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Time constraints and positional cues in the developing cerebellum regulate Purkinje cell placement in the cortical architecture

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Abstract

To elucidate the mechanisms that regulate neuronal placement and integration in the cerebellar circuitry, we assessed the fate of Purkinje cells transplanted to embryonic, juvenile and adult hosts, asking how architectural changes of the developing cortex influence their anatomical incorporation. Donor Purkinje cells navigate through the host parenchyma either along their natural migratory pathway or following unusual routes. In the latter case, donor neurons fail to orientate correctly and to establish the cortico-nuclear projection. Purkinje cells that follow the physiological route achieve the typical orientation and connectivity, but end displaced in the molecular layer if their arrival in the recipient cortex is delayed. Navigation routes and final settling of donor neurons vary with host age, depending on the ontogenetic construction of cortical layering, and particularly on the maturation of granule cells. The migratory behavior and homing of transplanted Purkinje cells is modified after external granular layer ablation, or neutralization of reelin signaling produced by granule cells. Therefore, although the cerebellar milieu remains receptive for Purkinje cells even after the end of development, correct placement of donor neurons depends on the timing of their migration, related to cerebellar developmental dynamics and granule cell layering.

Keywords: Neuronal differentiation; Neuronal migration; Granule cell; Purkinje cell; Neural progenitors; Reelin; Neural transplantation; Cell replacement

Introduction

During CNS development, environmental signals and positional cues interact with intrinsic properties of progenitor cells to regulate their specification and acquisition of mature neuronal phenotypes, in terms of morphology and functional integration in neural circuits. Cell transplantation represents the most appropriate approach to dissect the relative contribution of extrinsic signaling and cell-autonomous mechanisms in these processes. By applying this method, several studies demonstrated that neural progenitors are able to settle and differentiate according to the host environment in different regions of the immature CNS, but this ability is gradually 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, donor cells integrate in a morphologically correct manner in active neurogenic sites (Lois and Alvarez-Buylla, 1994; Gage, 2000; Emsley et al., 2005) or under particular conditions that may recapitulate ontogenetic processes (Macklis, 1993; Magavi et al., 2000; MacLaren et al., 2006). Although these studies provided important information on the responsiveness of donor cells to the recipient milieu, much less attention has been paid

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to developmental changes of the CNS environment that influence the acquisition of a phenotype. Here, we applied a heterochronic transplantation approach to ask how architectural changes of the developing CNS influence neuronal placement and integration.

The cerebellum is particularly suitable to address these issues 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, Purkinje cells, with their long-distance projections and highly stereotypic alignment and orientation in the cortical architecture, provide an ideal model to investigate the role of environmental mechanisms involved in cell positioning.

Purkinje cells transplanted to the adult cerebellum incorporate in the cortical network, but fail to establish cortico-nuclear connections (Sotelo and Alvarado-Mallart, 1987a,b, 1991; Triarhou, 1996, 1997; Rossi et al., 1992, 1994; Carletti and Rossi, 2005). In contrast, when grafted to embryonic hosts these neurons achieve full integration and connectivity (Carletti et al., 2002). To elucidate the developmental mechanisms underlying the placement of Purkinje cells into the cerebellar cortex, we compared the fate of embryonic Purkinje cells grafted to recipient cerebella at embryonic, postnatal and adult stages of maturation. Namely, we asked how the architectural changes that occur during the development of the cerebellar cortex influence the migratory routes followed by the donor cells towards their final destination, and their ability to settle into the host network. We show that Purkinje neurons migrate through the cerebellar parenchyma either along natural migratory pathways or following unusual routes. Nevertheless, their navigation and final settling vary with host age, depending on the structural evolution of the cerebellar environment, associated with the development of mature cortical layering.

Materials and methods

Animals and surgical procedures

All experiments were performed on Wistar rats or FVB mice (Harlan, San Pietro al Natisone, Italy). Donor cells for transplantation were obtained from transgenic mice or 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; Okabe et al., 1997; Ito et al., 2001). L7-GFP BAC transgenic mice (Gensat Project, Rockefeller University, NY), in which the reporter gene is selectively expressed by Purkinje cells, were used for the reelin experiments. All surgical procedures were carried out under deep 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 work (Leto et al., 2006).

Donor cell generation

Donor cells were isolated from embryonic cerebellar primordium of β-actin-EGFP mice (embryonic day 12, E12) and rats (E14) as described previously (Jankovski et al., 1996; Carletti et al., 2002). Embryos were removed by caesarean section from deeply anaesthetized timed-pregnant females, rapidly decapitated, and dissected in saline solution with 0.6% glucose (dissection medium). The cerebellar anlage was separated from the embryonic CNS and mechanically dissociated to a single-cell suspension, which was centrifuged and resuspended at a final concentration of 5×10^4 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, 2004). Briefly, timed-pregnant E16 rats were deeply anaesthetized, and the uterine horns were exposed. The embryonic CNS was identified under transillumination, and 2 μl of the cell suspension were 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 killed at different postnatal ages (P1, $N=5$; P7, $N=2$; P10, $N=2$; P30, $N=4$). In an additional set of cross-species transplantation experiments, E14 rat cells were grafted to E14 mouse hosts ($N=3$) and E12 mouse cells were grafted to E16 rat hosts ($N=6$). All these animals were examined at P30.

Transplantation to postnatal hosts

P1 or P8 pups were cryoanesthetized in melting ice, whereas adult (P30) rats were deeply anaesthetized 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 ($N=5$) and P8 ($N=12$) grafts were sacrificed 1 month post-transplantation. In P1 grafts, hosts were killed either 7 days ($N=7$), 10 days ($N=5$) or 1 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 ($N=7$) at P4 and P5, control animals ($N=6$) receiving vehicle alone. Small tissue blocks dissected from E14 cerebella of β-actin-EGFP rats were gently placed on the cerebellar surface of P8 pups. These grafts were positioned on the recipient cerebellar surface, with no damage to the host tissue (Rossi et al., 1992, 1994), in order to test effect of EGL on the migratory behavior of donor Purkinje and granule cells.

Organotypic cultures and reelin interference experiments

FVB mice at P9 were processed and parasagittal slices made as above (see Donor cell generation). The slices were placed on millicell inserts (Millipore, Bedford, MA) and maintained in previously described cell culture conditions (Zagrebelsky et al., 1998). At the same time, small pieces of E12 cerebellar tissue were dissected from L7-GFP BAC transgenic mice (Gensat Project, Rockefeller University, NY), and juxtaposed to the EGL of the P9 slice. After 2–3 days *in vitro* the intrinsic fluorescence of embryonic Purkinje cells became detectable. At this time to assess the role played by the reelin pathway in the inhibitory action of granule cell layer against Purkinje cell migration, the medium was supplemented with either the Src family inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; 20 μM/1:500, Calbiochem, Jossin et al., 2003), the function-blocking antibody against reelin CR50 (1:200, prepared as in Nakajima et al., 1997), or the corresponding control