SMN-dependent intrinsic defects in Schwann cells in mouse models of spinal muscular atrophy

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Low levels of survival of motor neuron (SMN) protein lead to spinal muscular atrophy (SMA). The major pathological hallmark of SMA is a loss of lower motor neurons from spinal cord and peripheral nerve. However, recent studies have revealed pathological changes in other cells and tissues of the neuromuscular system. Here, we demonstrate intrinsic, SMN-dependent defects in Schwann cells in SMA. Myelination in intercostal nerves was perturbed at early- and late-symptomatic stages of disease in two mouse models of SMA. Similarly, maturation of axo–glial interactions at paranodes was disrupted in SMA mice. In contrast, myelination of motor axons in the corticospinal tract of the spinal cord occurred normally. Schwann cells isolated from SMA mice had significantly reduced levels of SMN and failed to express key myelin proteins following differentiation, likely due to perturbations in protein translation and/or stability rather than transcriptional defects. Myelin protein expression was restored in SMA Schwann cells following transfection with an SMN construct. Co-cultures of healthy neurons with diseased Schwann cells revealed deficient myelination, suggestive of intrinsic defects in Schwann cells, as well as reduced neurite stability. Alongside myelination defects, SMA Schwann cells failed to express normal levels of key extracellular matrix proteins, including laminin α2. We conclude that Schwann cells require high levels of SMN protein for their normal development and function in vivo, with reduced levels of SMN resulting in myelination defects, delayed maturation of axo–glial interactions and abnormal composition of extracellular matrix in peripheral nerve.

INTRODUCTION

Proximal spinal muscular atrophy (SMA) is a leading genetic cause of infant mortality in humans, with an incidence of 1 in 6000 live births (1). SMA is an autosomal recessive condition and, in its most severe forms, disease onset occurs before 6 months of age with death from respiratory distress usually within 2 years. The major pathological characteristic of SMA is loss of lower alpha motor neurons from the ventral horn of the spinal cord, resulting in progressive muscle denervation, skeletal muscular atrophy—particularly of the proximal muscles—and eventual paralysis. Peripheral nerve abnormalities are also a notable feature of SMA in human patients, including reduced conduction velocities, abnormal axonal membrane conductance and disrupted Schwann cells/myelin (2–5).

SMA is primarily caused by homozygous deletion of, or mutations in, the survival of motor neuron 1 (SMN1) gene (6). Its full-length protein product, SMN, is a ubiquitous and essential cellular protein. In humans, there are two SMN genes, SMN1 and SMN2. However, the SMN2 gene produces considerably less full-length SMN protein than the SMN1 gene meaning that it cannot fully compensate when full-length SMN protein from the SMN1 gene is lost. Historically, low levels of SMN were thought to selectively target lower motor neurons. However, with the emergence of SMA animal models and a clearer appreciation of disease pathology in human patients, it has become apparent that reduced levels of SMN have effects on multiple cell types and tissues, albeit to varying degrees (7).

Glia cells fulfil crucial roles in the development, functionality and survival of neurons in both the central and peripheral

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nervous systems. Indeed, there is growing evidence that non-neuronal cells play an important role in regulating neurodegeneration (8). For example, astrocytes and microglia are known to contribute to motor neuron loss during the pathogenesis of amyotrophic lateral sclerosis (9). Additionally, hereditary peripheral neuropathies, commonly associated with axonal atrophy and degeneration, can result from genetic defects targeting myelin proteins associated with Schwann cells (10). The contribution of multiple cell types, including neighbouring non-neuronal glial cells, to neurodegeneration in a range of disorders thereby reveals the potential for non-cell autonomous regulation of neuronal dysfunction and degeneration in SMA. Given the important role that myelinating Schwann cells play in regulating the form and function of lower motor neurons in peripheral nerve (11), alongside data from previous studies reporting alterations in expression levels of myelin genes in microarray screens on SMA mouse spinal cord (12,13), we set out to determine whether intrinsic defects in myelinating Schwann cells contribute to disease pathogenesis in SMA.

RESULTS

Abnormal myelination of peripheral nerve in SMA mice in vivo

To establish whether preliminary indications of defects in myelin gene expression in SMA mouse spinal cord (12,13) were indicative of widespread defects in myelination of peripheral nerve, we first carried out an ultrastructural analysis of myelination in intercostal nerves from the ‘severe’ mouse model of SMA. Intercostal nerves were chosen for these initial experiments because muscles innervated by lower motor neurons in peripheral nerve (11), alongside data from previous studies reporting alterations in expression levels of myelin genes in microarray screens on SMA mouse spinal cord (12,13), we set out to determine whether intrinsic defects in myelinating Schwann cells contribute to disease pathogenesis in SMA. Using a range of in vivo and in vitro approaches we demonstrate defects in myelination and extracellular matrix composition in peripheral nerve from SMA mouse models, occurring as a result of intrinsic, SMN-dependent defects in Schwann cells.

Quantitative analyses confirmed a higher proportion of unmyelinated large diameter axons in the SMA mice (Fig. 1c) as well as a significant increase in the average G-ratio (Fig. 1d). This indicates a reduction in average myelin sheath thickness relative to the axon diameter. The increase in average G-ratio was observed across the full range of axonal calibres (Fig. 1e), with no evidence for reduced axon diameters in the SMA mice (Fig. 1e).

To confirm that the deficient myelination observed in peripheral nerve was a common phenotype across genetically distinct SMA mouse models, we next asked whether similar myelination defects could be detected in ‘Taiwanese’ SMA mice. Qualitative assessment of intercostal nerves from both early-symptomatic (P7) and late-symptomatic (P11) animals revealed thinner myelin sheaths surrounding a subpopulation of axons. As in the ‘severe’ SMA model, we found no evidence for widespread demyelination/dysmyelination or axon degradation, and quantitative analyses showed that nerves from SMA mice had significantly greater G-ratios compared with littermate control mice across a spread of axonal calibres (Fig. 1f and g). Importantly, our observation of a significant thinning of the myelin sheath in SMA mice at P7 (the time point where overt disease symptoms become detectable in our colony of ‘Taiwanese’ mice) confirms that this phenotype is present at all stages of the disease and is not simply an ‘end-stage’ phenomenon.

To determine whether the myelination defects we observed were affecting all nerves equally we next evaluated myelination in the sciatic nerve, a more distal nerve that is less affected in SMA mouse models. Qualitative and quantitative assessment of myelination in sciatic nerve from early-symptomatic (P7) animals revealed no significant differences between SMA mice and littermate controls (Fig. 2a–c). Thus, at an early-symptomatic time point where significant myelination defects were observed in intercostal nerves, other distal nerves in the same animal remained unaffected.

Given that upper motor neuron involvement is rarely observed in human SMA patients, we also wanted to determine whether the myelination phenotypes we observed in peripheral nerve were also present in motor tracts of the spinal cord. Motor axons are specifically located in the dorsal corticospinal tract (dCST) of mice (17–19) and ultrastructural analyses of these motor axons revealed no difference in myelination between ‘Taiwanese’ SMA and littermate control mice (Fig. 2d–f). Thus, in keeping with the restriction of neuromuscular pathology to peripheral nerve in human patients, the defective myelination phenotype observed in SMA mice was similarly restricted to subpopulations of affected peripheral nerves.

Next, we wanted to determine whether the morphological defects in myelination observed in SMA mice were accompanied by perturbations in the expression of key myelin proteins, such as myelin protein zero (MPZ), peripheral myelin protein 22 (PMP22) and myelin basic protein (MBP) (20). We measured the levels of MPZ, PMP22 and MBP using fluorescent western blotting in both affected intercostal nerves and less-affected sciatic nerves from SMA mice at P11 (15,21). We observed a significant reduction in levels of both MPZ and PMP22 levels in intercostal nerves from SMA mice, with a concomitant increase in levels of MBP (Fig. 3a) (Supplementary Material, Fig. S1). Increases in MBP levels have previously been shown to occur when myelination processes are perturbed (see discussion).
Figure 1. Abnormal myelination of peripheral nerve in two mouse models of severe SMA. (A) Representative low-power (top panels) and high-power (bottom panels) electron micrographs of intercostal nerves from late-symptomatic (P5) ‘severe’ SMA mice and littermate controls. Large diameter (>1 μm) axons lacking myelin sheaths (white arrows in upper panels) and axons with thinner myelin sheaths were more commonly observed in SMA mice (e.g. five large diameter, unmyelinated axons are present in the SMA example compared with two in the control). (B) No difference in the number of degenerating axons between control and SMA intercostal nerves from ‘severe’ SMA mice at P5 (P > 0.05, Mann–Whitney test; N = 3 mice per genotype). (C) Significant increase in the numbers of unmyelinated large diameter (>1 μm) axons in ‘severe’ SMA mice at P5 (P < 0.05). (D and E) Significantly higher average G-ratio in intercostal nerves from ‘severe’ SMA mice at P5 compared with littermate controls, indicating thinner myelin sheaths relative to the axon diameter (d; ***P < 0.001; two-tailed, unpaired t-test), with higher G-ratios across the range of axon calibres (e) (N = 3 mice per genotype, n > 50 independent nerve fibre measurements per genotype). (F) Intercostal nerves from ‘Taiwanese’ SMA mice also had a significantly higher average G-ratio at both early- (P7) and late-symptomatic (P11) time points compared with littermate controls (**P < 0.01; ***P < 0.001; two-tailed, unpaired t-test) (G) Higher G-ratios across the range of axon calibres in ‘Taiwanese’ SMA mice at P11 (N = 3 mice per genotype, n > 50 independent nerve fibre measurements per genotype). Scale bars = 5 μm (upper panels of a), 1 μm (lower panels of a).
In contrast, MPZ levels remained unchanged, and both PMP22
and MBP were expressed at higher levels in sciatic nerve from
SMA mice (Fig. 3a). These findings therefore provided molecu-
lar data to support our previous morphological evidence for per-
turbations in myelination in intercostal nerve, whilst confirming
previous studies suggesting that myelination is not overtly
affected in the sciatic nerve of SMA mice (22). In keeping
with our previous ultrastructural analyses, the severity of mye-
lination defects was not consistent throughout all peripheral
nerves of the body, with intercostal nerves being particularly
affected in SMA mice.

One possible explanation for the changes we observed in
levels of key myelin proteins in SMA peripheral nerve was
that low levels of SMN disrupted ongoing dynamic changes in
the temporal expression of myelin proteins occurring during
normal postnatal development. To address this, we carried out
a temporal analysis of MPZ, PMP22 and MBP protein expres-
sion levels in both peripheral nerves over the first month of life
(Fig. 3b and c), suggesting that altered levels of myelin proteins
observed in SMA directly reflect disruption of ongoing develop-
mental processes in early postnatal mice.

Alongside post-natal regulation of myelination, Schwann
cells are responsible for forming axo–glial interactions at para-
nodes in the first few weeks of life (23,24). Paranodes develop at
the onset of myelination, with initial interactions forming closest
to the Node of Ranvier before extending towards the juxtapara-
node (25). To determine whether maturation of paranoval axo–
glial interactions was also affected in SMA, we examined nodes
of Ranvier and paranodes in single teased-axon preparations.

We used sciatic nerve preparations for these analyses as inter-
costal nerves from neonatal mice are too fragile to produce
sewed single fibre preparations. Nodes of Ranvier and paranodes
were present in all mice examined at both pre/early (P6) and late-
symptomatic (P10) time points, with both axonal (Caspr) and
glial (NFASC155) paranodal proteins clustering normally
(Fig. 4). However, paranodal length was significantly reduced
in SMA nerves at both time points (Fig. 4), indicating a develop-
mental delay in paranodal maturation. Nodal length was not
affected (Fig. 4). Taken together, these experiments demon-
strated that low levels of SMN protein lead to defects in myelin-
ation and maturation of axo–glial interactions at paranodes in
peripheral nerves in SMA mice. Interestingly, these subtle
defects in axo–glial maturation were present even in peripheral
nerves where gross defects in myelination was not observed
(i.e. the sciatic nerve).

SMA-derived Schwann cells develop normally
pre-differentiation

It remained possible that the myelination defects we observed in
SMA mice were occurring as a secondary consequence of path-
ology in neighbouring motor neurons, rather than as a result of
primary pathology intrinsic to the Schwann cells. To determine
whether Schwann cells were intrinsically affected by reduced
levels of SMN, we modified established protocols to allow isola-
tion of murine Schwann cells from ‘Taiwanese’ SMA mice and
littermate controls at an early-symptomatic time point (P8) (see
methods). This approach allowed us to assess Schwann cells
from SMA mice in the absence of any influence from neighbouring neurons. The purity of Schwann cell cultures (>98% from both control and SMA mice) was confirmed by immunolabelling for the Schwann cell markers S100 calcium-binding protein (S100) and glial fibrillary acidic protein (GFAP), neither of which are expressed by fibroblasts (Supplementary Material, Fig. S2).

In vitro preparations of isolated Schwann cells revealed strong SMN expression in cells from control mice, but significantly reduced levels in cells from SMA mice (Fig. 3d and e). Schwann cells derived from SMA mice that had de-differentiated to an immature, non-myelinating phenotype were stable in culture, with no evidence for activation of apoptotic pathways (Supplementary Material, Fig. S3a and b). Moreover, Schwann cells derived from SMA mice showed normal expression of core molecular markers of Schwann cells (26–31), including SRY-box containing gene 10 (SOX10),

Figure 3. Disruption of key peripheral myelin proteins in vivo. (A) Quantitative fluorescent western blotting revealed differential alterations in the expression of myelin proteins in peripheral nerves (I/C, intercostal nerve; sciatic, sciatic nerve) from late-symptomatic (P11) ‘Taiwanese’ SMA mice compared with littermate controls (all ***P < 0.0001; N = 3 mice per genotype). (B and C) Quantitative fluorescent western blotting for myelin proteins in intercostal (b) and sciatic (c) nerves from healthy wild-type mice revealed dynamic temporal alterations in the expression during the first days and weeks of life. (D) Representative confocal micrographs of isolated Schwann cells from ‘Taiwanese’ SMA mice and littermate controls, immunolabelled to reveal SMN protein. SMN was strongly expressed in Schwann cells from control mice, but was markedly reduced in primary cultures of SMA-derived Schwann cells (**P < 0.001; N = 4 mice/cultures per genotype, two fields of view imaged per culture, >150 cells quantified per field of view) (all tests two-tailed, unpaired t-test).
SRY-box containing gene 2 (SOX2) and POU domain class 3 transcription factor 1 [POU3F1 (or OCT6)] (Fig. 5a–f). Thus, immature Schwann cells isolated from SMA mice were stable and expressed the normal complement of markers associated with immature (non-myelinating) Schwann cells.

SMA-derived Schwann cells fail to respond normally to myelination cues

We next wanted to establish whether the in vivo myelination defects we observed in SMA mice were due to differences in the intrinsic ability of Schwann cells to respond to differentiation signals when SMN levels were reduced. Differentiation of myelinating Schwann cells from immature Schwann cells can be induced in vitro by the addition of dbCAMP and NRG1, resulting in the strong expression of myelin-related markers, such as MPZ (32). Transcriptional activation of MPZ gene expression in Schwann cells requires the co-operative binding of both SOX10 and early growth response 2 [EGR2 (or KROX20)] to MPZ promoter elements (28,33,34).

One hundred and twenty hours after induction of differentiation MPZ levels were significantly less elevated in SMA-derived Schwann cells compared with control Schwann cells (Fig. 6c). Thus, low levels of MPZ in SMA Schwann cells were not caused by low levels of SMN impacting on gene transcription. This suggests that SMN regulates MPZ expression in Schwann cells by mediating protein translation and/or stability.

To determine whether the myelination defects we observed were SMN dependent and reversible, we transfected SMA-derived Schwann cells with an SMN expression construct driven by a Schwann cell-specific promoter (pMPZ-SMN) (35). At 90 h post-differentiation cultures were transfected with pEHHG (a GFP-expression, non-SMN carrying, control construct) or pEHHG and pMPZ-SMN. MPZ levels were quantified 30 h later (equivalent to a total of 120 h post-differentiation). We observed a significant increase in MPZ levels in SMA-derived Schwann cells transfected with pMPZ-SMN (Fig. 6d and e), confirming that the Schwann cell phenotypes previously observed were occurring as a direct result of reduced SMN levels. Moreover, these defects were reversible when SMN levels were restored.

Figure 4. Delayed maturation of axo–glial interactions at the paranode in SMA mice. (A and B) Representative confocal micrographs of teased sciatic fibres from ‘Taiwanese’ SMA mice at P6 (pre/early symptomatic) showing shorter paranodes, indicating delayed development of axo–glial interactions. Paranodes were immunolabelled with antibodies against pan-neurofascin (green in a) and caspr (green in b). Nodes (red) were immunolabelled with ankhrin-G. Nodal and paranodal regions are highlighted with arrowheads and arrows, respectively. (C and D) No change in nodal length (c) in SMA mice, but a significant reduction in paranodal length (d) (*P ≤ 0.05). (E and F) Representative confocal micrographs of teased fibres from mice at P10 (late-symptomatic), indicating a continued delay in maturation of axo–glial interactions. (G and H) No significant change in nodal length (g) in SMA mice, but a significant decrease in paranodal length (h) (*P ≤ 0.05; all N = 3 mice per genotype, with 50–100 nodes analysed per nerve; two-tailed, unpaired t-test).
to differentiation cues. Schwann cell cultures derived from SMA mice revealed increased expression of caspase-3 compared with control cells ($P \leq 0.0001$; Supplementary Material, Fig. S3c and d), indicating that apoptotic pathways were being initiated when the cells were directed towards a myelinating phenotype. In addition, we examined whether defects in cell proliferation in SMA Schwann cells could be contributing to the phenotype. We measured BrdU incorporation rates in SMA and control Schwann cells, but there was no difference between the genotypes (Supplementary Material, Fig. S3e).

To determine whether levels of key Schwann cell transcription factors were altered in SMA Schwann cells undergoing differentiation we examined levels of SOX10, SOX2 and POU3F1 at 72 h after induction of differentiation. SOX10 and SOX2 levels were significantly lower in SMA Schwann cells (Supplementary Material, Fig. S4a–d), whereas levels of POU3F1 remained unchanged (Supplementary Material, Fig. S4e and f). Although we previously demonstrated that reduced levels of MPZ observed in SMA Schwann cells were unlikely to be occurring due to perturbations in transcription, these results indicate widespread defects in core responses to differentiation cues in SMA Schwann cells.

We also examined levels of Notch1 in SMA Schwann cells post-differentiation, given the known role of this signalling molecule in EGR2-dependent regulation of post-natal myelination (38). Notch1 levels were significantly increased in SMA Schwann cells (Supplementary Material, Fig. S5), suggesting that disruption of Notch1 signalling may contribute, at least in part, to the myelination defects in SMA.

Figure 5. Immature Schwann cells isolated from peripheral nerve of SMA mice develop normally pre-differentiation. (A, C and E) Representative high-magnification confocal micrographs of isolated Schwann cells showing no significant change in cell lineage markers, SOX10, SOX2 and POU3F1 in SMA-derived Schwann cells compared with cells derived from control littersates. (B, D and F) Bar charts showing no significant difference in SOX10, SOX2 or POU3F1 levels in SMA- and control-derived Schwann cells (unpaired two-tailed t-tests; $N = 4$ mice/cultures per genotype, two fields of view imaged per culture >150 cells quantified per field of view).

Deficient myelination of wild-type neurons by Schwann cells derived from SMA mice in vitro

Next, we wanted to establish whether intrinsic deficiencies present in Schwann cells from SMA mice were sufficient to cause defective myelination of otherwise healthy neurons. We therefore established co-cultures of Schwann cells from SMA or control mice with neurons generated from wild-type embryonic mouse dorsal root ganglia (DRG). DRGs were used for these experiments as cultures of motor neurons were not sufficiently robust to perform co-culture experiments. Moreover, we had already demonstrated myelination defects in a mixed (motor and sensory) nerve in SMA mice, and previous reports have demonstrated alterations to both motor and sensory axons in SMA mice (16).

Preliminary qualitative analysis of co-cultures with phase-contrast microscopy at 40 days suggested that preparations with SMA-derived Schwann cells did not show the same level of myelin protein expression as those containing littermate control-derived Schwann cells. To quantify this, we fixed cover slips at 42 days and immunocytochemically labelled neurites (with antibodies against neurofilament medium protein) and myelin protein (with antibodies against MBP). There was a significant reduction in the percentage of neurites with a corresponding myelin protein label in co-cultures with SMA-derived Schwann cells compared with those with littermate control-derived Schwann cells (Fig. 7). Thus, Schwann cells with low levels of SMN protein had lower levels of MBP expression indicating a reduced ability to myelinate otherwise healthy axons, suggesting that at least some of the myelination abnormalities...
we observed in SMA mice in vivo were due to intrinsic defects in Schwann cells.

Influence of SMA Schwann cells on neuronal stability and the extracellular matrix

Alongside defects in myelination and maturation of axo–glial interactions, we wanted to establish whether SMA Schwann cells exerted any influence on the stability of neighbouring neurons. We therefore quantified neurite densities in DRG/Schwann cell co-cultures established with either SMA-derived or control Schwann cells. Whereas neurite density remained stable in co-cultures of healthy neurons and Schwann cells from control mice, there was a significant reduction in neurite density in co-cultures with SMA-derived Schwann cells (Fig. 8a and b). We found no evidence for axonal fragmentation in our co-cultures, suggesting that there was a gradual loss of neurites over time (possibly through the ‘dying-back’ pathway known to be activated in SMA) (39), rather than a rapid wave of Wallerian degeneration.

To investigate the mechanism through which SMA Schwann cells had a detrimental effect on otherwise healthy neighbouring neurons, we first evaluated whether SMA-derived Schwann cells modulated neurite stability by secreting a neurotoxic factor, as

Figure 6. Defective, SMN-dependent, responses to myelination cues in Schwann cells isolated from SMA mice. (A) Representative high-magnification confocal micrographs of differentiated Schwann cells immunolabelled with antibodies against myelin protein MPZ (red) with cell nuclei labeled with TOPRO3 (blue). MPZ levels were noticeably lower in SMA-derived Schwann cells. (B) Bar graph showing a significant reduction of MPZ level expression in SMA-derived Schwann cells 120 h post-differentiation (***P < 0.001; two-tailed, unpaired t-test; N = 4 cultures per genotype, 2 fields of view imaged per culture, >25 cells quantified per field of view). (C) Representative high-magnification confocal micrographs of primary Schwann cell cultures transfected with pEHHG (non-SMN transgene) alone (Control and SMA) or pEHHG/pMPZ-SMN (SMA). Cells were immunolabelled with antibodies against MPZ (red) and cell nuclei were labeled with TOPRO3 (blue). GFP expression was used to confirm the expression from the pEHHG plasmid (not shown). (D) Bar chart showing a significant restoration of MPZ levels in SMA-derived Schwann cells transfected with pMPZ-SMN compared with pEHHG alone (*P ≤ 0.05). (E) No significant difference in the MPZ gene expression levels in control and SMA differentiated Schwann cells (at 120 h post-differentiation; two-tailed, unpaired t-test; N = 3 mice per genotype, each sample analysed in triplicate).
has been shown to occur in diseased astrocytes in ALS (40). Healthy neurons from wild-type embryonic mouse DRGs were exposed to conditioned differentiation media originating from either SMA-derived or control-derived Schwann cell cultures (exposed to isolated Schwann cells for at least 48 h prior to use). Phase-contrast images were then taken at 6, 24 and 72 h following addition of the conditioned medium. There was no detrimental effect on neurite density when cultured with either SMA-derived or control-derived Schwann cell conditioned media (Fig. 8c and d), suggesting that SMA-derived Schwann cells did not influence neuronal viability by secreting a neurotoxic factor.

An alternative mechanism through which Schwann cells could conceivably influence neuronal viability in SMA is...
through disruption to the extracellular matrix (ECM). Previous microarray studies on SMA mouse spinal cord have identified altered expression levels of several core components of the ECM, including laminin alpha 2 (LAMA2) (12,13). Laminin proteins have previously been shown to exert a strong influence on the viability of motor neurons in SMA, likely by regulating extracellular cues controlling translation (41), and Schwann cells play an important role in establishing and maintaining the ECM in the peripheral nerve, in part through expression of laminins (42,43). To determine whether Schwann cells could be influencing neuronal viability in SMA by modulating the composition of the ECM, we examined LAMA2 expression in both intercostal (affected) and sciatic (less affected) nerve from early- and late-symptomatic ‘Taiwanese’ SMA mice. Qualitative analysis of sciatic nerve showed no change in LAMA2 levels at an early-symptomatic (P7) time point (Fig. 9a), but a notable reduction in the levels of LAMA2 in SMA mice at late-symptomatic time point (Fig. 9b). LAMA2 levels were already robustly reduced in intercostal nerves from SMA mice by P7 (Fig. 9c) and remained low at late-symptomatic time points (data not shown). Subsequent analysis of LAMA2 expression in isolated Schwann cells revealed that this deficit was due, at least in part, to a significant reduction in LAMA2 expression in Schwann cells (Fig. 9d–f). As with our experiments examining the regulation of MPZ levels in Schwann cells, there was no significant reduction in LAMA2 gene expression in SMA-derived Schwann cells, again suggesting that low levels of SMN were modulating LAMA2 levels via post-translational mechanisms rather than gene transcription (Fig. 9g). Taken together, these findings suggest that defects in Schwann cell expression of ECM factors known to be critical for the stability of neurons may in part explain the reduced neurite stability in our previous co-culture experiments. Perhaps more importantly, these experiments revealed an additional pathological consequence of low levels of SMN in SMA: defective expression of core ECM proteins in peripheral nerve, mediated at least in part by defective expression of ECM proteins in Schwann cells.

**DISCUSSION**

Increasing awareness of the consequences of low SMN levels for a wide range of cells and tissues has broadened our understanding of the contribution that non-neuronal cells can make to the pathogenesis of SMA (7). This study adds to the growing list of cells and tissues targeted by providing evidence for intrinsic defects in Schwann cells in SMA. Our experiments revealed defects in cellular and molecular aspects of myelination in affected peripheral nerves from mouse models of severe SMA. Delayed maturation of axo-glial interactions at paranodes, alongside perturbations in the expression of key myelin proteins, suggested that these defects resulted from disruption to myelination processes occurring during normal postnatal development. In **vitro** experiments demonstrated that Schwann cells isolated from SMA mice failed to respond normally to differentiation cues, leading to abnormal expression of key myelin proteins. By restoring SMN to Schwann cells we demonstrated that these defects were SMN-dependent and reversible. Neuron/Schwann cell co-culture experiments revealed that defects in the myelinating capacity of SMA cells Schwann cells were due to intrinsic deficiencies in the Schwann cells themselves. We also demonstrated that SMA-derived Schwann cells can have a detrimental influence on the stability of otherwise healthy neighbouring neurons. This influence was not mediated by secretion of a neurotoxic factor, but rather was likely due to Schwann cell-dependent defects in the ECM of peripheral nerve. Whilst it remains possible that...
some aspects of Schwann cell pathology in SMA occur as a secondary consequence of underlying pathology in neighbouring neurons, our findings suggest that low levels of SMN also induce primary, intrinsic changes in Schwann cells, with consequences for myelination, axo–glial interactions and development of the ECM in the peripheral nerve.

The pattern of cellular and molecular dysregulation of myelin pathways we report is entirely consistent with disruption to the normal developmental process of myelination that occurs in the early postnatal period. For example, the myelination defects we observed at the ultrastructural level in intercostal nerves were found to correlate with altered levels of key myelin proteins, including MPZ, the most abundantly expressed myelin gene (44).

Although it is known that transcription factors such as SOX10 and EGR2 are responsible for committing Schwann cells to a myelinating phenotype (45), and the levels of these factors...
were altered in SMA Schwann cells, our in vitro data suggested that reduced MPZ protein levels in SMA Schwann cells were not occurring as a result of transcriptional defects, as MPZ mRNA levels remained unchanged. This suggests that SMN protein is required in Schwann cells either to regulate translational control of key myelin proteins and/or to modulate protein turnover. Isolated Schwann cell preparations may therefore provide an ideal homogenous cell source in which to further investigate key biological properties of the SMN protein.

During normal development of peripheral nerve, myelination pathways depend on strict dosage of MPZ (46). Importantly, coordination of myelin gene expression can be affected by either the over-expression of, or lack of, MPZ (46,47). For example, PMP22 was shown to be expressed at lower levels in MPZ<sup>−/−</sup> mice, whereas in MPZ<sup>+/−</sup> mice both PMP22 and MBP were expressed at a higher level (47,48). Such responses indicate an attempt by the Schwann cell to maintain precise stoichiometric requirements for myelination (46) and confirm that cellular levels of MPZ are particularly vital to the myelination process. Thus, the reduced levels of MPZ that we observed in SMA Schwann cells likely represent a major disruption to the molecular regulation of myelination, and also provide an explanation for the increased levels of MBP observed in SMA peripheral nerve in vitro.

The discovery of intrinsic deficits in Schwann cells in SMA, with resulting consequences for myelination, ECM composition and neuronal stability, adds to a growing body of work demonstrating that glial cells can have an important role to play across a range of neurodegenerative disorders, including through non-cell autonomous pathways (e.g. ALS) (40). Indeed, one such recent study has demonstrated that there are morphological and cellular changes in astrocytes in an SMA mouse prior to overt motor neuron loss (49). The results we describe here are entirely consistent with such studies and confirm that glial cells have a clear non-cell autonomous role in diverse neurodegenerative diseases (8). Both Schwann cells and neurons depend upon the ECM for their development and stability and so the observation that LAMA2 was reduced in the peripheral nerve due to intrinsic defects in Schwann cells suggests a possible ECM-dependent mechanism through which Schwann cells influence neuronal development and stability in SMA. Laminin isoforms are known to regulate axon elongation and pre-synaptic differentiation and have been demonstrated to affect a number of phenotypes associated with neuronal pathology in SMA, including the differential regulation of local translation of β-actin in distal axons and growth cones (41,50), and local Ca<sup>2+</sup> transients in axonal growth cones (51).

There is currently no cure for SMA, but recent breakthroughs in translational research suggest that there are several approaches that may lead to new therapeutic options in the medium- to long term (52). For example, gene therapy technologies are being used to replace SMN1 or modify the splicing of SMN2. However, if they are to be successful, most of these therapies are likely to require the ability to specifically target affected cells during critical stages of disease pathogenesis. Our findings indicate that therapies capable of targeting SMN-dependent pathology in glial cells (alongside well-documented pathological changes in motor neurons and muscle) are likely to be required in order to ameliorate the full range of neuromuscular pathology observed in SMA.

**MATERIALS AND METHODS**

**Animal models**

Two SMA mouse models were used in this study: both were on a congenic FVB background and were established from breeding pairs originally purchased from Jackson Labs. The ‘severe’ SMA mouse model (Smn<sup>−/−</sup>;SMN2<sup>0/0</sup>) had a mean survival of 5 days (53). ‘Taiwanese’ SMA mice (Smn<sup>−/−</sup>;SMN2<sup>0/0</sup> mice carrying two SMN2 copies on one allele on a null murine Smn background) (54) were maintained following the breeding strategy devised by Riessland et al. (55) and had a mean survival of 11 days. Litters were retrospectively genotyped using standard PCR protocols [JAX<sup>®</sup> mouse resources (54)]. Wild-type CD1 mice were obtained from in-house breeding stocks at the University of Edinburgh. All mice were housed within the animal care facilities in Edinburgh under standard SPF conditions. All animal procedures and breeding were performed in accordance with Home Office and institutional guidelines. ‘Taiwanese’ SMA mice were used for all in vitro experiments.

**Electron microscopy**

Intercostal nerves, sciatic nerves and spinal cords were dissected then incubated for 48 h in 4% PFA: 2.5% glutaraldehyde at 4°C before post-fixation in 1% osmium tetroxide in 0.1 M phosphate buffer for 45 min. Following dehydration through an ascending series of ethanol solutions and propylene oxide, sections were embedded on glass slides in Durcupan resin. Regions to be used for assessment of myelination were then cut out using a scalpel and glued onto a resin block for sectioning. Ultrathin sections (60 nm) were cut and collected on formvar-coated grids (Agar Scientific, UK), stained with uranyl acetate and lead citrate in an LKB Ultrostainer and then quantitatively assessed in a Philips CM12 transmission electron microscope equipped with a Gatan digital camera. Individual axon profiles were measured using ImageJ. For each individual fibre, axon diameters and G-ratios were calculated as previously described (56). Unmyelinated axons were not included in calculations for G-ratios.

**Quantitative fluorescent western blotting**

Fresh tissue was dissected and frozen on dry ice. Protein was extracted in RIPA buffer (ThermoScientific) with protease inhibitor cocktail (Sigma) and quantitative western blots were performed using primary antibodies against MPZ (mouse, 1:10 000; gift), PMP22 (rabbit, 1:1000; Abcam) and MBP (mouse, 1:500; Abcam). Odyssey secondary antibodies were added according to the manufacturers’ instructions (goat anti-rabbit IRDye 680 and 800 and goat anti-mouse IRDye 680 and 800 depending on required combinations). Blots were imaged using an Odyssey Infrared Imaging System (Li-COR; Biosciences) at a resolution of 169 µM. Each blot was scanned and measured in triplicate to minimize user variability. MPZ and PMP22 antibodies recognize one band, 30 and 19 kDa, respectively. MBP antibody recognizes multiple isoforms resulting from alternative splice variants; a single isoform of ~21.5 kDa was quantified. Total protein levels were determined by incubation of each blot in a solution of Ponceau S.
Microscopy
Fluorescent images were captured using a Zeiss 710 laser-scanning confocal microscope (40 × objective; 1.4NA) or a standard epi-fluorescence microscope equipped with a chilled CCD camera (20 × or 40 × objective; 0.8NA; Nikon IX71 microscope; Hamamatsu C4742-95), as previously described (12). Images were prepared in Adobe Photoshop and all comparable images were subjected to identical editorial processes to ensure accuracy.

Immunohistochemical analyses of axo–glial interaction sites
Nerves were fixed for 30 min in 4% PFA before teasing single fibres in 0.1 M phosphate buffered saline (PBS) on 3-amino-propyltriethoxysilane-coated slides (Sigma) and incubated in a solution of 0.2% Triton X and 5% bovine serum albumin (BSA) for 1 h before overnight incubation in primary antibodies against pan-neurofascin (rabbit, 1:1000; Abcam), Caspr (rabbit, 1:100; gift) and ankyrin-G (goat, 1:500; Santa Cruz Biotechnology). After 3 × 20 min washes in 0.1 M PBS, teased fibres were incubated in a solution of swine anti-rabbit secondary antibody conjugated to FITC (1:40; Dako) and donkey anti-goat secondary antibody conjugated to Cy3 (1:500; Jackson ImmunoResearch) for 2 h. Following several washes, slides were coverslipped using Mowiol (Calbiochem). Parasnodes and nodes were analysed and measured using ImageJ.

Schwann cell culture
Schwann cells were isolated at post-natal Day 8 using a protocol modified from (57). Briefly, sciatic nerves and the brachial plexus were dissected, treated with 0.125% trypsin and 0.05% collagenase A, before re-suspension in basic growth medium containing 10−6 M insulin and plating out on 60 mm dishes coated with neat poly-l-lysine (PLL) and laminin. Schwann cells were purified using complement-mediated cytolysis to remove fibroblasts, then expanded in Schwann cell growth medium containing 10−7 M insulin to reach 80% confluency. Schwann cells were differentiated to a myelination-competent phenotype using a previously described protocol (32). Briefly, 5000 Schwann cells were grown in supplemented defined medium on coverslips coated with PLL and laminin. Differentiation was induced by the addition of NRG-1 and dbcAMP, prior to Schwann cell analysis at 72 or 120 h post-differentiation.

pMPZ-SMN construct generation
pMPZ-SMN, SMN under the control of the MPZ promoter, was created using a two-step cloning procedure from two starting plasmids (plasmid containing the full-length human SMN1 construct; gift; MPZ promoter plasmid, gift). Briefly, SMN was amplified using two overlapping forward primers that introduced Nhel and AscI sites, an ATG start site and a 5′FLAG tag (F1, 5′GCTAGCTAGCAGGGCGCAGCTAGGATTCAAGG ATGACGACGATAAAG 3′; F2, 5′AAGGATGACGACGATA AGGGAGGTGCGATGAGCAGCGGCGGC 3′) and a reverse primer that introduced BglII and AatII sites and a TGA stop site (R, 5′GGCTAGATCCCTGACTAATCTTGAAATG TGAACCCCTTCC 3′). Amplified products were digested using Nhel and BglII (New England Biolabs), ligated to an intermediate plasmid (pGL4.13, Promega) using T4 DNA ligase (Promega), then confirmed by sequencing. The intermediate SMN-containing plasmid and the MPZ-promoter plasmid were digested using AscI and AatII (New England Biolabs) and ligated using T4 DNA ligase. pMPZ-SMN constructs were confirmed by restriction digestion and sequencing.

Transfection of primary Schwann cells with pMPZ-SMN
The effect of restoring intrinsic SMN levels to Schwann cells was determined using the pMPZ-SMN plasmid. Ninety hours after-transfection, Schwann cells at 70–80% confluency were transfected with either a GFP-expressing, non-SMN transgene carrying, plasmid, pEHGG (58) or both pEHGG and pMPZ-SMN using Lipofectamine 2000 (Invitrogen). A total amount of 1 μg DNA was used per reaction, with 2.4 μl L2000. Thirty hours later cells were stained with Topro and MPZ and expression levels were quantified as described below.

Neuron-Schwann cell co-culture
Purified Schwann cells were grown in co-culture with purified dorsal root ganglion (DRG) neurons using an established protocol (59). Briefly, DRG neurons were dissected from the spinal cord of E14 CD1 mouse embryos, grown on matrigel-coated coverslips and purified using DRG purification medium for 3 days, before transfer to DRG growth medium for 2 days. This process was repeated two times prior to the addition of purified Schwann cells. DRG-Schwann cell co-cultures were maintained for 10 days in differentiation medium followed by a period of 30–42 days in myelination medium.

Immunocytochemical analysis of primary schwann cell cultures and DRG-schwann cell co-cultures
Analysis was carried out using a modified protocol from previously described work (57,59). Five thousand cells were plated on PLL/laminin-coated coverslips and cultured for either 48 h (pre-differentiation) or a further 72 or 120 h (post-differentiation). Cells to be treated with BrdU were differentiated for 96 h, then 10 μM BrdU (Sigma) added for 24 h. Cells were fixed with 4% PFA for 10 min, permeabilized using ice-cold methanol for 15 min (Schwann cells) or 0.25% Triton X in PBS for 30 min (DRG-Schwann cells), blocked in a solution of 1% BSA in PBS for 1 h before a 1 h incubation in primary antibodies against S100B (mouse, 1:100; Abcam), GFAP (rabbit, 1:1000; Abcam), SOX10 (goat, 1:50; Santa Cruz), SOX2 (Rabbit, 1:500; Chemicon), POUF31 (Goat, 1:50; Santa Cruz), EGR2 (rabbit, 1:100; Covance), caspase-3 (rabbit, 1:50; Cell Signalling), MBP (rabbit, 1:1000; gift), PMP22 (rabbit, 1:50; Abcam), neurofilament (2H3, mouse, 1:200; Developmental studies hybridoma bank), MPZ (mouse, 1:1000; gift); NOTCH1 (goat, 1:200; Santa Cruz), BrdU (mouse, 1:100; BD Biosciences) and LAMA2 (rat, 1:100; Abcam). After 6 × washes with 1% BSA in PBS, cells were incubated for 1 h in a solution of donkey anti-goat secondary antibody conjugated to Cy3, donkey anti-mouse secondary antibody conjugated to Cy3 (1:250; Jackson ImmunoResearch) and swine anti-rabbit antibody conjugated to FITC. Following several washes in PBS cells were incubated for 10 min with...
TOPRO-3 (1:500; Life Technology), washed with 1% BSA in PBS then cover slipped using Mowiol (Calbiochem) before imaging.

Protein labelling intensities were quantified using an established protocol (60). Briefly, levels were quantified from cells by acquiring micrographs with identical confocal settings, ensuring that signal from the brightest cells was not saturated. Quantitative analysis of signal intensity was carried out using the point analysis tool in ImageJ software. To measure neurite density, purified DRG neuron cultures, cultured either with or without control Schwann cells or SMA Schwann cells, were immunocytochemically labeled with antibody to NF medium. Three images were captured 250 μm from the DRG core in each culture. Each image was converted to binary and the total area covered by neurites quantified using ImageJ.

Quantitative analysis of gene expression
mRNA was extracted from Schwann cells 120 h post-differentiation using an RNeasy Microkit (Qiagen). Samples were checked for DNA contamination and concentration was determined using a nanodrop spectrophotometer (Thermo Scientific). cDNA was made from 100 ng RNA using the High Capacity cDNA Reverse Transcription kit (Invitrogen). Primers were designed that amplified MPZ, LAMA2, SOX2 and SOX10 (MPZ-F, 5′ CTGGTCCAGTGAATGGGTCT 3′; MPZ-R, 5′ ATGACAATGGAGCCATCCTT 3′; LAMA2-F, 5′ CTCGAAGGCTCCAGACTC 3′; LAMA2-R, 5′ GCATTGGTTGAGTA TGATGTC 3′; SOX2-F, 5′ GAACGCCTTCACTATGGTATGGT 3′; SOX2-R, 5′ TCTCGGTTCAGGACAAAGT 3′; SOX10-F, 5′ GACCAGTACCTCACCTCA 3′; SOX10-R, 5′ GGATGTGCTCTTTTGTGGTC 3′). Two mouse housekeeping genes (GAPDH and OAZ1) were used (GAPDH-F, 5′ CTGGTCCAGTGAATGGGTCT 3′; GAPDH-R, 5′ CTGGTCCAGTGAATGGGTCT 3′; SOX10-F, 5′ ATCCTCAACAGCCTACTGGT 3′; SOX10-R, 5′ CGGACGCCGTTACTACGC 3′). For realtime detection an ABI7000 machine (Applied Biosystems) was used. cDNA was amplified using 0.5–1 μM primer with the DyNAmo Flash SYBR Green qPCR kit (Thermo Scientific) and using a standard PCR programme with amplification at 95°C for 15 s, annealing at 60°C for 1 min and a 25 s extension. The equation where raw data was 1 + EChmin−Ct was used to determine relative expression levels for each sample. Raw values obtained for all genes of interest were normalized using the geomean of the two housekeeping genes.

Immunohistochemistry on intercostal and sciatic nerve
Whole rib cages were fixed for 30 min in 4% PFA, then halved at the sternum. Each half was incubated overnight in 30% sucrose. Rib cages were embedded in a 1:1 solution of 30% sucrose and OCT embedding matrix (CellPath). Whole sciatic nerves were prepared similarly. Ten micrometer sections were prepared on slides before incubation in primary antibodies against LAMA2 (rat, 1:100, Abcam). After 6 × 5 min washes in 0.1 ml PBS, sections were incubated in a solution of goat anti-rat secondary antibody conjugated to (1:200; Jackson ImmunoResearch). Slides were coverslipped using Mowiol (Calbiochem).

Statistical analysis
All data were collected into Microsoft Excel and analysed using GraphPad Prism software (specific statistical tests used for each comparison are detailed in the text). For all statistical analyses, P < 0.05 was considered to be significant. All data are expressed as mean ± SEM.

AUTHORS’ CONTRIBUTIONS
T.H.G. conceived the study; G.H., A.A., S.L.R., R.C.S. and T.H.G. designed and carried out the experimental work; G.H., A.A., S.L.R., R.C.S. and T.H.G. analysed data; all authors contributed to the writing of the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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