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Extracerebellar progenitors grafted to the neurogenic milieu of the postnatal rat cerebellum adapt to the host environment but fail to acquire cerebellar identities

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Abstract

Stem or progenitor cells acquire specific regional identities during early ontogenesis. Nonetheless, there is evidence that cells heterotopically transplanted to neurogenic regions of the developing or mature central nervous system may switch their fate to adopt host-specific phenotypes. Here, we isolated progenitor cells from different germinative sites along the neuraxis where GABAergic interneurons are produced (telencephalic subventricular zone, medial ganglionic eminence, ventral mesencephalon and dorsal spinal cord), and grafted them to the prospective white matter of the postnatal rat cerebellum, at the time when local interneurons are generated. The phenotype acquired by transplanted cells was assessed by different criteria, including expression of region-specific transcription factors, acquisition of morphological and neurochemical traits, and integration in the cerebellar cytoarchitecture. Regardless of their origin, all the different types of donor cells engrafted in the cerebellar parenchyma and developed mature neurons that shared some morphological and neurochemical features with local inhibitory interneurons, particularly in the deep nuclei. Nevertheless, transplanted cells failed to activate cerebellar-specific regulatory genes. In addition, their major structural features, the expression profiles of type-specific markers and the laminar placement in the recipient cortex did not match those of endogenous interneurons generated during the same developmental period. Therefore, although exogenous cells are influenced by the cerebellar milieu and show remarkable capabilities for adapting to the foreign environment, they essentially fail to switch their fate, integrate in the host neurogenic mechanisms and adopt clear-cut cerebellar identities.

Introduction

The different subdivisions of the mature central nervous system (CNS) derive from distinct domains along the neural tube that become progressively specified during embryogenesis. The acquisition of regional identities along the neuraxis involves the activity of precise combinations of transcription factors and signalling molecules, required to generate the varieties of local neurons and glia. According to this mechanism, the developmental potential of progenitor cells becomes progressively restricted in space and time, so that different neural phenotypes are produced following precise spatio-temporal schedules (Helms & Johnson, 2003; Jessell, 2000; Schuurmans & Guillemot, 2002).

Although neural progenitors and stem cells retain region-specific features even when placed *in vitro* (Zappone *et al.*, 2000; Hitoshi *et al.*, 2002), transplantation experiments show that exposure to heterotopic/heterochronic environmental conditions may induce these cells to acquire host-specific identities. This ability depends on intrinsic features of donor cells, such as their site of origin (Brustle *et al.*, 1995; Fishell, 1995; Olsson *et al.*, 1998) or developmental stage (Brustle *et al.*, 1995; Campbell *et al.*, 1995; Olsson *et al.*, 1997; Shen *et al.*, 2006), but also on the presence of active neurogenic processes in the recipient milieu (Campbell *et al.*, 1995; Vicario-Abejon *et al.*, 1995; Suhonen *et al.*, 1996; Hitoshi *et al.*, 2002). In spite of these findings, however, other studies indicate that heterotopically displaced cells maintain phenotypes typical of their native site (Na *et al.*, 1998; Carletti *et al.*, 2002; Liu *et al.*, 2009). As a consequence, it is still unclear whether and to what extent progenitors from one CNS region can actually acquire identities characteristic of another.

To address this issue, we compared the fate of cells from different CNS regions [lateral ganglionic eminence (LGE), telencephalic subventricular zone (SVZ), ventral mesencephalon (VM), dorsal spinal cord (DSC)], transplanted to the prospective white matter (PWM) of the early postnatal cerebellum. The PWM is a secondary germinal site that contains progenitors destined to produce the repertoire of cerebellar GABAergic interneurons and glia (Zhang & Goldman, 1996; Maricich & Herrup, 1999; Leto *et al.*, 2006; Grimaldi

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et al., 2009). The PWM environment provides instructive information to specify the mature phenotype and laminar position of both endogenous and transplanted cerebellar cells, according to precise spatio-temporal patterns (Leto *et al.*, 2009). Therefore, we investigated whether extracerebellar progenitors can be responsive to this neurogenic milieu and switch towards local fates.

Embryonic neocortical cells transplanted to the cerebellum *in utero* do not adopt host-specific identities (Carletti *et al.*, 2004), whereas progenitors from the SVZ implanted into the postnatal PWM develop cerebellar-like features (Milosevic *et al.*, 2008). To investigate the developmental processes and determine the mature phenotype of donor cells, here we applied a combination of complementary criteria, designed to define whether extracerebellar donors entrain in the recipient neurogenic process or develop according to their original ontogenetic programmes. Our observations indicate that extracerebellar cells are able to adapt some morphological and neurochemical features to foreign environmental constraints, but fail to switch their fate to true cerebellar identities.

Materials and methods

Animals and surgical procedures

All experiments were performed on Wistar rats (Harlan, San Pietro al Natisone, Italy). Donor cells for transplantation experiments were obtained from transgenic rats overexpressing the enhanced green fluorescent protein (GFP) under the control of the β-actin promoter (a generous gift from Dr M. Okabe, Osaka University, Osaka, Japan; Okabe *et al.*, 1997; Ito *et al.*, 2001). All surgical procedures were performed under deep general anaesthesia obtained either by hypothermia (in early postnatal pups; see De Marchis *et al.*, 2007) or by intraperitoneal administration of ketamine (100 mg/kg; Ketavet; Bayer, Leverkusen, Germany) supplemented by xylazine (5 mg/kg; Rompun; Bayer). The experimental plan was designed according to the European Communities Council Directive of 1986 (86/609/EEC), National Institutes of Health guidelines, and Italian law for the care and use of experimental animals (DL116/92), and was approved by the Italian Ministry of Health.

Transplantation experiments

Donor cells for heterotopic/heterochronic transplantation were isolated from different CNS regions at the age when local interneurons are generated: embryonic day 15 (E15) for LGE, postnatal day 2 (P2) for SVZ, E14 for VM and E13 for DSC. Embryonic LGE and postnatal SVZ cells are ontogenetically related (Wichterle *et al.*, 2001), and often showed similar features and fates following transplantation (see Results). Therefore, observations relating to these donors are described together, underlining specific differences.

Donor cells were intraparenchymally grafted to P1 cerebella, according to previously established procedures (Jankovski *et al.*, 1996; Carletti *et al.*, 2004; De Marchis *et al.*, 2007). Briefly, GFP-rat embryos were removed by caesarean section from deeply anaesthetized timed-pregnant females at appropriate gestation stages. The embryos were rapidly decapitated, and the CNS was dissected in saline solution with 0.6% glucose (dissection medium). Under a dissecting microscope, LGE, VM and DSC were carefully isolated from surrounding brain structures. The tissue blocks were mechanically dissociated to a single-cell suspension in the same dissection medium without proteolytic agents.

To isolate the postnatal SVZ, P2 pups were cryoanaesthetized in melting ice and rapidly transcardially perfused with 5 mL of dissection medium to wash out blood cells. The brain was dissected, placed in ice-cold dissection medium and cut by a vibratome into 250-μm-thick coronal slices. Tissue from the SVZ was isolated under a dissecting microscope and mechanically dissociated into a single-cell suspension. In all instances the donor cell suspensions were centrifuged and resuspended at a final concentration of 8×10^4 cells/μL. An aliquot was immediately examined under the microscope to assess cell viability and GFP expression.

Transplantation to postnatal hosts

For transplantation to the cerebellum, P1 rat pups were anaesthetized by hypothermia and maintained at 4°C during surgery (total duration, 15–20 min). The head was immobilized on a custom neonatal stereotaxic apparatus and the posterior surface of the cerebellum was exposed by removing small fragments of the occipital bone. Two microlitres of the cell suspension was pressure-injected into the parenchyma using a glass micropipette. Recipient animals were killed at 2 or 30 days after transplantation.

For homotopic transplants of LGE/SVZ cells into the SVZ, newborn rats (P1–P2) were anaesthetized by hypothermia (as above). The skull was exposed by a skin incision, and small holes were drilled through. Two microlitres of the cell suspension was pressure-injected in the SVZ of the lateral ventricle at stereotaxic coordinates of 0.7 mm anterior to bregma, 1.9 mm lateral to sagittal sinus and 1.5 mm depth, by means of a glass micropipette. These animals were killed 2 or 30 days after transplantation. In all instances, at the end of surgery the skin was sutured with 0.8-mm silk thread, the pups were quickly revitalized under a heat lamp and subsequently returned to the dam.

Dissociated cell cultures

Cells from the different CNS sites were dissociated according to the same technique described above and plated on 13-mm poly-L-lysine-coated glass coverslips at a density of 5×10^4 per cm^2. The cells were cultured in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) supplemented with B27 (Invitrogen), 0.5 mM glutamine (Invitrogen), 100 μg/mL streptomycin and 100 U/mL penicillin at 37°C with 5% CO_2. After 1 day, the cultures were fixed by 40 min of immersion in 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.2–7.4.

Histological procedures

Under deep general anaesthesia, recipient rats were transcardially perfused with 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.2–7.4 (500 mL for adult animals, 20–50 mL for juvenile animals). The brains were immediately dissected, stored overnight in the same fixative at 4°C and finally transferred in 30% sucrose in 0.12 M phosphate buffer. Brains were cut with a cryostat in 30-μm-thick parasagittal slices collected in PBS. The sections were incubated overnight at room temperature (RT) in PBS with 1.5% normal serum, 0.25% Triton X-100 and primary antibody and then for 1 h at RT in secondary biotinylated antibodies followed by a solution of streptavidin Texas Red conjugate (1 : 200; Invitrogen) or secondary fluoresceinated antibody (1 : 200; Vector Laboratories, Burlingame, CA). The following primary antibodies were used (Table 1): anti-parvalbumin (PV; 1 : 1500; Swant, Bellinzona, Switzerland), anti-neuronal-specific nuclear protein (NeuN; 1 : 500; Chemicon, Temecula, CA, USA), anti-neurogranin (NG, 1 : 250; Chemicon, Billerica, MA, USA); anti-GFP (1 : 700; Invitrogen), anti-calretinin (CR, 1 : 2500; Swant), anti-Lhx1 (1 : 1500; Santa Cruz Biotechnology, Santa Cruz, CA,

TABLE 1. List of primary antibodies used in the study

Antibody	Supplier and cat. no.	Species of origin	Immunogen	Specificity and controls
Parvalbumin	Swant 235	Mouse	Parvalbumin purified from carp muscles	IHC knock-out tissue, WB IHC pattern consistent with Bastianelli (2003)
NeuN	Chemicon MAB377	Mouse	Purified cell nuclei from mouse brain	WB (see data sheet) IHC pattern consistent with Weyer & Schilling (2003)
Neurogranin	Chemicon AB5620	Rabbit	Recombinant rat Neurogranin	WB (see data sheet) IHC pattern consistent with Singec *et al.* (2003)
GFP	Novus Biological NB600-597	Mouse	GST-GFP fusion protein, full-length amino acid sequence (246 amino acids, jellyfish *Aequorea victoria*)	ELISA, WB (see data sheet) Selective staining of GFP-expressing cells *in vitro* and *in vivo*
GFP	Invitrogen A11122	Rabbit	GFP isolated directly from *Aequorea victoria*	WB (see data sheet) Selective staining of GFP-expressing cells *in vitro* and *in vivo*
Calretinin	Swant 7699/3H	Rabbit	Recombinant human calretinin containing a 6-his tag at the N-terminal	WB (see data sheet) IHC pattern consistent with Bastianelli (2003) and Leto *et al.* (2006)
Lhx1	Santa Cruz sc-19341	Goat	Carboxy terminus of LHX1 of human origin	WB (see data sheet) IHC pattern consistent with Gross *et al.* (2002) and Pillai *et al.* (2007)
Pax2	Zymed	Rabbit	GST-Pax-2 fusion protein derived from the C-terminal domain (amino acids 188–385) of the murine Pax-2 protein	ELISA (see data sheet) IHC pattern consistent with Maricich & Herrup (1999) and Leto *et al.* (2006, 2009)
Dlx2	Produced by Dr Kazuaki Yashikawa	Guinea-pig	MBPDlx2 fusion protein (amino acids 1–154)	IHC pattern consistent with Brill *et al.* (2008)

IHC, immunohistochemistry; WB, Western blot.

USA), anti-Pax-2 (1 : 200; Zymed, San Francisco, CA, USA) and anti-Dlx2 (1 : 3000; produced by Dr Kazuaki Yoshikawa, Osaka University). Details about production and specificity tests for all the antibodies are given in Table 1. In some cases, 4′,6-diamidino-2-phenylindole (DAPI; Fluka, Buchs, Switzerland) was used to counterstain cell nuclei. The sections were mounted on microscope slides with Tris–glycerol supplemented with 10% Mowiol (Calbiochem, La Jolla, CA, USA) to reduce fading of fluorescence.

Data analysis

The histological preparations were examined by means of a Zeiss Axiophot light microscope (Karl Zeiss, Oberkochen, Germany), equipped with a Nikon DS-5M digital camera (Nikon Italy, Florence, Italy). The material was also examined with a Leica TCS SP5 confocal microscope (Leica Microsystems, Milan, Italy). Digital images were processed with Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA, USA) to adjust contrast and to assemble the final plates. Quantitative and morphometric evaluations were made using the Neurolucida software (MicroBrightField, Colchester, VT, USA) connected to an E-800 Nikon microscope via a colour CCD camera (Nikon Italy).

Donor cells in the host tissue were recognized by the intrinsic GFP expression and their phenotypic traits were investigated by morphometric evaluation and analysis of expression of type-specific markers. Cell quantification was done by sampling cells on at least 15 sections from at least two animals for each experimental condition. Because of the variable amounts of donor cells that engrafted in each transplant, results obtained from different cases of the same experimental set were usually pooled together. To evaluate the distribution of donor cells in the host cerebellum, samples of at least 500 GFP-positive cells for each donor population were classified according to their position in the deep nuclei, white matter or granular layer. The same analysis was also carried out for the different cell subsets, defined by the expression of neurochemical markers. These data are illustrated in Table 2, in which the relative frequencies of donor cells in each position are reported.

To estimate the frequency of donor neurons expressing different markers in the different cerebellar structures, we examined several series of cerebellar sections double-immunostained for GFP and one of the considered neurochemical markers (i.e. NeuN, CR, PV, NG). On these sections, we sampled all GFP-positive cells, recorded their position in the host tissue and estimated the number of cells that were also labelled for the marker under examination. The data reported in Figs 3D and 6A represent the percentage of neurons double-labelled

TABLE 2. Relative frequencies of extracerebellar neurons engrafted in different positions of the host cerebellum

	Deep cerebellar nuclei (%)	White matter (%)	Granular layer (%)
LGE donors			
Total	16.6	19.3	64.1
NG	2.3	42	55.7
NeuN	28.6	23.1	48.3
CR	2.9	2.9	94.2
PV	1.5	7.3	91.2
SVZ donors			
Total	3.9	32.3	63.8
NG	0	26.9	73.1
NeuN	14.3	24.3	61.4
CR	0	0	100
PV	0	0	100
VM donors			
Total	34.7	35.2	30.1
NG	25.4	32.8	41.8
NeuN	37.1	24.3	38.6
CR	94.1	5.9	0
PV	0	60	40
DSC donors			
Total	12.2	50.4	37.4
NG	0	29.1	70.9
NeuN	32.7	22.4	44.9
CR	37.5	18.8	43.7
PV	0	0	100

For each type of donor, the table reports the distribution of the whole population of grafted cells (Total) and that of the different subsets, defined by the expression of NG, NeuN, CR or PV. CR, calretinin; NeuN, neuronal-specific nuclear protein; NG, neurogranin; PV, parvalbumin.

for GFP and each examined marker out of the number of GFP-positive neurons sampled in the different regions of the host cerebellum. Because these analyses have been carried out on sections immunostained for one marker at a time, no information has been obtained about the co-expression of different markers in single GFP-positive neurons.

The cell body size of transplanted and endogenous neurons was measured by means of the Neurolucida system at 40 × magnification, on a sample of 1702 cells from nine animals.

The laminar position of grafted cells in the recipient cortex was recorded as the distance from the Purkinje cell layer (positive for the molecular layer, and negative for the granule cell layer; for details about this procedure see Leto *et al.*, 2009), and was represented as scatter diagrams that combine measures obtained from different animals of the same set.

To evaluate the orientation of the dendrites of endogenous or transplanted neurons relative to their position in the cerebellar cortical layering, by means of the Neurolucida system we measured the angle formed by the main dendritic trunks and the direction of the Purkinje cell layer. As shown in Fig. 4C, the soma of the examined neuron was centred on the axis of the Purkinje cell layer and the direction of the main dendrites was recorded. This procedure yielded a radial plot containing all the dendrites belonging to a given experimental set, which were subdivided into two broad categories: those with angles smaller than ± 45° (orientated along the direction of the Purkinje cell layer), and those with wider angles (orientated transversely to the direction of the Purkinje cell layer). This procedure was repeated separately for samples of endogenous Golgi and Lugaro cells and for grafted cells of the different donor populations that settled in the granular layer. The transplanted neurons were subdivided into two groups according to their position relative to the Purkinje cell layer: those placed within 50 μm from the row of Purkinje cell bodies and those located further away.

Statistical analysis

Statistical significance was assessed by a chi square test to analyse whether differences between data sets were significant. An unpaired Student's *t*-test was used when only two groups were analysed. In all instances, $P < 0.05$ was considered statistically significant.

Results

Extracerebellar donors were transplanted to P1 rat hosts. At this age, glial types as well as GABAergic interneurons of the cortex and deep nuclei are being generated in the PWM, whereas granule cells are produced by progenitors located in the external granular layer (Ramón y Cajal, 1911; Altman & Bayer, 1997; Carletti & Rossi, 2008). Although grafted cells yielded both neurons and glia (Carletti *et al.*, 2004; Milosevic *et al.*, 2008), our analysis was restricted to neuronal types. In addition, because donor cells did not engraft in the external granular layer and never acquired the phenotypic traits of granule cells, we primarily assessed whether they can differentiate into cerebellar GABAergic interneurons.

Progenitors for cerebellar inhibitory interneurons proliferate in the PWM, where they become specified to different mature identities (Zhang & Goldman, 1996; Maricich & Herrup, 1999; Leto *et al.*, 2009). To determine whether this neurogenic environment can direct the fate choice of extracerebellar progenitors towards local identities, we examined the behaviour of donor cells from the LGE, SVZ, VM and DSC, which are the origin of GABAergic interneurons destined to populate different regions of the mature forebrain, midbrain and hindbrain. To elucidate whether the transplanted cells acquired cerebellar identities, we applied a set of concurrent criteria, including: (i) expression of region-specific transcription factors, (ii) position occupied in the host cerebellar architecture, (iii) expression of mature type-distinctive markers and (iv) acquisition of type-distinctive morphological features. Furthermore, given the precise spatio-temporal sequence by which different categories of cerebellar neurons are generated and assigned to specific laminar positions (Altman & Bayer, 1997; Carletti & Rossi, 2008), we assessed whether transplanted cells shared the same fate and placement as endogenous elements generated during the same developmental period.

Expression of region-specific regulatory genes by donor cells exposed to the host cerebellar environment

In the developing CNS, regional identities are defined by the activity of specific combinations of transcription factors. Therefore, as a first index of the phenotype adopted by donor cells, we investigated whether they retained the expression of transcription factors typical of their site of origin or turned on regulatory genes characteristic of cerebellar development.

Dlx homeobox genes are crucial for the specification of forebrain GABAergic interneurons (Anderson *et al.*, 1997; Eisenstat *et al.*, 1999; Panganiban & Rubenstein, 2002). Among these genes, Dlx2 is expressed by cells derived from the medial and lateral ganglionic eminences, the origins of GABAergic interneurons of neocortex, hippocampus, basal ganglia and olfactory bulb (Panganiban & Rubenstein, 2002). Dlx2 is switched on at early developmental stages (Eisenstat *et al.*, 1999) and is maintained up to adulthood (Saino-Saito *et al.*, 2003), being strictly confined to forebrain derivatives.

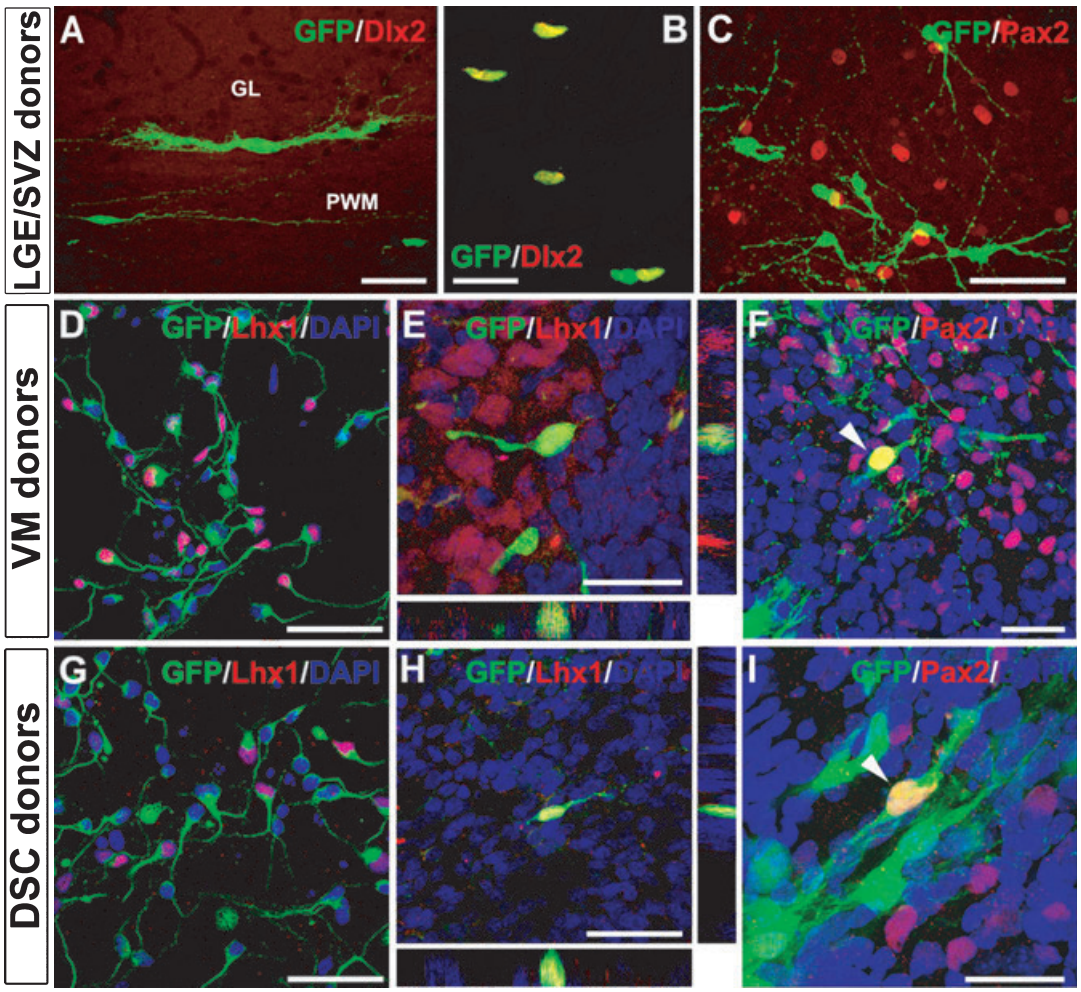

FIG. 1. Expression of region-specific transcription factors in extracerebellar cells *in vitro* and *in vivo*. LGE/SVZ-derived cells do not express Dlx2 (A) or Pax2 (C) 2 days after transplantation, but they maintain expression of Dlx2 when placed *in vitro* (B). VM- (D) and DSC-derived cells (G) express Lhx1 *in vitro* and maintain this expression in the recipient cerebellum (E and H respectively; 2 days after grafting). Some of these transplanted cells (indicated by arrowheads in F and I) are also labelled by anti-Pax2 antibodies. LGE, lateral ganglionic eminence; SVZ, subventricular zone; VM, ventral mesencephalon; DSC, dorsal spinal cord. Scale bars: 25 μm in E, F and I; 50 μm in A–D, G and H.

Expression of Dlx2 in LGE or SVZ progenitors grafted to the postnatal cerebellum was assessed by immunocytochemical labelling at 2 or 30 days after transplantation to evaluate the activity of the gene in donor cells at different maturation stages. In no instance did we observe any transplanted cell labelled by anti-Dlx2 antibodies (Fig. 1A), including both immature cells in the PWM and mature neurons throughout the recipient parenchyma [2 days after grafting (DAG), n = 158 cells/two transplants; 30 DAG, n = 267 cells/four transplants].

To rule out the possibility that Dlx2 expression was modified by cell dissociation, LGE/SVZ cells were plated *in vitro* and examined after 24 h. In this condition, most of the cells were labelled with anti-Dlx2 antibodies (Fig. 1B; 80.1%, n = 602/three cultures). Furthermore, to determine whether Dlx2 expression could be suppressed as a consequence of transplantation *per se*, we performed homotopic grafts in the postnatal forebrain. In line with previous reports (Fishell, 1995; Olsson *et al.*, 1997; De Marchis *et al.*, 2007), donor cells acquired morphologically identifiable phenotypes: LGE cells generated medium-sized spiny neurons in the striatum and interneurons in the olfactory bulb, whereas SVZ cells almost exclusively produced olfactory bulb interneurons (Supporting information, Fig. S1A and B). Donor cells were Dlx2-positive in the SVZ of the lateral ventricle (supporting Fig. S1C), along the rostral migratory stream (supporting Fig. S1D) and in the granule cell layer of the olfactory bulb (supporting Fig. S1E), but they were Dlx2-negative in the striatum (supporting Fig. S1F; Panganiban & Rubenstein, 2002). Therefore, homotopically transplanted LGE/SVZ cells express Dlx2 in an appropriate region-specific manner and acquire local phenotypes in different structures of the forebrain.

To assess whether LGE/SVZ cells grafted to postnatal cerebella upregulated host-specific genes, we examined the expression of Pax2, a selective marker of maturing cerebellar interneurons (Maricich & Herrup, 1999; Weisheit *et al.*, 2006). Also in this case, none of the grafted cells at any survival time was ever labelled by anti-Pax2 antibodies (Fig. 1C; 2 DAG n = 147 cells/two transplants; 30 DAG, n = 197 cells/three transplants).

The LIM-homeodomain transcription factor 1, Lhx1, is expressed during the genesis of inhibitory interneurons in the mesencephalon and spinal cord (Gross *et al.*, 2002; Müller *et al.*, 2002; Pillai *et al.*, 2007; Nakatani *et al.*, 2007). Moreover, Lhx1 and Pax2 interact when these interneurons acquire GABAergic identities (Pillai *et al.*, 2007). In the postnatal cerebellum, Lhx1 identifies granule cell progenitors

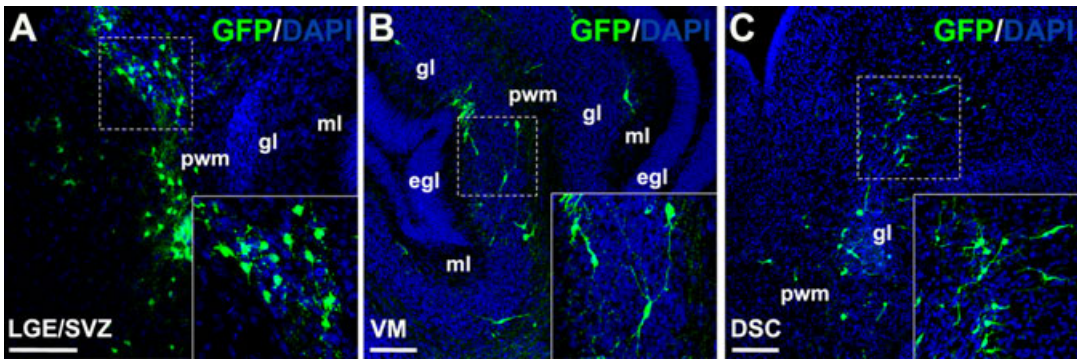

FIG. 2. Initial placement of donor cells in the recipient cerebellum 2 days after transplantation. Regardless of their origin, LGE/SVZ (A), VM (B) and DSC (C) progenitors are preferentially located in the host PWM and in the adjacent granular layer (gl). High-magnification images (insets) illustrate the morphological features of donor cells. DAPI, blue. Scale bars: 100 μm.

and developing Purkinje neurons, which remain positive in adulthood (Furuyama *et al.*, 1994; Hayes *et al.*, 2001; Zhao *et al.*, 2007). VM and DSC cells maintained expression of these markers *in vitro* (Fig. 1D and G, VM 43.9%, n = 1345 cells/three cultures; DSC 82%, n = 1228 cells/three cultures). Two days after transplantation to the cerebellum, Lhx1 was expressed by a fraction of VM (Fig. 1E; 8.9%, n = 212 cells/two transplants) and DSC cells (Fig. 1H; 22.5%, 256 cells/two transplants), but only a few of the latter were still positive at 30 days (VM, 0%, n = 154 cells/three transplants; DSC 3.6%, n = 166 cells/three transplants). On the other hand, at any time point anti-Pax2 antibodies only labelled rare VM- or DSC-derived neurons (Figs 1F and I; 2 DAG, DSC 12.7%, n = 267 cells/two transplants; VM 4.7%, n = 213 cells/two transplants; 30 DAG, VM 2%, n = 200 cells/two transplants, DSC 7.8%, n = 108 cells/two transplants).

Placement of extracerebellar interneurons in the recipient cerebellum

Cerebellar cells heterochronically transplanted to the developing cerebellum incorporate in the recipient PWM and acquire the same mature phenotypes and positions of the endogenous interneurons that are generated at the time of transplantation (Leto *et al.*, 2006, 2009). Therefore, exogenous progenitors that switch their fate towards cerebellar types are expected to comply with this ontogenetic schedule.

Regardless of their extracerebellar origin, 2 days after grafting the vast majority of GFP-positive cells were located in the PWM surrounding the deep nuclei or along the axis of the folia (Fig. 2A–C). Rare cells were present on the pial surface of the cerebellar cortex, but they were never seen within the external granular layer. The donor cells displayed morphological features of immature neurons and glia, undergoing migration or initial phases of differentiation (Fig. 2A–C). Thirty days after transplantation, mature neurons were present in wide areas of the recipient parenchyma, including cerebellar cortex, white matter and deep nuclei, with no clear differences among the different donor cell populations (Table 2). Concerning the laminar position in the cortex, transplanted cells were scattered throughout the whole granular layer, but totally absent from the molecular layer (Fig. 3A–C).

Neurochemical phenotype of extracerebellar interneurons in the recipient cerebellum

The placement of donor neurons in the deep nuclei and cortical granular layer, but not in the molecular layer, suggests that they may be only able to acquire certain cerebellar phenotypes. Thus, we examined the neurochemical profile and morphology of mature transplanted neurons (30 days after transplantation), relative to their position in the host cerebellum. In particular, we studied the expression of a panel of well-established markers that, alone or in specific combinations, are distinctive of different categories of cerebellar inhibitory interneurons (Fig. 3D–H; Bastianelli, 2003; Singec *et al.*, 2003; Leto *et al.*, 2008).

Concerning donor cells in the granular layer of the recipient cerebellar cortex, the majority of LGE-donors expressed NG (65.6% of 270 cells/eight transplants; Fig. 3D) or NeuN (61.6% of 239 cells/eight transplants; Fig. 3D), whereas PV- and CR-positive cells were less represented, being 14.4% (of 250 cells/eight transplants; Fig. 3D) and 8.7% (of 81 cells/eight transplants, Fig. 3D), respectively. SVZ-derived neurons showed a similar pattern of marker expression: NG-positive cells, 73.1% (of 91 cells/five transplants, Fig. 3D), NeuN-positive cells, 60% (of 116 cells/five transplants, Fig. 3D); CR-positive cells, 11.3% (of 79 cells/five transplants, Fig. 3D). PV-expressing cells were absent (no positive cells out of a sample of 98 cells/five transplants, Fig. 3D).

VM-derived cells yielded considerable amounts of NG- (62.3% of 108 cells/four transplants), NeuN- (67.9% of 103 cells/four transplants) and CR-positive neurons (35.3% of 48 cells/four transplants), plus a minor fraction of PV-expressing cells (8.5% of 70 cells/four transplants). By contrast, DSC donors also generated numerous NeuN-positive cells (53.1% of 92 cells/four transplants), whereas NG- (23.6% of 233 cells/four transplants), CR- (20.2% of 81 cells/four transplants) and PV-positive neurons were less frequent (11.7% out of 145 cells/four transplants). In spite of the differences among the various donor cell populations, many of them expressed CR and NG, markers of granular layer interneurons (CR is also expressed by a subset of the glutamatergic unipolar brush cells), whereas expression of PV, which is distinctive of molecular layer interneurons, was less frequent. On the other hand, expression of NeuN, which was common among the different types of donors, is unusual in granular layer interneurons (Weyer & Schilling, 2003; Leto *et al.*, 2008).

The morphology of donor interneurons is influenced by cerebellar cortical architecture

The different categories of cerebellar GABAergic interneurons are characterized by highly distinctive morphological features (Ramón y Cajal, 1911; Lainé & Axelrad, 2002), particularly relating to the dendritic and axonal patterns. A salient feature of granular layer interneurons is the orientation of their main dendrites relative to their

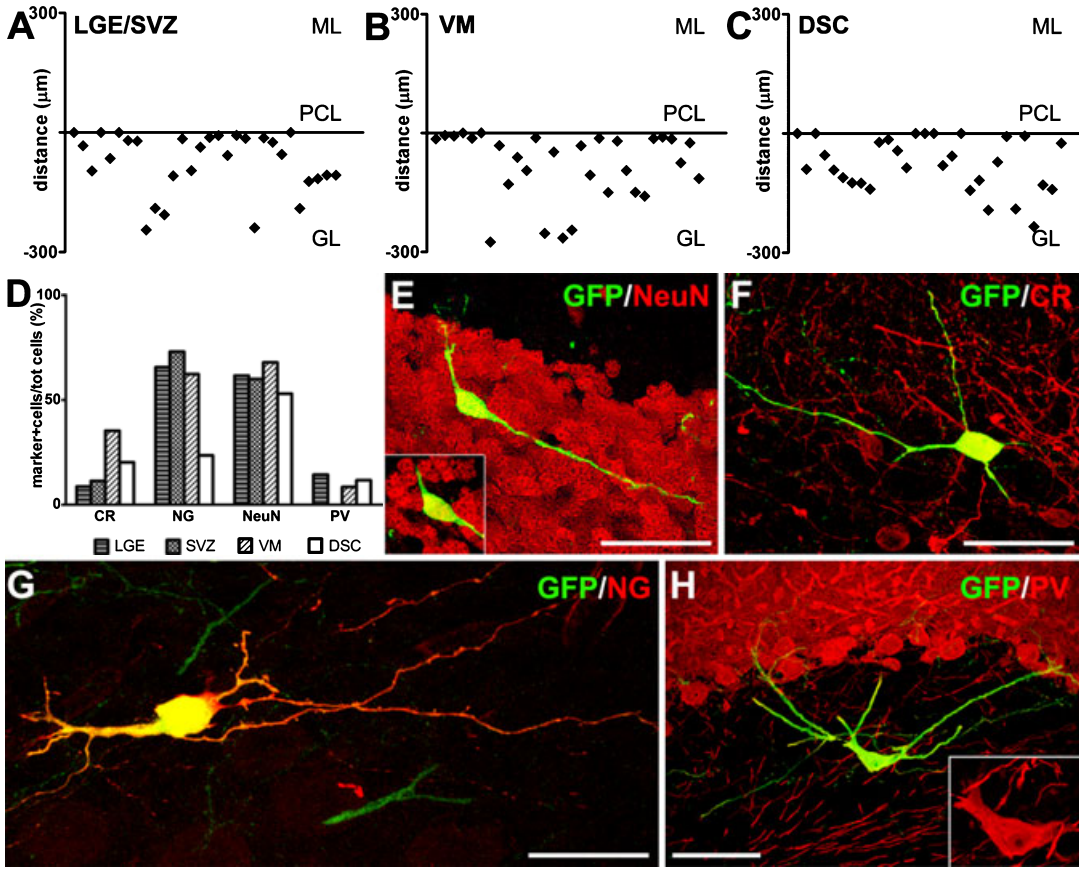

FIG. 3. Laminar distribution and neurochemical profile of extracerebellar interneurons after transplantation to the P1 cerebellum. (A–C) Laminar position of GFP-positive donor interneurons generated by LGE/SVZ (A), VM (B) and DSC (C) donors. (D) The fraction of GFP-positive cells double-labelled with CR, NG, NeuN or PV over the total number of donor neurons at 30 days' survival. Examples of donor interneurons labelled with NeuN (red in E), CR (red in F), NG (red in G) and PV (red in H) at 30 days after transplantation (see colour graphics on-line). Scale bars: 50 μm in E–H.

position deep within the layer (Golgi cells, Fig. 4B), or close to the row of Purkinje cell somata (Lugaro cells, Fig. 4A). This peculiar arrangement can be highlighted by mapping the dendritic orientation of endogenous interneurons (Fig. 4D–E): dendrites of Golgi neurons radiate from the cell body in all directions, whereas those of Lugaro cells are typically aligned to the Purkinje cell layer ($\chi^2_2 = 7.965$, $n = 96$, $P = 0.0186$).

We investigated whether extracerebellar donors also adopted the same structural arrangement. As shown in Fig. 4F and G, GFP-positive neurons in the granular layer developed the generic morphology of small to medium-sized multipolar neurons, which is consistent with, though not distinctive of, granular layer interneuron phenotypes. Accordingly, evaluation of cell body sizes showed no correspondence between transplanted cells and their endogenous counterparts (Fig. 5A and B; LGE/SVZ vs. Golgi, $\chi^2_4 = 18.37$, $n = 151$, $P = 0.001$; VM vs. Golgi, $\chi^2_4 = 10.01$, $n = 125$, $P = 0.0403$; DSC vs. Golgi, $\chi^2_4 = 15.58$, $n = 134$, $P = 0.0036$; LGE/SVZ vs. Lugaro, $t_{125} = 2.695$, $P = 0.008$; VM vs. Lugaro, $t_{141} = 10.51$, $P < 0.0001$; DSC vs. Lugaro, $t_{117} = 9.727$, $P < 0.0001$). To analyse dendritic orientation (Fig. 4H–P), grafted neurons were subdivided into two groups according to the distance of their cell bodies from the Purkinje cell layer (cut-off being at 50 μm). The result was clear-cut and consistent within all donor populations: neurons positioned deep in the layer had randomly orientated dendrites, whereas those located close to the Purkinje cell bodies displayed a clear preferential orientation along the direction of the layer (LGE/SVZ, $\chi^2_2 = 14.01$, $n = 181$, $P = 0.0009$, Fig. 4H–J; VM, $\chi^2_2 = 8.677$, $n = 59$, $P = 0.0131$, Fig. 4K–M; DSC, $\chi^2_2 = 8.970$, $n = 94$, $P = 0.0113$, Fig. 4N–P).

Finally, we examined the axons of transplanted neurons, although this analysis was hampered by the faint GFP staining of this neuronal compartment. Donor neurites terminated in the vicinity of the parent cell body, as expected for local interneurons, but their axonal fields appeared highly variable and never displayed the distinctive features and distribution of the axons of Golgi and Lugaro neurons. Of a sample of 50 GFP-positive axons, 31 comprised scarcely branched slender processes (Fig. 4Q), whereas 19 formed extensive networks of varicose chains, either extended throughout the granular layer (Fig. 4R) or restricted along the Purkinje cell layer (Fig. 4S).

Morphological and neurochemical phenotype of extracerebellar donors located in the deep cerebellar nuclei

The deep cerebellar nuclei (DCN) contain a population of GABAergic interneurons that share the same lineage with their cortical counterparts, and can be distinguished by their small size, multipolar shape and expression of NeuN and CR (Leto *et al.*, 2006). Donor cells from any of the different extracerebellar sources generated moderate amounts of neurons that settled in the deep nuclei (Table 2), and bore morphological features similar to those of their local counterparts. Indeed, in this position cell body size of VM or DSC donor cells matched that of endogenous interneurons, whereas LGE/SVZ cells

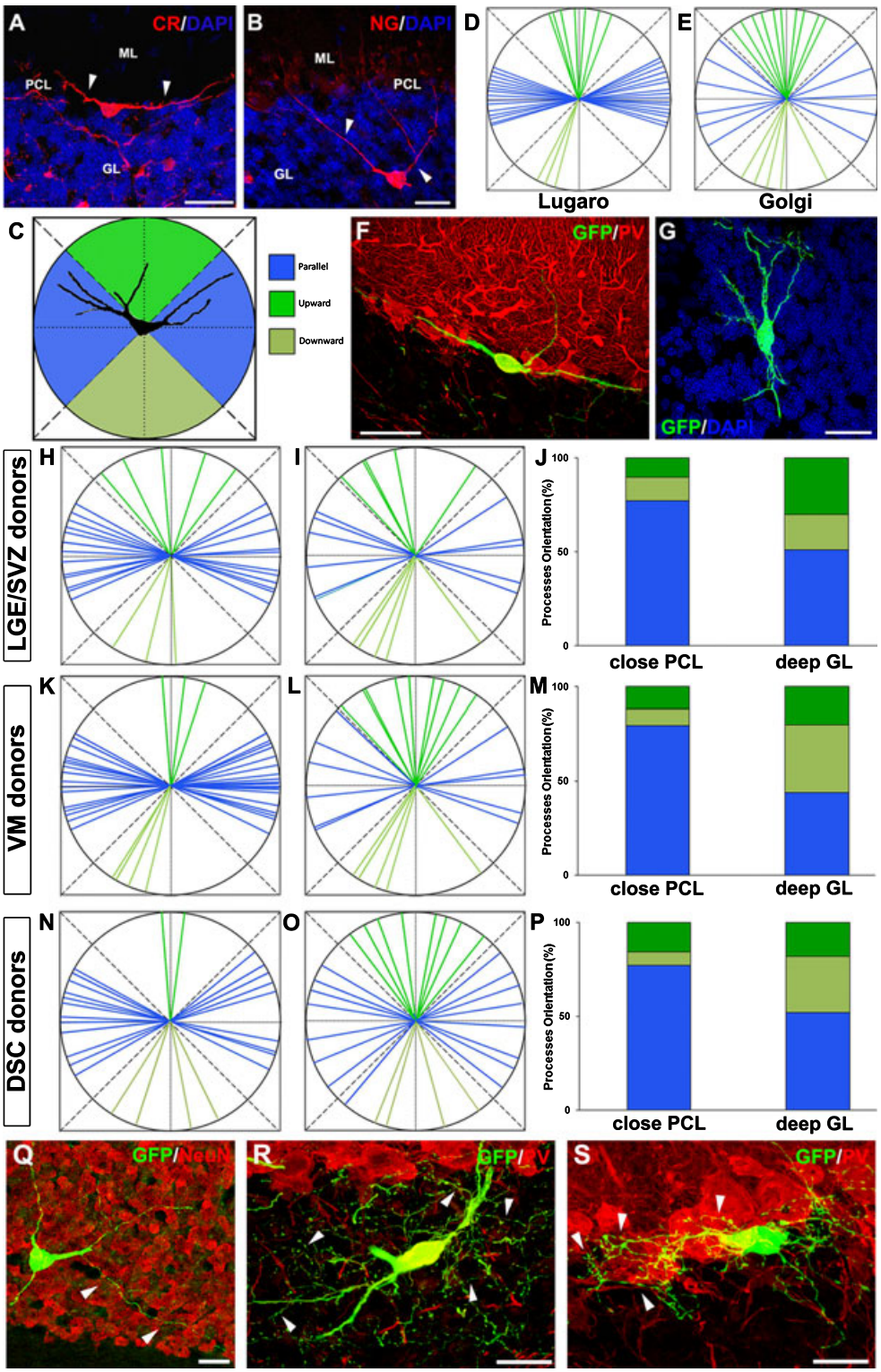

FIG. 4. Dendritic arrangement of endogenous and transplanted neurons in relation to their position in the granular layer. Examples of the typical dendritic orientation of Lugaro (dendrites indicated by arrowheads in A) and Golgi neurons (dendrites indicated by arrowheads in B). Analysis of the preferential dendritic arrangement of donor and host interneurons have been performed, as sketched in C, by evaluating their alignment relative to the Purkinje cell layer. This analysis shows that Lugaro cells have dendrites mainly orientated parallel to the Purkinje cell layer (D), whereas Golgi neurons have dendrites that radiate in all directions (E). (F, G) Examples of transplanted extracerebellar neurons placed close to the Purkinje cell layer (F) or deep into the granular layer (G). (H, I, K, L, N, O) The dendritic orientation achieved by LGE/SVZ- (H, I), VM- (K, L) and DSC- (N, O) derived neurons relative to their superficial (H, K, N) or deep (I, L, O) position in the granular layer. The histograms in J, M and P display the relative frequencies of dendrites orientated along or across the axis of the Purkinje cell layer in LGE/SVZ (J), VM (M) and DSC cells (P). (Q–S) Representative examples of axonal fields of transplanted neurons. Arrowheads point to some of the neurites or varicose terminal branches. Scale bars: 25 μm in Q–S; 50 μm in A, B, F, G, Q.

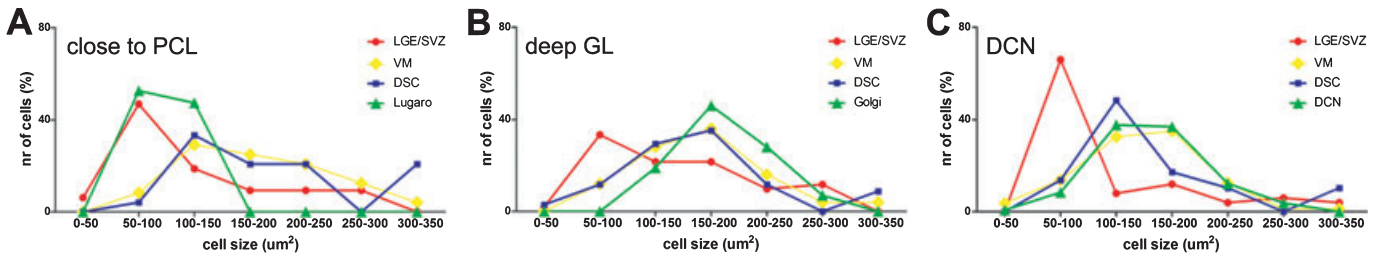

FIG. 5. Distribution of cell body sizes of endogenous and transplanted interneurons located in the superficial regions of the granular layer (A), in the depth of the same layer (B) or in the deep cerebellar nuclei (C). Endogenous interneurons, green; LGE/SVZ, red, lateral ganglionic eminence/subventricular zone; VM, yellow, ventral mesencephalon; DSC, blue, dorsal spinal cord; PCL, Purkinje cell layer; GL, granular layer; DCN, deep cerebellar nuclei (see colour graphics on-line).

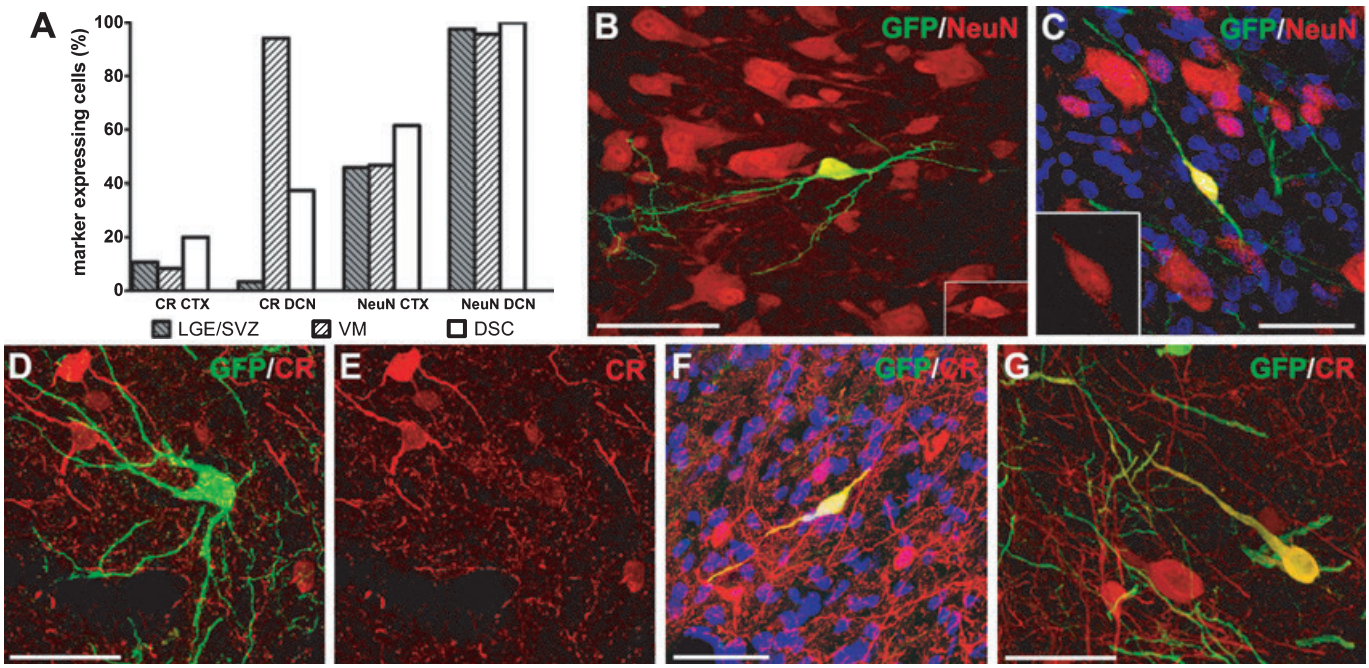

FIG. 6. Morphological and neurochemical phenotype of extracerebellar donors in the deep cerebellar nuclei. (A) Frequency of transplanted neurons in the cortex and in the deep nuclei that express CR or NeuN. (B, C) Examples of NeuN-positive cells (red), generated by LGE/SVZ (B) and VM (C) donors in the deep nuclei. (D, E) CR-negative LGE/SVZ neurons in the deep nuclei. (F,G) DSC (F) and VM (G) neurons double-labelled for GFP (green) and CR. CTX, cortex; DCN, deep cerebellar nuclei. DAPI, blue in C and F (see colour graphics on-line). Scale bars: 50 μm in B–G.

remained significantly smaller (Fig. 5C; LGE/SVZ vs. DCN, $\chi^2_4 = 69.79$, $n = 180$, $P < 0.0001$; DSC vs. DCN, $\chi^2_4 = 9.944$, $n = 188$, $P = 0.0414$; VM vs. DCN, $\chi^2_4 = 1.563$, $n = 210$, $P = 0.8155$). Similar to what was observed for cortically positioned cells, the vast majority of donor neurons expressed NeuN, regardless of their origin (Fig. 6A–C; Table 2). By contrast, expression of CR varied significantly among the different donor cell populations, being present in the vast majority of VM cells (94.1%), in 37% of DSC cells and only in 3.3% of LGE/SVZ cells (Fig. 6A and D–G; Table 2). Finally, a few cells also expressed PV and NG (Table 2), markers that are unusual for nuclear interneurons.

Discussion

Previous studies indicate that stem or progenitor cells heterotopically transplanted to neurogenic regions of the developing or mature CNS may switch their fate to host-specific phenotypes (Campbell *et al.*, 1995; Vicario-Abejon *et al.*, 1995; Suhonen *et al.*, 1996; Hitoshi *et al.*, 2002). Here, we examined the behaviour of cells isolated from different sites along the neuraxis and grafted to the PWM of the postnatal cerebellum, at the time when local interneurons and glia are generated. The behaviour and mature phenotype of donor cells have been investigated by examining a panel of morphological and neurochemical features to assess whether they entrained into local ontogenetic processes and acquired cerebellar phenotypes. Cells from different extracerebellar origins stably engrafted in the host cerebellum and developed mature neurons that, nonetheless, failed to achieve unequivocal traits of cerebellar interneurons. Thus, although the transplanted cells were able to adapt to the recipient environment, they essentially failed to adopt host-specific identities.

Extracerebellar cells engraft in the PWM but fail to switch on cerebellar-specific genes

The acquisition of host-specific phenotypes by exogenous cells is thought to be dependent upon the exposure to local neurogenic cues. In our experiments, the majority of extracerebellar donors initially

engrafted in the host PWM, whereas they consistently failed to settle in the external granular layer, the other germinative site active at the time of transplantation. This outcome may be partly due to mechanical constraints that favour the homing of implanted cells into particular positions of the recipient tissue. However, transplanted granule cell precursors readily integrate in the external granular layer (Snyder *et al.*, 1992; Gao & Hatten, 1994; Williams *et al.*, 2008), while maturing cerebellar interneurons are actively excluded (Vilz *et al.*, 2005). Therefore, the initial placement of the exogenous donors was not random, but reflected specific interactions with the recipient environment.

Although most of the grafted cells were exposed to the neurogenic milieu of the PWM (Leto *et al.*, 2006, 2009), they failed to express host-specific markers. Consistent with a previous report, in which SVZ-derived cells were implanted to P4 cerebella (Milosevic *et al.*, 2008), LGE/SVZ cells switched off the expression of Dlx2, a forebrain marker, but failed to upregulate Pax2, which is distinctive of cerebellar GABAergic interneurons. Site-specific regulation of Dlx2 was observed when these cells were transplanted to different telencephalic sites, indicating that expression of this gene is finely tuned by local cues. Both VM and DSC donors displayed patterns of expression of Lhx1 and Pax2 that were more consistent with maintenance of their native features rather than acquisition of cerebellar traits.

Previous studies show that expression of region-specific markers is maintained by stem cells dissociated and cultured *in vitro* (Zappone *et al.*, 2000; Hitoshi *et al.*, 2002). On the other hand, the ability to switch to host-specific expression following heterotopic transplantation is more controversial, although it appears to occur more easily when the cells are displaced along the dorso-ventral rather than the antero-posterior axis (Na *et al.*, 1998; Hitoshi *et al.*, 2002). Our observations show that the expression of region-specific transcription factors can be regulated by the recipient environment. Nevertheless, exogenous cells remain essentially unable to switch-on cerebellar-specific genes, indicating that they do not differentiate according to local ontogenetic mechanisms.

Extracerebellar neurons do not share the phenotype and position of their cerebellar counterparts

According to our previous observations on heterochronically transplanted cerebellar cells (Leto *et al.*, 2009), extracerebellar donors that entrain into the recipient neurogenic mechanisms and switch to local identities should eventually share the same phenotypes and distribution of endogenous interneurons generated at the time of transplantation. GABAergic interneurons produced in the P1 cerebellum comprise a majority of basket and stellate neurons, plus a minority of Golgi and Lugaro cells and sparse deep nuclei interneurons (Sekerková *et al.*, 2004; Leto *et al.*, 2006, 2009). The neurons derived from extracerebellar cells failed to develop unequivocally identifiable identities and were located in the deep nuclei and granular layer, but not in the molecular layer. Therefore, although the donor cells were initially placed in the host PWM, they did not acquire the same positions and phenotypes of age-matched cerebellar interneurons.

The absence of transplanted neurons from the host molecular layer suggests that donor progenitors are unable to differentiate into basket or stellate cells. Milosevic *et al.* (2008) described SVZ-derived donor neurons with morphological features reminiscent of basket cells, but failed to detect expression of parvalbumin, which is a landmark of this phenotype even in ectopic locations (Jankovski *et al.*, 1996; Carletti *et al.*, 2002; Leto *et al.*, 2009). Indeed, it is most likely that exogenous cells are unable to enter the molecular layer. This conclusion is consistent with previous studies, in which embryonic neocortical cells were grafted to the adult (Rossi *et al.*, 1994) or embryonic cerebellum (Carletti *et al.*, 2004). In particular, although Bergmann glia provide a highly conducive substrate for different types of transplanted cerebellar neurons across the molecular layer (Sotelo & Alvarado-Mallart, 1991; Grimaldi *et al.*, 2005; Carletti & Rossi, 2008; Carletti *et al.*, 2008; Williams *et al.*, 2008), they appear to be refractory to extracerebellar cells (Rossi *et al.*, 1994; Grimaldi *et al.*, 2005). As a consequence, regardless of their effective developmental potentialities, extracerebellar cells are prevented from following the migratory routes and differentiation processes typical of molecular layer interneurons (Simat *et al.*, 2007; Huang *et al.*, 2007).

Although donor cells were excluded from the molecular layer, they might be able to generate granular layer or deep nuclei interneurons. Extracerebellar neurons that settled in the granular layer showed a characteristic position-dependent orientation of their dendrites, which was similar to that of Lugaro and Golgi cells. Nevertheless, their axonal fields and perikaryal sizes did not match those of endogenous interneurons. In addition, their neurochemical features were often inconsistent with those of their host counterparts. Considering that the examined markers are not specific for the cerebellum, but are common in the native regions of donor cells, the observed immunostaining patterns suggest maintenance of original features rather than acquisition of local traits. In a similar paradigm of heterotopic transplantation, Milosevic *et al.* (2008) attributed cerebellar identities to SVZ-derived neurons, based on their morphological appearance. Also in that study, however, neurochemical profiles did not corroborate phenotype identification. Therefore, although exogenous donors may display structural features reminiscent of granular layer interneurons, they are clearly unable to develop unequivocal phenotypes.

The situation was somewhat different in the deep nuclei. Here, donor cells often showed morphological features and neurochemical profiles typical of local interneurons. Interestingly, the ability to acquire host-specific traits varied according to the distance between the cerebellum and the native region of donor cells: virtually all VM-derived neurons expressed CR, a distinctive marker for nuclear interneurons (Leto *et al.*, 2006), whereas the fraction of CR-positive cells was increasingly lower with DSC and LGE/SVZ donors. Also in this position, however, none of the donor-derived nuclear interneurons ever upregulated Pax2, suggesting that they acquired phenotypic traits typical of cerebellar neurons following alternative ontogenetic strategies.

Regional re-specification vs. cellular naturalization of exogenous donor cells in the host cerebellum

The different categories of cerebellar interneurons are generated through a multiple step process. Specification towards specific subtypes occurs while young postmitotic neurons sojourn in the PWM (Leto *et al.*, 2009). Thereafter, acquisition of mature traits, and notably axonal and dendritic patterns, is regulated by molecular cues or anatomical constraints present at the final destination (Sultan & Bower, 1998; Huang *et al.*, 2007). The exogenous cells engraft in the PWM but cannot entrain in the local neurogenic mechanism. Accordingly, these cells fail to upregulate cerebellar-specific regulatory genes (Milosevic *et al.*, 2008; present study), and acquire mature phenotypes and positions that are unrelated to age-matched host interneurons. On the other hand, donor cells are clearly sensitive to local cues that influence their gene expression, migratory behaviour, specific placement and phenotypic maturation. Transplanted neurons eventually develop a number of site-specific characters that, in some cases, reproduce distinctive features of local interneurons. Acquisition

of such host-specific traits is more frequent in the deep nuclei than in the cortex. It is likely that integration into the fine architecture of cortical circuits requires specialized developmental capabilities that cannot be faithfully reproduced by foreign neurons, whereas adaptation to the less complex anatomical arrangement of the cerebellar nuclei is more easily achieved.

In conclusion, our experiments show that the regional identity of donor cells cannot be easily modified, even after exposure to a foreign neurogenic milieu. Extracerebellar cells adapt to local constraints to become stably incorporated in the recipient tissue (Carletti *et al.*, 2004; Milosevic *et al.*, 2008; present study), but they fail to switch their fate towards full-blown cerebellar phenotypes. As a consequence, the morphological and neurochemical traits developed by the transplanted neurons are not determined by a mechanism of regional re-specification, but rather by a process of cellular naturalization, in which the foreign element retains fundamental characters of its origin, but also acquires some typical traits of the recipient site. It remains to be ascertained whether this process allows any meaningful integration of the transplanted neurons in the cerebellar network.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Dlx2 expression in LGE/SVZ progenitors homotopically grafted to the P1 SVZ.

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Abbreviations

CNS, central nervous system; CR, calretinin; DAG, days after grafting; DCN, deep cerebellar nuclei; Dlx2, distal less homeobox 2; DSC, dorsal spinal cord; EGL, external granular layer; GFP, green fluorescence protein; GL, granular layer; LGE, lateral ganglionic eminence; Lhx1, LIM homeobox 1; ML, molecular layer; NeuN, neuronal-specific nuclear protein; NG, neurogranin; Pax2, paired box gene 2; PV, parvalbumin; PWM, prospective white matter; SVZ, subventricular zone; VM, ventral mesencephalon.

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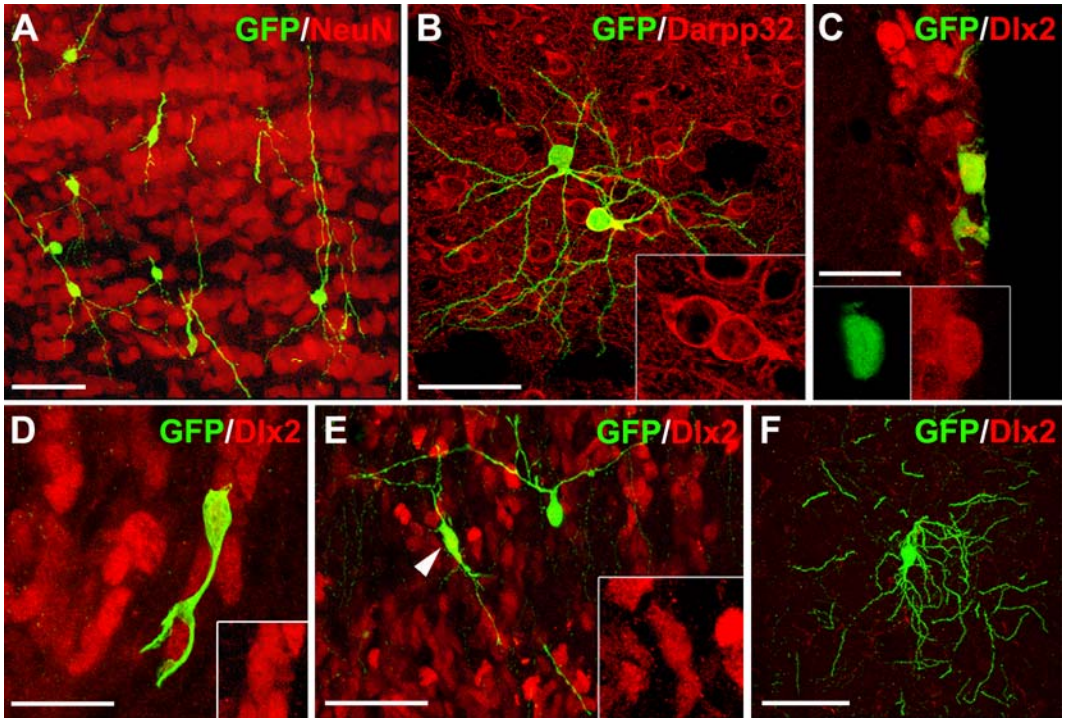

Fig. S1. A-F. Dlx2 expression in LGE/SVZ progenitors homotopically grafted in to the P1 SVZ. (A) Granule interneurons integrated in the olfactory bulb and stained by Neu-N antibodies (red). (B) medium spiny neurons in the striatum labeled by anti-Darpp-32 antibodies (red). (C-E) Dlx2-positive (red) donor cells (green) in the SVZ of the lateral ventricle (C), along the rostral migratory stream (D) and in the olfactory bulb (E). (F) displays a Dlx2-negative medium spiny neuron in the host striatum. Calibration bar: 50µm in A, B, E, F, 25µm in C, D.

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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

COMMENTARY
Nature over nurture
(Commentary on Rolando *et al.*)

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The acquisition of mature neuronal phenotypes by progenitors residing in different germinal sites along the neuraxis is thought to be regulated by the expression of region-specific combinations of transcription factors or proneural genes. Nevertheless, heterotopic transplantation experiments suggest that fate choices of uncommitted cells can be changed after exposure to a novel neurogenic environment. However, whether progenitors taken from one region of the CNS can switch their fate to acquire features typical of a foreign site has remained controversial. This issue has been recently addressed by James Goldman's group, by transplanting progenitors isolated from the forebrain subventricular zone to the prospective white matter (PWM) of the postnatal cerebellum (Milosevic *et al.*, 2008). As shown by the same group several years ago (Zhang & Goldman, 1996), the PWM is the germinal site where the different types of inhibitory cerebellar interneurons are generated. The transplanted forebrain cells failed to activate regulatory genes specific of cerebellar interneurons, such as Pax-2 (Maricich & Herrup, 1999). Nonetheless, they engrafted in the cerebellum and developed mature neurons, which were assigned to different categories of local interneurons, based on their morphology and localization. Hence, it was concluded that extracerebellar donors differentiate into cerebellar-like interneurons.

In the article published in this issue of EJN, Rolando *et al.* (2010) compared the developmental potentialities of progenitors from different sites along the neuraxis exposed to the postnatal cerebellar PWM. To identify the phenotypes acquired by donor cells, these investigators applied a set of concurrent criteria, including expression of region-specific transcription factors, morphological features, neurochemical profiles and position in the recipient architecture. Most importantly, starting from the recent work of Fernando Rossi and collaborators, showing that the phenotype and position of cerebellar interneurons are specified according to precise spatio-temporal patterns (Jankovski *et al.*, 1996; Carletti *et al.*, 2002; Leto *et al.*, 2006, 2009), Rolando *et al.* (2010) asked whether extracerebellar donors shared the same developmental phases and final fate of the cerebellar interneurons generated at the age when transplantation was done. Although the results of these experiments are partly consistent with those of Milosevic *et al.* (2008), the conclusions are quite different. The morphology, position and expression of type-specific markers in donor neurons did not correspond to those of their age-matched cerebellar counterparts. Furthermore, the morphological features of donor neurons that may be termed 'cerebellar-like' appeared to result from local interactions at the homing site rather than from the unfolding of a host-specific ontogenetic program. Interestingly, the acquisition of such features occurs more frequently when donor cells are derived from sites close to the cerebellum along the rostro-caudal extent of the neuraxis. Thus, although exogenous neurons stably engraft in the cerebellum and acquire some features reminiscent of local interneurons, it is clear that they develop according to their own native properties and fail to become integrated into the host ontogenetic mechanisms. Thus, the results reported by Rolando *et al.* (2010) indicate that changing the regional identity of neural progenitors is not an easy task.

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