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Neurobiology of Disease

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Neurobiology of Disease 30 (2008) 139–149

# Cerebellar granule cells transplanted *in vivo* can follow physiological and unusual migratory routes to integrate into the recipient cortex

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Received 29 October 2007; revised 4 January 2008; accepted 8 January 2008
Available online 26 January 2008

**CNS repair by cell transplantation requires new neurons to integrate into complex recipient networks. We assessed how the migratory route of transplanted granule neurons and the developmental stage of the host rat cerebellum influence engraftment. In both embryonic and postnatal hosts, granule cells can enter the cerebellar cortex and achieve correct placement along their natural migratory pathway. Donor neurons can also reach the internal granular layer from the white matter and integrate following an unusual developmental pattern. Although the frequency of correct positioning declines in parallel with cortical development, in mature recipients correct homing is more frequent through the unusual path. Following depletion of granule cell precursors in the host, more granule neurons engraft, but their ability for achieving correct placement is unchanged. Therefore, while the cerebellar environment remains receptive for granule cells even after the end of development, their full integration is partially hindered by the mature cortical architecture.**


*Keywords:* Neural transplantation; Cell replacement; Differentiation; Neuronal migration; Granule cell; Neural progenitors; Methylazoxymethanol acetate; Stem cells

## Introduction

CNS repair by neuronal replacement requires that new cells, either endogenous or transplanted, migrate to precise locations, acquire site-specific phenotypes and become functionally integrated into recipient networks (Rossi and Cattaneo, 2002). The outcome of these processes results from the interaction between the new cells and the surrounding microenvironment. Nonetheless, while major efforts have been aimed at unraveling biological properties of stem/progenitor cells that may be used as donors for replacement therapy, less is known about the mechanisms that regulate their integration into the recipient tissue.

Transplantation experiments have shown that anatomical incorporation of individual donor cells in the host tissue is highly influenced by the local environment. Neural progenitors can differentiate and integrate in a morphologically correct manner into different regions of the immature CNS, but this ability is lost as development advances (Das and Altman, 1971; McConnell, 1985, 1988; Nikkah et al., 1995; Brüstle et al., 1995; Campbell et al., 1995; Lim et al., 1997; Wichterle et al., 2001). In the adult, solid grafts can establish specific long-distance afferent/efferent connections with the host (Gaillard et al., 2007). In addition, complete anatomical incorporation of individual neurons in the recipient tissue and circuitry has been demonstrated in active neurogenic sites (Lois and Alvarez-Buylla, 1994; Gage, 2000; Emsley et al., 2005) or under particular conditions that recapitulate ontogenetic processes (Macklis, 1993; Magavi et al., 2000; MacLaren et al., 2006). Therefore, the extent of successful engraftment depends on specific conditions provided by the host tissue, but also on the ability of donor cells to reactivate developmentally-regulated mechanisms (Sotelo et al., 1994; Grimaldi et al., 2005).

The cerebellum is particularly suitable to investigate integration processes because it comprises a limited number of neuronal phenotypes, embedded in precisely patterned circuits and characterized by highly distinctive morphological and neurochemical features (Ramón y Cajal, 1911; Palay and Chan-Palay, 1974). In addition, the essential dynamics of cerebellar development are well-known (Altman and Bayer, 1997; Sotelo, 2004). Among cerebellar neurons, we examined granule cells, whose incorporation in the cortical network occurs during a long developmental time-window, through a well-known sequence of migratory and maturation phases (Ramón y Cajal, 1911; Altman and Bayer, 1997; Sotelo, 2004; Carletti and Rossi, 2008). Transplanted granule cells may successfully settle into immature cerebella (Gao and Hatten, 1994; Rosario et al., 1997; Carletti et al., 2002), whereas their fate in adult hosts is less clear (Grimaldi et al., 2005; Carletti and Rossi, 2005). Furthermore, little is known about the route that donor

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**Available online on ScienceDirect (www.sciencedirect.com).**

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doi:10.1016/j.nbd.2008.01.002

granule cells may follow to enter the recipient cerebellum, navigate to their final destination and integrate in the cortical network. To address these issues, we compared the behavior of granule neurons grafted to recipient cerebella at different stages of maturation, asking how developmental changes in the cortical architecture may influence their ability to incorporate into the host system. In addition, we examined the fate of granule cells transplanted to cerebella affected by degeneration of the external granular layer, to assess whether the loss of local precursors influences the engraftment of the donor neurons.

## Materials and methods

### *Animals and surgical procedures*

All experiments were performed on Wistar rats (Harlan, San Pietro al Natisone, Italy). Donor cells for transplantation were obtained from transgenic rats overexpressing the enhanced green fluorescent protein (EGFP) under the control of the β-actin promoter (a generous gift from Dr. M. Okabe, Osaka University, Osaka, Japan; (Ito et al., 2001)). All surgical procedures were carried out under general anesthesia obtained by intraperitoneal administration of ketamine (100 mg/kg; Ketavet; Bayer, Leverkusen, Germany) supplemented by xylazine (5 mg/kg; Rompun; Bayer) or diazepam (2.5 mg/kg; Roche, Mannheim, Germany). The experimental plan was designed according to the European Communities Council Directive of 1986 (86/609/EEC), National Institutes of Health guidelines, and the Italian law for care and use of experimental animals (DL116/92) and was approved by the Italian Ministry of Health. Some of the transplants examined in this study have been also used for another study (Leto et al., 2006).

### *Donor cell generation*

Donor cells were isolated from the embryonic cerebellar primordium of β-actin-EGFP rats (E14) as well as from the cortex of postnatal (P4) animals of the same species, as described previously (Jankovski et al., 1996; Carletti et al., 2002, 2004). Embryos were removed by caesarean section from anesthetized timed-pregnant females, rapidly decapitated, and dissected in saline solution with 0.6% glucose (dissection medium). Postnatal pups were cryoanesthetized in melting ice and rapidly transcardially perfused with 5 ml of dissection medium to wash out blood cells. The cerebellum was removed from the skull and cut using a tissue chopper into 300-μm-thick parasagittal slices, collected in the same medium. From such slices small tissue blocks were isolated from cortical folia. Embryonic or postnatal tissue blocks were mechanically dissociated to a single-cell suspension, which was centrifuged and resuspended at a final concentration of $5 \pm 10^4$ cells/μl. An aliquot was immediately examined under the microscope to assess cell viability and EGFP expression.

### *Transplantation in utero*

The surgical manipulation of rat embryos *in utero* was performed according to a previously described approach (Cattaneo et al., 1994; Carletti et al., 2002). Briefly, timed-pregnant E16 rats were anesthetized (ketamine/xylazine as above), and the uterine horns were exposed. The embryonic CNS was identified under transillumination, and 2 μl of the cell suspension was gently injected into the fourth ventricle by means of a glass capillary inserted through the uterine wall. The embryos were placed back into the abdomen for spontaneous delivery. Live-born recipient rats were perfused and examined at different postnatal ages (P1, $N=5$; P7, $N=2$; P10, $N=2$; P30, $N=4$).

### *Transplantation to postnatal hosts*

P1 or P8 pups were cryoanesthetized in melting ice, whereas P30 rats were anesthetized (ketamine/xylazine as above). The posterior surface of the cerebellum was exposed by removing small fragments of the occipital bone, and 2 μl of the cell suspension were injected into the parenchyma using a glass micropipette. The wound was sutured, and the animal was returned to its cage. The recipient animals for P30 ($N=8$) and P8 ($N=23$) grafts were sacrificed one month post-transplantation. In P1 grafts, hosts were killed either 7 days ($N=7$), 10 days ($N=5$) or one month ($N=9$) after grafting.

### *Transplantation in external granular layer (EGL)-depleted hosts*

Transient ablation of the EGL was induced by means of Methylazoxymethanol acetate (MAM), according to a previously established protocol (Bravin et al., 1995; Zagrebelsky and Rossi, 1999). Single MAM injections (20 mg/kg in saline, National Cancer Institute, Midwest Research Institute, Kansas City, MO) were made into postnatal rat pups ($N=5$) at P4 and P5, control animals receiving vehicle alone ($N=4$). Small tissue blocks dissected from E14 cerebella of β-actin-EGFP rats were gently placed on the cerebellar surface of P8 recipients, with no damage to the host tissue (Rossi et al., 1992, 1994), in order to examine the process of integration of transplanted granule cells in a hypogranular cerebellum. These animals were killed one month post-transplantation.

### *Histological procedures*

Under renewed anesthesia, recipient animals were transcardially perfused with 500 ml of 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.2–7.4. 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 until they sank. The brains were cut with a cryostat in 30-μm-thick parasagittal or frontal slices collected in PBS. The sections were immediately examined under the microscope, and immunohistochemically processed to detect the expression of different antigens: calbindin (1:1500, monoclonal or polyclonal; Swant, Bellinzona, Switzerland); parvalbumin (1:1500, monoclonal; Swant); zebrin II (1:200, monoclonal, a generous gift of Dr Richard Hawkes, Brochu et al., 1990); neuronal-specific nuclear protein (NeuN, 1:500, monoclonal; Chemicon, Temecula, CA); zic2 (1:10,000, a generous gift of Dr Stephen Brown, University of Vermont; Brown et al., 2003); Pax-2 (1:200, polyclonal, Zymed, San Francisco, CA); glial fibrillary acidic protein (GFAP; 1:1000, polyclonal, Dakopatts, Glostrup, Denmark); GABA (1:5000, monoclonal, Sigma); neuroglycan 2 (NG2; 1:200, Chemicon); vesicular glutamate transporter 1 (V-Glut-1, 1:1000; Sysy, Goettingen, Germany). In some cases we used anti-GFP antibodies (1:700, polyclonal or monoclonal; Invitrogen, Carlsbad, CA) to enhance the intrinsic fluorescence of transplanted cells. In addition, the proliferation of granule cell progenitors was visualized by means of anti-Ki67 antibodies (1:100, polyclonal, Abcam, Cambridge, UK) or BrDU incorporation after pulse injections (see details in Leto et al. 2006).

Incubation with primary antibodies was made overnight at room temperature in PBS with 1.5% normal serum and 0.25% Triton X-100.