Semaphorin 3A and 3F: key players in myelin repair in multiple sclerosis?

Anna Williams, Gabrièle Piaton, Marie-Stéphane Aigrot, Aisha Belhadi, Marie Théaudin, Franziska Petermann, Jean-Léon Thomas, Bernard Zalc and Catherine Lubetzki

Inserm, U711, Paris, F-75013 and Université Pierre & Marie Curie, Faculté de médecine, IFR 70, Paris, France

Correspondence to: Anna Williams, Department of Clinical Neurosciences, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK
E-mail: annacwilliams@yahoo.co.uk

The presence of demyelinated plaques in the central nervous system is the hallmark of multiple sclerosis (MS). Some plaques remyelinate but others do not, leaving permanent damage. The reasons for this failure of repair are many, but one possible reason is the lack of migration of oligodendrocyte precursor cells to the lesion. The guidance molecules Semaphorin 3A and 3F, already known to direct oligodendroglial migration in development, may also be active in controlling oligodendrocyte precursor cell migration in MS, and hence may determine the ability of plaques to remyelinate. Here, in MS tissue and an experimental model of demyelination, we demonstrate a local source of these molecules around active demyelinating lesions, but not chronic plaques. We also provide evidence for their up-regulation at a distance from the lesion, in the neuronal cell bodies corresponding to the demyelinated axons. We propose that both of these mechanisms influence remyelination.

Keywords: multiple sclerosis; demyelination; remyelination; myelin; semaphorin

Abbreviations: ISH = in situ hybridization; LPC = lysophosphatidylcholine; MS = multiple sclerosis; NP = neuropilin; PBS = phosphate buffered saline; PFA = paraformaldehyde


Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS mostly affecting young adults (Noseworthy et al., 2000). Despite heterogeneity in disease expression and severity, many patients accrue permanent neurological disability with chronic disease. Currently available therapies, mostly aimed at reducing the inflammatory component of the disease, only show partial efficacy in the relapsing-remitting forms of MS, where inflammation is predominant, whereas no drug has any proven effect on the secondary neurodegenerative phase of the disease.

Spontaneous remyelination occurs in MS (Perier and Gregoire, 1965; Prineas and Connell, 1979; Raine and Wu, 1993; Patrikios et al., 2006). However, repair is often limited to the periphery of the lesion around a largely demyelinated core. Such chronically demyelinated lesions differ in their oligodendroglial content (Lucchini et al., 1999; Chang et al., 2002; Charles et al., 2002; John et al., 2002; Kotter et al., 2006). Some only contain immature oligodendroglial cells, perhaps due to the presence of inhibitors of oligodendroglial maturation or myelination. Others contain no cells of the oligodendrocyte lineage, and this depopulation may be secondary to an oligodendrogliopathy, or to an exhaustion of the oligodendroglial pool related to successive attacks of demyelination, although this was shown not to be the limiting factor in experimental demyelination (Penderis et al., 2003). Alternatively, this lack of oligodendroglial cells may correspond to a defect of recruitment of oligodendroglial progenitors to the lesion.

The Semaphorins comprise of a large family of secreted and transmembrane proteins. Secreted Semaphorin 3A and 3F act as axonal guidance cues in the developing nervous system (Chisholm and Tessier-Lavigne, 1999). In addition, they act as chemotactic factors for oligodendroglial cells during development (Sugimoto et al., 2001; Spassky et al., 2002; Tsai and Miller, 2002). In the developing optic nerve, oligodendrocyte precursor cell migration from the brain towards the retina is guided by Semaphorin 3 molecules, Semaphorin 3A (Sema3A) as a repulsive signal and Semaphorin 3F (Sema3F) as an attractive signal (Spassky et al., 2002). Class 3 Semaphorins bind to a receptor complex consisting of Neuropilins (NPs) and the Plexin-A subfamily. Increasing evidence indicates that their
expression alters upon nervous system injury in rodents and in neuropathology.

Here we show that Sema3A and Sema3F transcripts are up-regulated in MS brain, as well as in experimental models of demyelination. We provide evidence that this increased expression is not only related to the glial infiltrate around active inflammatory lesions in the white matter but is also present in neurones, in response to axonal demyelination. Our data suggest that glial and neuronal expression of these guidance cues may influence oligodendrocyte precursor cell recruitment, and hence remyelination of lesions.

Material and methods

Human samples

All post-mortem tissues were obtained from the UK Multiple Sclerosis Tissue Bank at Imperial College London via a UK prospective donor scheme with full ethical approval (MREC/02/2/39). Confirmation of the diagnosis of MS for each case was provided by Dr F. Roncaroli (Consultant Neuropathologist, Imperial College London). A summary of the clinical history for each case was prepared by a clinical neurologist with an interest in MS (Dr R. Nicholas, Consultant Neurologist, Imperial College London). Snap frozen unfixed tissue blocks (2 x 2 x 1 cm) were cut at 10 μm and stored at −80 °C. The clinical and post-mortem details of the MS and control cases used in this study are given in Table 1, including case number, age at death, sex, type of MS, post-mortem delay and lesion characterization. The block numbers are given to locate the block in the brain. The blocks are numbered in a standard way by the brain bank. The brain is divided into anterior (A) and posterior (P) halves at the mamillary bodies. One centimetre coronal slices are then cut through the brain with the first slice anterior to the mamillary bodies named A1, and the second A2 etc. The first slice posterior to the mamillary bodies is named P1 etc. Each slice is then cut into 2 x 2 cm blocks identified by letters (A–E) in a vertical plane, with A as the left superior block, and by numbers in the horizontal plane (1–5) with 1 as the left superior block. Thus block A1B4 is 1 slice anterior to the mamillary bodies, fourth block from the left and 2nd block down.

Luxol Fast Blue/Cresyl Violet staining

Tissue sections were dehydrated in increasing concentrations of ethanol and then incubated overnight at 60 °C in 95% ethanol acidified with 10% acetic acid containing 0.1% Luxol Fast Blue. Differentiation was carried out using saturated lithium carbonate and then 70% ethanol. Cell nuclei were stained with Cresyl Fast Violet (10 min at 60 °C). Sections were then dehydrated and mounted.

In situ hybridization (ISH)

Eight riboprobes were generated that are specific to either rat or human forms of Sema3A, Sema3F, NP1 and NP2. Probes were made from the corresponding cDNAs using the Promega riboprobe kit (Promega, France), and labelling with digoxigenin-dUTP. ISH for both human and rat tissue was carried out as previously described (Spasovsky et al., 1998). However, human tissue was first fixed for 10 min in 4% paraformaldehyde (PFA) diluted in 4% paraformaldehyde (PFA) diluted in phosphate buffered saline (PBS), and we added an additional

Table 1 Details of MS and Control cases

<table>
<thead>
<tr>
<th>Patient and block number</th>
<th>Age (years)</th>
<th>Sex</th>
<th>MS cases</th>
<th>Post-mortem delay (h)</th>
<th>Pathological classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS127—P2C1</td>
<td>51</td>
<td>m</td>
<td>sp</td>
<td>21</td>
<td>Early active</td>
</tr>
<tr>
<td>MS122—P3D2</td>
<td>44</td>
<td>m</td>
<td>sp</td>
<td>16</td>
<td>Late active</td>
</tr>
<tr>
<td>MS249—P3D2</td>
<td>59</td>
<td>f</td>
<td>rr</td>
<td>8</td>
<td>Late active</td>
</tr>
<tr>
<td>MS36—A2A3</td>
<td>40</td>
<td>m</td>
<td>sp</td>
<td>10</td>
<td>Late active</td>
</tr>
<tr>
<td>MS203—P2B3</td>
<td>53</td>
<td>f</td>
<td>sp</td>
<td>17</td>
<td>Chronic active</td>
</tr>
<tr>
<td>MS107—A1B3</td>
<td>38</td>
<td>m</td>
<td>sp</td>
<td>18</td>
<td>Chronic active</td>
</tr>
<tr>
<td>MS154—A3C4</td>
<td>34</td>
<td>f</td>
<td>sp</td>
<td>12</td>
<td>Chronic active</td>
</tr>
<tr>
<td>MS36—A8B6</td>
<td>40</td>
<td>m</td>
<td>sp</td>
<td>10</td>
<td>Chronic active</td>
</tr>
<tr>
<td>MS100—A5C6</td>
<td>46</td>
<td>m</td>
<td>sp</td>
<td>7</td>
<td>Chronic active</td>
</tr>
<tr>
<td>MS53—A1B1</td>
<td>50</td>
<td>f</td>
<td>sp</td>
<td>12</td>
<td>Chronic</td>
</tr>
<tr>
<td>MS100—A1C5</td>
<td>46</td>
<td>m</td>
<td>sp</td>
<td>7</td>
<td>Chronic</td>
</tr>
<tr>
<td>MS94—A1B7</td>
<td>42</td>
<td>f</td>
<td>pp</td>
<td>11</td>
<td>Chronic</td>
</tr>
<tr>
<td>MS14—A1C4</td>
<td>52</td>
<td>f</td>
<td>sp</td>
<td>12</td>
<td>Chronic</td>
</tr>
<tr>
<td>MS102—A1D2</td>
<td>73</td>
<td>m</td>
<td>pp</td>
<td>20</td>
<td>Chronic</td>
</tr>
<tr>
<td>MS200—P4E3</td>
<td>44</td>
<td>f</td>
<td>sp</td>
<td>20</td>
<td>Chronic</td>
</tr>
<tr>
<td>MS200—P2E3</td>
<td>44</td>
<td>f</td>
<td>sp</td>
<td>20</td>
<td>Shadow plaque</td>
</tr>
<tr>
<td>MS36—A2C2</td>
<td>40</td>
<td>m</td>
<td>sp</td>
<td>10</td>
<td>Shadow plaque</td>
</tr>
<tr>
<td>MS104—A4C2</td>
<td>53</td>
<td>m</td>
<td>sp</td>
<td>12</td>
<td>Shadow plaque</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebrovascular disease</td>
</tr>
<tr>
<td>Cardiogenic shock</td>
</tr>
<tr>
<td>Cardiac failure</td>
</tr>
<tr>
<td>Old age</td>
</tr>
<tr>
<td>Cardiac failure</td>
</tr>
</tbody>
</table>

m = male; f = female; rr = relapsing remitting MS; pp = primary progressive MS; sp = secondary progressive MS.
microwave treatment of 5 min at 600 W in 0.01 M citric acid pH 6 before the addition of the probe. Sense probes were used as negative controls.

**Immunohistochemistry**

Immunohistochemistry was carried out after ISH. We carried out blocking for 1 h in PBS/0.3% triton/10% horse serum, and then added the primary antibody for 48 h in PBS/0.3% triton/5% horse serum. The source and dilution of the antibodies are as follows: NeuN (mouse IgG1, Euromedex, Souffelweyersheim, France) 1/200, GFAP (rabbit IgG, Dako, France) 1/100, Olig2 (rabbit IgG, Euromedex) 1/200, CD68 (mouse IgG1, Serotec, Cergy Saint Christophe, France) 1/50, ED1 (anti human CD68, mouse IgG1, Serotec) 1/50, MBP (rat IgG, Euromedex) 1/50, PLP (rat IgG, gift from K. Ikenaka, Okasaki, Japan) 1/5, activated caspase-3 (rabbit IgG, Ozyme, Saint Quentin Yvelines, France) 1/100, Sema3A (mouse IgG1, gift from A. Barzilai, Tel Aviv University, Israel) 1/1000. Appropriate fluorescent secondary antibodies obtained from Molecular Probes (Invitrogen, Cergy-Pontoise, France) were used at a concentration of 1/1000 in PBS/0.3% triton/5% horse serum. For Olig2 staining in human tissue, an additional biotinylation step was added: biotinylated anti-rabbit antibody (Sigma, Saint Quentin Fallavier, France) at 1/200 and fluorescein streptavidin (Molecular Probes) at 1/1000. For Sema3A staining, we used the universal immunostaining system (Immunotech, Marseille, France) for biotinylation, and DAB for revelation. Sections were stained with Hoechst solution (1 μg/ml) and mounted in Fluoromount-G (Clinisciences, Montrouge, France).

**Animal experiments**

All animal care and experiments were carried out according to European Community regulations and ethical policies. Animals were anaesthetized with an intraperitoneal injection of 10 mg/kg xylazine (Rompun, Bayer, France) and 100 mg/kg ketamine (Imalgene 500, Merieux, Lyon, France).

**Cortex and spinal cord injections**

Spinal cord injections were carried out at L1-2 level in 1-month old male Wistar rats. The vertebral column was fixed between metal bars on stereotaxic apparatus at the level L2. The vertebral spinal processes were removed and a laminectomy performed at this level. The meninges were pierced and a pulled glass needle (≈10–20 μm diameter) used to inject 1 μl of solution into the spinal cord at a depth of 1 mm, just lateral to the central blood vessel, at a rate of 0.25 μl/min using a peristaltic pump. The needle was not disturbed for another 4 min, to allow the injected solution to diffuse into the tissue, and then slowly withdrawn. Lysophosphatidylcholine (LPC) (Sigma) was used at a final concentration of 1%, and Fluorogold (Molecular Probes) at 4%, both diluted in PBS. Cerebral cortex injections were carried out in the same manner in the motor cortex of 3-month old male Wistar rats. Stereotoxic coordinates were 3.2 mm antero-posterior and 3.5 mm lateral from the bregma at a depth of 1.5 mm from the skull surface. We used three animals for each subgroup and perfused the rats with 4% PFA in PBS, 3, 7, 15 or 28 days after spinal cord injections or 1 week after cortical injections.

**Quantification and statistical analysis**

Quantification was carried out using Image J software. We quantified semaphorin-positive cells in normal appearing white matter or the lesion area. As the limit between plaque and periplaque is not distinct in active lesions, fields were chosen in comparison with Luxol fast blue stained sections to contain both plaque and periplaque. Positive cells were expressed as the percentage of the total number of cells in the field as defined by the total number of Hoechst-positive cells, to control for different tissue densities after fixation. Between 500 and 1500 cells were counted per lesion. A minimum area of 1.5 mm² was analysed for each lesion.

To quantify the number of neuronal cell bodies in the human cortex expressing the semaphorins, positive cells were expressed as a percentage of the total number of neurones in the field (cells positive for NeuN) to control for different tissue densities after fixation. Between 1000 and 1500 neurones were counted per case. The same system was used for quantifying the number of motor cortex neurones expressing semaphorins in the rat, but detection of the motor cortex was confirmed by the presence of Fluorogold positive cells.

We used either the Student’s t-test or an ANOVA as significance tests.

**Results**

**Classification of lesions**

Human MS brain blocks were classified according to the International Classification of Neurological Diseases Classification (www.icdns.org) (Table 2). We used nine blocks containing active lesions, of which four were classified as containing early or late active lesions, and five as chronic active lesions, six blocks containing chronic lesions and three blocks containing completely remyelinated lesions or shadow plaques.

**Increased Sema3A and Sema3F mRNA expression around and within active white matter MS lesions**

We first showed that in control adult brain white matter, Sema3A or Sema3F transcripts are not detected. In contrast, in MS brain, there were numerous cells

| Table 2 Pathological criteria used for classifying MS plaques |
|-----------------|-----------------|-----------------|-----------------|
| Classification  | CD68+ Inflammation | Myelin MBP+/PLP+ in the lesion | Lesion border on Luxol staining |
| Early active    | Extensive around and within lesion Yes | Diffuse |
| Late active     | Some around and within lesion Yes | Diffuse |
| Chronic active  | Only around the lesion No | Diffuse to sharp Sharp |
| Chronic         | Virtually absent No | Diffuse |
| Remyelinated    | Absent Yes | / |
positive for the two transcripts around and within demyelinating white matter lesions. However, this expression was strictly restricted to active plaques (Fig. 1A, C, E), whereas no expression was detectable in normal appearing white matter (NAWM) distant to active lesions, around or within chronically demyelinated lesions (Fig. 1B, D, F) or shadow plaques.

**Glial cells are the main source of Sema3A and Sema3F around and within active MS plaques**

We found that the majority of cells positive for either Sema3A or Sema3F around and within active lesions were either astrocytes (co-labelled with anti-GFAP antibody) or microglia (co-labelled with anti-CD68 antibody) (Fig. 2A–D). A mean of 58% ± 10.2 (SD) of Sema3A-positive cells around and within active lesions were astrocytes, and 20% ± 10.8 (SD) were activated microglia (Fig. 2G). For Sema3F, a mean of 55% ± 12.5 (SD) were astrocytes, and 17% ± 6.2 (SD) were activated microglia (Fig. 2H). In addition to this expression by astrocytes and microglia, 15% ± 7.8 (SD) of Sema3A-positive cells and 23% ± 9.6 (SD) of Sema3F-positive cells were Olig2-positive oligodendrocytes (Fig. 2E–H). Semaphorin positive neurones were also detected. However, as there were very few neuronal cell bodies found within or around white matter MS plaques, the semaphorin contribution from these cells is likely to be low on the basis of low numbers.

Expression of the Sema3A and Sema3F receptors, NP1 and NP2, was detected in a subpopulation of cells of the oligodendrocyte lineage as identified by co-expression of Olig2 (Fig. 2I and J). Olig2 was the only antibody which labelled oligodendroglial cells (including oligodendrocyte precursor cells) after tissue processing for ISH. Although Olig2 labels different stages of the oligodendroglial lineage, the small size of these cells was suggestive of oligodendroglial precursors. A mean of 24% ± 8.0 (SD) and 26% ± 8.8 (SD) of total oligodendroglial cells expressed NP1 and NP2 respectively. These neuropilin-receptor expressing oligodendroglial cells were mostly detectable in the plaque and periplaque area, and not in the NAWM. In addition to oligodendroglial cells, astrocytes, microglia and neurones also expressed the neuropilin receptors. These data demonstrate that oligodendroglial guidance cues, which are normally absent from adult white matter, are up-regulated within and around active MS lesions and that oligodendrocyte precursor cells bearing semaphorin receptors are likely to respond to these signals.

### Sema3A and Sema3F expression is heterogeneous between active MS lesions

Close inspection of the nine active lesions showed that there was variability in both the number of positive cells around and within the lesions, and the proportion of such cells which were either Sema3A or Sema3F positive. There was an increase in the number of cells positive for both semaphorin transcripts around and within active lesions. In addition, a higher number of cells expressing Sema3F (compared to Sema3A) was seen around and within the most inflammatory lesions (where CD68-positive cells represent more than 15% of the perilesional cells). In contrast, around and within less inflammatory active lesions, there was a predominance of cells expressing Sema3A (Fig. 3). Hence, inflammation is associated with an augmentation of attractive signals for oligodendrocyte precursor cells.
The number of cortical neurones expressing *Sema3A* and *Sema3F* mRNA is strongly increased in MS

In contrast to control white matter, we showed that class 3 Semaphorins are detected in adult control cortex (Giger et al., 1998), and that this expression is restricted to neurones, as shown by co-labelling with NeuN antibody (Fig. 4A). In the samples examined, there was a twofold increase in the percentage of neurones expressing each semaphorin in MS brain grey matter compared to control brain grey matter. In MS brain, 63% ± 6.3 (SD) of neurones expressed *Sema3A*, compared to 33% ± 6.6 (SD) in control brain, and 70% ± 13.3 (SD) of neurones in MS brain expressed *Sema3F* compared to 30% ± 2.9 (SD) in control brain (Fig. 4B). This up-regulation occurred in all MS grey matter, regardless of the activity of the white matter lesion seen in the block. As a control, we carried out ISH using a probe for glutamic acid decarboxylase (GAD) on both MS and control brain blocks and found no difference in neuronal expression of GAD between the two types of tissue (data not shown).

As cortical demyelination is frequent in MS patients, especially those with long-standing disease, we questioned whether this up-regulation of semaphorin mRNA was related to cortical plaques. The neuronal expression of *Sema3A* and *Sema3F* was not restricted to the vicinity of the cortical lesions, but appeared equally distributed between areas of cortical demyelination (Fig. 4C), and normal appearing cortical areas, even those areas apparently far from cortical plaques.
Sema3A and Sema3F mRNA up-regulation in MS brain is reproduced in experimental models of demyelination

To get further insight into semaphorin expression after demyelination, we turned to an experimental model of demyelination, with intra-spinal injection of the myelino-toxic agent LPC in adult rats. The number of cells expressing Sema3A and Sema3F was quantified around and within the demyelinated lesion.

After 3 days, Sema3A and Sema3F were already up-regulated around and within the demyelinated lesion, with means of 17.2% ± 5.3 (SD) of cells expressing Sema3A, and 11.3% ± 4.0 (SD) of cells expressing Sema3F, whereas expression after a saline injection did not differ from that in non-injected animals (Fig. 5A).

The majority of the expression was by glial cells. A mean of 64% ± 28.1 (SD) of Sema3A-positive cells around a demyelinating lesion were astrocytes, and 15% ± 3.4 (SD) were microglia, whereas for Sema3F-positive cells, 48% ± 9.5 (SD) were astrocytes, and 21% ± 6.8 (SD) were activated microglia. Some oligodendrocytes around the lesion were also seen to express these semaphorins—a mean of 9% ± 1.8 (SD) of Sema3A-positive cells were oligodendrocytes and 6% ± 4.1 (SD) for Sema3F. Expression of transcripts encoding the neuropilin receptors NP1 and NP2 was detected in oligodendroglial cells, with a mean of 18% ± 10.8 (SD) and 16% ± 12.6 (SD) of Olig2-positive oligodendroglial cells expressing NP1 and NP2, respectively, around the lesions (Fig. 5B–E), many with a morphology suggestive of oligodendrocyte precursor cells. The other cells expressing the neuropilin receptors around the lesions were astrocytes, microglia and neurones. Thus, these experimental results reproduce the up-regulation of guidance cues in MS brain white matter.

Furthermore, we looked at the expression of Sema3A and Sema3F around and within demyelinating (LPC) and traumatic (saline) lesions as a function of time (Fig. 5F). We found that Sema3A and Sema3F expression around and within the traumatic lesion produced by saline injection into the spinal cord was always low. However, around and within demyelinating lesions, the percentage of cells expressing Sema3A and Sema3F was significantly higher, reaching 17% of surrounding cells with Sema3A at days 3 and 15, and with a peak expression of Sema3F of almost 30% of surrounding cells at 7 days after lesion induction. By 28 days, a time when repair was almost complete, expression of both semaphorins had decreased but had not yet reached basal levels. Sema3F expression around and within demyelinating lesions during their recovery was higher than Sema3A expression, though this only reached significance at P = 0.05 (ANOVA). Thus, after spinal cord demyelination, semaphorin expression increases early and decreases later in line with process of repair and remyelination, and this is significantly more marked after demyelination than after traumatic insult.

Up-regulation of Sema3A and Sema3F in the motor cortex in response to spinal cord demyelination

To investigate whether demyelination induces semaphorin expression at a distance in the neuronal cell bodies corresponding to demyelinated axonal tracts, LPC was injected into the rat spinal cord to demyelinate the corticospinal tract. The corresponding area of the motor cortex was determined by the presence of Fluorogold,
injected with the myelinotoxic agent. This retrograde neuronal tracer is only taken up by the small percentage of cut axons, and not purely demyelinated axons, but still allowed delineation of the target area in the contralateral motor cortex (Fig. 6A). The percentage of neurones positive for Sema3A or Sema3F was quantified in the motor cortex 3, 7, 15 and 28 days after demyelination, and compared to that of animals injected with saline or non-injected animals.

**Fig. 4** MS brain shows an increased number of cortical neurones expressing Sema3A and Sema3F. (A) Combined immunohistochemistry with anti-NeuN antibody (red) and ISH with a Sema3A or Sema3F probe on sections of MS and control cerebral cortex. Sema3A or Sema3F mRNA are expressed by NeuN-positive neuronal cell bodies (examples marked by arrows). Scale bar: 40 μm. (B) Graph showing that there is a twofold increase in the percentage of neurones expressing Sema3A or Sema3F mRNA in MS cortex compared to control cortex (Mean ± SD). **P < 0.01 (t-test). (C) ISH for Sema3A or Sema3F combined with immunohistochemistry for MBP (green) in MS cerebral cortex. The dotted line indicates the border between cortical plaque (reduced MBP) and normal cortical myelination. The distribution of cells positive for Sema3A or 3F remains similar in both myelinated and poorly myelinated areas. Scale bar: 50 μm.
Fig. 5  Sema3A and Sema3F up-regulation around experimental demyelinating lesions in rat spinal cord. Rats were injected with LPC to induce demyelination of the dorsal corticospinal tracts in the spinal cord. Spinal cords were dissected 3, 7, 15 and 28 days later and used for ISH with riboprobes followed by immunolabelling with appropriate antibodies. (A) Graph at 3 days post lesion showing the percentage of cells expressing Sema3A or Sema3F around the lesion, or an equivalent region in LPC injected, saline injected and normal animals. Bars show means ± SD. Asterisks indicate statistical significance compared to saline injection and non-injected animals, **P < 0.01, *P = 0.01 (t-test). (B – E) Combined ISH with NP1 (B) and NP2 (D) riboprobes, and immunohistochemistry with anti-Olig2 antibody (C, E). Cells positive for NP1 or NP2 are falsely coloured in green in (C, E), and merged with a photo of the same field showing staining for Olig2 in red, showing that some Olig2-positive cells are also NP1 or NP2 positive (arrows). Scale bar: 10 μm. (F) Graph showing the time course of expression of Sema3A and Sema3F mRNA around spinal cord demyelinating lesions, compared to control (saline injection). Points are means ± SD ***P < 0.001 (ANOVA).
There was a clear increase in the percentage of neuronal cell bodies expressing both Sema3A and Sema3F in the motor cortex of rats injected with LPC, compared to those injected with saline and control non-injected rats (Fig. 6B, C). The peak expression was at 15 days when virtually all neurones expressed Sema3A (90% ± 6.2) and Sema3F (100% ± 16.1), and this was at least three-fold higher than in rats injected with saline (or non-injected rats). The peak expression appears later (15 days) than that around and within the demyelinating spinal cord lesion (3–7 days). At 28 days, although the expression was still prominent, it had begun to decrease. More distant cortical areas expressed basal levels of these semaphorins (8–10% of neurones positive), indicating that this up-regulation was a specific response rather than a global hemispheric response. Thus, in agreement with the observations in MS cortex, these results show that after a focal demyelination, Sema3A and Sema3F are up-regulated in the cortex, in the corresponding neuronal cell bodies and that this effect in the rat is maintained for at least a month after demyelination.

Due to technical limitations, we were unable to simultaneously detect the two semaphorin transcripts by double ISH, and turned to combined ISH and immunohistochemistry. Using an antibody for Sema3A we showed that neurones in rat motor cortex can indeed express the mRNA for Sema3F and stain positive for Sema3A protein, whereas others are positive for Sema3F and negative for the Sema3A protein (data not shown). Due to limitations in

Fig. 6 Sema3A and Sema3F are up-regulated in the contralateral motor cortex in response to distant corticospinal tract demyelination in the spinal cord. (A) LPC or saline was injected into the corticospinal tract of adult rats, and expression of Sema3A and Sema3F analysed in the contralateral motor cortex 3, 7, 15 and 28 days after operation. The target area of the motor cortex was identified by Fluorogold uptake by neuronal cell bodies (NeuN-positive). Scale bar: 10 μm. (B) Combined ISH and immunohistochemistry showing Sema3A and Sema3F expression in NeuN positive neurones of the motor cortex contralateral to the spinal cord demyelinated lesion (some cells marked with arrows). Scale bar: 10 μm. (C) Graph showing the percentage of motor cortex neurones expressing Sema3A and Sema3F mRNA as a function of time after a corticospinal tract demyelinating lesion in the spinal cord, compared to control (saline injection). Points are means ± SD ***P < 0.001 (ANOVA).
the quality of human post-mortem tissue, we have been unable to show this definitively in human MS tissue.

**Sema3A is not a signal for neuronal apoptosis in rat brain**

Neuronal expression of Sema3A in vitro is associated with apoptosis, as demonstrated by activated caspase 3 immunoreactivity in the same cells (Fankhauser et al., 2000; Shirvan et al., 2000; Bagnard et al., 2004; Ben-Zvi et al., 2006). To investigate whether semaphorin-expressing neurones were indeed engaged in the apoptotic pathway, we examined the expression of activated caspase 3 in rat brain after induction of an acute cortical demyelinating lesion. In these experiments, 7 days after the injection, there was a local up-regulation of Sema3A and Sema3F, expressed in neurones and infiltrating microglial cells around the lesion. Expression of activated caspase 3 was increased globally, even far from the demyelinated area. However, we found no neurones, either expressing Sema3A (or Sema3F) or not, which also showed activated caspase 3 immunoreactivity (data not shown), suggesting that, at least in our model, neuronal Sema3A does not appear to be either a marker or a promoter of neuronal apoptosis.

**Discussion**

Our study has shown that Sema3A and Sema3F, which act as oligodendrocyte precursor cell guidance cues during development, are up-regulated in MS brain and chemically induced demyelination. This expression differs between white and grey matter. In the white matter, expression is mostly glial and restricted to the vicinity of active demyelinated lesions whereas, in the grey matter, it is purely neuronal.

Semaphorin expression in MS white matter is only detected around active inflammatory lesions with a marked CD68-positive microglial infiltrate, suggesting that, even if expression is not solely microglial, it is related to the inflammatory process associated with plaque formation. In contrast, up-regulation of semaphorins, expressed in cortical neurones in MS and our experimental model of demyelination, is independent of the inflammatory activity of the plaque suggesting that it might be secondary to the demyelination of the axon downstream. In favour of this possibility, the cortical increased expression of semaphorins was mostly restricted to the contralateral motor cortex after corticospinal tract demyelination, and did not occur in other areas or after saline injection. This up-regulation may be either specifically related to the demyelinating process or may represent a response to axonal insult. In MS, there is frequently axonal pathology in addition to areas of demyelination (Ferguson et al., 1997; Trapp et al., 1998), especially in chronic disease, therefore not allowing us to discriminate between demyelination and axonal degeneration as triggers of the semaphorin up-regulation in humans. However, our experimental results suggest that demyelination is sufficient to induce semaphorin expression as, after LPC-induced spinal cord demyelination, cortical expression of semaphorins in the motor cortex is considerable, widespread and significantly higher than that of control rats injected with saline, which corresponds to a similar traumatic injury. This suggests that axonal demyelination itself induces an up-regulation of expression of semaphorin transcripts. Other traumatic or ischaemic experimental insults are also known to result in neuronal semaphorin up-regulation locally to the injury (Pasterkamp et al., 1999; Fujita et al., 2001; De Winter et al., 2002; Lindholm et al., 2004; Nitzan et al., 2006). In addition, an increase in Sema3A expression in the cortex detected by RT-PCR has been reported after spinal cord hemisection (Hashimoto et al., 2004).

What is the consequence of this up-regulation of semaphorin mRNA? In the perilesional white matter, microglia, astrocytes and also oligodendrocytes are able to release guidance cues locally. As for neuronal semaphorin expression, recent in vitro experimental data have demonstrated that Sema3A is transported and secreted along axons (de Wit et al., 2006). Thus, we hypothesize that semaphorins are able to travel along the axons to an area of demyelination and be secreted in this area, providing a second source of oligodendrocyte precursor cell guidance cues close to the demyelinated area.

We have demonstrated, both in human and murine tissue, that a subset of Olig2-positive oligodendroglial cells express the semaphorin receptors NP1 and NP2, and are thus able to respond to these signals. These cells are mostly located around white matter demyelinating lesions, and are probably oligodendrocyte precursor cells, as suggested by their relatively small size. However, we were not able to confirm this due to the difficulty in labelling cells with antibodies more specific for precursors after the ISH step. Recent data have demonstrated that such oligodendrocyte precursor cells persist in the adult murine brain, as well as in normal human and MS brain (Chang et al., 2002; Reynolds et al., 2002; Wilson et al., 2006). These data suggest that Sema3A and Sema3F, released at the site of the demyelinated lesion, are able to act on oligodendrocyte precursor cells expressing the corresponding receptors, and influence their recruitment towards the demyelinated lesion.

In MS tissue, white matter lesions defined as more active expressed proportionally more Sema3F compared to Sema3A. These lesions could thus be considered as more attractive towards oligodendrocyte precursor cells, hence more favourable to myelin repair, compared to less inflammatory lesions expressing proportionally more Sema3A. Similarly, in our experimental model of rat spinal cord demyelination, which spontaneously remyelinates, there was greater expression of Sema3F around and within the lesion compared to Sema3A. Such a positive influence of inflammation in MS plaques would be in good
agreement with the pro-(re)myelinating role of inflammation, recently emphasized. This pro-myelinating role of inflammation has been attributed to removal of myelin debris, but also, as suggested by recent in vivo results in experimentally induced demyelination, to an enhanced migration of oligodendrocyte precursor cells (Kotter et al., 2001; Foote and Blakemore, 2005; Li et al., 2005). However, the situation is further complicated by the fact that some cells express both Sema3A and 3F, hence inducing contradictory effects. The determining factor for the effect of these guidance cues may be the differential expression of NPI and NP2 receptors by oligodendrocyte precursor subpopulations, which may in turn be influenced by their different sites of origin (Spassky et al., 2000).

Recent data have proposed a neuroprotective role of Sema3A, after lipopolysaccharide (LPS) injection into rat brain, where Sema3A was up-regulated in cortical neurones, and thought to induce apoptosis of the infiltrating microglia, thus perhaps protecting neurones from their attack (Majed et al., 2006). These results are in agreement with the detection of activated caspase 3 immunoreactivity in astrocytes and activated microglia expressing the NPI receptor in our model of LPC-induced intra-cortical demyelination.

Myelin repair occurs after CNS demyelination. Whereas this myelin repair is efficient in most MS experimental models, repair failure is frequent in MS, leading to irreversible disability. The reasons for this repair failure are many, and not mutually exclusive [see review of Franklin (2002)]. Some recent data have suggested inter-individual heterogeneity of brain myelin repair capacities, contrasting with a relative homogeneity of repair amongst the different lesions from a single MS brain (Patrikios et al., 2006). A critical factor for myelin repair is the migration of oligodendrocyte precursor cells into demyelinated lesions. These adult oligodendrocyte precursor cells are disseminated within the normal appearing white matter, and the semaphorins are excellent candidates to influence their migration, similarly to their role in development. Their increased expression in MS tissue may favour the recruitment of oligodendrocyte precursor cells towards the demyelinated area if attractive guidance cues such as Sema3F are predominantly expressed, and thus favour remyelination. In contrast, oligodendrocyte precursor cell migration may be impaired if inhibitory guidance cues such as Sema3A are predominant, thus inhibiting remyelination. A better knowledge of these mechanisms may be key to understanding myelin repair in MS. The ultimate goal will be to manipulate the molecular environment around demyelinated lesions in MS to promote remyelination in them all.

**Acknowledgements**

We thank K. Ikenaka and A. Barzilai for gifts of antibodies. All tissue samples were provided by Prof. R. Reynolds of the UK Multiple Sclerosis Tissue Bank (www.ukmstissuebank.imperial.ac.uk), funded by the MS Society of Great Britain and Northern Ireland (registered charity 207495). The project was supported by Insmir and the French MS Society ARSEP. AW was funded by Medical Research Scotland. No author has a competing financial interest.

**References**


