).

Since SVZ forebrain progenitors in the postnatal brain differentiate into interneurons in the olfactory bulb, and since we grafted postnatal cells into postnatal animals at a point after Purkinje cell neurogenesis, we did not expect to see Purkinje-like neurons or large projection neurons in deep nuclei. However, we tested this possibility by labeling some of the sections with an antibody to calbindin, a calcium-binding protein expressed by Purkinje cells (Celio, 1990), but did not observe any transplanted cells that stained positively for the anti-calbindin antibody. We also did not observe any transplanted cells that resembled cerebellar granule cells, or that were labeled with an antibody to glutamate, the neurotransmitter expressed by granule cells (Kuhar et al., 1993; Pickford et al., 1989).

Thus, some of the transplanted neurons showed morphological and spatial characteristics of cerebellar interneurons, and expressed interneuron specific markers, such as GABA and CR, but did not express other markers specific for cerebellar interneurons, such as Lhx5 or Pax2. To determine if the transplanted cells expressed markers characteristic of olfactory bulb interneurons, their normal forebrain fate, we applied several antibodies, such as NeuN, expressed in olfactory bulb interneurons and progenitors (Bedard et al., 2002; Iwai et al., 2003), or antibodies for transcription factors distal-less homeobox gene 1 (Dlx1) and eomesodermin homolog (Tbr2), which are expressed by olfactory bulb interneurons (Kimura et al., 1999; Saino-Saito et al., 2004). NeuN labels granule cells, but does

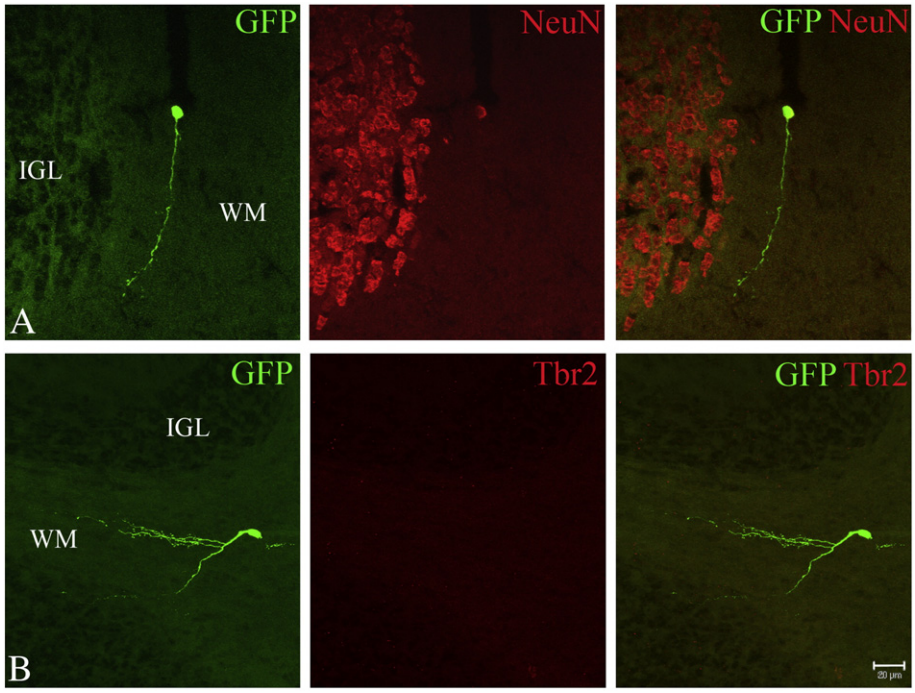

Fig. 4. Immunolabeling of transplanted neurons with antibodies to proteins expressed in olfactory bulb interneurons and their progenitors. (A) Neuron in white matter labeled with NeuN antibody (red). The only endogenous cerebellar neurons labeled with antibody to NeuN are granule cells in IGL; 21 dpt. (B) Transplanted neuron in cerebellar white matter is not labeled with antibody to Tbr2 (red), specific marker for olfactory bulb neurons; 21 dpt. Scale bar for A and B is 20 μm.

not label other subtypes of cortical interneurons in the cerebellum such as basket, stellate, Golgi, Lugaro or unipolar brush cells (Mullen et al., 1992; Weyer and Schilling, 2003). Tbr2 and Dlx1 are not expressed by any cerebellar cell (Gensat database, www.gensat.org), except unipolar brush cells, which can be labeled with anti-Tbr2 antibody (Englund et al., 2006). We found that only a few of the transplanted cells were labeled with NeuN, and these were found in the cerebellar white matter (Fig. 4A). Since only granule cells express NeuN in the cerebellum, and since we did not observe any transplanted cells exhibiting the morphological or immunochemical hallmarks of granule cells, we believe that NeuN expression indicates the retention of SVZ characteristics by a small proportion of transplanted cells. However, we did not detect any transplant cells that were labeled with other markers characteristic of interneurons in the olfactory bulb, such as Tbr2 (Fig. 4B) or Dlx1 (data not shown). Thus the SVZ cells had been shifted out of their normal patterns of expression after transplantation in the cerebellum.

However, the SVZ cells did not switch to a cerebellar fate since they did not express transcription or homeobox factors normally expressed by cerebellar interneurons. Other studies that have investigated the potential of forebrain SVZ or cerebellar progenitors by heterotypic transplantation method examined the fate of transplanted cells by expression of non-cell specific transcription factors (Aguirre et al., 2007) or in an *in vitro* environment (Klein et al., 2005). Specifically, Aguirre and colleges analyzed perinatal SVZ cells that had been grafted into the hippocampus with the pan-Dlx antibody, which labels Dlx1/2 and Dlx5/6 and is not specific for the host and/or donor cells in that study. Moreover, the upregulation of Math1, a transcription factor specifically expressed in cerebellar granule cells, was examined in neurospheres essay (Klein et al., 2005). Other studies limited the analysis of the expression profile in transplanted cells to markers such as CR, PV, calbindin, or pan-neuronal markers such as SMI32 and βtubulin, and morphology and spatial characteristics (Carletti et al., 2002; Jankovski and Sotelo, 1996; Klein et al., 2005). Thus, at present there are no conclusive results demonstrating the complete change in the expression profile of donor cells upon the transplantation into heterotypic host tissue.

Membrane properties of transplanted cells

Our immunocytochemical data indicates that SVZ forebrain cells differentiate into neurons and glia after transplantation into the cerebellum. We sought confirmation of neuronal identity by examining the membrane properties of the transplanted cells in their new environment. We obtained electrophysiological recordings from the GFP-positive cells in acute slices prepared from animals 2 to 6 weeks post transplantation to determine whether transplanted cells exhibited the membrane properties characteristic of neurons and glia. This data is summarized in Table 1 and representative recordings are shown in Fig. 5.

Whole-cell patch-clamp recordings were obtained from GFP-positive transplanted cells that we identified as neurons or glia based on their morphology and location in the cerebellum. All GFP-positive cells that had acquired the morphological phenotype of cerebellar interneurons (n=24), demonstrated clear neuronal

Table 1
Summary of electrophysiological analysis done on cells transplanted from forebrain SVZ into the cerebellum (transplanted cells), endogenous cerebellar cells (cerebellum control) and cells transplanted from the forebrain SVZ back into the same area and analyzed after they migrated into the olfactory bulb (forebrain control)

Transplanted cells	Cerebellum control	Forebrain control
9 experiments, 31 cells recorded	1 experiment, 8 cells recorded	4 experiments, 15 cells recorded
17 dpt and 4–7 wpt	13 dpi	26 dpi
4 astrocytes	1 astrocyte	1 astrocyte
1 Bergmann glia	1 Bergmann glia	
2 myelinating oligodendrocytes	1 myelinating oligodendrocyte	1 myelinating oligodendrocyte
24 cerebellar-like interneurons	1 basket cell 2 Lugaro cells 2 Golgi interneurons	9 periglomerular cells 4 interneurons (SVZ-SVZ transplant)

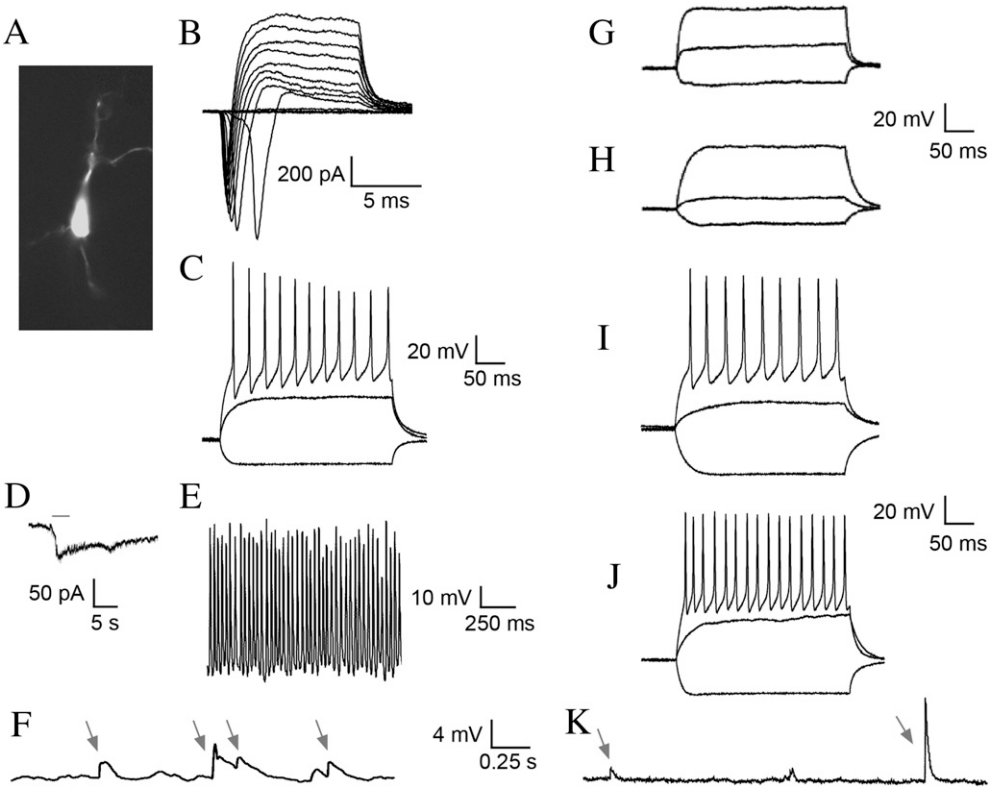

Fig. 5. Membrane properties of transplanted and endogenous cerebellar cells; 13 dpt–7 wpt. (A) Image of a transplanted SVZ cell in the cerebellum before electrophysiological recording. (B, C, E) Transplanted SVZ cells displayed typical current and voltage properties of mature interneurons including strong inward currents and repetitive non-accommodating action potentials. (D) Transplant cells responded to glycine (3 mM) in the presence of bicuculine (10 μM). (F) Transplanted neurons showed evidence of integration into host circuitry such as spontaneous action potentials (arrows). SVZ transplant cells that acquired the morphology of Bergmann glia (G) and oligodendrocytes (H), lacked neuronal membrane properties. (I) Host interneurons labeled with retroviral injections into the cerebellum displayed membrane properties similar to the transplanted SVZ cells. (J) SVZ cells that were re-transplanted into the cortical SVZ and recorded 5.5 weeks later after they had migrated into the olfactory bulb also displayed characteristic properties of mature interneurons, and exhibited spontaneous synaptic potentials (arrows) consistent with integration into host circuitry (K).

electrophysiological characteristics. This included strong voltage-gated inward sodium current, and responses to application of the neurotransmitter glycine in the presence of the $GABA_A$ receptor antagonist, bicuculine (Figs. 5A and D). The transplanted neurons exhibited membrane properties that are common to interneurons, including large after-hyperpolarizations, and the ability to fire repetitive non-accommodating action potentials at approximately 30 Hz both spontaneously and upon stimulation (Figs. 5C and E). These neuronal membrane properties were absent from the transplanted SVZ cells with the morphology of oligodendrocytes or astrocytes (Figs. 5G and H).

We compared the membrane properties of the SVZ transplant cells with that of host cells that had been labeled through injections of the GFP-expressing retrovirus delivered into the cerebellar white matter at P3/4. We recorded from Bergmann glia, astrocytes, oligodendrocytes, and interneuron subtypes such as Golgi, Lugaro, basket and stellate cells, as well as neurons in white matter. We found that the transplanted SVZ cells exhibited similar morphologies and membrane properties compared to those of host cells in the cerebellum (Fig. 5I).

We also compared the electrophysiological profile of SVZ cells transplanted into the cerebellum with that of the endogenous olfactory bulb interneurons, labeled by injection of GFP retrovirus into SVZ at P0/1and recorded at 26dpi. The migration and differentiation of infected perinatal forebrain SVZ cells has been examined in detail (Kakita and Goldman, 1999; Marshall et al., 2005; Suzuki and Goldman, 2003). In short, the SVZ cells differentiate into granule cells and periglomerular cells in the granule and glomerular layers of the olfactory bulb (Figs. 6A, B). Few GFP-positive glial cells can be detected in the olfactory bulb (Fig. 6C). We also examined the membrane potential of GFP-labeled SVZ cells that had been transplanted back into the cortical SVZ of age-matched littermates. The re-transplanted SVZ cells matured in the same manner as the endogenous SVZ cells, populating the granule and glomerular layers with granule (Fig. 6D) and periglomerular interneurons respectively, and glial cells in the granule layer (Fig. 6E) and white matter (Fig. 6F). Both endogenous GFP-positive cells in the olfactory bulb, as well as transplanted GFP-positive cells that had migrated from the site of transplantation in the cortical SVZ to the olfactory bulb exhibited the characteristic membrane properties of interneurons (Figs. 5J and K), which closely resembled that of the SVZ cells that had been transplanted in the cerebellum.

Transplanted cells are integrated into the cerebellar circuitry

We investigated if the SVZ cells transplanted into the cerebellum integrated into the cerebellar circuitry. We noted that several transplanted neurons showed evidence that was consistent with their integration into host circuitry, such as action potentials and spontaneous synaptic potentials (Figs. 5E and F). In addition, we immunolabeled sections prepared from SVZ transplanted cerebella and found that transplanted neurons were labeled with antibodies specific for presynaptic terminals, such as VAMP2 or bassoon, and postsynaptic terminals, such as PSD-95 (Fig. 7). Nerve terminals of GFP-positive cells, labeled with PSD-95 and VAMP2, were found in the internal granule cell layer. These data taken together point to the integration of at least some of the transplanted neurons into the host environment.

The transplanted SVZ cells that differentiated into neurons occupied the appropriate anatomic niches for cerebellar interneurons. The grafted SVZ cells eventually displayed the morphology of

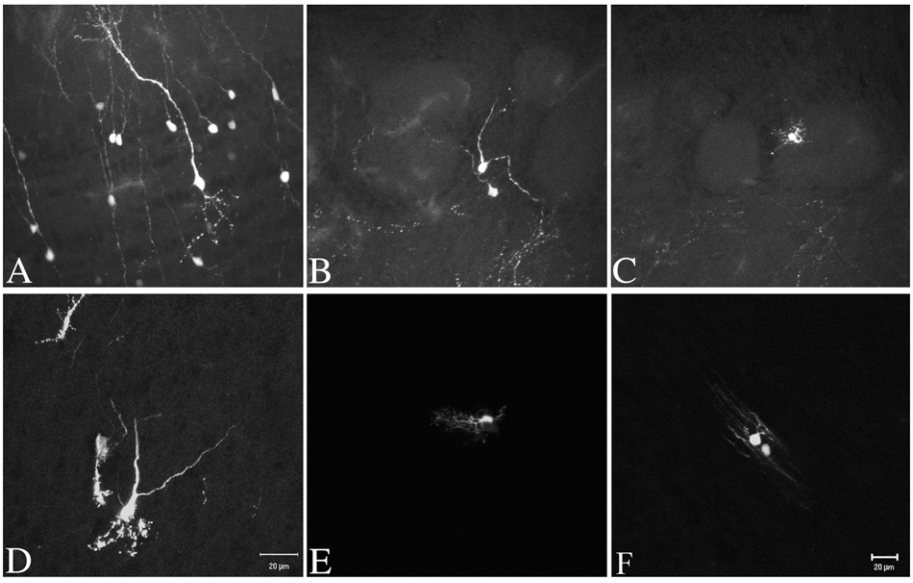

Fig. 6. Morphology and distribution of the endogenous olfactory bulb cells and forebrain SVZ cells transplanted back into the SVZ; 10 dpt to 7 wpt. (A–C) Examples of cells in the olfactory bulb labeled by injection of the GFP retrovirus into SVZ at P0/1. SVZ cells migrated and differentiated into the granule cells (A) in the olfactory bulb granule layer and periglomerular cells (B) in the glomerular cell layer. Small glial cells (C) can be occasionally found. (D–F) Cells infected with GFP retrovirus in the SVZ, isolated and transplanted back into forebrain SVZ differentiated into neuron (D) and small glia (E) in the glomerular layer, and oligodendrocyte in the olfactory bulb white matter (F). Scale bars for A–F is 20 μm.

cerebellar-like interneurons rather than those of olfactory bulb granule or periglomerular cells, indicating that the patterns of axonal and dendritic growth are not determined intrinsically in the neuron lineage, but are strongly molded by the local environment. Transplanted cells displayed the morphology of cerebellar-like interneurons for the layer in which they resided and some showed antigenic characteristic of cerebellar interneurons. The majority of the cells displayed membrane properties of interneurons, but our data does not allow us to classify the cells in a more detailed manner. It has been reported that transplantation slows down the maturation of cells (Buchet et al., 2002). We found that cells transplanted into the cerebellum mature into interneurons at least 1 week later then the endogenous cerebellar cells (Table 1). In addition, we found that SVZ cells transplanted back into the forebrain SVZ showed membrane properties of mature olfactory bulb neurons at later stage than cerebellar cells. These data prompted us to conclude that transplanted cells are indeed capable of maturing, but may follow the differentiation pace of olfactory bulb interneurons, which can proceed over a period of 7 weeks (Carleton et al., 2003).

Our data are consistent with a shift in the key properties of cells derived from the forebrain SVZ after transplantation into the cerebellum. An alternate possibility would be that neuronal progenitors transplanted from the SVZ do not differentiate physiologically into cerebellar interneurons. Their morphologies resemble endogenous cerebellar-like interneurons, and this could be the result of the spatial constraints defined by other cells in the local environment. However, if the "re-specification" was only a morphological one, we would expect the interneurons to express markers characteristic of their olfactory bulb interneuron lineage, such as Dlx1 (Gensat database, www.gensat.org), Tbr2 (Englund et al., 2005) or NeuN (Weyer and Schilling, 2003). We failed to label any of the transplanted interneurons with Dlx1 or Tbr2. The only marker of olfactory bulb interneurons and their progenitors that labeled transplanted cells in the cerebellum was NeuN, suggesting that at least some of the transplanted neurons do not assume area-specific phenotypes, but continue to exhibit a characteristic of olfactory bulb interneurons. Our data indicate that early postnatal SVZ cells in the neuronal lineage have already been specified as GABAergic interneurons, but can alter their morphology based on environmental factors. While some interneuronal progenitors from the forebrain SVZ have the potential to differentiate into cerebellar-like interneurons, more studies remain to be done in order to fully understand the signals that influence the maturation of these cells upon transplantation. Identifying the mechanisms involved in the re-specification of the forebrain SVZ might provide important information for future transplantation strategies for neurodegenerative disease and CNS repair treatment.

Experimental methods

Transplantation

Sprague Dawley pups were used in this study. Forebrain lateral SVZ progenitors were labeled by injection of replicant-deficient retrovirus encoding GFP on P0/1. The GFP virus was produced as previously described (Kakita and Goldman, 1999). The stereotaxic injection into the lateral forebrain SVZ was performed as described

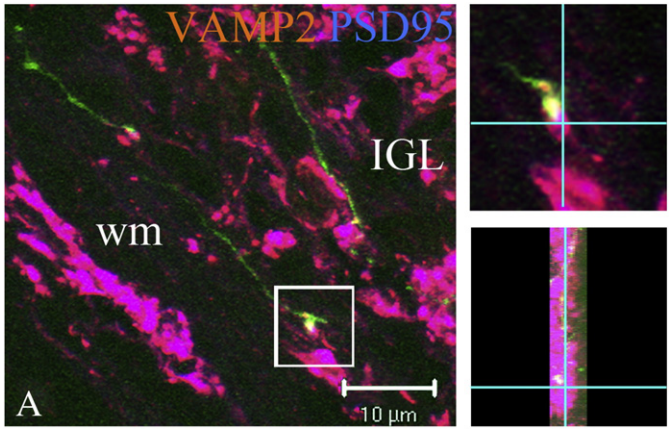

Fig. 7. Expression of synaptic proteins VAMP2 (red) and PSD95 (blue) in transplanted neurons; 21 dpt. (A) A z-stack image showing GFP-positive processes of transplanted cells labeled with VAMP2 (red) and PSD95 (blue). One section from the z-stack (1 μm-thick), representing the nerve ending in the boxed area in A, was magnified in Photoshop to show the expression of synaptic proteins, VAMP2 and PSD95. The image was also rotated for 180° along the *z*-axis, and it shows that the labeling is indeed in the nerve ending and not in the adjacent structure.

(Suzuki and Goldman, 2003). Two days after the GFP retrovirus injection, pups were anesthetized using 1 ml/g of mixture 3:1 of Ketamine and Xylazine, decapitated, and brains were removed and placed in ice-cold Leibowitz's L15 media. All products for cell culture were purchased from Life Sciences, Rockville, MD, unless stated otherwise. Forebrains were removed and cut in 900 μm-thick coronal slices on a tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, England). The lateral SVZ was dissected from these slices and cells were dissociated as described before (Milosevic and Goldman, 2004). The single cell suspension, composed of GFP-labeled and unlabeled cells, was sorted using a fluorescent activated cell sorter (BD FACSAria) and the GFP-positive cells were collected in Leibowitz's L-15 medium containing 10%FBS. Typically, 150,000–200,000 cells from 18 to 20 pups were collected. After a brief spin cells were re-suspended in a small volume (about 10 μl) of the same medium without FBS and 1 micro liter of cell suspension was injected into the cerebellar deep white matter or lateral forebrain SVZ of P3/4 rat pups. The detailed description of stereotaxic injections into the cerebellum and lateral SVZ have been published previously (Levison and Goldman, 1993; Milosevic and Goldman, 2002; Zhang and Goldman, 1996a). Pups were anesthetized, perfused with 4% paraformaldehyde, and brains were removed and fixed for 1 h up to overnight in the same fixative at 4 °C, then cryoprotected with 20% sucrose in PBS (pH 7.3), and frozen. Twenty to thirty micron-thick sections were cut and examined immediately or labeled with various glial- and neuronal-specific markers.

Immunocytochemistry

Immunocytochemical labeling was described elsewhere in detail (Milosevic and Goldman, 2002). Antibodies used in this study were: GABA (1/1000, Sigma, St. Lois, MO), calretinin (CR, 1/1000, Swant, Bellinzona, Switzerland), calbindin (1/1000, Sigma), chondroitin sulfate proteoglycan 2 (NG2, 1/400, gift from Dr. Stallcup), tyrosine hydroxylase (1/500, Sigma), glial fibrilary acidic protein (GFAP, 1/500, Dako, Carpinteria, CA), Ki67 (1/1000, Vector Labs, Burlingame, CA), glutamate (1/250, Sigma), brain lipid-binding protein (Blbp, 1/3000, gift from Dr. Heintz), glutamate receptor 1 (GluR1, 1/50, Chemicon, Tamecula, CA), and monoclonal antibodies were: NeuN (1/500 Chemicon), parvalbumin (PV, 1/3000, Sigma), synaptobrevin (VAMP21/500, Synaptic Systems, Goettingen, Germany), bassoon (1/100, StressGen, Ann Arbor, MI), PSD95 (1/500, Upstate, Charlottesville, VA), MAP2 (1/500, Sternberger Monoclonals, Lutherville, MD), paired box gene 2 (Pax2, 1/500, Zymed), LIM homeobox protein 5 (Lhx 5, 1/500, Developmental Studies Hybridoma Bank), eomezodermin homolog (Tbr2, 1/2000, gift from Dr. Hevner), distal-less homeodomain transcription factor (Dlx1, 1/500, USBiological)and myelin 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase, 1/500, Sternberger Monoclonals). Appropriate secondary antibodies were conjugated to TRITC (Southern Biotechnology Associates, Inc., Birmingham, AL) or Cy5 (Chemicon International Inc., Temecula, CA) and applied at the dilution of 1/200. Additionally, some sections were incubated for 5 minutes with the DNA-interactive agent DRAQ5 (Biostatus Limited, UK), applied at the dilution of 1/1000 in PBS. Fluorescent images were obtained using the confocal microscope LSM 510 Meta (Carl Zeiss, Inc., Thornwood, NY).

Electrophysiology

Acute slices for electrophysiological recordings of GFP-expressing cells in rat neocortex were prepared as described previously (Noctor et al., 2001). Briefly, the cerebellum was removed and sectioned parasagitally at 400 μm with a vibratome (Leica, Bannockburn, IL) in ice-chilled artificial cerebrospinal fluid (aCSF) bubbled continuously with 95/5% O_2/CO_2 containing (in mM): NaCl 125, KCl 5, NaH_2PO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2, $NaHCO_3$ 25, and glucose 20, pH 7.4, at 25 °C, 310 mOsm/l. For control recordings of olfactory bulb (OB) neurons, the OB was removed and sectioned coronally at 400 μm on a vibratome. Slices were allowed to recover for 1 h at room temperature in aerated aCSF before electrophysiological recordings began. The slices were transferred to a recording chamber on an Olympus BX50WI upright microscope and were perfused with aerated room temperature aCSF. GFP-expressing cells were identified under epifluorescence and whole-cell patch-clamp recordings performed using an EPC-9 patch-clamp amplifier (Heka Electronics, Mahone Bay, Canada) controlled by an Apple computer running Pulse v8.0 (Heka). Glass recording electrodes (5–7 M) were filled with (in mM): KCl 130, NaCl 5, $CaCl_2$ 0.4, $MgCl_2$ 1, HEPES 10, pH 7.3, and EGTA 1.1. Epifluorescent images of the recorded cells were collected using Scion Image, and arranged using Adobe Photoshop v7.0 (Adobe Systems). Electrophysiological responses were measured and analyzed using Pulse v8.0, and traces were arranged using Igor Pro v4.01 (WaveMetrics, Lake Oswego, OR), and Freehand v10.0 (Macromedia).

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