Growth and differentiation of cerebellar suspensions transplanted into the adult cerebellum of mice with heredodegenerative ataxia

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ABSTRACT

Cell suspensions from cerebellar primordia of 12-day mouse embryos were grafted into the cerebellum of 4-month-old Purkinje cell degeneration (pcd) mutant mice and examined 2–3 months later. In contrast to those of nontreated mutants, all of the grafted cerebella exhibited Purkinje cells that had migrated into the molecular layer, where they were clustered over its superficial two-thirds. These Purkinje cells develop flattened dendritic trees perpendicular to bundles of parallel fibers. Ultrastructural examination of their synaptic inputs and outputs disclosed that (i) as in normal cerebella, climbing fibers and axons from basket and stellate cells synapse on thick dendrites, whereas parallel fibers almost exclusively contact the distal spiny branchlets, and (ii) a substantial number of Purkinje cell axons reach their appropriate targets in the deep cerebellar nuclei, where they establish synaptic connections on large and small neurons. These results indicate that embryonic Purkinje cells grafted into the cerebellum of adult mice with heredodegenerative ataxia integrate themselves very specifically into the cerebellar circuitry of the recipient mouse, where they can replace the missing Purkinje cells. They also provide a morphological basis favoring the notion of functional restorative capabilities of neural grafts in systems in which neurons are connected in an almost point-to-point manner.

Most studies of embryonic neuron transplantation into adult animal brain have dealt, so far, with “global” systems (those in which a few nerve cells innervate very extensive terminal domains, as in monoaminergic systems or cholinergic cortical systems), in which grafting into selectively denervated target regions results in neuron growth and, more importantly, in a normal biochemical differentiation, in which the embryonic neurons are able to produce their appropriate neurotransmitters (1, 2). Functional recovery from this type of grafting (3, 4) does not necessarily imply the formation of specific synaptic contacts since it can result from transmitter release, acting in a paracrine fashion and diffusing over large distances on cells equipped with adequate receptors, as seems to be the case in the normal central nervous system (5).

Increasing evidence indicates that central neurons of “point-to-point” systems (those in which each nerve cell contacts no more than a few target neurons) can survive transplantation even when grafted heterotopically, as shown for cerebellar Purkinje cells (PCs) (6). It was postulated that in this case any reparative mechanism would be nonparacrine and would require specific synaptic input/output interactions between the transplanted neurons and the host. Our present experiments provide morphological evidence that, indeed, a normal pattern of synaptic investment is established by and on PCs previously transplanted as dissociated cell suspensions into the cerebellum of adult PC degeneration (pcd) mutant mice (pcd being an autosomal recessive mutation which causes the death of virtually all PCs 20–30 days postnatally; ref. 7). Our results show a remarkable ability of the grafted PCs to migrate into their proper cerebellar domain, the molecular layer (ML), left vacant by the intrinsic action of the pcd locus (8). Moreover, they develop dendritic trees, receive a synaptic investment, and issue terminals, all of which exhibit features comparable, at least qualitatively, to those observed in normal cerebella.

MATERIALS AND METHODS

Timed pregnant C57BL mice were purchased from Iffa Credo (Lyon, France). Mating day was considered as E0. Twelve-day embryos (E12) were donors. Embryos were dissected at room temperature in phosphate-buffered saline (pH 7) with glucose added (6 mg/ml). The cerebellar primordium was mechanically dissociated in culture medium without proteolytic agents (9). Cells were centrifuged (500 x g, 5 min), resuspended in culture medium, and used for transplantation. Graft recipients were three 4-month-old homozygous pcd mutant mice of the C57BL/cdJ strain (raised at the Pasteur Institute by J. L. Guénét). They were anesthetized with chloral hydrate and fixed in a stereotaxic frame. Two small, symmetrical craniotomies were performed in the occipital bone to expose the posterior surface of the cerebellum at the two borders between vermis and hemispheres. One-half to 1 μl of cell suspension (30,000 cells per μl) was injected with a 10-μl Hamilton syringe fitted to a glass micropipette (about 100 μm in diameter) through a plastic tube, so that each hemicerebellum contained one graft. Two to 3 months after this procedure, the mice were anesthetized with chloral hydrate and perfused through the heart with aldehyde fixatives for routine electron microscopy (mouse 1) and immunocytochemistry with antibodies selectively staining PCs (mice 2 and 3).

The two hemicerebella of mouse 1 were treated with 2% osmium tetroxide and embedded in Araldite in the parasagittal plane. One hemicerebellum of mouse 2 was sagittally sectioned on a Vibratome (Oxford) at about 30 μm and immunocytochemically stained with an antisera to vitamin D-dependent calcium-binding protein (CaBP), according to the peroxidase-antiperoxidase method (10). After treatment in 2% osmium tetroxide, the sections were embedded flat in Araldite. The remaining hemicerebellum and the two obtained from mouse 3 were soaked in 20% sucrose and sectioned either in the coronal or in the parasagittal plane with a freezing microtome at about 25 μm. Sections were immunohistochemically stained either with an antisera to CaBP (sagittal) or with an antisera to guanosine 3',5'-phosphate-dependent protein kinase (one coronal, one sagittal). Additional ungrafted cerebella from two pcd mice aged about 5 months were prepared for light microscopy immunostaining with the antisera to guanosine 3',5'-phosphate-dependent protein kinase. The procedure for immuno-

Abbreviations: PC, Purkinje cell; CaBP, vitamin D-dependent calcium-binding protein; ML, molecular layer.
histochemical staining was the same as that described elsewhere (11).

The percentage of the volume of cerebellar ML occupied by grafted PCs was calculated with stereological methods (12) from the three immunostained hemicerebella. Parallel fiber-PC spine synapse density was calculated from five sets of photomontages (final magnification, ×13,000) covering large areas (8350 μm²) of the two grafted hemicerebella. Control density was obtained in a similar manner from the cerebella of two C57BL mice about 6 months old.

**RESULTS**

As expected, the cerebella of the two untreated pcd mice were practically devoid of PCs. In guanosine 3',5'-phosphate-dependent protein kinase-immunostained material, a maximum of 100 PCs was present per cerebellum, occupying the proper location at the interface of the ML, and granular layers. Most of these cells were confined to the nodulus (43%); in the intermediate cortex they were very rare (5 or 6 per cerebellum).

**Distribution of Grafted PCs.** All six grafted suspensions provided PCs to the pcd mouse cerebellum. Distribution of the grafted cells followed a similar pattern: although many cells remained in the vicinity of the pipette track at the injection site (Fig. 1), the majority had migrated out and occupied exclusively the ML of the folia contiguous to this track (Fig. 1 C and D). Serial reconstructions of the three immunostained grafts (Fig. 1) illustrates that of mouse 2 showed a spread of transplanted cells on both sides of the injection site. Spreading was uneven, being greater in the ML of the affected folia (Fig. 1 A–F) than around the pipette track (Fig. 1 B–E). Each hemicerebellum measured about 4 mm in width (160 sections, 25 μm thick) and grafted cells were present in a radius of about 375–600 μm around the center of the pipette track. Thus, the majority of grafted PCs occupied the intermediate cortex with minimal involvement of the vermal and hemispheric cortices. Examination of 1-μm-thick plastic sections (mouse 1) showed that the large majority of cells invading the ML were PCs. Increased density of stellate and basket cells was not obvious, and only a few small clusters of granule cells were found in the ML.

Stereological analysis allowed evaluation of the proportion of the total ML volume per hemicerebellum occupied by the graft. This varied between 3% and 5% for each, indicating a very small homotopic restoration by the grafted PCs.

**PC Dendritic and Axonic Pattern.** Closer examination of the cortical regions containing PCs showed that these are always distributed in a three- to five-cell-deep zone within the upper two-thirds of the ML (Fig. 2 B and E). In sagittal sections the dendritic trees display a maximal extent and stop abruptly at the border with the granular layer (Fig. 2 A and B). Most PCs were monopolar, even though bi- and multipolar forms were also present. In areas like those illustrated in Fig. 2A, which correspond to the transplant border, the main PC dendrites were oriented parallel to the surface, invading for some distance the neuropil of the ML, which was devoid of this neuronal population. In transverse sections dendritic trees extend minimally (Fig. 2B). Thus, in the adult cerebellum grafted PCs develop extensive dendrites, which, although notably different from those of normal animals, exhibit features reminiscent of normal PCs: (i) they are flattened in the transverse plane and (ii) they have clear-cut proximal and distal compartments.

Guanosine 3',5'-phosphate-dependent protein kinase-immunoreactive fibers corresponding to PC axons were present mainly in the interposed and in the medial nuclei, whereas they were almost absent from the lateral nucleus, as expected from the location of grafted PCs. These fibers followed a rectilinear path and bore numerous varicosities (Fig. 2C).

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**Fig. 1.** Immunohistochemistry with the anti-CaBP antibody. Serial reconstruction (A–F) of a graft into one hemicerebellum. Each micrograph is taken at about a 7-section interval. The graft extends over 45 sections, from the lateral vermis (A) to the medial hemisphere (F). The center of the graft, marked by the maximal extension of the pipette track, is close to the section in D. Note the dispersion of immunoreactive PCs within the ML of several folia contiguous to the pipette track (A–F). (×45.)
They sometimes formed intricate nests covering immunonegative neuronal perikarya but primarily followed unreac
tive dendrites. Although the PC projection to the deep nuclei
was far less dense than in normal cerebellum, it was none
theless much more developed than in pcd mutants of the same
age. In these mutants we never observed bundles of
Guanosine 3',5'-phosphate-dependent protein kinase-positive
axons, which were present only occasionally in the deep
nuclear territory.

PC Synaptic Investment. As in normal cerebellum, ultra
structural identification of grafted PC perikarya and
dendrites was possible in material prepared for routine elec
tron microscopy (13). Immunocytochemical study not
only confirmed that within the cerebellum CaBP reactivity is
selective for PCs but also allowed the identification of PC
axons and axon terminals in the deep cerebellar nuclei.

Qualitatively, the only striking difference from normal PCs
was the absence of basket fibers and of their perisomatic
nests and pinceaux formations around the initial segment of
the PC axons. However, axon terminals of basket and stellate
cells did synapse on the somatic membrane and even occa
sionally on the surface of the initial segment (Fig. 3A). Almost
all thick primary dendritic branches received climbing fiber
inputs; their axon terminals established typical synapses on
the stubby spines (Fig. 3B). Also, this proximal dendritic
compartment received synaptic inputs from ML interneurons
on its smooth surface, mostly from stellate cells (Fig. 3B).
The distal dendritic compartment, composed of spiny
branchlets, was the postsynaptic domain of numerous par
allel fibers, which synapsed on dendritic spines (Fig. 3 A–D)
as in control cerebella. Some of the parallel fiber varicosities
in synaptic contact with distal spines of grafted PCs were still
attached to a thin band of dense material, a remnant of the
necrotic PC dendrites of the pcd mutant (Fig. 3D). Grafted
PCs thus appear to succeed in correctly segregating their
synaptic inputs, although not as completely as normal PCs,
since distal-like spines emerge occasionally from thick proxi
mal dendrites and contact parallel fiber varicosities.

To evaluate the importance of parallel fiber inputs to the
grafted PCs, the densities of the synapses between these
axons and distal dendritic spines in controls and in grafted
mutants were compared (Table 1; see also Materials and
Methods). The 47.4% decrease in density indicates that
grafted PCs receive only about half of the normal contingent
of parallel fiber synapses. This reduction could result par
tially from the already reduced number of granule cells at the
time of grafting, since the death of the mutant PCs starts
a slow secondary process of granule cell degeneration, like
that occurring in the cerebellum of the nervous (nr) mouse (15).
FIG. 3. Electron micrographs of the synaptic inputs and outputs of grafted PCs. (A) Part of a PC perikaryon and the initial segment of its axon (IS) are illustrated here. This neuron is located in the middle of the ML, and bundles of parallel fibers (open arrows) are in the vicinity of the initial segment. Note that axon terminals belonging to ascending collaterals of basket fibers and to stellate cells synapse on the perikaryon (arrows) and on the initial segment (arrowhead). A PC spiny branchlet (PCD) provides distal spines. One of them is contacted by a parallel fiber (asterisk). (x 16,000.) (B) A proximal dendritic segment gives off spines that are in synaptic contact with a climbing fiber varicosity (CF). Two axon terminals belonging to ascending collaterals of the basket fibers and/or to stellate cells also synapse on the smooth surface of the dendrite (arrows). Note that in the neuropil three distal spines of PC dendrites are contacted by parallel fibers (asterisks). (x 19,000.) (C) Parallel fiber varicosities synapse as distal spines of PC dendrites. One of these varicosities (PF$_1$) is very much enlarged and contacts four spines (numbered 1–4), suggesting a process of terminal sprouting (14). The other (PF$_2$) is of normal size and synapses on only one spine (asterisk). A stellate cell dendrite (SD) receives synaptic inputs from parallel fibers (arrows), one of them being the enlarged varicosity (PF$_1$). (x 29,000.) (D) A parallel fiber varicosity, undoubtedly from the pcd mouse because it is still attached to the dark debris (star) belonging to a degenerated PC dendrite, is in synaptic contact (arrowhead) with a spine of a distal PC dendrite. (x 22,000.) (E) Immunohistochemistry with the anti-CaBP antibody. A PC axon terminal, labeled with electron-dense immunoprecipitate, synapses on the perikaryon of a large neuron (DN) of the anterior interposed nucleus. (x 24,000.)
Interactions between host cerebellar interneurons and grafted PCs can also occur. Although parallel fiber–PC synapse density is reduced by as much as 48%, it is unlikely that synapses are established between grafted granule cells and grafted PCs for the following reasons. (i) The number of granule cells in the injection track and in the ML is very low, suggesting that most of the grafted stem cells from the external granular layer have failed to proliferate. This could result from the fast migration of embryonic PCs to the ML since it is likely that a normal proliferation of granule cells (17) requires the close proximity of PCs to the external granular layer. (ii) Some of the parallel fibers synapsing on PC spines remain attached to dark debris from mutant PCs, indicating that they belong to the recipient cerebellum. (iii) The number of these synapses is too high to account for the possibility that they arise from granule cells that could have succeeded in migrating to the granular layer. The most obvious difference between synaptic investment of control and grafted PCs is that in the latter basket fibers do not form perisomatic nests and pinceau formations, probably because of the abnormal location of the PC perikarya and because of the behavior of basket cell axons in PC-deprived cerebellum, which prevents interactions between them (the axons remain clustered together, forming "empty baskets," which persist as long as the animal lives; see ref. 14). Nonetheless, inhibitory inputs from basket and stellate cell axons establish abundant synaptic contacts on PC perikarya and primary dendrites. Thus, local inhibitory input is partially restored on grafted PCs.

Although in this study grafted PCs occupy no more than 5% of the total ML volume, data obtained in our laboratory with solid transplants indicate that this ratio can be improved. It is necessary now to determine what PC input/output synaptic density would be sufficient to compensate, at least partially, the cerebellar deficit of pcd mice.

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Table 1. Density of parallel fiber–PC synapses

| Material | No. of fibers | ML surface measured, μm² | No. of parallel fiber–PC synaps per 100 μm² | No. of parallel fiber–PC synaps per | Terminals were and in absent, several with dendritic
|          |              |                         |                                         | parallel fibers                     |
|----------|--------------|--------------------------|----------------------------------------|------------------------------------|--------------------------------------|
| Control  | 766          | 3950                     | 19 (= 100%)                            |                                    | Synapses Established by Grafted PC Terminals. These axon terminals were assessed in the interposed nuclei immunostained with CaBP antibody. All CaBP-reactive profiles were axons and axon terminals. They were infrequent, even absent, in some of the sections analyzed. However, when present, several of them were grouped in close proximity, suggesting that they belong to the terminal cluster of a single axon. These terminals are ovoid varicosities, roughly 3 μm in largest diameter, packed with pleomorphic vesicles (Fig. 3E); they establish Gray 2 synaptic junctions with the perikarya of large (Fig. 3E) and small neurons as well as with main dendritic segments. Given their morphological features and their CaBP reactivity, these terminals—which are absent in untreated pcd mouse cerebellum—can be considered as the nuclear projections of grafted PCs.

**DISCUSSION**

The present results demonstrate long-term survival of grafted PCs in the cerebellum of adult pcd mice. Each grafted cell suspension provided numerous PCs that survived for a much longer time than those of the host, corroborating the notion of an intrinsic action of the pcd gene, as suggested by previous analyses of chimere mice (8).

Most striking is the fact that the majority of surviving PCs are found in the ML, at some distance from the transplant, taking over the territory left vacant by the genetic defect. Thus, embryonic PCs are able to migrate and are specifically attracted to regions rich in adult presynaptic partners deprived of their normal targets. These PCs develop dendritic trees different from those of control animals but manifesting two of their main features: (i) a monopolarar disposition and (ii) a segregation of synaptic inputs into proximal and distal compartments. Since PC dendrite acquisition during normal development is the result of an interplay between genetic factors and cellular interactions (namely, synaptic) taking place during dendritic growth (16), the formation of dendritic trees by the grafted PCs must also result from a similar interplay. This implies that PCs invade the mutant ML shortly after their transplantation and that adult postsynaptically deprived axons must start synaptogenesis immediately after the arrival of these still immature neurons. Thus, embryonic neurons would replace degenerated cells of a corresponding population and differentiate dendrites under the influence of a surrounding adult axonal field.

The survival of embryonic neurons in adult brains is a commonly established fact. However, whether such neurons are able to integrate themselves into the recipient brain and to replace the missing links of a pathological neuronal network is still unknown—but this would be the only way to assess the reparative function of neural grafting, at least when dealing with point-to-point systems. Data reported here clearly show that this integration can occur, since grafted PCs not only receive specific synaptic inputs but also send projections toward the deep nuclei of the host cerebellum. The presence of climbing fiber varicosities synapsing on a majority of the primary dendrites further supports the notion that specific interactions are established between the host brain and the grafted neurons.