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Preventive motor training but not progenitor grafting ameliorates cerebellar ataxia and deregulated autophagy in *tambaleante* mice

Elisa Fucà [a,b,*], Michela Guglielmotto [b], Enrica Boda [a,b], Ferdinando Rossi [a,1], Ketty Leto [b,2], Annalisa Buffo [a,b,2]

[a] Department of Neuroscience Rita Levi-Montalcini, University of Turin, Italy
[b] Neuroscience Institute Cavalieri Ottolenghi, Regione Gonzole, 10043 Orbassano, Turin, Italy

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ABSTRACT

Treatment options for degenerative cerebellar ataxias are currently very limited. A large fraction of such disorders is represented by hereditary cerebellar ataxias, whose familiar transmission facilitates an early diagnosis and may possibly allow to start *preventive* treatments before the onset of the neurodegeneration and appearance of first symptoms. In spite of the heterogeneous aetiology, histological alterations of ataxias often include the primary degeneration of the cerebellar cortex caused by Purkinje cells (PCs) loss. Thus, approaches aimed at replacing or preserving PCs could represent promising ways of disease management. In the present study, we compared the efficacy of two different *preventive* strategies, namely cell replacement and motor training. We used *tambaleante* (*tbl*) mice as a model for progressive ataxia caused by selective loss of PCs and evaluated the effectiveness of the preventive transplantation of healthy PCs into early postnatal *tbl* cerebella, in terms of PC replacement and functional preservation. On the other hand, we investigated the effects of motor training on PC survival, cerebellar circuitry and their behavioral correlates. Our results demonstrate that, despite a good survival rate and integration of grafted PCs, the adopted grafting protocol could not alleviate the ataxic symptoms in *tbl* mice. Conversely, preventive motor training increases PCs survival with a moderate positive impact on the motor phenotype.

1. Introduction

Cerebellar ataxias are a heterogeneous group of neurological disorders characterized by lack of motor coordination and imbalance associated with other typical clinical signs such as tremors, muscular hypotonia, oculomotor and speech impairment (Klockgether, 2007). To date, there are no effective cures for ataxias and the few pharmacological therapies tested persistence so far have revealed poor efficacy (Marmolino and Manto, 2010; Ilg et al., 2014). Since Purkinje cell (PC) loss is the common and cardinal feature of these disorders (Sugawara et al., 2007), therapeutic strategies aimed at replacing and/or preserving these cells may represent promising options. However, approaches based on neurotransplantation at the symptomatic stages of the disease have been challenged by the low receptiveness for full integration of grafted cells of the mature nervous tissue (Carletti et al., 2002 and Carletti et al., 2008; Carletti and Rossi, 2005). The integration of PC progenitors into host cerebellar cortex has been achievable only when progenitors have been grafted into very immature cerebella (Carletti et al., 2008). Thus, the window of opportunity for utilisation of grafting as a therapeutic approach precedes the common onset of the signs or symptoms of the pathology. Given this rationale, *preventive* neurotransplantation could be considered as an effective replacement therapy in the large fraction of hereditary cerebellar ataxias, wherein familiar transmission facilitates an early genetic diagnosis.

Among the non-invasive treatment options, motor exercise has been demonstrated to trigger neuroprotective mechanisms (Van Kummer and Cohen, 2015) and alleviate ataxic symptoms in both preclinical and clinical studies (Uhlendorf et al., 2011; Synofzik and Ilg, 2014). However, the extent of this amelioration, as well as its endurance, is profoundly affected by factors such as the disease severity and areas of neurodegeneration, and becomes more limited with the progression of the pathology (Rabe et al., 2009; Miyai 2012a, b). Motor training at early and asymptomatic stages, when significant neurodegeneration and functional deterioration are absent, might exert more efficacious and lasting effects on the onset and progression of neurodegeneration, as well on the promotion of plasticity changes supportive for motor functions, thereby affecting the course of the disease. Therefore, the present study attempts to understand and compare the therapeutic effects of cell replacement or motor training as *preventive* approaches

Abbreviations: CB, calbindin; CN, cerebellar nuclei; PC, Purkinje cell; PNN, perineuronal net; *tbl*, tambaleante mutation; WT, wild type.

* Corresponding author.
E-mail addresses: elisa.fuca@unito.it (E. Fucà), annalisa.buffo@unito.it (A. Buffo).

[1] Deceased 24 January 2014.
[2] Co-last authors.

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for cerebellar ataxia in homozygous *Tambaleante* (*tbl*) mice (Wassef et al., 1987).

Tbl mice exhibit a severe ataxic phenotype starting from two months of age, associated with abundant loss of PCs. The first sporadic manifestations of PC loss can be detected as early as at one month of age (Mashimo et al., 2009). The subsequent massive degeneration of PCs has been attributed to the alteration of the HERC1 E3 ubiquitin ligase, which leads to overactive autophagic processes (Mashimo et al., 2009). PC degeneration in *tbl* mice occurs later compared to the other well-known spontaneous cerebellar mutation models, namely, the Purkinje cell degeneration and Lurcher mice (Mullen et al., 1976; Phillips, 1960). Thus, *tbl* mice represent an ideal opportunity to study preventive approaches in a timeline where PC degeneration has not yet begun. The uniqueness of this approach is its ability to showcase the effects of any adopted strategy specifically on the onset of the disease. Additionally, although signs of autophagy occur in extracerebellar neurons (Ruiz et al., 2016), consequential neurodegenerative processes only occur in PCs in *tbl* mice (Mashimo et al., 2009; Ruiz et al., 2016). This allows the investigation of the effects of preventive therapies on degenerative processes of a well-defined neuronal target.

In this study, we examined the effects of cell transplantation in postnatal *tbl* mice, when healthy PC progenitors are able to fully develop and integrate into host cerebellum. Next, we executed a protocol of preventive motor training daily to juvenile *tbl* mice until the day of sacrifice. We investigated the impact of such approaches on ataxic symptoms and neuronal degeneration. Our results demonstrate that, in spite of good survival and integration into host cerebellar cortex, grafted PCs do not alleviate the ataxic phenotype of *tbl* mice. In contrast, motor training attenuates ataxic symptoms and promotes PC survival with preservation of cerebellar circuitries and mitigation of autophagy deregulation.

2. Material and methods

2.1. Animals and surgical procedures

Tambaleante heterozygous (+/−) mice were bred to produce wild-type (WT) and homozygous null littermates. Genomic DNA of transgenic mice was extracted from tail snips using standard protocols and examined by PCR-based genotyping using primers for WT and null alleles (Mashimo et al., 2009). Day of vaginal plug detection was defined as embryonic day 0 (E0), and the day of birth was considered as postnatal day 0 (P0). Pups were anesthetized by hypothermia.

Donor cells were isolated from the cerebellar primordium of E11 β-actin–GFP mice and grafted into the cerebellum of WT or mutant P1 littermates, as described (Jankovski et al., 1996; Carletti et al., 2002). The posterior surface of the cerebellum was exposed by removing a small fragment of the occipital bone. Two μl of the single-cell suspension (final concentration 5×10^4 cells/μl), obtained by mechanical dissociation was pressure-injected through a glass capillary into the parenchyma of cerebellar hemispheres in the attempt to increase the possibility for deep nuclei re-innervation from the grafted PCs. The wound was sutured, and the animal was returned to its cage. The recipient animals were sacrificed at 2 months post-transplantation.

All procedures on experimental animals were in accordance with the European Communities Council Directive (2010/63/EU), the National Institutes of Health guidelines, and the Italian Law for Care and Use of Experimental Animals (DL26/14) and were approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin.

2.2. Training procedures

Starting from P17, a group of *tbl* mice and their WT littermates underwent a protocol of motor training 5 days/week until the day of sacrifice (postnatal day 60). Motor training consisted of motor exercise (3′ of climbing activity on cage and 20′ of running wheel) and 6 crossing of a wooden beam bar with a rectangular section (1 cm × 1,5 cm), 1 m in length, placed 80 cm above a foam carpet.

2.3. Behavioral assays

2.3.1. Rotarod test

The accelerated rotarod test was used to assess motor coordination and function (rotarod apparatus: Ugo Basile; five flanges divide the five 5.7 cm lanes, enabling five mice to be simultaneously on test, the cylinder diameter was 3.5 cm). Testing was performed once for month for mice of each group. Animals were put on the rotarod until the latency to fall off reached the total time of 300 s, with constant acceleration of 5.5 and maximum speed of 60 rpm. We performed three trials per day with 3–5 min intervals, on three consecutive days; the averages of the three sessions were considered. During pauses between trials, mice were allowed to rest in their home cages (adapted from Mashimo et al., 2009).

2.3.2. Beam walking test

Beam walking test was used to assess balance capabilities. Testing was performed once for month for mice of each group. The apparatus consisted of a motionless wooden beam with a rectangular section (1 cm × 3 cm), 1 m in length, placed 80 cm above a foam carpet. At the onset of the single trial, each animal was placed at the middle of the beam, its body axis being perpendicular to the beam long axis. We recorded the time spent to complete each cross and the number of slips made during the passages to the wooden bar, for a final number of 5 crossings to the bar. Then, for each animal, the walking speed and the frequency of slips were calculated; after 5 consecutive unsuccessful attempts to stay on the bar, the test was considered failed (adapted from Hilber and Caston, 2001). At two months of age, all *tbl* mice failed the test. After training number of slips and crossing time was calculated only for mice that completed the test.

2.4. Histological procedures

Under deep general anaesthesia, mice were transcardially perfused with 250 ml of 4% paraformaldehyde in 0.12 M phosphate buffer (pH 7.2–7.4). The brains were immediately dissected, postfixed overnight at 4 °C and transferred to 30% sucrose in 0.12 M phosphate buffer. The cerebella were then embedded and frozen over dry ice in OCT (Tissue-Tek), sectioned in the parasagittal plane at 30 μm using a cryostat, and collected in PBS. Immunofluorescent staining of wild-type and mutant sections was performed in parallel to minimize inter-experiment variability. Primary antibodies were dissolved in PBS, with 1.5% normal serum and 0.25% Triton X-100, and incubated overnight at 4 °C with: anti-calbindin (CB, 1:1500, Swant) to label PCs; anti-green fluorescent protein (GFP, 1:700, Invitrogen) and anti-GFP (1:700, Aves Labs) to enhance the GFP fluorescent signal of transplant-derived cells; anti-SMI32 (1:500, Sternberger) to label neurons in cerebellar nuclei (CN); anti-vesicular transporter for glutamate 1 - Vglut1 (1:1000, Synaptic System), anti-vesicular transporter for glutamate 2 - Vglut2 (1:1500, Synaptic System). For perineuronal nets (PNNs) visualization, sections were transferred to a solution containing biotinylated *Wisteria floribunda* agglutinin (WFA, 1:200, Sigma TM) for 2 h at room temperature and then in streptavidin-Alexa Fluor 633. The sections were exposed or 1 h at room temperature to secondary species-specific antibodies conjugated with Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 647 (1:500; Invitrogen) or Cy3 (1:500; Jackson ImmunoResearch) or biotinylated secondary antibodies followed by streptavidin-Texas Red conjugate (1:200; Invitrogen). 4″,6″-diamidino-2-phenylindole (DAPI, Fluka) was used to counterstain cell nuclei. Finally, the sections were mounted in Mowiol (Calbiochem).