Motor and sensory neuropathy due to myelin infolding and paranodal damage in a transgenic mouse model of Charcot–Marie–Tooth disease type 1C

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Charcot–Marie–Tooth disease type 1C (CMT1C) is a dominantly inherited motor and sensory neuropathy. Despite human genetic evidence linking missense mutations in SIMPLE to CMT1C, the in vivo role of CMT1C-linked SIMPLE mutations remains undetermined. To investigate the molecular mechanism underlying CMT1C pathogenesis, we generated transgenic mice expressing either wild-type or CMT1C-linked W116G human SIMPLE. Mice expressing mutant, but not wild type, SIMPLE develop a late-onset motor and sensory neuropathy that recapitulates key clinical features of CMT1C disease. SIMPLE mutant mice exhibit motor and sensory behavioral impairments accompanied by decreased motor and sensory nerve conduction velocity and reduced compound muscle action potential amplitude. This neuropathy phenotype is associated with focally infolded myelin loops that protrude into the axons at paranodal regions and near Schmidt–Lanterman incisures of peripheral nerves. We find that myelin infolding is often linked to constricted axons with signs of impaired axonal transport and to paranodal defects and abnormal organization of the node of Ranvier. Our findings support that SIMPLE mutation disrupts myelin homeostasis and causes peripheral neuropathy via a combination of toxic gain-of-function and dominant-negative mechanisms. The results from this study suggest that myelin infolding and paranodal damage may represent pathogenic precursors preceding demyelination and axonal degeneration in CMT1C patients.

INTRODUCTION

Charcot–Marie–Tooth disease (CMT), also known as hereditary motor and sensory neuropathy, encompasses a genetically heterogeneous group of inherited disorders of the peripheral nervous system (PNS) (1,2). CMT is categorized into the demyelinating type, which accounts for 80% of CMT cases and the axonal degeneration type, which accounts for 20% of CMT cases (3). CMT type 1C (CMT1C) is a dominantly inherited, demyelinating type of peripheral neuropathy characterized by slowed motor and sensory nerve conduction velocity with typical CMT clinical symptoms, including progressive motor weakness and sensory loss (4–8). Human genetic studies have revealed that CMT1C is linked to missense mutations in small integral membrane protein of lysosome/late endosome [SIMPLE; also known as lipopolysaccharide-induced TNF-α factor (LITAF)], a ubiquitously expressed protein of unknown function (4–8). Our recent study indicates that endogenous SIMPLE is an early endosomal membrane protein (9) rather than a lysosomal/late endosomal protein as previously suggested (10). We found that CMT1C-linked mutations map in and around the transmembrane domain of SIMPLE and that these mutations cause mislocalization of SIMPLE protein from the early endosome to the cytosol in cultured cells (9). The in vivo role of CMT1C-linked SIMPLE mutations remains undetermined.

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Molecular analysis of the genotype–phenotype relationship in hereditary peripheral neuropathy is essential for a mechanistic understanding of CMT pathogenesis. The identified linkage of heterozygous SIMPLE missense mutations to autosomal dominant CMT1C (4–8) raises two possibilities: CMT1C may be due to haplo-insufficiency of SIMPLE or dominant effects of the SIMPLE mutants. To distinguish these possibilities and determine the in vivo role of the identified human SIMPLE mutations, we generated transgenic mice expressing CMT1C-linked human SIMPLE W116G mutant or human SIMPLE wild-type (WT) protein. Characterization of these transgenic mice reveals that expression of human SIMPLE W116G mutant, but not human SIMPLE WT, causes a late-onset motor and sensory neuropathy in mice that recapitulates key clinical features of CMT1C disease. SIMPLE W116G mutant mice exhibit motor and sensory nerve conduction defects and impaired motor and sensory performance. The observed motor and sensory impairments are associated with focally infolded myelin loops that protrude into the axons at paranodal regions and near Schmidt–Lanterman incisures of motor and sensory nerves. By immunohistochemical analysis, we show that the SIMPLE W116G mutant protein, like endogenous SIMPLE protein, is localized in non-compact myelin cytoplasmic regions of myelinating Schwann cells but is absent in axons. Our findings support that SIMPLE mutation causes CMT1C pathogenesis via a combination of toxic gain-of-function and dominant-negative mechanisms.

**RESULTS**

**Generation of transgenic mice expressing human SIMPLE WT or SIMPLE W116G mutant**

To investigate the pathogenic role of CMT1C-linked SIMPLE mutation in vivo, we generated transgenic mice expressing either N-terminal hemagglutinin (HA)-tagged human SIMPLE W116G mutant or SIMPLE WT protein under the control of the human cytomegalovirus (CMV) promoter (Fig. 1A). The CMV promoter was used to drive ubiquitous expression of SIMPLE transgenes because endogenous SIMPLE is a ubiquitously expressed protein (4,9). Transgenic founder mice were identified by PCR analysis of tail genomic DNAs (Fig. 1B) and bred with FVB/N mice to establish transgenic lines on a pure FVB/N background. The expression of HA-tagged human SIMPLE W116G mutant or SIMPLE WT protein was confirmed by immunoblot analysis of sciatic nerves (Fig. 1E) and Schwann cell lysates (Supplementary Material, Fig. S1) from transgenic mice using our anti-SIMPLE antibody that recognizes both mouse and human SIMPLE proteins (9).

We established three transgenic lines expressing human SIMPLE W116G mutant and selected the line with the highest expression of SIMPLE mutant protein (Fig. 1E) for the experiments described below. To exclude the possibility of integration site effects, we performed genome walking PCR and sequencing analyses to identify the transgene integration site in this SIMPLE W116G transgenic line. We found that the SIMPLE W116G transgene inserted into a non-coding region on mouse chromosome 14 between genes *Thrb* (thyroid hormone receptor beta) and *Nr1d2* (nuclear receptor subfamily 1, group D, member 2) (Fig. 1C). This integration site was confirmed by PCR reactions using primers located in the flanking sequences of the integration site at both 5′-end and 3′-end of the SIMPLE W116G transgene (Fig. 1C and D). Quantitative immunoblot analysis indicated that the expression level of human SIMPLE W116G mutant protein relative to the level of endogenous mouse SIMPLE protein in sciatic nerves is 22% for homozygous (SIMPLE<sup>W116G/W116G</sup>) mice and 10% for heterozygous (SIMPLE<sup>W116G/+</sup>) mice at 3 months of age (Fig. 1E and F).

We also established four transgenic lines expressing human SIMPLE WT protein and selected the line with an expression level similar to that in the SIMPLE W116G mutant mice (Fig. 1E; Supplementary Material, Fig. S1) for the analyses described below. The expression level of human SIMPLE WT protein relative to the level of endogenous mouse SIMPLE protein in sciatic nerves is 22% for homozygous (SIMPLE<sup>WT/WT</sup>) mice and 11% for heterozygous (SIMPLE<sup>WT/+</sup>) mice at 3 months of age (Fig. 1E and F). We found that the expression levels of human SIMPLE WT and SIMPLE W116G mutant proteins in sciatic nerves of the transgenic mice did not change significantly between 3 months and 1 year of age (Fig. 1E–G).

**Endogenous SIMPLE is localized to early endosomes in myelinating Schwann cells but is absent in myelin sheath or axons**

The cellular and subcellular localization of endogenous SIMPLE protein in myelinated peripheral nerves remains poorly characterized. Therefore, we performed immunofluorescence confocal microscopic analyses to determine endogenous SIMPLE protein distribution in sciatic nerves from adult non-transgenic mice and rat. We found that endogenous SIMPLE exhibits a punctate staining pattern, and double immunostaining analyses revealed that SIMPLE is localized in myelinating Schwann cells labeled by various Schwann cell markers (Fig. 2A and C–E) but not in axons labeled by neurofilament H (Fig. 2B). We observed no colocalization of SIMPLE with the compact myelin marker myelin basic protein (MBP) (Fig. 2A), indicating that SIMPLE is not a structural component of myelin sheath. We found that SIMPLE is localized in the Dlg1-positive abaxonal cytoplasmic region (Fig. 2C) at the outer side of the compact myelin (Fig. 2A) and in the adaxonal cytoplasmic region next to myelin-associated glycoprotein (MAG)-positive membrane (Fig. 2D) at the inner side of the compact myelin facing the axon (Fig. 2A and B). Our results also showed that SIMPLE is enriched in the cytoplasmic regions at Schmidt-Lanterman incisures (Fig. 2C and D) and paranodal domains (Fig. 2E).

Consistent with our previous report that SIMPLE is an early endosomal membrane protein (9), we observed a substantial colocalization of SIMPLE with the early endosome marker Rab5 (Fig. 2F). Triple immunostaining analyses confirmed the colocalization of SIMPLE with Rab5 in S100-positive myelinating Schwann cells (Fig. 2G). To further characterize the membrane localization of SIMPLE in sciatic nerves, we performed density gradient fractionation analysis and found that SIMPLE co-fractionated with Rab5-positive early endosomal membranes, but not with MAG-positive plasma membranes.
membranes derived from the inner surface of myelin sheath, Schmidt-Lanterman incisures and paranodal loops (Fig. 2H). Together, these results indicate that SIMPLE is localized to the early endosomes in non-compact myelin cytoplasmic regions of myelinating Schwann cells but is absent in myelin membranes or axons.

SIMPLE W116G mutant, but not SIMPLE WT, is partially mislocalized from the membrane to the cytosol in myelinated peripheral nerves of transgenic mice

Next, we performed double labeling immunofluorescence confocal microscopic analyses to assess the localization of human SIMPLE WT and SIMPLE W116G proteins in sciatic nerves from SIMPLEWT/WT and SIMPLEW116GW116G transgenic mice. We found that HA-tagged SIMPLE WT and SIMPLE W116G mutant proteins, like the endogenous mouse SIMPLE protein, are localized in non-compact myelin cytoplasmic regions of myelinating Schwann cells such as Schmidt-Lanterman incisures (Fig. 3A) but not in axons (Fig. 3B). These results confirm that the human SIMPLE WT and SIMPLE W116G proteins are both targeted in a similar manner as the endogenous SIMPLE protein to the cytoplasm of myelinating Schwann cells in our transgenic mice.

Despite similar staining patterns, the punctate staining of HA-SIMPLE W116G mutant in SIMPLEW116GW116G mice
was weaker than that of HA-SIMPLE WT in SIMPLEWT/WT mice (Fig. 3A). Because there was no significant difference between HA-SIMPLE WT and HA-SIMPLE W116G protein levels in these mice (Fig. 1C–E), the weaker staining raised the possibility that SIMPLE W116G mutation may cause SIMPLE mislocalization to the cytosol in transgenic mice as we have previously observed in transfected cells (9). To test this possibility, we performed subcellular fractionation analyses to compare the localization of SIMPLE WT and mutant proteins in sciatic nerves from SIMPLEWT/WT and SIMPLEW116G/W116G mice. We found that human SIMPLE WT, like endogenous mouse SIMPLE, was exclusively localized to the membrane fraction in SIMPLEWT/WT mice. In contrast, human SIMPLE W116G protein was partially mislocalized to the cytosol in the SIMPLEW116G/W116G mice (Fig. 3C). These results, together with the immunostaining data (Figs 2 and 3A and B), support that CMT1C-linked SIMPLE W116G mutation causes partial mislocalization of human SIMPLE protein from the early endosomal membrane to the cytosol in myelinating Schwann cells. Our subcellular fractionation analyses showed that endogenous mouse SIMPLE protein remained fully associated with the membranes in SIMPLEW116G/W116G mice (Fig. 3C), indicating that the membrane localization of endogenous mouse SIMPLE protein in myelinated peripheral nerves was not affected by the expression of human SIMPLE W116G mutant protein.

**SIMPLE W116G mutant mice, but not SIMPLE WT transgenic mice, exhibit motor and sensory impairments**

SIMPLE W116G mutant and SIMPLE WT transgenic mice were viable, fertile and born according to expected Mendelian ratios. They developed normally, and necropsy of these mice showed normally formed organs without any abnormality at 3, 12 and 15 months of age. Histological analysis revealed abnormalities in peripheral nerves (described later) but not in brain or spinal cord of SIMPLE W116G mutant mice (Supplementary Material, Fig. S2 and data not shown). Although no observable behavioral abnormality was observed at the age of 3 and 6 months, SIMPLEW116G/W116G mice showed...
In addition to motor abnormalities, we observed that SIMPLE WT/W116G mice occasionally self-mutilated their tails (Fig. 4D), which could be a consequence of paresthesia reported in patients with CMT1C (5,8). Analysis of nociceptive sensory function by the tail-flick test revealed that SIMPLE WT/W116G mice (Fig. 4E), but not SIMPLE WT/WT mice (Supplementary Material, Fig. S3D), performed significantly worse than their non-transgenic littermates, suggesting impaired sensory function. Together, these results indicate that SIMPLE WT/W116G mice have motor and sensory impairments that are consistent with the clinical features of human patients with CMT1C motor and sensory neuropathy (4–8).

SIMPLE W116G mutant mice, but not SIMPLE WT transgenic mice, have motor and sensory nerve conduction defects

The pathophysiological hallmark of demyelinating CMT is slowed motor nerve conduction velocity (MNCV <38 m/s) (2,19–21). Human CMT1C patients carrying SIMPLE W116G or other mutations show reduced MNCVs with 100% penetrance (4–8). To determine whether SIMPLE W116G mutant mice recapitulate this CMT1C phenotype, we performed electrophysiological analyses of motor nerve conduction in mutant mice and controls at 3 months and 1 year of age. In 3-month-old animals, the MNCVs and compound muscle action potential (CMAP) amplitudes were not significantly different in SIMPLE WT/W116G mice compared with their non-transgenic littermates (Fig. 5B and C). At 1 year of age, however, SIMPLE WT/W116G mice showed significantly reduced MNCVs (Fig. 5A and B) and CMAP amplitudes (Fig. 5A and C) compared with their non-transgenic littermates. The 1-year-old SIMPLE WT/W116G mice also had significantly lower MNCVs than those of the non-transgenic controls (Fig. 5B), although their CMAP amplitudes were not significantly altered (Fig. 5C). In contrast, SIMPLE WT/WT and SIMPLE WT/+ mice showed no significant difference in the MNCV or the CMAP amplitude compared with the non-transgenic controls at 1 year of age (Supplementary Material, Fig. S4A and B).

In addition to motor nerve conduction defects, electrophysiological analyses of tail sensory nerve conduction also revealed a significant decrease in the sensory nerve conduction velocity (SNCV) in the SIMPLE WT/W116G/W116G and SIMPLE WT/W116G/+ mice compared with their non-transgenic littermates at the age of 1 year but not 3 months (Fig. 6A and B). Although the sensory nerve action potential (SNAP) amplitudes of the SIMPLE WT/W116G/W116G and SIMPLE WT/W116G/+ mice did not significantly differ from those of the non-transgenic controls (Fig. 6C), we found that sensory nerve action potentials could not be evoked in 2 of 11 SIMPLE WT/W116G/W116G mice tested (Fig. 6A and D). The inability to evoke sensory nerve action potentials in a small percentage of CMT1C patients has been previously reported (4) and is correlated with a loss of sensory function. Interestingly, one of the mice with no evoked sensory nerve action potentials exhibited self-injurious behavior shown in Figure 4D, suggesting that the self-mutilation behavior may be resulted from a loss of sensory nerve conduction. In contrast to the SIMPLE WT/W116G mice, SIMPLE WT/WT and SIMPLE WT/+ mice
did not show any difference in the SNCV or the SNAP amplitude compared with the non-transgenic controls at 1 year of age (Supplementary Material, Fig. S4C and D). Together, these results indicate that SIMPLE W116G mutant mice, but not SIMPLE WT transgenic mice, display motor and sensory nerve conduction defects that are consistent with the electrophysiological findings from human CMT1C patients (4–8).

**Figure 4.** Impaired motor and sensory performance in SIMPLE W116G mutant mice. (A) When suspended by the tail, an abnormal phenotype of hind-limb clamping and occasional limb/body flexing was seen in SIMPLE W116G mice but not in non-transgenic control (CTRL) or SIMPLE WT mice at 1 year of age. (B) Quantitative analysis of the percentage of mice with the hind-limb clamping phenotype in 1-year-old SIMPLE W116G, SIMPLE WT and control (CTRL) mice (n = 7–13 mice per genotype). (C) Impaired rotarod performance in SIMPLE W116G and SIMPLE WT mice compared with the non-transgenic control (CTRL) mice. The latency was plotted versus the trial number. Data represent mean ± SEM. (n = 6–10 mice per genotype). *P < 0.05 versus the control. (D) Self-mutilation of the tail in a SIMPLE W116G mouse was shown, which was suggestive of paresthesia. (E) Impaired tail-flick response in SIMPLE W116G mice compared with the non-transgenic control (CTRL) mice at 1 year of age. Data represent mean ± SEM. (n = 6–10 mice per genotype). *P < 0.05 versus the control.

**SIMPLE W116G mutation causes peripheral nerve dysmyelination with myelin infolding and reduced axon caliber**

To investigate the pathological changes underlying the observed motor and sensory neuropathy phenotype in SIMPLE W116G mice, we performed histological analyses of sciatic nerves, ventral roots and dorsal roots from the mutant mice and the control mice. At 3 months of age, SIMPLE W116G mice showed no obvious abnormality in the myelin or axonal structure of peripheral nerves (Supplementary Material, Fig. S5A–C). However, analysis of semithin cross sections of peripheral nerves from 1-year-old SIMPLE W116G mice revealed abnormal Schwann cell–axon units with focally infolded myelin sheaths that appeared as single, double or triple internal myelin rings within a myelinated axon (Fig. 7A–C). Myelin infolding was observed in both motor and sensory nerves, and the infolding was more prominent in sciatic nerves which are more distally located with respect to the neuronal cell bodies (Fig. 7A) compared with ventral and dorsal roots which are more proximally located (Fig. 7B and C). The myelin infolding phenotype appeared to be specific to the peripheral nerves, as no myelin infolding was observed in myelinated nerves from the central nervous system.
Myelin infoldings originate from the paranodal regions and near Schmidt-Lanterman incisures

To further characterize the pathological changes in SIMPLE\textsuperscript{W116G/W116G} mice, we performed electron microscopic analyses of sciatic nerves from the mutant mice and control mice at 12–15 months of age. The results revealed that, unlike non-transgenic controls (Fig. 8A), SIMPLE\textsuperscript{W116G/W116G} nerves showed abundant myelin abnormalities with focally folded structures (Fig. 8B–I). Interestingly, we found only myelin infoldings but no myelin outfoldings. Some myelin infoldings were observed in the orientation perpendicular to the nerve axis with myelin sheath that protrudes into a compressed axon (Fig. 8B). More frequently, myelin infoldings were observed in the orientation parallel to the nerve axis, which appeared in cross sections as one or more internal myelin rings inside the myelinated axons of both large and small caliber nerve fibers (Fig. 8C–F). The internal myelin rings had the same number of myelin lamellae and periodicity as the myelin sheath surrounding the axon (Fig. 8F–H), suggesting that the internal myelin rings are invaginated loops of the myelin sheath.

Light microscopic and electron microscopic analyses of longitudinal sciatic nerve sections revealed that the infolded myelin loops predominantly originated from the myelin sheath at the paranodal regions (Fig. 9C–H) and the internodal regions adjacent to Schmidt-Lanterman incisures (Figs 8I and 9B) in SIMPLE\textsuperscript{W116G/W116G} mice. In contrast, myelin infolding was virtually absent in the longitudinal sciatic nerve sections from the control mice (Figs 8J and 9A).

SIMPLE W116G mutation disrupts the integrity of Schwann cell–axon units and nodes of Ranvier

Ultrastructural analysis by electron microscopy revealed that SIMPLE\textsuperscript{W116G/W116G} axons with myelin infoldings were often displaced, deformed or constricted (Fig. 8B–F), consistent with the reduced axon caliber and axon area (Fig. 7F and G) from the morphometric analysis of semithin sections. Moreover, loss of myelin compaction (Fig. 8M) and widened spacing of the Schmidt-Lanterman incisures (Fig. 8K and L) were occasionally observed. We also observed signs of axonal damage and axonal degeneration (Fig. 8M–P). Quantitative analysis revealed a small but significant increase in the percentage of sciatic nerves undergoing axonal degeneration in SIMPLE\textsuperscript{W116G/W116G} mice compared with the control mice at 12–15 months of age (Fig. 8S). In addition, a small but significant increase in the percentage of sciatic nerves with demyelinated axons was also observed in the SIMPLE\textsuperscript{W116G/W116G} mice compared with the control mice (Fig. 8Q, R and T).

Given the prominent presence of myelin infoldings at the paranodal regions (Fig. 9C and D), we analyzed SIMPLE\textsuperscript{W116G/W116G} nodes of Ranvier and paranodal regions in more detail. Analysis of longitudinal sciatic nerve sections revealed that myelin infolding did not affect the two sides of the node symmetrically in most cases (Fig. 9C, D and G), although occasionally myelin infolding can be observed in both sides of the node (Fig. 9E). Paranodal regions with myelin infolding often exhibited signs of dys/demyelination and
axonal damage (Fig. 9C and E–G). Non-compacted myelin whorls were found to extend from the compact myelin into the axon (Fig. 9C, E and F), suggesting a loss of myelin compaction at the paranodal region that may impair axonal function and integrity. We observed the accumulation of electron-dense organelles, mainly mitochondria, in the axoplasm of the paranodal region next to the infolded myelin, likely as a result of impaired axonal transport (Fig. 9E and F). Our data suggest that the focally infolded myelin loops, particularly those protruding deep into the axons perpendicularly (Fig. 9C, E and F) may physically block axonal transport, leading to axonal degeneration observed in SIMPLEW116G/W116G sciatic nerves (Fig. 8M–P and S).

Electron microscopic analysis showed that, despite myelin infolding, axoglial junction and Schwann cell microvilli appeared largely intact and the myelin sheath is properly lined by Schwann cell basal lamina (Fig. 9E–H), indicating that the overall nodal architecture is intact in the SIMPLEW116G/W116G nerves. However, we observed that the nodes of Ranvier with myelin infolding often showed paranodal retraction leading to a substantially larger nodal gap (Fig. 9G and H). The enlarged nodal gaps were also observed in the semithin longitudinal sciatic nerve sections (Fig. 9D) of 1-year-old SIMPLEW116G/W116G mice. Quantitative analysis indicated that the nodal gap length of SIMPLEW116G/W116G nerves is significantly longer than that of the control nerves (Fig. 9I).

To further characterize SIMPLE W116G mutation-induced changes at nodes of Ranvier, we performed immunofluorescence confocal microscopic analyses of sciatic nerves from 1-year-old SIMPLEW116G/W116G and control mice. As expected, the control nerves showed normal nodal organization with three clearly segregated, axonal compartments: the node, marked by the clustering of sodium (Na⁺) channels; the paranode, labeled by the staining of the adhesion molecule Caspr and the juxtaparanode, defined by the presence of potassium (Kv1.2) channels (Fig. 9J and K). In SIMPLEW116G/W116G mutant nerves, although Na⁺ channels remained tightly clustered at the nodes, the distribution of the paranodal marker Caspr was altered, with elongated Caspr-positive structures that extended into the juxtaparanodal region (Fig. 9J). The distribution of the juxtaparanodal marker Kv1.2 channels was also altered, with elongated Kv1.2-positive structures that extended into the paranodal region and the internodal region (Fig. 9K). The elongation of Caspr and Kv1.2 clusters affected the two sides of the node asymmetrically (Fig. 9J and K). Double immunostaining with anti-Caspr and anti-Kv1.2 antibodies revealed a substantial overlap of the paranodal and juxtaparanodal markers (Fig. 9K), indicating the segregation of the paranodal and juxtaparanodal compartments is disrupted in the mutant axons. Quantitative analysis indicated that 73% of nodes of Ranvier in SIMPLEW116G/W116G sciatic nerves had the paranode-juxtaparanode overlap phenotype.
Myelin infoldings precede axonal defects in the peripheral nerves of SIMPLE W116G mutant mice

To determine whether myelin infoldings or axonal defects appeared first in SIMPLE WT116G/W116G mice, we stained the sciatic nerves from 8-month-old and 1-year-old SIMPLE WT116G/W116G and control mice with Nile red, a fluorescent lipophilic dye that strongly labels lipid-rich structures such as myelin. Fluorescence confocal microscopic analysis of Nile red-labeled nerves showed myelin infoldings at paranodal regions of sciatic nerves in both ages of SIMPLE WT116G/W116G mice that are largely absent in the age-matched control mice (Fig. 10A). Quantitative analysis revealed that the percentage of paranodes with myelin infolding is significantly increased in the 1-year-old mutant mice compared with the 8-month-old mutant mice (Fig. 10B). In addition, Nile red staining analysis revealed a significant increase in the percentage of nodes with enlarged nodal gaps in SIMPLE WT116G/W116G mice compared with the control mice at 1 year but not at 8 months of age (Fig. 10A and C).

Next, we performed immunostaining analyses with anti-Caspr antibodies to assess age-dependent changes in the paranodal axonal compartments of sciatic nerves from 8-month-old and 1-year-old SIMPLE WT116G/W116G and control mice. We found that, consistent with the enlarged nodal gap (Figs 9D, G, I and 10A, C), the gap between Caspr-positive paranodes was significantly enlarged in 1-year-old, but not in 8-month-old, SIMPLE WT116G/W116G nerves compared with the age-matched control mice (Fig. 10D and E). Furthermore, the axonal Caspr-positive structures were significantly elongated in 1-year-old, but not in 8-month-old, mutant mice compared with the control mice (Fig. 10D, F and G). Together, these results provide evidence supporting that myelin infolding is a pathogenic event that precedes axonal defects and nodal disorganization.

DISCUSSION

Despite human genetic evidence linking heterozygous SIMPLE missense mutations to dominantly inherited CMT1C (4–8), it is unclear whether these mutations cause peripheral neuropathy via haplo-insufficiency of SIMPLE or dominant effects of the SIMPLE mutants. Our finding of the motor and sensory neuropathy phenotype by transgenic expression of human SIMPLE W116G mutant protein on a
WT SIMPLE background in mice supports that human CMT1C disease is caused by dominant effects of the SIMPLE mutant rather than haplo-insufficiency of WT SIMPLE protein. Consistent with this view, a recent study reported that SIMPLE knockout mice developed normally and did not show a neuropathy phenotype (22). The lack of a neuropathy phenotype in our control transgenic mice, which express human SIMPLE WT protein at an equivalent level to that of human SIMPLE W116G protein in the mutant transgenic mice, indicates that the neuropathy phenotype of the SIMPLE W116G mutant mice is the consequence of the SIMPLE mutation rather than an overexpression of SIMPLE protein. Our finding of the SIMPLE W116G transgene integration into a non-coding genomic region (Fig. 1C and D) supports that the neuropathy phenotype in the SIMPLE mutant mice is not due to an integration site effect.

Figure 8. Electron microscopic analysis of myelin abnormalities and axonal degeneration in SIMPLE W116G mutant mice. Ultrastructural analysis of sciatic nerves from 12- to 15-month-old mice showed myelin and axon abnormalities of SIMPLE W116G/W116G mice (B–I and K–R) that are virtually absent in the control (CTRL) mice (A and J). The most frequent type of myelin abnormality in the SIMPLE W116G/W116G nerves is the focally infolded myelin that protrudes into the axon (B) and the presence of one or more infolded myelin loops within myelinated axons of both large and small calibers (C–F). The infolded myelin loops (H) and the myelin sheath (G) of the axon have the identical periodicity and number of lamellae. Schwann cell cytoplasmic vesicles (G) were indicated by an arrow. Longitudinal section showed infolded myelin loop originating from the myelin sheath near the Schmidt-Lanterman incisures (I). Loss of myelin compaction and widened spacing of the Schmidt-Lanterman incisures were occasionally observed in SIMPLE W116G/W116G nerves in longitudinal (K) and cross sections (L) but were absent in the control (J). The Schmidt-Lanterman incisures (I–L) were indicated by arrows, and myelin infolding (I) was indicated by arrowhead. SIMPLE W116G/W116G nerve fibers with various stages of axonal degeneration (M–P), intramyelin edema (Q) and demyelinated axons (R) were also occasionally observed. Scale bar, 2 μm (A–F, I–R) or 200 nm (G and H). (S and T) Quantification of the percentage of sciatic nerves undergoing axonal degeneration (S) and demyelination (T) showed a significantly increased axonal degeneration and demyelination in SIMPLE W116G/W116G mice compared with the control (CTRL) at 12–15 months of age. For each quantification, 10 randomly selected fields (50–100 nerve fibers per field) from 3 to 5 ultrathin cross sections of sciatic nerve per mouse were analyzed, and 3 mice were analyzed per genotype. Data represent mean ± SEM. ∗P < 0.05 versus the control.
motor impairments accompanied not only by a reduced MNCV (which is characteristic of demyelinating CMT) but also by a reduced CMAP amplitude (which is indicative of axonal degeneration). Furthermore, as reported in CMT1C patients (4–8), SIMPLE\textsuperscript{W116G/W116G} mice also exhibited sensory impairments accompanied by a reduced SNCV. In addition, similar to CMT1C where a subgroup of patients with the SIMPLE W116G mutation have no evoked SNAP and show paresthesia (4), we found that a small percentage of SIMPLE\textsuperscript{W116G/W116G} mice lost the ability to evoke SNAP and showed self-mutilating behavior which could be caused by the loss of sensory nerve conduction.

CMT1C is a rare form of hereditary motor and sensory neuropathy and biopsy material is scarce. Therefore, our knowledge of the pathology of human CMT1C is very limited. The only published pathological result is a semithin cross section showing the presence of onion-bulb formations in the sural nerve biopsy material from a CMT1C patient (6). We did not find obvious evidence for onion-bulb formations in the peripheral nerves of SIMPLE\textsuperscript{W116G/W116G} mice. A similar lack of onion-bulb formations has been reported in several other mouse models of human CMT (23–25), which could be due to the shorter life span of mice, shorter lengths of nerves in mice and/or some other species difference between mice and humans (26).

Despite the lack of onion-bulb formations, we found that the motor and sensory functional impairments in SIMPLE\textsuperscript{W116G/W116G} mice are associated with focally infolded myelin loops that protrude into the axons at paranodal regions and near
Our finding that myelin infolding is often linked to constricted axons with signs of impaired axonal transport suggests that focally infolded myelin may physically block axonal transport. Our analyses revealed that myelin infolding is also associated with altered distribution of the paranodal and juxtaparanodal axonal molecules and a widened nodal gap at the nodes of Ranvier. In addition to myelin infolding and paranodal damage, we have identified a small but significant percentage of peripheral nerves with signs of demyelination and axonal degeneration. These pathological changes provide a structural basis for the observed motor and sensory nerve conduction defects, including reduced MNCV and SNCV, decreased CMAP amplitude and a loss of evoked SNAP in SIMPLEW116G/W116G mice.

Our data show that myelin infolding appears in peripheral nerves of SIMPLEW116G/W116G mice at 8 months of age, prior to the development of paranodal axonal defects and nodal disorganization. The percentage of paranodal regions with myelin infolding is significantly increased as the mutant mice get older to the 1 year of age and develop the motor and sensory neuropathy with paranodal axonal defects and nodal disorganization. Based on our results, we propose that myelin infolding and paranodal damage may represent pathogenic precursors leading to demyelination and axonal degeneration in CMT1C patients, and these pathological changes should be considered as major factors contributing to the CMT1C motor and sensory neuropathy.

Figure 10. Myelin infolding precedes axonal damage in SIMPLE W116G mutant mice. (A) Nile red-stained teased sciatic nerve fibers from 8-month-old (8 months) and 1-year-old (1 year) SIMPLEW116G/W116G mice showed myelin infolding at the paranodal regions and near Schmidt-Lanterman incisures and occasional widening of the node of Ranvier that are absent in the control (CTRL) mice. Myelin infolding (arrowhead), Schmidt-Lanterman incisures (arrow) and nodal gap (bracket) were indicated. Scale bar, 20 μm. (B and C) Quantification of the percentage of paranodal regions with myelin infolding (B) and the percentage of nodes with enlarged nodal gap (C) showed age-dependent increases in myelin infolding and nodal disorganization in SIMPLEW116G/W116G mice compared with the CTRL mice. For each analysis in (A–C), 20–25 randomly selected nodes of Ranvier per mouse were analyzed, and three mice were analyzed per genotype/age group. Representative images were shown. Data represent mean ± SEM. *P < 0.05 versus the control, #P < 0.05 versus the 8-month-old mice for the corresponding genotype. (D) Teased sciatic nerves from the indicated 8-month-old (8 months) and 1-year-old (1 year) SIMPLEW116G/W116G and control (CTRL) mice were stained with anti-Caspr (green) antibody. Caspr lengths (red brackets), Caspr gap (yellow brackets) and myelin infolding (arrowhead) were indicated. Scale bar, 5 μm. (E–G) Quantification of the Caspr gap distance (E), Caspr length (F) and the percentage of paranodes with elongated Caspr staining (G) showed significantly enlarged Caspr gap (E) and longer Caspr length (F and G) in 1-year-old mutant nerves, but not in 8-month-old mutant nerves, compared with the CTRL mice. For each analysis in (D–G), 10–20 randomly selected nodes of Ranvier per mouse were analyzed, and three mice were analyzed per genotype/age group. Representative images were shown. Data represent mean ± SEM. *P < 0.05 versus the control, #P < 0.05 versus the 8-month-old mice for the corresponding genotype.
suggest that abnormal myelin folding and nodal disorganization may represent convergent pathogenic mechanisms leading to demyelinating peripheral neuropathy.

Our immunohistochemical analysis reveals that human SIMPLE W116G transgene, like endogenous SIMPLE, is expressed in myelinating Schwann cells but not in axons. Furthermore, the SIMPLE W116G mutant protein is partially mislocalized from the early endosomal membrane to the cytosol in the SIMPLEWT116G/W116G mice. These results, together with our published data showing that the W116G mutation promotes SIMPLE protein misfolding (9,39), support the hypothesis that SIMPLE mutation causes peripheral neuropathy via a combination of toxic gain-of-function and dominant-negative pathogenic mechanisms.

We recently reported that SIMPLE interacts and colocalizes with endosomal sorting complex required for transport (ESCRT) components STAM1, Hrs and TSG101 on early endosomes and functions with the ESCRT machinery in the control of endosomal cargo sorting to the lysosome for degradation (40). We showed that CMT-linked SIMPLE mutants, including SIMPLE W116G mutant, are loss-of-function mutants that act in a dominant-negative manner to impair endosome-to-lysosome trafficking in the presence of WT SIMPLE (40). Consistent with the critical role of endosome-to-lysosome trafficking in signaling attenuation (41), we found that SIMPLE mutant-induced trafficking impairment leads to prolonged signaling of neuregulin-1 (NRG1)-activated ErbB2/ErbB3 receptors to downstream pathways in Schwann cells (40). A NRG1-ErbB downstream pathway that functions in the regulation of myelin membrane growth is the phosphotyrosylinositol 3 kinase (PI3K)-Akt-mTOR-signaling pathway, and hyperactivation of this signaling pathway in Schwann cells has been shown to cause the formation of focally folded myelin loops and progressive peripheral neuropathy (42). These results, together with the data from this study, support a model in which SIMPLE W116G mutant disrupts endosome-to-lysosome trafficking and signaling attenuation of NRG1-activated ErbB2/ErbB3 receptors, causing prolonged activation of downstream signaling pathways such as the PI3K-Akt-mTOR pathway in Schwann cells, thereby leading to myelin infolding and paranodal damage, ultimately resulting in demyelinating peripheral neuropathy. In conclusion, our findings obtained from this work provide new insights into the pathogenic mechanisms of CMT1C-linked SIMPLE mutations. The transgenic mouse model of CMT1C generated in this study should facilitate the investigation of CMT pathogenesis and therapeutics.

MATERIALS AND METHODS

Generation and genotyping of SIMPLE WT and SIMPLE W116G transgenic mice

Conventional molecular biological techniques were used to generate the transgenic constructs encoding HA-tagged human SIMPLE WT and SIMPLE W116G mutant under the control of the human CMV promoter in the pCHA vector (43). After digestion with Mlu I and Nat I, the linearized SIMPLE transgene DNAs were microinjected into pronuclei of fertilized embryos from the FVB mice and then implanted in pseudo-pregnant female FVB/N mice. Transgenic founder mice were identified by the PCR analyses of genomic DNAs isolated mouse tails using two independent sets of transgene-specific primer pairs and then bred with FVB/N mice to established SIMPLE WT and SIMPLE W116G transgenic lines on a pure FVB/N background. Zygosity of SIMPLE WT and SIMPLE W116G transgenic mice was determined by a well-established protocol using SYBR Green real-time quantitative PCR (Q-PCR) of tail genomic DNAs and comparative Ct (2AACt) analysis as described (44–46). Expression of HA-tagged SIMPLE WT and SIMPLE W116G proteins in transgenic mice was determined by immunoblot and immunostaining analyses using anti-SIMPLE antibody and anti-HA antibody.

Determination of transgene integration site

For identification of the transgene integration site in the SIMPLE W116G transgenic line used in this study, genomic DNAs were isolated from liver tissues of SIMPLE W116G transgenic mice and then subjected to genome walking PCR analyses using a series of transgene-specific primers and the degenerate random tagging primers provided by APAgene™ Gold-RT Genome Walking Kits (Bio S&T, Inc., Montreal, Canada) in accordance to the manufacturer’s instructions. The PCR products were either cloned or directly sequenced to identify the transgene integration site. The identified integration site was confirmed by PCR analyses shown in Figure 1D using the following primers: A (5′-CATTATCACGGTTATTGTCTC), B (5′-GTCCCTATTGGCGTTACTATGG), C (5′-TGACATCGCATTTGCTAGTAG) and D (5′-GGTAATAGAAGGAGGTTAGGAG).

Antibodies

The generation and characterization of rabbit polyclonal anti-SIMPLE antibody was described in our previous study (9). Other antibodies used in this study include the following: anti-HA (12CA5), anti-actin (Millipore), anti-Rab5 (BD Transduction), anti-S100 (Sigma Aldrich), anti-Dig1 (Enzo), anti-MBP (Millipore), anti-MAG (Millipore), anti-NF-H (Millipore), anti-Caspr (Abcam), anti-pan-Na+, (Sigma) and anti-Kv1.2 (NeuroMab). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc.

Teased nerve fibers, Nile red staining and immunofluorescence confocal microscopy

Mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Sciatic nerves were dissected and post-fixed in 4% paraformaldehyde overnight at 4°C and washed in 0.1 M phosphate buffer. Individual fibers were separated in glycerol on glass slides and were immersed in cold acetone for 10 min. Teased fibers were then rehydrated in PBS and incubated at room temperature for 30 min in a blocking solution containing 10% horse serum, 0.5% Triton X-100 and PBS. For Nile red staining, teased fibers were mounted in Nile red solution (0.5 mg/ml in acetone) diluted 1000× in 75% glycerol as described (13). For immunostaining, primary antibodies were diluted in the blocking solution and

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were incubated with the teased fibers overnight. After washing, teased fibers were incubated with the appropriate secondary antibodies conjugated to FITC, Texas Red or Cy5, and were mounted with ProLong Gold (Invitrogen). Confocal images were acquired in room temperature with the Nikon Eclipse Ti confocal microscope equipped with 60 × 1.4 oil immersion objectives and the Nikon EZ-C1 software.

**Western blot analysis**

Primary Schwann cells were isolated from postnatal day 2 (P2) to P4 non-transgenic, SIMPLE WT and SIMPLE W116G mutant mice and cultured using established protocols as described (9,47,48). For western blot analysis, sciatic nerves or primary Schwann cells were homogenized in 1% SDS and then subjected to SDS–PAGE. The proteins were transferred onto nitrocellulose membranes and probed with the indicated antibodies. Antibody binding was detected by using the enhanced chemiluminescence (ECL) system (Amer sham Biosciences). The expression levels of SIMPLE proteins were quantified by measuring the intensity of SIMPLE and actin bands from the immunoblot images using the ImageJ software as described previously (9).

**Subcellular fractionation analyses**

Subcellular fractionation of sciatic nerve homogenates into cytosol and membrane fractions were performed as described (9). For density gradient fractionation analysis, the membrane fraction from sciatic nerves was resuspended in fractionation buffer and was then placed on a 10–30% linear Optiprep (Sigma) gradient and centrifuged for 20 h at 125 000 g in a SW41 rotor (Beckman Coulter) as described previously (9). After centrifugation, the gradient was harvested into 250 μl fractions by an Auto Densi-Flow gradient harvester (Labconco).

**Behavioral tests**

For the rotarod test, mice were placed on a standard rotarod apparatus (Columbus instruments) and acclimated to a non-accelerating rotarod at an initial speed of 1.25 rpm for 30 min. At the beginning of each trial, rotarod speed was accelerated at the rate of 1 rpm/min, and the latency for a mouse to fall off the accelerating rotarod was recorded. Each animal was tested six times with a 5 min of rest between each trial. For the tail-flick test, mouse tails were immersed in a water bath containing 52°C water as the nociceptive stimulus, and the latency between tail immersion and tail-flick response was measured blindly from recorded videos. The test was repeated three times with a 15 min of rest between each trial, and the mean latency of each mouse over three trials was used for statistical analysis.

**Electrophysiology**

SIMPLE WT and W116G mutant mice and their non-transgenic littermates were analyzed at 3 months and 1 year of age. Mice were anesthetized with chloral hydrate (400 mg/kg of body weight, i.p.) and placed under a heat lamp to avoid hypothermia. Nerve conduction studies were performed using standard equipment (Nicolet Viking Quest). For sciatic nerve motor conduction assays, stimuli were given at the sciatic notch and at the ankle, and recording electrodes were inserted into interosseous muscles of the left foot, whereas a ground electrode was inserted subcutaneously at the tail. For tail nerve sensory conduction assays, the recording electrodes were placed at the base of the tail, keeping the anode and the cathode about 5 mm apart, and with a ground electrode placed subcutaneously 2 cm distal. Stimuli were given 4 cm distal and sensory nerve conduction was averaged over 20 stimuli.

**Histological analysis and electron microscopy**

Mice were anesthetized followed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Dorsal roots, ventral roots and sciatic nerves were harvested and postfixed by immersion in 4% paraformaldehyde and 2% glutaraldehyde overnight at 4°C. Nerves were then washed in 0.1 M phosphate buffer and embedded in paraffin. Cross and longitudinal semithin (0.5 μm) sections were stained with toluidine blue and were analyzed by light microscopy (Olympus). For ultrastructural studies, nerves were prepared for electron microscopy by postfixation with OsO4 followed by en bloc staining with uranyl acetate. Cross and longitudinal ultrathin (100 nm) sections were cut and stained with uranyl acetate and lead nitrate. Grids containing the ultrathin nerve sections were examined by a Hitachi H-7500 transmission electron microscope.

**Morphometric analyses**

Quantitative analyses of focally folded myelin, number of axons, axon roundness, axon diameter and axon area were performed from 10 randomly selected fields of semithin sciatic nerve cross sections (150–400 nerve fibers per field, 40 × magnification) per mouse with three mice analyzed per genotype. The number of nerve fibers with focally infolded myelin (internal myelin loops) and the total number of fibers on each field were counted, and the percentage of nerve fibers with myelin infoldings was calculated. Axon roundness, axon diameter and axon area were measured by using the ImagePro software (Media Cybermetics). Axon roundness, an index which measures the circularity of axons, was calculated according to the formula [roundness = perimeter²/(4 × π × area)]. A roundness index of 1 represents a perfect circle, and the roundness index increases as the shape of an axon in the cross section becomes less circular.

Myelin thickness and G-ratio were determined as previously described (49) from electron microscopic images (3000 × magnification) for 50 randomly selected sciatic nerve fibers from three mice per genotype by using the ImagePro software (Media Cybermetics). Quantitative analyses of axonal degeneration and demyelination were performed from 10 randomly selected fields of sciatic nerve electron microscopic images (50–100 nerve fibers per field, 3000 × magnification) per mouse with three mice analyzed per genotype. The number of nerve fibers with degenerating axons or demyelinated axons and the total number of fibers on each field were evaluated.
counted, and the percentage of nerve fibers with degenerating axons or demyelinated axons was calculated.

Quantitative analysis of the nodal gap length was performed for longitudinal sciatic nerve sections (40× magnification) for 12–15 nodes of Ranvier from three mice per genotype, and the nodal gap length was measured by using the ImageJ software. Quantitative analysis of the percentage of paranodal regions with myelin infolding and the percentage of nodes with enlarged nodal gap were performed as described previously (16) from Nile red fluorescence confocal microscopic images (60× magnification) for 20–25 randomly selected nodes of Ranvier per mouse with three mice analyzed per genotype/age group. Quantitative analysis of the Caspr gap distance, Caspr length and the percentage of paranodes with elongated Caspr cluster were performed as described previously (16) from immunofluorescence confocal microscopic images (60× magnification) for 10–20 randomly selected nodes of Ranvier per mouse with three mice analyzed per genotype/age group. All quantitative analyses were performed in a blinded manner.

Statistical analysis

Data were subjected to statistical analyses by Student’s t-tests or one-way analysis of variance using the SigmaPlot software (Systat Software, Inc.). Results are expressed as mean ± SEM. A P-value of <0.05 was considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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