Purkinje cell fusion and binucleate heterokaryon formation in multiple sclerosis cerebellum

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A major conceptual consideration in both endogenous and therapeutic central nervous system repair is how damaged (or senescent) neurons, given their often enormously complex and extensive network of connections, can possibly be replaced. The recent observation of fusion of circulating bone marrow cells with, in particular, cerebellar Purkinje cells, as well as the subsequent formation of stable heterokaryons, offers a tantalizing potential solution to this difficulty. Here, we have explored Purkinje cell fusion and heterokaryon formation in the human brain and the influence of central nervous system inflammation. We analysed post-mortem cerebellum tissue from patients who had multiple sclerosis and from appropriate controls. Purkinje cells were analysed for heterokaryon formation using immunohistochemistry techniques and chromosome composition using fluorescence in situ hybridization. For the first time in humans we show a disease-related increase in Purkinje cell fusion and heterokaryon formation. We have shown that heterokaryon formation takes place in control subjects, and that the frequency of this event is considerably increased in patients with multiple sclerosis, the prototypical inflammatory brain disease, with ~0.4% of Purkinje cells being binucleate heterokaryons. No mononucleate polyploid Purkinje cell heterokaryons were found. The observation that heterokaryon formation in the cerebellum occurs as part of the central nervous system inflammatory reaction suggests a potential mechanism of neural repair. It also suggests an exciting new avenue for therapeutic intervention, as enhancement or manipulation of fusion events may have a therapeutic role in cellular protection in multiple sclerosis.

Keywords: Purkinje cells; multiple sclerosis; fusion; heterokaryon; cerebellum

Abbreviations: DAPI = 4′,6′-diamidino-2-phenylindole; MBP = myelin basic protein

Introduction

Over the past decade, the unexpected observation has emerged that cells derived from bone marrow can contribute genetic material to cells of the adult human brain (Mezey et al., 2003; Weimann et al., 2003a; Cogle et al., 2004). Within the CNS, migration and fusion of bone marrow-derived stem cells with neuronal cells to form heterokaryons are seen, predominantly involving Purkinje cells within the cerebellum (Johansson et al., 2008; Nygren et al., 2008), although appearing extremely infrequent under normal biological conditions (Weimann et al., 2003a, b) (Fig. 1). The biological relevance of cell fusion is, as yet, unclear, but it is postulated to represent a physiological phenomenon to introduce healthy nuclei or functional genes into aged or degenerating cells (Magrassi et al., 2007). Indeed, in organs other than the brain, fusion represents a significant process by
which degenerating cells with genetic damage can be rescued, thus promoting their survival (Lagasse et al., 2000; Vassilopoulos et al., 2003; Willenbring et al., 2004).

The observation that chronic inflammation in rodents promotes migration and infiltration of bone marrow-derived stem cells to the site of brain injury suggests a mechanism by which the body targets sites for neural repair (Chopp and Li, 2002; Mahmood et al., 2003, 2005; Johansson et al., 2008; Nygren et al., 2008). Interestingly, inflammation not only promotes migration and infiltration of bone marrow-derived stem cells to sites of brain injury (Chopp and Li, 2002; Mahmood et al., 2003, 2005) but also increases the frequency of Purkinje cell fusion (Johansson et al., 2008; Nygren et al., 2008; Nern et al., 2009; Kemp et al., 2011). This raises the possibility that fusion represents a means of cell-mediated neuroprotection or rescue of highly differentiated cell types that cannot be replaced in adults (Blau, 2002; Singec and Snyder, 2008). Immunological and inflammatory factors, recruiting endogenous stem cells and stimulating fusion, could help limit the loss of structurally highly complex neurons such as Purkinje cells (Johansson et al., 2008).

Purkinje cells are some of the largest neurons in the human brain. Their axons are the sole outputs from the cerebellar cortex, and the extensive dendritic network from a single Purkinje cell can receive synaptic inputs from as many as 200,000 parallel fibres (Tyrrell and Willshaw, 1992). They are therefore critical for normal cerebellar function (Kozorovitskiy and Gould, 2003). Traditionally, Purkinje cells, in both rodents and humans, are mononucleate diploid cells that lack the ability to undergo cell division (Zecevic and Rakic, 1976; Mann et al., 1978; Miyata et al., 1999). They are generated only during early brain development and in contrast to other neurons; there is no evidence of their generation after birth (Weimann et al., 2003a). Purkinje cell atrophy resulting from a range of toxic, autoimmune, genetic and neurodegenerative insults therefore leads to irreversible reduced motor function and ataxia. It could therefore be postulated that any preservation in Purkinje cell numbers through fusion events in humans could have a large clinical significance.

In mice, it has been shown that transgenes carried by bone marrow-derived cells are stably expressed following fusion events with Purkinje cells in genetic models of Purkinje cell degeneration (Chen et al., 2011). An understanding of the mechanisms underlying cell fusion events and heterokaryon formation might therefore lead to techniques to manipulate these mechanisms and the opportunity to introduce functional ‘donor’ cell genes or engineer ‘donor’ cells to express factors that may increase Purkinje cell survival in patients with cerebellar degeneration and disease.

Here, we have explored in human tissue whether Purkinje cell fusion and heterokaryon formation in the brain is affected in multiple sclerosis, a prototypical CNS inflammatory disease. We analysed post-mortem cerebellum tissue from patients who had multiple sclerosis and from appropriate controls. Purkinje cells were analysed for heterokaryons using immunohistochemistry techniques and chromosome composition using fluorescence in situ hybridization. Following in-depth evaluation, binucleate Purkinje cell heterokaryons were evident in all patients with multiple sclerosis and in a proportion of control patients. A considerable increase in the frequency of heterokaryons was detected in patients with multiple sclerosis. We believe this is important to multiple sclerosis repair mechanisms and suggest that this process may be a useful therapeutic target for human inflammatory diseases of the CNS or for a wide range of neurodegenerative conditions of the cerebellum.

Materials and methods

Patients

Post-mortem cerebellum samples from six patients with multiple sclerosis and five control patients were obtained through collaboration with the UK Multiple Sclerosis Tissue Bank at Imperial College, London, UK. All patients had been clinically diagnosed as having multiple sclerosis, and the diagnosis had been confirmed during neuropathological autopsy examination. Control cerebellum samples were derived from

![Figure 1](https://academic.oup.com/brain/article-abstract/135/10/2962/296350)

**Figure 1** Heterotypic cell fusion is the result of fusion between two different cell types without subsequent chromosomal loss. Cellular fusion can result in either the absence or amalgamation of nuclear material to form binucleate and mononucleate heterokaryons, respectively.
patients who had died from causes other than neurological disease (Table 1). All tissues were collected with the donors’ informed consent via a prospective donor scheme. At death, brains were removed, fixed in neutral-buffered formalin and tissue blocks embedded in paraffin. For this study, 10- and 30-μm sections were cut from cerebellar tissue of each patient and mounted onto glass slides. In 10-μm sections, only half of a Purkinje cell nucleus could be included; thicker sections were deemed to be impractical for epifluorescent screening of Purkinje cell heterokaryons before further evaluation using confocal microscopy.

Case histories for all six patients with multiple sclerosis were reviewed, although incomplete. Four of six were documented to have moderate to severe ataxia. All patients had established primary or secondary progression before death.

Post-mortem cortex samples from six patients with multiple sclerosis were also obtained through collaboration with the UK Multiple Sclerosis Tissue Bank. Paraffin-embedded 10-μm sections were obtained from both the frontal and parietal cortex. Lesion location and activity were characterized using methods described below.

Characterizing cerebellar lesions

To characterize multiple sclerosis lesions, immunostaining was performed to identify the centres and borders of the lesions and lesion activity. Cerebellar sections (10-μm thick) were immunostained with antibodies to myelin basic protein (MBP) (1:3200, Serotec) and human leukocyte antigen (HLA)-DP, -DQ and -DR (1:800, Dako). To examine signs of cellular apoptosis, sections were also immunostained with antibodies to cleaved caspase 3 (Asp175) (1:800, Cell Signaling). Sections were deparaffinized in Clearene, dehydrated in 100% ethanol, hydrated in distilled water and immersed in 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity, rinsed and microwaved in sodium citrate buffer (0.01 M, pH 6.0, 5 min) or EDTA buffer (1 mM, pH 8, 10 min) as appropriate and rinsed in PBS. Non-specific binding was blocked with VECTASTAIN Elite ABC complex (PK-6200, Vector Laboratories) followed by a 20-min incubation with 3,3’-diaminobenzidine (DAB) and 0.01% hydrogen peroxide. Sections were washed in water, immersed in copper sulphate and 50 mM ammonium acetate for 1 h at room temperature prior to microwaving in sodium citrate buffer (0.01 M, pH 6.0, 5 min). Purkinje cells were labelled by single or double immunostaining with mouse anti-calbindin-D28K (1:500, Sigma-Aldrich) and rabbit anti-cleaved caspase 3 (Asp175) (1:400, Cell Signaling). Non-specific binding was blocked with 10% normal goat serum (20 min). After addition of the primary antibody, sections were incubated overnight at 4°C. The sections were then rinsed in PBS before incubation for 20 min with secondary antibody (Biotinylated Universal Antibody, Vector Laboratories) and 20 min with VECTASTAIN Elite ABC complex (PK-6200, Vector Laboratories) followed by a 10-min incubation with 3,3’-diaminobenzidine enhancer (4 min), counterstained with haematoxylin, dehydrated, cleared and mounted. For light imaging, images were acquired using an Olympus IX70 microscope coupled with Image-Pro Plus software.

Immunofluorescence labelling

Sections (10 or 30 μm) were deparaffinized, hydrated and washed, as above. To reduce auto-fluorescence, sections were incubated in 5 mM copper sulphate and 50 mM ammonium acetate for 1 h at room temperature prior to microwaving in sodium citrate buffer (0.01 M, pH 6.0, 5 min). Purkinje cells were labelled by single or double immunofluorescence using mouse anti-calbindin-D28K (1:500, Sigma-Aldrich) and rabbit anti-cleaved caspase 3 (Asp175) (1:400, Cell Signaling). Non-specific binding was blocked with 10% normal goat serum diluted in PBS containing 0.1% Triton X-100. Sections were incubated at 4°C overnight with primary antibodies. Sections were then washed in PBS and incubated for 30 min in the dark with Alexa Fluor488 goat anti-mouse (1:500) or Alexa Fluor555 goat anti-rabbit (1:500, Invitrogen) before being washed in PBS and mounted in

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### Table 1 The characteristics of both multiple sclerosis and control patients used within this study for heterokaryon analysis

<table>
<thead>
<tr>
<th>Patient Sex</th>
<th>Age (years)</th>
<th>MS/control</th>
<th>Lesion Tissue preservation</th>
<th>Duration of disease (years)</th>
<th>Classification of MS</th>
<th>Cause of death</th>
<th>Sex</th>
<th>Age (years)</th>
<th>MS/control</th>
<th>Lesion Tissue preservation</th>
<th>Duration of disease (years)</th>
<th>Classification of MS</th>
<th>Cause of death</th>
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<tr>
<td>1 MS</td>
<td>78</td>
<td>Control</td>
<td>Negative</td>
<td>42</td>
<td>Chronic inactive</td>
<td>Metastatic carcinoma of bronchus</td>
<td>F</td>
<td>78</td>
<td>MS</td>
<td>Control</td>
<td>42</td>
<td>Chronic inactive</td>
<td>Metastatic carcinoma of bronchus</td>
</tr>
<tr>
<td>2 MS</td>
<td>64</td>
<td>Control</td>
<td>Negative</td>
<td>36</td>
<td>Chronic active</td>
<td>Gastrointestinal bleed/obstruction</td>
<td>F</td>
<td>64</td>
<td>MS</td>
<td>Control</td>
<td>36</td>
<td>Chronic active</td>
<td>Gastrointestinal bleed/obstruction</td>
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<tr>
<td>3 MS</td>
<td>49</td>
<td>Control</td>
<td>Negative</td>
<td>18</td>
<td>Chronic inactive</td>
<td>Chronic renal failure, heart disease, general decline</td>
<td>F</td>
<td>49</td>
<td>MS</td>
<td>Control</td>
<td>18</td>
<td>Chronic inactive</td>
<td>Chronic renal failure, heart disease, general decline</td>
</tr>
<tr>
<td>4 MS</td>
<td>21</td>
<td>Control</td>
<td>Negative</td>
<td>31</td>
<td>Active</td>
<td>Bronchopneumonia</td>
<td>M</td>
<td>21</td>
<td>MS</td>
<td>Control</td>
<td>31</td>
<td>Active</td>
<td>Bronchopneumonia</td>
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<tr>
<td>5 Control</td>
<td>55</td>
<td>Control</td>
<td>Negative</td>
<td>10</td>
<td>Chronic active/active</td>
<td>Bronchopneumonia</td>
<td>M</td>
<td>55</td>
<td>Control</td>
<td>Control</td>
<td>10</td>
<td>Chronic active/active</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>6 MS</td>
<td>16</td>
<td>Control</td>
<td>Negative</td>
<td>6</td>
<td>Secondary progressive</td>
<td>Chronic progressive</td>
<td>M</td>
<td>16</td>
<td>MS</td>
<td>Control</td>
<td>6</td>
<td>Secondary progressive</td>
<td>Chronic progressive</td>
</tr>
<tr>
<td>Mean</td>
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<td>13</td>
<td>23</td>
<td></td>
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In situ hybridization

Sections (10 μm) were deparaffinized in Clearene, dehydrated in 100% ethanol and placed in 0.2 N HCl for 10 min. The sections were then washed in water before immersing them in heat pretreatment solution with disodium EDTA (Zymed SPO-Light® Tissue Heat Pretreatment Solution, Invitrogen) at 85°C for 10 min. After washing with water, proteins were digested for 60 min at room temperature using pepsin A (Digest-All 3 protease, Invitrogen). The sections were then washed in water and 2 × saline-sodium citrate buffer, fixed in 1% formaldehyde solution, dehydrated through an ethanol series and air dried. The fluorescent in situ hybridization probes (Vysis CEP® X Spectrum Orange®/Y SpectrumGreen® Direct Labelled Fluorescent DNA Probe, Abbott Molecular Inc) were prepared according to the manufacturer’s recommendations and then applied directly to the tissue sections. DNA was denatured at 73°C for 10 min and then renatured with fluorescent in situ hybridization probes by incubating overnight at 42°C. The following day, slides were washed in 0.4 × saline-sodium citrate buffer for 2 min at 73°C and 2 × saline-sodium citrate/0.1% Tween® 20 for 1 min at room temperature. The sections were then dehydrated through an ethanol series, air dried and mounted in VECTASHIELD® medium containing the nuclear dye DAPI (H-1200, Vector Laboratories).

Microscopy

For identification of binucleate Purkinje cell heterokaryons, cerebellar sections immunolabelled for calbindin-D28K were viewed at ×63 using a Leica SP5-AOBS confocal laser scanning microscope attached to a Leica DM 16000 inverted epifluorescence microscope. Each section was scanned along the entire length of the Purkinje cell layer, situated between the granular cell layer and molecular layer, for Purkinje cell bodies containing two separate nuclei. At least 3000 Purkinje cells from each patient sample were examined, allowing for the determination of the frequency of binucleate Purkinje cells. All binucleate Purkinje cells were confirmed on the confocal microscope by obtaining serial sections throughout the whole Purkinje cell body. All Z-stack and 3D imaging was created using both Leica Application Suite Advanced Fluorescence software and Velocity 3D image analysis software (PerkinElmer).

For X/Y chromosomal enumeration and identification of mononucleate polyplody Purkinje cells, fluorescent in situ hybridization-labelled cerebellar sections were viewed at ×100 using a Nikon C1 confocal microscope and EZ viewer software. Using confocal microscopy, 1000 Purkinje cells from each patient sample were scanned throughout the entire cell body to observe the X/Y chromosomal frequency. For all epifluorescence imaging, images were acquired using a Leica DM6000 B epifluorescence microscope and merged with Leica Application Suite Advanced Fluorescence software.

Statistical analysis

Counting data between patient groups were analysed using unpaired t-tests. Values from at least three independent experiments are expressed as mean ± standard error of the mean (SEM), taking $P < 0.05$ to represent statistical significance.

Results

Cerebellar lesions

Areas of cerebellar demyelination within the arbor vitae were readily identified in the sections derived from patients with multiple sclerosis using immunolabelling for MBP (Fig. 2). No demyelination or other cerebellar lesions were seen in the serial sections of control sections. In control sections immunolabelled for the macrophage/microglial markers HLA-DP, -DQ and -DR (Fig. 2), few or no positive cells were demonstrable in the cerebellum. In cases with multiple sclerosis (Fig. 2), HLA-DP-, -DQ- and -DR-positive cells were scattered throughout the cerebellum but were abundant in areas of demyelination and were particularly prominent towards the plaque edge, where they had enlarged perikarya with thicker processes. In non-demyelinated areas of the cerebellum, positive cells were less numerous and more evenly distributed. Of the six cases with multiple sclerosis, three cerebellum tissue blocks were classified as having active lesions and the other three as inactive lesions (Table 1).

Cerebellar apoptosis

The presence of cerebellar inflammation and demyelination in sections derived from patients with multiple sclerosis led us to investigate whether Purkinje cells in the cerebellum were undergoing apoptotic cell death. Using cleaved (active) caspase 3 as an indicator of apoptosis, positive Purkinje cells were demonstrable in the cerebellum of these patients (Fig. 3). An almost complete absence of cleaved caspase 3 staining was observed in control sections (data not shown).

Quantification of binucleate Purkinje cell heterokaryons

To determine whether binucleate Purkinje cells were present in the human cerebellum of patients with multiple sclerosis and controls, cerebellar tissue from post-mortem brains was first screened. Sections were scanned along the entire length of the Purkinje layer, and Purkinje cells were readily identified based on calbindin-D28K positivity and their unique morphology and location (Fig. 4). The nucleus of each cell was identified using DAPI labelling. As previously reported by Weimann et al. (2003a), the thickness of the 10-μm sections analysed encompassed less than half of the Purkinje cell nucleus but was necessary to allow for the identification of heterokaryons by epifluorescence microscopy in thousands of cells before confocal further analysis. Using epifluorescence microscopy, we examined ≥3000 Purkinje cells from each patient (total = 20,239 Purkinje cells) and control (total = 18,855) for the presence of two nuclei. The location of each suspected binucleate Purkinje cell was noted and subsequently scanned using confocal microscopy to acquire 0.25-μm serial sections throughout the entire Purkinje cell body.

Following in-depth confocal evaluation and serial reconstruction, binucleate Purkinje cells were found to be present in all six patients with multiple sclerosis and in two control patients.
A substantial increase in the frequency of binucleate Purkinje cells was detected in patients with multiple sclerosis (mean \pm SEM: 0.376\% \pm 0.036), compared with control patients (mean \pm SEM: 0.024\% \pm 0.006, \textit{P} < 0.0001, Fig. 5). To further dissect the results, patients with multiple sclerosis were split into groups depending on the presence of active or inactive cerebellar lesions. Again, the frequency of binucleate Purkinje cells in both the multiple sclerosis active (mean \pm SEM: 0.359\% \pm 0.032) and inactive (mean \pm SEM: 0.392\% \pm 0.071) lesion groups was significantly greater than in control patients (\textit{P} < 0.001). In contrast, the differences in the frequency of binucleate Purkinje cells between active and inactive lesion groups were not significant (\textit{P} = 0.696, Fig. 5). To further validate our findings, using immunofluorescence/confocal methods identical to those used on cerebellar sections, we examined cortical tissue for signs of cellular fusion identifying neuronal cells using βIII-tubulin and DAPI labelling.

Samples from six patients (~1000 cells/patient) were examined: three derived from pathologically uninvolved tissue and three containing active lesions. This cohort included patients whose cerebellar tissue was also evaluated for heterokaryon formation. No binucleate neurons were found in any of the cortical tissue samples.

Two distinct types of nuclear morphology were evident in binucleate Purkinje cells (Figs 6 and 7). In patients with multiple sclerosis, the vast majority (mean \pm SEM: 90.87\% \pm 3.10) of binucleate cells contained one large Purkinje cell-like nucleus with disperse chromatin and a large nucleolus, and one smaller nucleus (a little more than half the normal size) with compact chromatin. Much less commonly seen, the second type of binucleate Purkinje cell contained two equal-sized nuclei. In control patients, of the four binucleate heterokaryons found, all (100\%) contained one large Purkinje cell-like nucleus and one smaller nucleus. There

Figure 2  Cerebellar sections from patients with multiple sclerosis (MS) show typical lesions with loss of myelin and inflammatory cell infiltration. Representative human cerebellar sections derived from a control (A and B) and a patient with multiple sclerosis (C–F). Sections are DAB (brown) immunolabelled with MBP (A, C and E) and HLA-DP, -DQ and -DR (B, D and F) and counterstained with haematoxylin (blue). The outlined areas in C and D are magnified in E and F, respectively. Scale bar = 10 mm.

Figure 3  Purkinje cells within the cerebellum of patients with multiple sclerosis show signs of apoptosis. Representative human cerebellar sections derived from a patient with multiple sclerosis. Sections are DAB (brown) immunolabelled with cleaved caspase 3 (Asp175) and counterstained with haematoxylin (blue). The outlined area in A is magnified in image B. Active caspase 3-positive cells in A are indicated by red arrows. Scale bar = 300 µm.
was no consistent pattern of orientation of the two nuclei in relation to one another within the cell body of binucleate Purkinje cells. Furthermore, in an attempt to rule out that binucleate Purkinje cells are undergoing apoptosis, although cleaved caspase 3-positive Purkinje cells were observed within the cerebellum of patients with multiple sclerosis, we did not find any binucleate Purkinje cells expressing this apoptotic cell marker.

Quantification of (mononucleate) polyploidy Purkinje cell heterokaryons

Cerebellar sections from both multiple sclerosis and control patients were further analysed for indicators of cell fusion by attempting to identify Purkinje cells with a single nucleus containing a sex chromosomal complement other than the...
expected normal XX or XY diploid karyotype (males: XXY, XYX, XYY, XX or YY; females: XXXX or XXX). Fluorescent in situ hybridization was used for visualizing nuclear chromosomal content. As shown in Fig. 8, in sections from males and females, X and Y chromosomes can be visualized using confocal microscopy. In situ hybridization using fluorescently labelled X and Y probes yielded red and green signals, respectively. Overlaying the red and green channels allowed determination of the characteristic autofluorescent cytoplasm of the Purkinje cell. Sections were scanned along the entire length of the Purkinje layer, and Purkinje cells were readily identified based on their unique morphology and location (Fig. 8). Using confocal microscopy, we examined 1000 mononucleate Purkinje cells from each patient and control for their X/Y chromosome content. Each Purkinje cell was manually scanned using confocal microscopy throughout the entire z-axis of the Purkinje cell body. In all cerebellar sections, from both multiple sclerosis and control patients, no mononucleate Purkinje cell was shown to have more than the expected ‘correct’ diploid karyotype. It was also noted that no section from a female patient was shown to contain the green-labelled Y chromosome. In addition, Purkinje cells did not always contain two sex chromosomes because of the thickness of the 10-μm sections analysed encompassing less than half of the entire Purkinje cell nucleus.

Discussion

Here, we show for the first time in humans that Purkinje cell fusion and heterokaryon formation in the brain are influenced by CNS disease. Not only have we shown heterokaryon formation in control patients under non-invasive conditions, we have also shown a considerable increase in the frequency of heterokaryon formation in patients with multiple sclerosis, an inflammatory condition of the CNS. If indeed fusion and heterokaryon formation represents a mechanism for Purkinje cell regeneration or preservation in disease states such as multiple sclerosis, then the significantly increased frequency of fusion events observed in the

Figure 6 A binucleate Purkinje cell heterokaryon containing two large ‘Purkinje-like’ nuclei with dispersed chromatin. (A–C, front view; D–F, rear view) A 3D image of a binucleate Purkinje cell immunofluorescently labelled with calbinin-D28K (green) and DAPI nuclear stain (blue). The outlined box in A is magnified in B and C. The outlined box in D is magnified in E and F. The outlined circles in C and F indicate locations of nuclei within the binucleate Purkinje cell. (G–I) Sequential confocal slices (side and top views) through the binucleate Purkinje cell showing locations of each nucleus within the cell.
diseased brains in this study could indicate the potential for functional improvement in in vivo cerebellar processes.

In a landmark study, Weimann et al. (2003a) found that bone marrow-derived stem cells contributed to Purkinje cells in the brains of adult females who had received bone marrow from male donors for the treatment of haematological malignancies. Subsequently, other studies in humans have shown that bone marrow cells contribute to a number of different neuronal and glial cell populations in the CNS, including Purkinje cells (Mezey et al., 2003; Cogle et al., 2004). In these studies, little or no sign of Purkinje cell heterokaryon formation was evident; thus, fusion was considered unlikely, and transdifferentiation of bone marrow cells was proposed to be the more likely explanation. A number of groups have used bone marrow chimerism in rodents, transplanting fluorescently tagged bone marrow stem cells into lethally irradiated or non-irradiated mice, thereby demonstrating that the transplanted cells contribute to some of the Purkinje cells in the cerebellum, where they can remain for months post-infusion (Priller et al., 2001; Alvarez-Dolado et al., 2003; Weimann et al., 2003b; Corti et al., 2004; Magrassi et al., 2007; Wiersema et al., 2007; Johansson et al., 2008; Nygren et al., 2008; Espejel et al., 2009; Nern et al., 2009; Chen et al., 2011). These studies also provide compelling evidence for fusion of bone marrow-derived cells with Purkinje cells based on the formation of bi/mononucleate heterokaryons (Fig. 1). Furthermore, they have shown that bone marrow stem cell and Purkinje cell fusion-like events can lead to reprogramming of the donor nucleus, resulting in the expression of Purkinje cell-specific genes (Johansson et al., 2008), and even developing into electrically active neurons with functional synaptic formation in the cerebellum (Bae et al., 2007).

Under normal physiological conditions, Purkinje cell fusion seems to occur at low levels (Weimann et al., 2003a, b), although it may increase with age (Wiersema et al., 2007). It is, however, debated whether many of these studies were conducted under physiological conditions, as the patients or animals were subjected to highly invasive regimens involving either irradiation or...
chemotherapeutic drugs to remove the host haemopoietic system (Priller et al., 2001; Alvarez-Dolado et al., 2003; Weimann et al., 2003a, b; Corti et al., 2004; Magrassi et al., 2007; Wiersema et al., 2007; Johansson et al., 2008; Nygren et al., 2008; Espejel et al., 2009; Chen et al., 2011). To address this problem in rodents, parabiosis experiments have revealed that fusion can occur without irradiation (Johansson et al., 2008). In humans, others have not found binucleate or polyploid Purkinje cells in non-transplanted individuals (Nern et al., 2009). In the current study, we also screened Purkinje cells from five control patients who presented no neuropathological changes at post-mortem and had not received any prior bone marrow transplantation. Although we did not observe any mononucleate polyploid Purkinje cells in our control population, we did observe a low frequency of binucleate Purkinje cells in four of the control patients (a single binucleate Purkinje cell was found in each of the four patients). At such low frequencies, a role for Purkinje cell fusion under normal physiological conditions seems negligible or lends to the hypothesis that Purkinje cell heterokaryons are unstable, and fusion is only a transient phenomenon (Nern et al., 2009).

Inflammation following tissue damage seems to play an important regulatory role in heterokaryon formation. In rodents, studies have demonstrated fusion and heterokaryon formation between bone marrow stem cells and Purkinje cells, with increased levels occurring in the context of either CNS or systemic inflammation (Johansson et al., 2008; Nern et al., 2009). An increase in fusion events is postulated to occur as the result of inflammation-induced migration and infiltration of cells, linked to increased permeability of the blood–brain barrier, with fusion between the infiltrating cell nucleus and the Purkinje cell (Johansson et al., 2008; Nygren et al., 2008). Alternatively, fusion events may be, in part, mediated as a secondary effect of infiltrating cells and/or soluble factors on local cell populations, such as microglia and astrocytes (Kemp et al., 2011). Taking advantage of the inflammatory nature of multiple sclerosis, we investigated whether the presence of CNS inflammation influenced Purkinje cell heterokaryon formation in humans. In keeping with the control patients, we did not observe any mononucleate polyploid Purkinje cells in the multiple sclerosis cohort. We did, however, observe binucleate Purkinje cells in all patients with multiple sclerosis who were examined, with ~0.4% of Purkinje cells being binucleate. This heterokaryon formation was substantially higher than the numbers found in control patients. The observation that inflammation in the CNS promotes migration and infiltration of donor cells to the site of cerebellar injury to undergo fusion with Purkinje cells tentatively suggests a mechanism by which the body targets sites for neural repair and/or protection (Chopp and Li, 2002; Mahmood et al., 2003, 2005).

We did not, however, observe any differences in heterokaryon formation between sections containing active or chronic cerebellar lesions, nor did we observe heterokaryon formation occurring preferentially at lesion sites. In rodents, a correlation between the extent of macrophage or microglial infiltration in the cerebellum and heterokaryon formation is not seen (Johansson et al., 2008). Together, these results suggest that heterokaryon formation may be independent of local inflammatory cell infiltration and may rely more on systemic cues. The finding of similar numbers of heterokaryons in sections with active compared with inactive lesions might relate to the necessary limitation of human autopsy studies, namely, the ‘single snapshot’ nature of the samples in a disease of several decades’ duration, and also to the complexity of tissue changes in multiple sclerosis. It is now clear that even in areas of brain where no lesions are present (‘normal appearing’ white or grey matter), there are subtle pathophysiological changes, particularly in the microglia (Gray et al., 2008), presumably resulting from multiple sclerosis-related systemic cues, and it may be these changes rather than infiltrating inflammatory cells that triggered heterokaryon formation.

In agreement with observations in animal studies, we found that the structure of the two nuclei within human Purkinje cells differed between heterokaryons (Weimann et al., 2003b). The majority of heterokaryons had one large ‘Purkinje-like’ nucleus with dispersed chromatin and one smaller nucleus with a dense chromatin.
structure. The others had two large ‘Purkinje-like’ nuclei. Normally, Purkinje cells are mononucleate diploid cells (Zecevic and Rakic, 1976; Mann et al., 1978; Miyata et al., 1999). It is hypothesized that once fusion has occurred, the small dense donor nuclei are progressively reprogrammed, causing them to become, over time, less dense and compact, and finally assume the morphology of the Purkinje nucleus (Weimann et al., 2003b). The observed difference in nuclear morphology between heterokaryons may therefore be an indication of heterokaryon maturity.

The biological relevance of Purkinje cell fusion and subsequent nuclear reprogramming, either in vitro or in vivo, is, as yet, unclear, but this may represent a physiological phenomenon to introduce healthy nuclei or functional genes into aged or degenerating cells (Magrassi et al., 2007; Valadi et al., 2007). Indeed, in this study, we found significant levels of Purkinje cells in the cerebellum of patients with multiple sclerosis expressing cleaved caspase 3, indicating they are undergoing apoptotic cell death. Transgenes carried by normal bone marrow-derived cells are stably expressed following fusion events with genetically abnormal Purkinje cells, promoting their survival (Chen et al., 2011). Certainly it could be hypothesized that the rescue of highly complex cells within the brain, through fusion events, seems a more efficient evolutionary mechanism compared with complete cell replacement.

Although further studies are warranted to improve our understanding of Purkinje cell heterokaryon formation, the results presented in this study strongly support the notion that pathophysiological mechanisms associated with multiple sclerosis have a role in promoting Purkinje cell fusion and heterokaryon formation in the cerebellum. Whether the donor nuclei involved in the fusion processes observed in this study are indeed bone marrow-derived, as demonstrated in rodent studies, although probable, still needs to be established. Our study further supports the notion that immunological factors involved in the inflammatory process may have a significant role in limiting the loss of structural neurons, such as Purkinje cells, which cannot be replaced in adult life (Johansson et al., 2008). No binucleate neurons were found in cortical tissue, therefore giving further indication that multiple sclerosis disease processes—either the presence (at some time) of local inflammation or systemic immune cues—promotes cellular fusion specifically in Purkinje cells within the cerebellum. Degeneration of the cerebellum, and particularly Purkinje cells therein, occurs in many neurological disorders, including multiple sclerosis, spinocerebellar ataxias, stroke, metabolic disturbances and cancer. Even in multiple sclerosis, the overall proportion of heterokaryon formation in the Purkinje cell layer might be considered small, at ~0.4%—indeed too small to be functionally relevant. We would argue otherwise, for several reasons. Firstly, our methods of identifying binucleate cells are intrinsically always likely to yield an underestimate of the true proportion. Secondly, it is suggested that the binucleate nature of fused cells is transient; such cells may commonly extrude or degrade ‘excess’ chromosomal material and therefore re-emerge as mononucleate cells, even though their nuclear material now is bone marrow derived. In this way, quantifying binucleate cells reflects only a snapshot of a process that could, in principle, ultimately account for any proportion of Purkinje cells. Thirdly, a small proportion might, optimistically, be amplified to a functionally relevant number by therapeutic interventions yet to be identified, and fourthly, it is highly likely that, given functional redundancy in the Purkinje cell layer, rather small changes in cell numbers could have disproportionate functional consequences. It could therefore be postulated that preservation of Purkinje cell numbers through harnessing fusion events could have clinically valuable effects.

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