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Astrocyte activation via Stat3 signalling determines the balance of oligodendrocyte versus Schwann cell remyelination

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Abbreviations used in the manuscript:

Stat3- Signal Transducer and Activator of Transcription 3

CNS - Central Nervous System

OPC- Oligodendrocyte Progenitor Cell

GFAP- Glial Fibrillary Acidic Protein

GFAP-STAT3-CKO - conditional astrocytic (GFAP promoter dependent) phosphorylated Stat3 knockout mouse

PNS - Peripheral Nervous System

Pdgfra - Platelet Derived Growth Factor Receptor Alpha

NG2 - Neural/Glial antigen 2

fl/fl - denotes a floxed region of a gene (flanked with loxP sites)

T12 /T13 - Thoracic levels 12 /13

PBS - phosphate buffered saline

Olig2 - Oligodendrocyte transcription factor 2 protein

CC1 (Apc) - Adenomatous Poliposis Coli protein

Iba1 - Ionized calcium Binding Adaptor molecule 1

pStat3- phosphorylated Stat3 protein

Tyr705- tyrosine 705

Aldh1l1- Aldehyde dehydrogenase family 1 member L1 protein

MOM - mouse on mouse blocking reagent

DIG - digoxigenin

plp - proteolipid protein mRNA

mpz - myelin protein zero mRNA

SSC - saline sodium citrate buffer

AP - alkaline phosphatase

NBT/BCIP - Nitro Blue Tertrazolium/ 5-Bromo-4-Chloro-3-Indolyl Phosphate

DPX - Dibutyl Phthalate in Xylene

dpl - days post lesion

BMP- Bone Morphogenetic Protein

Abstract

Remyelination within the Central Nervous System (CNS) is most often the result of oligodendrocyte progenitor cells (OPCs) differentiating into myelin-forming oligodendrocytes. In some cases, however, Schwann cells, the peripheral nervous system myelinating glia, are found remyelinating demyelinated regions of the CNS. The reason for this peripheral type remyelination in the CNS and what governs it is unknown. Here we have used a conditional astrocytic phosphorylated Signal Transducer and Activator of Transcription 3 knockout (GFAP-STAT3-CKO) mouse model to investigate the effect of abrogating astrocyte activation on remyelination following lysolecithin-induced demyelination of spinal cord white matter. We show that oligodendrocyte mediated remyelination is decreased and Schwann cell remyelination is increased in lesioned knockout mice in comparison to lesioned controls. Our study shows that astrocyte activation plays a crucial role in the balance between Schwann cell and oligodendrocyte remyelination in the CNS, and provides further insight into how Schwann cells remyelinate CNS axons.

Introduction

The adult mammalian central nervous system (CNS) is remarkably efficient at replacing myelin forming cells following primary demyelination [1]. This regenerative process is called remyelination. While in most circumstances the new myelin forming cells are oligodendrocytes, the myelinating cells of the CNS, it is also well-established in both experimental models and clinical disease that remyelination can be mediated by Schwann cells, the myelinating cells of the peripheral nervous system (PNS) [2-8]. New remyelinating oligodendrocytes are generated from a population of neural progenitor cells widely distributed throughout the adult CNS [9-11]. These cells can be identified using a range of markers, of which two commonly used are Pdgfra and NG2, and are generally referred to as oligodendrocyte progenitor cells (or OPCs) [12]. In the past, it was assumed that all Schwann cells engaged in remyelinating CNS axons were derived from PNS Schwann cells, and entered the CNS through breaches in the astrocytic *glia limitans* [13, 14]. While this is certainly a source of some CNS Schwann cells [10], transplantation [15] and, more especially, genetic fate mapping studies [10] have revealed that large numbers of CNS Schwann cells are derived from OPCs.

What remains unclear is how and why Schwann cell remyelination occurs within the CNS. Clues are provided by the anatomical features associated with areas of the CNS in which either oligodendrocyte or Schwann cell remyelination occurs. The most consistently observed feature is the astrocyte status: oligodendrocyte remyelination occurs in regions where astrocytes are present, restoring a complete CNS glial environment, while Schwann cell remyelination occurs where astrocytes are absent, resulting in patches of tissue that resemble the PNS [16-19]. Indeed, the extent of Schwann cell remyelination is directly proportional to the extent of astrocyte absence.

This is most clearly seen when comparing the remyelination of ethidium bromide-induced spinal cord demyelination, where there is initially extensive astrocyte loss and hence a high proportion of Schwann cell remyelination, with remyelination of lysolecithin induced spinal cord demyelination, which is more sparing of astrocytes and hence has a smaller Schwann cell contribution to remyelination [20].

The extent of Schwann cell remyelination has been experimentally manipulated using cell transplantation approaches, where, for example, the extent of Schwann cell remyelination of ethidium bromide-induced spinal cord demyelination can be reduced by astrocyte transplantation [21, 22]. However, whether astrocyte responses play a physiological role in determining the balance of central versus peripheral types of remyelination has not been tested in experiments where the endogenous astrocyte response is altered. In this study we took advantage of the central role of phosphorylation of the transcription factor Signal Transducer and Activator of Transcription 3 (Stat3) in the astrocyte response to CNS injury [23] to address the hypothesis that the proportion of Schwann cell remyelination following experimental demyelination is dependent on astrocyte activation within the lesion. Using a conditional Cre-loxP approach, we were able to specifically prevent Stat3 phosphorylation in astrocytes following focal toxin-induced demyelination and demonstrate that not only did this lead to a reduced astrocyte response, it also led to an impairment in OPC activation and resulted in an increased level of Schwann cell remyelination and a decreased level of oligodendrocyte remyelination, thereby demonstrating a central role of astrocyte activation in determining the nature of CNS remyelination.

Materials and Methods

Animals

The mouse line containing a conditional phosphorylated Stat3 (pStat3) knockout in astrocytes (GFAP-STAT3-CKO) on a C57BL6 background was kindly provided by Dr. Michael Sofroniew, University of California, Los Angeles [23]. Ablation of the activated form of Stat3 in astrocytes was achieved by conditional Cre-loxP recombination. In this line, the Cre recombinase is expressed under the mouse GFAP promoter (GFAP-Cre). Recombination occurs at loxP sites flanking exon 22 (the phosphorylation site containing exon) of the stat3 gene (stat3$^{fl/fl}$). As a result, phosphorylation of Stat3, crucial for its function, does not occur. Experimental animals were bred using homozygous stat3$^{fl/fl}$ males and heterozygous Cre expressing females (GFAP-Cre+/-:stat$^{fl/fl}$). The resulting GFAP-STAT3-CKO mice showed normal development and were fertile. Both male and female mice were used in the experiments with non-Cre expressing littermates (stat$^{fl/fl}$) used as controls. Experiments were performed in compliance with UK Home Office regulations and institutional guidelines.

Toxin induced demyelination

Focal spinal cord demyelination was created as previously described [24]. Briefly, 8-10 week old mice were anaesthetized with isoflurane and the spinal cord was exposed at the level of T12/T13 by removing the soft tissue between the vertebrae. 1µl of 1% L-a-lysophosphatidylcholine (lysolecithin – Sigma-Aldrich, Gilingham, UK) in sterile saline was injected using a Hamilton syringe fitted with a fine glass tip into the ventral spinal cord white matter (Fig.1.A). At designated time points after injection, mice were terminally anaesthetized with an overdose of pentobarbital before being perfused

transcardially with either 4% paraformaldehyde for immunohistochemistry, or 4% glutaraldehyde for resin embedding and electron microscopy.

Immunohistochemistry

Immunohistochemistry was performed on 12μm thick frozen sections. Where required, heat mediated antigen retrieval with 10mM sodium citrate buffer (pH 6) was performed prior to standard protocol for indirect immunofluorescence staining. Briefly, slide mounted sections were washed with phosphate buffered saline (PBS, pH 7.4), and blocked with 5% normal donkey serum and 0.1% triton X-100 in PBS for 1 hour at room temperature. Sections were then incubated with primary antibodies diluted in blocking solution overnight at 4 ºC. The following primary antibodies were used: Olig2 1:200 (Millipore, Watford, UK), CC1 1:100 (Apc, Calbiochem - San Diego, CA), Iba1 1:500 (Wako - Osaka, Japan), pStat3 (Tyr705) 1:100 (Cell Signaling Technology - Beverly, MA), Aldh1l1 1:100 (clone N103/39 – Neuromab, Davis, CA). For Apc and Aldh1l1 staining on lesion sections, Mouse On Mouse (MOM) blocking reagents (Vector Labs) were used to reduce nonspecific background according to manufacturer's instructions. Staining was visualised with Alexa Fluor conjugated secondary antibodies (1:500, Invitrogen, Paisley, UK). The slides were counterstained with 0.1% Sudan Black (Sigma-Aldrich, Gillingham,UK) to reduce background and reveal lesion area and subsequently mounted in FluorSave™ Reagent (Calbiochem, San Diego, CA). The images were acquired using the Zeiss Axio Observer fluorescence microscope.

In situ Hybridization

In situ hybridisation of digoxigenin (DIG) labelled cRNA probes for myelin protein zero (*mpz*) and myelin proteolipid protein (*plp*) was performed as previously described [26]. Briefly, sections were hybridised with DIG-labelled cRNA probes at 65°C overnight and

subjected to a standard wash protocol (50% formamide, 1X SSC, 0.1% tween-20, 65°C, 3X 30min) to remove non-specific binding of probes. The target bound probes were detected by alkaline phosphatase (AP) conjugated anti-DIG antibody, and visualised as purple precipitate after incubation in NBT/BCIP solution according to manufacturer's instructions (Roche, Lewes, UK). The slides were dehydrated with ascending concentration of ethanol, cleared with xylene and mounted in dibutyl phthalate in xylene (DPX). Images were acquired with the Zeiss Axio Observer microscope.

Electron microscopy

Animals were perfused with 4% glutaraldehyde in PBS containing 0.4mM CaCl2. The spinal cord was coronally sliced at 1mm thickness and treated with 2% osmium tetroxide overnight before being subjected to a standard protocol for epoxy resin embedding [24]. Lesions were localised on semithin 1µm sections stained with toluidine blue. Ultrathin sections of the lesion site were cut onto copper grids and stained with uranyl acetate before being examined with a Hitachi H-600 Transmission Electron Microscope.

Quantification and Statistics

For each animal, three demyelinated lesion sections, separated by approximately 120 µm, were selected from within the central region of the lesion. For immunostaining, the outline of each lesion was defined based on the increase in cellularity inside the lesion, as visualised by Hoechst 33342 counterstain. For in situ hybridisation, the outline was defined based on the lesioned tissue texture, using Zeiss AxioVision software. The numbers of marker-positive cells inside the lesions were manually counted using ImageJ, and normalized against the lesion area. The average of 3 sections was used

for each lesioned animal. For each group, 4-5 animals were used. To compare differences between the control and experimental group, a two way ANOVA followed by Bonferroni post test was used and the threshold for statistical significance was set at $p<0.05$.

Results

Expression of phosphorylated Stat3 in astrocytes is reduced in GFAP-STAT3-CKO mice following toxin-induced CNS demyelination

Phosphorylation of Stat3 is increased in multiple CNS cell types during injury [25-27]. To verify this in our demyelination model we examined the expression of phosphorylated Stat3 (pStat3) in control, non-Cre expressing lesioned and unlesioned mice, using a pStat3 specific antibody. To obtain a focal demyelinating lesion, animals were injected with 1% lysolecithin solution into the ventral spinal cord (Fig.1.A). In normal unlesioned spinal cord, very few cells were found to express pStat3 (not shown). In contrast, there was a marked increase in pStat3 expression 5 days post lesion (dpl), (Fig.1.B). The pStat3 levels remained elevated at 14 and 21 dpl, albeit somewhat decreased from the expression at 5dpl (Fig.1.B). The staining was most intense within the nucleus (Fig.1.D, inset). pStat3+ cells were a variety of cell types, including CD11b+ macrophages/microglia, Olig2+ oligodendrocyte lineage cells and Gfap+ astrocytes (Fig.1.E,F,M-O).

Phosphorylation of Stat3 plays a key role in mediating astrocyte responses to CNS injury [23]. To further explore the astrocyte-specific role of pStat3 in lysolecithin-induced demyelination, we used a conditional Stat3 knockout mouse model in which Cre recombinase was expressed under the GFAP promoter. The Cre recombinase excised

the floxed Stat3 exon 22 containing the phosphorylation site (Tyr 705) involved in Stat3 activation [23], resulting in a mutant unable to phosphorylate Stat3 in astrocytes (GFAP-STAT3-CKO mice). We first investigated pStat3 expression in lesioned GFAP-STAT3-CKO mice. As expected, the number pStat3 expressing astrocytes, as examined by colabelling with astrocyte markers Gfap (Fig.1.C-I) and aldehyde dehydrogenase 1 (Aldh1l1) [28] surrounding pStat3+ nuclei, was significantly lower in the mutants compared to controls (Fig.1.J-L).

Demyelination-associated astrogliosis is reduced in GFAP-STAT3-CKO mice

Lysolecithin demyelination is characterised by an abundance of astrocytes within the lesioned area (Fig.1.C-L; Fig.2.A-D). In control mice, we observed an increase in Gfap immunoreactivity within and beyond the demyelinated area (the latter determined by the lipophilic dye Sudan Black) (Fig.2.A, B). An increase in Gfap expression was also seen in demyelinated areas of spinal cord in GFAP-STAT3-CKO mice, when compared to unlesioned tissue surrounding the lesioned areas (Fig.1.H; Fig.2.C,D), although the intensity of the staining was lower than in control animals (not shown). Because Gfap staining localises to astrocytic processes that often form a tangled mesh in injured tissue, rendering quantification of individual positive cells challenging, we instead compared the areas occupied by reactive astrocytes in control and GFAP-STAT3-CKO spinal cord lesions, as defined by a clear boundary of increased Gfap reactivity. We found the relative area of reactive astrocytes over total lesion area to be reduced in GFAP-STAT3-CKO mice at both 5 and 14 dpl (Fig.2.E). This was verified by counting the number of Aldh1l1+ cells in demyelinated areas, which revealed a significant 40-60% reduction in GFAP-STAT3-CKO mice compared to controls at all three survival time points examined (Fig.2.G).

Reducing Stat3 activation in astrocytes does not alter macrophage responses to demyelination

Abrogation of Stat3 activation results in spreading of inflammation following a spinal cord crush injury [23, 29]. To assess the influence of astrocytic Stat3 knockout in our demyelination model, we examined the microglia/macrophage response in GFAP-STAT3-CKO mice following demyelination by immunostaining for Ionized calcium Binding Adaptor molecule 1 (Iba1). Iba1+ cells were found to be present at high density throughout the lesion (Fig.3). Since it is not feasible to quantify microglia/macrophage cellular density due to the fused pattern of immunostaining in lesions, we measured normalised mean optical density to represent the extent of microglia/ macrophage infiltration in the demyelinated area. There was no difference in either Iba1 intensity or area of Iba1+ cell infiltration at all the time points examined (Fig.3.C, D).

The attenuated astrocyte response reduces oligodendrocyte remyelination

Astrocytes are known to influence OPCs during CNS remyelination [21, 30-32]. We therefore assessed the impact of the conditional astrocytic pStat3 ablation on oligodendrocyte remyelination, by comparing the distribution of oligodendrocyte lineage cells using different markers expressed at specific stages of lineage progression in control and mutant lesioned mice. In control animals, Olig2+ cell numbers had increased at 5 dpl (when OPCs are actively recruited to the lesion), had increased further at 14 dpl (when differentiation is ongoing) and remained high at 21 dpl (when remyelination is near completion) (Fig.4.A,B and E). In GFAP-STAT3-CKO animals, the density of Olig2+ cells

was comparable to controls at 5 dpl, but had significantly reduced at 14 and 21 dpl (Fig.4.C-E).

Increased expression of the transcription factor Sox2 is a marker of OPC activation (unpublished observations). The density of total Sox2+ cells, and Sox2+/Olig2+ colabelled cells in the demyelinated area were significantly lower in the GFAP-STAT3-CKO group compared to controls at 5 and 14 dpl, suggesting impaired OPC activation (Fig.4.F,G). There was also a reduction in Sox2+ cells that did not express Olig2, which are likely to be astrocytes and is consistent with the data in Fig. 2G. The reduced density of Olig2+ and Olig2+/Sox2+ cells was mirrored by reduced expression of mature oligodendrocyte markers CC1 and *plp* at 14 and 21 dpl (Fig.5).

Schwann cell CNS remyelination is increased as a result of attenuated astrogliosis

The mutual exclusivity of astrocyte presence and Schwann cell remyelination within the same region of repairing demyelinated lesion led us to reason that an attenuated astrocytic response could lead to increased Schwann cell remyelination in GFAP-STAT3-CKO mutants. Indeed, compared to lesioned controls, the GFAP-STAT3-CKO spinal cord white matter lesions showed increased areas of periaxin antigenicity and increased density of myelin protein zero (*mpz*) mRNA expression at 14 and 21 dpl, indicating increased Schwann cell remyelination (Fig.6.A-I). The increased number of remyelinating Schwann cells was confirmed by analysing semithin resin sections and performing electron microscopy, where Schwann cells could be readily recognised by their typical 'signet ring' morphology and relatively thicker myelin sheath (Fig.6.J-O). Notably, in the GFAP-STAT3-CKO lesions, Schwann cell remyelinated areas contained more demyelinated axons than oligodendrocyte remyelinated areas at all the time

points examined (Fig.6.M,N). Since toxin-induced demyelination invariably undergoes complete remyelination it is likely that these few remaining demyelinated axons will eventually undergo remyelination.

Discussion

In this study, we used a transgenic conditional astrocytic pStat3 knockout model to show that altering astrocyte activation significantly influences the response to CNS demyelination injury. This model had been previously used to study astrogliosis and scar formation in spinal cord trauma [23]. In accordance with this previous study, we found that abrogating astrocyte activation decreased the astrocytic response in the lesion. However, unlike in the trauma model, we found no effect of pStat3 manipulation in astrocytes on macrophage responses [29]. Crucially, our study focused on remyelination and revealed that oligodendrocyte remyelination was reduced and Schwann cell remyelination increased in lesioned GFAP-STAT3-CKO mice. Our findings therefore constitute the first direct proof of the indispensable role of astrocyte activation in the balance between Schwann cell and oligodendrocyte remyelination in the CNS.

The intriguing phenomenon of Schwann cell remyelination within the CNS has been recognised for many decades, yet its mechanisms and functions remain obscure. It is now known, as a result of genetic fate mapping strategies that, contrary to the previously held belief, many of the Schwann cells that appear in the CNS are not immigrants from the peripheral nervous system but are instead derived from CNS progenitor cells [10]. It has also been recognised that Schwann cell remyelination often occurs around blood vessels and within areas of damaged CNS from which astrocytes are absent [16, 19, 33]. This observation has led to the hypothesis that the presence or absence of astrocytes determines if CNS progenitor cells support remyelination of demyelinated axons by becoming an oligodendrocyte or a Schwann cell. Our results clearly reveal a central role for astrocytes in determining the balance of central versus

peripheral type remyelination. It remains unclear how astrocytes exert this effect. One proposed hypothesis is that members of the bone morphogenetic protein (BMP) family induce OPCs to become Schwann cells and this is prevented in the presence of astrocytes by astrocyte-derived inhibitors of BMP signalling, with OPCs becoming oligodendrocytes instead. However, while there is some evidence that the fate of transplanted OPCs can be influenced by prior treatment *in vitro* with BMPs [34, 35] there is no compelling evidence for such mechanism *in vivo*. Since the current STAT3 knockout takes place at the first appearance of GFAP expression during development it is possible that there are long-term changes in the environment that contribute to the shift in remyelination type. However, if such changes do exist they would only seem to be revealed following injury since in neither our study nor in previous studies on astrocyte STAT3-null animals have phenotypic changes been identified in the absence of injury. Thus, exactly how OPCs become Schwann cells in the CNS in the absence of astrocytes remains to be fully explored.

What is the functional significance of Schwann cells myelinating CNS axons? There are two main functions of myelin – to allow rapid saltatory conduction and to help maintain axon health and integrity [36]. It has been evidence for many years that Schwann cell myelination restores saltatory conduction to demyelinated CNS axons, and from this perspective it appears to make no difference which type of myelin surrounds the axons [37, 38]. However, the relative effect of peripheral versus central type myelin on axonal integrity is entirely unknown. Schwann cells and oligodendrocytes differ in a number of ways - they develop from different tissues, use different strategies to myelinate target axons, produce different extracellular components and assemble molecularly distinct nodes and paranodes [39]. Moreover, key differences have been shown in their metabolic relationships with the axons they ensheath [40]. It is possible therefore that in the

context of recovery from CNS demyelinating injury, Schwann cell CNS remyelination may have distinctive physiological advantages over oligodendrocyte remyelination, although this hypothesis remains to be tested. Certainly, in the context of immune mediated damage directed against epitopes specific to oligodendrocytes and their myelin, one might imagine that Schwann cell would be resistant to direct injury and that therefore this form of remyelination might protect against subsequent oligodendrocyte-directed immune attack.

Our study has shown that substantial reduction of pStat3 mediated astrocyte activation is a sufficient prerequisite to sway the remyelination process towards PNS type remyelination in the CNS. This evidence will help to further elucidate the development and function of peripheral type CNS remyelination, which presents not only an intriguing biological phenomenon but also an interesting and unexplored therapeutic possibility.

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Figure legends

Figure 1. Expression of pStat3 following focal demyelination in spinal cord white matter is reduced in GFAP promoter controlled conditional Stat3 knockout mice.

Control and mutant animals were injected with lysolecithin to create a focal demyelination lesion (A). The quantification of total numbers of pStat3+ cells and pStat3/Gfap double positive cells in control and GFAP-STAT3-CKO lesions, is shown in graphs (B) and (C). Image sets (D-I) show immunostaining of lesioned ventral spinal cord white matter at 14 days post lesion (dpl). D and G show merged immunostaining for phosphorylated Stat3 (pStat3), and the nuclear dye Hoechst 33342. The dotted line marks the border of the demyelinated area, as demarcated by increased cellularity shown by Hoechst staining (inset in D). Colabelling with pStat3 and astrocyte marker Gfap is shown in E and H, with boxed area in each image magnified in F and I respectively. Representative images show colocalization of an alternative astrocyte marker Aldh1l1 and pStat3 in demyelinated areas at 14 dpl, in control (J) and GFAP-STAT3-CKO (K) animals, with quantification shown in (L). pSTAT3 is also expressed in other types of cells, including CD11b+ macrophages/ microglia (M,N) and Olig2+ oligodendrocyte lineage cells (O) within lesions. Mean ± SEM, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$. Arrowheads in images F and J-O indicate cells labelled with both markers. Scale bar in D represents 100 μm for D-I , and 25μm for J and K.

Figure 2. Astrocyte response to demyelination is attenuated in GFAP-STAT3-CKO mice.

Images (A-D) illustrate areas of lysolecithin-induced demyelination in ventral spinal cord white matter at 14 days post lesion (dpl), immunolabelled for astrocyte marker

Gfap and nucleus dye Hoechst 33342 (A,C), with lesion areas demarcated by counterstaining with Sudan Black (B,D). The relative total area occupied by immunoreactive Gfap staining in demyelinated lesions, and the average length of astrocytic processes in control and GFAP-STAT3-CKO mice are quantified in graphs (E) and (F). The number of astrocytes inside the lesion area identified by the astrocyte marker Aldh1l1 are shown in (G). Dotted lines in images mark the lesion boundaries. Mean ± SEM, *, p<0.05, **, p<0.01 ***, p<0.001. Scale bar represents 100 μm for all images.

Figure 3. Macrophage and microglia infiltration following demyelination in GFAP-STAT3-CKO mice is similar to that in controls.

The microglia/macrophages in demyelinated area at 5 dpl were visualised with antibody against macrophage/microglia marker Ionized calcium Binding Adaptor molecule 1 (Iba1) in control (A) and GFAP-STAT3-CKO mice (B). Mean optical density was used to compare Iba1 immunoreactivity between controls and mutants across time points (C). (D) shows the average macrophage/microglia infiltration area at different survival times. Measurements were taken form transverse sections of the lesion that had the largest area of lesion (identified by increased cellularity). Mean +/- SEM. Scale bar represents 50μm for both images.

Figure 4. The response of oligodendrocyte lineage cells to lysolecithin induced spinal cord demyelination in GFAP-STAT3-CKO mice.

Images show demyelinated areas of ventral spinal cord lesions at 14 dpl, from control and GFAP-pSTAT3-CKO mice, double stained for oligodendrocyte lineage marker Olig2 and transcription factor Sox2, a marker for activated OPCs (A-D). B and D are the enlarged areas marked by orange rectangle boxes in A and C respectively. Densities of

single labelled and colabelled cells are compared in (E), (F) and (G). Arrows indicate double-labelled cells. Mean ± SEM, *, p<0.05, **, p<0.01, *** p<0.001. Scale bar represents 100 μm for images A and B.

Figure 5. Oligodendrocyte remyelination is reduced in GFAP-STAT3-CKO mice. Remyelination by oligodendrocytes in control and GFAP-pSTAT3-CKO mice at 14 and 21dpl was assessed by immunostaining for the differentiated oligodendrocyte marker CC-1 (A and B) and *in situ* hybridization for proteolipid protein mRNA (*plp*), (D and E). Images depict representative examples of demyelinated lesions at 21dpl. The cell densities for each marker at selected survival times are compared in (C) and (F). Mean ± SEM, **, p<0.01, ***, p<0.001. Scale bar represents 100μm for all images.

Figure 6. Schwann cell remyelination is increased in GFAP-STAT3-CKO mice. Remyelinating Schwann cells from control and GFAP-STAT3-CKO mice at 21 dpl were examined by Periaxin immunostaining (A-D), and *mpz* mRNA *in situ* hybridisation (F-H). Dotted lines mark demyelinated areas. (E) and (I) show quantifications of relative area of positive Periaxin immunofluorescence and cells containing *mpz* mRNA, respectively. Semithin resin sections from control (J) and STAT3CKO (K) lesioned mice were stained with toluidine blue. (M) and (N) are enlarged boxed areas from (J) and (K) respectively. Areas of oligodendrocyte remyelination are marked with yellow letter 'O', and that of Schwann cell with the red letter 'S'. In (L) and (O), examples of axons remyelinated by oligodendrocytes are marked by yellow arrows; green arrows point to examples of axons that were not demyelinated; cyan arrows indicate poorly remyelinated axons, whereas red arrows mark typical morphology of myelinating Schwann cells. These observations were further verified by electron microscopy with examples shown in (L) - control and (O) - GFAP-STAT3-CKO. Inset in (O) shows an

enlarged view of boxed area in (O), depicting the typical structure of myelinating Schwann cells. Mean ± SEM, *, $p<0.05$, **, $p<0.01$, *** $p<0.001$. Scale bar in A represents 100μm for A-D. Scale bar in F represents 100μm for F and G. Scale bar in J represents 100μm for J-M. Scale bar in L represents 5μm for N and O.