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Neurogenesis in the chronic lesions of multiple sclerosis

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Subcortical white matter in the adult human brain contains a population of interneurons that helps regulate cerebral blood flow. We investigated the fate of these neurons following subcortical white matter demyelination. Immunohistochemistry was used to examine neurons in normal-appearing subcortical white matter and seven acute and 59 chronic demyelinated lesions in brains from nine patients with multiple sclerosis and four controls. Seven acute and 44 of 59 chronic multiple sclerosis lesions had marked neuronal loss. Compared to surrounding normal-appearing white matter, the remaining 15 chronic multiple sclerosis lesions contained a 72% increase in mature interneuron density, increased synaptic densities and cells with phenotypic characteristics of immature neurons. Lesion areas with increased neuron densities contained a morphologically distinct population of activated microglia. Subventricular zones contiguous with demyelinated lesions also contained an increase in cells with phenotypes of neuronal precursors. These results support neurogenesis in a subpopulation of demyelinated subcortical white matter lesions in multiple sclerosis brains.

Keywords: multiple sclerosis; white matter neurons; neurogenesis

Abbreviations: DAB = diaminobenzidine; MAP2 = microtubule-associated protein 2; MHC = major histocompatibility complex; nNOS = neuronal nitric oxide synthase; NPY = neuropeptide Y; PLP = proteolipid protein; SVZ = subventricular zone

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Introduction

In the past 15 years, there has been a paradigm shift in research on multiple sclerosis. Although myelin and the oligodendrocyte are the primary targets of inflammatory demyelination, permanent disability in multiple sclerosis patients is now attributed to the impact that this process has on the viability of axons and neurons (Bjartmar *et al.*, 2003). Numerous studies have confirmed the extent of axonal damage in white matter lesions (Ferguson *et al.*, 1997; Trapp *et al.*, 1998), as well as the extent of grey matter demyelination and injury to neurons (Brownell and Hughes, 1962; Kidd *et al.*, 1999; Peterson *et al.*, 2001). In addition, there is extensive cognitive impairment in multiple sclerosis patients (Rao *et al.*, 1991; Beatty, 1993), which may also be attributed to the effect of demyelination on neuronal function. Therefore, the mechanisms of neuronal injury and identification of potential targets for neuroprotection have become important areas of research focus.

Study of neuronal injury in grey matter is difficult to perform because of the high neuronal density in these areas. An alternative location more conducive to analysis of neurons is the subcortical white matter. Neurons in this region are less dense than in grey matter, but display an extensive network of dendrites and receive ultrastructurally confirmed synaptic input (Kostovic and Rakic, 1980; Chun and Shatz, 1989; Okhotin and Kalinichenko, 2003). White matter neurons regulate vascular tone (Okhotin and Kalinichenko, 2003) based upon their expression of the vasoconstrictors, somatostatin (Kostovic *et al.*, 1991) and neuropeptide Y (NPY; Kuljis and Rakic, 1989; Delalle *et al.*, 1997), and neuronal nitric oxide synthase (nNOS), the enzyme that synthesizes the vasodilator, nitric oxide (Okhotin and Kalinichenko, 2003). Subcortical white matter neurons are readily identifiable in species with large white matter volume, such as primates and cats (Kostovic and Rakic, 1980; Chun and Shatz, 1989; Okhotin and Kalinichenko, 2003).

Table 1 Features of multiple sclerosis patients studied

ID no.	Age (years)/ gender	Race	PMI (h)	Type of multiple sclerosis	Disease duration (years)	EDSS	Lesion type and number		
							Active	Chronic decreased neurons	Chronic increased neurons
1	53/M	C	17	SPMS	15	9.5	2	0	0
2	46/M	C	3	SPMS	23	8	4	10	0
3	63/F	C	5	SPMS	9	8	1	0	0
4	77/F	C	5	SPMS	46	8	0	13	1
5	61/F	A	10	SPMS	35	9.5	0	17	13
6	52/M	C	5	SPMS	25	7.5	0	1	1
7	56/M	C	3	SPMS	33	9.5	0	1	0
8	57/F	C	6	PPMS	15	6.5	0	1	0
9	43/M	C	3	RRMS	1	6	0	1	0

Race: C = Caucasian; A = African-American; PMI = post mortem interval. Type of multiple sclerosis: SP = secondary progressive; PP = primary progressive; RR = relapsing remitting.

Neurons are not a prominent feature in subcortical white matter of adult mice and rats (Robertson *et al.*, 2000; Clancy *et al.*, 2001).

The present report investigates the fate of subcortical white matter neurons in the lesions of multiple sclerosis. Our studies document the destruction of neurons during inflammatory demyelination of subcortical white matter. While many chronically demyelinated lesions also showed marked neuronal loss, we identified 15 lesion areas that contain a 72% increase in the density of cells that express unambiguous neuronal markers and that receive ultrastructurally confirmed synaptic input. These results support neurogenesis in a subpopulation of demyelinated subcortical white matter lesions in multiple sclerosis brains.

Materials and Methods

Tissue

The brains of nine patients with multiple sclerosis and four individuals without neurological disease were obtained by a rapid autopsy protocol. The procurement of these tissues was approved by Cleveland Clinic Institutional Review Board. Tables 1 and 2 list the characteristics of these patients who were followed up at the Cleveland Clinic. The brains were sliced (1 cm thick) and fixed in 4% paraformaldehyde. Sixty-six demyelinated regions were macroscopically identified, removed and cryoprotected as described previously (Trapp *et al.*, 1998; Chang *et al.*, 2000). The lesions examined were all located within cerebral subcortical white matter without extension into grey matter. The demyelinated lesions were characterized as active or chronic, based upon the distribution of myelin proteins and staining for the major histocompatibility complex (MHC) Class II molecules, as described previously (Bo *et al.*, 1994).

Immunohistochemical analyses

Tissue blocks were sectioned (30 μm thick) on a sliding microtome, microwaved in 10 mM citric acid buffer (pH 6.0) for 5 min, incubated in 3% hydrogen peroxide and 1% Triton X-100 in phosphate-buffered saline for 30 min and immunostained by the avidin–biotin complex procedure with diaminobenzidine (DAB), as described previously (Trapp *et al.*, 1998; Chang *et al.*, 2000). Sections used for double-labelling experiments were pretreated as above and incubated with two primary antibodies for 3–5 days followed by fluorescently conjugated and biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1–2 h.

Table 2 Features of control patients studied

Age (years)/ gender	Race	PMI (h)	Proximate cause of death
65/F	A	7	Acute myocardial infarction
47/F	A	15	Disseminated intravascular coagulation
52/F	A	12	Polymicrobial sepsis
65/M	C	11	Acute pericardial hemorrhage

Race: A = African-American; C = Caucasian; PMI = post mortem interval.

Antibodies

Sections were immunostained with antibodies specific for the following molecules: proteolipid protein (PLP, Agmed, Bedford, MA), MHC Class II, leukocyte common antigen, synaptophysin (all from Dako, Glostrup, Denkmark), PSA-NCAM (BD Pharmigen, San Diego, CA), nNOS (monoclonal, BD Biosciences, San Jose, CA), microtubule-associated protein 2 (MAP2), calretinin (both from Sigma, St Louis, MO), NeuN, calbindin, Dlx2, nNOS (polyclonal), NPY, somatostatin, GAD67, parvalbumin (all from Chemicon, Temecula, CA), myelin basic protein (MBP, Sternberger Monoclonals, Baltimore, MD), Hu clone 16A11 (abcam, Cambridge, MA) and Iba1 (Ito *et al.*, 1998, monoclonal antibody produced by Trapp Laboratory).

Microscopy

Sections were examined with an Axiophot microscope (Carl Zeiss, Thornwood, NY) equipped with a Magnafire digital camera

(Optronics, Goleta, CA) or with a laser scanning confocal microscope (Leica Microsystems, Exton, PA) (Trapp *et al.*, 1998; Chang *et al.*, 2000). Confocal laser intensity was adjusted to eliminate 'bleed-through'. The images presented are stacks of 12–30 optical sections that were scanned synchronously and compiled using Scion image (Scion, Frederick, MD). Contrast and colour balance of digital images were processed using Photoshop 7.0 (Adobe systems, San Jose, CA).

Definition and quantification of neurons

For brightfield immunohistochemistry, only MAP2-positive cells with unambiguous neuronal morphology were counted as neurons. MAP2 is particularly suited to this analysis, because there is normally a low density of reactivity in the white matter. Other neuronal markers, such as Class III beta-tubulin (Tuj1) and neurofilament protein, show abundant immunoreactivity in axons of the white matter, making it impossible to recognize the small population of neuronal cell bodies in this region. MAP2-positive neurons had large cell bodies (typically 20 μm) and one or more dendrites (typically 50 μm or longer).

Brightfield preparations of single-labelled sections were used for quantification of immunohistochemistry. Neuronal numbers were quantified within the demyelinated area as well as in surrounding normal-appearing white matter present in the same section but positioned closer to the cortex (Fig. 4C). Comparison of lesion with non-lesion area of the same sections controlled for any possible regional variation in subcortical white matter neuronal density. Since neuronal densities are always greater closer to the cortex (Akbarian *et al.*, 1996) (Fig. 4B and C), this analysis also permitted the most stringent test of differences between lesion and non-lesion areas. NeuN-positive and MAP2-positive neurons were counted in lesion and control areas measuring $\sim$1 mm^2. For large lesions, the region of highest neuronal density was counted. NeuN-positive cells were also quantified in subventricular zones (SVZs) contiguous with demyelinated subcortical white matter and compared to densities in SVZ contiguous with myelinated white matter present in the same section (Fig. 5A). Such areas were present adjacent to 22 of the 59 lesions examined. Cell numbers were expressed as cells per millimeter length of SVZ. SVZ cell number was also counted in a total of three regions from two control brains. Differences were compared using the Student's *t*-test. $\alpha = 0.05$.

Electron microscopic immunohistochemistry

Sections (30 μm thick) from lesions of multiple sclerosis containing increased neuronal densities were immunostained with synaptophysin antibodies and DAB as a substrate for detection of specific staining. Areas containing punctate synaptophysin staining were placed in 2.5% glutaraldehyde and 4% paraformaldehyde for 48 h, osmicated and embedded in Epon by standard procedures. Blocks were sectioned on a Leica ultramicrotome, placed on grids and examined in a Phillips H-100 electron microscope.

Results

Subcortical white matter neurons

Subcortical white matter neurons were identified with antibodies to MAP2, a cytoskeletal protein enriched in dendrites and somas of mature neurons. The morphology and distribution of white matter neurons in multiple sclerosis normal-appearing white matter are identical to controls. Morphologically, these MAP2-positive neurons have fusiform cell bodies and one to three primary dendrites with additional arborizations that are oriented parallel to myelinated axons (Fig. 1A and B). Subcortical white matter neurons express markers of cortical interneurons such as calretinin (Fig. 1C) and calbindin (Fig. 4H), but do not express glutamic acid decarboxylase or parvalbulmin (Supplementary Fig. 1) (DeFelipe, 1997; Okhotin and Kalinichenko, 2003). White matter neurons also express a variety of vasoactive molecules, which supports their involvement in the regulation of vascular tone (Okhotin and Kalinichenko, 2003). Specifically, subsets of white matter neurons express nNOS, the enzyme that synthesizes the vasodilatory molecule nitric oxide (Fig. 1D), as well as the vasoconstrictors, NPY (Fig. 1E) and somatostatin (Fig. 1F).

Inflammatory demyelination destroys white matter neurons

Seven areas of acute demyelination in subcortical white matter were identified by the absence of the myelin proteins (Fig. 2A) and abundant MHC Class II-positive cells within the demyelinated area (Fig. 2B). The MHC Class II-positive cells were round, lipid-filled phagocytic macrophages, indicative of recent demyelination (Fig. 2C, Supplementary Fig. 2). When sections from these acute lesions were stained for MAP2 and NeuN, neurons were rarely detected. When present, most had fragmented MAP2-positive dendrites and shrunken perikarya (Fig. 2D). While we cannot exclude the possibility that some neurons within white matter lesions might survive, these observations indicate that most, if not all, white matter neurons are destroyed during inflammatory demyelination of subcortical white matter.

Fifty-nine chronic lesions of multiple sclerosis were identified by a lack of myelin protein staining (Fig. 2E) and paucity of MHC Class II-positive round, phagocytic macrophages (Fig. 2F). When these chronic lesions were examined for neurons, 44 of the 59 had markedly reduced neurons (Fig. 2G) and dystrophic changes in residual ones (Fig. 2H). In addition, MAP2-positive dendrites that should have extended into the lesions stopped at the borders (Fig. 2G). These data provide additional support for the concept that most, if not all, subcortical white matter neurons are destroyed during inflammatory demyelination in multiple sclerosis brains.

Chronic lesions with increased neuronal densities

Fifteen of the 59 chronic lesions of multiple sclerosis studied contained areas with increased density of MAP2-positive neurons relative to adjacent non-lesion areas (Fig. 3A–C). These lesions were present in three of the nine multiple sclerosis brains examined. MAP2-positive neurons had a mature appearance, but dendrites of these cells were not

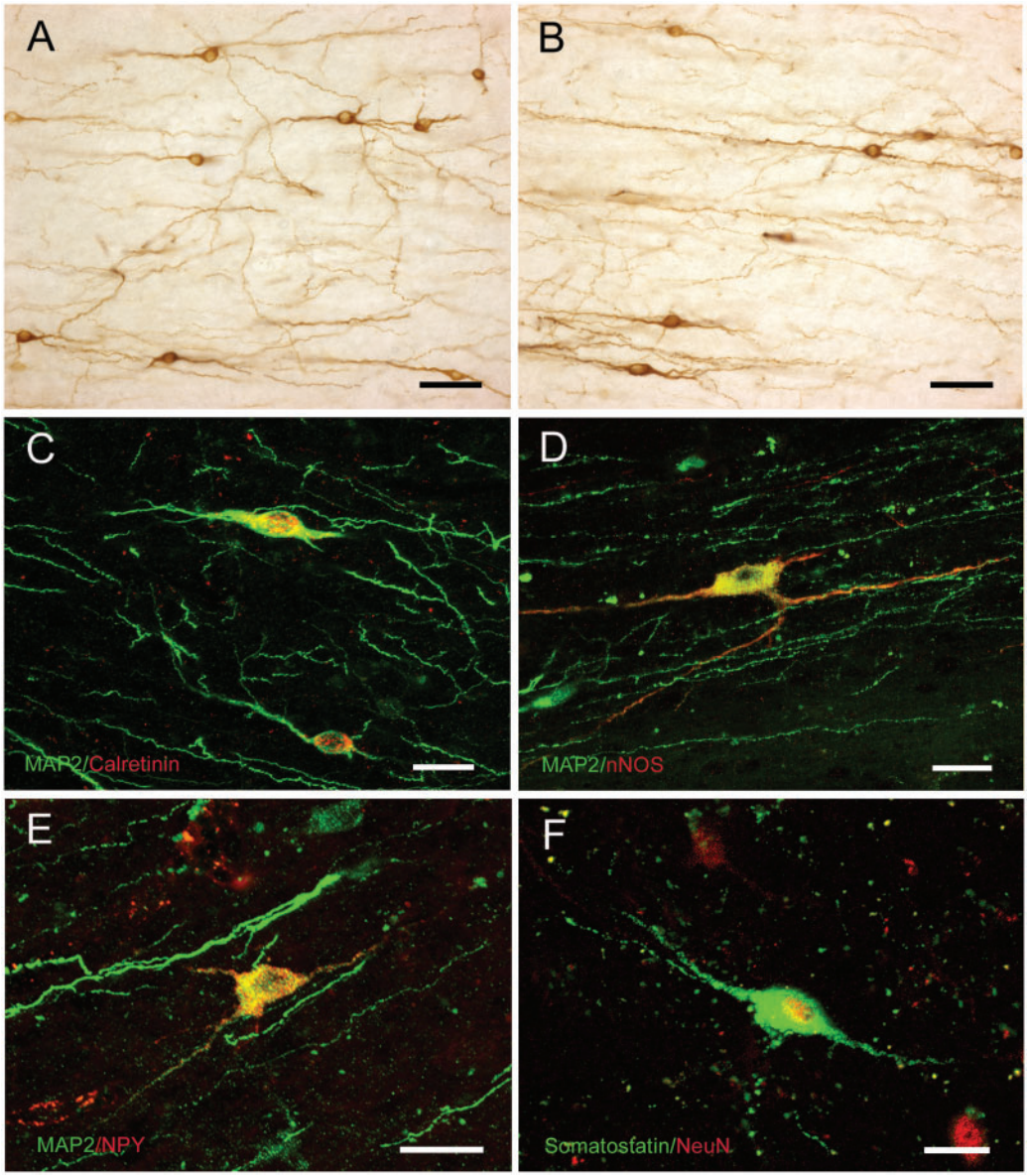

Fig. 1 Morphology and immunophenotype of white matter neurons. MAP2-positive neurons in subcortical white matter have fusiform cell bodies and one to three primary dendrites with additional arborizations that are oriented parallel to myelinated axons (**A** and **B**). Images are from multiple sclerosis normal-appearing white matter; neuronal morphology and distribution are identical to controls. White matter neurons also express other neuronal markers, such as calretinin, a marker of interneurons (**C**, red), nNOS (**D**, red) and vasoactive molecules, NPY (**E**, red) and somatostatin (**F**, green). White matter neurons also express the neuronal nuclear antigen, NeuN (**F**, red). Green immunostain in **C**–**E** is MAP2. (**C**–**E**) Multiple sclerosis normal-appearing white matter. (**F**) Multiple sclerosis lesion (with increased neurons). Scale bars: (**A** and **B**) 50 μm; (**C**–**F**) 20 μm.

always oriented parallel to axons as in normal-appearing subcortical white matter (cf. Fig. 3D and E). Lesions with increased neuronal density also had increased density of MHC Class II-positive cells (Fig. 3B). These cells had relatively large cell bodies, prominent, stout processes (Fig. 3F and G), and did not contain myelin degradation products. Their morphology was distinct from the MHC Class II-positive microglia with small cell bodies and short thin processes in chronic lesions with decreased neurons (Fig. 3H), and from the phagocytic macrophages present in active lesions (Fig. 2C).

By double-label immunofluorescence and confocal microscopy, MAP2-positive white matter neurons were also NeuN-positive. Compared to normal-appearing white matter, lesions with increased neuronal densities contained an additional population of cells that were NeuN-positive and MAP2-negative (Fig. 4A, arrowheads). All NeuN-positive cells were also positive for Hu (Supplementary Fig. 3). Expression of NeuN and Hu begins in post-mitotic immature neurons (Pincus *et al.*, 1998; Sarnat *et al.*, 1998). Therefore, the NeuN-positive/MAP2-negative cells most likely represent a population of immature cells, which are

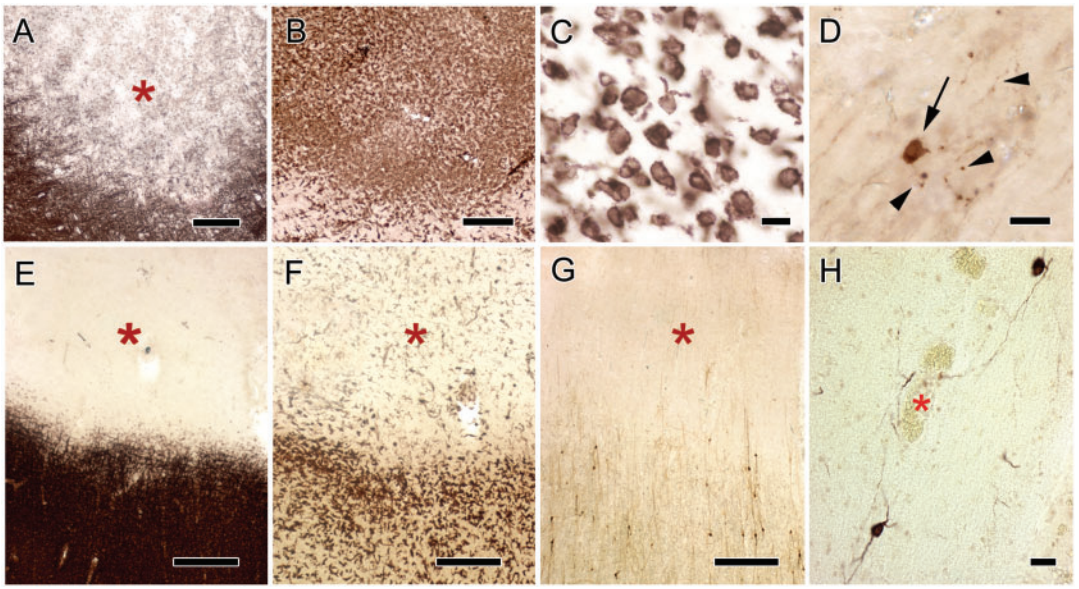

Fig. 2 Inflammatory demyelination is associated with decreased numbers of white matter neurons. Acute (**A**–**D**) and chronic (**E**–**H**) lesions are characterized using immunohistochemistry for PLP (**A** and **E**) and MHC Class II (**B**, **C** and **F**). Normal myelin protein stain is characterized by diffuse intense immunoreactivity as shown in the lower portion of panels **A** and **E**. Normal MHC Class II immunoreactivity is shown in lower portion of panel **F**. An active lesion of multiple sclerosis characterized by loss of myelin (**A***), myelin debris within the demyelinated area and an even distribution of MHC Class II-positive macrophages throughout the lesion (**B** and **C**). All active lesions had decreased neurons. Residual MAP2-positive neurons had shrunken perikarya (**D**, arrow) and fragmented dendrites (**D**, arrowheads). A chronic lesion shows loss of myelin (**E***) and increased MHC Class II-positive cells at the lesion border (**F**). White matter neurons are greatly reduced in most chronic multiple sclerosis lesions (**G**, MAP2). nNOS-positive neurons with shrunken perikarya (**H**) extend processes to blood vessels (**H***). Scale bars: (**A**, **B**, **E**–**G**) 200 μm; (**C**, **D** and **H**) 20 μm.

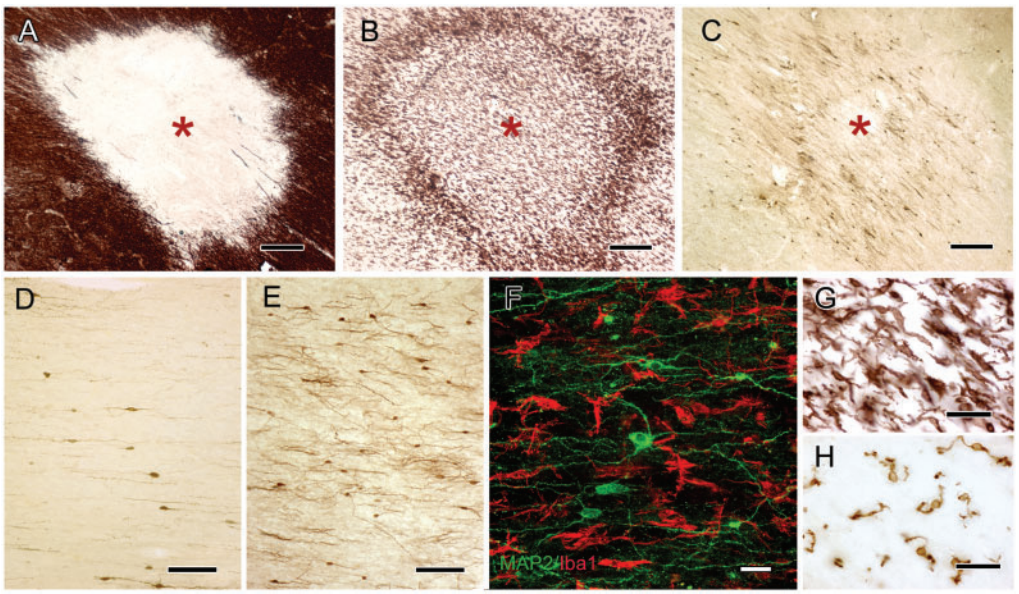

Fig. 3 A subset of chronic multiple sclerosis lesions shows an increased density of white matter neurons. The area of demyelination is demonstrated by lack of PLP immunostaining (**A**). Leukocyte common antigen immunostaining demonstrates the lesion border as well as increased staining within the lesion (**B**). Increased neuronal density is demonstrated by MAP2 staining (**C**). Neurons in non-lesion white matter extend long processes that run along axons (**D**, MAP2). Neurons that are increased in white matter lesions extend shorter dendrites (**E**, MAP2). Double-label immunofluorescence and confocal microscopy show that MAP2 immunoreactivity is not present in microglial cells indicating that the increased MAP2 immunoreactivity in these lesions is not due to phagocytosis by microglia (**F**, MAP2, green, microglial marker, Iba1, red). Microglial cell morphology predicts whether lesions will contain increased neurons. In multiple sclerosis lesions containing increased neurons, microglia are abundant and have large cell bodies and multiple thick processes (**F**, Iba1, red, **G**, MHC Class II). In lesions where neurons are absent, microglia density is low, and these cells extend few thin processes (**H**, MHC Class II). Scale bars: (**A**–**C**) 200 μm; (**D** and **E**) 100 μm; (**F**–**H**) 20 μm.

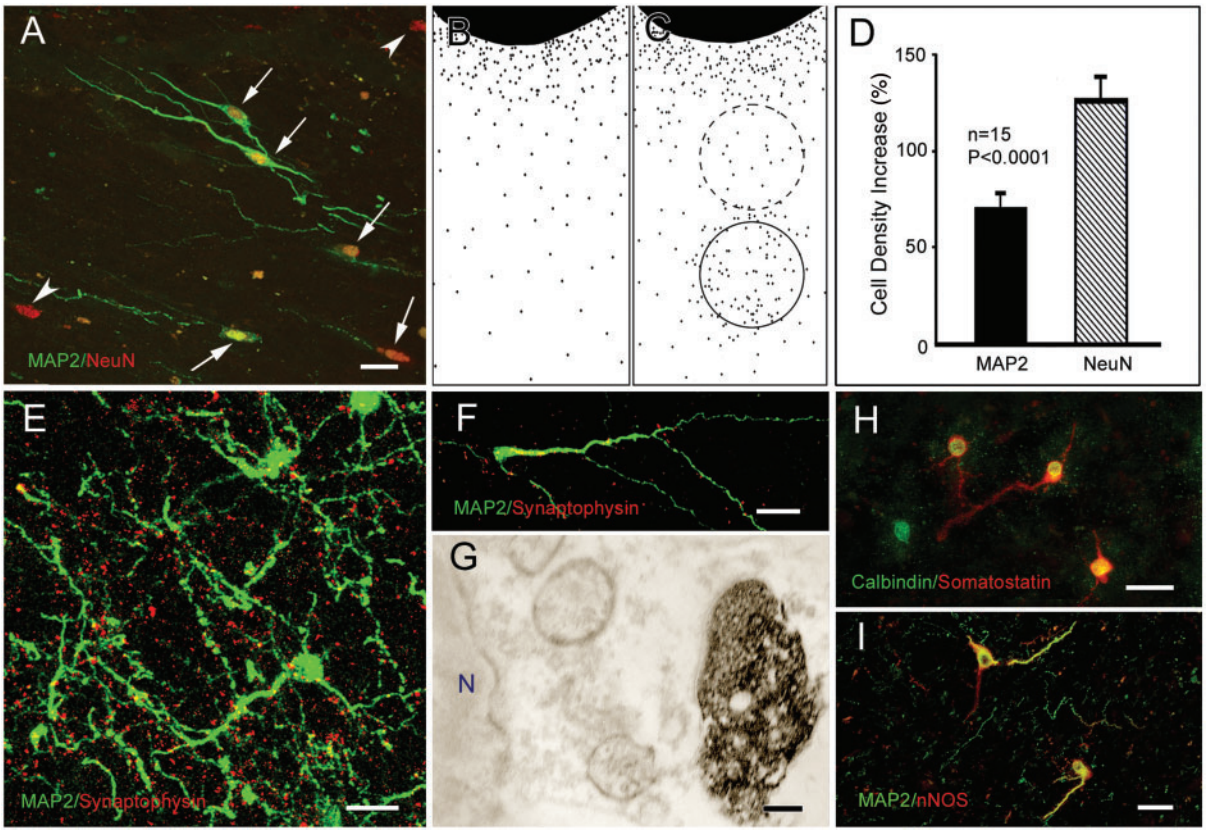

Fig. 4 Evidence for neurogenesis in multiple sclerosis lesions. Confocal image of a section double-labelled for MAP2 (**A**, green) and NeuN (**A**, red). All MAP2-positive cells are NeuN-positive (**A**, arrows), but some NeuN-positive cells are MAP2-negative (**A**, arrowheads). Schematic diagrams illustrate the normal distribution of white matter neurons (**B**) and the appearance of multiple sclerosis lesions with increased neurons (lower circle in **C**). In 15 of 59 lesions, MAP2-positive cell density is increased 72% (**D**) compared to locations closer to the cortex (upper circle in **C**). NeuN-positive cell density is increased 130% when compared to normal white matter (**D**). Synaptophysin-positive (red) puncta closely appose MAP2-labelled (green) neuronal soma and dendrites in a multiple sclerosis lesion (**E**) and in control white matter (**F**). Neurons in the lesions radiate highly branched dendrites and receive an increased density of synaptophysin-positive puncta compared with control neurons. Electron microscopy of synaptophysin-stained multiple sclerosis lesion with increased neuronal density (**G**) demonstrates that synaptophysin immunoreactivity is present in membrane-bound structures apposed to neurons consistent with presynaptic endings (dark staining on right, N = neuronal nucleus). Neurons in multiple sclerosis lesions also express proteins specific for interneurons including calbindin (**H**, green), somatostatin (**H**, red) and nNOS (**I**, red, MAP2, green). Scale bars: (**A**, **E**, **F**, **H** and **I**) 20 μm; (**G**) 250 nm. Error bars: SEM.

committed to the neuronal lineage, but have not differentiated into neurons that polarize their processes into mature dendrites and axons. The 15 lesions with increased neurons were immunostained with antibodies to MAP2 and NeuN, and their density compared to normal-appearing white matter located closer to the cerebral cortex (Fig. 4B and C). The density of MAP2-positive neurons in lesions was increased by 72% and NeuN-positive cell density was increased by 130% ($P < 0.0001$, Fig. 4D). These data support an increase in both mature NeuN-positive/MAP2-positive neurons and immature NeuN-positive/MAP2-negative neurons in a subset of chronic lesions in multiple sclerosis brains.

Since neurons in subcortical white matter receive synaptic input (Kostovic and Rakic, 1980), we investigated whether demyelinated regions with increased neuronal densities also contained increased synapses by double-labelling sections with antibodies specific for MAP2 and synaptophysin, a protein enriched in synaptic vesicles (Fig. 4E and F). MAP2-positive neuronal perikarya and dendrites in normal-appearing white matter were closely apposed by putative synaptic boutons as identified by punctate synaptophysin staining (Fig. 4F). Compared to normal-appearing white matter, lesions with increased neuronal density contained a dramatic increase in punctate synaptophysin staining apposing both neuronal perikarya and dendrites (Fig. 4E). To validate synaptic innervation of these neurons at the ultrastructural level, 30 μm thick sections were immunostained with synaptophysin antibodies using DAB as a chromogen, embedded in Epon, and sectioned for electron microscopic examination. Ultrastructural preservation was adequate for localization of DAB signal in thin sections. The punctate staining identified at the light microscopic level was confined to small, round to oval, membrane-bound structures containing vesicles and met ultrastructural criteria for synaptic

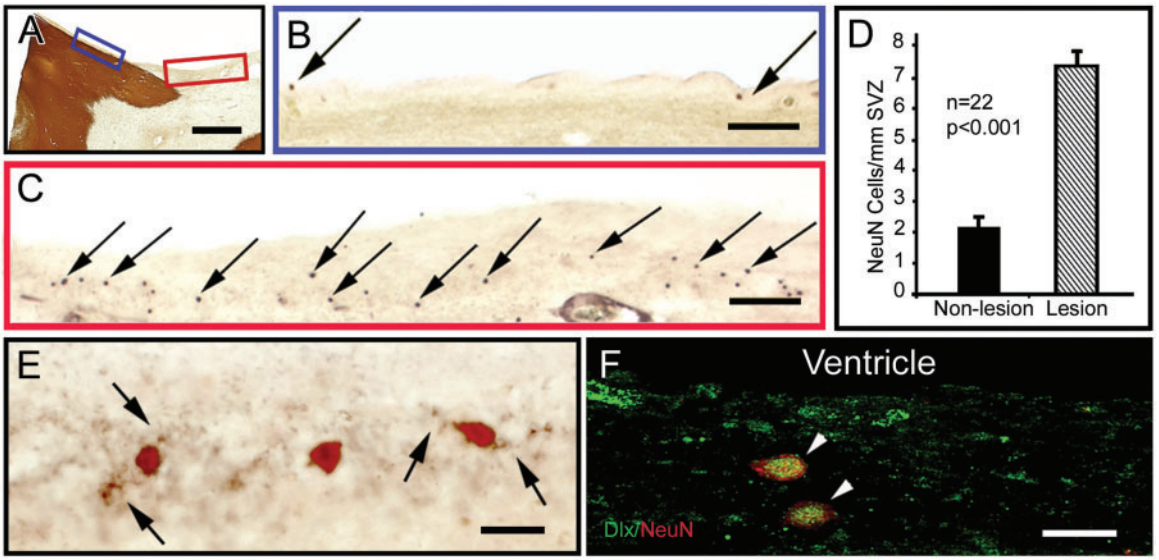

Fig. 5 Immature neurons are increased in the SVZ bordering multiple sclerosis lesions. NeuN-positive cells were quantified in the SVZ bordering periventricular white matter lesions (**A**, red box) and adjacent non-lesion areas (**A**, blue box). NeuN-positive cells are rare in the non-lesion SVZ (**B**, arrows) but frequent in the adjacent lesion SVZ (**C**, arrows). There is a 3.5-fold increase of NeuN-positive cell density in the SVZ of the lesions compared to the non-lesion area (**D**). Some NeuN-positive cells (**E**, NovaRed, **F**, red) in the SVZ bordering a periventricular white matter lesion have PSA-NCAM-positive processes (**E**, brown cytoplasmic staining, DAB, arrows) and Dlx2-positive nuclei (**F**, green, NeuN, red). Scale bars: (**A**) 2 mm; (**B** and **C**) 200 μm; (**E** and **F**) 20 μm. Error bars: SEM.

endings (Fig. 4G). The synapses identified were symmetric axodendritic and axosomatic synapses typical of interneuronal innervation. No axospinous or asymmetric synapses were seen. This provides unambiguous ultrastructural evidence that the increased cells recognized by MAP2 and NeuN staining are neurons. In addition to MAP2 and NeuN, the neurons within these lesions showed functional differentiation as demonstrated by expression of calbindin, somatostatin and nNOS (Fig. 4H and I).

The subventricular zone as a source of cells committed to neurogenesis

To investigate whether the SVZ is a possible source of neurons in multiple sclerosis brains, we compared the number of NeuN-positive cells in SVZs that border demyelinated regions and normal-appearing white matter in 22 sections (Fig. 5A). To control for possible differences based upon brain location, the non-lesion data were obtained from the same section as the lesion data. In SVZs that border normal-appearing white matter, an average of two NeuN-positive cells was detected per millimeter length of SVZ (Fig. 5B), and 1.3 cells/mm were detected in SVZ from normal control subjects (data not shown). In contrast, there was a 3.5-fold increase ($P<0.001$) in NeuN-positive cells per millimeter of SVZs that bordered demyelinated white matter (Fig. 5C and D). The NeuN-positive cells were Hu-positive (Supplementary Fig. 3). Some were PSA-NCAM-positive and extended a few processes from small round cell bodies (Fig. 5E). Since progenitor cells committed to interneuron production express the transcription factor Dlx2 (Flames and Marin, 2005), we double-labelled sections with NeuN and Dlx2. Most NeuN-positive cells in the SVZ expressed Dlx2 (Fig. 5F). Collectively, these data support the SVZ as one source of new neurons in multiple sclerosis brains.

Discussion

This report establishes that subcortical white matter interneurons are destroyed during the inflammatory demyelination that occurs in the brains of individuals with multiple sclerosis. In response to this neuronal loss, some chronically demyelinated brain regions contained neurons at densities that, on average, exceeded those in normal-appearing white matter by 72%. These neurons express immunophenotypic markers of fully differentiated interneurons, have the shape of mature neurons and receive ultrastructurally confirmed synapses. Demyelinated brain regions with increased neuronal densities also contain a morphologically distinct population of activated microglial cells and a significant increase in cells with characteristics of immature neurons that may originate, in part, from adjacent SVZs. These findings support the possibility that white matter interneurons can be replaced following their destruction.

All acute lesions and 44 of 59 chronic lesions analysed in the present study had marked neuronal loss and dystrophic changes in residual neurons. This susceptibility of white matter neurons to inflammatory demyelinating environments has not been recognized previously. It cannot be mediated by loss of myelin sheaths because interneurons are not myelinated. As proposed for axonal transection in acute white matter lesions (Trapp *et al.*, 1998), it is likely that white

matter neurons are vulnerable to the inflammatory demyelinating environment. Indeed, the inflammatory microenvironment contains a variety of substances that could potentially injure neurons, including proteolytic enzymes, cytokines, oxidative products and free radicals produced by activated immune and glial cells (Thery *et al.*, 1991; Chao *et al.*, 1992; Giulian *et al.*, 1993; Moore and Thanos, 1996; Cardona *et al.*, 2006). In addition, demyelination of the cerebral cortex occurs without significant influx of hematogenous leukocytes and frank destruction of neurons (Peterson *et al.*, 2001). It is therefore possible that mechanisms that prevent influx of hematogenous leukocytes during grey matter demyelination evolved as a means to protect neurons from destruction.

An important question regarding these studies is the mechanism by which the increased neuronal densities occur. It is possible that an increase in white matter neurons could occur if cortical blocks were oriented tangentially and then sectioned into or close to cortical layers of adjacent sulci. Since we carefully orient our tissue blocks and maintain grey matter–white matter orientations through serial sections, we are confident that increased neuronal densities occur in demyelinated subcortical white matter. Chronic white matter lesions often have extensive cell loss. In gross brain slices, there is often parenchymal collapse such that the thickness of tissue within a lesion is reduced relative to the adjacent non-lesion tissue. It is possible that atrophy could increase the density of neurons that may have survived acute demyelination. However, there was no consistent difference in gross atrophy between the chronic lesions with and without increased neuronal densities. Therefore, parenchymal atrophy and/or survival of pre-existing neurons cannot account for the observation of increased neurons in a subset of chronic lesions.

We also considered whether the increased neuronal density arises from migration of mature neurons into the lesion. There is no precedence for migration of mature neurons in the adult CNS. Nevertheless, recent data has documented the translocation of synaptically connected interneurons in the early postnatal rat dentate gyrus (Morozov *et al.*, 2006). We do not favour this hypothesis for our data in the mature human nervous system for two reasons. First, if mature neurons were migrating from another location, it would also be expected that a decrease in neuronal density would occur outside of the lesion, which does not appear to be the case. Second, because of the low density of white matter neurons in adult control brain, a migration model would require neurons to translocate distances that are much greater than that observed in the post-natal rat dentate gyrus. Furthermore, the extensive dendritic branches of white matter neurons present a major obstacle to migration.

We favour the interpretation that increased neuronal densities arise from neurogenesis. Our studies demonstrate an increase in the absolute number of cells with unambiguous neuronal phenotype based upon morphology, antigen expression and ultrastructural evidence of synaptic connections. These phenotypes are similar to the interneurons normally found in subcortical white matter and support functional replacement of previously destroyed neurons. These results fulfill several criteria proposed to support identification of new neurons (Rakic, 2002*a*, *b*). Additional criteria for neurogenesis include evidence for recent neuronal cell proliferation (Rakic, 2002*a*, *b*). BrdU incorporation was used to provide evidence of hippocampal neurogenesis in post-mortem brains of patients who had received BrdU as part of a cancer research protocol (Eriksson *et al.*, 1998). We have no knowledge that multiple sclerosis patients have received BrdU premortem, and the possibility of performing such studies on a prospective basis is unlikely for ethical reasons. The proliferation marker, PCNA, was used to support neurogenesis in post-mortem human Huntington disease brains (Curtis *et al.*, 2003). We have been unable to reliably localize proliferation antigens, such as PCNA or Ki-67 in cells expressing phenotypic markers of immature neurons. Multiple sclerosis patients die 20–40 years after the clinical onset of the disease, and the inflammatory demyelination to which white matter neurons are susceptible predominates during the early stages. Newly generated neurons may have exited the cell cycle and migrated to their destination decades prior to the patient's death; so, it would not be surprising that proliferation antigens are no longer detectable.

What are the possible sources for new neurons in chronic multiple sclerosis lesions? It is possible that the MAP2-positive neurons differentiated from immature cells normally present in adult white matter. One candidate cell expresses the chondroitin sulfate proteoglycan, NG2, and platelet-derived growth factor receptor alpha (Chang *et al.*, 2000; Nunes *et al.*, 2003). While characterized as a glial progenitor cell (for review, see Nishiyama, 2007) that can be present in chronic multiple sclerosis lesions (Chang *et al.*, 2000), these cells also have the ability to produce interneurons during rodent brain development (Aguirre *et al.*, 2004; Dayer *et al.*, 2005), and when isolated from adult human brain (Nunes *et al.*, 2003). Another source is the SVZ. The SVZ is the source of all progenitor cells during brain development, and the adult mammalian brain contains multipotent progenitor/stem cells that can proliferate *in vivo* and produce neurons, astrocytes and oligodendrocytes *in vitro* (Pincus *et al.*, 1998; Johansson *et al.*, 1999; Kukekov *et al.*, 1999; Roy *et al.*, 2000; Alvarez-Buylla and Garcia-Verdugo, 2002; Sanai *et al.*, 2004). In the SVZ adjacent to demyelinated lesions, the density of NeuN-positive cells was increased, and these cells express PSA-NCAM and Dlx2, markers expressed by committed neuronal progenitor cells (Flames and Marin, 2005; Bonfanti, 2006). These observations are consistent with reports that brain injury or destruction can induce progenitor cell proliferation in the SVZ of rodents and primates (Calza *et al.*, 1998; Nait-Oumesmar *et al.*, 1999; Jin *et al.*, 2001; Arvidsson *et al.*, 2002; Nakatomi *et al.*, 2002; Parent *et al.*, 2002*a*, *b*; Tonchev *et al.*, 2003, 2005; Tattersfield *et al.*, 2004).

The present study also suggests that demyelinated white matter produces signals that either prevent or enhance the ability of neurons to repopulate lesions. We observed an association between an abundant and morphologically distinct population of activated microglia and regions of multiple sclerosis lesions with increased neuronal densities. Whether this association is a cause or consequence of increased neurons is unknown; however, a recent report suggests that activated microglia can promote neurogenesis from neural stem cells *in vitro* (Walton *et al.*, 2006). This raises the possibility that activated microglia provide a positive signal for neuronal differentiation and/or survival. In summary, we have presented data that support neurogenesis in the lesions of multiple sclerosis. We recognize that we cannot eliminate the possibility that neurons with extensive dendritic processes migrated over substantial distances to the lesion area. If this is the case, we argue that such a phenomenon is unprecedented in the adult mammalian brain and is of bigger impact to the neuroscience field than our preferred interpretation of neurogenesis.

Supplementary material

Supplementary material is available at *Brain* online.

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