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ORIGINAL PAPER

Embryonic Cerebellar Graft Morphology Differs in Two Mouse Models of Cerebellar Degeneration

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Abstract

Cerebellar diseases causing substantial cell loss often lead to severe functional deficits and restoration of cerebellar function is difficult. Neurotransplantation therapy could become a hopeful method, but there are still many limitations and unknown aspects. Studies in a variety of cerebellar mutant mice reflecting heterogeneity of human cerebellar degenerations show promising results as well as new problems and questions to be answered. The aim of this work was to compare the development of embryonic cerebellar grafts in adult B6CBA Lurcher and B6.BR pcd mutant mice and strain-matched healthy wild type mice. Performance in the rotarod test, graft survival, structure, and volume was examined 2 months after the transplantation or sham-operation. The grafts survived in most of the mice of all types. In both B6CBA and B6.BR wild type mice and in pcd mice, colonization of the host's cerebellum was a common finding, while in Lurcher mice, the grafts showed a low tendency to infiltrate the host's cerebellar tissue. There were no significant differences in graft volume between mutant and wild type mice. Nevertheless, B6CBA mice had smaller grafts than their B6.BR counterparts. The transplantation did not improve the performance in the rotarod test. The study showed marked differences in graft integration into the host's cerebellum in two types of cerebellar mutants, suggesting disease-specific factors influencing graft fate.

Keywords Ataxia · Cerebellar degeneration · Lurcher mouse · Neurotransplantation · Pcd mouse

Introduction

Hereditary cerebellar degenerations represent a wide group of diseases [1] that may have a detrimental impact on patients' movements, cognitive functions, and affectivity (for review see [2–7]). The effectiveness of therapy for cerebellar ataxias, particularly degenerative ones, is mostly insufficient. Currently, routine treatment that would significantly restore cerebellar functions does not exist [1, 8]. The variability of human hereditary cerebellar ataxias having different pathogenesis complicates the development of effective therapy to target the pathogenic process. One of the hopeful therapies for cerebellar degenerations could be neurotransplantation. However, this approach still needs in-depth investigation before safe and reasonable clinical use [9, 10].

Variability of human cerebellar degenerations is also reflected in a wide spectrum of animal models [11]. Comparison of graft development and therapeutic benefits in various mouse models of cerebellar degenerations could help to notify potential specifics of human diseases that would require specific therapeutic approaches or represent limitations of the therapy. Lurcher and Purkinje cell degeneration (pcd) mutant mice belong among the most frequently used animal models of cerebellar degeneration. Despite different pathogenesis, both mutations result in similar neuropathological changes in the cerebellum, with almost complete, early onset, and rapid loss of Purkinje cells.

Lurcher mice [12] are heterozygous for the semi-dominant gain-of-function *Grid2*^{Lc} mutation in the δ2

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glutamate receptor (GluRδ2) gene [13] that is expressed predominantly by Purkinje cells [14]. The mutation changes the receptor into a leaky membrane channel, which chronically depolarizes the cell membrane [13]. Therefore, cell-autonomous cell death of Purkinje cells is a primary effect of the mutation [15, 16]. It has been alternatively described as apoptotic [13, 17], necrotic [18], autophagic [19, 20], or even mediated via multiple cell death pathways [21]. Purkinje cell reduction can be detected by postnatal days 8–10; about 95% of Purkinje cells die between postnatal day 8 and 25, and at the age of 60 days, only 1% of these cells survive [22]. Degeneration of Purkinje cells is followed by a fast and massive reduction of cerebellar cortex interneurons and inferior olive neurons, due to secondary target-related cell death [15, 16, 22, 23]. On the other hand, degeneration of the deep cerebellar nuclei is relatively mild in Lurcher mice [24]. Recently, analogous point gain-of-function mutation of the *Grid2* has been found in humans [25].

Pcd mice are homozygous for the *Agtpbp1*$^{pcd/J}$ mutations in the gene encoding cytosolic ATP/GTP binding protein 1 (synonyms: cytosolic carboxypeptidase-like protein, CCP1, Nna1) that is intensively expressed in cerebellar Purkinje cells, olfactory bulb mitral cells, and retinal photoreceptors [26]. Rapid and almost complete degeneration of Purkinje cells is of apoptotic [27] or autophagic [28] mechanisms. Purkinje cell loss starts at postnatal day 20 [29]. By postnatal day 28, it is nearly complete in most parts of the cerebellum and, at the age of 7 weeks, only a few Purkinje cells remain in the nodulus [30]. The degeneration of cerebellar granule cells is secondary to the loss of Purkinje neurons (as in Lurcher mice) and is exponentially progressive [31, 32]. Cerebellar nuclei are only slightly reduced in size [33]. Inferior olivary neurons start to disappear between postnatal day 17 and 23 and, by postnatal day 300, the reduction has reached 49% [31]. Pcd mice also suffer from slow, progressive degeneration of the retina and olfactory bulb mitral cells [30, 34–36], and degenerative changes in the thalamus [37].

In both Lurcher and pcd mice, transplantation of embryonic cerebellar tissue has been intensively investigated [38–43]. While in pcd mice, motor function improvement after intracerebellar transplantation of cerebellar cell suspension has been reported by Triarhou et al. [44, 45], in Lurchers, only mild improvement of gait parameters was seen and graft morphology did not promise any strong specific functional effect [46]. However, direct comparison of graft development and volume in Lurcher and pcd mice, using identical experimental procedures has not yet been done.

The aim of this study was to compare survival and morphology of embryonic cerebellar grafts in adult Lurcher and pcd mutant mice and wild type control mice. The effect of transplantation on motor performance was also assessed.

Materials and Methods

Animals and Design of the Experiment

Lurcher mutant and wild type mice of the B6CBA strain and pcd mutant and wild type mice of the B6.BR strain (aged 90–120 days at the time of surgery, both males and females in approximately a 1:1 ratio) were used. B6CBA wild type mice served as controls for Lurcher mutants; B6.BR wild type mice were used as controls for pcd mutants. The mice were kept in standard conditions with a 12:12-h light/dark cycle (light period from 6 a.m. to 6 p.m.), at a temperature of 22–24 °C, with commercial pellet diet and water available ad libitum. Donor embryos were obtained by cross-breeding of enhanced green fluorescent protein (EGFP)-positive C57BL/6-Tg (ACTB-EGFP)1Osb/J mice with host strain (B6CBA or B6.BR) wild type mice.

The mice were treated with transplantation of EGFP-positive embryonic cerebellar cell suspension. To investigate the effect of the transplantation on motor abilities, a group of age-matched B6CBA Lurcher mice and B6.BR pcd mice was subjected to a sham-operation, and also analogous groups of mice were left intact without any surgery. For *n* see Table 1. After an 8-week survival period, the mice were examined for motor abilities and euthanized for histological examination of the grafts.

All experimental procedures were performed in compliance with EU guidelines for scientific experimentation on animals and with the permission of the Ethical Commission of the Faculty of Medicine in Pilsen. All efforts were made to minimize the number of animals used and their suffering.

Transplantation

Transplantation procedure was analogous to that described earlier [47]. Donor females with conception-timed pregnancies were euthanized by overdose of thiopental on the 12th day of gestation, and the embryos were removed. The cerebella of EGFP-positive embryos were dissected in the form of two pieces of tissue that were then pooled in a cold aqueous solution of 0.9% sodium chloride and 0.6% glucose, which also served as a vehicle for graft administration. The embryonic cerebella were incubated with trypsin at 37 °C for 10 min, washed with the vehicle, and mechanically dissociated with pipet trituration to prepare cell suspension. Cell concentration was adjusted to 50,000 cells/μl.

The host mice were anesthetized with an intraperitoneal administration of ketamine (100 mg/kg body weight) and xylazine (16 mg/kg body weight). The cell suspension was injected bilaterally (3 μl per site) into the host cerebellum (injection coordinates were 6.3 mm posterior to the bregma, 1.6 mm lateral, 3.1 mm below the bregma), using a glass microcapillary. The injection speed was 0.5 μl/min.

Table 1 Number of intact mice and sham-operated mice used as controls for motor function evaluation and number of graft-treated mice. Number of mice in which the graft volume was estimated and mean coefficient of graft volume error (coefficient of error, CE) in individual experimental groups of mice

	No. of intact mice	No. of sham-operated mice	No. of graft-treated mice	No. of mice examined for graft volume	CE
B6CBA Lurcher	20	17	17	15	0.145
B6CBA wild type	22	–	18	15	0.106
B6.BR pcd	21	17	20	12	0.072
B6.BR wild type	19	–	20	12	0.075

Thereafter, the capillary was left in situ for 5 min and then slowly extracted. Sham-operated mice were treated with the vehicle without the cells injected, using the same procedure.

Motor Function Examination

Motor skills were examined on the rotarod (RotaRod Advanced, TSE Systems GmbH, Germany). For the test, the cylinder diameter was 3.5 cm, rod width 8 cm. Rotation speed accelerated from 0 to 60 RPM within 6 min. Thereafter, the trial was stopped. Fall latencies were measured. Four trials per day-session were performed with 16 min intertrial intervals and the values were averaged. The test was repeated for five consecutive day-sessions (D1–D5). For groups tested on the rotarod, see Table 1. Among graft-treated mice, only those with bilaterally surviving grafts were involved in the analysis of rotarod test results (for *n* see Table 2; note that one pcd and two B6.BR wild type mice were excluded from this analysis for technical reasons). We compared intact mutants with their intact strain-matched wild type controls to see the basal impairment of motor skills caused by the cerebellar degeneration. Then we compared graft-treated mutants with their strain-matched mutant sham and intact controls, as well as sham-operated mutants with intact mutants. Finally, we compared graft-treated wild type mice with their strain-matched wild type intact controls, to see potential negative effects of the transplantation.

Table 2 Graft survival. Number and percentage of mice with surviving grafts inside the host's cerebellum or in contact with the host cerebellum (both, bilateral, and unilateral graft presence is considered a surviving graft) and number and percentage of mice with bilateral graft survival. In one B6.BR pcd mouse, the graft was localized on the brainstem having no contact with the host's cerebellum

	No. of mice examined	No. of mice with surviving grafts	% of graft survival	No. of mice with bilaterally surviving graft	% of bilateral graft survival
B6CBA Lurcher	17	17	100	14	82.4
B6CBA wild type	18	18	100	15	83.3
B6.BR pcd	20	19	95	12	60
B6.BR wild type	20	19	95	19	95

Histological Examination

After completing the rotarod test (eight weeks after transplantation), the mice were sacrificed by overdosing with thiopental and transcardially perfused with Ringer's solution and 4% phosphate-buffered paraformaldehyde (pH 7.4). The brains were stored for 2 h in 4% phosphate-buffered paraformaldehyde for post-fixation and then incubated in sucrose for cryoprotection. Frontal 40 μm frozen sections were prepared. Graft identification and assessment of graft localization and presence of interactions between graft and host tissue (graft sprouting) were done in native specimens, based on the natural green fluorescence of EGFP.

Immunohistochemistry was used to identify Purkinje cells with anti-calbindin staining and to identify astrocytes with anti-glial fibrillary acidic protein (GFAP) staining in selected free-floating sections. For anti-calbindin staining, the sections were incubated with anti-calbindin primary antibody (ab 11426, Abcam, Cambridge, UK; dilution 1:1000) overnight at room temperature and with AlexaFluor 594 secondary antibody (ab 150076, Abcam, Cambridge, UK; dilution 1:400) for 2 h at room temperature. For anti-GFAP staining, sections were incubated with anti-GFAP primary antibody (clone G-A-5 Cy3 conjugate, Sigma-Aldrich, Saint Louis, USA, dilution 1:800) overnight at 4 °C.

The slices were visualized using an Olympus BX41 fluorescent microscope (Olympus Corporation, Japan) and an Olympus Fluoview FV 10i confocal laser scanning microscope (Olympus Corporation, Japan). Photographs for volume estimation were acquired using an Olympus BX41 fluorescent microscope with an UPlanFl 4×/0.13 objective and an Olympus DP70 digital camera (Olympus Corporation, Japan).

Graft Volume Estimation

The volume of the graft was estimated in mice in which all slices containing the graft were available in adequate quality for EGFP-area estimation or in which only single slices were missing (for *n* see Table 1). Values for the missing samples

were estimated using previous slices. The slices containing the EGFP fluorescent tissue were recorded using an Olympus BX41 fluorescent microscope with an UPlanFl 4×/0.13 objective and an Olympus DP70 digital camera (Olympus Corporation, Japan).

Graft volumes were estimated with ImageJ software with the same procedure that we used previously [47], based on the point grid method and Cavalieri principle. In the slices, the graft was considered the EGFP fluorescing area. The coefficient of volume error (coefficient of error, CE) was estimated using the procedure proposed by Gundersen and Jensen [48, 49] to inform about variability of estimated volume values and accuracy of the stereological estimation (relative error of the estimation, for details see [48, 49]).

Statistics

Statistical analyses were performed using R software [50]. All statistical analyses were extended with permutation approaches (10,000 Monte-Carlo permutations) to avoid dependency on assumption of parametric methods.

Data with binomial response variable (qualitative parameters characterizing graft survival and morphology) were analyzed by permutation test of generalized linear models (GLMs) with quasi-binomial distribution and logit-link function. Post hoc comparisons were evaluated by permutation test of differences at groups′ coefficients derived from the GLMs.

Effects of cerebellar degeneration (wild type or cerebellar mutant—any of pcd and Lurcher), strain (B6.BR or B6CBA), and their interaction (cerebellar degeneration: strain) on graft volume were analyzed by permutation two-way ANOVA. Post hoc comparisons were done using the permutation t test.

Repeated measurements from the rotarod test were analyzed by permutation test of linear mixed-effects model (LME) with AR1 autocorrelation structure modeling serial within-subjects autocorrelations [51]. The LME was performed using the nlme package in R [52]. As we detected substantial heteroscedasticity in model residuals, a square root transformation of the response variable was applied and the model was refitted. Post hoc comparisons were performed using the permutation t test.

In all cases, post hoc tests were followed by false discovery rate correction for multiple comparisons [53]. The results are presented as number of mice and percentage for qualitative data (graft survival, presence of graft features) and as mean ± standard error of the mean (S.E.M) for quantitative data (graft volume, rotarod). In all cases, $P < 0.05$ was considered statistically significant.

Results

Graft Survival and Structure

The grafts survived in most of the mice and, in most of them, the grafts were found bilaterally (Table 2, Fig. 1a). There was only one B6.BR wild type mouse in which we did not find any EGFP-positive tissue. In one pcd mouse, the graft was localized on the brainstem and did not have any contact with the host's cerebellum. Thus, there were no significant differences in general graft survival frequency. However, for bilateral graft survival, a significant effect of degeneration factor was found ($F_{(1,\ 71)} = 4.216$, $P < 0.04$) and it was seen in a lower percentage of B6.BR pcd mice than in B6.BR wild type mice ($P = 0.037$).

The grafts contained cells having the shape and size characteristic of Purkinje cells in the vast majority of the mice (Table 3). In selected specimens, calbindin positivity of such cells was verified immunohistochemically (Fig. 1b, c). There were no significant differences in EGFP-positive Purkinje cell presence between individual groups of mice.

Both B6CBA and B6.BR wild type mice and pcd mutant mice showed EGFP-positive fiber sprouting from the graft into the host's cerebellum (Fig. 2a) and/or colonization of the host's cerebellar cortex by EGFP-positive cells, having the size and shape typical of Purkinje cells (Fig. 2b) in most cases. On the other hand, most of the Lurcher mice showed strictly delimited grafts (Fig. 2c). For detailed data, see Table 4.

Fiber sprouting from the graft into the host's cerebellum (Fig. 2a) was not so frequent in B6CBA wild type and Lurcher mice and B6.BR wild type mice, but was found in all B6.BR pcd mice in which the graft was in contact with the cerebellum (Table 4). Thus, significant effects of degeneration ($F_{(1,\ 69)} = 9.523$, $P = 0.002$) and strain ($F_{(1,\ 69)} = 20.535$, $P < 0.001$) factors, as well as of their interaction ($F_{(1,\ 69)} = 26.223$, $P < 0.001$), were found. The frequency of fiber sprouting presence was significantly higher in pcd B6.BR mice than in B6.BR wild type mice ($P < 0.001$) and than in B6CBA Lurcher mice ($P < 0.001$). The difference in fiber sprouting frequency between Lurcher and wild type B6CBA mice was not statistically significant. In pcd mutant mice, the fibers grew from the graft mass into the white matter sometimes up to a distance of 0.5 mm, or were directed towards the cerebellar nuclei (Fig. 2a).

Colonization of the host cerebellum by graft-derived Purkinje cells (Fig. 2b) was significantly dependent on degeneration ($F_{(1,\ 69)} = 11.039$, $P = 0.002$) and strain ($F_{(1,\ 69)} = 62.121$, $P < 0.001$) factors, but not on their interaction. This phenomenon (Table 4) was much less frequent in B6CBA Lurcher mice, compared with their wild type B6CBA littermates ($P = 0.024$), as well as with B6.BR pcd mutants ($P < 0.001$). There was no difference in graft

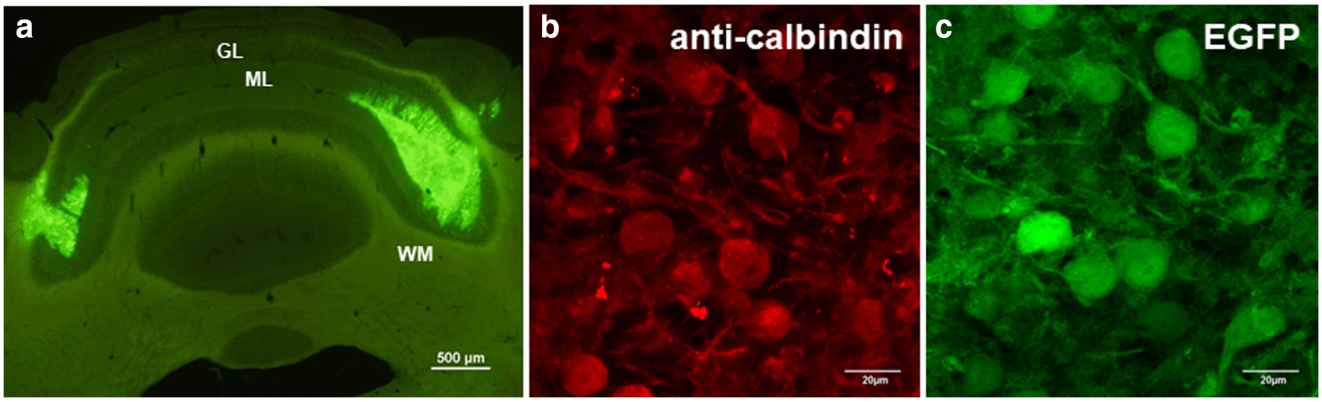

Fig. 1 Bilateral EGFP fluorescent graft in the cerebellum of a B6CBA wild type mouse (**a**), anti-calbindin immunostaining (**b**), and EGFP fluorescence of the graft in the same field (**c**) in a B6.BR pcd mouse. WM cerebellar white matter, ML molecular layer of the cerebellar cortex, GL granular layer of the cerebellar cortex

colonization between B6.BR pcd mice and B6.BR wild type mice. The difference between B6CBA wild type mice and B6.BR wild type mice was not statistically significant. Graft-derived Purkinje cells mostly colonized the host's cerebellar cortex, but not the white matter (Fig. 1a, Fig. 2B).

Many astrocytes (GFAP-positive cells) grew through the grafts in all mice. Most of these astrocytes came from the host tissue, because they were not EGFP-positive (Fig. 3).

Graft Volume

Mouse strain factor ($F_{(1,\ 50)} = 18.279$, $P = 0.001$) but not degeneration factor had a significant effect on the graft volume. In B6CBA mice, the graft volume was always smaller than in B6.BR mice, regardless of whether these were wild type or carried the mutation causing Purkinje cell demise (Fig. 4). On the other hand, no significant differences between cerebellar mutants and their wild type littermates were seen (Fig. 4). For CE see Table 1.

Rotarod Test

Fall latencies in the rotarod test were significantly dependent on the experimental group factor ($F_{(9,\ 163)} = 244.771$, $P < 0.001$), day-session ($F_{(4,\ 652)} = 79.522$, $P < 0.001$), and their interaction ($F_{(36,\ 652)} = 2.283$, $P < 0.001$). The rotarod test confirmed marked motor disability in both Lurcher and pcd mutants, compared with their wild type controls (for untreated, intact mice: B6CBA strain, for all day-sessions $P < 0.001$; B6.BR strain, for all day-sessions $P < 0.001$; compare Fig. 5a with Fig. 5b and Fig. 5c with Fig. 5d). No effect of the transplantation was observed (Fig. 5). Only sham-operated pcd mice achieved slightly longer latencies than the intact controls (Fig. 5c).

Discussion

In this work, we compared the development of embryonic cerebellar cell suspension grafts in Lurcher and pcd mice, two models of hereditary cerebellar degeneration with similar morphology but different pathogenesis. The grafts survived well in the wild type, Lurcher mutant as well as in pcd mice for 2 months. This complies with our previous findings that in Lurcher mice, as in wild type animals, both solid and cell suspension embryonic cerebellar grafts can survive for 6 months [46, 47, 54]. This suggests that the long-term survival of cerebellar grafts is not a problem, even in cerebellar mutants, and that cerebellar degeneration does not significantly influence overall graft survival rate. Thus, there should be sufficient time for graft development, for manifestation of the host tissue niche impact, and for potential graft integration.

In our previous studies, we have described differences in graft morphology between Lurcher mutant and wild type mice [46, 47]. The present experiment confirmed that, unlike wild type mice, Lurcher mutants mostly have strictly delimited grafts, lacking a tendency to invade the host's cerebellum. Nevertheless, in this study, pcd mice had an even higher frequency of fiber sprouting from the graft than wild type mice,

Table 3 Presence of EGFP-positive (graft-derived) Purkinje cells (PC) in the grafts

	No. of mice examined	Presence graft-derived PC	% of graft-derived PC presence
B6CBA Lurcher	17	16	94.1
B6CBA wild type	18	15	83.3
B6.BR pcd	20	20	100
B6.BR wild type	19	19	100

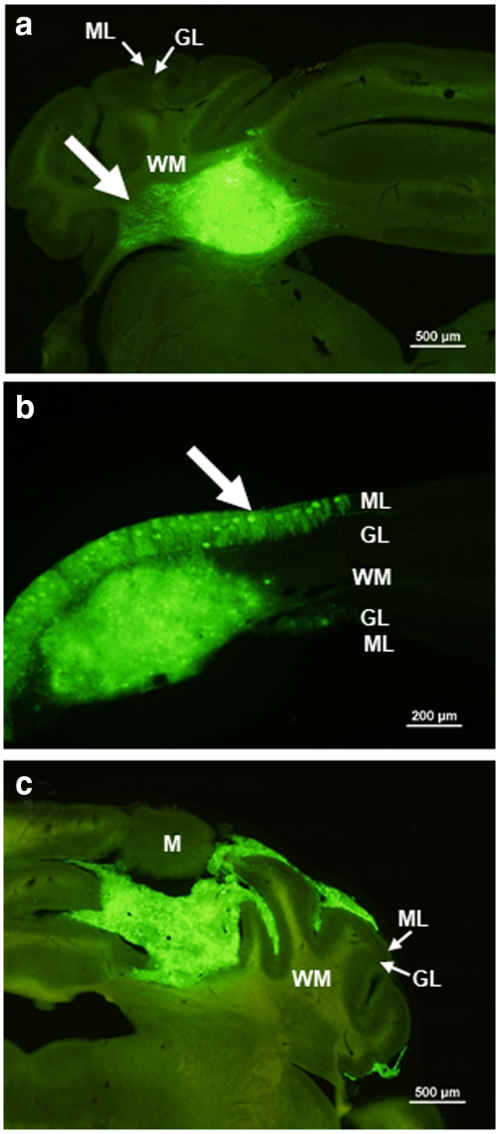

Fig. 2 A graft with EGFP-positive fibers (indicated by the large arrow) growing into the host's cerebellar tissue in a pcd mouse (**a**), colonization of the cerebellar cortex adjacent to the graft by EGFP-positive cells having features of Purkinje cells (indicated by the large arrow) in a pcd mouse (**b**), and strictly restricted graft, showing no infiltration of the host's cerebellar tissue in a Lurcher mouse (**c**). Native EGFP fluorescence. M mesencephalon, WM cerebellar white matter, ML molecular layer of the cerebellar cortex, GL granular layer of the cerebellar cortex

and Purkinje cells migrated from the graft and colonized the host's cerebellum in all of them. Thus, strict delimitation of the graft is not a problem in pcd mutants. In pcd mice, integration of the graft and motor performance improvement has also been described by Triarhou et al. [44, 45]. Tomey and Heckroth [41] pointed out differences of graft survival rate between Lurcher and pcd mice, deduced from a comparison of their results in Lurcher mice [41] and graft survival in pcd mice reported earlier by Sotelo and Alvarado-Mallart [55, 56]. On the contrary, in our recent study, we achieved graft survival of a high percentage in both types of mutants under identical experimental conditions.

Furthermore, we have shown here that cerebellar degeneration does not have any impact on graft size, either in B6CBA Lurcher or in B6.BR pcd mice. Nevertheless, both B6CBA mutant and wild type mice had smaller grafts than their B6.BR counterparts. This means that there were strain differences, rather than the effect of cerebellar degeneration on the graft size. We previously found strain differences in graft size also between B6CBA and C3H wild type mice [47]. On the other hand, C3H Lurcher mutants had smaller grafts than C3H wild type controls in that study [47]. It suggests that the effect of cerebellar degeneration could be manifested only in combination with other factors, such as some strain-specific traits.

Our finding that Lurcher mice have a low rate of graft integration, while graft survival is the same as in pcd and healthy mice, suggests that the local tissue niche has a different impact on the cells inside the graft mass and on the single cells and fibers dispersed in the host's tissue. We can hypothesize that the graft develops its own environment. Elements leaving it are in direct contact with the tissue changed by the neurodegenerative process that might decrease its neurogenicity and reduce the level of signals attracting immature cell migration and fiber sprouting (for review see [57]). Migration of graft-derived Purkinje cells selectively into the host's cerebellar cortex and fiber sprouting towards the cerebellar nuclei which we have observed in pcd and wild type mice underlines the importance of specific host's tissue signals, as pointed out by Sotelo and Alwarado-Mallart [58]. Modulation of graft development by local tissue-specific signals and by pathological changes in the host tissue has been described in several cerebellar mouse mutants [38, 59–61]. Our recent comparison of Lurcher and pcd mice showed that relatively similar cerebellar degenerations could have substantially different impacts on graft development, and thereby confirmed the crucial importance of the local niche of the diseased tissue.

The question is, in which aspects does the adult Lurcher mutant cerebellum niche differ from the niche of the pcd mutant cerebellum and healthy cerebellum. The adult Lurcher mouse cerebellum contains a higher level of brain-derived neurotrophic factor [46] and a higher density of capillaries [62] than the healthy cerebellum. Nevertheless, it is not known what the role of these changes in the degenerative process is, or whether they are just secondary consequences [63]. Thus, their impact on the graft might be ambiguous [60]. For pcd mice, analogous data are not available. Inflammation and oxidative stress accompanying the degenerative process may also play a role [64, 65]. Finally, as reduction of the cerebellum is distinctly more profound in Lurcher than in pcd mice [66], the size of the degenerated cerebellum could also contribute to the differences in development of intracerebellar grafts.

Table 4 Presence of graft-host interactions. Number and percentage of mice in which EGFP-positive fiber sprouting from the graft and/or host's cerebellum colonization by EGFP-positive Purkinje cells (PC) were found and number and percentage of mice in which both these phenomena were detected. For these parameters, mice with surviving grafts being in contact with the host cerebellum were examined

	No. of mice examined	Fiber sprouting	Colonization by PC	Fiber sprouting and colonization by PC
B6CBA Lurcher	17	2 (11.8%)	5 (29.4%)	2 (11.8%)
B6CBA wild type	18	5 (27.8%)	14 (77.8%)	5 (27.8%)
B6.BR pcd	19	19 (100%)	19 (100%)	19 (100%)
B6.BR wild type	19	5 (26.3%)	19 (100%)	5 (26.3%)

As expected, the recent study confirmed severe motor deficits in both Lurcher and pcd mutant mice using the rotarod test. However, neurotransplantation therapy did not influence the performance of cerebellar mutant mice in this test. This is in agreement with our previous studies, in which only mild amelioration of some gait parameters, but no improvement in the rotarod test was found in Lurcher mice [46, 47]. Thus, we failed to prove a specific functional effect of the graft in our experiments. However, recovery of motor performance has been described by Triarhou et al. [44, 45] in pcd mice, after injection of wild type cerebellar cell suspension into the cerebellar nuclei, suggesting that pcd mutant mice have the capability of improving their motor functions with either specific or non-specific mechanisms. Lurcher mice, on the other hand, have difficulties in reducing their motor deficits after they lose the Purkinje cells. The only marked therapeutic effect of neurotransplantation therapy in Lurcher mice was mediated by rescuing their intrinsic Purkinje cells, by means of grafting mesenchymal stem cells in the neonatal period, as reported by Jones et al. [67].

Besides pcd mice [44, 45], cerebellar grafts alleviated motor deficit in the spinocerebellar ataxia type 1 mouse model [68]. On the other hand, substitution of Purkinje cells by grafting of their progenitors has been shown to

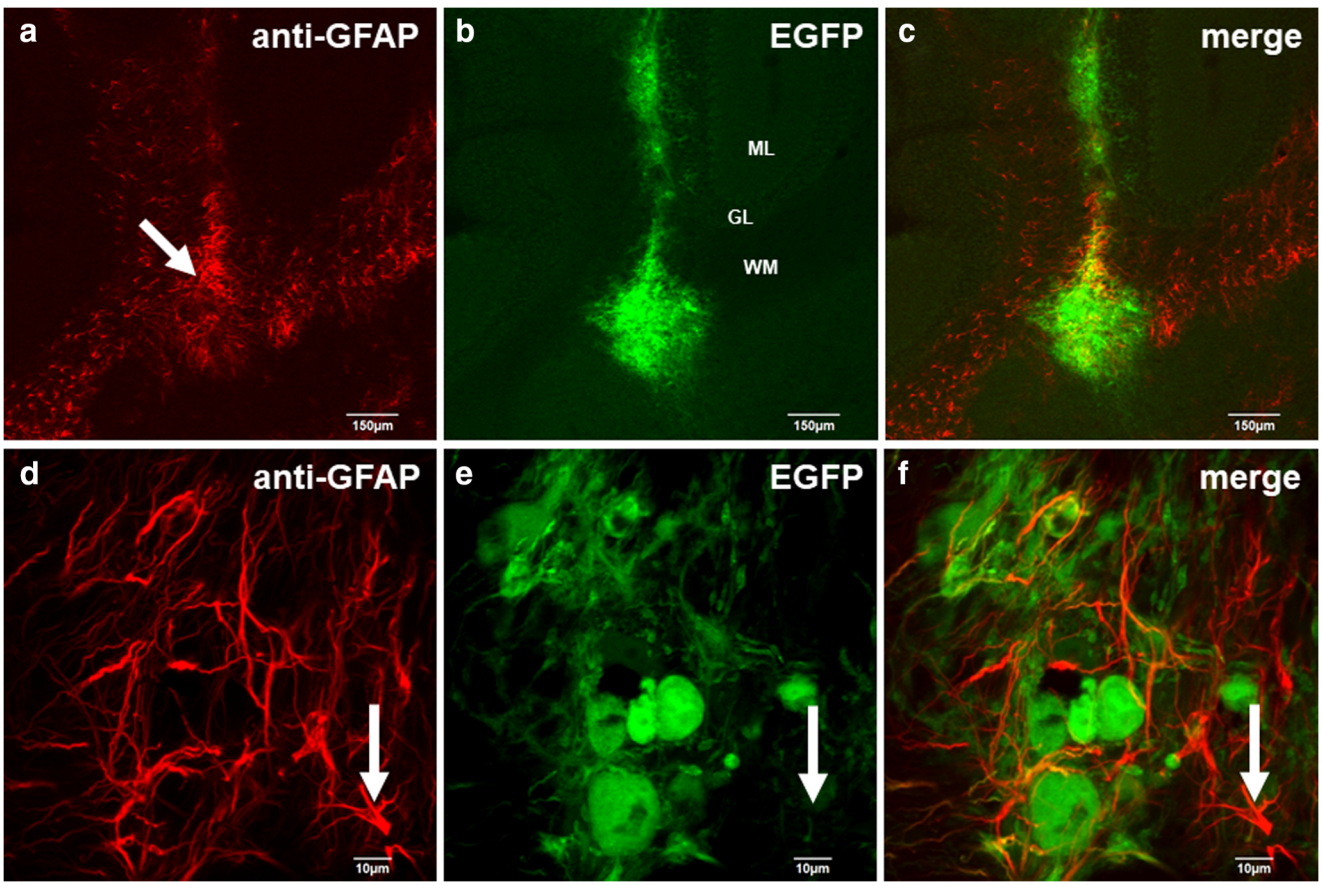

Fig. 3 Anti-GFAP immunofluorescence (**a**, **d**), EGFP fluorescence of the graft in the same field (**b**, **e**), and merged images (**c**, **f**) in wild type mice. The arrows indicate accumulation of astrocytes in the graft area (**a**) and an example of an EGFP-negative astrocyte (**d**, **e**, **f**). WM cerebellar white matter, ML molecular layer of the cerebellar cortex, GL granular layer of the cerebellar cortex

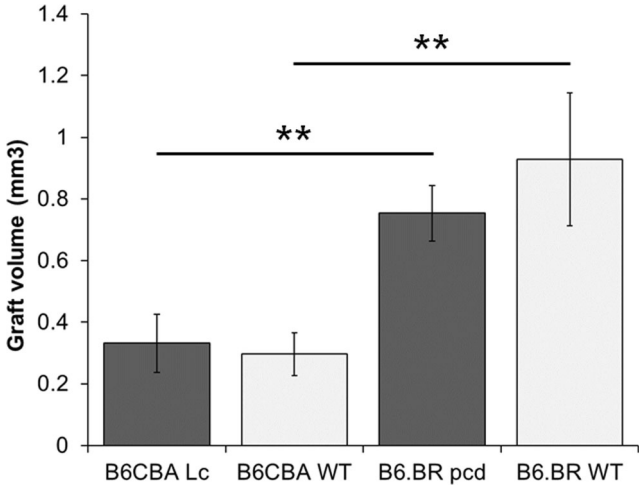

Fig. 4 Mean graft volume in B6CBA Lurcher (Lc) mice, B6CBA wild type (WT) mice, B6.BR pcd mice, and B6.BR wild type (WT) mice. Error bars represent SEM. $^{**}P < 0.01$

fail to ameliorate cerebellar ataxia in tambaleante mice [69]. Tambaleante mice, however, showed improvement capacity since preventive motor training increased Purkinje cell survival and had a moderately positive effect on motor performance [69]. Thus, comparing the results of Purkinje cell substitution in several cerebellar mutant mice suggests that there might be substantial differences in graft integration and functional impact, dependent on some degeneration-specific factors.

The limiting factor for functional recovery after intracerebellar graft injection could also be the presence of extracerebellar damage, represented by secondary inferior olive degeneration in adult Lurchers [22], and by degeneration of the inferior olive, retina, olfactory bulb mitral cells, and thalamus in pcd mice [30, 35–37]. Pcd mice, indeed, suffer from degenerative changes in more regions than Lurcher mice, but degeneration of the inferior olive, the source of climbing fibers' reduction, is milder and slower than in Lurchers [22, 31]. Extracerebellar damage and disease manifestations also accompany some human cerebellar diseases [70, 71]. Despite similar morphology of the cerebellar degeneration, Lurcher and pcd mutants differ substantially in the extent and character of their functional impairments, such as deficits in execution or acquisition of conditioned eyelid response, respectively [72, 73], or in their performance in the spatial navigation tasks [74–76]. This suggests that there can be differences in character of cerebellar circuit disorder between these two mutants having an impact on functional integration of the grafts. Furthermore, the cerebellum has its functional topography, and the degeneration of Purkinje cells is a diffuse process in both mutants. Thus, the question is whether potential synaptic integration of grafted cells into the circuits could reflect such topography and whether the graft could restore or support the function of all parts of the mutant cerebellum or all the complex cerebellar functions.

Functional effects of neurotransplantation therapy for cerebellar pathologies might involve various mechanisms, including specific cell substitution, support of cerebellar reserve, and maintenance of the restorable state, as proposed by

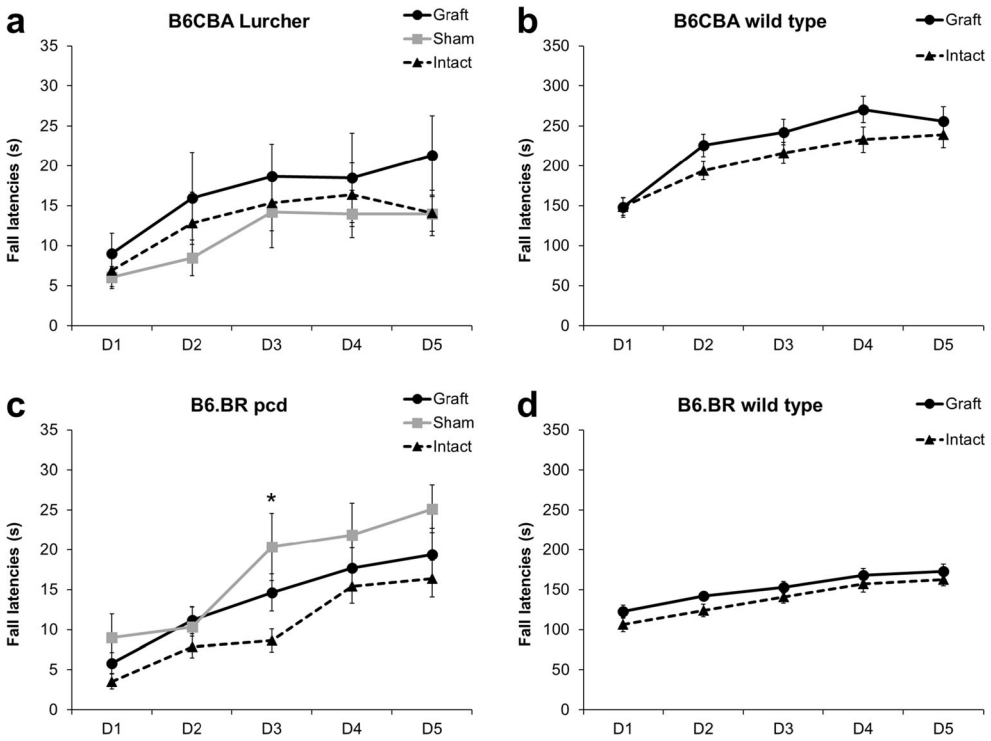

Fig. 5 Mean fall latencies in the rotarod test (day-sessions D1–D5) in **a** B6CBA Lurcher (Lc) mice, **b** B6CBA wild type (WT) mice, **c** B6.BR pcd mice, and **d** B6.BR wild type (WT) mice. Error bars represent SEM. $^{*}P < 0.05$ for sham-operated vs. intact mice

Mitoma and Manto [8]. Nevertheless, it seems that functional recovery might be more difficult to achieve by neurotransplantation in some diseases than in others, because of different pathological conditions (for review see [9, 10, 77]).

Conclusion

This study was the first direct comparison of graft survival, volume, and morphology in Lurcher and pcd mice. We can conclude that (1) none of the cerebellar degenerations had a strong effect on survival of the embryonic cerebellar graft; and (2) the adult Lurcher mutant cerebellum, but not the cerebellum of pcd mice, had a negative impact on graft integration. This finding suggests that there might be disease-specific factors influencing the development and integration of cerebellar grafts. These factors might determine the success or failure of neurotransplantation therapy in particular types of cerebellar degeneration. Identification of such factors would help to determine diseases treatable with neurotransplantation, or to develop approaches modulating these factors in the patient's cerebellum prior to grafting.

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Conflict of Interest The authors declare that they have no conflict of interest.

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