THE RE-EXPRESSION OF THE HOMEODOMAIN TRANSCRIPTION FACTOR Gtx
DURING REMYELINATION OF EXPERIMENTALLY INDUCED DEMYELINATING LESIONS IN YOUNG AND OLD RAT BRAIN

F. J. SIM,*† G. L. HINKS† and R. J. M. FRANKLIN†‡

*Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK
†Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK
‡To whom correspondence should be addressed. Tel.: +44-1223-337642; fax: +44-1223-337610.
E-mail address: tfj1000@cam.ac.uk (R. J. M. Franklin).

Abstract—Since myelination and remyelination both involve investing an axon with a myelin sheath, a plausible hypothesis is that the two processes involve the expression of similar transcription factors. In this study we have addressed this hypothesis by comparing the expression of messenger RNA of Gtx, a homeodomain transcription factor expressed within oligodendrocytes during myelination, with the expression of messenger RNAs of the major myelin proteins, myelin basic protein and proteolipid protein during remyelination of experimentally induced demyelination in the adult rat brain. We have found a close temporal and spatial association between the expression patterns of the three messenger RNA species during remyelination. By comparing the expression patterns in rapidly remyelinating lesions in young adult rats with slowly remyelinating lesions in old adult rats, we have shown that Gtx messenger RNA expression follows the reappearance of myelin basic protein and proteolipid protein messenger RNAs regardless of the rate of remyelination. This observation demonstrates a clear association between the expression of Gtx messenger RNA and myelin repair. We have also shown that there is a decrease in constitutive levels of expression of myelin basic protein, proteolipid protein and Gtx messenger RNA in old adults compared with young adults.

Taken together, our results indicate that Gtx, which has multiple binding sites in the promoter regions of both myelin basic protein and proteolipid protein genes, may have a similar role in the regulation of myelin protein gene expression during remyelination as has been proposed in myelination. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: ageing, demyelination, in situ hybridization, myelin basic protein, oligodendrocyte, proteolipid protein.

The mechanisms involved in the regenerative process of remyelination following CNS demyelination are relatively poorly understood. Given that both the developmental process of myelination and that of remyelination share the similar objective of investing an axon with a myelin sheath, studies on myelination have provided useful clues to unravelling the events occurring during remyelination.6,7 Using this approach it has been possible to show that many of the cell signalling strategies, such as growth factors and adhesion molecules, that play a role in orchestrating myelination are re-expressed during remyelination.10,16,20,24

The processes of oligodendrocyte maturation and the formation of new myelin sheaths during remyelination appear to be associated with a similar pattern of expression of the myelin protein genes myelin basic protein (MBP) and proteolipid protein (PLP) as occurs during myelination.13,14,29,31,32 In this study, we have addressed whether the re-expression of MBP and PLP during remyelination is associated with the re-expression of their putative regulatory factor Gtx.

Gtx is a member of the homeodomain family of transcription factors, which is specifically expressed by oligodendrocytes but not in oligodendrocyte precursors, astrocytes, or Schwann cells, and has a restricted expression pattern confined to white matter tracts in the CNS and germ cells in the testis.1,17 A role for Gtx in the regulation of MBP and PLP gene expression has been proposed on the basis of its DNA binding properties, an onset of expression in postnatal rat CNS that coincides with that of the MBP and PLP, and the decreased levels of expression in the hypomyelinated CNS of the myelin-deficient rat.1,19 To address whether Gtx may be similarly associated with MBP and PLP gene expression during remyelination we have compared the temporal and spatial patterns of expression of Gtx mRNAs with those of the two myelin protein genes following experimentally induced demyelination in adult rat cerebellar white matter.20 In order to more closely establish a relationship between Gtx expression and remyelination we have compared patterns of expression in rapidly remyelinating lesions in the white matter of young adult rats with the slower remyelination in aged adult rats.25 If Gtx mRNA were truly associated with remyelination then one would predict that the pattern of Gtx expression should follow the reappearance of MBP and PLP mRNA expression that is required for remyelination, regardless of the rate at which this occurs.

EXPERIMENTAL PROCEDURES

Creation of ethidium bromide lesions in the caudal cerebellar peduncles of the adult rat

Female Sprague–Dawley rats (“young adults”), eight to 10 weeks, ~200 g; “old adults”, ex-breeders, >12 months, >280 g) were used in all experiments. Demyelination was induced unilaterally or bilaterally by stereotaxic injection of 4 μl of 0.01% ethidium bromide (EB) into the caudal cerebellar peduncles using the method described, in detail, by Woodruff and Franklin.30 Anaesthesia was induced using a neuroleptanalgesic combination (0.7 ml/kg Hypnorm; 0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone, Janssen Pharmaceuticals), and diazepam (3 mg/kg, Phoenix Pharmaceuticals). The rats were placed into a stereotaxic instrument, and using standard coordinates determined from a stereotaxic atlas,22 4 μl of 0.01% EB (Sigma Chemical Co.,
in situ hybridization was performed using a standard protocol as previously described. Oligonucleotides were end-labelled with [35S]dATP (1000 Ci/mmol; New England Nuclear, Boston, MA) using terminal deoxynucleotidyl transferase, purified using Sephadex columns (Microbiotip6; Bio-Rad, Hemel Hempstead, UK) and dithiothreitol was added to a final concentration of 50 mM prior to dilution in hybridization buffer. Lesion-containing sections of hindbrain were hybridized overnight in hybridization buffer, containing 3000 c.p.m./µl [35S]dATP-labelled oligonucleotide probes for all probes except PLP/DM20, which only contained 1500 c.p.m./µl. Excess unbound and non-specifically bound probe was removed using standard stringency washes: 1× standard saline citrate buffer (SSC) at room temperature for 30 min and then 1× SSC at 55°C for 30 min. Slides were then rinsed in 1× SSC, dehydrated in ethanol and air-dried. Sections were then exposed to autoradiographic film for a probe-specific period before development. Care was taken to standardize procedures for control and experimental sections at all stages.

Quantification of mRNA expression

The in situ hybridization autoradiograms were analysed using the MCID image analysis system (MCID, Model M4, Toronto, Canada) using a similar protocol to that previously described with minor modifications. Areas of altered gene expression associated with the lesion (Fig. 1, region x) were sampled, providing an averaged optical density reading across the lesion from which the background film levels (region z) were deducted. This value was subjected to a correction factor (CF) to normalize for differences between autoradiographic films. Using the observation that normal constitutive expression levels (region y) did not vary within age groups (data not shown), the individual CFs were calculated from the ratio of mean y to the individual section y. All measurements were taken within the linear range of optical density levels, throughout the lesion region, and the measurements for each animal were processed independently. The data were processed with a spreadsheet (Microsoft Excel 97) and statistically analysed using the Kruskal–Wallis test for multiple measures, followed by Mann–Whitney post-hoc pairwise comparisons for data at individual time points (Graphpad Software). P < 0.01 was taken as the minimum level of statistical significance.

RESULTS

In order to compare expression of Gtx mRNA with extent of remyelination, we examined the expression of the myelin protein genes, MBP and PLP/DM20, which we have previously shown to coincide with the appearance of myelin sheaths during myelination and remyelination. If Gtx mRNA expression were truly associated with remyelination then one would predict that the relationship between Gtx expression and myelin protein gene expression would be preserved irrespective of the rate of remyelination. An opportunity to test this is provided by the age-related decrease in the rate of remyelination that follows gliotoxin-induced demyelination. Thus, in order to confirm the relationship between Gtx expression and remyelination we compared expression patterns in rapidly remyelinating lesions induced in the white matter of young adult rats with the slower remyelination that occurs in aged adult rats.

Demyelination is associated with a rapid reduction in all three mRNA transcripts

At two days following injection, lesion positioning was verified using solochrome cyanine, a myelin stain (Fig. 2a), and in situ hybridization performed on serial sections. MBP, PLP/DM20 (henceforth simply PLP) and Gtx mRNA were reduced compared with normal expression levels in the region associated with the injection site (Fig. 3). By five days, there was a clearly defined region within which MBP, PLP and Gtx mRNAs could not be detected (Figs 2, 3). At subsequent survival times, age-related differences in the patterns of expression of these genes were apparent.
Reappearance of myelin basic protein and proteolipid protein/DM20 mRNAs following demyelination reflects the different rates of remyelination in young and old animals

Young animals, myelin basic protein and proteolipid protein. In young animals, MBP and PLP mRNA expression was first observed at seven days as weak spots around the rim of the lesion (Fig. 3, arrowheads). Subsequent expression then followed a similar pattern for both mRNAs. Typically, expression was then seen as a crescent at the lesion edge that by 21 days formed a complete ring around the lesion centre. At 28 days, at which time the lesion is fully remyelinated, expression of both mRNAs reached peak levels (Fig. 4, MBP 60% and PLP 121% above control levels) and filled all or most of the lesion. Elevated expression levels were still seen at the final time point of 66 days, but were less intense than at 28 days. At this stage, both MBP and PLP mRNA expression had declined to 86% of peak expression levels but remained significantly elevated relative to normal expression.

Old animals, myelin basic protein. The age-related difference in remyelination observed by morphological analysis was manifested as changes in the timing and intensity of expression of MBP and PLP mRNAs. In old rats the reappearance of MBP mRNA expression occurred at 10 days (Fig. 3, arrowhead), three days after its reappearance in young rats. The subsequent rate of mRNA accumulation was also reduced in aged rats (Fig. 4). MBP mRNA levels were significantly reduced compared with young animals at days 10, 21 and 28 days post-injection. For example, at 28 days, when the lesion is fully remyelinated in young animals, the mean level of mRNA was only 22% above control levels and 38% less than at the younger age, and was distributed as a ring of increased expression around the lesion. This closely matched the difference previously reported using a histological analysis of remyelination, where at this stage there was 38% less oligodendrocyte remyelination in old animals compared with young animals. The spatial distribution of MBP mRNA at 28 days closely matched the location of remyelinated sheaths. At the last survival time (66 days), when remyelination is complete in aged rats, MBP expression filled the entire lesion and the level of expression was 56% higher than at 28 days. This is similar to the 50% increase seen in the extent of remyelination between 28 and 63 days that has been detected histologically.

Old animals, proteolipid protein. Unlike MBP, small spots of PLP mRNA re-expression could be found at seven days in both young and old animals (Fig. 3). However, although the timing of initial re-expression of PLP was not different in old animals, the subsequent accumulation of PLP mRNA was much slower. Thus, in old animals the control levels were reached three days after they were reached in young animals, and levels of expression were significantly reduced compared with young animals at all subsequent time points (Fig. 4). The distribution of PLP mRNA in old animals resembled the pattern of MBP mRNA re-expression in this age group (Fig. 3). In contrast to MBP, peak levels of PLP mRNA were observed at 28 days. However, at 28 days, PLP mRNA was not found throughout the lesion as in young animals but was only observed around the lesion rim. PLP mRNA remained
Fig. 3. Expression patterns of MBP, PLP and Gtx mRNAs during remyelination of EB-induced demyelination of the caudal cerebellar peduncle in both young and old adult animals. Sections through the centre of the lesion were hybridized with $^{35}$S-labelled oligonucleotide probes using a standard *in situ* hybridization protocol. Representative autoradiograms demonstrate the resulting hybridization signal at two, five, seven, 10, 14, 21, 28 and 66 days post-injection. Arrowheads indicate the initial point at which re-expression of each mRNA could be detected. Scale bar = 500 µm.
Fig. 4. Changes in mean relative optical density (ROD) measurements (±S.E.M.) for MBP, PLP and Gtx mRNA expression within EB-induced lesions at between two and 66 days following lesion-induction in young and old adult animals. At the following time points the lesion ROD was significantly different from saline-injected controls \(P < 0.01\): young animals, all time points except 10 (MBP, PLP and Gtx) and 14 days (MBP and Gtx); old animals, all time points except 14 (MBP), 21 (MBP and Gtx) and 28 days (Gtx).
significantly elevated above control levels at 66 days (Fig. 3). Although the level of PLP expression had at this stage dropped below the 28-day level, PLP mRNA was expressed throughout the lesion, consistent with the distribution of new myelin sheaths.

Gtx is expressed with a similar pattern to both myelin basic protein and proteolipid protein during CNS remyelination in both young and old adult rats

Having established the expression patterns for MBP and PLP mRNA during remyelination we then compared these to the expression patterns of the mRNA of the transcription factor Gtx. Similar to MBP and PLP, Gtx mRNA was reduced at two days post-injection (Fig. 3) and at five days was undetectable in both young and old animals (Figs 2d, 3). The reappearance of Gtx expression during remyelination of the demyelinated area occurred with a similar timing to that of the myelin protein genes. Thus, in young animals, only on rare occasions were small spots of Gtx mRNA expression found at seven days after injection, with clearer expression apparent at 10 days after injection (Fig. 3), representing a delay of re-expression of up to three days relative to initial myelin protein gene re-expression. Thereafter, the mean Gtx mRNA expression levels rose progressively, reaching a peak at 28 days. At the late time point, Gtx mRNA had declined to 86% of peak expression levels and remained significantly above normal levels (Fig. 4). The spatial distribution of Gtx mRNA was similar to both MBP and PLP (Fig. 3), although in the middle of the lesion there was occasionally a central region from which Gtx was absent that corresponded to an area of the lesion generally remyelinated by Schwann cells.

In old animals, weak spots of elevated Gtx mRNA expression were first detected at 14 days (Fig. 3), seven days after initial expression in young animals. There was also a delay in the subsequent accumulation of Gtx mRNA expression compared with young animals, indicated by the significantly lower expression at every time point after 10 days. Similar to MBP, there was no peak in the Gtx expression at the survival times examined, the final survival time representing the highest mean level of expression achieved (Fig. 4).

In both the young and old animals, the patterns of Gtx mRNA expression bore a closer resemblance to that of MBP rather than PLP message expression. To examine this more closely, the profiles for each probe were compared after scaling expression to the highest level observed (Fig. 5). Expression of Gtx message did not increase more rapidly than either MBP or PLP message in the young animals. In the old animals the expression of PLP slightly proceeded that of Gtx and MBP. In neither the young nor old animals did the initial expression precede that of either of the two myelin protein genes (Figs 3, 5).

In some animals, particularly young animals, there was a clear increase in MBP and PLP mRNA expression compared at five and seven days after saline injection. However, a Kruskal–Wallis test performed on all the data indicated that there were no significant changes in MBP, PLP or Gtx expression in the saline-injected animals (Fig. 3).

Constitutive expression of myelin basic protein, proteolipid protein and Gtx mRNAs are lower in the white matter of old adult rats than young adult rats

An interesting additional observation that we made on the brain sections subjected to MBP, PLP and Gtx in situ hybridization was the lower levels of background expression in the old animals compared with the young animals (Table 1). This phenomenon was observed in sections that were subjected to in situ hybridization at the same time, and therefore can be considered to represent differences in levels of constitutive expression.

**DISCUSSION**

Although some progress has been made in recent years in elucidating the transcriptional regulation of the developmental process of myelination, there have been fewer investigations of the transcriptional regulation of the related regenerative process of remyelination. It is not clear, for example, to

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td>0.227 ± 0.005</td>
<td>0.209 ± 0.005</td>
</tr>
<tr>
<td>PLP</td>
<td>0.160 ± 0.005</td>
<td>0.093 ± 0.004</td>
</tr>
<tr>
<td>Gtx</td>
<td>0.067 ± 0.002</td>
<td>0.052 ± 0.003</td>
</tr>
</tbody>
</table>
what extent the same transcription factors used in the two processes are the same, as would be predicted by the recapitulation hypothesis of remyelination.\textsuperscript{5,7} In this study, we have correlated the expression of a myelination-associated transcription factor Gtx with the process of remyelination following experimentally induced demyelination. We have also shown that an age-related decrease in the constitutive levels of expression of MBP and PLP mRNA expression in normal white matter correlates with a corresponding decrease in the constitutive levels of Gtx mRNA expression.

Using a gliotoxin model of demyelination/remyelination in the adult rat CNS we have shown a specific pattern of expression of Gtx mRNA that closely parallels the initial demyelination-associated loss and subsequent reappearance of the transcripts of the principal myelin proteins MBP and PLP. Thus, the reappearance of Gtx mRNA closely follows the process of remyelination since the expression of MBP and PLP mRNAs following demyelination correlates with the reappearance of new myelin sheaths within the lesion.\textsuperscript{14,25} The expression pattern of Gtx mRNA compared with those of MBP and PLP is similar not only in terms of the rate of accumulation (Fig. 5) but also in terms of the spatial distribution of expression (Fig. 3), first appearing at the edge of the lesion and gradually extending throughout the oligodendrocyte-remyelinated area. It is noticeable that Gtx mRNA is not expressed before either MBP or PLP mRNA, providing further evidence that Gtx expression is confined to late stages of the oligodendrocyte lineage. A further significant point is that the correlation between Gtx and both MBP and PLP is similar regardless of the rate of accumulation of the latter, an observation made possible because of the decrease in the rate of remyelination that occurs with age. Furthermore, the levels of expression of Gtx mRNA remain high within the lesion relative to background, much as those of MBP and PLP do even after remyelination is complete. In old animals there is a greater separation in the timing of reappearance of PLP and MBP than occurs in the young animals. In this situation it is interesting that Gtx more closely follows MBP than PLP, perhaps indicating a greater role in the regulation of MBP rather than PLP despite both genes containing the appropriate Gtx binding sequences. Taken together, these results suggest that Gtx is an important regulator of MBP and possibly PLP mRNA expression during remyelination in much the same way as it appears to be in myelination. This conclusion means that, at least in terms of MBP and PLP expression, the concept of remyelination using similar transcriptional mechanisms to those operating in development (the recapitulation hypothesis) is broadly upheld. Although Gtx mRNA reappearance corresponds with remyelination, expression was difficult to detect with this technique and therefore of limited use as an indicator of remyelination.

Our results on the re-expression of MBP and PLP mRNAs following demyelination of the caudal cerebellar peduncles concur with our earlier studies on gliotoxin-induced demyelination in the spinal cord.\textsuperscript{3,4} In that the rate and distribution of re-expression correlate closely with the reappearance of new myelin sheaths.\textsuperscript{25} In this study, we have followed the difference in remyelination that occurs in young and old animals. Our results indicate that the start of reappearance of MBP and PLP mRNAs is slightly delayed in old animals and thereafter there is a slower rate of accumulation. Whether the slower rate of MBP and PLP accumulation occurs because of a slower rate of recruitment of oligodendrocyte progenitors into the lesion or whether there is slower differentiation of recruited cells remains to be established. A few of the caudal cerebellar peduncle axons that are demyelinated by ethidium bromide injection are remyelinated by Schwann cells.\textsuperscript{25,30} Since both MBP and PLP mRNA are expressed in these cells, it is not possible to distinguish the areas of oligodendrocyte remyelination from those of Schwann cell remyelination.\textsuperscript{5,9} However, the extensive expression of Gtx, which is not expressed in Schwann cells, throughout the lesion and the close association between Gtx mRNA and MBP and PLP mRNA expression suggest that Schwann cell remyelination in the lesion sections taken for analysis was not extensive. We also observed a slight increase in MBP and PLP mRNA expression in the saline-injected control animals in which there was no demyelination. The degree of increase was greater in the young animals than in the old animals. This change in levels of myelin protein gene expression is similar to that which has been reported to occur in intact oligodendrocytes around areas of trauma and may represent a non-specific “reactive” change in these cells.\textsuperscript{5}

An interesting aspect of this study is that in both young and old animals the levels of MBP and PLP mRNA expression remain higher than background even after remyelination is complete at 28 days in young animals and 66 days in old animals. These high levels of expression, presumably in cells that have recently differentiated into myelinating oligodendrocytes, are similar to those that occur in development-mental myelination where there is a progressive reduction in the levels from the peaks achieved at the time of myelination.\textsuperscript{3,27,29,33} There are two possible explanations for the higher levels of expression in the remyelinated area. Firstly, it is known that cell proliferation is required for remyelination and that the remyelinated area contains more myelinating oligodendrocytes than were present before the area was demyelinated.\textsuperscript{15,23} If oligodendrocytes were expressing similar amounts of MBP or PLP mRNA then the increased numbers of cells would account for the higher levels of myelin protein gene expression. However, since the myelin sheaths of remyelination are smaller than those of normal myelination, the total amount of myelin in the remyelinated area will be less than was present prior to demyelination in spite of the increased numbers of cells. The second possibility is that the levels of expression reflect the age of the myelinating cell, those in the remyelinated area being much younger than that in the surrounding white matter. Consistent with an age-related decline in the levels of expression in myelinating oligodendrocytes is the difference in the constitutive levels of MBP and PLP expression in normal white matter between the young adult and old adult rats (Table 1). This decrease in constitutive levels of expression of these two myelin genes is very similar to that which occurs in the peripheral nervous system where there is an age-related decrease in the expression of MBP and the peripheral myelin specific protein, P0.\textsuperscript{18} Interestingly, the constitutive level of Gtx mRNA is also decreased in the older animals, suggesting that this putative regulator of MBP and PLP mRNA expression may play a role in the age-related changes in their expression. Does the age-related change in constitutive expression have any bearing on the slower rate of re-expression during remyelination? This seems unlikely since the new myelin sheaths are almost certainly derived from newly
formed oligodendrocyte that have differentiated from recruited oligodendrocyte progenitors. The new myelin is therefore approximately the same age in both young and old animals. Thus, as expected the MBP mRNA levels within the remyelinated area is the same following demyelination in both young and old animals at 66 days. However, the same is not true for PLP where there is a significant difference between the young and old animals at 66 days. This discrepancy is difficult to resolve. One possibility is that the high levels of PLP in the young animals is due to the presence of high number of oligodendrocyte lineage cells generated during remyelination that fail to fully differentiate. Consistent with this explanation is the appearance of PLP before MBP during remyelination in both ages, but especially the old animals, suggesting that it is expressed at an earlier stage of oligodendrocyte maturation than MBP. Moreover, our PLP oligonucleotide probe also recognizes DM20, which according to some authors is expressed by oligodendrocyte progenitors.\textsuperscript{2,26}

An understanding of transcriptional regulation in remyelination is important not only for unravelling the factors controlling this process, but also because manipulation of transcriptional regulation may provide a means of intervening in the pathogenesis of white matter disease.\textsuperscript{21} The results of this study indicate that Gtx has a similar relationship to MBP and PLP expression in remyelination as occurs in myelination, and provides information on the transcriptional regulation of remyelination. An eventual aim of studies on the cellular and molecular mechanisms of remyelination is to identify ways in which this process can be enhanced or rendered more efficient. It now remains to establish the extent to which manipulating the expression of Gtx may be a means of enhancing the process of new myelin sheath formation following demyelinating episodes in the CNS.

Acknowledgements—This work was supported by the Medical Research Council and the Wellcome Trust.

REFERENCES


*(Accepted 12 May 2000)*