**Endogenous cerebellar neurogenesis in adult mice with progressive ataxia**

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**Abstract**

**Objective:** Transplanting exogenous neuronal progenitors to replace damaged neurons in the adult brain following injury or neurodegenerative disorders and achieve functional amelioration is a realistic goal. However, studies so far have rarely taken into consideration the pre-existing inflammation triggered by the disease process that could hamper the effectiveness of transplanted cells. Here, we examined the fate and long-term consequences of human cerebellar granule neuron precursors (GNP) transplanted into the cerebellum of Harlequin mice, an adult model of progressive cerebellar degeneration with early-onset microgliosis.  

**Methods:** Human embryonic stem cell-derived progenitors expressing Atoh1, a transcription factor key to GNP specification, were generated in vitro and stereotaxically transplanted into the cerebellum of preataxic Harlequin mice. The histological and functional impact of these transplants was followed using immunolabeling and Rotarod analysis.  

**Results:** Although transplanted GNPs did not survive beyond a few weeks, they triggered the proliferation of endogenous nestin-positive precursors in the leptomeninges that crossed the molecular layer and differentiated into mature neurons. These phenomena were accompanied by the preservation of the granule and Purkinje cell layers and delayed ataxic changes. In vitro neurosphere generation confirmed the enhanced neurogenic potential of the cerebellar leptomeninges of Harlequin mice transplanted with exogenous GNPs.  

**Interpretation:** The cerebellar leptomeninges of adult mice contain an endogenous neurogenic niche that can be stimulated to yield mature neurons from an as-yet unidentified population of progenitors. The transplantation of human GNPs not only stimulates this neurogenesis, but, despite the potentially hostile environment, leads to neuroprotection and functional amelioration.

**Introduction**

Inherited cerebellar ataxias, a heterogeneous group of neurodegenerative disorders characterized by progressive degeneration and resulting in impaired balance, gait, and movement coordination,\(^1\) are a major cause of disability and reduced life-span, with few treatment options. The Harlequin (Hq) mouse is a spontaneous genetic mouse model of cerebellar ataxia with an 80–90% reduction in the mitochondrial protein Apoptosis-inducing factor,\(^2\) which leads to progressive ataxia with selective cerebellar granule neuron loss and subsequently, Purkinje cell death.\(^2\) Although the first signs of ataxia begin at 4–5 months of age, the degenerative process is already underway by 2 months, with visible signs of mitochondrial degeneration in both granule and Purkinje neurons.
accompanied by widespread inflammatory changes including astrogliosis and microgliosis. Thus, the type and temporal characteristics of the neuronal loss observed as well as the combination of inflammatory and neurodegenerative changes make the Hq mouse eminently suitable for the study of such cerebellar ataxias, and cell-replacement strategies aimed at treating them.

The capacity of embryonic stem cells (ESCs) to self-propagate in vitro and differentiate into functional neurons in response to extrinsic cues has raised the hope that neurodegenerative diseases in which specific subtypes of neurons progressively degenerate could be treated by stem cell-based replacement strategies. In the present study, we asked whether human ESC-derived granule neuron precursors (GNPs) generated in vitro could survive in vivo and induce histological and functional improvements in the Hq mouse. Surprisingly, GNPs transplanted into the cerebellum of preataxic Hq mice reduced neurodegenerative changes, slowed the progression of ataxia, and stimulated endogenous neurogenesis from a previously unsuspected niche in the cerebellar leptomeninges.

Materials and Methods

Animals and ethics statement

B6CBACA-Aw/J-A-Pdc8Hq/J mice (Jackson Laboratory, Bar Harbor, Maine, USA) were housed under a 12 h light/dark cycle with food and water ad libitum, and care taken to ensure that ataxic animals could access food and water despite their weakness. Animal procedures were designed to minimize suffering, approved by the Debré-Bichat National Ethics Committee (Project No. 2010-13/676-0018) and conformed to French laws on animal protection.

Generation of Atoh1-driven NLS-GFP-expressing cells from undifferentiated hESCs

The human HUES-4 and HUES-7 lines from Harvard were used at passages 35–55, and verified for normal karyotype and pluripotency. For Atoh1-driven NLS-GFP (green fluorescent protein)-expressing cell generation, cells were stably transfected with the pJ2XnGFP-Hygro plasmid derived from pJ2XnGFP, kindly provided by Prof. Johnson (Dallas, TX). Briefly, pJ2XnGFP was modified by the introduction of the hygromycin gene between the Kpn1 and Xho1 restriction sites, linearized and transfected using Lipofectamine 2000 (Life Technologies, Saint Aubin, France) under feeder-free conditions. Hygromycin-resistant clones were generated and used for neuronal differentiation.

Differentiation of hESCs into GNPs and mature granule neurons

Cells were grown in six-well plates on inactivated mouse embryonic fibroblasts (iMEFs) with daily changes of hESC medium (Knockout-DMEM, 20% Knockout-SR, 1× penicillin-streptomycin, 1× NEAA, 1× GlutaMax, 1× β-mercaptoethanol, 10 ng/mL bFGF [all from Life Technologies]). For neural induction, hESCs were seeded onto low-aderherence plates to allow embryoid body (EB) formation. EBs were grown in hESC medium without bFGF for 8 days and 10 μmol/L retinoic acid (Sigma-Aldrich, St. Louis, MO, USA) was added on days 4 and 6. On day 8, EBs were cultured on Matrigel-coated plates for 6–10 days in DMEM/F12 with 200 mmol/L glutamine, antibiotics, 20 ng/mL bFGF, 1× Insulin-Transferrin-Selenium (Life Technologies). After 6–10 days, neurospheres (NS) were manually isolated, plated on coated Petri plates and cultured in DMEM/F12 with 1× N2, 1× B27, 100 ng/mL FGF8, 50 ng/mL Wnt3a, 20 ng/mL BMP6, 100 ng/mL BMP7, and 100 ng/mL GDF7 (all from R&D Systems, Minneapolis, MN, USA) for the first 4 days (NS+) with the addition of 100 ng/mL Shh for another 4 days (NS+8). Cells were then treated with 0.025% Trypsin/EDTA at 37°C for 2–3 min to make single cell suspensions. For final differentiation (FD), 10^5 cells were grown on coated coverslips in 24-well plates in Neurobasal medium containing 1× N2, 1× B27, 100 ng/mL FGF8, 50 ng/mL Wnt1, 50 ng/mL Wnt3a, 20 ng/mL BMP6, 100 ng/mL BMP7, 100 ng/mL GDF7, 100 ng/mL Shh, and 20 ng/mL Jag1. The medium was changed every alternate day.

RNA extraction and quantification of relative gene expression by real-time PCR

Total RNA was extracted from cells at NS+4, NS+8, and FD+7 stages using an RNA extraction Kit (Qiagen, Courtaboeuf, France) and used to synthesize cDNA (Bio-Rad, Marnes-la-Coquette, France). Real-time PCRs were performed as previously reported, in triplicate with negative and internal (HPRT) controls. Primers for quantitative real-time PCR are given in Table 1.

Cell transplantation and immunofluorescence

Twenty-four hours before transplantation, mice were injected intraperitoneally with 10 mg/kg cyclosporin (Novartis, Basel, Switzerland). Stereotaxic injections of GNPs into the cerebellum were carried out as previously described. Following injection, Alzet pumps (DURECT, Cupertino, CA, USA) filled with cyclosporin (10 mg/kg per day) were transplanted subcutaneously.
and replaced monthly. Procedures for sample preparation and immunolabeling were performed as described. All antibodies and concentrations used are listed in Table 2. For BrdU incorporation, mice received BrdU intraperitoneally (50 mg/kg) every alternate day for 30 days, starting at 5 months, and sacrificed at 7 months. After antigen retrieval, sections were treated with 2 mol/L HCl for 30 min followed by 0.1 mol/L Sodium Borate for 5 min, and normal labeling performed.

**Rotarod assay**

Control \((n = 12)\) and GNP-treated \((n = 14)\) mice were placed on a computer-driven Rotarod device (Imetronic) accelerating linearly from 4 to 40 rpm over a 5-min period, following the protocol initially adapted by Klein for Hq mice. The latency to fall was averaged from the results of three trials. Data were expressed as a percentage of the initial performance for each animal.

**Culture of leptomeninges**

Leptomeninges from the cerebellar surface were stripped mechanically, collected in PBS, centrifuged at 500 g and resuspended in Neurobasal medium containing 1× N2-supplement, 1× B27, 200 mmol/L glutamine, antibiotics and 10 ng/mL bFGF. On Day 10, the growing leptomeninges were trypsinized into small pieces and plated onto coverslips coated with 100 μg/mL polyornithine and 10 μg/mL laminin (Sigma) in Neurobasal medium containing 1× N2 supplement, 1× B27, 200 mmol/L glutamine, antibiotics and 50 ng/mL BDNF (R&D Systems). Meninges were cultured for an additional 10 days with a change of medium every alternate day.

**Electrophysiology**

Experiments were performed on differentiated stem cell cultures. Cells were continuously superfused in recording solution consisting of (in mmol/L): NaCl, 124; KCl, 3; NaHCO3, 26; NaH2PO4, 1.25; CaCl2, 2; MgSO4, 1; glucose, 10. Visually guided whole-cell recordings were obtained from the soma of neuron-like cells using patch electrodes (4–6 MΩ) that contained in (mmol/L): KCl, 140; HEPES, 10; NaCl, 8; EGTA, 0.5; Mg-ATP, 4; Na-GTP, 0.3. Voltage was recorded on-line using current-clamp techniques. Data were analyzed using the LTP Program.

**Results**

**Human GNP generation**

Because Atoh1 is key to the control of cerebellar granule cell differentiation, we reasoned that transplanting...
GNPs at a stage when Atoh1 expression is maximal would enhance our chances of obtaining efficient differentiation in vivo. To identify this stage, we generated HUES-4 cells-expressing GFP localized to the nucleus (NLS-GFP) under the Atoh1 promoter. ESCs were first induced to differentiate following the sequence of embryoid bodies (EB), proliferative neural stem cells (NS), differentiating GNPs (NS+4 and NS+8), and mature granule neurons (FD+7).

Figure 1. Differentiation of human embryonic stem cells (ESCs) into mature cerebellar granule neurons. (A) Design of the six step-differentiation procedure used to generate mature cerebellar neurons in vitro. Undifferentiated cells were grown into embryoid bodies (uEBs) using low-adherence dishes, a neural fate induced with retinoic acid (RA; iEBs), and grown into heterogeneous neurospheres (NS) from which typical neural rosettes developed (NS+4), proliferated (NS+8) and differentiated into neurons (FD+7). (B and C) Immunocytochemistry and cell counts of mature cerebellar granule neurons using early (TuJ1, Zic1, Zic2) and mature neuron-specific markers (MAP2, GABA<sub>AR</sub>-a6). Scale bar: 25 μm. (D) Electrophysiological analysis of a mature cerebellar granule neuron at FD+7 showing a spontaneous action potential. Voltage was recorded online using current-clamp techniques. Data were analyzed using the LTP Program.®
(Fig. 1A). After 30–40 days in culture, almost all TuJ1-positive cells were also positive for the mature neuronal marker MAP2, indicating that neuronal maturation had been properly achieved (Fig. 1C). In addition, a significant number of neurons expressed markers for cerebellar granule cells such as Zic1 (70.8%), Zic2 (28.3%), and GABA\textsubscript{A}R-\textsubscript{6} (50.6%) (Fig. 1B and C). To examine the functionality of the neurons produced, we performed electrophysiological measurements in vitro. Cells were recorded in current-clamp mode to assess whether they were able to generate an action potential. Spontaneous action potentials were systematically recorded in the long T-shaped processes and ovoid cell bodies typical of granule cells (Fig. 1D). These data indicate that human ESCs can be differentiated into granule neurons capable of generating action potentials in vitro. Next, ESCs were analyzed for GFP expression. GFP expression was maximal at NS+8 but decreased drastically at FD+7 (Fig. 2A), consistent with 50–65% higher Atoh1 expression at NS+8 than at NS+4 or FD+7 (Fig. 2B). Accordingly, Pax6, a marker of proliferative GNPs, as well as Zic1 and Zic2 were highly expressed at NS+8. These data were confirmed by immunocytochemistry (Fig. 2B and C) and reproduced in a second cell line (HUES-7). We therefore selected the NS+8 stage for subsequent GNP transplantation experiments.

**GNP-transplanted Hq mice display better cerebellar preservation**

To evaluate the long-term effects of GNP transplantation into the degenerating cerebellum, NS+8 GNPs were...
injected into the cerebellum of 3-month-old preataxic mice and cerebella were analyzed 2 and 4 months after transplantation. Control groups consisted of sham-operated preataxic mice injected with PBS alone. At 2 months post-transplantation, the density of NeuN-positive nuclei in the granule cell layer appeared similar between control and GNP-treated animals, although slightly more nuclei were counted in folia surrounding the transplanted area (folia III–VI) in the GNP-treated group (Fig. 3A). While calbindin labeling showed that most Purkinje cells were still present at this age (Fig. 3B), Fluoro-Jade B labeling revealed that many of these were undergoing degeneration in control animals only, suggesting that Purkinje cells were protected from degeneration in GNP-treated animals (Fig. 4).

At 4 months posttransplantation, the number of NeuN-positive nuclei was greatly reduced in the granule cell layer in the control group, but neuronal density was fairly well preserved in GNP-treated animals, with NeuN-positive cell numbers comparable to those seen 2 months posttransplantation (Fig. 3C). Similarly, in control mice, more than half the Purkinje cells had disappeared 4 months after surgery, while this layer was better preserved in GNP-treated animals (Fig. 3D). Together, these data indicate that GNP transplantation resulted in the significant long-term protection of both granule and Purkinje neurons in Hq mice.

**GNP-transplanted Hq mice display improved sensorimotor coordination**

To measure sensorimotor coordination in Hq mice, we used the same accelerating rotarod test originally described.\(^2\) Mice were placed on a rotarod accelerating linearly from 4 to

**Figure 3.** Cerebellar structure and behavioral analysis of GNP-transplanted Hq mice 2 months (8 weeks) and 4 months (16 weeks) posttransplantation. (A) NeuN labeling and cell counts 2 months (8 weeks) after GNP transplantation. Cerebellar folia are numbered and white asterisk indicates the site of injection. White rectangles indicate the area from folia III and VI that are shown in higher magnification. Different cerebellar layers are also indicated: WM (white mater), GL (granule cell layer), ML (molecular layer). No significant difference is seen in the number of granule neurons in the cerebellum of 5-month-old control (\(n = 5\)) versus GNP-treated Hq mice (\(n = 5\)). (B) Calbindin-D28k labeling and cell counts 2 months (8 weeks) after GNP transplantation. White asterisk indicates the site of injection. White rectangles indicate the area from folia III and VI that are shown in higher magnification. Different cerebellar layers are also indicated: WM (white mater), GL (granule cell layer), PC (Purkinje cell layer) and ML (molecular layer). No significant difference is seen in the number of Purkinje cells in the cerebellum of 5-month-old control versus GNP-treated Hq mice. Scale bars: 1 mm for whole cerebella and 25 \(\mu\)m for magnifications. Rotarod analysis indicates that motor coordination decreases similarly in the two groups up to this time point. (C) NeuN labeling and cell counts 4 months (16 weeks) after GNP transplantation show that a significantly greater number of granule neurons are protected from death in the GNP-treated group (\(n = 5\)) as compared to the control group (\(n = 6\)). Cerebellar folia are numbered and white asterisk indicates the site of injection. White rectangles indicate the area from folia III and VI that are shown in higher magnification. Different cerebellar layers are also indicated: WM (white mater), GL (granule cell layer), ML (molecular layer). (D) Calbindin-D28k labeling and cell counts 4 months (16 weeks) after GNP transplantation show that a significantly greater number of Purkinje cells are protected from death in the GNP-treated group as compared to the control group. White asterisk indicates the site of injection and white rectangles indicate the area from folia III and VI that are shown in higher magnification. Different cerebellar layers are also indicated: WM (white mater), GL (granule cell layer), PC (Purkinje cell layer) and ML (molecular layer). Quantitative data are expressed as means ± SEM for each group. Results were compared using the Mann–Whitney U-test (GraphPad Prism Software). Significant effects are indicated by asterisks (*\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\)). Scale bars: 1 mm for whole cerebella and 25 \(\mu\)m for magnifications. Rotarod analysis shows that motor coordination decreases less rapidly in the GNP-treated group (\(n = 14\)) from 12 weeks posttransplantation onward when compared with the control group (\(n = 12\)). Results were compared using a 2-way ANOVA (GraphPad Prism Software).
40 rpm over a 5-min period, and the latency to fall recorded weekly for 18 weeks after GNP transplantation. No significant difference was seen during the first 10 weeks of the test. However, GNP-treated Hq mice performed significantly better than control mice starting 12 weeks post-transplantation (Fig. 3). Interestingly, 7.5-month-old GNP-treated animals (i.e., 18 weeks posttransplantation) performed as well as 6-month-old controls, indicating a slowing of the progression of ataxia. In agreement with the neuronal preservation seen at this age, these data show that GNP transplantation also improved sensorimotor performance.

**GNP transplantation is associated with local microgliosis**

To determine the fate of the transplanted cells in vivo, we labeled cerebellar sections of a second batch of animals with an anti-human nucleus (AHN) antibody that specifically recognizes the nuclei of human cells, or with the anti-SC121 antibody, which stains the cytoplasm of human cells. One week posttransplantation, human cells were detected in all animals. One month posttransplantation, human cells were still detected in three of six animals, and had migrated locally throughout the injected folia (IV–V) and, to a lesser extent, into adjacent folia (III and VI). Many cells were positively labeled by a human-specific antibody to the progenitor marker nestin. Co-labeling for AHN and doublecortin (DCX), β-III tubulin (TuJ1) or MAP2 revealed that these GNPs were also capable of...
differentiating into neurons (Fig. 5). Co-labeling with AHN and the cell-cycle-dependent antigen Ki67 revealed few proliferative cells (Fig. 6A) and no tumor formation was observed. In contrast, we found few or no human cells in the three other animals, indicating that the GNPs had not survived up to this time point in all cases. At this age, strong microglial activation occurs in the cerebellum of Hq mice. Accordingly, Iba1-positive microglia/macrophages were found throughout the cerebellum, and were particularly abundant around the transplanted area (Fig. 6A and B), suggesting that the GNPs had triggered acute local microgliosis. Consistent with this, labeling for the activation-associated macrophage mannose receptor, CD206, was also increased in transplanted cerebella (Fig. 6B). Interestingly, while transplantation of either dead GNPs or human fibroblasts (hFibs) resulted in a similar Iba1 reaction in Hq mice, GNPs did not trigger any detectable microgliosis when transplanted into wild-type animals (Fig. S1). This indicates that the local microgliosis observed is specific to the Hq phenotype in reaction against stranger cells. Moreover, no human cells were detected in GNPs-treated Hq mice beyond 2 months, whereas they were still abundant in wild-type or immunedeficient Nude mice (Fig. S2). This suggests that the strong transplantation-induced microglial reaction had likely led to human cells elimination by this time point.

Endogenous neurogenesis occurs in GNP-transplanted Hq mice

At 2 months posttransplantation (i.e., at 5 months of age), a large number of cells positive for mouse nestin was detected throughout the molecular and granule cell layers in all GNP-treated animals (compared to almost none in controls), predominantly in the injected folia (Fig. 7A), suggesting that human cells had activated endogenous nestin-positive cerebellar progenitors. The same phenomenon was observed following dead GNPs or hFibs transplantation but was not seen in GNP-treated wild-type mice, indicating that this reaction was specific to Hq mice but was not specifically due to GNPs (Fig. S1).

To determine if endogenous neurogenesis could occur in GNP-treated Hq animals, mice were given BrdU injections intraperitoneally every alternate day for 30 days, starting at 5 months, and sacrificed at 7 months (Fig. 7B). Confocal microscopic analysis of the granule cell layer showed several nuclei positive for both BrdU and NeuN, indicating that endogenous neurogenesis had occurred (Fig. 7C). The number of BrdU-positive neurons was significantly higher in folia adjoining the injected folia (Fig. 7D), consistent with nestin labeling at earlier stages. Thus, endogenous neurogenesis occurs in

Figure 6. Ki67 labeling of transplanted GNPs at 1 month and reactive microglia at 1 and 2 months posttransplantation. (A) Immunohistochemistry of GNP-transplanted Hq cerebella 1 month posttransplantation shows that human cells express Ki67 only weakly and do not form tumors but are massively surrounded by macrophages-expressing Iba1. Scale bar: 25 μm. (B) Immunohistochemistry of GNP-transplanted and control Hq cerebella 2 months posttransplantation shows strong immunoreactivity for the macrophage-associated markers Iba1 and CD206 in the GNP-treated group only, indicating that a strong microgial reaction persists in the transplanted area, although human cells are not present anymore. Scale bar: 1 mm. (C) Quantitative data are expressed as means ± SEM for each group. Results were compared using the Mann–Whitney U-test (GraphPad Prism Software). Significant effects are indicated by asterisks (**P < 0.01, ***P < 0.001).
Figure 7. Neural induction and endogenous neurogenesis in GNP-transplanted Hq mice 2 and 4 months after transplantation. (A) Immunohistochemistry of GNP-treated and control Hq cerebella 2 months posttransplantation shows strong labeling with a mouse-specific antibody to the neural marker nestin in the GNP-treated group only. High magnification images of the GNP-treated cerebellum show that nestin-expressing cells are present in the molecular layer (ML) as well as in the granular layer (GL). Scale bars: 1 mm for whole cerebella, 50 μm for lower panels and 25 μm for high magnification images (B) Intraperitoneal BrdU injection procedure. GNP-treated Hq animals received BrdU injections every alternate day between 2 and 3 months after transplantation, and were sacrificed 1 month later, that is, 4 months after transplantation. (C) Immunohistochemistry of GNP-treated/BrdU-injected Hq cerebella shows that a number of BrdU-positive cells express the neuronal marker NeuN and are found in the granular layer 4 months after transplantation. Scale bars: 1 mm for whole cerebella, 25 μm for high magnification. (D) Quantification of NeuN/BrdU-positive cells in different folia from GNP-treated/BrdU-injected Hq mice. Quantitative data are expressed as means ± SEM for each group. Results were compared using the Mann–Whitney U test (GraphPad Prism Software). Significant effects are indicated by asterisks (** P < 0.01). Scale bar: 25 μm.
GNP-transplanted mice in the folia close to the transplanted site.

**The cerebellar leptomeninges of GNP-transplanted Hq mice contain neural progenitors**

To determine the origin of these nestin-positive progenitors, we looked for proliferating cells 2 months posttransplantation. While no Ki67-positive cells were identified in the cerebellum of age-matched wild-type mice, the leptomeninges of all Hq mice, including nonoperated and PBS-injected controls, displayed a number of Ki67-positive cells, which increased significantly in GNP-treated animals (Fig. 8A and B). Moreover, a number of Ki67-positive cells were positive for mouse nestin (Fig. 8B). These data indicate that the cerebellar leptomeninges of Hq mice contain nestin-positive neural progenitors that are actively proliferating, a process potentiated by GNP transplantation.

Since nestin-positive processes stretched radially across the molecular layer in a pattern typical of Bergmann glia, we asked whether these cells displayed other characteristics of this specialized population of cerebellar radial glia. In the mature cerebellum, Bergmann glia expresses the calcium-binding protein S100-beta, whereas during development they express the radial glial marker RC2 in addition to nestin. Surprisingly, the cell bodies of nestin-positive cells were located both at the pial surface and within the molecular layer and were negative for S100-beta, while cell bodies positive for S100-beta, belonging to typical adult Bergmann glia, were located in between the Purkinje cells as expected, and were nestin-negative (Fig. 8C). In keeping with these results, nestin and S100-beta were almost never colocalized in these radial projections. In addition, these putative Bergmann glia were also negative for both the developmental markers RC2 and Sox2 (not shown), suggesting that they correspond to a third type of leptomeningeal Bergmann glia with progenitor potential in adulthood.

**The cerebellar leptomeninges of Hq mice are neurogenic in vitro**

To confirm in vitro the neurogenic potential of the cerebellar leptomeningeal progenitors identified in Hq mice, we stripped the leptomeninges from the cerebellar surface of 5-month-old wild-type and Hq animals and cultured them in neural stem cell medium in the presence of bFGF for 10 days. Leptomeninges from wild-type animals curled up and adopted an irregular shape after 2–4 days in vitro (DIV2–4), and finally degenerated. In contrast, leptomeninges from Hq mice rapidly formed dense floating spheres, the edges of which became refractive around DIV9 (Fig. 9). The number of neurospheres generated from GNP-treated Hq mice was systematically higher than from nonoperated Hq mice. At DIV17–20, the neurospheres as well as most of the cells migrating out of them expressed nestin. At a distance from the neurospheres, many cells also expressed MAP2 (Fig. 9). These findings indicate that the cerebellar leptomeninges of Hq mice have neurogenic potential in vitro that can be potentiated by human GNP transplantation, and confirm that this layer is the source of the neural progenitors seen in Hq mice in vivo.

**Discussion**

In this study, we asked whether human ESC-derived progenitors could survive and replace or protect dying neurons in a clinically relevant context in which microglial activation is high and the degenerative process has already begun. In the Hq mouse, microgliosis starts around 1–2 months of age, long before the first clinical signs of ataxia. Our GNPs were thus injected into a strongly inflammatory environment, which could explain the rapid microglial recruitment to the transplantation site and the elimination of transplanted cells within 2 months. A similar rapid loss of transplanted human cells has been observed in the brain of other animal models of neurological disorders, where transplanted cell survival is very low (less than 5%), irrespective of the immunosuppressive strategy or source of stem cells used. Consistent with this, GNP transplantation into the cerebellum of wild-type animals, where preexisting microgliosis is absent, resulted in prolonged survival, integration and differentiation in our study, a finding confirmed by observations in immunodeficient nude mice (Fig. S2).

Regardless of the loss of transplanted cells, however, we observed significant improvements at both the histological and sensorimotor levels in GNP-treated Hq mice. This suggests that before or during their elimination, GNPs had released factors or triggered processes that were neuroprotective at the local level. This hypothesis is consistent both with our observation that endogenous neuronal loss was less pronounced in the folia adjoining the transplantation site than in more rostral or caudal ones, and with previous reports of the lack of survival of transplanted cells in the brain even though structural or functional rescue had taken place.

Interestingly, however, Hq mice, unlike wild-type adult mice, appeared to possess progenitors capable of differentiating into mature neurons in the cerebellum in vivo. To our knowledge, this is the first time that such a phenomenon has been convincingly shown to occur in the adult rodent cerebellum, although there has been indirect evidence for...
the existence of multipotent progenitors in the adult cerebellum and new neuronal cells have been reported in the atypical cerebellum of peripubertal rabbits.

Another unexpected and important finding of our study was that these progenitors were located in the cerebellar leptomeninges, the thin meningeal membranes surrounding the brain and spinal cord, of adult Hq mice, a finding confirmed by the generation of neurospheres capable of differentiating into neurons from these membranes. While the leptomeninges are in direct contact with the nervous tissue, they are not simply protective membranes but have been shown to penetrate neural tissue and contribute to Central Nervous System (CNS) homeostasis by secreting several trophic factors. Leptomeninges have been recently identified as sites of stem cell recruitment in response to ischemia or brain damage, suggesting that they also serve as a niche for neural precursors in adulthood during periods of CNS distress. Although these studies focused on specific injuries of the cerebral cortex, they support our observations in the cerebellum of mice undergoing neurodegenerative changes.

The fact that nontreated Hq mice also displayed nestin-positive progenitors and cell proliferation in vivo and neurosphere-generating capacity in vitro is highly interesting, since it suggests that the pathological process might itself trigger the compensatory production of new cells, whether or not these cells succeed in replacing dying neurons. This is the case, for example, in epilepsy, Alzheimer’s disease, Huntington disease, and Parkinson disease. In addition, the insult to the brain caused by the introduction of exogenous cells may itself augment endogenous neurogenesis, as has been shown for mesenchymal stem cells. The pro-neurogenic mechanism of neuronal injury or degeneration could be mediated by inflammatory processes. A growing number of studies show, for example, that acute microglial activation, initially thought to be strictly detrimental to neuronal survival, can influence adult neurogenesis and even exert beneficial neurogenesis-enhancing effects.

Further work is now required to characterize the molecular processes that trigger these unexpected endogenous mechanisms in the adult cerebellum, and to exploit
the neurogenic and neuroprotective potential of these processes in pathological situations.

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**Conflict of Interest**

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Neural induction and microgliosis reaction in transplanted Hq mice 2 months after transplantation. Immunohistochemistry of cerebellar folia 2 months post-transplantation showing labeling for mouse nestin (upper panel) and the microglia marker Iba1 (lower panel). Strong labeling for nestin is detected in GNP-treated Hq mice, dead GNP-treated Hq mice and in hFib-treated mice but not in PBS-treated Hq mice (control) and GNP-treated Wild-type mice. Similarly, a local microglial reaction is detected in GNP-treated Hq mice, dead GNP-treated mice and in hFib-treated mice but not in PBS-treated Hq mice (control) and GNP-treated Wild-type mice. Higher magnifications shown correspond to white squares. The different cerebellar layers are labeled: ML, molecular layer; GL, granule cell layer; WM, white matter. Scale bars: 100 μm (lower magnifications) and 50 μm (higher magnifications).

Figure S2. Detection of human GNPs in the cerebellum of wild-type and nude mice 2 months after transplantation. Immunohistochemistry of cerebellar folia of wild-type and nude mice 2 months post-transplantation using either the human cytoplasmic SC121 antibody (left) or doublecortin (DCX, right). Scale bars: 1 mm (whole cerebellum), 100 μm (medium magnification), 25 μm (higher magnification).