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Mutant mouse cerebellum does not provide specific signals for the selective migration and development of transplanted Purkinje cells

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Embryonic cerebellum transplanted to adult Purkinje cell degenerate mice was assessed for integration and Purkinje cell migration by using the antigenic markers Thy-1 and Leu-4. It was found that the grafted cells migrated into the host's molecular layer, but there was no evidence for specific migration of Purkinje cells. Furthermore, grafted cells were found to form normal cerebellar cyto-architecture only with other grafted cells and not with the host's cells.

The functional replacement of populations of neurons in the central nervous system, lost through trauma or disease, could be best achieved if grafted embryonic neurons, of a similar phenotype, could precisely localize and integrate into the deficient regions of the host. Recent transplantation studies in the Purkinje cell deficient mutant [8, 9, 17–21] indicate that this type of replacement may be possible. In this model, it has been reported that Purkinje cells migrate out of an embryonic cerebellar graft and re-locate into Purkinje cell deficient areas [17, 18]. To explain this process, it has been proposed firstly, that the mutant host produces neurotropic signals specific for cells of the lost phenotype [17, 18, 20] and secondly, that the adult host's neural cells interact with the migrant embryonic neurons, by a process resembling normal development [17], to re-establish cerebellar structure and function [8, 9, 19, 20]. To investigate whether cell-type specific tropism and donor–host interactions occur in this model, we have used a Purkinje cell marker, Leu-4 [10], in conjunction with Thy-1 allelic markers to unequivocally demarcate host from donor neurons [5, 12]. Our studies show that Purkinje cells comprise a small proportion of neural cells that migrate into the host, and that their organization into normal cerebellar cyto-architecture only occurs in areas comprised predominantly of donor tissue. These findings argue against the release of specific neurotropic factors by the host and suggest that graft morphogenesis is independent of the host's environment.

Mice homozygous for the Purkinje cell degeneration mutation (*pcd/pcd*) lose virtually all their Purkinje cells by 4 weeks postnatally resulting in gross ataxia [14]. The selective loss of a single morphologically distinct cell type in the cerebellum has made it an ideal experimental animal in which to study the efficacy and mechanisms of neural replacement by embryonic tissue. In our experiments, a single cell suspension was prepared, as previously described [6], from cerebellar anlagen of E12 C57Bl mice (Thy-1.1 positive). A 4-μl aliquot, containing approximately 5×10^5 cells, was injected, to a depth of 2 mm, into the dorsal aspect of the right cerebellar lobe of 11, 3-month-old *pcd/pcd* mutant host C57Bl/cdJ mice (Thy-1.2 positive). Animals were sacrificed 63–136 days after grafting, and immediately perfused with ice-cold 10% sucrose solution. The cerebella containing the grafts were removed and blocked in OCT embedding compound (Tissue-Tek, Miles, USA), and then rapidly frozen in isopentane cooled by liquid nitrogen. Blocks were later sectioned and immunostained as described below.

As the donor and recipient strains of mice contain different alleles of the Thy-1 gene, Thy-1.1 and Thy-1.2, respectively, and as this molecule is expressed predominantly on the body and processes of the majority of neurons [3], it is possible to identify host and donor cerebellar neurons independently using immunohistochemistry. (Figs. 1c,d, and 2a,c show specificity of antibodies; also see refs. 5 and 12.) In addition to Thy-1, Purkinje cells

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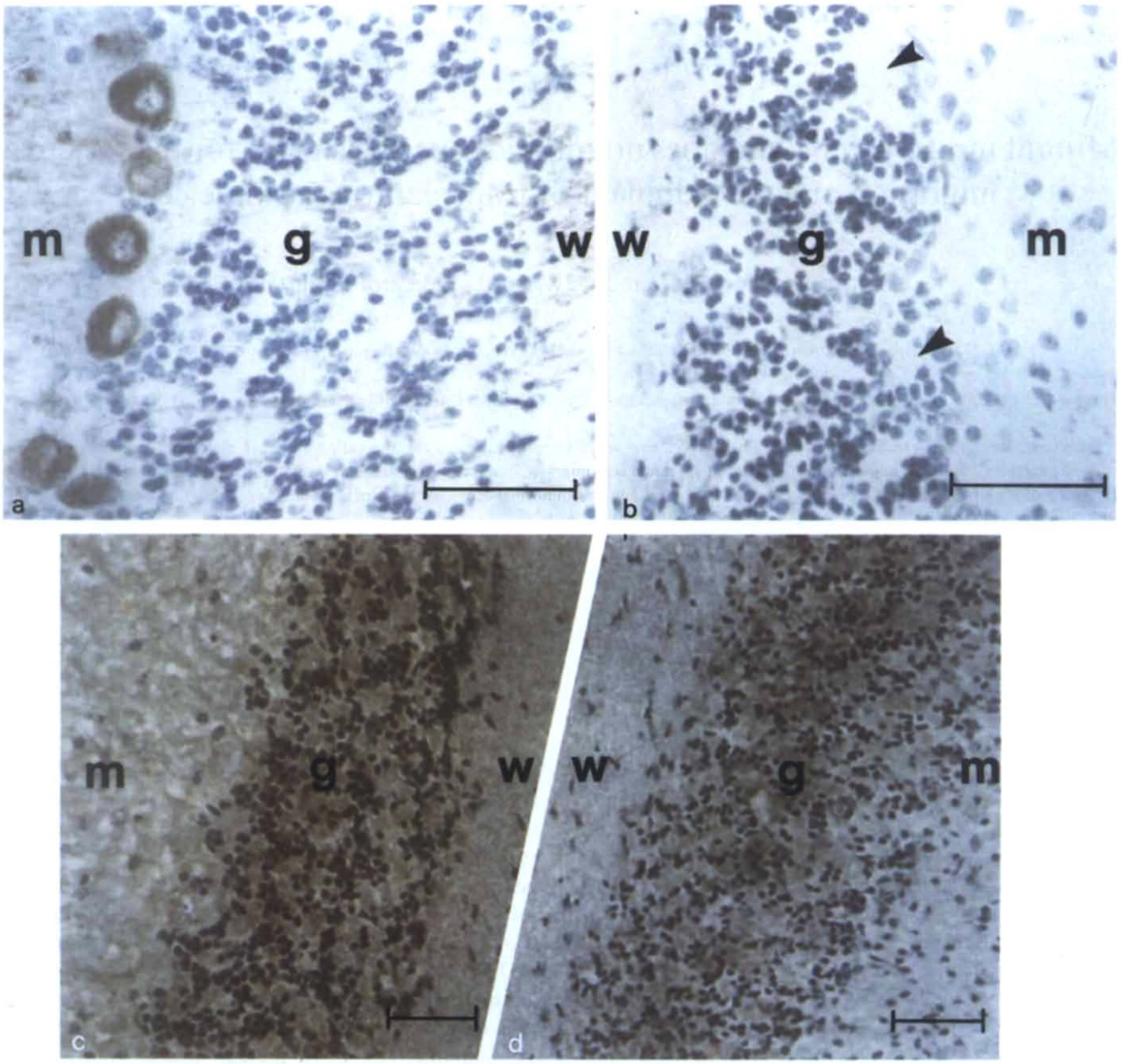

Fig. 1. Immunoperoxidase-stained sections showing the presence of Leu-4 positive Purkinje cells in the cerebellum of normal (a) but not mutant *pcd/pcd* mice (b; arrows indicate space previously occupied by Purkinje cells). Thy-1 is expressed in the cerebellum of both the normal (c; Thy-1.1) and mutant (d; Thy-1.2) mice. m, molecular layer; g, granular layer; w, white matter. Bar = 100 μm.

were identified by an anti-Leu-4 antibody which specifically binds to all Purkinje cells in the mouse cerebellum (see Fig. 1a and ref. 10), and is not found in mutant cerebellum (Fig. 1b). Immunohistochemistry was carried out on 8-μm serial, cryostat sections. The sections were placed on gelatinised slides, air-dried and then fixed in 100% acetone for 5 min at room temperature. Slides were then placed in a humidified chamber and washed in phosphate-buffered saline (PBS) and blocked for 20 min in PBS containing 2% normal sheep serum and 0.1% bovine serum albumin. Slides were then incubated for 2 h at room temperature with 100 μl of one of the monoclonal antibodies: rat anti-Thy-1.1 (OX-7, Sera-Lab, UK) diluted 1:200; mouse anti-Thy-1.2 (30H12, Becton Dickinson, USA) undiluted supernatant; or a biotinylated mouse anti-human Leu-4 (CD3, Becton Dickinson) diluted 1:100. The primary antibody was washed off in PBS and the slides re-dipped in blocking solution prior to incubation for 90 min with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody:

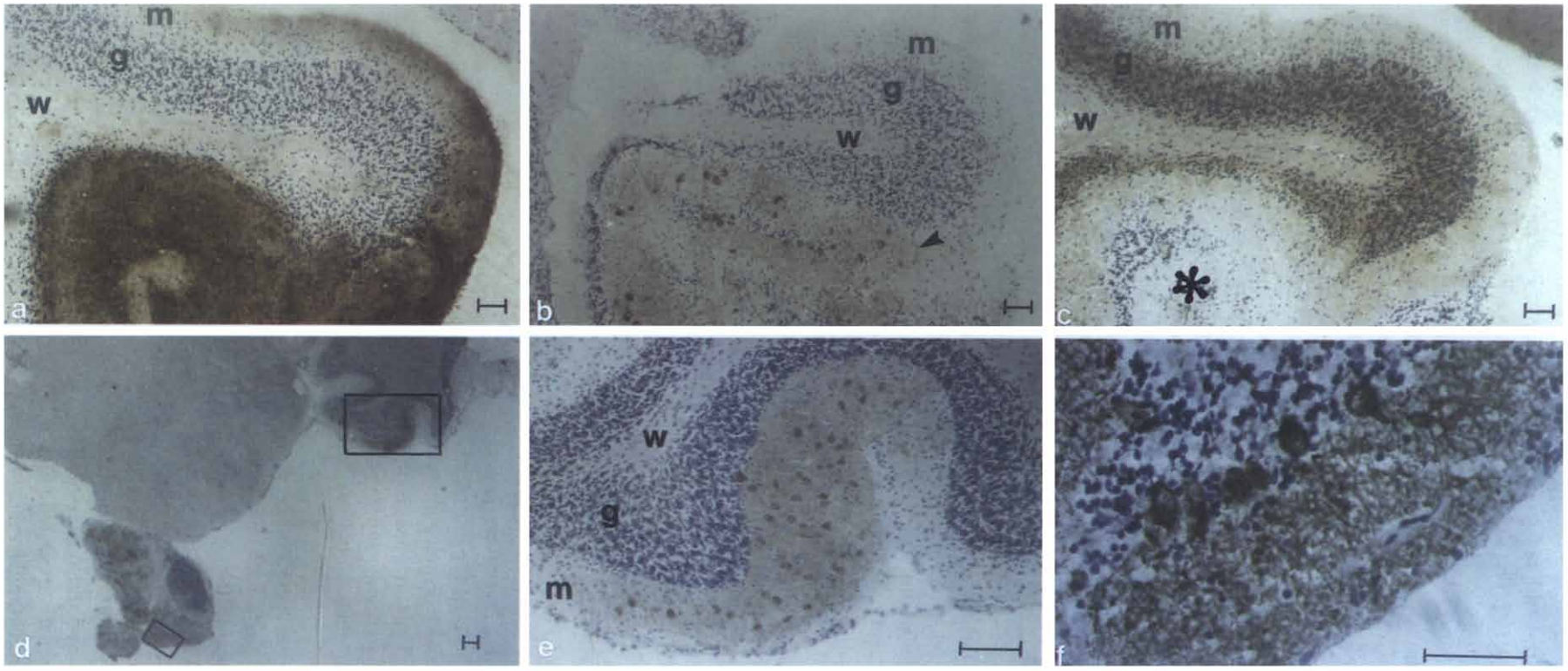

Fig. 2. Immunoperoxidase-stained sections of grafted cerebellum. Serial sections (a–c) show migration of donor neurons, as identified by Thy-1.1 staining (a), into the molecular layer (m) of a folia of the host's cerebellum (host tissue identified by Thy-1.1 staining in c). Although donor neurons have migrated a considerable distance from the body of the graft (body of graft shown to be centered around asterisk in c, was identified by its failure to stain for Thy-1.1, and strong staining for Thy-1.2), the Leu-4 positive Purkinje cells have only migrated a relatively short distance (b; extent of migration indicated by arrow), demonstrating that the migrating neuronal population is not restricted to Purkinje cells. In areas where Purkinje cells had migrated into the host's molecular layer (d, large boxed area, magnified in e; both stained for Leu-4 expression), they were unevenly distributed throughout its width and were not aligned with the host's granular layer (g), as normally occurs (see Fig. 1a). However, in areas comprised solely of donor tissue, best illustrated by an ectopic graft (d, small boxed area, magnified in f), the Leu-4 positive cells were aligned with what appears to be donor granule cells. w, white matter. Bar = 100 μm.

sheep anti-mouse Ig (DAH, Silenus, Australia) diluted 1:50, goat anti-rat Ig (Chemicon, USA) diluted 1:100, or streptavidin (Caltag, USA) diluted 1:100. The slides were again washed in PBS and incubated for 4–7 min with a freshly prepared solution containing 0.1% of the chromogen. 3,3′-diaminobenzidene tetrahydrochloride (Dakopatts, USA), and 15 μl of 30% (w/v) H_2O_2, in mt-PBS. Sections were then counterstained with haematoxylin and mounted.

Although the donor and recipient strains are on the same genetic background, they do differ at minor histocompatibility loci, however, no signs of immunological rejection were observed (unpublished observations) in contrast to that previously reported with major histocompatibility loci differences [12, 16].

Removal of the grafts 63–136 days after transplantation revealed that they were all primarily contained within the cerebellum (Fig. 2a–f), although in some animals a portion of the graft had developed ectopically (see Fig. 2d,f). In all grafts, regions were found in which donor neurons, as identified by Thy-1.1 staining (Fig. 2a), had migrated away from the body of the graft into the molecular layer of the host's cerebellum, identified by Thy-1.2 staining (Fig. 2c), often traversing the perimeter of a single cerebellar folia (Fig. 2a). Neurons that had migrated farthest from the body of the graft were found to be distributed toward the pial surface (Fig. 2a), indicating that graft migration occurred initially beneath the pial surface and later extended into the molecular layer, as previously suggested [20].

We examined whether the migrating Thy-1.1 positive cells were Purkinje cells by immunostaining consecutive sections for the presence of Leu-4 positive cells. This revealed that the majority of Thy-1-expressing cells migrating in the molecular layer were not Purkinje cells (compare Fig. 2b with Fig. 2a). It was found that Purkinje cells present in the host's molecular layer were largely confined to regions closest to the body of the graft, indicating they had migrated only a short distance into the host's molecular layer (Fig. 2b). Thus, it appears that neuronal migration into the host's molecular layer is not limited to Purkinje cells, a finding that strongly argues against cell-type specific tropism.

The finding of Thy-1 positive neurons, other than Purkinje cells, migrating into the host's molecular layer suggested that the Purkinje cell organization observed in all 11 grafts may result from interactions with donor neurons rather than with host neurons. This was confirmed by the observation that whereas Leu-4 positive Purkinje cells in the vicinity of the host's granule cells remained disorganized (Fig. 2e), unaligned and did not associate with the host granular layer, those juxta-apposed to clusters of donor neurons, resembling granule cells, were closely aligned (Fig. 2f). As shown in Table I, the percentage of Purkinje cells in each graft aligned with donor granule cells was far greater (mean = 18.4%) than with host granule cells (mean = 0.45%). Organization of grafted cells into tissue of 'normal' cerebellar cyto-architecture also occurred in ectopic grafts (Fig. 2d,f), including cerebellar grafts placed in the frontal cortex (data not shown). This indicates that the environment of the mutant host had little influence on the morphogenesis of the grafted cerebellar cells.

These findings strongly suggest that the mutant host's milieu does not influence the organization of grafted embryonic Purkinje cells, but rather it is the interaction between donor neural cells that leads to their ordered differentiation. This concept of autonomous development is supported by the observation that neural precursor cells continue to differentiate in vitro away from their environment [1, 15], and also by recent findings showing that early neural development can be regulated by the endogenous production of growth factors [4, 7]. This latter finding suggests that the growth and differentiation of embryonic grafts may be dependent on autocrine factors produced within the graft, and not by putative factors derived from the host tissue.

In light of these findings, there appears to be a need to re-examine, using markers to positively distinguish host and donor neurons, whether synaptic connections occur between donor Purkinje cells and the host's stellate or basket cells as previously reported [20, 21]. Nevertheless,

TABLE I

ASSOCIATION OF PURKINJE CELLS WITH HOST OR DONOR GRANULE CELLS

Successive 8-μm cryostat sections through the graft, 64 μm apart, were stained with anti-Leu-4 antibody as described in Fig. 1, counterstained and counted. Granule cells in the sections were categorized as host or donor by staining adjacent sections with either Thy-1.1 or Thy-1.2. Purkinje cells were categorized as being aligned if they were within 2 μm of granule cells.

Number of Purkinje cells per graft	Percentage of Purkinje cells aligned with donor granule cells	Percentage of Purkinje cells aligned with host granule cells
596	8.1	0
1764	27.8	0.3
1497	20.4	1.0
120	0	0
2350	34.2	0.3
5104	21.1	2.3
1397	21.3	0
2147	13.5	0
1000	0	0.5
3636	28.2	0
894	27.7	0.5

electrophysiological studies [8, 9] indicate that synaptic connections do occur between grafted Purkinje cells and the host's climbing fibers. However, as connectivity has been shown also to occur between Purkinje cells residing in solid cerebellar grafts and the host [2], this may reflect the ability of host axons to penetrate the graft rather than the donor cells integrating into the hosts neuropil. Host innervation of graft has led to functional repair in the retinal [13] and striatal [11] systems, and, given our findings, it may be the predominant, if not exclusive, mechanism by which neuronal repair can be achieved.

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