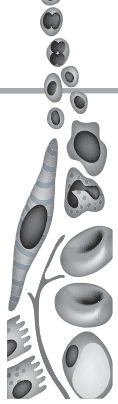

Disease-responsive neural precursor cells are present in multiple sclerosis lesions

Aims: Spontaneous tissue repair occurs in multiple sclerosis (MS), but the origin of remyelinating cells remains obscure. Here we explore the hypothesis that endogenous neural precursors are involved in MS disease processes. **Materials & methods:** We studied postmortem brain and spinal cord samples from MS patients using immunocytochemical techniques. **Results:** We show that cells co-positive for nestin and musashi-1 are not merely present in lesions, but found in markedly increased numbers (up to fivefold). Small numbers of nestin-positive cells show direct evidence of proliferation, co-staining for Ki67; some also coexpress glial fibrillary acidic protein or oligodendrocyte progenitor markers (NG-2 or PDGF-α receptor), or the early neuronal marker doublecortin, consistent with transition from neural precursors. **Conclusions:** These findings suggest that endogenous neural precursors react to disease processes in MS.

KEYWORDS: multiple sclerosis, nestin, neural precursor, oligodendrocytes, regeneration, remyelination

Heidi Snethen[1], Seth Love[2] & Neil Scolding[1†]

[†]Author for correspondence:
[1]Department of Neurology, University of Bristol Institute of Clinical Neurosciences, Frenchay Hospital, Bristol, BS16 1LE, UK
Tel:. +44 117 970 1212;
Fax: +44 117 975 3824;
E-mail: n.j.scolding@bristol.ac.uk
[2]Department of Neuropathology, University of Bristol Institute of Clinical Neurosciences, UK

A detailed understanding of spontaneous repair processes in human CNS disease, and in particular of the potential contribution of endogenous precursors, should underpin any future development of cell therapies in conditions such as multiple sclerosis (MS) and indeed in other neurodegenerative diseases. Although the capacity of the brain for limited repair was recognized by Cajal, intensive interest has been rekindled only relatively recently, and the mechanisms involved remain poorly understood.

In MS, where damage to oligodendrocytes and their myelin sheaths causes demyelination in lesions throughout the CNS, spontaneous myelin repair was first identified over 40 years ago [1]. More recent studies have suggested that remyelination occurs earlier and more extensively than initially realized [2]. However, the origin of remyelinating cells in MS remains unclear [3–5]. Schwann cells make a limited contribution [6], but most is achieved by oligodendrocyte lineage cells. These could, theoretically, originate from surviving differentiated cells in lesions, or from local or inwardly migrating endogenous oligodendrocyte progenitors. Another, more recently emerging possibility is that endogenous stem cells, perhaps better termed multipotential neural precursors, contribute to the pool of remyelinating cells in MS [4,7–9].

The study of neural precursors in human disease is complicated by the absence of specific markers. Nestin, employed in many experimental studies, is expressed by such cells, but is not wholly specific [10]. However, the co-expression of other markers, such as the RNA-binding protein musashi-1, can help confirm neural precursor identity [11]. Additionally, the transient co-expression by differentiating precursors of nestin combined with lineage-specific markers such as glial fibrillary acidic protein (GFAP; astrocytes), NG2 (oligodendrocyte progenitors) or doublecortin (immature neurons) can offer indicative 'retrospective' evidence of precursor cell presence. Here we study MS lesions, and show that cells with the immunophenotype of neural precursors are present in MS, and expand in number substantially in response to the disease. Only a minority, however, show evidence of oligodendroglial differentiation. We believe our findings have significant implications for stem cell therapies and for our basic understanding of neurodegenerative disease.

Methods

Tissue sections

A total of 25 postmortem cerebrum and spinal cord samples from seven patients (four male and three female) with MS were obtained from the Multiple Sclerosis Society Tissue Bank (Imperial College, London, UK). The mean age of patients was 56 years (range: 35–72 years).

Each tissue sample had been histologically examined, and all lesions classified by Dr Federico Roncaroli (MS Tissue Bank). Lesions were observed in seven samples of cerebrum, one classified as active, two as chronic active and four as inactive (Table A1). Two of the remaining samples of cerebrum contained shadow plaques; the other four appeared

future medicine part of fsg

normal. Nine of the spinal cord samples contained lesions: one active, two chronic active and six inactive. Three spinal cord samples appeared normal. All 25 samples contained some areas of normal-appearing white matter (NAWM).

A total of 12 brain samples and three spinal cord samples from four control cases (two male and two female; mean age: 67 years; range: 35–92 years) was also studied. Causes of death included cardiac failure, carcinoma of the tongue and myeloid leukemia.

CNS tissue samples were divided into seven distinct classes:

- Macroscopically normal control patient white matter
- NAWM from MS patients
- Lesion tissue from the brain of MS patients
- Control spinal cord (also normal macroscopically)
- Normal-appearing spinal cord white matter (NASC) from MS patients
- Lesion tissue within the spinal cord of MS patients
- Shadow plaques within the cerebrum of MS patients

Sectioning

Cryostat sections 7 µm thick were cut from each sample, picked up on 3-aminopyltriethoxysilane-coated slides, air-dried for 48 h, then stored at -80°C until required. Sections were removed as required from cold storage and immediately fixed in acetone at 4°C for 10 min.

Solochrome cyanine & hematoxylin & eosin staining

Sections from all 40 samples (MS and control tissue) were stained with solochrome cyanine to locate lesions. After fixation in acetone at 4°C, sections were rinsed in tap water and immersed in solochrome cyanine for 10 min. Sections were washed in running water for 5 min to intensify staining and differentiated in 10% iron alum. After they had been rinsed thoroughly, sections were counterstained with Kernecktrot solution for 15 min, dehydrated in industrial methylated spirits (IMS), cleared in xylene and mounted in DPX (BDH Laboratories, Poole, UK).

Hematoxylin and eosin staining was also performed on sections from all samples to aid in the observation of lymphocyte infiltration. Sections were stained with Harris' hematoxylin for 1 min, 'blued' in tap water, dehydrated and counterstained with eosin (Surgipath, Peterborough, UK) for 2 min. Sections were then briefly rinsed in IMS, cleared in xylene and mounted in DPX.

Diaminobenzidine single staining

Immunoperoxidase staining for nestin was carried out on three sections from each sample on separate occasions. Sections incubated without primary antibody or with a mouse IgG-matched isotype antibody acted as negative controls. After acetone fixation, sections were washed in phosphate-buffered saline (PBS) and permeabilized in methanol at -20°C for 10 min. Sections were then immersed in 3% hydrogen peroxide/methanol for 30 min to block endogenous peroxidase and washed in running water for 10 min. Sections were covered for 20 min with normal blocking serum (Vector Universal Elite ABC Kit, Vector Laboratories, made up according to manufacturer's recommendations) and incubated with primary antibody overnight. Two primary anti-nestin antibodies were initially tested: human-specific mouse monoclonal IgG1 antibody and rabbit polyclonal antibody (both Chemicon, Livingston, UK). Both were diluted 1:400 in PBS before use: comparison of these two antibodies showed no significant difference in immunohistochemical or immunofluorescent staining. All results shown were from experiments using mouse IgG1 anti-nestin antibody. Rat anti-musashi-1 (a gift from Professor Okano, Tokyo) was used at a dilution of 1:500 in PBS and mouse IgG2 anti-MBP (Pharmingen, Oxford, UK) was diluted 1:200.

After 12 h, sections were washed in PBS and incubated with biotinylated secondary antibody (Vector Universal Elite ABC Kit) for 20 min, washed in PBS and incubated with ABC reagent (Vector Universal Elite ABC Kit) for 20 min (both made up according to manufacturers' recommendations). Sections were washed again before incubation with diaminobenzidine (DAB) solution for 10 min. DAB solution was made up in 5 ml de-ionized water using reagents from the DAB substrate kit (Vector laboratories; 100µl solution buffer, 200 µl DAB reagent, 100 µl H_2O_2). Sections were washed again before incubation with DAB solution for 10 min (DAB substrate kit; Vector laboratories). Sections were counterstained with Harris' hematoxylin (BDH Laboratories) for 30 s and blued under cold running water for 3 min. Sections were dehydrated, cleared with xylene and mounted in DPX.

Immunofluorescence staining

Immunofluorescence staining was used for double labeling. After acetone fixation, sections were washed in PBS and permeabilized in methanol at -20°C for 10 min. Sections were washed again in PBS, rinsed in de-ionized water and immersed in 5 mM copper sulfate/50 mM ammonium acetate solution for 45 min to reduce autofluorescence (this step was omitted when using the anti-Ki67 antibody). Sections were re-rinsed in de-ionized water, washed in PBS and incubated for 1 h with 10% normal goat serum (NGS)/PBS to block nonspecific binding. Primary antibodies were added to the sections and incubated at 4°C overnight (the exceptions being anti-PDGF-αR, which was incubated for 72 h, and the anti-Ki67 antibody, which was incubated at room temperature for 1 h). Primary antibodies were diluted in 10% NGS (except goat anti-PDGF-αR antibody [R&D Systems, Abingdon, UK], for which horse serum was used to block sections and dilute antibody). The other primary antibodies were rabbit polyclonal anti-GFAP (1:400), rabbit anti-NG2 polyclonal (1:200), rabbit anti-collagen IV polyclonal (1:100; all Chemicon), goat polyclonal anti-PDGFαR (1:40; R & D Systems), rat anti-musashi-1 IgG (1:40; Professor Okano, Tokyo), guinea pig polyclonal anti-doublecortin (1:500; Novus Biological) and rabbit polyclonal IgG anti-Ki67 (1:100; Affinity BioReagents) as a proliferation marker [12]. After incubation with primary antibody, sections were washed in PBS and incubated with secondary antibodies for 45 min in the dark (in runs using the anti-Ki67 antibody, 0.1% triton-X100 was added to the wash PBS to aid cleaning). Secondary antibodies included goat anti-rabbit IgG conjugated to Alexa Fluor 488 nm, goat anti-rabbit IgG-Alexa Fluor 546nm, goat anti-mouse IgG1-Alexa Fluor 488nm, goat anti-mouse IgG-Alexa Fluor 546nm, goat anti-mouse IgM-Alexa Fluor 546nm, goat anti-rat IgG-Alexa Fluor 488 nm, goat anti-rat IgG-TRITC (1:500, Southern Biotech, Cambridge, UK), donkey anti-goat IgG-Alexa Fluor 488nm and goat anti-guinea pig IgG-Alexa Fluor 568nm (all Alexa Fluor conjugates from Cambridge Bioscience, Cambridge, UK; diluted 1:2000 in 10% NGS). Sections were washed in PBS and incubated with 10% NGS for 5 min. Nuclei were counterstained with 1 µg/ml Hoechst 33342 for 3 min. Sections were then washed in PBS and mounted in Vectashield aqueous mountant.

As a positive control for proliferation studies, fresh frozen human lymph node was sectioned and fixed in the same way as brain tissue and anti-Ki67 immunofluorescence staining was performed to confirm the ability of this antibody to detect the nuclei of proliferating cells (results not shown).

Cell counting & analysis

To assess the extent of nestin immunoreactivity, nestin-stained tissue sections were viewed by light microscopy (Olympus IX70), at a magnffication of x400. Counts were obtained from three sections from each block. In normal-appearing samples, three fields were selected at random from the NAWM in each section. For samples containing lesion tissue or shadow plaques, three fields within the lesioned or remyelinated area and three in NAWM were selected. A total cell count and a count of nestin-positive cells were obtained for each field. To allow for variation in total cell number between individual patients, the number of nestin-positive cells was expressed as a percentage of total cell count. SPSS 12.0.1 was used for analysis and statistics. Data were analyzed using a general linear model for repeated measures with a Tukey HSD *post-hoc* test for multiple comparisons of the percentage of nestin-expressing cells within the different tissue classifications studied (control brain, control spinal cord, NAWM, NASC, brain lesions and spinal cord lesions).

Sections were viewed using a Leica DM6000B fluorescence microscope, with FW4000 software. In addition to counts of total cells and number of nestin-positive cells, the number of cells expressing the second marker and the number of cells that co-expressed both markers were also counted.

Results

Nestin-positive cells are present in MS lesions

We studied CNS tissue from normal control samples and from patients with MS. In control brain or spinal cord tissue, few nestin-positive cells were present (a mean of 5.6% of all cells; **Table 1**; **Figures 1D, 2 & 3A**). Double labeling for nestin and collagen-IV showed that most were endothelial, as reported elsewhere (**Figure 1B**) [13–15]. Similarly, in NAWM and in NASC from patients, only small numbers of nestin-positive cells were seen, and the great majority of these were endothelial (65–100% in the brain co-expressing collagen-IV; 36–52% co-expressing collagen IV in the spinal cord; **Figures 1B & 3B**), although close to lesion sites (and not seen in control tissue), a few nestin-positive collagen-IV-negative cells with multiple processes were observed.

Table 1. Descriptive statistics, including means and ranges for the total numbers of nestin-positive cells (per high power field), and the percentage of cells positive for nestin (of total cell number).

Tissue type	Mean (%)	Range (%)	Minimum (%)	Maximum (%)	Standard deviation
Control white matter brain	**4.84 (5.66)**	4.13 (5.24)	3.27 (2.86)	7.40 (8.10)	0.99 (1.47)
MS NAWM	**5.45 (5.63)**	6.11 (7.91)	3.00 (2.86)	9.11 (10.77)	1.94 (2.13)
MS brain lesion	**20.02 (28.19)**	40.89 (46.31)	8.89 (8.30)	49.78 (54.62)	11.62 (14.42)
Shadow plaques	**18.93 (25.02)**	5.11 (12.53)	17.00 (19.76)	22.11 (32.29)	2.78 (6.50)
Control spinal cord	**5.88 (10.58)**	5.02 (5.71)	3.89 (8.49)	8.91 (14.20)	2.67 (3.15)
MS spinal cord NAWM	**10.74 (14.84)**	19.76 (28.38)	4.11 (5.29)	23.87 (33.67)	5.90 (9.55)
MS spinal cord lesion	**23.35 (33.93)**	23.78 (58.40)	12.00 (19.38)	35.78 (77.77)	6.20 (17.72)

NAWM: Normal-appearing white matter; MS: Multiple sclerosis.

Within MS lesions, however, there was a highly significant increase in both the percentage and absolute number of nestin-positive cells: 26.5% of cells in cerebral lesions versus 5.6% in NAWM ($p < 0.001$), or a mean of 20 cells per field compared with 4.8 (a four-to-five-fold increase), and 23.4 cells per field in spinal cord lesions compared with 5.9 per field in NASC ($p < 0.001$; Table 1; Figures 1A, 2 & 3C, D, F). Only approximately 25% of nestin-expressing cells (range: 19.4–33%) within lesions were collagen-IV positive, indicating that increased angiogenesis in MS lesions [13,16] did not explain the increase in nestin-expressing cells. Nestin-positive/collagen-IV-negative cells fell into two morphologically distinct groups: small, round, densely stained cells and larger cells with multiple processes (Figure 3D & F). Much smaller numbers of both types were also present in NAWM.

In NASC, there were more nestin-positive cells (mostly the round, densely-staining type) than in brain NAWM (Table 1) but this difference was not significant ($p = 0.074$). One control case and several MS spinal cord samples showed nestin-expressing cells apparently converging from the edge of the cord, most notably at the ventral edge (Figure 3G & H). In the MS samples, this bore no obvious relationship to lesions. Most of these circumferential nestin-expressing cells co-expressed musashi-1. Single staining studies showed a morphology suggesting nestin-expressing and musashi-1-expressing cells are the same population (Figure 3G & H).

Although our case numbers are small, we did not find any relationship between numbers of nestin-positive cells and patient age. We observed no clear pattern of distribution of these cells within lesions: they appeared evenly dispersed throughout lesions, with no tendency to occur either at the lesion border or the plaque center.

Differences in nestin expression in active, chronic active and inactive brain lesions were seen but did not reach statistical significance (active: 16 cells per field [range: 14–17] expressed nestin; inactive: 21 cells per field [range: 12–29]; chronic active: 17 [range: 9–23]). Two shadow plaques (from different cases) were identified by myelin pallor on solochrome cyanine staining and confirmed by the demonstration of abnormally thin myelin sheaths in MBP-immunostained sections. Each showed an increase in the relative numbers of nestin-expressing cells (means of 18 and 22 cells per field) compared with corresponding NAWM from MS brains (four and seven cells per field; Figures 1F, G & 2), although these cell numbers were more akin to chronic inactive lesions than acute. In shadow plaques, many nestin-expressing cells showed an interlacing elongated morphology with multiple processes, which was not seen elsewhere (Figure 1F).

Nestin/musashi-1 co-staining

Immunofluorescence labeling with nestin and a second marker also known to label neural precursors, musashi-1, showed similar staining patterns (Figures 1C, D & 3G, H). A mean of 65.8% of NASC nestin-expressing cells (range: 61–71%) and 71.1% in NAWM (range: 57–84%) expressed musashi-1. Likewise, not all musashi-1-positive cells expressed nestin; although there was some variation between cases, most showed 50–100% musashi-1-positive cells to co-express nestin (but as few as 23% in one instance). Within lesions, including shadow plaques, the proportion of nestin-expressing cells co-expressing musashi-1 was higher, often approaching 90%.

Relationship of nestin-positive cells to astrocytes

In NAWM, approximately 30% of all cells were GFAP-positive astrocytes, but very few (mean of 0.3%; range: 0–1.3%) co-expressed nestin. This differed markedly in MS brain lesions, including shadow plaques, where up to 64.2% (mean of 35%; range: 6–64%) of

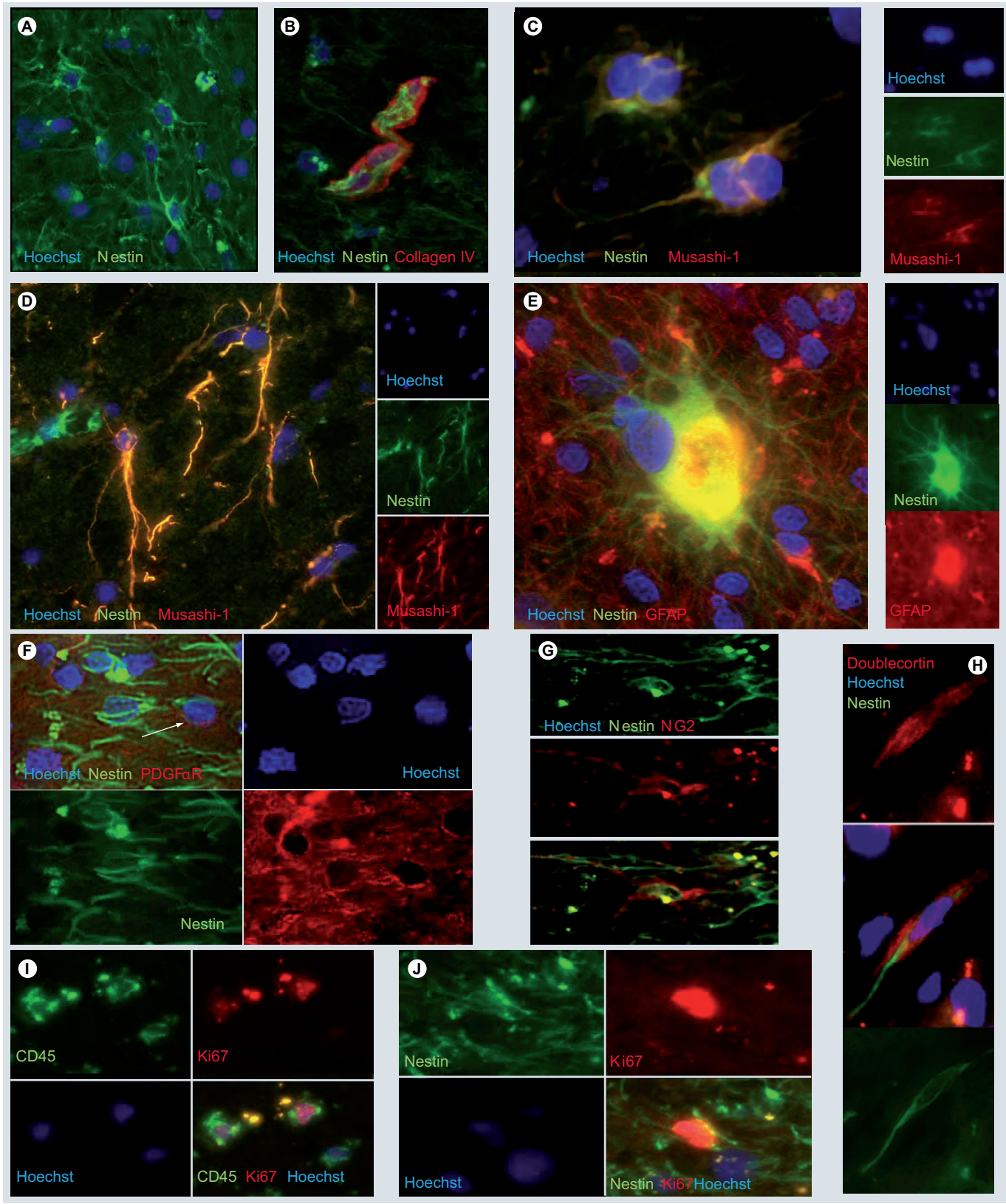

Figure 1. Neural precursors in normal and multiple sclerosis-affected CNS. **(A–C)** Multiple sclerosis (MS) lesion tissue (**A**: MS98 A1C4; **B & C**: MS53 P3C5) showing nestin-positive single stained cells **(A),** and nestin-positive double-stained cells co-positive for collagen V **(B)** or for musashi **(C)**. **(D)** Control spinal cord (C25 SC3) nestin-musashi co-positive cells. **(E & H–J)** MS lesion tissue from brain (MS154 P3C3). **(F & G)** Shadow plaque from MS affected brain (MS170 P2A2). As indicated, **(E–H & J)** show nestin-staining cells co-positive for GFAP, PDGF-α, NG2, doublecortin or Ki67, respectively, while **(I)** shows proliferating CD45/Ki67 co-positive cells. Magnification ×400. All images show nestin in green and Hoechst nuclei stain in blue, other antibodies as indicated.

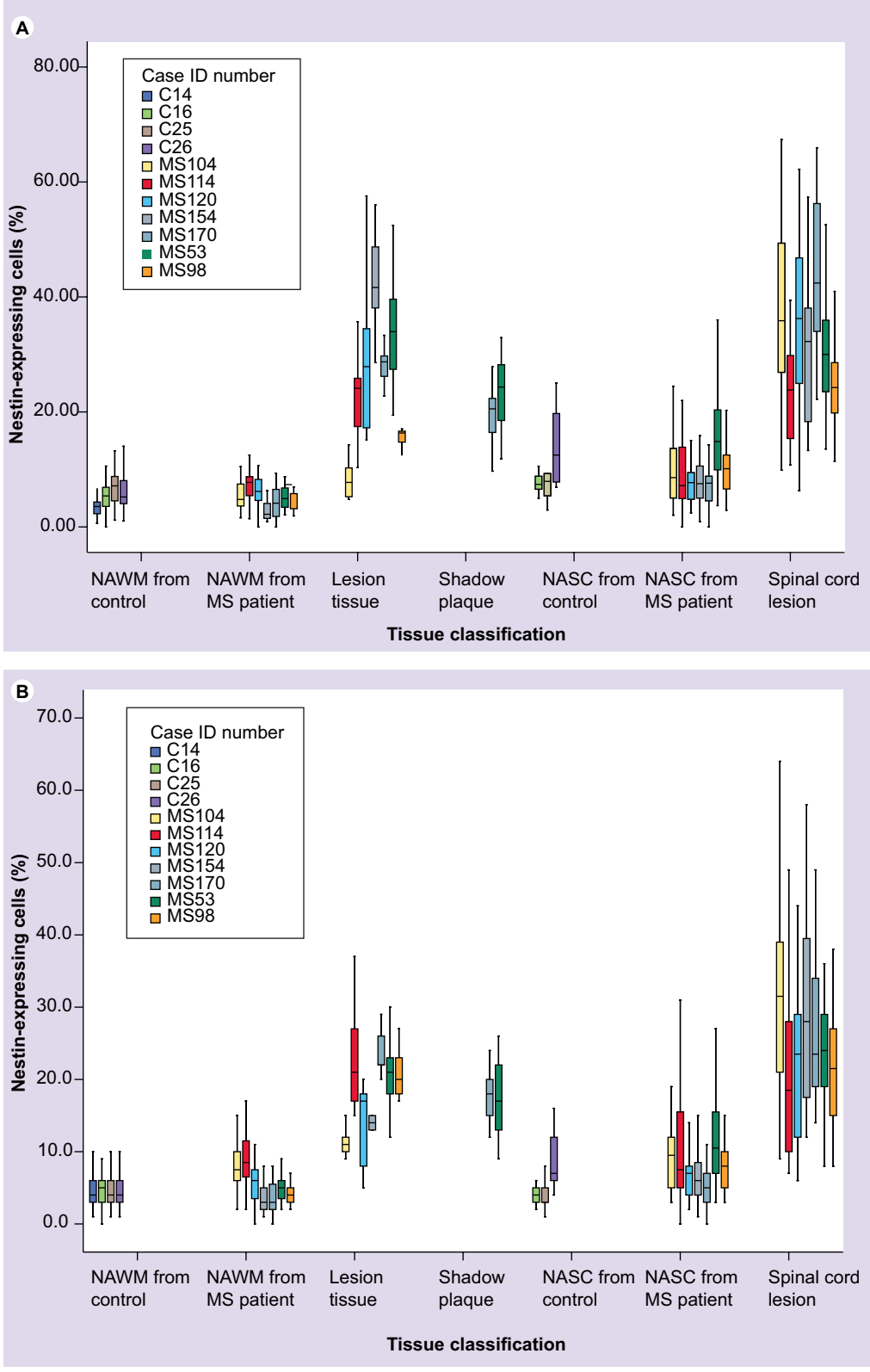

Figure 2. Quantitation of nestin-positive cells in multiple sclerosis tissue. Box-and-whisker plot showing **(A)** the percentage of cells and **(B)** the absolute number of cells/high power field that are positive for nestin in normal-appearing tissue, MS lesion tissue and shadow plaques from white matter and spinal cord. Each case is represented by a different colour bar. C denotes a control case and MS a multiple sclerosis case.
MS: Multiple sclerosis; NASC: Normal-appearing spinal cord white matter; NAWM: Normal-appearing white matter.

GFAP-positive astrocytes co-expressed nestin, and 63.9% of nestin-expressing cells stained for GFAP (Figure 1E). Spinal cord showed less variation. In NASC, a mean of 28% (range: 10–48%) of GFAP-positive astrocytes co-expressed nestin; in spinal lesions, 32% (range: 17–47%).

Relationship of nestin-positive cells to oligodendrocyte lineage cells

The increase in nestin-expressing cells was consistent with the possibility that they might generate oligodendrocyte progenitor cells (OPCs) for remyelination. Immunofluorescent labeling for either of two OPC surface markers, NG2 and PDGF-αR, showed NG2- and PDGF-αR-expressing cells to be present in lesions (including shadow plaques) as previously reported [17–20]. Within lesions, OPCs were found in greater numbers in those areas that also contained most nestin-expressing cells. Double labeling revealed that nestin-positive cells co-expressing NG2 or PDGF-αR were also present, although in very small numbers (<1%; Figure 1F & G).

Relationship of nestin-positive cells to neuronal lineage cells

We double stained tissue with antibodies to nestin and doublecortin, a marker of immature neurons [21,22], to pursue the hypothesis that some neural precursors in lesions might have differentiated not along glial pathways but into neuronal cells. We found small numbers of nestin–doublecortin co-positive cells in MS lesions (Figure 1H). The proportion varied, but a mean of 13.0% of nestin-positive cells (range: 0–43%) within lesions were doublecortin positive.

Proliferation of nestin-positive cells in MS lesions

We used Ki67 immunolabeling to disclose proliferation of cells within MS tissue, and in particular to assess whether any nestin-positive cells might be identified in the process of division [12]. We found significant numbers of Ki67-positive cells, but the great majority of these were nestin negative. Further immunocytochemical investigation revealed that most Ki67-positive cells were co-positive for the leukocyte marker CD45 (Figure 1I), suggesting these were infiltrating inflammatory cells (or possibly activated microglia). However, we also found very small numbers of nestin-positive cells in MS lesions that were clearly co-positive for Ki67 (Figure 1J; a mean of 0.1 cells per field; range: 0–0.56.

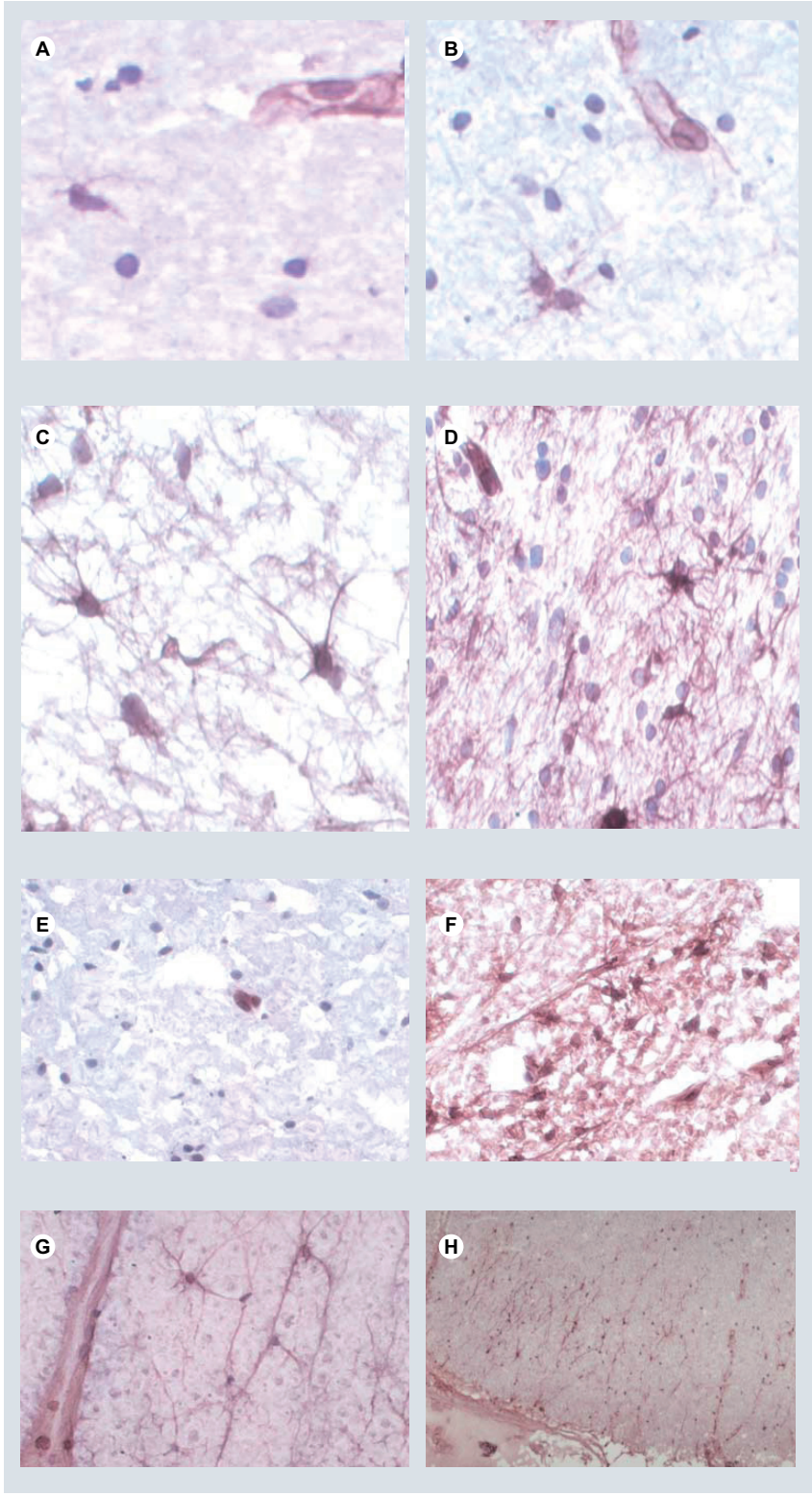

Figure 3: Nestin-expressing cells stained using mouse anti-nestin antibody and visualized with diaminobenzidine peroxidase technique. (A) Control white matter (C25 A4B4). **(B)** Normal-appearing white matter from multiple sclerosis (MS) case (MS170 P2A2). **(C)** and **(D)** MS lesion (MS154 P3C3). **(E)** Normal-appearing spinal cord white matter from MS case (MS154 Scc1). **(F)** Spinal cord MS lesion (MS154 Scc1). Two morphologically distinct types of nestin-positive cells are apparent. **(G & H)** Anterior edge of control spinal cord (C25 SC3). **(G)** Nestin; **(H)** musashi-1. Magnification ×400.

Discussion

We have demonstrated a marked increase in the number of nestin-positive cells in acute MS lesions. The majority of these are co-positive for musashi-1. Our results are consistent with the hypothesis that endogenous neural precursors react locally to disease processes in MS. The presence of nestin–GFAP co-staining cells, and of small numbers of nestin-positive cells that co-stain for markers of neuronal or oligodendroglial lineages, together with the identification of small numbers of proliferating nestin-positive cells, support this proposal.

Nestin antibody does not exclusively mark neural stem cells: normal endothelia and some astrocytes can be nestin positive [14]. In our study, the absence of differentiated cell markers, including endothelial collagen-IV and GFAP, in approximately one third of nestin-positive cells in lesions rather supports neural precursor identity. Furthermore, musashi-1 (although also not absolutely specific as a neural stem cell marker) was expressed in the majority (50–74%) of nestin-expressing cells (excluding collagen IV-expressing endothelia), further suggesting that they are indeed neural precursor cells.

Adult neural precursors were assumed to be restricted to the subependymal zone and hippocampus [23,24]. Subsequently, nestin-positive multipotent neural precursors were grown in cell culture from various areas of the adult human CNS [25–27]. This might be explained as a cell culture artefact. However, more recent studies indicate that this potential is realized not just by *in vitro* conditioning, but also *in vivo* following tissue damage. In experimental models of stroke, trauma and other disorders, large numbers of nestin-positive neural precursors appear in response to tissue injury [28–32]. Finally, it has been confirmed that this process also occurs in the adult human brain following infarction [33–35], sub-arachnoid hemorrhage [36], epilepsy [37], and in Parkinson's [38], Alzheimer's [39] and Huntington's diseases [40,41]. The molecular mechanisms underlying the induction (and suppression) of neurogenesis outside the classical neurogenic regions of the adult CNS are beginning to emerge [42–44].

In experimental demyelinating disease, proliferation of OPCs precedes the generation of new oligodendrocytes and remyelination [45–47]. In other (viral) demyelinating models, endogenous neural stem cells are a major source of proliferating remyelinating cells [48] and subventricular zone neural progenitors contribute to oligodendrogliogenesis in the adult mouse [8,49]. There is also evidence that cellular activation and increased gliogenesis occur in the human subventricular zone in MS [50,51]. Others have demonstrated neurogenesis in response to inflammatory demyelination in the rodent spinal cord [7,52].

Could endogenous neural stem cells therefore contribute to the pool of remyelinating oligodendrocyte progenitors previously demonstrated in MS lesions [17–20,53], some of which appear to proliferate [18], although (probably more mature) O4-positive cells do not [54]? The presence of nestin/musashi co-positive cells, their fivefold increase in lesions and the demonstration of nestin–Ki67 co-positive cells, their close juxtaposition within lesions to OPCs, and the finding of small numbers of nestin/NG2 and nestin/PDGF-αR co-expressing cells (particularly considering the probable very short transitional stage of nestin/PDGF-αR co-expression, with nestin expression being limited to perhaps just a few days [20,30] during neural precursor differentiation to lineage-restricted OPC) are all consistent with this possibility. The presence of cells co-positive for nestin and doublecortin or GFAP also supports the possibility that neural precursors were present in lesions. It has in fact been suggested that, being only very transiently expressed, the demonstration of doublecortin alone is a reliable and specific marker for adult neurogenesis [55–57].

Our findings differ slightly from those of Nait-Oumesmar *et al.*, whose study used polysialic acid-NCAM as a precursor marker, and disclosed, using β-tubulin as a marker, no neuronal differentiation. In both studies, small numbers of proliferating cells, and small numbers of putative precursor cells co-staining with early oligodendroglial markers were found. We did not find a relationship between the number of cells of putative neural precursor origin and proximity to the sub- or peri-ventricular area, but the number of cells identified in our study was small and we certainly could not exclude such an origin.

Others have suggested an alternative (or additional) origin for neural precursors in the adult human diseased brain – namely, the injured and reactive parenchymal astrocyte. It is now clear that GFAP can be an exception to the rule that 'mature' cell markers are not expressed in stem or precursor cells; GFAP-positive 'astrocytes' (type B cells) in the sub- (or peri-) ventricular zone and in the hippocampus exhibit stem cell properties [58], particularly in the adult [59–61] (including human [62]) brain. However, it is also possible that reactive, nestin-expressing parenchymal astrocytes in regions distant from the subventricular zone may also assume neural precursor

behavior. This was first suggested over a decade ago by McKay and Brustle and colleagues [63] and supported by subsequent studies exploring various types of tissue injury [30,32,64,65]. There is, in short, evidence that there is a 'multipotent astrocyte found throughout the postnatal CNS amenable to de-differentiation and stem cell-like behavior' [66].

Regardless of their origin, the large numbers of precursor cells, and evidence of proliferation, together with the far greater proportion of GFAP-positive nestin-expressing cells than those expressing oligodendrocyte-lineage markers, implies that environmental signals in MS lesions are such as to encourage sufficient numbers to accumulate (through division and/or migration), but may not be conducive for determining an oligodendroglial fate for neural precursors. Nestin–GFAP co-positive cells could thus represent 'default' pathway cells useful for no more than scar formation. It remains conceivable, however, that they may play a more subtle but positive role – helping to reprogram repair through various so-called noncanonical reparative mechanisms exhibited by stem cells [67,68], including immune modulation [69–71], neuroprotection [72,73], growth factor production [74–76], reduced scar formation and/or other effects on local macro- and microglia [68].

Our results may help shift the focus of attention away from endogenous OPCs towards parenchymal neural precursors in understanding myelin repair in MS. The challenge becomes to dissect and define the relative contribution that each potential mechanism of reprogramming repair makes to spontaneous remyelination, in addition to understanding the mechanisms of endogenous neural precursor recruitment and proliferation. Thus, a more complete explanation of the frequent failure of tissue repair in MS may emerge, which will help the development of stem cell-based therapies or small-molecule interventions that promote or supplement their activity.

Acknowledgements

Tissue was generously provided by the UK Multiple Sclerosis Society Tissue Bank and we are grateful for this help and assistance, and particularly to the consenting patients and their carers. We are also grateful for the support of the North Bristol NHS Trust, and to H Okano, Department of Physiology, Keio University School of Medicine, Tokyo, Japan, for the generous provision of musashi-1 antibody.

Financial & competing interests disclosure

This research was generously funded by the UK Multiple Sclerosis Society. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Endogenous neural progenitors are present in the normal adult brain and spinal cord.
- Studying brain and spinal cord tissue from patients dying with multiple sclerosis (MS), we show that these cells appear to react to disease processes in MS, markedly increasing in numbers.
- Our results are consistent with differentiation of these cells partly into astrocytes, and rather less so into neuronal cells, but only very rarely along the oligodendroglial lineage.
- Further studies could valuably explore the origins of these cells, and whether parenchymal astrocytes might respond to disease by exhibiting stem cell properties.
- A better understanding of the cell biology of repair in MS, and other neurodegenerative diseases, is vital for the development of regenerative therapies.

Bibliography

1 Perier O, Gregoire A: Electron microscopic features of multiple sclerosis lesions. *Brain* 88(5), 937–952 (1965).

2 Bruck W, Kuhlmann T, Stadelmann C: Remyelination in multiple sclerosis. *J. Neurol. Sci.* 206(2), 181–185 (2003).

3 Franklin RJ: Why does remyelination fail in multiple sclerosis? *Nat. Rev. Neurosci.* 3(9), 705–714 (2002).

4 Zhao C, Fancy SP, Magy L, Urwin JE, Franklin RJ: Stem cells, progenitors and myelin repair. *J. Anat.* 207(3), 251–258 (2005).

5 Rice CM, Halfpenny C, Scolding NJ: Cell therapy in demyelinating diseases. *NeuroRx* 1, 423–415 (2004).

6 Itoyama Y, Webster HD, Richardson-EP J, Trapp BD: Schwann cell remyelination of demyelinated axons in spinal cord multiple sclerosis lesions. *Ann. Neurol.* 14(3), 339–346 (1983).

7 Brundin L, Brismar H, Danilov AI, Olsson T, Johansson CB: Neural stem cells: a potential source for remyelination in neuroinflammatory disease. *Brain Pathol.* 13(3), 322–328 (2003).

8 Picard-Riera N, Decker L, Delarasse C *et al.*: Experimental autoimmune encephalomyelitis mobilizes neural progenitors from the subventricular zone to undergo oligodendrogenesis in adult mice. *Proc. Natl Acad. Sci. USA* 99(20), 13211–13216 (2002).

9 Chandran S, Hunt D, Joannides A, Zhao C, Compston A, Franklin RJ: Myelin repair: the role of stem and precursor cells in multiple sclerosis. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 363(1489), 171–183 (2008).

10 Lendahl U, Zimmerman LB, McKay RD: CNS stem cells express a new class of intermediate filament protein. *Cell* 60(4), 585–595 (1990).

11 Kaneko Y, Sakakibara S, Imai T *et al.*: Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. *Dev. Neurosci.* 22(1–2), 139–153 (2000).

12 Gerdes J, Schwab U, Lemke H, Stein H: Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int. J. Cancer* 31(1), 13–20 (1983).

13 Mokry J, Cizkova D, Filip S *et al.*: Nestin expression by newly formed human blood vessels. *Stem Cells Dev.* 13(6), 658–664 (2004).

14 Gu H, Wang S, Messam CA, Yao Z: Distribution of nestin immunoreactivity in the normal adult human forebrain. *Brain Res.* 943(2), 174–180 (2002).

15 Holley JE, Gveric D, Newcombe J, Cuzner ML, Gutowski NJ: Astrocyte characterization in the multiple sclerosis glial scar. *Neuropath. App. Neurobiol.* 29(5), 434–444 (2003).

16 Kirk S, Frank JA, Karlik S: Angiogenesis in multiple sclerosis: is it good, bad or an epiphenomenon? *J. Neurol. Sci.* 217(2), 125–130 (2004).

17 Chang A, Nishiyama A, Peterson J, Prineas J, Trapp BD: NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *J. Neurosci.* 20(17), 6404–6412 (2000).

18 Maeda Y, Solanky M, Menonna J, Chapin J, Li W, Dowling P: Platelet-derived growth factor-α receptor-positive oligodendroglia are frequent in multiple sclerosis lesions. *Ann. Neurol.* 49(6), 776–785 (2001).

19 Scolding NJ, Franklin RJM, Stevens S, Heldin CH, Compston DAS, Newcombe J: Oligodendrocyte progenitors are present in the normal adult human CNS and in the lesions of multiple sclerosis. *Brain* 121, 2221–2228 (1998).

20 Wolswijk G: Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells. *J. Neurosci.* 18(2), 601–609 (1998).

21 Francis F, Koulakoff A, Boucher D *et al.*: Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. *Neuron* 23(2), 247–256 (1999).

22 Gleeson JG, Minnerath SR, Fox JW *et al.*: Characterization of mutations in the gene doublecortin in patients with double cortex syndrome. *Ann. Neurol.* 45(2), 146–153 (1999).

23 Kukekov VG, Laywell ED, Suslov O *et al.*: Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. *Exp. Neurol.* 156(2), 333–344 (1999).

24 Johansson CB, Svensson M, Wallstedt L, Janson AM, Frisen J: Neural stem cells in the adult human brain. *Exp. Cell Res.* 253(2), 733–736 (1999).

25 Nunes MC, Roy NS, Keyoung HM *et al.*: Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nat. Med.* 9(4), 439–447 (2003).

26 Mayer EJ, Carter DA, Ren Y *et al.*: Neural progenitor cells from postmortem adult human retina. *Br. J. Ophthalmol.* 89(1), 102–106 (2005).

27 Arsenijevic Y, Villemure JG, Brunet JF *et al.*: Isolation of multipotent neural precursors residing in the cortex of the adult human brain. *Exp. Neurol.* 170(1), 48–62 (2001).

28 Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O: Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat. Med.* 8(9), 963–970 (2002).

29 Azari MF, Profyris C, Zang DW, Petratos S, Cheema SS: Induction of endogenous neural precursors in mouse models of spinal cord injury and disease. *Eur. J. Neurol.* 12(8), 638–648 (2005).

30 Douen AG, Dong L, Vanance S *et al.*: Regulation of nestin expression after cortical ablation in adult rat brain. *Brain Res.* 1008(2), 139–146 (2004).

31 Kernie SG, Erwin TM, Parada LF: Brain remodeling due to neuronal and astrocytic proliferation after controlled cortical injury in mice. *J. Neurosci. Res.* 66(3), 317–326 (2001).

32 Lie DC, Dziewczapolski G, Willhoite AR, Kaspar BK, Shults CW, Gage FH: The adult substantia nigra contains progenitor cells with neurogenic potential. *J. Neurosci.* 22(15), 6639–6649 (2002).

33 Jin K, Wang X, Xie L *et al.*: Evidence for stroke-induced neurogenesis in the human brain. *Proc. Natl Acad. Sci. USA* 103(35), 13198–13202 (2006).

34 Minger SL, Ekonomou A, Carta EM, Chinoy A, Perry RH, Ballard CG: Endogenous neurogenesis in the human brain following cerebral infarction. *Regen. Med.* 2(1), 69–74 (2007).

35 Macas J, Nern C, Plate KH, Momma S: Increased generation of neuronal progenitors after ischemic injury in the aged adult human forebrain. *J. Neurosci.* 26(50), 13114–13119 (2006).

36 Sgubin D, Aztiria E, Perin A, Longatti P, Leanza G: Activation of endogenous neural stem cells in the adult human brain following subarachnoid hemorrhage. *J. Neurosci. Res.* 85(8), 1647–1655 (2007).

37 Gonzalez-Martinez JA, Bingaman WE, Toms SA, Najm IM: Neurogenesis in the postnatal human epileptic brain. *J. Neurosurg.* 107(3), 628–635 (2007).

38 Yoshimi K, Ren YR, Seki T *et al.*: Possibility for neurogenesis in substantia nigra of parkinsonian brain. *Ann. Neurol.* 58(1), 31–40 (2005).

39 Jin K, Peel AL, Mao XO *et al.*: Increased hippocampal neurogenesis in Alzheimer's disease. *Proc. Natl Acad. Sci. USA* 101(1), 343–347 (2004).

40 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. USA* 100(15), 9023–9027 (2003).

41 Curtis MA, Penney EB, Pearson J, Dragunow M, Connor B, Faull RL: The distribution of progenitor cells in the subependymal layer of the lateral ventricle in the normal and Huntington's disease human brain. *Neuroscience* 132(3), 777–788 (2005).

42 Jiao J, Chen DF: Induction of neurogenesis in nonconventional neurogenic regions of the adult central nervous system by niche astrocyte-produced signals. *Stem Cells* 26(5), 1221–1230 (2008).

43 Jiao JW, Feldheim DA, Chen DF: Ephrins as negative regulators of adult neurogenesis in diverse regions of the central nervous system. *Proc. Natl Acad. Sci. USA* 105(25), 8778–8783 (2008).

44 Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH: Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J. Neurosci.* 19(19), 8487–8497 (1999).

45 Reynolds R, Cenci di Bello I, Dawson M, Levine J: The response of adult oligodendrocyte progenitors to demyelination in EAE. *Prog. Brain Res.* 132, 165–174 (2001).

46 Reynolds R, Dawson M, Papadopoulos D *et al.*: The response of NG2-expressing oligodendrocyte progenitors to demyelination in MOG-EAE and MS. *J. Neurocytol.* 31(6–7), 523–536 (2002).

47 Watanabe M, Toyama Y, Nishiyama A: Differentiation of proliferated NG2-positive glial progenitor cells in a remyelinating lesion. *J. Neurosci. Res.* 69(6), 826–836 (2002).

48 Vana AC, Lucchinetti CF, Le TQ, Armstrong RC: Myelin transcription factor 1 (Myt1) expression in demyelinated lesions of rodent and human CNS. *Glia* 55(7), 687–697 (2007).

49 Menn B, Garcia-Verdugo JM, Yaschine C, Gonzalez-Perez O, Rowitch D, varez-Buylla A: Origin of oligodendrocytes in the subventricular zone of the adult brain. *J. Neurosci.* 26(30), 7907–7918 (2006).

50 Nait-Oumesmar B, Picard-Riera N, Kerninon C, Baron-Van EA: The role of SVZ-derived neural precursors in demyelinating diseases: From animal models to multiple sclerosis. *J. Neurol. Sci.* (2008) (Epub ahead of print).

51 Nait-Oumesmar B, Picard-Riera N, Kerninon C *et al.*: Activation of the subventricular zone in multiple sclerosis: evidence for early glial progenitors. *Proc. Natl Acad. Sci. USA* 104(11), 4694–4699 (2007).

52 Danilov AI, Covacu R, Moe MC *et al.*: Neurogenesis in the adult spinal cord in an experimental model of multiple sclerosis. *Eur. J. Neurosci.* 23(2), 394–400 (2006).

53 Chang A, Tourtellotte WW, Rudick R, Trapp BD: Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. *N. Engl. J. Med.* 346(3), 165–173 (2002).

54 Wolswijk G: Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells. *J. Neurosci.* 18(2), 601–609 (1998).

55 Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG: Transient expression of doublecortin during adult neurogenesis. *J. Comp. Neurol.* 467(1), 1–10 (2003).

56 Couillard-Despres S, Winner B, Schaubeck S *et al.*: Doublecortin expression levels in adult brain reflect neurogenesis. *Eur. J. Neurosci.* 21(1), 1–14 (2005).

57 Hua R, Doucette R, Walz W: Doublecortin-expressing cells in the ischemic penumbra of a small-vessel stroke. *J. Neurosci. Res.* 86(4), 883–893 (2008).

58 Doetsch F: The glial identity of neural stem cells. *Nat. Neurosci.* 6(11), 1127–1134 (2003).

59 Imura T, Kornblum HI, Sofroniew MV: The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. *J. Neurosci.* 23(7), 2824–2832 (2003).

60 Richardson RM, Broaddus WC, Holloway KL, Sun D, Bullock MR, Fillmore HL: Heterotypic neuronal differentiation of adult subependymal zone neuronal progenitor cells transplanted to the adult hippocampus. *Mol. Cell Neurosci.* 28(4), 674–682 (2005).

61 Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A: Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97(6), 703–716 (1999).

62 Sanai N, Tramontin AD, Quinones-Hinojosa A *et al.*: Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. *Nature* 427(6976), 740–744 (2004).

63 Lin RC, Matesic DF, Marvin M, McKay RD, Brustle O: Re-expression of the intermediate filament nestin in reactive astrocytes. *Neurobiol. Dis.* 2(2), 79–85 (1995).

64 Lang B, Liu HL, Liu R, Feng GD, Jiao XY, Ju G: Astrocytes in injured adult rat spinal cord may acquire the potential of neural stem cells. *Neuroscience* 128(4), 775–783 (2004).

65 Laywell ED, Rakic P, Kukekov VG, Holland EC, Steindler DA: Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc. Natl Acad. Sci. USA* 97(25), 13883–13888 (2000).

66 Steindler DA, Laywell ED: Astrocytes as stem cells: nomenclature, phenotype, and translation. *Glia* 43(1), 62–69 (2003).

67 Rice CM, Scolding NJ: Adult stem cells – reprogramming neurological repair? *Lancet* 364(9429), 193–199 (2004).

68 Martino G, Pluchino S: The therapeutic potential of neural stem cells. *Nat. Rev. Neurosci.* 7(5), 395–406 (2006).

69 Pluchino S, Zanotti L, Rossi B *et al.*: Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 436(7048), 266–271 (2005).

70 Muraro PA, Douek DC, Packer A *et al.*: Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. *J. Exp. Med.* 201(5), 805–816 (2005).

71 Einstein O, Grigoriadis N, Mizrachi-Kol R *et al.*: Transplanted neural precursor cells reduce brain inflammation to attenuate chronic experimental autoimmune encephalomyelitis. *Exp. Neurol.* 198(2), 275–284 (2006).

72 Madhavan L, Ourednik V, Ourednik J: Grafted neural stem cells shield the host environment from oxidative stress. *Ann. NY Acad. Sci.* 1049, 185–188 (2005).

73 Ryu JK, Kim J, Cho SJ *et al.*: Proactive transplantation of human neural stem cells prevents degeneration of striatal neurons in a rat model of Huntington disease. *Neurobiol. Dis.* 16(1), 68–77 (2004).

74 Guo X, Du J, Zheng Q *et al.*: Expression of transforming growth factor β1 in mesenchymal stem cells: potential utility in molecular tissue engineering for osteochondral repair. *J. Huazhong. Univ. Sci. Technolog. Med. Sci.* 22(2), 112–115 (2002).

75 Kim DH, Yoo KH, Choi KS *et al.*: Gene expression profile of cytokine and growth factor during differentiation of bone marrow-derived mesenchymal stem cell. *Cytokine* 31(2), 119–126 (2005).

76 Mayer H, Bertram H, Lindenmaier W, Korff T, Weber H, Weich H: Vascular endothelial growth factor (VEGF-A) expression in human mesenchymal stem cells: autocrine and paracrine role on osteoblastic and endothelial differentiation. *J. Cell Biochem.* 95(4), 827–839 (2005).

Appendix

Table A1. Summary of anatomical site and histological description of sample blocks as provided by multiple sclerosis Brain Bank.

MS case number	Block	Description
MS053	SCB4	• Thoracic spinal cord • Myelin pallor with no obvious loss of myelin (?retrograde degeneration)
	A3C3	• Frontal lobe, close to L ventricle • No obvious demyelination
	SCD4	• Lumbar cord • Inactive plaque with peripheral rim of microglia with increased class II expression at the level of one of the anterior horns
	P3C5	• Parietal lobe, close to subventricular zone • Inactive plaque, mild upregulation of class II on microglia • Part of the tissue area of demyelination is at the edge of the section
MS098	A1C4	• Frontal lobe, close to subventricular zone • Chronic active plaque within the striatum • Marked upregulation of class II on microglia at the edge of the plaque
	A1C3	• Frontal lobe, close to subventricular zone • Prominent upregulation of class II on microglia in both the gray and white matter with no loss of myelin • There is some myelin pallor (incomplete demyelination or shadow plaque?)
	SC1	• Thoracic cord • Chronic active plaque at the level of one posterior horn
MS104	P2D2	• Temporal lobe • Chronic active plaque with some macrophages still actively phagocytosing myelin
	A4B2	• Frontal lobe • No obvious demyelination
	SC1	• Cervical cord • Some active demyelination at the level of one lateral column • Some macrophages are noted within the plaque
	SC3	• Thoracic cord • No loss of myelin

Table A1. Summary of anatomical site and histological description of sample blocks as provided by multiple sclerosis Brain Bank (cont.).

MS case number	Block	Description
MS114	P4C5	• Parietal lobe, close to subventricular zone • Inactive white matter plaque at the edge of the section
	P4B5	• Parietal lobe, close to subventricular zone • No demyelination
	SC8	• Lumbar cord • Small inactive plaque at the level of one of the lateral columns
	SC6	• Thoracic cord • No obvious demyelination
MS120	P3C2	• Parietal lobe • Small inactive plaque with a few microglial cells at the edge of the section
	P2B3	• Parietal lobe • No obvious demyelination
	SC5	• Thoracic cord • Inactive plaque at the level of one the lateral columns with no microglia
	SC3	• Cervical cord • Inactive plaque at the level of one the lateral columns with no microglia
MS154	P3C3	• Parietal lobe close to subventricular zone • Some confluent white matter and subcortical active plaques with varying levels of class II upregulation on microglia • One plaque shows numerous macrophages and no spared myelin
	SCC4	• Lumbar cord • Diffuse bilateral inactive demyelination more prominent in the lateral columns • Focal upregulation of class II on microglia
	SCC1	• Thoracic cord • Chronic active plaque in one of the lateral columns
MS170	P2D3	• Temporal lobe • Periventricular inactive plaque with mild upregulation of class II on microglia mostly within the plaque
	P2A2	• Parietal lobe • White matter tissue area of myelin discolouration with mild microglia activation • Possible shadow plaque
	SC1	• Cervical cord • One inactive plaque involving one of the lateral columns with mild microglia infiltrates • Some discolouration, more consistent with retrograde tract degeneration, on contralateral surface