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Intracerebellar application of P19-derived neuroprogenitor and naive stem cells to Lurcher mutant and wild type B6CBA mice

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Neurotransplantation has great potential for future treatments of various neurodegenerative disorders. Preclinically, the Lurcher mutant mouse represents an appropriate model of genetically-determined olivocerebellar degeneration. The aim of the present study was to assess survival of naïve and neurally differentiated P19 carcinoma stem cells following transplantation into the cerebellum of Lurcher mice and wild type littermates.

Material/Methods: Adult normal wild type (n=51) and Lurcher mutant mice (n=87) of the B6CBA strain were used. The mean age of the animals at the time of transplantation was 261.5 days. Suspension of naive and neurally differentiated P19 carcinoma stem cells was injected into the cerebellum of the mice. In the Lurcher mutants, 2 depths of graft injection were used. Three weeks after implantation the brains of experimental animals were examined histologically.

Results: Survival of neuroprogenitor grafts at a depth of 1.6 mm was significantly higher in wild type *vs.* Lurcher mutant mice. In wild type mice, the typical graft localization was in the middle of the cerebellum, whereas in Lurcher mice the graft was never found inside the degenerated cerebellum and was primarily localized in the mesencephalon.

Conclusions: We conclude that the appearance and low survival rate of cerebellar P19 carcinoma stem cell grafts in the Lurcher mutant mice weigh against the therapeutic value of this cell line in preclinical studies of neurodegeneration.

key words: **carcinoma stem cells • cerebellum • Lurcher • neuroprogenitor cells • neurotransplantation**

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Background

Neurotransplantation has great potential for the future treatment of various neurological/neurodegenerative diseases. Preclinical studies have utilized several approaches and different stem cell lines. Nevertheless, determination of appropriate stem cell lines as the ideal source for brain-specific restorative neural grafts remains an unsolved problem. Second, it is necessary to elucidate cellular mechanisms by which stem cell transplantation leads to functional recovery and structural reorganization.

The biological risks of carcinoma stem cells are obvious due to their potential to form donor-derived malignant tumors in the host. On the other hand, it has been previously shown that, when differentiated, carcinoma-derived stem cells lose tumorigenic capacity [1]. Furthermore, it has been previously demonstrated that transplantation of neurons derived from a human teratocarcinoma-derived cell line are potentially beneficial in several animal models of neurological disorders [1], including ischemia-induced injury [2], spinal cord injury [3], stroke [4–6], Parkinson's disease [7], Huntington's disease [8] and amyotrophic lateral sclerosis [1].

In light of the above, there exists a wide spectrum of human cerebellar ataxias with different pathogenetic etiologies [9,10], as well as established animal models of cerebellar disorders [11–13]. Despite several therapeutic strategies for spinocerebellar ataxias, none of them is effective enough to stop the degenerative process with substantial recovery of diminished cerebellar function [14,15]. Promising approaches include gene transfer into the cerebellum to prevent the degeneration of Purkinje cells [16] and neurotransplantation to provide restorative cells and/or trophic effect of immature grafts (for review, see [17]).

The Lurcher mutant mouse represents one of the most frequently used natural models of genetically-determined olivocerebellar degeneration [18]. They are heterozygotes (+/Lc), carrying a mutation in the glutamate receptor delta2-subunit gene [19], which is predominantly expressed by cerebellar Purkinje cells [20]. Lurcher mice suffer from a virtually complete postnatal loss of Purkinje cells and a decrease in the number of cerebellar granule, basket and stellate cells and inferior olive neurons [21,22]. The reduction in the Purkinje cell number can be detected from postnatal day 8 (P8) and the degeneration is finalized at the P90 [21]. The death of Purkinje cells is a primary effect of the mutation that changes the altered receptor into a leaky membrane channel, leading to chronic depolarization of the cells [19]. The degeneration of the other cerebellar cells and inferior olive neurons is a secondary consequence of the disappearance of target of their axons – the Purkinje cells [23,24] – and affects 90% of the granule cells and 70% of the inferior olive neurons [21].

Homozygous mutants (Lc/Lc) are not viable due to the massive loss of brainstem neurons during prenatal development and they die at birth [25,26]. Homozygous wild type littermates of Lurcher mice (+/+) are completely healthy and serve as controls. In contrast, heterozygous Lurcher mice suffer from cerebellar ataxia [27–29], a deterioration of spatial learning or orientation [30–33], and abnormalities in conditioned eyelid response [32,34]. For review, see [35–37]. We have recently shown that naive carcinoma stem cells and neuroprogenitors derived from these cells are able to survive in a normal mouse cerebellum, although there are some differences in growth character of these 2 types of grafts [38]. Accordingly, the aim of the present study was to assess survival of naive and neurally differentiated P19 carcinoma stem cells following transplantation into the cerebellum of Lurcher mice and wild type littermates.

Material and Methods

Experimental procedures are identical to those detailed in previous studies [38] and are briefly described below.

Stem cell culture and neurodifferentiation

Embryonic carcinoma (EC) stem cells of the P19 line isolated from a teratocarcinoma induced in the C3H/He strain of mice [39] were purchased from the European Collection of Cell Culture, Wiltshire, UK. They were genetically modified to express the green fluorescent protein (GFP) so that they could subsequently be detected after transplantation in histological sections using direct fluorescent microscopy [38].

Undifferentiated EC cells were cultured on gelatin-coated tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 0.05 mM beta-mercaptoethanol, 100 i.u. /ml penicillin, and 0.1 mg/ml streptomycin (all GIBCO BRL, Chemos CZ, Prague, Czech Republic) [40].

For neurodifferentiation, the EC cells were cultured under serum-free conditions in DMEM/F12 (1:1) media supplemented with a mixture of insulin, transferrin, selenium (ITS) and antibiotics. For the first 2 days, the cells were treated with retinoic acid (RA, $c=5\times10^{-7}$ M) to induce neurogenesis and then they were cultured for 1 day without RA [40]. At that point, they were used as neuroprogenitors for transplantation.

We have previously described the characterization of naive P19 cells and neuroprogenitors in the stage in which they were grafted by immunocytochemical, Western blot and quantitative reverse transcriptase real-time PCR analyses [38,41].

Graft preparations

For transplantation of naive P19 cells or neuroprogenitors, the cells were isolated by trypsin, which was neutralized by adding DMEM with serum and then centrifuged and resuspended in DMEM to give a final cell concentration of 50 000 viable cells/µl. The cells were then grafted within 1 hour.

Transplantations

Transplantations were performed under general anaesthesia (Ketamine 100 mg/kg bw and Xylazine 16 mg/kg bw). A hole (2 mm in diameter) was drilled in the occipital bone (Bregma – 6.5–7.0 mm, midline), and 1 µl of cell suspension (a total amount of 50 000 cells) was injected with a Hamilton syringe at a constant speed of 0.5 µl/min. The tip of the needle was inserted 1.6 mm under the surface of the cerebellum in wild type mice and 1.6 or 1.2 mm in Lurcher mice. In the Lurcher mouse, the cerebellum is flattened, necessitating a lower depth of implantation. During the injection

of the cell suspension, the tip was elevated 0.1 mm. Upon completion of the administration, the needle remained *in situ* for 5 minutes to prevent the return of the cells out of the host head. Finally, the wound was sutured by 1 layer with Chirlac rapid (Chirmax GmbH) and disinfected.

Histological examination

Mice were sacrificed 21 days after the transplantation by a lethal dosage of Thiopental and were transcardially perfused with phosphate-buffered saline solution (pH 7.4) and 4% phosphate-buffered paraformaldehyde. Fixed brainstem and cerebellar blocks were sectioned using a cryostat (40 µm frontal sections). The grafts were detected according to their GFP fluorescence in the native sections under a fluorescent microscope. Sections were counter-stained with hematoxylin-eosin or according to the Nissl technique to visualize the histological structure of the graft and surrounding host tissue [38].

Detection and quantification of Purkinje cells and astrocytes was achieved via immunohistochemical staining utilizing a rabbit anti-calbindin-D-28K antiserum (Sigma-Aldrich, St. Louis, USA) and a mouse monoclonal glial fibrillary acidic protein (GFAP) antiserum (clone G-A-5, Sigma-Aldrich, St. Louis, USA), respectively.

In the immunohistochemical analyses, sections were incubated in PBS with 10% normal serum, 0.5% Tween and 0.1% FBS (foetal bovine serum) for 1 hour at room temperature. After washing in PBS, the sections were incubated overnight at 4°C with the primary antibody (dilution of GFAP 1:800 and anti-calbindin 1:1000). After a PBS wash, GFAP labelling was completed and the sections were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen, Eugene, USA). Sections incubated with anti-calbindin antiserum were washed in PBS and subsequently incubated with the secondary antibody, Alexa Fluor 568 goat anti – rabbit IgG (final dilution 1:400, Invitrogen, Eugene, USA), for 2 hours at room temperature. After a PBS wash, sections were mounted in ProLong Gold antifade reagent with DAPI and viewed under an epifluorescent microscope (Olympus BX 41, Olympus Czech Group, Prague, Czech Republic).

Statistical analysis

The number and percentage of mice in which the graft survived (quantified by counting GFP-positive cells) and the frequency of the occurrence of various features of the grafts (localization of the graft, the destruction of the tissue, and the characteristic growth of the graft) were assessed. The differences between the groups of mice were evaluated using Fisher's test. In all cases, the differences were considered significant at the $p<0.05$ level.

Animals

All animal experiments described in the present study were performed in full compliance with the EU guidelines for scientific experimentation on animals and with the permission of the Ethics Commission of the Faculty of Medicine at Charles University, Pilsen.

Adult normal wild type and Lurcher mutant mice of the B6CBA strain were used; 43 wild type mice were taken from the previous study [38]. The final number of wild type mice used in the present study was 51. The number of Lurcher mutant mice employed in the study was 87. The mean age of the animals at the time of transplantation was 261.5 days (SD=64.3 days, minimum =156 days, maximum =385 days). The mice were reared under standard conventional conditions with 12:12 hours light: dark cycle (6 am – 6 pm) and temperature 22–24°C. Water and food were available *ad libitum*. The mice were housed in plastic cages with metal mesh cover (11×25 cm, 14 cm high for 1–2 mice or 18×25 cm, 14 cm high for 2–4 mice). Twenty-five wild type mice (12 males and 13 females) were treated with naive P19 cells and 26 wild type animals (13 males and 13 females) received neuroprogenitor cells. In 25 Lurcher mice (12 males and 13 females) naive P19 cells were injected at the depth of 1.6 mm under the surface and in 18 Lurcher mice (9 males and 9 females) received neuroprogenitor cells injected at the depth of 1.2 mm. Twenty-five Lurcher mice (13 males and 12 females) received neuroprogenitor cells injected at the depth of 1.6 mm and 19 Lurcher mice (9 males and 10 females) received neuroprogenitor cells injected into the depth of 1.2 mm.

Results

Graft survival

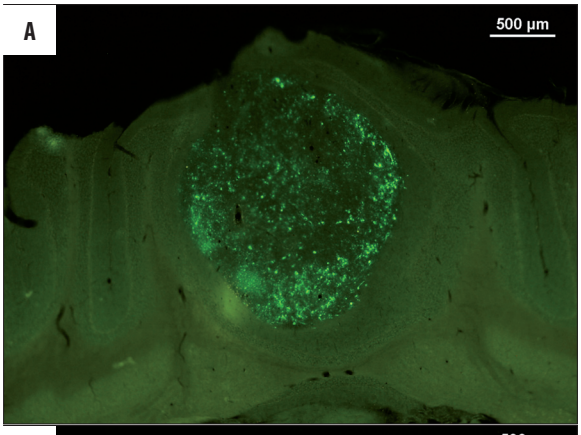
The number and percentage of experimental animals with viable grafts are listed in Table 1. Notably, the number of viable neuroprogenitor cellular grafts at an injection depth of 1.6 mm was significantly higher in wild type as compared to Lurcher mutant mice ($p=0.0078$).

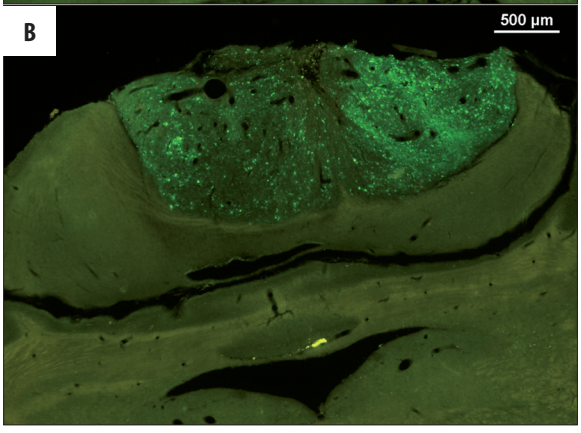
Graft localization

Both P19 cell and neuroprogenitor grafts formed a separate mass containing GFP-positive cells dispersed in non-fluorescent tissue, as previously described for wild type mice [38].

In 11 and 7 wild type mice injected with neuroprogenitor and naive P19 cells, respectively, the graft was typically localized to the vicinity of the injection site in the middle of the cerebellum (Figure 1A). In 2 wild type mice injected with neuroprogenitor cells, viable grafts were localized to the mesencephalon. In a single animal, a viable graft was localized to an area between the cerebellum and the mesencephalon and was strictly delimited against both of these structures.

In Lurcher mice, viable grafts were localized to the mesencephalon and were never observed to be inside the cerebellum (Figure 1B). In 2 Lurcher mice injected with neuroprogenitor cells at a depth of 1.6 mm, viable grafts were observed to be in contact with the border between the mesencephalon and the cerebellum, with the mass of the graft strictly delimited against the cerebellum (Figure 1C). In a single Lurcher mouse injected with naive P19 cells at a depth of 1.6 mm, a large viable graft was observed in the mesencephalon and a small piece of fluorescent graft tissue was found at an angle between the pons and the cerebellum with no signs of direct contact with the cerebellum. Finally, in a single Lurcher mouse injected with neuroprogenitor cells at a depth of 1.2 mm, GFP-positive cells were observed in the mesencephalon and also on the surface of the medulla oblongata.





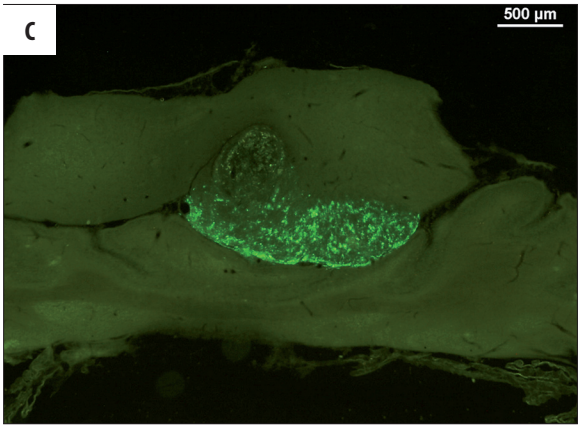


Figure 1. (**A**) Intracerebellar graft from a wild type mouse that received naive P19 stem cells. (**B**) Intramesencephalic graft from a Lurcher mouse that received naive P19 stem cells injected at the depth of 1.6 mm. (**C**) Graft localized between the mesencephalon and the cerebellum in a Lurcher mouse treated with neuroprogenitors injected at the depth of 1.6 mm. Cellular populations in grafts are detected by GFP-positive fluorescence.

Localization parameters of viable cerebellar and mesencephalic neurografts are depicted in Table 2. Statistically significant differences were observed in the cerebellar localization of viable grafts in Lurcher mutants in comparison to wild type littermates (for neuroprogenitor graft, depth 1.6 mm p=0.0063, for naive P19 cells, depth 1.6 mm p=0.0101).

Table 2. Tabulated number of wild type (WT) and Lurcher mutant (Lc) mice with viable cerebellar or mesencephalic neurografts.

Graft localisation		
Experimental group	**Cerebellum**	**Mesencephalon**
WT neuroprogenitors – 1.6 mm	11	2
WT naive P19 cells – 1.6 mm	7	1
Lc neuroprogenitors – 1.6 mm	0	4
Lc naive P19 cells – 1.6 mm	0	4
Lc neuroprogenitors – 1.2 mm	0	7
Lc naive P19 cells – 1.2 mm	0	2

Graft structure

Immunohistochemical analyses demonstrated that viable grafts contained numerous GFAP-positive cells (Figure 2B, D), at least some of which were GFP-positive (Figure 2A, C). Calbindin-positive cells co-expressing GFP fluorescence were not observed in viable grafts.

Table 1. The number of wild type (WT) and Lurcher mutant (Lc) mice with surviving grafts (column 1), extinct grafts (column 2) and the percentage of mice with surviving grafts (column 3).

Graft survival			
Experimental group	**Surviving grafts**	**Extinct grafts**	**Percentage of animals with surviving graft**
WT neuroprogenitors – 1.6 mm	14	12	53.8%
WT naive P19 cells – 1.6 mm	8	17	32.0%
Lc neuroprogenitors – 1.6 mm	4	21	16.0%
Lc naive P19 cells – 1.6 mm	4	21	16.0%
Lc neuroprogenitors – 1.2 mm	7	12	36.8%
Lc naive P19 cells – 1.2 mm	2	16	11.1%

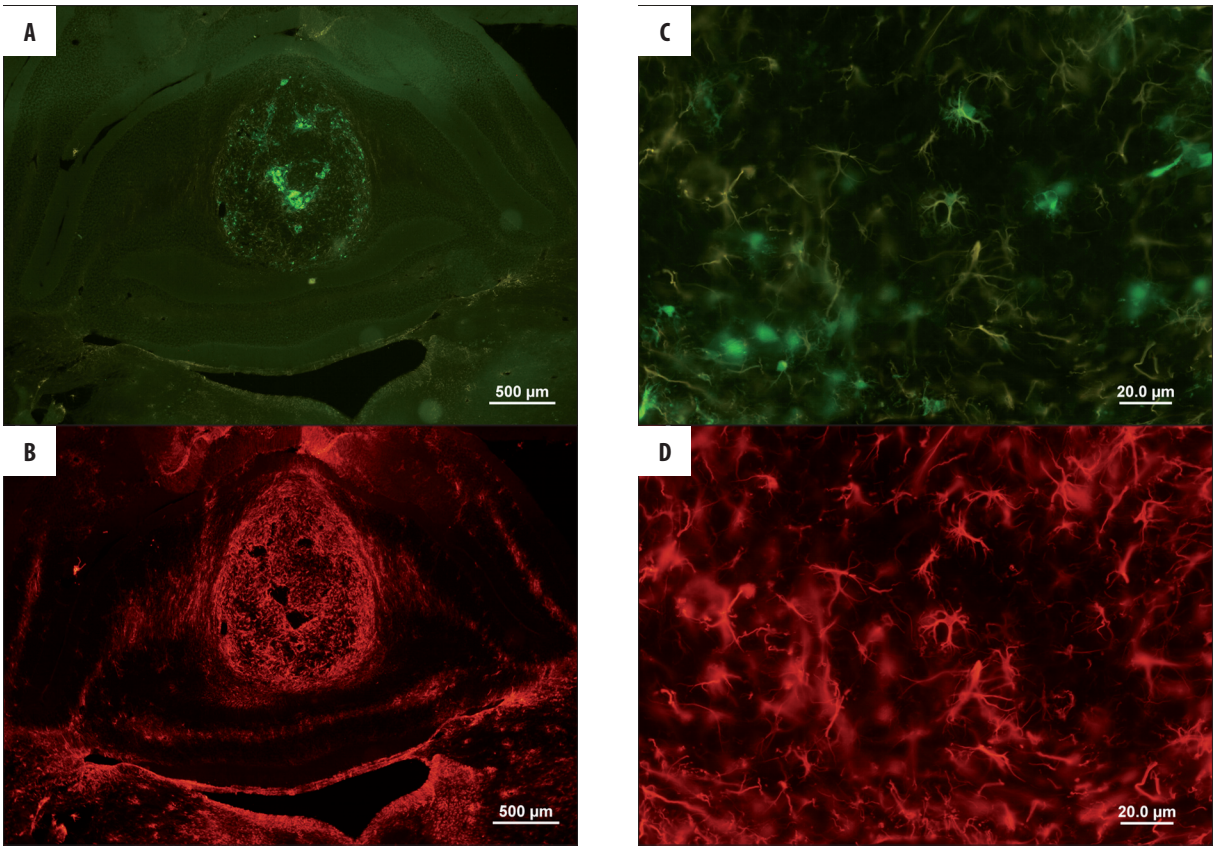


Figure 2. The graft in a wild type mouse treated with neuroprogenitors. GFP-positive fluorescence (**A, C**) and GFAP immunohistochemistry in the same slice and position (**B, D**).

Table 3. Tabulated number of wild type (WT) and Lurcher mutant (Lc) mice with observable tissue destruction inside viable grafts and with viable grafts exhibiting expansive morphological characteristics.

Character of the graft				
Experimental group	Destruction		Expansion	
	Yes	No	Yes	No
WT neuroprogenitors – 1.6 mm	6	8	4	10
WT naive P19 cells – 1.6 mm	0	8	8	0
Lc neuroprogenitors – 1.6 mm	1	3	2	2
Lc naive P19 cells – 1.6 mm	0	4	4	0
Lc neuroprogenitors – 1.2 mm	1	6	2	5
Lc naive P19 cells – 1.2 mm	0	2	2	0

In some of the animals, tissue destruction was observable inside viable grafts, whereas other viable grafts exhibited expansive morphological characteristics [38]. The tabulated number of viable grafts exhibiting these morphological characteristics is depicted in Table 3.

In wild type mice, expansive character was more frequent in mice that received naive P19 cells than in those treated with the neuroprogenitors (p=0.0017). Differences in intragraft tissue destruction in viable grafts originating from naive P19 cell and the neuroprogenitor cell injections to wild type mice did not reach statistical significance. Due to the low number of surviving grafts in Lurcher mice, it was not possible to perform reliable statistical analysis of various morphological features of the grafts.

Discussion

The present study confirms and elaborates previous preliminary findings that have described cerebellar transplantation of naive P19 carcinoma stem cells and P19-derived neuroprogenitors in wild type mice [38] by including similar analyses in Lurcher mutant mice. Our major observations indicate that in Lurcher mice graft survival is quite poor and survival

rate of the neuroprogenitor grafts is significantly lower in comparison to wild type mice when injected at the same tissue depth. The low number of surviving grafts did not allow for a more detailed comparison of morphological characteristics of the 2 types of grafts in Lurcher mice. We can conclude, however, that naive P19 cell grafts typically exhibited expansive morphological characteristics without intragraft tissue destruction in both wild type and Lurcher mice. In these parameters, Lurcher mice did not differ significantly from wild type individuals. The characteristic features of the 2 types of grafts have already been discussed elsewhere [38].

The main finding of the present study was the observed regional difference in graft localization between wild type and Lurcher mice. It is notable that in Lurcher mice, viable grafts were localized to the mesencephalon and were never observed to be inside the cerebellum. This may be attributable to technical limitations due to the flattened cerebellum of the Lurcher mutant mouse. In the Lurcher mouse cerebellum, there is much less space for viable graft development than in wild type animals and the cerebellum could be easily missed by the tip of the injection needle. Moreover, the depth of cell injection suitable for wild type mice could be too deep for the Lurcher cerebellum. These anatomical factors could also play a role in the difference in neuroprogenitor graft survival between Lurcher mutant and wild type mice. On the other hand, an apparent doubling of survival rate of the neuroprogenitor grafts when injected at the lower depth of 1.2 mm *vs.* 1.6 mm, together with a lower graft survival in Lurcher as compared to wild type mice, suggests that the niche of the Lurcher cerebellum is less suitable for the grafted cells than the adjacent mesencephalon. These intrinsic difficulties suggest that neurodegenerative changes of the cerebellar tissue could have a negative impact on the fate of stem cells grafted into the cerebellum of adult Lurcher mice.

The area of the graft contained numerous astrocytes, at least some of which were donor-derived. Additionally, we have no evidence that the grafted cell (naive embryonic carcinoma cells or neuroprogenitors) differentiated into the Purkinje cells, the main cell type that is missing in Lurcher mutant mice.

Survival of embryonic carcinoma stem cells in Lurcher mice is much lower than the survival of embryonic cerebellar tissue. Tomey and Heckroth [42], who transplanted a suspension of embryonic cerebellar cells, found surviving graft in 50% of both young and adult Lurcher mice 1–2 months after transplantation. The survival of solid embryonic cerebellar graft 3–9 weeks after the surgery was 80–90% in Lurcher mice and it did not differ from wild type mice [43]. However, cells grafted in the form of suspension are in direct and closer contact with the host tissue than cells inside the solid grafts. Therefore, the influence of the host tissue on grafted cells development is stronger in cell suspension than in solid grafts. Solid grafts are not as suitable as cell suspension for an investigation of the impact of the host environment on the fate of the grafted cells [44]. In addition, the solid embryonic cerebellar graft mediated controversial behavioural effects in Lurcher mice [45].

Conclusions

The appearance and low survival rate of the grafts do not show any promise for a sufficient therapeutic effect on cerebellar function that has deteriorated due to degenerative disease in Lurcher mice. This is in contrast to the findings of benefit of transplantation of neurons derived from the human teratocarcinoma cell-line in animal models of several other neurological diseases [1,6].

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