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Extensive fusion of haematopoietic cells with Purkinje neurons in response to chronic inflammation

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Abstract

Transplanted bone marrow derived cells (BMDCs) have been reported to fuse with cells of diverse tissues^{1–13}, but the extremely low frequency of fusion has led to the view that such events are biologically insignificant. Nonetheless, in mice with a lethal recessive liver disease (tyrosinaemia), transplantation of wild type BMDCs restored liver function by cell fusion and rescued the mice from death^{3,9}, indicating that cell fusion can have beneficial effects. Here we report that chronic inflammation resulting from severe dermatitis or autoimmune encephalitis leads to robust fusion of BMDCs with Purkinje neurons and formation of hundreds of binucleate heterokaryons, a 10–100 fold higher frequency than previously reported^{8,10,11,14}. Single haematopoietic stem cell transplants showed that the fusogenic cell is in the haematopoietic lineage and parabiosis experiments revealed that fusion can occur without irradiation. Species-mismatched bone marrow transplants resulted in activation of dormant rat Purkinje neuron-specific genes in BMDC nuclei post-fusion with mouse Purkinje neurons consistent with nuclear reprogramming. The precise neurological role of these heterokaryons awaits elucidation, but their frequency in brain after inflammation is clearly much higher than previously appreciated.

Although fusion of like cells has long been known to accompany the normal development of a number of tissues such as skeletal muscle, bone and the placenta^{15–17}, recent evidence from numerous laboratories indicates that bone marrow derived cells (BMDC) can fuse with

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AUTHORS CONTRIBUTIONS

C.B.J. Planned the project, executed all experiments, generated confocal-images, analyzed the data, wrote the manuscript and put together the figures.

S.Y. Participated in the EAE experiment.

K.K. Carried out the bone marrow transplantations (BMT) and helped with the parabiosis.

C.H. Helped with the immunohistochemistry and generated confocal-images.

R.D. Contributed to the data analysis and to the manuscript.

S.Y.C. Generated single-HSC transplanted mice.

L.S. Contributed to the writing of the manuscript.

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H.M.B. Planned the project, analyzed the data and wrote the manuscript.

cells of a number of tissues including skeletal muscle, liver, intestine, and brain^{1, 3, 4, 8–12}. However, the low incidence of fusion, which increases somewhat with tissue damage^{2, 4, 18}, has raised questions regarding its significance¹⁹.

In the brain, fusion of BMDCs appears to be restricted to the complex Purkinje neurons of the cerebellum^{8, 11, 14}. Such fusion events are not limited to rodents, but have also been observed in humans¹⁰. Purkinje neurons have extensive dendritic arbours with synapses to hundreds of thousands of other neurons. These neurons cannot be regenerated; all Purkinje neurons are present at birth and no stem cells are known to exist in the adult cerebellum. Accordingly, we hypothesized that the specificity of fusion of BMDCs for this particular cell type in the cerebellum could have biological significance, in which case conditions should exist that would yield higher frequencies and expression of dormant Purkinje genes in the BMDC nucleus would be expected.

Bone marrow (BM) consists of a heterogeneous cell population that includes stem cells, progenitors, and mature cells belonging to the haematopoietic, mesenchymal, and endothelial lineages. To test if cells of the haematopoietic lineage fused with Purkinje neurons, we transplanted lethally irradiated mice with single haematopoietic stem cells (HSCs) genetically labelled with green fluorescent protein (GFP)⁷. Two mice displaying robust peripheral blood (PB) reconstitution (34 and 48% GFP⁺ cells, respectively) were found to have GFP⁺ Purkinje neurons, demonstrating that derivatives of a single HSC can form cerebellar heterokaryons (Fig. 1a–c) at a low frequency similar to that observed with mice transplanted with whole BM (Fig. 1d). Thus, haematopoietic cells constitute the fusogenic cell type.

To track the cells that produce heterokaryons by bone marrow transplantation (BMT), myeloablation by lethal irradiation is required to generate a vacant niche for transplanted GFP⁺ cells to occupy. Lethal irradiation, however, is associated with transient cerebral inflammation and is known to disrupt the blood brain barrier (BBB) potentially leading to the extravasation of blood borne cells into the central nervous system (CNS)^{20, 21}. To address this concern, parabiosis was used to generate GFP⁺ blood chimerism without irradiation, a procedure which entails surgically joining two mice, the recipient wild type mouse and a donor mouse ubiquitously expressing GFP (Fig. 1e)²². Parabiotic mice develop a common anastomosed circulatory system leading to blood chimerism within a week from the surgery²². The contribution of GFP⁺ cells to Purkinje neurons in the wild type partner was analyzed at various time-points ranging from 12–58 weeks post-surgery (Fig. 1 and 2, and data not shown). While only rare cerebellar heterokaryons were detected after 12 weeks of parabiosis (data not shown), cerebella of mice harvested after 20–26 weeks consistently contained several GFP⁺ Purkinje neurons (mean: 24±14) and all heterokaryons expressed the Purkinje neuron-marker calbindin (Fig. 1f, g). These data provide evidence that the formation of cerebellar heterokaryons is not a result of the BMT procedure.

To distinguish whether cerebellar heterokaryons are (1) transient and constantly produced *de novo* or (2) stable and persist long-term, we designed an experiment using the parabiosis model. We reasoned that during parabiosis, blood chimerism would be approximately 50%, whereas following separation from the GFP⁺ partner, the chimerism of the separated non-

GFP⁺ partner would fall rapidly as reported by others²². Detection of heterokaryons in this partner post-separation would suggest long-term heterokaryon survival, as new heterokaryon formation would not be possible. Two parabionts without dermatitis were joined for 38 weeks at which time the blood chimerism was 51 and 56%, respectively. Five weeks post separation the blood chimerism had dropped to 8 and 12%, respectively, and by 20 weeks post-separation, the chimerism was somewhat lower, 6 and 10% respectively. In both cerebella, heterokaryons were detected suggesting long-term survival (Fig. 1h). However, we cannot definitively rule out fusion and *de novo* heterokaryon production after separation, because GFP⁺ cells were still present in the circulation, albeit in greatly reduced proportions.

In contrast to the low frequency of heterokaryons observed in the majority of parabionts, we detected several hundred GFP⁺ Purkinje neurons per mouse in some parabionts. In four parabionts (20–24-week-old), we detected 266±30 heterokaryons and in one 58-week-old parabiont as many as 596 heterokaryons were detected (Fig. 2). An analysis of different parts of the cerebellum that are functionally and anatomically distinct, the vermis and the larger hemispheres, did not reveal clustering or preferential localization when the relative size of these regions was taken into account (Supplementary Information, Fig. S1). A common feature of these mice was evidence of persistent dermatitis with erythematous large ulcers, initiated around the scar on the dorsal flank encompassing an area between 30 to 40 mm² (Fig. 2b). These ulcers were commonly infected with bacteria that likely contributed to the chronic inflammation. Idiopathic ulcerative dermatitis (IUD) of unknown aetiology is a well-recognized disease in aged C57BL/6 mice. This disorder manifests with a prevalence of 4.1% as a pruritic dermatitis leading to self-mutilation, dermal ulceration, necrosis, and fibrosis²³. Chronic dermatitis induces an increase in circulating monocytes and neutrophils, suggesting that its effects are not solely local. The presence of systemic inflammation in the parabionts was assessed as the frequency of myelomonocytic cells (CD11b⁺ and/or Gr-1⁺ cells) in the PB of healthy pairs or pairs with dermatitis (Fig. 2c, d). A considerable increase in circulating myelomonocytic cells was detected in parabionts with dermatitis (mean: 78±4%) compared to normal parabiotic mice of the same age (mean: 30±4%). By contrast, very few GFP⁺ microglia were detected in the cerebella of parabionts with or without dermatitis, in agreement with the findings of others²⁴, suggesting that the BBB are not compromised in these mice and that microglia are not the fusogenic haematopoietic cell type under these conditions (Figs. 1f and 2e, f). However, other cells cannot be ruled out, such as lymphocytic cells which play a central role in chronic inflammation, particularly in dermatitis.

To determine if dermatitis triggers cerebellar heterokaryon formation independently of parabiosis, C57BL/6 mice were irradiated and transplanted with GFP⁺ BM. The frequency of cerebellar heterokaryons was analyzed in the subset of transplanted mice that developed significant dermatitis by 52–54 weeks of age. In five of these mice several hundred cerebellar heterokaryons were detected (mean: 336±115) suggesting that their induction by chronic inflammation is independent of parabiosis (Fig. 3a). Consistent with our findings in parabiotic mice with dermatitis, an increase in myelomonocytic cells in PB provided additional evidence of ongoing inflammation, (mean: 64±10% [n=5]) compared to healthy

BMT mice of the same age (mean: $36 \pm 4\%$ [$n=3$]). These studies clearly implicate dermatitis and chronic inflammation in heterokaryon formation, in both parabionts and BMT mice.

A limitation of the dermatitis model is that the onset of the skin lesion is sporadic and cannot be controlled. We therefore sought a means to induce inflammation in a controlled manner. Further, we wished to test the role of bone marrow derived (BMD) macrophages in the brain, which are known to have the capacity to fuse in certain settings forming giant cells, for example in the mouse model of amyotrophic lateral sclerosis²⁵. We postulated that factors that caused an increase and activation of macrophages/microglia would lead to increased numbers of cerebellar heterokaryons. To test this hypothesis, we performed experiments using a well characterized compound, lipopolysaccharide (LPS), an endotoxin with a strong immunogenic response associated with leukocyte extravasation *in vivo* and activation of microglia leading to secretion of cytokines, chemokines, and prostaglandins²⁶. Three 20-week-old BMT mice were chronically exposed to LPS before being sacrificed. Although we detected an abundance of perivascular GFP⁺ BMD macrophages/microglia in the cerebellum and the cerebral cortex (Fig. 3b) as well as a minor increase in circulating myelomonocytic cells (mean: $38 \pm 5\%$) compared to the control group (mean: $26 \pm 4\%$), there were surprisingly few cerebellar heterokaryons (mean: 5 ± 1). Thus, consistent with our previous findings in muscle²⁷, increased accumulation of perivascular macrophages as well as activation of microglia is not indicative of increased cerebellar heterokaryon formation due to a stochastic process. The lack of correlation between the frequency of microglia and heterokaryons is consistent with the findings that in parabiotic animals only rare cerebellar GFP⁺ microglia are detected, yet large numbers of cerebellar heterokaryons.

In a second approach to control the onset of inflammation, we performed experiments using experimental autoimmune encephalitis (EAE), a mouse model of the human disease multiple sclerosis. EAE is associated with extravasation of monocytic cells and T-lymphocytes, followed by demyelination, axonal damage and neuronal death^{28–31}. As a result, both cerebellar oligodendrocytes and Purkinje neurons are lost. We postulated that a combination of CNS inflammation and stress to Purkinje neurons in the cerebellum might create a microenvironment conducive to cerebellar heterokaryon formation. Six mice received a BMT from GFP⁺ donors, followed 31 weeks later by injection of the immunogenic myelin oligodendrocyte glycoprotein (MOG) to induce EAE. In these animals, hundreds of GFP⁺ heterokaryons with typical Purkinje neuron morphology (mean: 402 ± 300 , [mouse #0127 was not included in the mean]) were detected within 44 to 65 days post-immunization (Fig. 4a–d) compared to the control group (mean: 13 ± 3). Indeed, as many as 2,364 cerebellar heterokaryons were found in a single cerebellum. Due to the progressive cerebellar destruction characteristic of the disease, the numbers of heterokaryons detected are variable and may be underestimates of the total frequency. A considerable increase in circulating myelomonocytic cells was detected in the EAE group (mean: $71 \pm 19\%$) compared to the control group (mean: $41 \pm 4\%$), indicative of systemic inflammation. These data demonstrate that endogenous factors associated with a well-characterized inflammatory CNS condition (EAE), leads to an increase in the frequency of cerebellar heterokaryons.

A major question concerns the fate of the fused BMDC nucleus after fusion. Cerebellar heterokaryons are binucleated cells formed naturally by the fusion of a BMDC to a Purkinje

neuron, wherein each nucleus initially expresses its own distinct repertoire of genes. In these heterokaryons, both the nucleus of the BMDC and the Purkinje neuron are exposed to transcription factors specific to the other fusion partner, potentially leading to activation of dormant genes. In theory, due to equivalent chromosomal input, each of the two individual nuclei could persist in expressing its own nuclear program. Alternatively, one program might dominate causing the other nucleus to express previously silent genes, but it is unclear whether the neuronal or the haematopoietic programs would be dominant. To determine whether endogenous patterns of gene expression were altered in the nuclei of BMDCs following fusion with Purkinje neurons, we performed a BMT between two different species followed by tissue- and species-specific reverse-transcriptase (RT)-PCR. Despite immunological differences, transplants of rat BM into mice lead to the engraftment and reconstitution of the mouse BM and PB with rat cells, a method that has been extensively used for studying tolerance in skin graft experiments³². Lethally irradiated female C57BL/6 mice were transplanted with whole BM from Sprague-Dawley rats ubiquitously expressing transgenic GFP³³. Recipient PB reconstitution was efficient (mean: 96±2%; Fig. 5a, c), and transplanted mice survived up to 1.5 years. Rat BMDCs fused to form numerous chimeric heterokaryons (mean: 74±59) per cerebellum within 22 weeks (Fig. 5b, c). To determine if these heterokaryons were binucleated, we scored one hundred GFP⁺ Purkinje neurons in cerebella of four mice post BMT. In all cases in which the soma was intact allowing nuclei to be distinguished and scored, two nuclei were clearly evident (Supplementary Information, Fig. S2). To further examine the fate of the BMDCs nuclei post-fusion, a species-specific transcription profile of rat-mouse chimeric Purkinje heterokaryons was generated using total RNA from whole cerebella of rat BMT mice. Primers were tested for tissue and species specificity (Supplementary Information, Fig. S3, S4). The rat Purkinje neuron-specific transcripts Purkinje cell protein 2 (*Pcp2*)¹¹, vitamin D-dependent calcium-binding protein (*Calb1*)¹¹, neuron voltage-gated potassium channel protein 1 (*Kcnc1*)³⁴ and G-substrate, (*Gsbs*)³⁵, were detected in mouse cerebella after rat BMT (Fig. 5d). Confirmation that markers reported to be neural-specific did not detect gene products in other tissues, such as BM and PB proved to be critical (Supplementary Information, Fig. S3a–c). In addition, using immunohistochemistry, haematopoietic markers such as CD45, CD11b, Iba1, and F4/80 were not detected as described in our previous studies of cerebellar heterokaryons¹¹, providing evidence for repression of haematopoietic gene expression. Although activation of a Purkinje neuron-specific transgene, *Pcp2-GFP* was previously detected¹¹, these data provide the first evidence of activation of expression of endogenous neuron-specific genes in BMDC nuclei after fusion in cerebellar heterokaryons. Taken together these results demonstrate that the nuclei of BMDCs in Purkinje heterokaryons activate previously silent genes typical of mature Purkinje neurons and repress haematopoietic genes, consistent with the hypothesis that they are reprogrammed to a Purkinje neuron fate.

Several groups including ours have now shown that BMDCs can contribute to non-haematopoietic tissues by cell fusion^{1–13} but the low incidence of fusion events under normal conditions has raised questions regarding their significance during life. Here we demonstrate that under specific conditions of stress, exemplified by chronic inflammation, the frequency of fusion is markedly increased and hundreds of heterokaryons are detected per cerebellum using diverse experimental paradigms. It has been postulated that cerebellar

heterokaryon formation is the consequence of experimental manipulations associated with the BMT procedure, such as lethal irradiation leading to damage of the BBB and to the induction of a “cytokine storm”³⁶. However, we detected Purkinje neuron heterokaryons in surgically joined mice (parabionts) which develop PB chimerism in the absence of irradiation and maintain a normal BBB demonstrating that heterokaryon formation is independent of irradiation. In addition, we show here that the fusogenic cell type is a haematopoietic derivative by detecting heterokaryons in the cerebella of mice transplanted with a single GFP⁺ HSC. However, despite clear signs of systemic inflammation, including an increase in myelomonocytic cells in the PB, no correlation was observed between infiltration of donor macrophages/microglia and the formation of heterokaryons in cerebella. Finally, activation of endogenous Purkinje neuron-specific genes such as *Calb1*, *Pcp2*, *Kcnc1* and *Gsbs* in BMDC nuclei after fusion together with the absence of haematopoietic proteins (CD45, CD11b, F4/80 and Iba1) demonstrates that the nuclei of BMDCs in Purkinje heterokaryons activate previously silent genes typical of mature Purkinje neurons and repress haematopoietic genes, consistent with the hypothesis that they are reprogrammed to a Purkinje neuron fate.

The unexpected finding that immunologic factors such as inflammation can modulate heterokaryon formation in the cerebellum supports the notion that such factors could play a role in limiting the loss of structural neurons, such as Purkinje neurons, that are presently impossible to replace in adults. Mimicking this phenomenon by directed fusion may constitute a functional therapeutic strategy. The unprecedented increase in heterokaryons in the brain reported here in response to inflammation was unexpected, and an elucidation of its role clearly warrants further attention.

METHODS

Animals

GFP⁺CD45.1 C57BL/6 and C57BL/6 mice were either bred in-house or from Jackson Laboratory. Sprague Dawley rats ubiquitously expressing GFP (kind gift of Dr Okabe, Osaka University) were bred in-house and maintained in a pathogen-free environment with free access to water and food in a 12 h light/dark environment.

All animal experiments were approved by the University of British Columbia Animal Care Committee as well as approved by the Administrative Panel on Laboratory Animal Care at the Stanford University School of Medicine.

Bone marrow transplantation

All mice were bone marrow transplanted at six to eight week-old as described in Weimann et al.¹¹.

Perfusion of mice and harvesting of cerebellum

Mice were sacrificed and perfused at various times after BMT as described in Johansson et al.³⁷. Briefly, mice were deeply anesthetized and immediately perfused with PBS followed by ice-cold 4% paraformaldehyde in phosphate buffer and cryoprotected in sucrose/

phosphate buffer solution overnight (O/N). The cerebellum was sectioned at 30, 50, or 60 μm for antibody staining using a sliding microtome (SM2000R; Leica, IL).

Immunofluorescence

Free-floating sections were incubated O/N at 4°C with rabbit anti-GFP (1:2000; Molecular Probes, OR) and mouse anti-calbindin (1:1000; Sigma, MO) followed by washing and incubation of appropriate secondary antibodies (goat anti-mouse Alexa 546; 1:1000 and goat anti-rabbit Alexa 488; 1:1000, Molecular Probes) O/N at 4°C and mounted with ProLong Gold (Molecular Probes)¹¹.

Quantification of cerebellar heterokaryons

The entire cerebellum was sectioned and scored for cerebellar heterokaryons, identified based on the typical Purkinje neuron morphology and the co-expression of GFP and calbindin.

Isolation and transplantation of single haematopoietic stem cells into mice

Isolation and transplantation of single HSCs (SPKLS, side population, c-Kit⁺, Lineage⁻, and Sca1⁺) was performed as previously described in Corbel et al.⁷. The chimerism in single HSC transplanted mice was assessed as in Corbel et al.⁷.

Isolation and transplantation of rat bone marrow into mice

Bone marrow was isolated from 8- to 10-week-old GFP⁺ Sprague Dawley rats by flushing femurs and tibia with PBS supplemented with 2% FBS and 2 mM EDTA. Red blood cells were lysed for 10 minutes in an erythrocyte lysis buffer, collected, and resuspended in PBS supplemented with 2% FBS and 2 mM EDTA. Recipient female mice (8- to 10-week-old) were lethally irradiated with 2 doses of 4.8 Gy 3 hr apart. Each irradiated recipient received 5×10^6 unfractionated rat BM cell-suspension by tail vein injection within 2 hr of the second irradiation dose.

Parabiosis

Parabiotic pairs were generated as described previously^{38, 39}. Briefly, female C57BL/6 mice of equal size and age (8–16 week-old) were anesthetized and shaved on either their right or left side. The shaved skin was cleaned and an incision was made from the olecranon to the knee joint. A double 4-0 silk suture and tie was used to join the opposing olecranon and knee joints, and opposing dorsal and ventral skins were closed by 9 mm wound clips (Becton-Dickinson, NJ). Each pair was housed separately. The degree of PB chimerism as well as assessment of myelomonocytic cells was evaluated by retro-orbital or tail vein bleeding followed by analysis on a FACSCalibur flow cytometer (Becton-Dickinson) using rat anti-mouse CD45-PE, CD11b-biotin and Gr-1-biotin antibodies and streptavidin-APC (all from eBioscience, CA).

LPS treatment of bone marrow transplanted mice

Three BMT female C57BL/6 mice were treated, twenty weeks post-BMT with 1,2 mg/kg LPS (LPS from *E. coli* 0111 B4, Sigma) by intraperitoneal injection for four weeks before they were perfused and analyzed.

Experimental autoimmune encephalomyelitis induction

EAE was induced as described in Youssef et al.⁴⁰ in 37–39-week-old female C57BL/6 mice previously transplanted with BM from GFP⁺CD45.1 C57BL/6 mice at 6- to 8-week of age. Briefly, mice were immunized with 100 µg of myelin oligodendroglial glycoprotein (MOG) p35–55 in the flanks. MOG was dissolved in PBS and mixed with complete Freund's adjuvant containing 2 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, NJ). On the day of immunization and 48 h later mice were injected intravenously with 75 ng of *Bordetella pertussis* toxin in PBS. Mice are examined daily for clinical signs of EAE and scored as in Youssef et al.⁴⁰. The mice were perfused as described in Johansson et al.³⁷ at either 44 or 65 days post EAE induction.

RNA extraction

The cerebellum (rat or mouse) was rapidly removed and placed in RNAlater (Ambion, TX) on ice. RNA was extracted using RNeasy Plus Mini Kit (Qiagen, CA) according to the manufacturer's protocol for animal tissue. Briefly, 25–30 mg of the cerebellum was homogenized in 700 µL RLT buffer in Lysis Matrix D tube in a Fastprep FP 120 apparatus (MP Biomedicals, OH), speed 6.5 for 45 seconds. The supernatant was transferred to a QIAshredder- followed by a gDNA Eliminator spin column and RNA was collected using the RNeasy spin column and eluted in RNase-free water. RNA was further treated with RNase-free DNase for 20 minutes followed by RNA cleanup and stored at –80°C. Total RNA from rat and mice PB and BM was extracted as above but homogenized by vortexing the cells in the RLT buffer for 1 minute after first been lysed in red blood cell lysis buffer for 5 minutes on ice.

RT-PCR

Total RNA (500 ng) was reverse transcribed using Superscript First-Strand synthesis system according to the manufacturer's recommendation (Invitrogen, CA) in a final volume of 20 µL. The reaction was carried out with 5 minutes at 65°C, 2 minutes at 42°C, 50 minutes at 42°C, 15 minutes at 70°C followed by 20 minutes at 37°C. Tissue- and species-specific primers were designed by aligning mouse and rat RNA using NCIB BLAST (bl2seq). The primers designed for brain-, including Purkinje neuron-specific genes were tested for tissue- as well as species- specificity by using 1 µL cDNA from either rat or mouse PB, BM and cerebellum. Four primers were tissue- and species-specific for the following genes *Calb1*, *Pcp2*, *Kcnc1* and *Gsbs*. To detect rat genes in mice BMT with rat BM, the PCR reaction contained 4 µL of RT product, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.2 mM of each primer, and 2.0 units Platinum Taq DNA Polymerase was amplified using a Peltier Thermal Cycler (MJ Research, CA). Cycling conditions were 3 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 59 or 63°C, and 60 seconds at 72°C. The PCR products were run on a 1.5% agarose gel with ethidium bromide. Following primers 5'-3' were used, rat

Gsbs forw., AAAAGACACACCTGCCTTCCATG, rev.,
 AAGGTGGGGCTACAAAGCAACT, 316 bp; rat *Kcnc1* forw.,
 ACTCTAACCACCACCGAGAACC, rev., GCATAGTTCTTGGTTTTCCGAGC, 294 bp;
 rat *Calb1*, forw. TAGGAATTGTAGAGTTGGCCCAT, rev.,
 GGCAAGATCTGTTCCGGTACAG, 550 bp; rat *Pcp2*, forw.,
 GCTTCTTCAACCTGCTGAG, rev., GAGGGCTGAGTGTCCCG, 220 bp; rat *Gapdh*
 forw., ATCATCTCCGCCCTTCCGCT, rev., CTTTAGTGGGCCCTCGGCC, 480 bp;
 mouse *Gapdh*, forw., TAACATCAAATGGGGTGAGGCC, rev.,
 CCTTCAGTGGGCCCTCAGAT, 550 bp.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Progeny of a single HSC can form heterokaryons with Purkinje neurons and the formation of cerebellar heterokaryons is independent of variables associated with bone marrow transplantation. (a) Schematic of the experimental design for BMT. Bone marrow from GFP mice was harvested and enriched for HSCs by FACS. A single HSC was transplanted with 10^6 helper cells into a lethally irradiated wild type mouse. (b) A high power laser-scanning confocal image from a cerebellar sagittal section of a mouse transplanted with a single HSC. Immunohistochemistry with specific antibodies demonstrates the presence of GFP in a single cerebellar heterokaryon (green/yellow) co-expressing calbindin (red/yellow) in Purkinje neurons. The GFP⁺ Purkinje neuron is located in the Purkinje cell layer (PCL) with dendrites extending into the molecular layer (ML). The only output connection from the cerebellum to the cerebellar nucleus is mediated by a single axon (arrow) descending through the granular cell layer (GCL). (c, d) Quantification of heterokaryon formation in the single HSC and whole BM transplantation paradigm, respectively. (e) Schematic of the experimental design to form parabionts. Age- and weight-matched female mice (a wild type, recipient and a transgenic, ubiquitously expressing GFP, donor) are surgically joined from the olecranon to the knee. (f) Immunohistochemistry demonstrates the presence of two heterokaryons, (GFP⁺ Purkinje neurons), the fusion product of a GFP⁺ BMDC and a Purkinje neuron expressing calbindin (yellow/red). (g) Quantification of cerebellar heterokaryons formed in conjoined parabionts without ulcerative dermatitis for 20 or 26 weeks. No gross pathology was detected in these mice such as dermatitis. (h) Quantification of cerebellar heterokaryons following separation of parabionts. Parabionts were conjoined

for 38 weeks before being separated for 20 weeks, during which blood chimerism fell from 51 and 56% to 6 and 10%, respectively. Although *de novo* heterokaryon production cannot be ruled out since some GFP⁺ cells remained present in the circulation, the presence of heterokaryons when GFP⁺ cells were markedly reduced in the circulation is consistent with their persistence over time. Scale bars (a) 50 μm and (b) 100 μm .

Figure 2.

Idiopathic ulcerative dermatitis modulates cerebellar heterokaryons.

A subset of parabionts developed a chronic dermatitis, consistent with idiopathic ulcerative dermatitis. **(a, b)** Hematoxylin and eosin (H&E) staining of skin biopsies from a parabiont without dermatitis **(a)** and a parabiont with severe dermatitis **(b)**. **(a)** Normal skin with all dermal structures, a continuous two-three-cell layer, epidermis (ED), dermis (DE), subcutaneous fat, and panniculus carnosus (PC). **(b)** Severe dermatitis. The skin is characterized by ulceration, serocellular crusting (SC) total loss of the epidermis and necrosis of the superficial dermis. Normal dermal structures are absent and the dermis is severely thickened by fibrosis and chronic inflammation extending to the panniculus carnosus (PC). **(c, d)** Representative FACS plots of PB from a normal mouse **(c)** and from a mouse with dermatitis **(d)** demonstrating the presence of inflammatory cells in normal mice and in mice with dermatitis. The peripheral blood was stained for CD45PE, CD11b-biotin-APC and Gr-1-biotin-APC and analyzed with a FACSCalibur using Flow-Jo and CD45⁺ cells were plotted as CD11b/Gr-1⁺ and GFP⁺. **(e, f)** Cerebellar sagittal sections from a mouse (#21) with severe dermatitis. Immunohistochemistry with antibodies, to GFP (green) and to calbindin (red) demonstrates that numerous heterokaryons have been formed in the cerebellum. **(f)** A high-power laser-scanning confocal image of heterokaryons shows their normal morphology with GFP⁺ cell bodies, dendrites extending into the molecular layer and an axon descending through the granular cell layer. Scale bars **(a, b)** 200 μ m, **(e)** 100 μ m, and **(f)** 20 μ m.

Mouse (id)	Time after BMT (weeks)	Blood chimerism (%)	Heterokaryons (n)
#002	54	99	317
#004	54	99	518
#005	54	99	363
#177	52	98	262
#762	52	98	220

Mouse (id)	Time after BMT (weeks)	Blood chimerism (%)	Heterokaryons (n)
#914	20	98	4
#916	20	99	6
#917	20	99	5

Figure 3.

Heterokaryon formation in bone marrow transplanted mice with inflammation due to idiopathic ulcerative dermatitis increased but not in LPS induced inflammation.

(a, b) Immunohistochemistry of cerebellar sagittal sections with antibodies to GFP (green) and to calbindin (red) show several green/yellow heterokaryons. (a) Several hundred heterokaryons were detected in cerebella of BMT mice 52–54 weeks post transplantation. (b) Few heterokaryons were formed in the cerebella of mice treated for four weeks with intraperitoneal injections of LPS. Note the increased infiltration of GFP⁺ macrophages/microglia cells in BMT and the LPS treated mice relative to parabionts in (Fig. 1f, 2e). Scale bar 100 μ m.

BMT mice with EAE

Mouse (id)	Time after BMT (weeks)	Duration of EAE (days)	Blood chimerism (%)	Heterokaryons (n)
#2120	40	0	98	10
#2121	"	"	99	12
#2122	"	"	99	18
#2123	"	"	99	12
#0120	44	44	98	185
#0121	"	"	98	844
#0123	"	"	98	220
#0089	47	65	98	176
#0127	"	"	98	2364
#0176	"	"	99	586

Figure 4.

Increased heterokaryon formation in mice with experimental autoimmune encephalitis (EAE).

(a–d) Immunohistochemistry of cerebellar sagittal sections with antibodies to GFP (green) and to calbindin (red). (a) Low power laser-scanning confocal image shows extensive infiltration of GFP⁺ macrophages/microglia cells and a heterokaryon with a GFP⁺ cell body (arrow) in sagittal section from mouse #0121. (b) High power laser-scanning image shows a heterokaryon (arrow in a) with dendrites in the molecular layer and a descending axon. Several macrophages/microglia cells are present around the heterokaryon and in the granular cell layer. (c) Low power laser-scanning image from an area with a major loss of Purkinje neurons in a sagittal section from mouse #0127 in one region with no GFP⁺ cell bodies (arrows) but not in a neighboring region with a number of GFP⁺ cell bodies (box). (d) High power confocal image of the boxed area in (c) shows several heterokaryons with complex dendrites and many synaptic spines intermingled with macrophages/microglia cells despite the intense inflammation. Scale bar in (a, c) 100 μm, and (b, d) 20 μm.

Figure 5. Rat neural gene expression in heterokaryons of mice transplanted with rat bone marrow. (a) Representative FACS analysis of PB of a mouse after BMT with rat BM ubiquitously expressing GFP. More than 93% of the PB is derived from the rat BM. (b) Immunohistochemistry with antibodies to GFP (green) and calbindin (red) demonstrate rat-mouse chimeric heterokaryons and rat macrophage/microglia cells in a sagittal section from the cerebellum (mouse #933). (c) Quantification of cerebellar heterokaryons in mice BMT with rat BM. (d) RT-PCR products were resolved on an agarose gel. RT-PCR products show the expression of the rat Purkinje neuron genes *Calb1*, *L7/pcp-2*, and *Gsbs* in addition to *Kcnc1* from mice transplanted with rat BM. Primers for rat and mouse *Gapdh* were used as internal controls. Scale bar 100 μ m.