A genetic mouse model of adult-onset, pervasive central nervous system demyelination with robust remyelination

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Adult-onset demyelinating disorders of the central nervous system represent the most common neurological abnormalities in young adults. Nevertheless, our understanding of disease pathogenesis and recovery in demyelinating disorders remains incomplete. To facilitate investigation into these processes, we have developed a new mouse model system that allows for the induction of diptheria toxin A subunit expression in adult oligodendrocytes, resulting in widespread oligodendrocyte loss and demyelination of the central nervous system. These mice develop severe ataxia and tremor that correlates with impaired axonal conduction in the spinal cord. Strikingly, these animals fully recover from their motor and physiological defects and display extensive oligodendrocyte replenishment and widespread remyelination. This model system demonstrates the robust reparative potential of myelin in the central nervous system and provides a promising model for the quantitative assessment of therapeutic interventions that promote remyelination.

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

Abbreviations: dpi = days post-injection; DT = diptheria toxin; DT-A = A subunit of diptheria toxin; MBP = myelin basic protein; OPC = oligodendrocyte progenitor cell; PLP = proteolipid protein; RT-PCR = real time-polymerase chain reaction; SSEP = spinal somatosensory evoked potentials

Introduction

Myelin facilitates rapid axonal conduction. In addition, myelin and the myelin-producing glial cells play a critical role in supporting axonal integrity, although the mechanisms by which they do this are not well understood (Nave and Trapp, 2008). In multiple sclerosis, continuous demyelination due to remyelination failure leads to chronically demyelinated axons that eventually degenerate and contribute to disease progression (Franklin and Ffrench-Constant, 2008; Trapp and Nave, 2008). Therefore, enhancing the endogenous remyelination potential of the CNS is a promising therapeutic approach to promoting functional recovery and axonal survival in multiple sclerosis.
Several animal models of demyelination have been developed to study remyelination in multiple sclerosis and other demyelinating disorders. Some approaches utilize the application of chemicals with disruptive effects (Blakemore and Franklin, 2008), such as cuprizone, lyssolecithin and ethidium bromide, whereas others, such as experimental autoimmune encephalomyelitis, murine hepatitis virus and Theiler’s murine encephalomyelitis, rely on an immune response against a myelin antigen (Mix et al., 2008), and the Theiler’s murine encephalomyelitis (Miller and Rodriguez, 1995) and murine hepatitis (Matthews et al., 2002) viruses, respectively. A number of studies using these models have shown that the myelin-repair process is carried out by adult oligodendrocyte progenitor cells (OPCs), an abundant cell type in the CNS (Franklin and Ffrench-Constant, 2008). Nevertheless, each of these models has its own set of complications and caveats that have made it difficult to discern whether the demyelinating agent is directly affecting oligodendrocytes or the axons.

Additionally, while the transgenic expression of the thymidine kinase gene of the herpes simplex virus 1 (HSV1-TK) in oligodendrocytes (Mathis et al., 2000) has been useful for inducing demyelination during the initial myelination phase, this model is less useful for studies in adult animals. CNS demyelination and neurological symptoms have also been described in cats fed a diet of irradiated chow during gestation (Duncan et al., 2009). Although remyelination and functional recovery is observed by 3–4 months after they return to a normal diet, the aetiology of demyelination in this model is unknown.

Oligodendrocyte ablation using diphtheria toxin (DT) is also possible. The A subunit of DT (DT-A) induces cell death by catalysing the inactivation of elongation factor 2 (Maxwell et al., 1986; Palmiter et al., 1987; Collier, 2001). To date, two different approaches have been used to successfully ablate oligodendrocytes with DT. The first approach utilizes the injection of DT into naturally resistant mice that transgenically express the human DT receptor in the targeted cell type, which renders them DT-sensitive (Buch et al., 2005). In addition to demyelination, these DT-treated mice demonstrate significant axonal defects and inflammation in the CNS that could be problematic when studying remyelination. A second approach takes advantage of the Cre/loxP recombination system to induce the expression of DT-A inside targeted cells (Brockschneider et al., 2004, 2006). Although this approach has been used to efficiently eliminate developing oligodendrocytes, which results in early mouse lethality, it has not been used in adult animals.

We report here the generation of a new mouse model of CNS demyelination wherein the ablation of oligodendrocytes is accomplished via cell-specific activation of DT-A expression in adult animals. In the ROSA26-eGFP-DTA line utilized in this study, the DT-A coding sequence has been inserted into the ROSA26 locus and is preceded by a floxed eGFP gene, Neo cassette and strong transcriptional stop sequence, which prevent DT-A expression (Ivanova et al., 2005). In mice also carrying the ROSA26-eGFP-DTA; PLP/CreERT female; Rosa26-stop-EYFP; PLP/CreERT double mutants were treated with intra-peritoneal injections of 1 mg of 4-hydroxytamoxifen (H-6278, Sigma) per day, or with sunflower seed oil (Sigma) as a control, for 5 consecutive days. The 4-hydroxytamoxifen was dissolved in a dimethylsulphoxide: ethanol:oil (4:6:90) mixture at a concentration of 10 mg/ml, as described previously (Masahira et al., 2006). Both male and female ROSA26-eGFP-DTA; PLP/CreERT mice were used, except that analysis after 21 days is limited to male mice only. Unless otherwise noted, ROSA26-eGFP-DTA littermates were used as control mice in all experiments involving the tamoxifen-treated PLP/CreERT; ROSA26-eGFP-DTA (DTA) mice.

**Materials and methods**

**Mice**

ROSA26-eGFP-DTA (Ivanova et al., 2005) and ROSA26-stop-EYFP (Srinivas et al., 2001) mice were purchased from The Jackson Laboratory and genotyped according to the protocols described on the vendor’s website: jaxmice.jax.org. The genotyping protocol for the PLP/CreERT transgenic mice has been previously described (Doerflinger et al., 2003). PLP/CreERT hemizygotes were bred either to ROSA26-eGFP-DTA or to ROSA26-stop-EYFP homozygous mice to generate PLP/CreERT; ROSA26-eGFP-DTA and PLP/CreERT; ROSA26-stop-EYFP mice, respectively. All mice used were housed under pathogen-free conditions and all animal studies were conducted in compliance with the University of Chicago’s Animal Care and Use Committee (IACUC) guidelines.

**Tamoxifen injections**

Five- to seven-week-old PLP/CreERT; ROSA26-eGFP-DTA and PLP/CreERT; ROSA26-stop-EYFP double mutants were treated with intra-peritoneal injections of 1 mg of 4-hydroxytamoxifen (H-6278, Sigma) per day, or with sunflower seed oil (Sigma) as a control, for 5 consecutive days. The 4-hydroxytamoxifen was dissolved in a dimethylsulphoxide: ethanol:oil (4:6:90) mixture at a concentration of 10 mg/ml, as described previously (Masahira et al., 2006). Both male and female PLP/CreERT; ROSA26-eGFP-DTA mice were used, except that analysis after 21 days is limited to male mice only. Unless otherwise noted, ROSA26-eGFP-DTA littermates were used as control mice in all experiments involving the tamoxifen-treated PLP/CreERT; ROSA26-eGFP-DTA (DTA) mice.

**Antibodies**

The following primary antibodies were used: the mouse monoclonal antibodies against CC-1 (1:50; Calbiochem), GFAP (1:1000; Covance) and BrdU (1:200; Novocastra); the rat monoclonal antibodies against PDGF-Rα (1:50; clone APAS, Millipore), CD11b (1:50; clone 5C6, Serotec) and BrdU (1:250; clone BU1/75 (ICR1), Abcam); the rabbit polyclonal antibodies against GFP (1:500; Invitrogen) and cleaved...
Caspase-3 (1:250; Cell Signaling) and the sheep polyclonal antibody against BrdU (1:100; Abcam).

Histology, immunohistochemistry and electron microscopy analysis

Histology, immunohistochemistry and electron microscopy analysis were performed as previously described (Dupree et al., 1998; Traka et al., 2008). TUNEL staining was performed using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore) according to the procedure described in the manufacturer’s protocol.

BrdU injection and detection

BrdU was injected intraperitoneally, in two doses (50 mg/kg) spaced 2 h apart, into tamoxifen-treated or oil-treated PLP/CreER<sup>2</sup>; ROSA26-eGFP-DTA mice at 5 days post-injection (dpi). Forty-eight hours later, the mice were deeply anaesthetized by intraperitoneal injections of Avertin (tribromoethanol, Sigma) at a dose of 500 mg/kg and perfused with 4% paraformaldehyde (pH 7.3) in 0.1 M Millonigs buffer. CNS tissues were processed into frozen sections for immunohistochemistry as previously described (Khaing and Blum, 2003).

Real time PCR analysis

Total RNA was extracted from the tissues of DTA and control mice at 7, 14, 21, 35 and 70 dpi using the TRizol reagent (Invitrogen). Real time-polymerase chain reaction (RT-PCR) analysis was performed as previously described (Traka et al., 2008) and the 5′-CAAACTCTTTGGCGTCTTTC-3′ (sense) and 5′-CTTAAACGGTTTCCG CCTGTC-3′ (antisense) primers were used for the amplification of the DT-A transcript. For quantitative RT-PCR analysis, the expression levels of Myelin basic protein (Mbp) and Plp mRNA were normalized according to the internal control Gapdh. The sequences of all primers and oligonucleotides used were previously described (Traka et al., 2008).

Flow cytometry

Total CNS cells were isolated from the brain and spinal cords of individual mice perfused with 20 ml of phosphate-buffered solution. Single-cell suspensions were prepared as previously described (McMahon et al., 2005). Flow cytometric analysis was performed on cells from individual animals (eight mice per group). Cells were stained with anti-CD11b-Pacific Blue (clone M1/70) and anti-CD45-APC-Cy (clone 30-F11) (BD Bioscience). A total of 5 × 10<sup>6</sup> viable cells were analysed per individual sample using a BD Canto II cytometer (Becton Dickinson), and the data were analysed using BD FACSDiva<sup>TM</sup> version 6.1 software (BD Bioscience).

Electrophysiology

Animals anaesthetized by intraperitoneal injections of Avertin (100–250 mg/kg) were laid prone and immobilized. Temperature was maintained at ≥32°C. An incision was made from the lower cervical to midthoracic region. For the recording of spinal somatosensory evoked potentials (SSEP), a needle electrode was inserted into the T5–T6 or the L4–L5 interspinous space, with a reference electrode placed in the subcutaneous tissue just proximal to the recording electrode. A grounding electrode was placed at the tail. The unilateral tibial nerve was stimulated at the ankle with rectangular pulses of 0.2 ms at 1 Hz. The potentials were averaged 25–30 times at a band-pass filter setting of 20 Hz to 2 kHz. Amplitude (Amp), peak latency (Lat) and ΔLat were measured. The ΔLat reflects the conduction time from the lower lumbar (cauda equina/lumbar roots) to midthoracic cord. Data do not include recordings from animals with an absent response at the T5–T6 level.

Morphometric analysis of the optic nerve

Electron microscopy images (×2900 magnification) of optic nerve cross sections at the prechiasmatic level were used for morphometric analysis. In order to quantify myelin thickness, axonal and fibre circumferences were measured using ImageJ software (NIH) and the corresponding g-ratios (axonal diameter/fibre diameter) were calculated. Five non-overlapping electron microscopy images were collected from three control and three DTA mice at 70 dpi and g-ratios were calculated from 150 axons per genotype. To identify the percentage of unmyelinated axons, we used five non-overlapping electron microscopy images from DTA mice and controls at 56 and 70 dpi (n = 3 mice/genotype per time point). Only axons with a diameter of ≥0.16 μm (Dangata and Kaufman, 1997) were included in the analysis. We also used 14–31 non-overlapping electron microscopy images per mouse to calculate the mean axonal density of optic nerves from DTA mice and littermate controls at 21 and 70 dpi (n = 3 mice/genotype per time point). To calculate the total number of axons per mouse, we multiplied the mean axonal density by the total surface area of the corresponding toluidine blue-stained (1-μm thick) cross section, which was measured by ImageJ.

Spinal cord axonal counts

We used toluidine blue-stained cross sections of the spinal cord at the level of the lumbar enlargement to calculate axonal densities within the ventral, lateral and dorsal columns of the white matter in DTA and control mice at 70–77 dpi (n = 3 mice/genotype). We counted the total number of axons on images acquired with an ×100 oil objective from 6315 μm<sup>2</sup> fields within each area (two random images per area) using an automated image-analysis program in the ImageJ software. Data are shown as mean axonal density/1000 μm<sup>2</sup>.

Rotarod testing

We measured the motor coordination and balance of DTA (n = 5) and littermate control (n = 4) male mice on the accelerating rotarod (Colombus Instruments). At 28 dpi, the DTA and control mice were trained to maintain themselves on the rotarod at a constant speed (5 rpm), and then were tested once a week (four trial sessions) for 7 consecutive weeks by monitoring the time (latency) that each mouse spent on the rod as it rotated in accelerating speed mode (5–65 rpm) during 5 min trial sessions.

Statistical analysis

Data from DTA and littermate control mice were compared using the unpaired one-tailed (unless two-tailed is noted) Student’s t-test. The alpha level was set at 0.05 or 0.01 and differences were considered to be statistically significant when P < alpha. The same approach was used to compare data from the tamoxifen-treated and oil-treated PLP/CreER<sup>2</sup>; ROSA26-stop-EYFP mice.
Results

Widespread oligodendrocyte cell loss in the CNS of tamoxifen-treated PLP/CreER$^T$; ROSA26-eGFP-DTA mice

PLP/CreER$^T$; ROSA26-eGFP-DTA (DTA) male and female mice were treated at 5–7 weeks of age with intraperitoneal injections of tamoxifen for 5 contiguous days to induce recombination of the ROSA26-eGFP-DTA allele (Ivanova et al., 2005) and subsequent expression of DT-A in oligodendrocytes (Fig. 1A). We confirmed that CreER$^T$ recombination is activated specifically in oligodendrocytes by observing that, in tamoxifen-treated PLP/CreER$^T$; ROSA26-stop-EYFP reporter mice, a high percentage (~95%) of enhanced yellow fluorescent protein-expressing cells in CNS white matter-rich areas also express CC-1, an oligodendrocyte marker (Supplementary Fig. 1). At 7 and 14 dpi, as measured from the first day of injections, RT-PCR analysis using primers flanking the loxP-enclosed region of the ROSA26-eGFP-DTA allele (Fig. 1A) amplified the ~650-bp recombination product from the brains of the DTA mice (Fig. 1B). No product was amplified from the brains of DTA mice at 21 dpi (Fig. 1B), suggesting that all DT-A expressing oligodendrocytes had disappeared by this stage. Furthermore, we performed TUNEL staining and immunostaining for the active form of Caspase-3 on brain sections of DTA and control mice at 3, 5 and 7 dpi and found that increased numbers of apoptotic cells were present in the white matter-rich area of the corpus callosum in DTA mice as early as 5 dpi, indicating that oligodendrocyte death occurs soon after Cre recombination is induced in these cells (Supplementary Fig. 2). Detection of the mature oligodendrocyte cell marker CC-1 via immunostaining and quantification of CC-1+ cells at 7, 14 and 21 dpi showed that significant oligodendrocyte loss had occurred in all CNS areas of DTA mice by 21 dpi (Fig. 1C). Oligodendrocyte loss in DTA mice is preceded by a dramatic drop in Mbp and Plp mRNA expression in the brain that is observable at 7 dpi and persists through 21 dpi (Fig. 1D).

As early as 7 dpi, increased numbers of BrdU-positive cells were found in different CNS areas, such as the brainstem (not shown) and the cervical cord (Fig. 1E), of DTA mice. The BrdU signal was observed in subpopulations of oligodendrocytes and adult OPCs, which were identified by CC-1 and PDGFR$\alpha$ staining, respectively, in the cervical cord of DTA mice (Fig. 1F), but not in controls (not shown). Approximately half of the BrdU-labelled cells in the cervical cord of the DTA mice were stained positive for CC-1 (56 ± 10%, \( n = 3 \)), whereas around one-third of the BrdU+ cells were stained positive for PDGFR$\alpha$ (35 ± 12%, \( n = 3 \) mice). Despite the observed proliferation, no dramatic changes in PDGFR$\alpha+$ cell numbers were found in the CNS (not shown) at 35 dpi, suggesting that the adult OPCs induced to proliferate shortly after oligodendrocyte loss rapidly differentiate into mature oligodendrocytes.

Phenotype progression in DTA mice

The DTA mice display no symptoms until 14 dpi, when they are characterized by a wobbly, uncoordinated gait that progressively worsens. Nearly all females develop hind-limb paralysis with tremor that is lethal by 21 dpi, while ~70% of male DTA mice are less affected and survive beyond 21 dpi (Supplementary Movie 1). Interestingly, we found no differences in the degree of oligodendrocyte loss between mice demonstrating the lethal and survival phenotypes.

The surviving DTA males develop severe tremor and ataxia by 35 dpi (Supplementary Movie 2), and then demonstrate a gradual recovery that culminates in full attenuation of the phenotype at around 70 dpi (Supplementary Movie 3). Oligodendrocyte cell numbers in all regions of the DTA CNS were slightly higher at 35 than at 21 dpi, and reached numbers comparable to those of control animals by 70 dpi (Fig. 2A). Also, the expression levels of Mbp and Plp mRNA showed changes that mirrored the recovery in oligodendrocyte numbers (Fig. 2B). The oligodendrocyte replenishment observed in DTA mice at 70 dpi likely results from the proliferation and differentiation of adult OPCs that occurs around 35 dpi, as significantly higher numbers of PDGFR$\alpha+$ cells were detected in different DTA CNS areas at this time point (Fig. 2C).

We screened DTA mice for CNS myelin defects subsequent to oligodendrocyte loss and observed, by toluidine blue staining, the presence of vacuoles in CNS white matter-rich regions at 21 dpi. The vacuolation was most prominent in the cerebellar white matter and brainstem and less so in the spinal cord and optic nerve (Fig. 3A). The phenotype observed in DTA mice correlates well with the degree of CNS vacuolation, as both increase in severity from 21 to 35 dpi, then are attenuated by 70 dpi (Fig. 3A). Observation of the cervical cord white matter by electron microscopy at 21 dpi revealed that the vacuoles are mostly periaxonal and are formed by the splitting of the myelin sheath lamellae (Fig. 3B). Despite the vacuolation, the myelin surrounding the majority of axons appeared to be intact and the myelin proteins MBP and myelin-associated glycoprotein were expressed at normal levels in the brain at this stage (results not shown). Myelin damage demonstrated further progression in the cervical cord white matter at 35 dpi, when increased myelin debris (results not shown) and completely demyelinated axons (Fig. 3B) were present. In addition, the large-diameter demyelinated axons looked swollen and contained increased numbers of mitochondria (Fig. 3B). Despite these abnormalities, axons in the cervical cord of the DTA mice at 35 dpi did not show increased immunostaining for the axonal injury-marker amyloid precursor protein betaA4 (APP) (results not shown). Myelin appeared to be significantly repaired in this area by 70 dpi, when many axons were found to be surrounded by thin myelin, a hallmark of remyelination (Fig. 3B).

Oligodendrocyte loss triggers either microglial activation or monocyte/macrophage infiltration of various CNS areas of DTA mice at 35 dpi, as demonstrated by increased staining for CD11b, a marker expressed in both microglial and monocytes/macrophages (Fig. 4A). In the naive CNS, microglial cells can be identified by CD45$^lo$/CD11b$^lo$/MHCI$^{-}$ expression via flow cytometry, while CNS-infiltrating monocytes/macrophages exhibit a more activated phenotype characterized by CD45$^hi$/CD11b$^hi$/ MHCI$^+$ expression (Hickey and Kimura, 1988; Sedgwick et al., 1991). Therefore, to determine whether the increase in CD11b$^hi$
cells was due to the proliferation of resident microglia, the infiltration and proliferation of peripheral monocytes or a combination of the two (Ponomarev et al., 2005), total CNS cells were isolated from the brains and spinal cords of individual littermate controls and DTA mice and the expression levels of CD45 and CD11b were analysed via flow cytometry (Fig. 4B–E). A 5.7-fold increase in the total number of cells isolated from the CNS of DTA mice (4.813 ± 0.4290 × 10^6, n = 8) as compared with littermate...
Figure 2 Recovery of oligodendrocyte numbers and myelin gene-expression levels in DTA mice at 70 dpi. (A) Quantification of CC-1 positive cells in the brainstem, cerebellum, cervical cord grey matter and optic nerve showed that oligodendrocyte numbers were significantly reduced in DTA mice at 21 dpi, then increased slightly in all areas at 35 dpi and reached values comparable to controls everywhere at 70 dpi. (n = 4, *P < 0.013, **P < 0.006, two-tailed t-test). (B) Changes in Mbp and Plp mRNA expression levels in the brain of DTA mice over time correlated with the variation in oligodendrocyte numbers (n = 3–4, *P < 0.047, **P < 0.001, two-tailed t-test). (C) Counts of PDGFRα-stained cells in the brainstem and cerebellum of DTA mice at 21, 35 and 70 dpi demonstrated that adult OPC numbers were significantly increased at 35 dpi as compared with controls (n = 3–4, *P < 0.04, two-tailed t-test). Graphs in A–C indicate mean ± SD.
controls ($0.843 \times 10^5 \pm 0.1034 \times 10^5$, $n=8$, $P<0.0017$) was observed. Calculation of the total number of CD45$^{hi}$ and CD45$^{lo}$ cells showed that the total number of CD45$^{hi}$ cells was increased 5.9-fold compared with littermate controls, while the total number of CD45$^{lo}$ cells was increased 19-fold (Fig. 4D). Additionally, analysis of the numbers of CD45$^{lo}$/CD11b$^+$ and CD45$^{lo}$/CD11b$^+$ cells showed a 5.6-fold increase in the former and a 24.3-fold increase in the latter (Fig. 4E), suggesting that

\[\text{controls: } 0.843 \times 10^5 \pm 0.1034 \times 10^5, \quad n=8, \quad P<0.0017\]

\[\text{increase in CD45$^{hi}$ cells: 5.9-fold, CD45$^{lo}$ cells: 19-fold}\]

\[\text{increase in CD45$^{lo}$/CD11b$^+$: 5.6-fold, CD45$^{lo}$/CD11b$^+$: 24.3-fold}\]
CNS-resident CD45lo/CD11b+ microglia may be proliferating within the CNS to a much greater extent in response to DT-A-mediated demyelination than infiltrating CD45hi/CD11b+ monocytes/macrophages. We also determined that there was no alteration in the percentage of T cells (CD4+ and CD8+), B cells (CD19+/B220+) and dendritic cells (CD11c+) present in the DTA CNS (results not shown).

A robust astroglial response to demyelination was also detected by GFAP immunostaining in all CNS areas examined in DTA mice at 35 dpi (Supplementary Fig. 3).

Figure 4 Microglial cell numbers are markedly increased in the CNS of DTA mice. (A) Immunostaining for the microglial and monocyte/macrophage cell marker CD11b showed that positive staining (red) was significantly increased in all DTA CNS areas examined at 35 dpi as compared with 21 and 70 dpi. Cell nuclei were counterstained with DAPI (blue). (B–E) Total CNS cells were isolated from the brains and spinal cords of individual littermate control and DTA mice (n=8) at 35 dpi. The cells were enumerated and analysed via flow cytometric analysis, gating on live cells for the presence of CD45+ cells and gating on the CD45hi (blue) versus CD45lo (purple) populations for the presence of CD11b+ cells. Flow plots from a representative littermate control mouse, shown in B, and a DTA mouse, in C, are presented. The total number of CD45hi and CD45lo cells, shown in D, and the total number of CD45lo/CD11b+ and CD45hi/CD11b+ cells, shown in E of control versus DTA animals are shown. The data are presented as the average of the cell counts from eight mice per group ± SEM in D and E. Scale bar: 100 µm (A).
Quantification of demyelination and remyelination in DTA mice

Electron microscopy analysis of myelination levels in the DTA optic nerve over time revealed that unmyelinated axons were much more numerous at 56 dpi as compared with controls, and that the number of unmyelinated axons decreased drastically by 70 dpi, when ~73% of the axons were found to be thinly myelinated (Fig. 5A and B), confirming that myelin repair occurred during this period. Calculation of the mean $g$-ratio (axon diameter/fibre diameter) showed that optic nerve axons were significantly hypomyelinated in DTA mice at 70 dpi as compared with controls, demonstrating average $g$-ratios of 0.89 and 0.80, respectively (50 fibres/mouse, $n = 3$ mice/genotype; $P < 1.80 \times 10^{-6}$). Increased $g$-ratios were found across the axon diameter spectrum (Fig. 5C), indicating a reduction in the relative thickness of the myelin sheath for all axon sizes. Furthermore, axonal degeneration and loss secondary to oligodendrocyte ablation was not observed in DTA mice, which showed normal axonal numbers in the optic nerves at 21 and 70 dpi (Fig. 5D). In addition normal axonal density numbers were found within the ventral, lateral and dorsal columns of the lumbar cord white matter in DTA mice at 70–77 dpi (Table 1).

Table 1 Axonal counts/1000 $\mu$m$^2$ in the white matter columns of the lumbar cord in DTA mice at 70–77 dpi

<table>
<thead>
<tr>
<th>Column</th>
<th>Mice, $n$</th>
<th>Control, mean (SD)</th>
<th>DTA, mean (SD)</th>
<th>P</th>
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</thead>
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<tr>
<td>Ventral</td>
<td>3</td>
<td>148 (19)</td>
<td>166 (12)</td>
<td>0.11</td>
</tr>
<tr>
<td>Lateral</td>
<td>3</td>
<td>175 (9)</td>
<td>168 (15)</td>
<td>0.26</td>
</tr>
<tr>
<td>Dorsal</td>
<td>3</td>
<td>192 (12)</td>
<td>191 (24)</td>
<td>0.47</td>
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Figure 5 Quantification of myelin and axonal defects in the optic nerves of DTA mice. (A) Electron microscopy analysis of the optic nerve revealed that the extensive myelin loss observable in DTA mice at 56 dpi is repaired by 70 dpi, at which point many axons were found to be thinly myelinated. (B) Although the numbers of unmyelinated axons were significantly higher in DTA mice at both time points, there were ~70% fewer unmyelinated axons at 70 dpi as compared with 56 dpi (**$P < 1.30 \times 10^{-5}$, $n = 3$). (C) Linear regression analysis comparing the $g$-ratios and axonal diameters of myelinated axons in the optic nerve showed thinner myelin for all sizes of axons in DTA mice at 70 dpi (black dots, $n = 3$) as compared with controls (grey dots, $n = 3$). (D) The total numbers of axons in the optic nerves of DTA mice were found to be similar to control values at 21 and 70 dpi ($P > 0.27$, $n = 3$). Graphs in B and D indicate mean ± SD. Scale bar: 2 $\mu$m (A).
Figure 6 Assessment of phenotypic progression in DTA mice. (A) SSEP waveforms. Each SSEP trace represents the average of 25–30 evoked responses recorded from the L4–L5 intervertebral space (low lumbar level) or T5–T6 intervertebral space (mid-thoracic level) following stimulation of the tibial nerve at the ankle. (B) Statistical analysis of the SSEP parameters: peak latency (Lat) and Δlatency (ΔLat, difference between the T5–T6 and L4–L5 peak latencies) and amplitude (Amp). In DTA mice at 35 dpi, peak latencies are prolonged and Δlatencies are increased, while amplitudes are decreased, indicating severe conduction defects, both in the PNS and CNS of these mice. At 70–77 dpi, the amplitudes observed in DTA mice reach values comparable to controls, but though improved in comparison to 35 dpi values, a milder defect in peak latency time lingers. At 35 dpi, three of the eight DTA mice did not demonstrate recordable T5–T6
Quantitative assessment of phenotype progression in DTA mice

To assess axonal conduction in DTA mice during phenotype progression, SSEPs were recorded. SSEP evaluation was not possible for DTA mice at 21 dpi, as no response could be recorded from the low lumbar and mid-thoracic levels upon stimulation of the tibial nerve at different levels, suggesting that conduction blocks were probably occurring both in the PNS and CNS of these animals. We were able to obtain SSEP responses from ~60% of the DTA mice at 35 dpi (Fig. 6A); these were characterized by prolonged peak latencies, increased Δlatencies (central conduction time) and decreased amplitudes (Fig. 6B), indicating that conduction was significantly compromised in both the PNS and CNS of these animals. Nevertheless, by 70–77 dpi, axonal conduction appeared to improve in these mice (Fig. 6A); the evoked potential amplitudes were similar to those of control animals and the increased peak latencies and Δlatencies seen at 35 dpi were ameliorated (Fig. 6B).

Lastly, the motor performance of DTA mice was assessed using the accelerating rotarod test, which was administered weekly starting at 35 dpi. The DTA mice failed to perform on the rotarod at 35 and 42 dpi, began showing a gradual improvement at 49 dpi and achieved latencies comparable to those of their littermate controls by 77 dpi (Fig. 6C). Overall, these data corroborate the histological, cellular, molecular and electrophysiological evidence demonstrating that CNS remyelination and functional recovery occurs in DTA mice by 70–77 dpi.

Discussion

The DTA mouse is a new genetic model of demyelination that allows for the ablation of oligodendrocytes at pre-selected ages, thereby facilitating the study of the mechanisms that control remyelination in the CNS.

Although DT-A has been shown to be a very potent cytocidal agent (Yamaizumi et al., 1978), we found that it does not cause the immediate death of all oligodendrocytes, since the toxin message remained detectable in the brains of DTA mice at 14 dpi. Nevertheless, the oligodendrocyte loss that occurs by 7 dpi appears to induce oligodendrocyte replenishment of these cells by the differentiation of recently proliferated OPCs. This response is not sufficient to replenish the full number of cells lost, however, leading to the dramatically low oligodendrocyte numbers observed at 21 dpi. Because oligodendrocyte replenishment and ablation occur simultaneously, it is not possible to determine the full extent of oligodendrocyte loss in these animals.

White matter vacuolation due to splitting of the myelin sheath lamella appears to be the immediate result of DTA-mediated oligodendrocyte cell death. Although the vacuoles do not disrupt the organization of nodal regions (not shown), they may contribute to conduction block. Further disruption to conduction could also be due to PNS myelin defects, as minor Schwann cell loss and PNS myelin abnormalities were also observed in the DTA mice (not shown). These combined defects likely explain why no SSEP responses could be recorded in the DTA mice at 21 dpi. Significant myelin degeneration and loss did not become obvious in most CNS areas until 35 dpi. Although myelin clearance is known to occur more slowly in the CNS compared with the PNS following injury (Vargas and Barres, 2007), our observations are unexpected, given that a large fraction of CNS myelin sheaths were deprived of support from oligodendrocyte cell bodies well before demyelination was initiated. A possible explanation for our observations is that the accumulation of myelin damage is delayed due to the slow turnover rate of myelin components in the adult brain (Benjamins and Morell, 1978; Benjamins et al., 1984). In addition, macrophage recruitment and myelin clearance might be delayed in CNS white matter since the protein translation-inhibitory function of DT-A likely reduces proinflammatory chemokine and cytokine secretion by the dying oligodendrocytes.

The severe myelin damage observed in DTA mice at 35 dpi significantly impaired the propagation of action potential along myelinated CNS fibres, as indicated by the abnormal Δlatencies and amplitudes of the SSEP responses recorded in these animals. In addition, the absence of a response at the T5–T6 level, but not the L4–L5 level, was found in ~40% of these mice, indicating a central conduction failure. These mice also demonstrated severe ataxia and tremor, and showed a total lack of motor coordination and balance when evaluated by rotarod test. The amelioration of the conduction impairment and the restoration of motor function as assessed by SSEP and rotarod assays, respectively, at 77 dpi are probably due to improvements in myelin integrity: at this point, oligodendrocyte numbers and myelin gene-expression levels approached control values, vacuoles were largely absent and axons were remyelinated throughout the CNS. Nevertheless, the remyelination process continues beyond 77 dpi; preliminary data indicate that CNS hypomyelination improves in DTA mice as they get older, although myelin thickness never fully returns to normal (not shown).

T cell infiltration has been reported in the CNS of mice over-expressing PLP (Ip et al., 2006) and in mice with peroxisome-deficient oligodendrocytes (Kassmann et al., 2007). It is assumed that primary oligodendrocyte dysfunction, which leads to accumulation of protein, cholesterol (Ip et al., 2006) and lipid breakdown products (Kassmann et al., 2007) triggers T
cell activation and attraction, but the exact mechanism of this phenomenon is not yet well understood. Although T cell infiltration cannot be excluded in the CNS of older DTA mice, the DTA model has demonstrated that oligodendrocyte loss and demyelination are not sufficient to cause activation and CNS infiltration by T cells during the most active disease period, as evidenced by the inability to detect these cells at 21 and 35 dpi by immunohistochemical staining or flow cytometric analysis (not shown). Moreover, the blood–brain barrier remains intact in DTA mice during this period, as the CNS was impermeable to Evans blue dye (not shown).

Importantly, an increased percentage of CD45^hi/CD11b^+ cells was found in the CNS of the DTA animals at 35 dpi, indicating that oligodendrocyte loss drives the proliferation of the resident microglia to a much greater extent than the infiltration and proliferation of peripheral monocytes/macrophages at this stage. Therefore, it is likely that resident microglia are primarily responsible for the phagocytosis of myelin debris, which is detected at increased levels at this age. Clearance of myelin debris has been shown to abrogate the inhibitory environment that blocks the reparative action of adult OPCs (Kotter et al., 2006), the major cell type that mediates remyelination, as demonstrated by studies of multiple sclerosis rodent models (Franklin and Ffrench-Constant, 2008). It also has been proposed that microglial activation, which occurs after injury, is essential for myelin repair, since activated microglia secrete factors that induce the rapid proliferation of adult OPCs (Franklin and Ffrench-Constant, 2008). Consistent with this hypothesis, an increase in adult OPCs was found at 35 dpi. It seems likely that these cells are responsible for the replenishment of oligodendrocytes and remyelination that occur in DTA mice by 70 dpi.

Interestingly, oligodendrocyte ablation, and the resulting demyelination, does not cause axonal loss in the optic nerve or spinal cord of DTA mice. Nevertheless, mitochondrial abnormalities were found in the large-diameter, demyelinated spinal cord axons during the peak of the disease, at 35 dpi, indicating mitochondrial dysfunction. This axonal defect also occurs in multiple sclerosis and it is assumed to be an adaptive response to the higher energy demand of the axons caused by demyelination (Witte et al., 2010). Despite the axonal abnormalities observed in the DTA mice at 35 dpi, no axonal loss was found in these mice at 70–77 dpi. Moreover, the absence of significant axonal loss is further supported by the full motor recovery of these mice on the rotarod, as well as by the normalized SSEP amplitudes recorded from their spinal cords. A possible explanation for the preservation of axons in the DTA model is that, unlike most demyelination models, in DTA mice the insult strictly targets oligodendrocytes and does not exert a direct toxic effect on the axons. Additionally, the robust oligodendrocyte replenishment and remyelination that occurs in the DTA model likely protects the demyelinated axons from severe damage and loss. Furthermore, the absence of T cell infiltration in the DTA model suggests that axonal loss may be primarily mediated by inflammatory Th1/17 cells in experimental autoimmune encephalomyelitis and multiple sclerosis, where significant axonal loss has been observed (Trapp and Nave, 2008). The DTA mice should provide a useful model system to test the hypothesis that oligodendrocytes provide trophic support to axons that could be independent of myelination (Nave, 2010).

Also, compared with other multiple sclerosis animal models, the DTA mouse is more applicable to target validation for drug development, as the demyelination and remyelination events are highly reproducible, they occur at distinct time points and they are both clearly associated with a quantitative behavioural readout. Overall, the DTA mouse model shows that the CNS has a robust innate ability to remyelinate. Nevertheless, our preliminary data indicate that older DTA mice fail to remyelinate their demyelinated axons and to recover from their motor defects (not shown). Thus, this model system should allow for the exploration of factors that contribute to the age-related decline in remyelination potential. Similarly, the DTA model should allow for an investigation of gender-related differences in the response to oligodendrocyte loss. It is unclear why male DTA mice display a far better survival potential than females. This observation raises the possibility that gender-related differences in body size and/or estrogen levels are responsible for the increased vulnerability of female animals to oligodendrocyte loss.

Given the robust remyelination potential of the DTA mice, it is unclear why remyelination fails in multiple sclerosis. Disease-related immune-mediated inflammatory factors could be responsible for depleting the adult OPCs or impairing the differentiation and maturation of these cells in multiple sclerosis lesions. In addition, the remyelination potential of adult OPCs could be affected by non-disease associated factors such as age, genetic background and gender (Franklin and Ffrench-Constant, 2008). The flexibility of the inducible DTA mouse model will enable the assessment of the impact of each of these factors on remyelination. Importantly, the DTA model provides a valuable resource for the screening of therapeutic strategies with the potential to promote myelin repair in multiple sclerosis.

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Supplementary material

Supplementary material is available at Brain online.

References

An oligodendrocyte ablation mouse model

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