



Endogenous cerebellar neurogenesis in adult mice with progressive ataxia

Manoj Kumar, Zsolt Csaba, Stéphane Peineau, Rupali Srivastava, Sowmyalakshmi Rasika, Shyamala Mani, Pierre Gressens, Vincent El Ghouzzi

► To cite this version:

Manoj Kumar, Zsolt Csaba, Stéphane Peineau, Rupali Srivastava, Sowmyalakshmi Rasika, et al.. Endogenous cerebellar neurogenesis in adult mice with progressive ataxia. *Annals of Clinical and Translational Neurology*, 2014, 1 (12), pp.968-981. <10.1002/acn3.137>. <hal-02342658>

HAL Id: hal-02342658

<https://hal.science/hal-02342658v1>

Submitted on 8 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

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

Manoj Kumar, PhD^{1,2}, Zsolt Csaba, PhD^{1,2}, Stéphane Peineau, PhD^{1,2,3},
Rupali Srivastava, PhD^{1,2,4}, S Rasika, PhD^{1,2}, Shyamala Mani, PhD⁵,
Pierre Gressens, MD, PhD^{1,2,6}, Vincent El Ghouzzi, PhD^{1,2}

1. Inserm, U1141, Paris, France
2. Université Paris Diderot, Sorbonne Paris Cité, UMRS 1141, Paris, France
3. MRC centre for Synaptic Plasticity, School of Physiology and Pharmacology, Bristol, United Kingdom
4. National Brain Research Centre, Manesar, India
5. Centre for Neuroscience, IISC, Bangalore, India
6. Centre for the Developing Brain, Department of Division of Imaging Sciences and Biomedical Engineering, King's College London, King's Health Partners, St. Thomas' Hospital, London, United Kingdom

Correspondence to: Dr Vincent El Ghouzzi
Address: Inserm U1141, Hôpital Robert-Debré, 48 Boulevard Sérurier,
F-75019, Paris, France.
E-mail: vincent.elghouzzi@inserm.fr
Phone: +331 40031973, Fax: +331 40031995

Running head: Adult cerebellar neurogenesis in Harlequin mice

Key words: Embryonic stem cells, granule neurons, ataxia, adult neurogenesis, leptomeninges

Abbreviations: ESCs = Embryonic Stem Cells; Hq = Harlequin mouse; GNPs = Granule Neuron Progenitors; MAP2 = Microtubule Associated Protein 2

Number of characters in the title: 72

Number of characters in the running head: 42

Number of words in the abstract: 246

Number of words in the body of the manuscript: 3378

Number of figures: 9 + 2 supplementary figures

Number of tables: 2

Conflict of interest: None

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 stereotactically transplanted into the cerebellum of pre-ataxic 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 coordination¹, 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 factor², which leads to progressive ataxia with selective cerebellar granule neuron loss and subsequently, Purkinje cell death². 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 microgliosis³. 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 pre-ataxic 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

B6CBACaAw-J/A-Pdc8Hq/J mice (Jackson Laboratory) were housed under a 12h 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 N°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 line generation, cells were stably transfected with the pJ2XnGFP-Hygro plasmid derived from pJ2XnGFP, kindly provided by Prof. Johnson (Dallas, TX,⁴). Briefly, pJ2XnGFP was modified by the introduction of the hygromycin gene between the Kpn1 and Xho1 restriction sites, linearized and transfected using Lipofectamine 2000 (Invitrogen) 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 6-well plates on inactivated mouse embryonic fibroblasts (iMEFs) with daily changes of hESC medium (Knockout-DMEM, 20% Knockout-SR, 1X penicillin-streptomycin, 1X NEAA, 1X GlutaMax, 1X β -mercaptoethanol, 10 ng/ml bFGF (all from Invitrogen)). For neural induction, hESCs were seeded onto low-adherence plates to allow embryoid body (EB) formation. EBs were grown in hESC medium without bFGF for 8 days and 10 μ M retinoic acid (Sigma) 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 mM glutamine, antibiotics, 20 ng/ml bFGF, 1X Insulin-Transferrin-Selenium (Invitrogen). After 6-10 days, neurospheres (NS) were manually isolated, plated on coated Petri plates and cultured in DMEM/F12 with 1X N2, 1X 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) for the first 4 days (NS+4) 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 1X N2, 1X 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) and used to synthesize cDNA (Bio-Rad). Real-time PCRs were performed as previously reported, in triplicate with negative and internal (HPRT) controls⁵. 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 ciclosporin (Novartis). Stereotaxic injections of GNPs into the cerebellum were carried out as previously described⁵. Following injection, Alzet pumps (DURECT) filled with ciclosporin (10 mg/kg/day) were transplanted subcutaneously and replaced monthly. Procedures for sample preparation and immunolabeling were performed as described⁵. 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 M HCl for 30 min followed by 0.1 M 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 4-40 rpm over a 5-minute period, following the protocol initially adapted by Klein for Hq mice². 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 700 rpm and resuspended in Neurobasal medium containing 1X N2-supplement, 1X B27, 200 mM 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 1X N2 supplement, 1X B27, 200 mM 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 mM): 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 MOhms) that contained in (mM): 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^{36,}

³⁷.

RESULTS

Human granule neuron precursor (GNP) generation

Because *Atoh1* is key to the control of cerebellar granule cell differentiation^{5,6}, 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) (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_AR- $\alpha 6$ (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 pre-ataxic mice and cerebella were analyzed 2 and 4 months after transplantation. Control groups consisted of sham-operated pre-ataxic 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 post-transplantation, 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 post-transplantation (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 described². Mice were placed on a rotarod accelerating linearly from 4-40 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 post-transplantation) 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 post-transplantation, human cells were detected in all animals. One month post-transplantation, human cells were still detected in 3 out of 6 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 3 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 mice³. 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 (Suppl. Fig 1). 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 immunodeficient Nude mice (Suppl. Fig 2). 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 post-transplantation (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 ([Suppl. Fig 1](#)).

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 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 post-transplantation. While no Ki67-positive cells were identified in the cerebellum of age-matched wild-type mice, the leptomeninges of all Hq mice, including non-operated 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* (DIV 2-4), 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 non-operated 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 ataxia³. 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 strategy⁷ or source of stem cells used⁸⁻¹². Consistent with this, GNP transplantation into the cerebellum of wild-type animals, where pre-existing microgliosis is absent, resulted in prolonged survival, integration and differentiation in our study, a finding confirmed by observations in immunodeficient nude mice ([Supplementary Fig 2](#)).

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^{13, 14}.

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 cerebellum¹⁵⁻¹⁷ and new neuronal cells have been reported in the atypical cerebellum of peripubertal rabbits¹⁸.

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 CNS homeostasis by secreting several trophic factors¹⁹. Leptomeninges have been recently identified as sites of stem cell recruitment in response to ischemia^{20, 21} or brain damage^{19, 22, 23} 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 non-treated 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 epilepsy²⁴, Alzheimer's disease²⁵, Huntington disease²⁶, and Parkinson disease²⁷. 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^{13, 28, 29}. 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^{30, 31}, can influence adult neurogenesis and even exert beneficial neurogenesis-enhancing effects³²⁻³⁵.

Further work is now needed 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.

ACKNOWLEDGEMENTS

We are grateful to Prof. Jane E. Johnson, Southwestern Medical Center, Texas, for providing us with the pJ2XnGFP construct, which was modified and used in the construction of the cell line, to Eva Pipiras for assistance with HUES karyotyping and to Cécile Martel for invaluable support. This study was supported by the Institut National pour la Santé et la Recherche Médicale (Inserm), the Centre National de la Recherche Scientifique (CNRS), the Université Paris7, the DHU PROTECT and grants from IFCPAR/CEFIPRA (projects N°3803-3 and N°4903-2), the French National Research Agency (project ANR-09-GENO-007), the Princesse Grâce de Monaco Foundation and the Roger de Spoelberch Foundation. MK was supported by the Inserm and ANR contract N°ANR-09-GENO-007 to VEG.

REFERENCES

1. Hersheson J, Haworth A, Houlden H. The inherited ataxias: genetic heterogeneity, mutation databases, and future directions in research and clinical diagnostics. *Hum Mutat.* 2012 Sep;33(9):1324-32.
2. Klein JA, Longo-Guess CM, Rossmann MP, et al. The harlequin mouse mutation downregulates apoptosis-inducing factor. *Nature.* 2002 Sep 26;419(6905):367-74.
3. El Ghouzzi V, Csaba Z, Olivier P, et al. Apoptosis-inducing factor deficiency induces early mitochondrial degeneration in brain followed by progressive multifocal neuropathology. *J Neuropathol Exp Neurol.* 2007 Sep;66(9):838-47.
4. Lumpkin EA, Collisson T, Parab P, et al. Math1-driven GFP expression in the developing nervous system of transgenic mice. *Gene expression patterns : GEP.* 2003 Aug;3(4):389-95.
5. Srivastava R, Kumar M, Peineau S, et al. Conditional induction of Math1 specifies embryonic stem cells to cerebellar granule neuron lineage and promotes differentiation into mature granule neurons. *Stem Cells.* 2013 Apr;31(4):652-65.
6. Ben-Arie N, Bellen HJ, Armstrong DL, et al. Math1 is essential for genesis of cerebellar granule neurons. *Nature.* 1997 Nov 13;390(6656):169-72.
7. Jablonska A, Janowski M, Lukomska B. Different methods of immunosuppression do not prolong the survival of human cord blood-derived neural stem cells transplanted into focal brain-injured immunocompetent rats. *Acta neurobiologiae experimentalis.* 2013;73(1):88-101.
8. Barker RA, Dunnett SB, Faissner A, Fawcett JW. The time course of loss of dopaminergic neurons and the gliotic reaction surrounding grafts of embryonic mesencephalon to the striatum. *Exp Neurol.* 1996 Sep;141(1):79-93.
9. Ekdahl CT, Kokaia Z, Lindvall O. Brain inflammation and adult neurogenesis: the dual role of microglia. *Neuroscience.* 2009 Feb 6;158(3):1021-9.
10. Emgard M, Hallin U, Karlsson J, Bahr BA, Brundin P, Blomgren K. Both apoptosis and necrosis occur early after intracerebral grafting of ventral mesencephalic tissue: a role for protease activation. *Journal of neurochemistry.* 2003 Sep;86(5):1223-32.
11. Guzman R, Uchida N, Bliss TM, et al. Long-term monitoring of transplanted human neural stem cells in developmental and pathological contexts with MRI. *Proc Natl Acad Sci U S A.* 2007 Jun 12;104(24):10211-6.

12. Hicks AU, Lappalainen RS, Narkilahti S, et al. Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery. *Eur J Neurosci*. 2009 Feb;29(3):562-74.
13. Munoz JR, Stoutenger BR, Robinson AP, Spees JL, Prockop DJ. Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. *Proc Natl Acad Sci U S A*. 2005 Dec 13;102(50):18171-6.
14. Titomanlio L, Bouslama M, Le Verche V, et al. Implanted neurosphere-derived precursors promote recovery after neonatal excitotoxic brain injury. *Stem Cells Dev*. 2011;20(5):865-79.
15. Alcock J, Scotting P, Sottile V. Bergmann glia as putative stem cells of the mature cerebellum. *Medical hypotheses*. 2007;69(2):341-5.
16. Kamphuis W, Mamber C, Moeton M, et al. GFAP isoforms in adult mouse brain with a focus on neurogenic astrocytes and reactive astrogliosis in mouse models of Alzheimer disease. *PLoS One*. 2012;7(8):e42823.
17. Klein C, Butt SJ, Machold RP, Johnson JE, Fishell G. Cerebellum- and forebrain-derived stem cells possess intrinsic regional character. *Development*. 2005 Oct;132(20):4497-508.
18. Ponti G, Peretto P, Bonfanti L. A subpial, transitory germinal zone forms chains of neuronal precursors in the rabbit cerebellum. *Dev Biol*. 2006 Jun 1;294(1):168-80.
19. Decimo I, Fumagalli G, Berton V, Krampera M, Bifari F. Meninges: from protective membrane to stem cell niche. *American journal of stem cells*. 2012;1(2):92-105.
20. Nakagomi T, Molnar Z, Nakano-Doi A, et al. Ischemia-induced neural stem/progenitor cells in the pia mater following cortical infarction. *Stem Cells Dev*. 2011 Dec;20(12):2037-51.
21. Nakagomi T, Molnar Z, Taguchi A, et al. Leptomeningeal-derived doublecortin-expressing cells in poststroke brain. *Stem Cells Dev*. 2012 Sep 1;21(13):2350-4.
22. Bifari F, Decimo I, Chiamulera C, et al. Novel stem/progenitor cells with neuronal differentiation potential reside in the leptomeningeal niche. *Journal of cellular and molecular medicine*. 2009 Sep;13(9B):3195-208.
23. Ninomiya S, Esumi S, Ohta K, et al. Amygdala kindling induces nestin expression in the leptomeninges of the neocortex. *Neuroscience research*. 2013 Feb;75(2):121-9.
24. Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci*. 1997 May 15;17(10):3727-38.
25. Jin K, Galvan V, Xie L, et al. Enhanced neurogenesis in Alzheimer's disease transgenic (PDGF-APP^{Sw},Ind) mice. *Proc Natl Acad Sci U S A*. 2004 Sep 7;101(36):13363-7.

26. Curtis MA, Penney EB, Pearson AG, et al. Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain. *Proc Natl Acad Sci U S A*. 2003 Jul 22;100(15):9023-7.
27. Wang S, Okun MS, Suslov O, et al. Neurogenic potential of progenitor cells isolated from postmortem human Parkinsonian brains. *Brain Res*. 2012 Jun 29;1464:61-72.
28. Lee H, Kang JE, Lee JK, Bae JS, Jin HK. Bone-marrow-derived mesenchymal stem cells promote proliferation and neuronal differentiation of Niemann-Pick type C mouse neural stem cells by upregulation and secretion of CCL2. *Human gene therapy*. 2013 Jul;24(7):655-69.
29. Xin H, Li Y, Cui Y, Yang JJ, Zhang ZG, Chopp M. Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2013 Nov;33(11):1711-5.
30. Ekdahl CT, Claassen JH, Bonde S, Kokaia Z, Lindvall O. Inflammation is detrimental for neurogenesis in adult brain. *Proc Natl Acad Sci U S A*. 2003 Nov 11;100(23):13632-7.
31. Monje ML, Toda H, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. *Science*. 2003 Dec 5;302(5651):1760-5.
32. Belarbi K, Rosi S. Modulation of adult-born neurons in the inflamed hippocampus. *Frontiers in cellular neuroscience*. 2013;7:145.
33. Gemma C, Bachstetter AD. The role of microglia in adult hippocampal neurogenesis. *Frontiers in cellular neuroscience*. 2013;7:229.
34. Nikolakopoulou AM, Dutta R, Chen Z, Miller RH, Trapp BD. Activated microglia enhance neurogenesis via trypsinogen secretion. *Proc Natl Acad Sci U S A*. 2013 May 21;110(21):8714-9.
35. Ziv Y, Ron N, Butovsky O, et al. Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat Neurosci*. 2006 Feb;9(2):268-75.
36. Anderson WW, Collingridge GL. The LTP Program: a data acquisition program for on-line analysis of long-term potentiation and other synaptic events. *J Neurosci Methods*. 2001 Jul 15;108(1):71-83.
37. Anderson WW, Collingridge GL. Capabilities of the WinLTP data acquisition program extending beyond basic LTP experimental functions. *J Neurosci Methods*. 2007 May 15;162(1-2):346-56.

TABLES

Table 1: Primers and annealing temperatures used for real-time PCR

Primer	Sequence forward/reverse (5'-3')	Amplicon size (bp)	Annealing temp (°C)
Pax6	AGGTATTACGAGACTGGCTCC / TCCCGCTTATACTGGGCTATTT	104	60
Atoh1	GAGTGGGCTGAGGTAAAAGAGT / GGTCGGTGCTATCCAGGAG	151	60
Zic1	CAGTATCCCGCGATTGGTGT / GCGAACTGGGGTTGAGCTT	139	60
Zic2	CCGTTCCAGTGTGAGTTTGAG / CTTGCAGAGATAGGGCTTATCG	105	60
Hprt	GGTGAAAAGGACCCACGA / TCAAGGGCATATCCTACAACA	261	60

Table 2: List of primary and secondary antibodies used for immunofluorescence

Antibody	Company	Catalogue	Dilution
Primary			
Laminin	Sigma	L9393	1:2000
Ki67	BD	556003	1:250
MAP2	Abcam	ab11268	1:400
Zic1	Abcam	ab72694	1:200
Zic2	Abcam	ab12072	1:200
hNestin	Millipore	MAB5326	1:200
mNestin	Millipore	MAB353	1:200
AHN	Millipore	MAB1281	1:200
TuJ1	Covance	435P-0250	1:1000
Atoh1 (Math1)	Abcam	ab22270	1:200
Pax6	Millipore	MAB5552	1:200
NeuN	Millipore	MAB377	1:500
Calbindin 28	Swant	300	1:2000
Iba1	Wako	19-19741	1:1000
CD206	Serotec	MCA2235	1:50
BrdU	BD	347580	1:100
S100	Abcam	ab41548	1:3000
GABA _A R- α 6	Millipore	AB5610	1:1000
MAP2	Millipore	AB5622	1:500
Ki67	Abcam	Ab16667	1:200
SC121	Stemcellsinc	STEM121	1:1000
DCX	Abcam	ab18723	1:200
Secondary			
Donkey Anti-Mouse IgG (AF 488)	Invitrogen	A-21202	1:1000
Donkey Anti-Mouse IgG (AF594)	Invitrogen	A-21203	1:1000
Donkey Anti-Rabbit IgG (AF488)	Invitrogen	A-21206	1:1000
Donkey Anti-Rabbit IgG (AF594)	Invitrogen	A-21207	1:1000
Donkey Anti-Rat IgG (AF594)	Invitrogen	A-21209	1:1000

FIGURE LEGENDS

Figure 1. Differentiation of human ESCs into mature cerebellar granule neurons

(A) Design of the 6 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-C) Immunocytochemistry and cell counts of mature cerebellar granule neurons using early (TuJ1, Zic1, Zic2) and mature neuron-specific markers (MAP2, GABA_AR- α 6). Scale bar: 25 μ m. (D) Electrophysiological analysis of a mature cerebellar granule neuron at FD+7 showing a spontaneous action potential. Voltage was recorded on-line using current-clamp techniques. Data were analyzed using the LTP Program^{36, 37}.

Figure 2. Identification of the stage with maximum Atoh1 expression

(A) An observation of various differentiation stages of a modified HUES-4 line stably expressing green fluorescent protein localized to the nucleus under the control of the Atoh1 promoter shows that NS+8 neurospheres express the highest levels of GFP. Scale bar: 100 μ m. (B) Real time PCR and cell counting of early transcription factors expressed in GNPs confirm that the NS+8 stage corresponds to the stage at which the highest levels of Atoh1, Pax6, Zic1 and Zic2 are seen. Quantitative data are expressed as means \pm SEM for each group. Results were compared using a one-way ANOVA (GraphPad Prism Software). Significant effects are indicated by asterisks (*: $p < 0.05$, **: $p < 0.01$). (C) Immunocytochemistry for Atoh1, Pax6, Zic1 and Zic2 in NS+8 neurospheres. Scale bar: 25 μ m.

Figure 3. Cerebellar structure and behavioral analysis of GNP-transplanted Hq mice 2 months (8 weeks) and 4 months (16 weeks) post-transplantation

(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: 1mm for whole cerebella and 25 μm 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 \pm 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 μm for magnifications. Rotarod analysis shows that motor coordination decreases less rapidly in the GNP-treated group ($n = 14$) from 12 weeks post-transplantation onward when compared with the control group ($n = 12$). Results were compared using a 2-way ANOVA (GraphPad Prism Software).

Figure 4. Detection of neurons undergoing degeneration using Fluoro-Jade B

Fluoro-Jade B, a high-affinity fluorescent marker specific for degenerating neurons, allows for the detection of the degenerative process and stains both neurites and cell bodies. Fluoro-Jade B staining of GNP-treated and control Hq cerebella 2 months post-transplantation shows Purkinje cell bodies undergoing degeneration in the control group only (white arrows) Scale bar: 1 mm.

Figure 5. Detection and neuronal differentiation of human GNPs in the cerebellum of Hq mice 1 week and 1 month after transplantation

Immunohistochemistry of GNP-treated Hq cerebella using the human cytoplasmic SC121 antibody indicates that human GNPs survive one week after they have been transplanted (first inset). The co-labeling of human GNPs with the anti-human nucleus antibody (AHN) and neuronal antibodies shows that in animals in which GNPs have survived for 1 month post-transplantation (half of the animals), a number of human cells are labeled by antibodies to human nestin (which does not recognize mouse nestin), doublecortin (DCX), TuJ1, MAP2 and the granule cell transcription factors Zic1 and Zic2. Scale bar: 1 mm (whole cerebellum) and 25 μm (magnifications).

Figure 6. Ki67 labeling of transplanted GNPs at 1 month and reactive microglia at 1 and 2 months post-transplantation

(A) Immunohistochemistry of GNP-transplanted Hq cerebella 1 month post-transplantation shows that human cells express Ki67 only weakly and do not form tumors but are massively surrounded by macrophages expressing Iba1. Scale bar: 25 μ m. (B) Immunohistochemistry of GNP-transplanted and control Hq cerebella 2 months post-transplantation shows strong immunoreactivity for the macrophage-associated markers Iba1 and CD206 in the GNP-treated group only, indicating that a strong microglial reaction persists in the transplanted area, although human cells are not present anymore. Scale bar: 1mm. (C) Quantitative data are expressed as means \pm 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 post-transplantation 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: 1mm for whole cerebella, 50 μ m for lower panels and 25 μ m 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 one month later, i.e. 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: 1mm for whole cerebella, 25 μ m 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 \pm 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 μ m.

Figure 8. Neural progenitors in GNP-treated Hq mice stem from cerebellar leptomeninges 2 months after transplantation

(A) Immunohistochemistry of Hq cerebella (control and GNP-treated) and WT cerebellum (GNP-treated) 2 months post-transplantation shows the presence of Ki67-positive nuclei at the pial surface of cerebellar folia (white arrows). Scale bar: 50 μ m. **(B)** Co-labeling of Ki67-positive nuclei for mouse nestin shows that some progenitors are still able to proliferate in GNP-treated Hq cerebella (white arrows) and likely stem from the leptomeninges. AR: Arachnoid, PM: Pia mater, ML: Molecular layer. Scale bar: 25 μ m. Cell counts indicate that GNP-treated Hq animals had more Ki67-positive nuclei than control or non-operated Hq mice and that the latter two groups had more Ki67-positive nuclei than wild-type animals (WT). Quantitative data are expressed as mean percentages of WT values \pm SEM for each group. Results were compared using a one-way ANOVA (GraphPad Prism Software). Significant effects are indicated by asterisks (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). **(C)** Immunohistochemistry of GNP-treated Hq cerebella 2 months after transplantation for mouse nestin (green) and the adult Bergmann glial marker S100-beta (red) shows that the two populations, despite their similar morphologies, are distinct (see high magnification insets), and that nestin-positive cells are located either at the pial surface (PM), or in the molecular layer (ML). Scale bar: 25 μ m.

Figure 9. The cerebellar leptomeninges of Hq mice have a neurogenic potential *in vitro*

Cerebellar leptomeninges from 5-month-old Hq mice ($n = 5$) are able to survive and differentiate in Neurobasal medium *in vitro*, while those from wild-type animals degenerate ($n = 6$). After several days in culture on Matrigel-coated wells (Day *In Vitro* DIV17-20), leptomeninges from Hq mice form neurospheres that give rise to neuron-like cells. When dissected from GNP-treated Hq mice ($n = 4$), leptomeninges generate a greater number of neurospheres. Results were compared using a one-way ANOVA (GraphPad Prism Software). Significant effects are indicated by asterisks (**: $p < 0.01$). Staining of neurospheres and surrounding cells shows nestin expression. A number of neuron-like cells at a distance from the neurospheres start to express the neuronal marker MAP2 from DIV20. Scale bars: 50 μm , except for immunofluorescence images where they are equivalent to 25 μm .

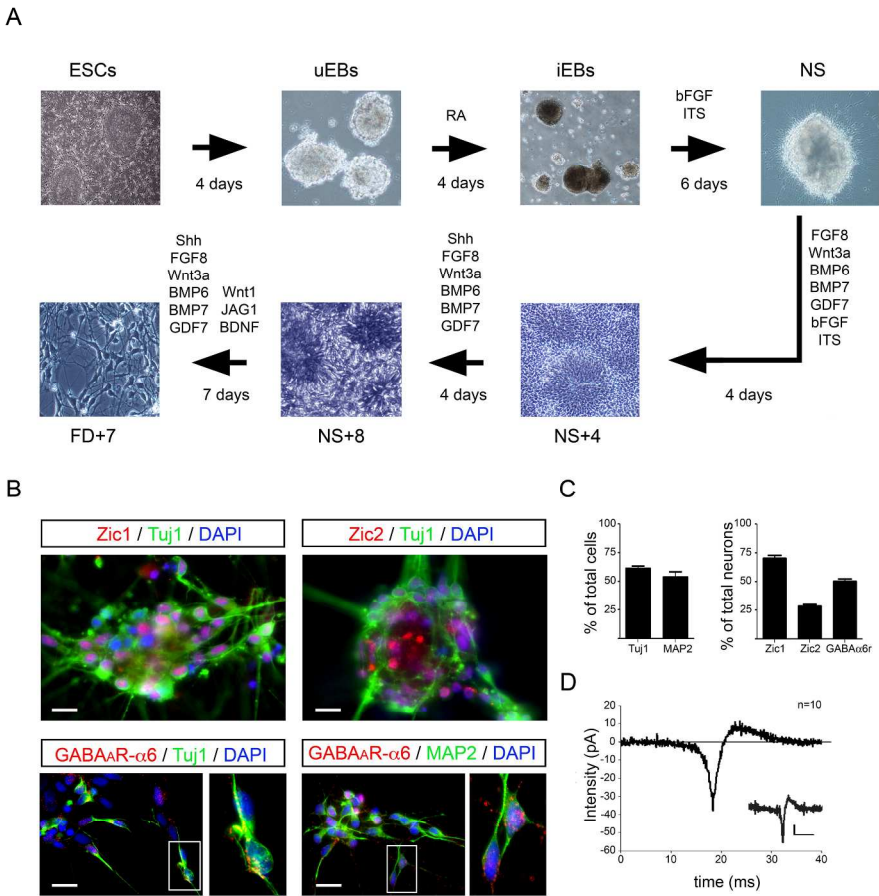
Supplementary Figure 1. Neural induction and microgliosis reaction in transplanted Hq mice 2 months after transplantation

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 Hq 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 mater. Scale bars: 100 μm (lower magnifications) and 50 μm (higher magnifications).

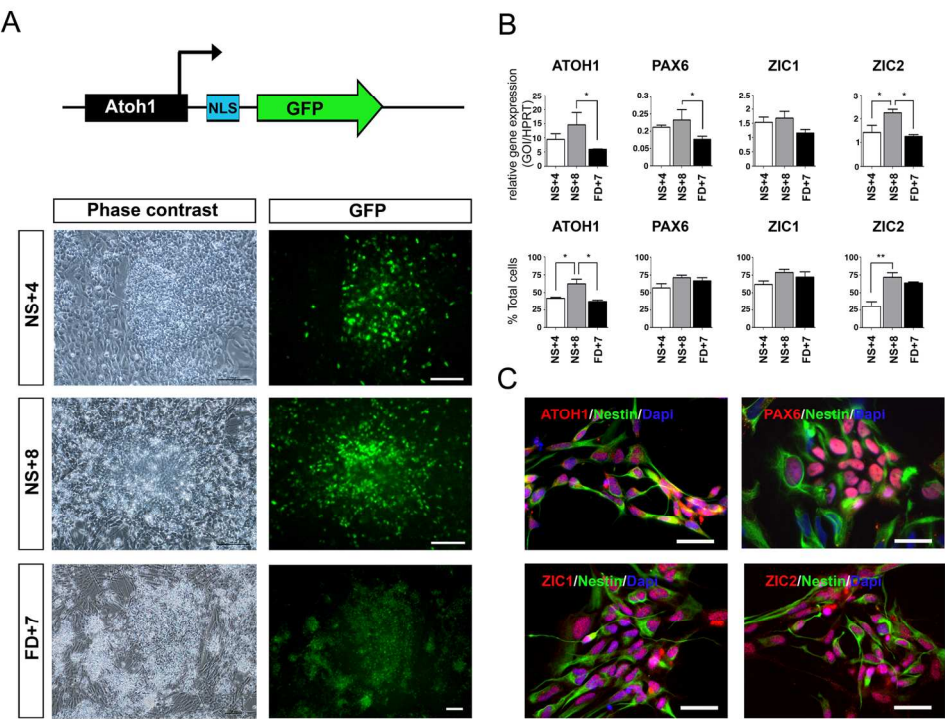
Supplementary Figure 2. Detection of human GNPs in the cerebellum of wild-type and nude mice 2 months after transplantation

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).



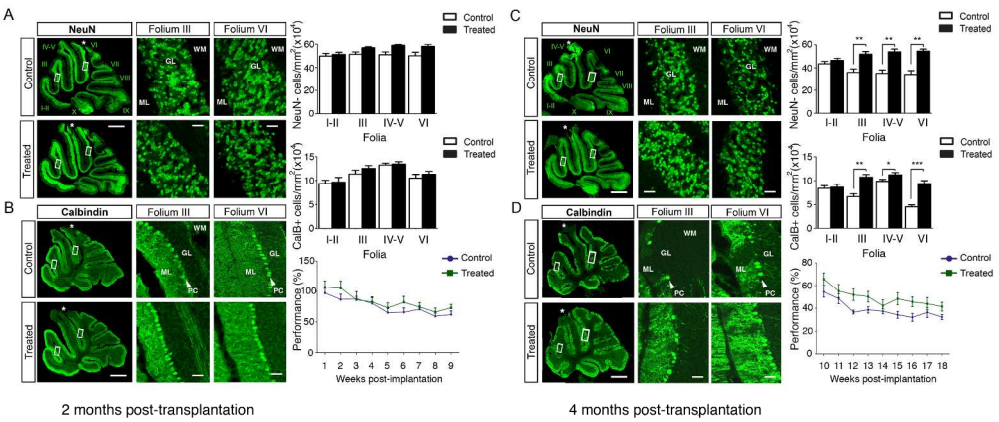
Kumar et al, Figure 1

228x306mm (300 x 300 DPI)



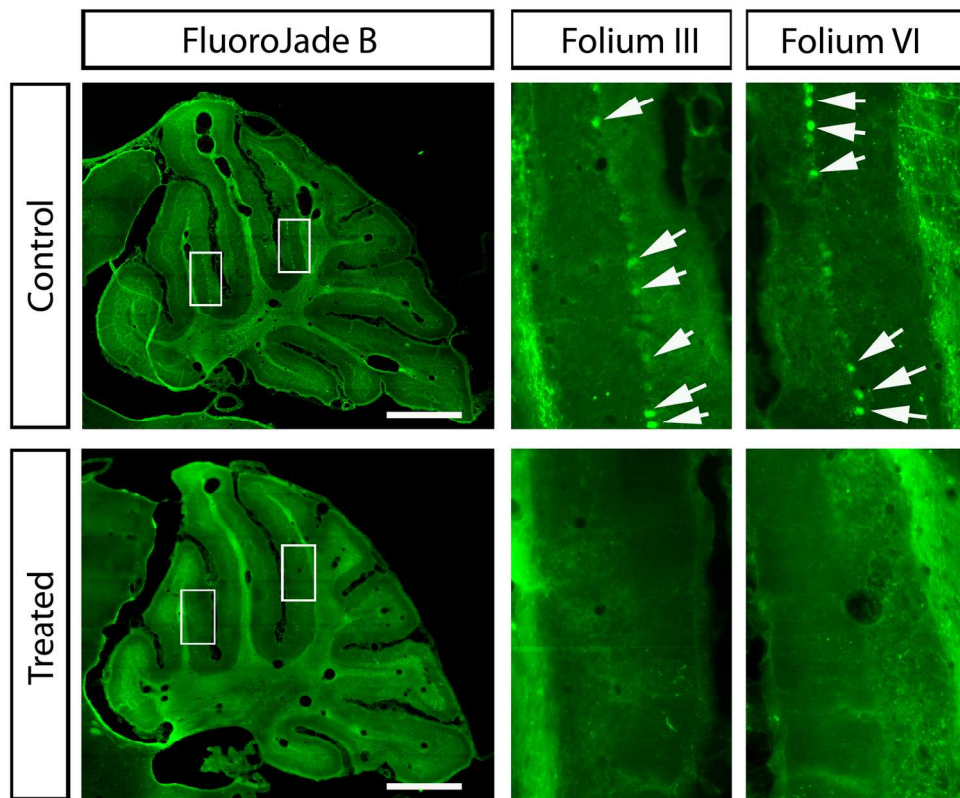
Kumar et al, Figure 2

170x160mm (300 x 300 DPI)



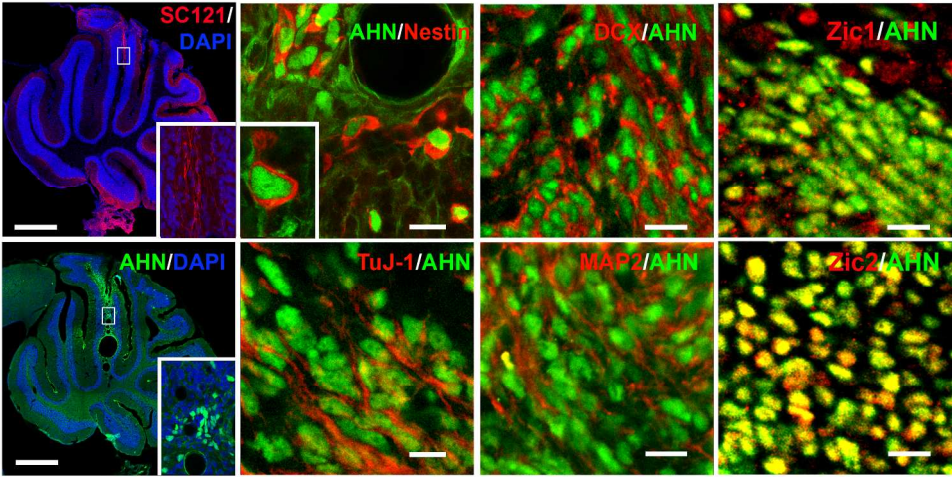
Kumar et al, Figure 3

289x149mm (300 x 300 DPI)



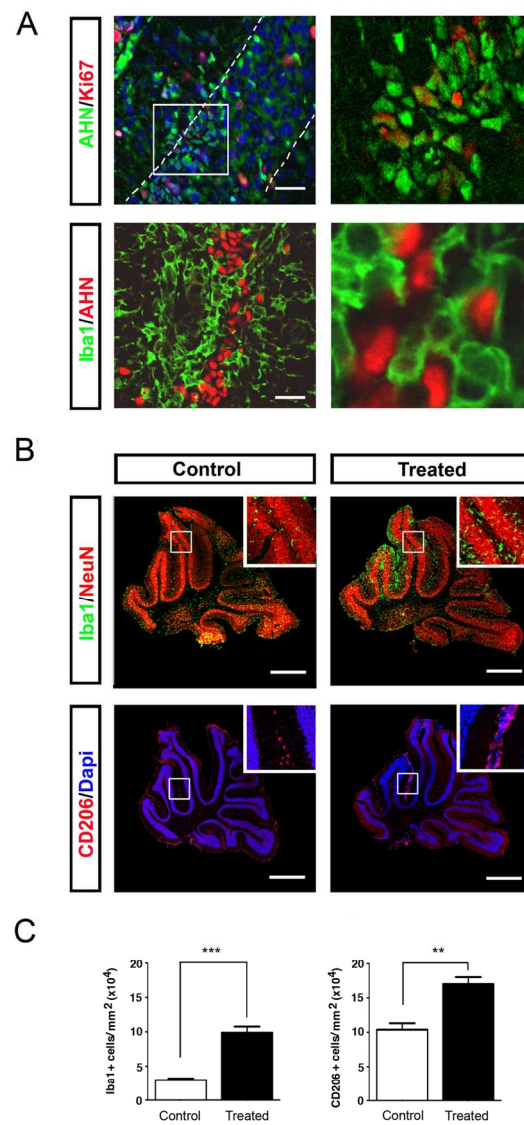
Kumar et al, Figure 4

164x175mm (300 x 300 DPI)



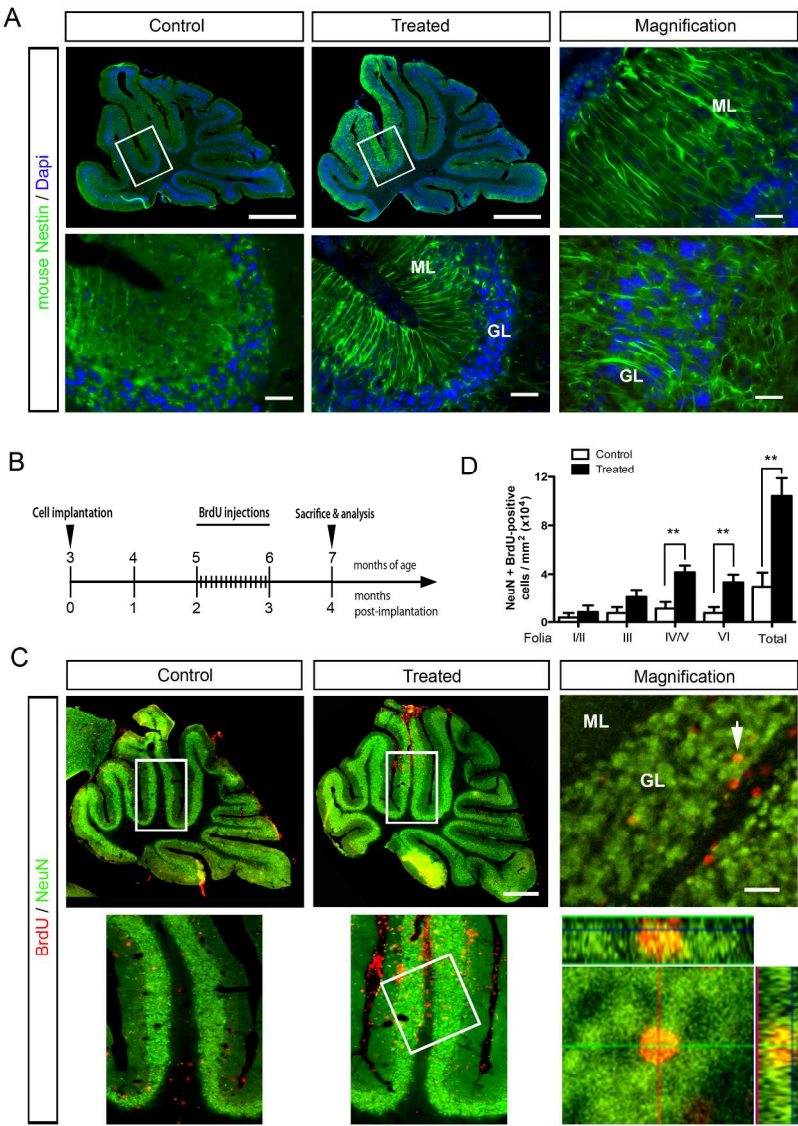
Kumar et al, Figure 5

225x202mm (300 x 300 DPI)



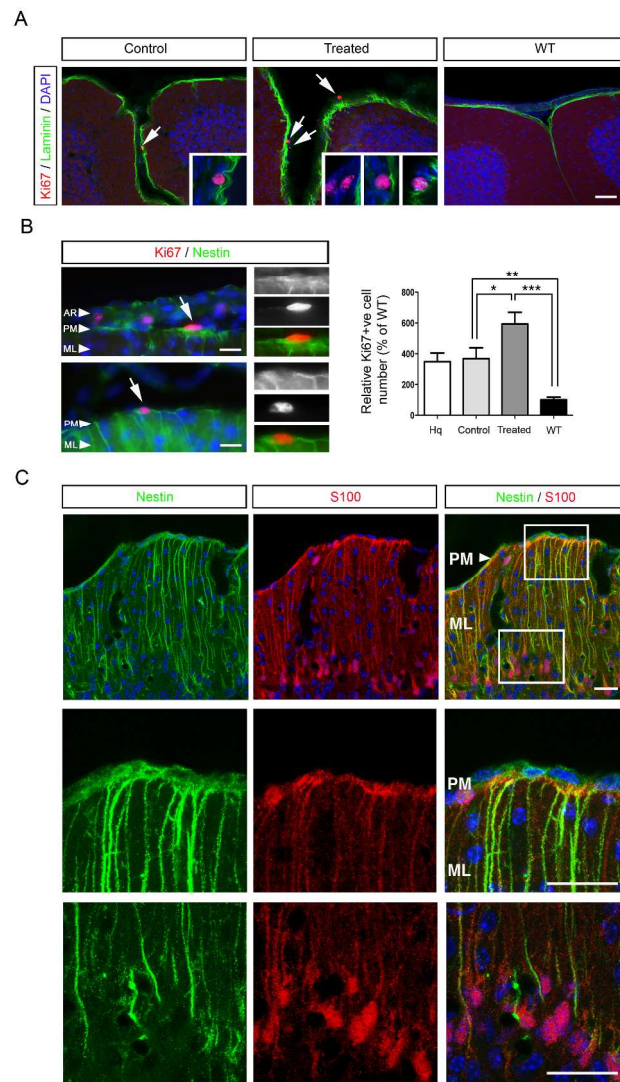
Kumar et al, Figure 6

129x186mm (300 x 300 DPI)



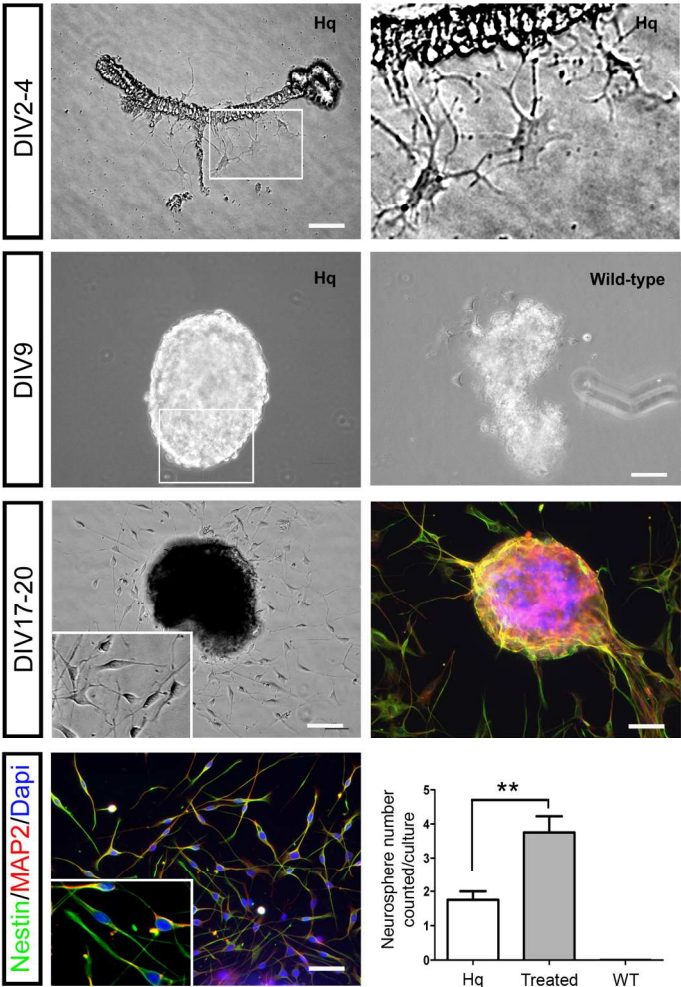
Kumar et al, Figure 7

199x280mm (300 x 300 DPI)



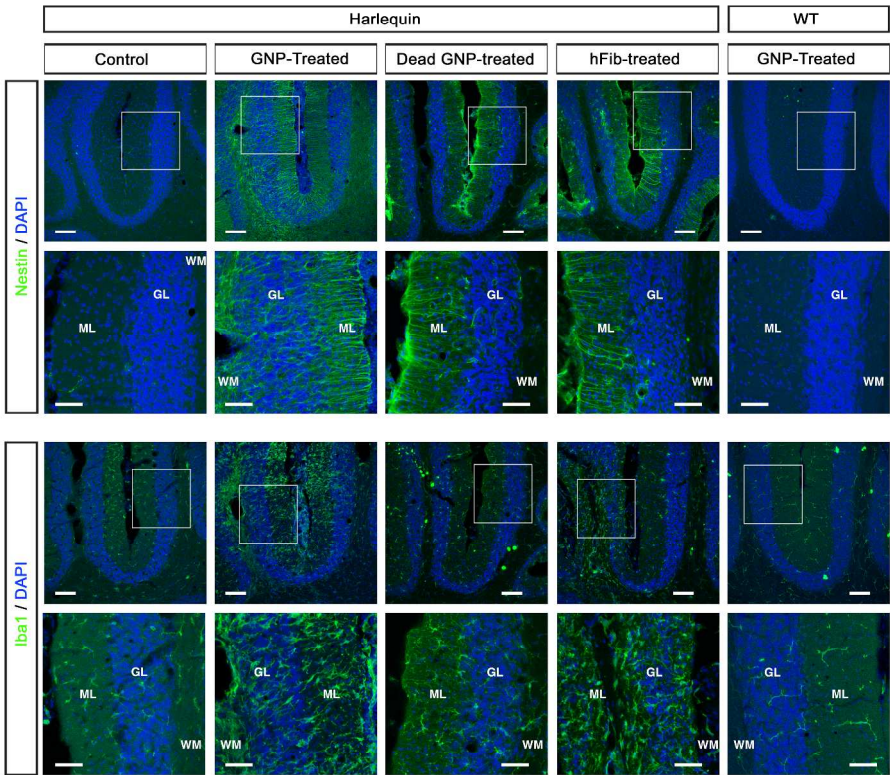
Kumar et al, Figure 8

199x362mm (300 x 300 DPI)



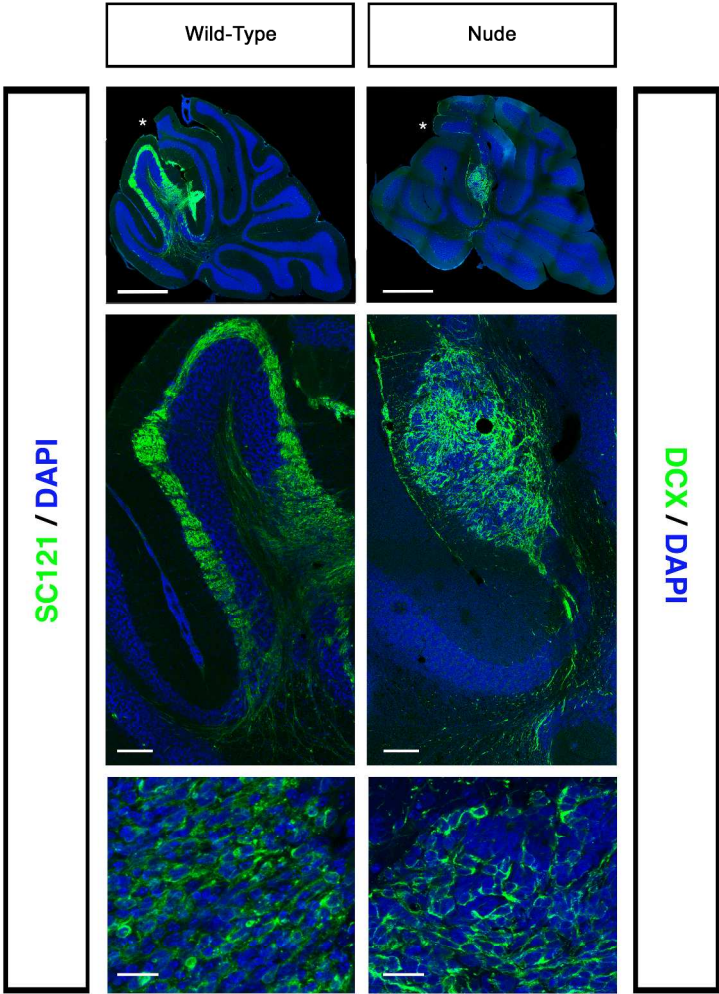
Kumar et al, Figure 9

122x199mm (300 x 300 DPI)



Kumar et al, Supplementary Figure 1

399x457mm (300 x 300 DPI)



Kumar et al, Supplementary Figure 2

299x400mm (300 x 300 DPI)