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Laminin γ1 is critical for Schwann cell differentiation, axon myelination, and regeneration in the peripheral nerve

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Laminins are heterotrimeric extracellular matrix proteins that regulate cell viability and function. Laminin-2, composed of α2, β1, and γ1 chains, is a major matrix component of the peripheral nervous system (PNS). To investigate the role of laminin in the PNS, we used the Cre–loxP system to disrupt the *laminin γ1* gene in Schwann cells. These mice have dramatically reduced expression of laminin γ1 in Schwann cells, which results in a similar reduction in laminin α2 and β1 chains. These mice exhibit motor defects which lead to hind leg paralysis and tremor. During development, Schwann cells that lack laminin γ1 were present in peripheral nerves, and proliferated and underwent apoptosis similar to control mice. However, they were unable to differentiate and synthesize myelin proteins, and therefore unable to sort and myelinate axons. In mutant mice, after sciatic nerve crush, the axons showed impaired regeneration. These experiments demonstrate that laminin is an essential component for axon myelination and regeneration in the PNS.

Introduction

Myelination of nerves is essential for proper function of the nervous system, predominantly by allowing faster conduction velocity of action potentials. In the peripheral nervous system (PNS), bundles of axons are assembled during embryonic development. After birth, premyelinating Schwann cells separate these bundles by extending processes between the axons. Myelinating Schwann cells establish a 1:1 ratio with axons, and envelop them in a myelin sheath. Nonmyelinating Schwann cells extend cytoplasmic processes between axons and separate them, but do not form a myelin sheath (Mirsky and Jessen, 1999).

The ECM has been implicated in myelination in the PNS (R. Bunge, 1993), and laminins are components of the ECM that appear to be especially important. Studies in cell culture have suggested that laminin plays a critical role in myelination (Doyu et al., 1993; Fernandez-Valle et al., 1993; Fernandez-Valle et al., 1994; Podratz et al., 2001; Tsiper and Yurchenco, 2002). In vivo studies have also suggested that laminin is important. Mutations in the *laminin α2* gene in mice (*dy/dy* and dy^{2J}/dy^{2J}; Xu et al., 1994; Sunada et al., 1995) or humans (*CMD*; Helbling-Leclerc et al., 1995) result in muscular dystrophy and a minor peripheral myelination defect (Bradley and Jenkison, 1973; Madrid et al., 1975; Perkins et al., 1981). In this case, because the mutations also affect the muscle, the phenotype is a combination of effects on the nerve and the muscle (Kuang et al., 1998). Other evidence suggesting a possible role for laminin in myelination stems from the observation that β1 integrin, a component of many laminin receptors, is necessary for proper myelination of peripheral nerves (Feltri et al., 2002).

These results suggest that laminin might be important for myelination of peripheral nerves, but the mechanism for laminin action in vivo has not been elucidated. Laminins are heterotrimeric proteins composed of an α, β, and γ chain. At present, five α chains, three β chains, and three γ chains have been identified, and of the possible 45 potential trimeric molecules that could be generated from these chains, 15 have been observed (Colognato and Yurchenco, 2000; Grimpe et al., 2002). Laminin γ1 chain is one of the most abundant chains and is present in all known laminin isoforms except laminin-5 and laminins 12–15 (Grimpe et al., 2002). Previous studies have shown that global disruption of *laminin γ1* gene leads to early (E5.5) embryonic lethality (Smyth et al., 1999).

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Abbreviations used in this paper: CaMKIIα, calcium/calmodulin-dependent protein kinase II α; MBP, myelin basic protein; neo^R, neomycin resistance; PNS, peripheral nervous system.

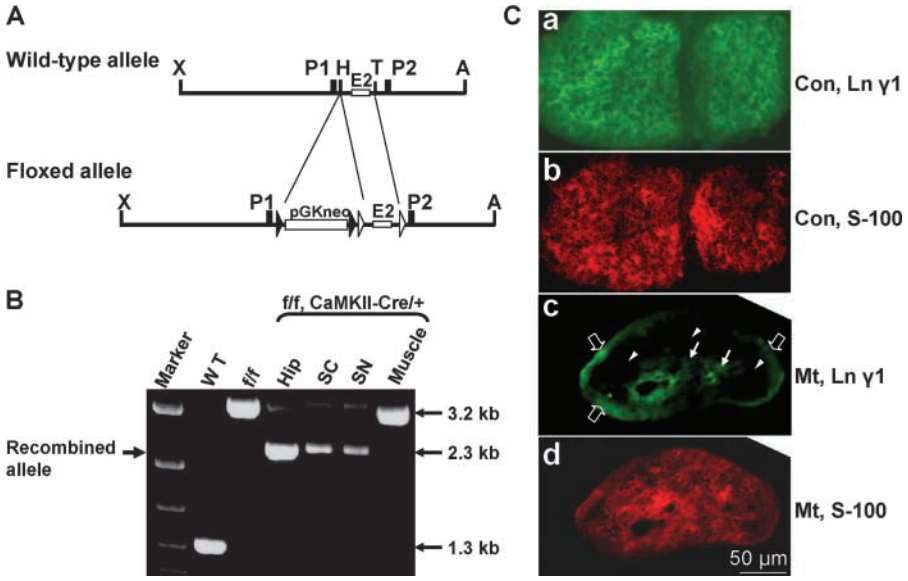

Figure 1. **Recombination of a floxed *laminin γ1* allele by a CaMKIIα promoter-Cre transgene leads to reduced expression of laminin γ1 in the sciatic nerve.** (A) Details of the *laminin γ1* gene targeting construct. The two loxP sites (open triangles) were inserted into the *laminin γ1* gene to flank exon 2 (E2). The neomycin resistance gene pGKneo (long open rectangle) used for ES cell selection was flanked by two frt sites (closed triangles). The P1 and P2 primer sites were used in B to monitor recombination by PCR. Restriction endonuclease sites are indicated (A, AvrII; H, HindIII; T, Tth111I; X, XcmI). (B) PCR analysis of tail DNA from wild-type and homozygous *fLAMγ1* mice (f/f), and of DNA from various tissues from *CaMKII/Cre:fLAMγ1* mice (f/f, CaMKII-Cre/+). The primers used allow amplification of the wild-type allele and the unrecombined and recombined *fLAMγ1* allele. In tissues where recombination takes place, both unrecombined and recombined alleles are detected because there are some cells (e.g., vascular tissue) in which recombination does not occur. Recombination is prominent in the hippocampus (Hip) as expected, and also in the spinal cord (SC) and sciatic nerve (SN); but it is minimal in the skeletal muscle from the leg. (C) Transverse sections of sciatic nerve from control and mutant mice at P1 were double stained for laminin γ1 (a and c) and S-100 (b and d). (a) The nerve from a control animal shows strong laminin γ1 staining; (c) nerve from a mutant mouse shows greatly reduced expression of laminin γ1 (arrowheads) but there was still some expression (closed arrows). Laminin expression in the epineurium is not affected (open arrows). Even though laminin γ1 expression is dramatically decreased in mutant sciatic nerve, the S-100 staining was similar to control (b and d). Con, control; Mt, mutant.

To investigate the function of laminin γ1 in development and in adults in vivo, and to avoid early embryonic lethality, we created mice with a *laminin γ1* gene containing loxP recombination sites flanking an essential exon. The *laminin γ1* gene in these mice can be disrupted by tissue specific expression of the Cre recombinase. To address the function of laminin γ1 in myelination, we disrupted its expression in Schwann cells, the myelinating cell in the PNS. Disruption of laminin γ1 expression in Schwann cells prevented their differentiation and axon myelination, whereas those Schwann cells that escaped recombination during development produced laminin γ1 and could normally myelinate axons. The sciatic nerve in the mutant mice exhibited impaired regeneration after injury. There results indicate that the production of laminin in Schwann cells is necessary for proper myelination and regeneration of peripheral nerves.

Results

Generation of mice that carry a floxed *laminin γ1* (*fLAMγ1*) allele

To accomplish tissue-specific disruption of the *laminin γ1* gene, genomic DNA containing the 5′ region of the *laminin γ1* gene was isolated, and loxP sites were inserted into intron 1 and intron 2 by standard molecular biological procedures (Fig. 1 A). We also inserted into intron 1 a neomycin resistance (neo^R) gene for selection of the transformed cells. This neo^R gene was flanked by frt sites that would allow its removal by the flp recombinase because in some cases the presence of the neo^R gene in the intron leads to a hypomorphic phenotype even in the unrecombined allele (Meyers et al., 1998). This DNA construct was electroporated into mouse ES cells (line E14), and the cells that had undergone homologous recombination were identified. The *fLAMγ1* (floxed *laminin γ1*) allele was analyzed to verify that the DNA construct had correctly inserted into the wild-type *laminin γ1* gene, and that no other chromosomal changes had occurred. PCR and Southern blot analyses demonstrated that the *laminin γ1* gene had been correctly targeted (Fig. 1 B and not depicted).

These targeted ES cells were used to create chimeric mice via standard blastocyst injection, and the chimeras were bred to obtain mice heterozygous for the *fLAMγ1* allele. By standard breeding, we generated mice homozygous for the *fLAMγ1* allele. These mice showed no altered phenotype, indicating that the presence of the neo^R gene and the loxP sites in the introns was not deleterious.

Disruption of laminin γ1 expression in Schwann cells

Next, we created mice that were homozygous for the *fLAMγ1* allele and also carried a transgene with the calcium/calmodulin-dependent protein kinase II α (CaMKIIα) promoter, driving expression of the Cre recombinase (Dragatsis and Zeitlin, 2000). Mice of this genotype (*CaMKII/Cre:fLAMγ1*, referred to hereafter as mutant mice) exhibited hind leg paralysis and tremor. Analysis of these mice revealed significant recombination in the hippocampus, spinal cord, and peripheral nerves (e.g., the sciatic nerve), but little to no recombination in other organs such as the muscle or heart (Fig. 1 B). Further PCR analyses indicated that the CaMKIIα promoter in this transgenic line was active as early as E17.5 in nerves (unpublished data). These and previous results (Dragatsis and Zeitlin, 2000) showed that the expression of Cre in this transgenic line was not as hippocampal-specific as in other *CaMKIIα* lines (Tsien et al., 1996).

Expression of laminin γ1 was reduced in the hippocampus and spinal cord, but the morphology of these regions was similar to control mice (unpublished data). In contrast, the mutant nerves exhibited dramatic changes at the cellular and molecular levels (see Figs. 5 and 8). Therefore, we focused our analysis on the PNS.

To analyze laminin γ1 expression in the mutant PNS during development, we compared laminin γ1 protein expression in control (mice homozygous for *fLAMγ1* without *Cre* or heterozygous for both *fLAMγ1* and *Cre* were used as control) and in mutant sciatic nerves by immunostaining (Fig. 1 C). There was extensive laminin γ1 expression in the control sciatic nerve at P1, but greatly reduced expression in the mutant nerves (Fig. 1 C, a and c). To investigate if the decrease of laminin γ1 expression was due to the absence of Schwann cells in the mutant nerves, we stained for S-100, a marker of Schwann cells. This staining revealed that the level of S-100 was similar in control and mutant nerves, showing that Schwann cells are present (Fig. 1 C, b and d).

Because the CaMKIIα promoter is known to be active in neurons, it was possible that the lack of laminin γ1 expression in the sciatic nerve was due to reduced expression by axons. Therefore, we examined laminin γ1 expression in axons of control animals at E15.5, E17.5, E19.5, and P1. This development period is important for axonal sorting and myelination. During this period, Schwann cells proliferate and subdivide axonal bundles; but some axons have not yet been wrapped by Schwann cells, allowing us to examine them for axonal laminin expression. Investigation of those axonal regions showed that there was no detectable laminin γ1 expression by axons (Fig. 2), and the laminin expression was always associated with Schwann cells. Moreover, Schwann cells are known to be a major source of laminin in nerves (Cornbrooks et al., 1983; M. Bunge, 1993). Finally, when the

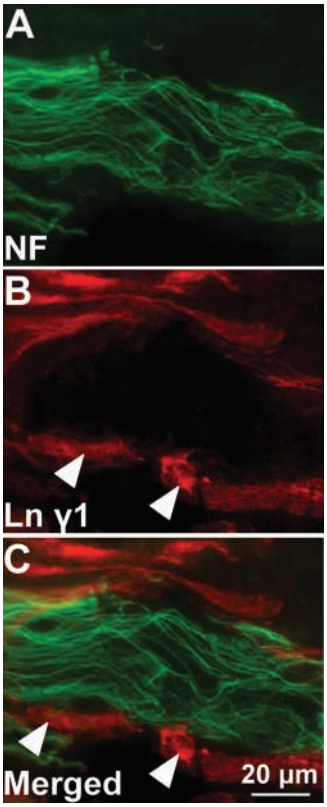

Figure 2. **Nerve axons do not express laminin γ1.** E17.5 control embryo sections were stained for neurofilament (A) and laminin γ1 (B). The sciatic nerves were identified by the location of the neurofilament staining. At this developmental stage, some axonal bundles have not yet been sorted by Schwann cells (A and C, green), and these axons do not show laminin γ1 staining (B). However, the epineurium and surrounding tissues showed strong laminin γ1 staining (B and C, arrowheads).

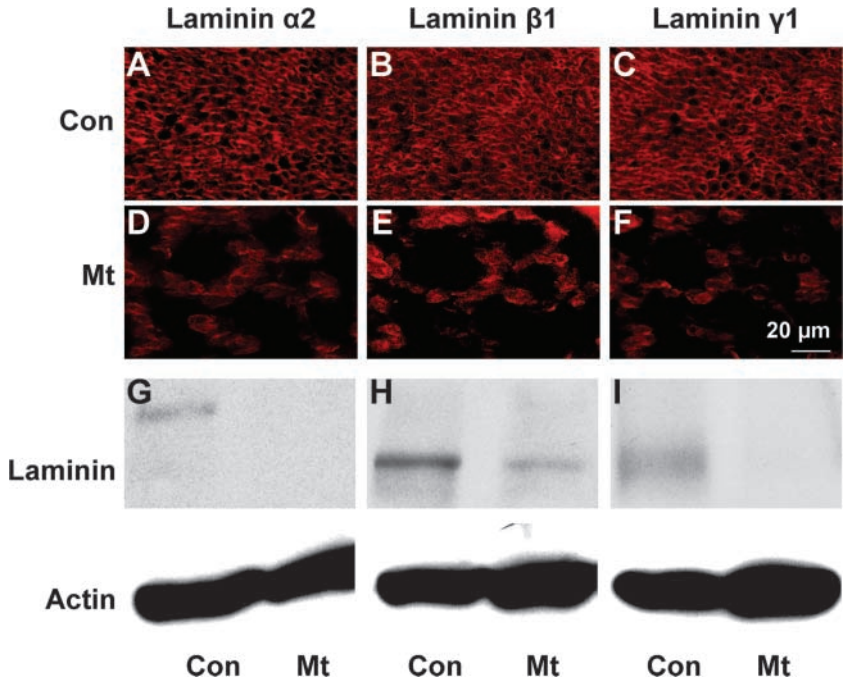

Figure 3. **Reduced expression of laminin α2, β1, and γ1 chains in the sciatic nerve of *CaMKII/Cre:fLAMγ1* mice.** Serial transverse sections of control (A–C) and mutant (D–F) mouse sciatic nerves at P28 were stained for laminin α2 (A and D), β1 (B and E), and γ1 (C and F). The expression of these laminin chains (components of laminin-2) were dramatically reduced in the mutant mice and showed similar staining patterns on adjacent sections (compare D, E, and F). Western blot analysis using the same monoclonal antibodies showed that expression of laminin α2 (G), β1 (H), and γ1 (I) in mutant sciatic nerves was dramatically reduced. Con, control sciatic nerve; Mt, mutant sciatic nerve.

laminin γ1 gene was disrupted in Schwann cells using Cre driven by the P_0 promoter, a promoter specific for Schwann cells, there was also no laminin γ1 staining in the axons (Yu, W.-M., personal communication). These results demonstrate that Schwann cells are the major source of laminin in the nerves and that reduced expression of laminin in the mutant nerves is due to recombination in Schwann cells.

Further analysis showed that although laminin γ1 expression was dramatically reduced in many Schwann cells, this reduction was not complete with some Schwann cells still synthesizing laminin γ1 (Fig. 1 C, c; and Fig. 3, C and F). Immunostaining using serial sciatic nerve sections and laminin chain-specific monoclonal antibodies for the α2 (Fig. 3, A and D) and β1 (Fig. 3, B and E) chains also showed greatly reduced expression, and the staining patterns for all three chains were similar (Fig. 3, compare D, E, and F). Furthermore, Western blot analysis of sciatic nerve extracts showed a dramatic reduction of expression of laminin α2 (Fig. 3 G), β1 (Fig. 3 H), and γ1 (Fig. 3 I), indicating that without laminin γ1 the laminin α2 and β1 chains did not accumulate. Thus, the mutant mice were viable, but laminin-2 (α2 β1 γ1) expression in the Schwann cells was reduced.

Laminin deficiency in peripheral nerves leads to motor dysfunction and paralysis

The mutant mice appeared normal for 2–3 wk after birth. However, by ~4 wk, they began to exhibit hind leg weakness. This weakness was manifested by a difficulty in walking, unsteadiness in their gait and inability in using their hind legs to grasp. By 3 mo of age, most of the affected mice had almost completely paralyzed hind legs and muscular atrophy (Fig. 4). They also showed a severe tremor that persisted even under deep anesthesia. In the most severely affected animals, the motor defects were also obvious in the front legs, which could not support their body weight (Fig. 4). The gross phenotype is similar to that observed in mice with a conditional disruption of the β1 integrin gene in Schwann cells (Feltri et al., 2002).

Schwann cells lacking laminin cannot sort and myelinate axons

As mentioned in the Introduction, during postnatal development, myelinating Schwann cells isolate single axons from the axon bundles that are formed during embryogenesis. Therefore, we compared control and mutant sciatic nerves at various times after birth. In control mice at P1, there were many large caliber axons that were subdivided and beginning to be myelinated, but there were still some bundles of unsorted axons (Fig. 5 A, and see Fig. 8 A). In contrast, the mutant nerve already had a different morphology (Fig. 5 B, and see Fig. 8 B), with many fewer subdivided axons. The difference in axonal sorting and myelination between the control and mutant sciatic nerves became progressively more dramatic with time (Fig. 5, C–H). In P5, P15, and P28 nerves, large bundles of unsorted axons persisted in mutant sciatic nerves, whereas in control nerves, the axons became sorted and myelinated.

The defect in axon sorting and myelination was also obvious in both the dorsal and ventral root of the spinal cord of the *CaMKII/Cre:fLAMγ1* mice (Fig. 5, I–L), but was greater in the ventral root of spinal cord, suggesting that motor axons are more affected than sensory axons by the loss of laminin. In laminin α2 mutant mice such as *dy/dy* dystrophic mice and dy^{2J}/dy^{2J} mice, the effect in roots is greater than in the nerve (Stirling, 1975; Weinberg et al., 1975), whereas in the *laminin γ1* mutant mice, the roots were similarly affected as the nerve (Fig. 5, H, J, and L).

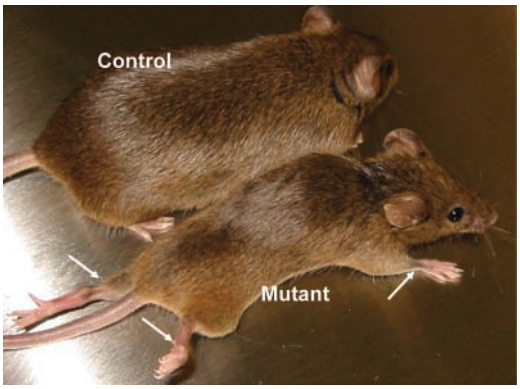

Figure 4. ***CaMKII/Cre:fLAMγ1* mice have motor dysfunction.** The mouse on the left was homozygous for the *fLAMγ1* allele, but did not contain the *CaMKII-Cre* gene. Mice of this genotype or mice heterozygous for the *fLAMγ1* allele and carrying the *CaMKII-Cre* gene were always normal. The mouse on the right (Mutant) was homozygous for the *fLAMγ1* allele and carried the *CaMKII-Cre* gene. These mice were smaller and exhibited muscle weakness and in most cases complete paralysis of the legs (arrows) and muscular atrophy.

The CaMKII promoter is known to be active in some neurons. Although our results above (Fig. 2) indicated that there was no laminin expression in sciatic nerve axons, it was possible that some of the observed phenotype was due to effects on neurons. To address this question, we have performed initial experiments using the P_0 promoter, which is Schwann cell specific (Feltri et al., 1999, 2002), to drive Cre expression. The nerves in *P_0/Cre:fLAMγ1* mice showed a similar defect in axon sorting and myelination as the *CaMKII/Cre:fLAMγ1* mice (Fig. 5, M and N). This result shows that the phenotype in the *CaMKII/Cre:fLAMγ1* mice is due to lack of expression in the Schwann cells.

Although the mutant sciatic nerves were under myelinated, there were some Schwann cells that had normal myelination. Further studies showed that the Schwann cells that formed myelin also had laminin (see Fig. 7). This hypomorphic phenotype was presumably due to the fact that the CaMKIIα promoter is not highly active in the PNS, and some Schwann cells escape recombination. This fortuitous circumstance has allowed us to study laminin γ1–positive and –negative cells in the same nerve (see Figs. 7 and 8).

To further study the details of the myelination defect in laminin γ1−/− mice, we examined sciatic nerves from control and mutant mice at P1 by electron microscopy. This analysis showed that at this age Schwann cells from control mice had already extensively sorted axons (large axons had been sorted to the outside of the axonal bundles) and begun the process of myelination (see Fig. 8 A). In contrast, the

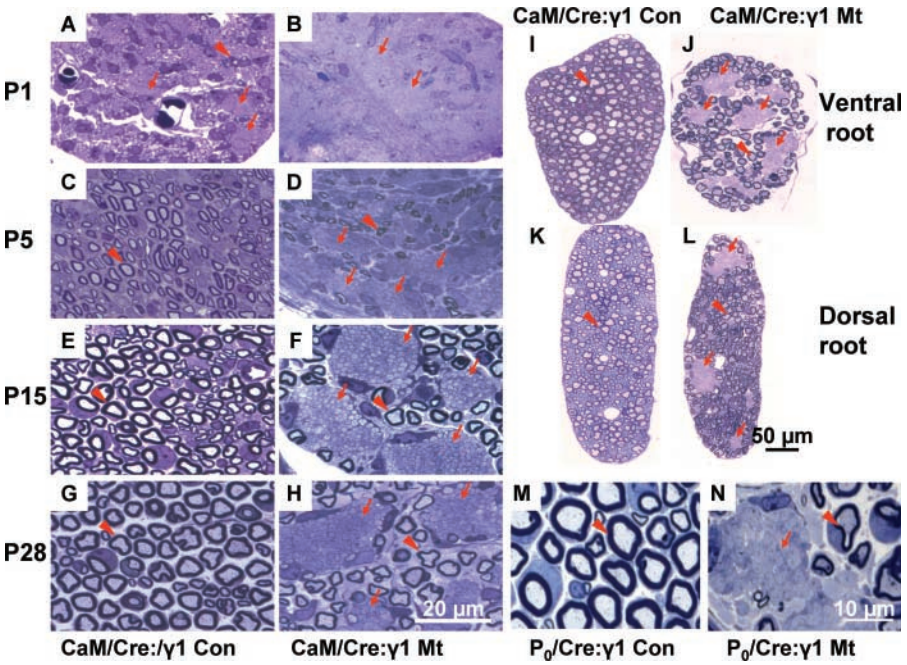

Figure 5. **Nerves from *CaMKII/Cre:fLAMγ1* and *P0/Cre:fLAMγ1* mice have defective axonal sorting and myelination.** (A–H) Transverse sections of sciatic nerve from control (A, C, E, and G) and *CaMKII/Cre:fLAMγ1* mutant mice (B, D, F, and H) were examined at various ages by Richardson's staining. The age of the mouse is indicated on the left of each panel. In the controls, even though there were some unsorted axonal bundles in P1 (A, arrows), the axons were gradually subdivided and myelinated by Schwann cells (A, C, E, and G, arrowheads), and by P28 the myelination was complete (G). In contrast, in the mutant mice, the axonal subdivision and myelination was impaired and there were unsorted axon bundles (B, D, F, and H, arrows); and only a few axons became myelinated (D and F, arrowheads). By P28, there were large bundles of unsorted axons and few myelinated fibers (H, arrowhead). (I–L) Comparison of changes in ventral and dorsal spinal root in adult mutant mice. Transverse sections were stained with Richardson's staining. Both ventral (motor) and dorsal (sensory) roots were affected, but the ventral root appeared more severe. The roots and the nerve were similarly affected (H, J, L, and controls in I and K). Sciatic nerves from *P_0/Cre:fLAMγ1* mutant mice at P28 (N, control in M) showed a similar phenotype to those from *CaMKII/Cre:fLAMγ1* mice. Arrows, unsorted axonal bundles; arrowheads, myelinated axons.

mutant nerves showed large bundles of unsorted axons comixed with very few sorted and myelinated axons, even though many Schwann cells were present (see Fig. 8 B). This ultrastructural analysis further confirmed that in mutant nerves Schwann cells populate the nerve, but fail to sort and myelinate axons.

Schwann cells in *CaMKII/Cre:fLAMγ1* nerves have normal proliferation and apoptosis during development

During late embryonic and early postnatal stages, Schwann cells proliferate vigorously, interact with axons, and subdivide them. Laminin is thought to be a mitogen for Schwann cells in vitro and important for cell viability (McGarvey et al., 1984; Baron-Van Evercooren et al., 1986; Chen and Strickland, 1997). Therefore, one possible mechanism for the defect in axonal sorting and myelination in mutant nerves could be a defect in Schwann cell proliferation and viability during these essential development stages. For this reason, we compared Schwann cell proliferation and viability in control and mutant sciatic nerves during these stages. Fetuses at E17.5 and E19.5 from control or mutant mice were labeled with BrdU to reveal cell proliferation, and their sciatic nerves were analyzed by immunohistochemistry. Control and mutant nerves showed extensive and similar cell division (Fig. 6, A, B, and E). Both types of nerves at E17.5, E19.5, and P5 were also analyzed for apoptosis. There was little apoptosis in late embryogenesis and in early postnatal life, and the amount was similar in both control and mutant nerves (Fig. 6, C–E). Further statistical analysis showed that Schwann cell BrdU incorporation and apoptosis between control and mutant mice at E17.5, E19.5, and P5 were not significantly different. These results indicate that Schwann cells in mutant sciatic nerves populate the nerve and proliferate similarly as in control and do not undergo more cell death during development.

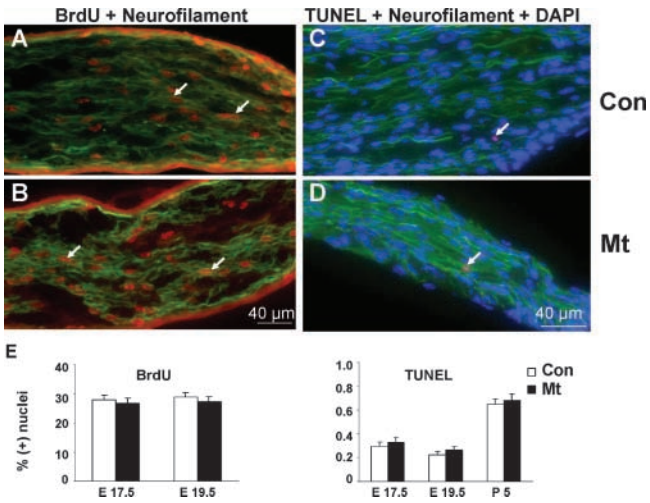

Figure 6. **Schwann cells in *CaMKII/Cre:fLAMγ1* nerves have normal proliferation and apoptosis during development.** Longitudinal sciatic nerve sections of control (A) and mutant embryos (B) at E19.5 were stained for BrdU (red) and neurofilament (green) after a 1-h pulse of BrdU, and the images were merged. In C and D, the sciatic nerve sections at E19.5 were stained with TUNEL (red), for neurofilament (green) and counterstained with DAPI (blue), and the images were merged. Schwann cells in mutant mice had similar nuclei BrdU incorporation as controls, and populated the nerve (A and B, arrows). Statistical analysis using the Mann-Whitney U test revealed no significant difference in percentage of BrdU-incorporated nuclei between control and mutant embryos (E). TUNEL staining showed that the ratio of positive nuclei was similar in mutant and the controls at E17.5, E19.5, and P5 (E). The TUNEL-positive nuclei always overlapped with DAPI staining (C and D, arrows). Error bars represent the SEM.

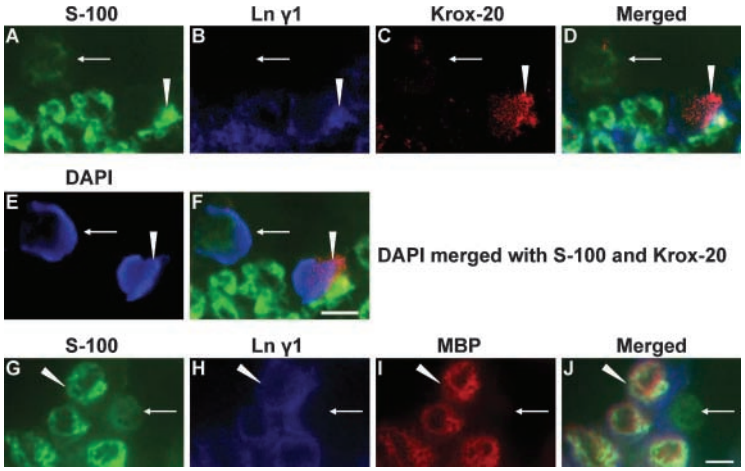

Figure 7. **Schwann cells that lack laminin γ1 are present in nerves but do not differentiate.** Sections of sciatic nerve from P28 d mutant mice were stained for S-100 (A and G), laminin γ1 (B and H), Krox-20 (C), and MBP (I). The images in A–C are merged in D. After photography, the sections were counterstained with DAPI, which is much stronger than the previous laminin staining using the same fluorescence dye, and therefore only showed DAPI staining (E). The DAPI-stained images were merged with S-100 (A) and Krox-20 (C) stained images and shown in F. The staining showed an S-100–positive Schwann cell (A, arrow) that did not express laminin γ1 (B, arrow) and did not show nuclei Krox-20 staining (C–F, arrow). An S-100–positive Schwann cell with its nuclei shown on this section (A and E, arrowheads) did express laminin γ1 (B, arrowhead) and showed nuclear Krox-20 staining (C, D, and F, arrowhead). The nuclei of the rest of the Schwann cells not indicated in A were not on this section. The correlation of laminin γ1 expression and MBP synthesis was also compared. An S-100–positive Schwann cell (G, arrow) did not express laminin γ1 (H, arrow) and did not produce MBP (I and J, arrow), whereas the rest of the S-100–positive Schwann cells (G, arrowhead) expressed laminin γ1 and produced MBP (H–J, arrowhead). Bars, 5 μm.

Schwann cells lacking laminin are present in nerves but do not differentiate

If Schwann cells in mutant mice populate the nerve, proliferate, and die normally, then why can't they perform their normal function of sorting and myelinating the axons? One possibility was that Schwann cells that lack laminin γ1 were not able to differentiate, and therefore could not sort and myelinate axons. To address this possibility, sciatic nerve sections of mutant mice were triple stained for S-100, a Schwann cell marker, laminin γ1, and Krox-20, a transcription factor important for induction of myelination (Topilko et al., 1994). The S-100 staining showed that there were abundant Schwann cells in the mutant nerves (Fig. 7 A). Schwann cells that were stained by laminin γ1 always showed Krox-20 staining in their nuclei (Fig. 7, B–F). However, some Schwann cells lacked laminin γ1 (Fig. 7, A and B). Those Schwann cells that lacked laminin γ1 expression were also negative for laminin α2 (unpublished data). Thus, Schwann cells that lack laminin expression can migrate along the axon and populate the nerve. We compared the staining pattern for laminin γ1 and Krox-20 expression in the nuclei and found that Schwann cells that did not produce laminin γ1 also did not show detectable Krox-20 in their nuclei (Fig. 7, A–F). This result indicates that laminin expression is important for Krox-20 induction and initiation of myelination process. Next, we compared the relationship between laminin γ1 expression and synthesis of myelin basic protein (MBP), a major myelin component. The results show that laminin γ1 expression is also important for MBP synthesis. Schwann cells expressing laminin γ1 produced MBP, whereas Schwann cells that did not express laminin γ1 also lacked MBP (Fig. 7, G–J). Examination of many laminin γ1–negative Schwann cells revealed that their S-100 expression level was slightly reduced (Fig. 7, A, B, G, and H); the reason for this phenomenon was not clear. Investigation of many mutant nerves at P28 showed a 100% correlation between Schwann cells that did not produce laminin γ1 and those that did not produce Krox-20 and MBP and failed to myelinate. Further analysis revealed that Schwann cells that lack laminin expression did not produce other myelin proteins such as P_0 (unpublished data). These results were also supported by ultrastructural analysis of the mutant nerves, which showed that Schwann cells that lack laminin γ1 were present in nerves but had a discontinuous basal lamina, were arrested at the premyelination stage, and therefore did not form myelin (see the next paragraph and see Fig. 8, C and D). These results indicate that the production of laminin is essential for Schwann cell differentiation and synthesis of myelin proteins.

Laminin γ1 is necessary for Schwann cell basal lamina formation during development

Studies in vitro have suggested that basal lamina formation is important for myelination, and it is known that laminin is a major component of the basal lamina. In a serum-free culture system, without induction of basal lamina assembly, Schwann cells proliferate but fail to differentiate and cannot form myelin. Therefore, the basal lamina is essential for the initiation of myelination in vitro (Carey et al., 1986; Clark and Bunge, 1989; Eldridge et al., 1989). To investigate the role of laminin γ1 in basal lamina formation and the function of the basal lamina in Schwann cell differentiation and myelination in vivo, we examined the Schwann cells associated with unsorted axonal bundles. In P15 mutant sciatic nerve, the unsorted axonal bundles have a mixture of different sized axons, and the Schwann cells closely associated with unsorted axonal bundles lack a continuous basal lamina (Fig. 8, C and D, S2). Even though such Schwann cells sent abnormally thick processes between axons, they failed to sort and myelinate the axons. In contrast, an adjacent Schwann cell (Fig. 8 C, S1) had a continuous basal lamina surrounding the cell membrane (Fig. 8 D, arrowheads, fuzzy material) and formed myelin. This result shows that laminin γ1 is essential for formation of a continuous basal lamina, which in turn is critical for myelination in vivo.

Regeneration of sciatic nerve after injury is impaired in the mutant mice

Because the laminin γ1−/− sciatic nerves are partially myelinated, and there were large bundles of naked, unsorted axons,

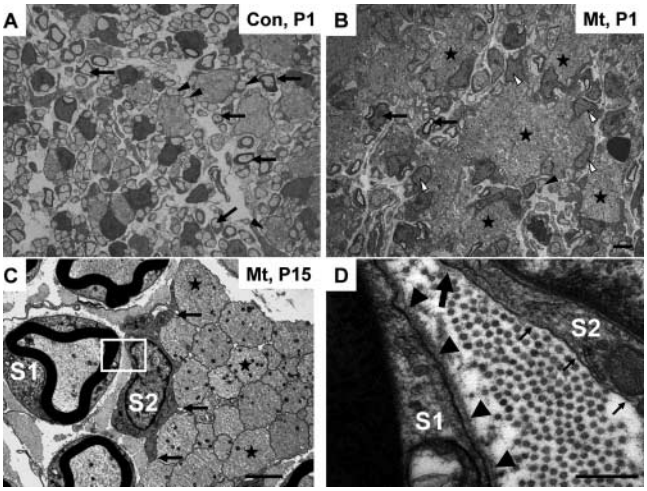

Figure 8. **Ultrastructure of *CaMKII/Cre:fLAMγ1* sciatic nerve shows impairment of axon sorting and basal lamina formation.** Electron microscopy of transverse sections of control (A) and mutant sciatic nerves (B) at P1. In control, many axonal bundles were sorted by Schwann cells, large axons have been segregated at the peripheral of the bundles (A, arrowheads) and many of them have formed a 1:1 relationship with a Schwann cell (A, arrows). In contrast, in the mutant nerve, the sorting was impaired, there were large axonal bundles (stars) but very few axons had been sorted (B, closed arrowhead) or formed a 1:1 relationship with a Schwann cell (B, arrows). Note that there were many Schwann cells in the mutant sciatic nerve (B, open arrowheads). At P15 in the mutant nerve, the unsorted axonal bundles contained different sized axons (C, stars). The Schwann cell closely associated with the naked axonal bundles (S2) sent abnormally thick processes between axons (arrows) but did not make myelin. Immediately adjacent to S2 is a Schwann cell (S1) with normal myelin. (D) Higher magnification of the boxed region in C shows that the myelinating Schwann cell S1 had a continuous basal lamina (arrowheads) attached to the cell membrane, whereas Schwann cell S2 did not form a continuous basal lamina (denuded areas indicated by thin arrows) and only had small patches of ECM attached to the cell membrane (thick arrow). Bars: (A and B) 2 μm; (C) 1 μm; (D) 0.5 μm.

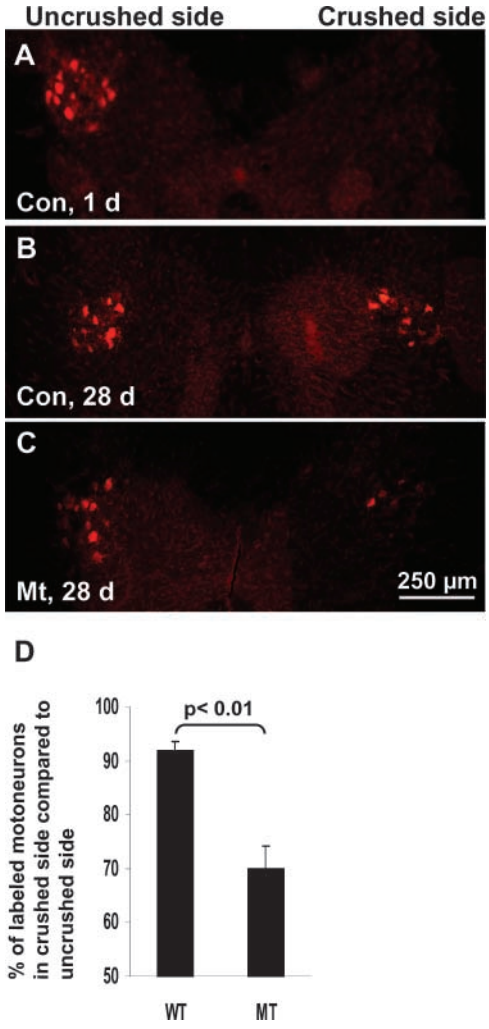

Figure 9. **Mutant sciatic nerves with reduced laminin γ1 expression are defective in axonal regeneration after injury.** 1 or 28 d after sciatic nerve crush, the nerves were cut 7 mm distal to the lesion, and a crystal of the retrograde tracer fluororuby was applied to the cut nerve. On the uncrushed side, the sciatic nerve was cut at a similar place as the crushed side and a crystal of fluororuby was applied. 3 d later the spinal cords were analyzed for labeled motoneurons. (A) 1 d after crush, there were no labeled motoneurons on the crushed side but many on the uncrushed side, indicating that the crush was complete and there was no leakage of the dye. (B) 28 d after crush in the wild-type animal, most of the motoneurons on the crushed side were labeled (compare with the uncrushed side). (C) In contrast, in the mutant mice, the crushed side showed many fewer labeled motoneurons compared with the uncrushed side. (D) The percentage of labeled motoneurons in the crushed side compared with the uncrushed side in six control and six mutant mice was quantitated, and the difference between control and mutant animals was significant.

we examined if the reduced matrix production would have consequences for regeneration of the nerve after injury. Adult control and CaMKII/Cre:fLAMγ1 sciatic nerves were crushed, and after 28 d, the retrograde dye fluororuby was applied distal to the injury. By determining the number of labeled motoneurons in the spinal cord, we could evaluate functional nerve regeneration after injury. There was dye transfer in both control and mutant nerves in the uninjured side as expected. Immediately after crush, there was no dye transfer (Fig. 9 A), reflecting the completeness of nerve crush and no leakage of the dye to the surrounding muscles. After 28 d, the control nerve showed significant regeneration (Fig. 9 B) compared with the uninjured side, whereas this process was greatly reduced in mutant nerves (Fig. 9, C and D). These results indicate that laminin γ1 affects the capacity of axons to regenerate after injury.

Discussion

This work shows that laminin synthesis by Schwann cells is essential for myelination. Without laminin, Schwann cells can migrate along the axons, populate the peripheral nerve, and proliferate normally, but cannot differentiate into a myelinating phenotype. These results should be considered from various points of view: (1) How do these results add to our knowledge of the role of laminin γ1 in PNS in vivo? (2) Which receptors does laminin use to affect Schwann cell differentiation? (3) How does laminin affect axon regeneration?

Function of laminin γ1 in the developing PNS

The function of laminin γ1 in the nervous system is associated with neurite outgrowth and axon guidance (Lander et

al., 1985; Luckenbill-Edds, 1997; Liesi et al., 2001; Wiksten et al., 2003). Recent work has also shown that it is essential for mossy fiber regeneration in the hippocampus (Grimpe et al., 2002). Laminin γ1 has been speculated to be important in the PNS, but there has been no direct in vivo study. Our results revealed several essential roles for laminin γ1 in the PNS. First, the expression of laminin γ1 is required for the presence of other laminin chains. The major laminin expressed in the PNS is laminin-2, which is composed of α2, β1, and γ1 chains. In the mutant sciatic nerve where laminin γ1 is absent, we could not detect laminin α2 and β1 chains, indicating that without laminin γ1 the other chains for laminin-2 do not accumulate. The reason for the absence of other laminin chains is not clear, but could be transcriptional, translational, or posttranslational.

Second, laminin γ1 is essential for axonal radial sorting and myelination. During later embryonic and early postnatal stages, laminin γ1 is strongly expressed in Schwann cells. A possible mechanism for laminin γ1 in axon sorting and myelination is that laminin binds to its receptors, such as integrins, and activates signaling pathways that result in the activation of the program to sort axons and initiate myelination. The laminin γ1–negative Schwann cells lacked nuclei Krox-20 staining, indicating the initiation of myelination was blocked. Nickols et al. (2003) recently showed that NF-κB activation is important for axon sorting and myelination; blocking NF-κB activation attenuated myelination, but laminin synthesis and basal lamina formation was not affected. This result suggests that laminin expression is an upstream event in axon sorting and myelination (Fig. 10).

Third, laminin γ1 is required for the formation of a continuous basal lamina during peripheral nerve development. The mutant Schwann cells lacked a continuous basal lamina, whereas in the same stage in the control nerves, all Schwann cells examined formed a continuous basal lamina. The Schwann cells without a continuous basal lamina also lacked a myelin sheath during development. At P28, there was an exact correlation between Schwann cells that did not produce laminin γ1 and those that did not produce MBP and did not myelinate, indicating that laminin is essential for Schwann cell terminal differentiation. These functions of laminin in the developing PNS may depend on each other (Fig. 10).

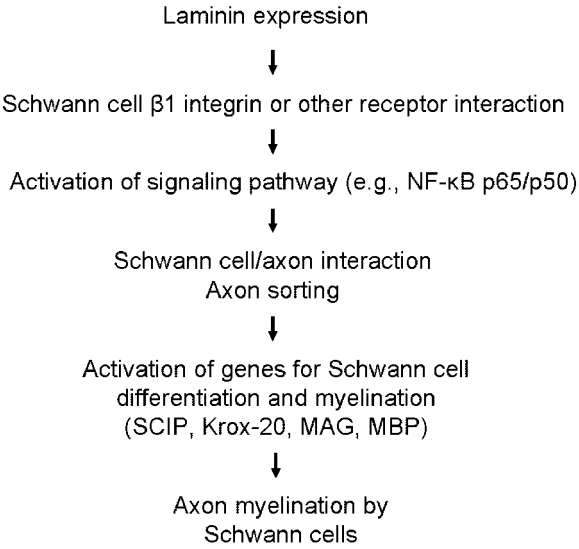

Figure 10. **Model of laminin function in peripheral nerve myelination.** During peripheral nerve development, laminin expressed in Schwann cell binds to integrin or other receptors. This may activate signaling pathways such as NF-κB p65/p50, allow proper axon sorting by Schwann cells, and activate genes for differentiation and myelination such as SCIP and Krox-20.

Receptors for laminin that control myelination

ECM proteins interact with surface receptors to activate signaling pathways that influence cell viability and function. There are three established integrin receptors for laminin on Schwann cells, α1β1, α6β1, and α6β4 (Einheber et al., 1993; Colognato et al., 1997; Yurchenco et al., 1997). Dystroglycan also functions as a laminin receptor (Previtali et al., 2001), and mice lacking this protein in Schwann cells exhibit dysmyelination (Saito et al., 2003). Work in cell culture and in vivo has shown that interfering with β1 integrin function can inhibit myelination (Podratz et al., 2001; Relvas et al., 2001; Feltri et al., 2002). The similarity between the phenotypes in mice with a Schwann cell defect in β1 integrin (Feltri et al., 2002) and laminin γ1 indicate that this integrin is playing a central role in laminin signaling. Whether β1 integrin is paired with α1 or α6 in the PNS remains to be determined. However, because α1 knockout mice do not have a myelination phenotype, whereas α6 knockout mice have fewer myelin forming oligodendrocytes in the CNS (Colognato et al., 2002), α6 may be the more likely partner.

There appears to be a subtle difference in the response of Schwann cells to the absence of β1 integrin and laminin γ1. In the absence of β1 integrin, Schwann cells could still produce MBP due to a switch to other receptors such as α6β4 and dystroglycan (Feltri et al., 2002). In contrast, Schwann cells that lack laminin γ1 were never observed to differentiate or myelinate axons.

Laminin γ1 in nerve regeneration

The regeneration of peripheral nerves after injury is known to depend on Schwann cells in areas distal to the injury (Fawcett and Keynes, 1990). It is thought that the Schwann cells provide a guide by which regrowing axons can precisely extend and reenervate the appropriate targets. In the distal areas, axons degenerate, and so Schwann cells cannot be supported by axon-generated molecules. Meier et al. (1999) have proposed an elegant hypothesis whereby Schwann cells at early time are dependent on axons to ensure a proper ratio of myelinating cells to axons. However, once mature, the Schwann cells become axon independent to facilitate axon regrowth in case of injury. If laminin is part of the autocrine loop that helps Schwann cells become axon independent, then the absence of this matrix would lead to more rapid Schwann cell death after Wallerian degeneration, which would in turn inhibit axon regeneration.

A role of laminin γ1 in nerve regeneration in the CNS has been previously investigated by blocking its expression with antisense DNA oligonucleotides. Although most CNS axons cannot regenerate, hippocampal mossy fiber axons severed in the early postnatal rat can (Zimmer and Gahwiler, 1987).

In hippocampi in which laminin γ1 expression had been blocked, glial repair of the lesion was unaffected, but most regenerating fibers did not cross the lesion, and if they did, they had shorter extensions and a more convoluted path (Grimpe et al., 2002). In our experiments with peripheral nerves, even 28 d after injury, the functional axon regeneration in mutant animals was still much less than the control. These combined results indicate that the laminin γ1 protein has a critical function in axon regeneration.

Materials and methods

Generation of mice with floxed *laminin γ1* gene

An AvrII–XcmI genomic DNA fragment containing exon 2 of the *laminin γ1* gene was isolated from a BAC-vector (Genome System; Fig. 1). The fragment was cloned into the PKS+II vector. The loxP cassette plasmid DNA was provided by G.R. Martin (University of California, San Francisco, San Francisco, CA). The upstream loxP site with the PGKneo gene selection cassette was inserted into a HindIII site 332 bp upstream of exon 2 (in intron 1). The downstream loxP site was inserted into a Tth111I site 235 bp downstream of exon 2 (in intron 2). The whole construct was sequenced, and showed correct insertion of the cassette and loxP sites into *laminin γ1* gene fragment (allele designated *fLAMγ1*).

E14 embryonic stem cells were grown on feeders and electroporated with 40 μg of linearized targeting vector. After 8–10 d in neomycin selection medium, colonies were picked, expanded, and screened by PCR and Southern blot. For PCR screening, the upstream primer was specific for the second loxP site, and the downstream primer was located in exon 3 of the *laminin γ1* gene and outside of the targeting vector. The positive clones were analyzed by further PCR using several different primer sets that allowed determination of the position of the two loxP sites and the location of targeting vector insertion into the wild-type *laminin γ1* gene. Furthermore, the positive clones were confirmed by Southern blot, which also showed correct targeting of the vector into the wild-type *laminin γ1* gene. The positive clones were used for injection of C57Bl/6 blastocysts. Chimeric mice were mated to C57Bl/6 mice, and the offspring carrying the floxed allele were bred to homozygosity. All experiments on the mice were performed in accordance with Animal Welfare guidelines at the Rockefeller University.

Generation and analysis of Cre-mediated recombination in *CaMKII/Cre:fLAMγ1* and *P_0/Cre:fLAMγ1* mice

Homozygous *fLAMγ1* mice were mated with either *CaMKIIα-Cre* transgenic mice line R1ag#5 (a gift from A. Morozov and E.R. Kandel, Columbia University, New York, NY; Dragatsis and Zeitlin, 2000) or with *mP_0TOTA*(Cre) transgenic mice (a gift from L. Feltri and L. Wrabetz, San Raffaele Scientific Institute, Milan, Italy; Feltri et al., 1999). The double heterozygous mice (*fLAMγ1/+:CaMKII-Cre/+* or *fLAMγ1/+:P_0-Cre/+*) were mated with homozygous *fLAMγ1* mice. Genomic DNA prepared from tails was analyzed by PCR (primer positions shown in Fig. 1 A). The primer sequence for P1 was 5′-CTC AGA GCT GGC TTC TCA C AT-3′ and for P2 was 5′-GAT TTT CAA AGA AGC AGA GTG TG-3′. The PCR conditions were 94°C for 2 min, followed by 35 cycles (94°C for 45 s, 56°C for 45 s, and 72°C for 3 min and 30 s), and 10-min extension at 72°C. The PCR products were the following: wild-type allele, 1.3 kb; *fLAMγ1* allele, 3.2 kb; recombined allele, 2.3 kb (Fig. 1 B).

Immunohistochemistry

Mutant and control mice at different ages were injected intraperitoneally with atropine (0.6 mg/kg of body weight) and were anesthetized deeply with 2.5% avertin (0.02 ml/gram of body weight). After cold PBS perfusion, the sciatic nerves were dissected and cryosections were prepared. For immunostaining, the sections were fixed in 4% PFA in 0.1 M phosphate buffer for 30 min. After washing in PBS, they were blocked in 0.3% Triton X-100 and 5% normal goat serum in PBS. The primary antibodies used were rabbit anti–S-100 and rat anti–laminin α2 (Sigma-Aldrich), rat anti–laminin β1 and γ1 (Chemicon), rabbit anti-human MBP (DakoCytomation), mouse anti-BrdU (Roche), rabbit anti–Krox-20 antibody (Babco), and rabbit anti–neurofilament H (Chemicon). The sections were incubated with primary antibody in 0.3% Triton X-100 and 3% normal goat serum in PBS at 4°C overnight. After rinsing in PBS, the sections were incubated with the appropriate secondary antibodies for 2 h. The sections were rinsed in PBS, coverslipped, and examined using fluorescent microscopy (model Axioskop 2; Carl Zeiss MicroImaging, Inc.). For the triple fluorescent immunostaining, the sections were first incubated with rat anti–laminin γ1 antibody and rabbit anti-MBP or Krox-20 antibody at 4°C overnight and incubated with the appropriate secondary antibodies. The rabbit anti–S-100 antibody (Sigma-Aldrich) was labeled with a fluorescein rabbit IgG labeling kit (Molecular Probes). The sections were incubated with this FITC-labeled S-100 antibody for 2 h, rinsed, and fixed in 4% PFA in 0.1 M phosphate buffer. After washing, the sections were coverslipped and examined. Photographs were taken using an AxioVision System.

Western blot analysis

Control and mutant mice at P28 were perfused with cold PBS. The sciatic nerves were removed, frozen in dry ice immediately, homogenized in 2% Triton-X 100, 0.5 M NaCl, 10 mM Tris, pH 7.4, and 1 mM PMSF (Sigma-Aldrich) using a dounce homogenizer on ice, incubated for 30 min, and centrifuged. The extracts were run on a reducing 4–15% SDS-PAGE, blotted to PVDF membrane (Immobilon-P; Millipore), incubated using primary antibody (rat anti–laminin α2 mAb, 1:1,000, Sigma-Aldrich; rat anti–laminin β1 mAb, 1:1,500, Chemicon; rat anti–laminin γ1 mAb, 1:1,000, Chemicon) overnight at 4°C; incubated with HRP-labeled goat anti–rat secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at RT, and stained using SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.). After stripping, the membranes were reblotted with mouse antiactin antibody (Sigma-Aldrich), 1:8,000.

BrdU incorporation assay

Assays for BrdU incorporation were performed as described by Feltri et al. (2002). In brief, double heterozygous female mice (*fLAMγ1/+* and *CaMKII-Cre/+*) were mated with homozygous *fLAMγ1* male mice. 17 or 19 d after detection of a vaginal plug, they were injected intraperitoneally with 100 μg BrdU/gram of body weight. 1 h later, the fetuses were dissected and frozen in dry ice, and the tails were cut for genotyping. The fetuses were thawed at RT; and their sciatic nerves were dissected, embedded in OCT, and processed for sectioning. The sciatic nerve sections were fixed in cold methanol, treated with 2 N HCl for 15 min at 37°C, and neutralized with 0.1 M sodium borate, pH 8.5, for 10 min. Sections were incubated with mAb anti-BrdU (Roche) and the appropriate secondary antibody. The sections were further stained with rabbit anti–neurofilament H antibody and visualized using FITC-conjugated goat anti–rabbit IgG secondary antibody to stain the axons. The nuclei were counterstained with DAPI. Only cigar-shaped nuclei were counted, and double-labeled nuclei (both BrdU and DAPI) were determined. At each time point, six control and six mutant embryos were analyzed. The differences in the percentage of BrdU incorporated nuclei between control and mutant sciatic nerves were analyzed by the Mann-Whitney U test.

TUNEL staining

For TUNEL staining, the In Situ Cell Death Detection kit (Roche) was used. Cryo-sciatic nerve sections from E17.5, E19.5, and P5 mutant and control mice were fixed in 4% PFA in PBS, pH 7.4, for 20 min, and permeabilized in 0.1% Triton X-100/0.1% sodium citrate on ice for 2 min. The sections were stained with In Situ Cell Death Detection kit (Gavrieli et al., 1992). Nuclei counterstaining was performed with DAPI. Double-labeled nuclei were determined. At each time point, six control and six mutant animals were analyzed. The differences in the percentage of TUNEL-positive nuclei in control and mutant sciatic nerves were analyzed by the Mann-Whitney U test.

Richardson's staining and EM analysis

Mice at different ages were anesthesized and the sciatic nerve was exposed. 3% glutaraldehyde in 0.1 M pb, pH 7.2, was applied to the nerve to immerse it for 5 min. The nerve was dissected and immersed in 3% glutaraldehyde in 0.1 M pb for 24 h, postfixed in 2% osmium tetroxide solution, and embedded in resin. Semi-thin sections were cut and stained with Richardson's staining, which stains proteins. For the EM analysis, ultra-thin sections were cut on a Reichert-Jung Ultracut E microtome and poststained with uranyl acetate and lead. Sections were examined and photographed on a JEOL100CXII at 80 kV.

Surgery

Mutant or control mice 8–10 wk old were injected intraperitoneally with atropine (0.6 mg/kilogram of body weight) and anesthetized deeply with 2.5% avertin (0.02 ml/gram of body weight). The sciatic nerve was exposed in the upper thigh and crushed with No. 5 Dumont Mirror finish forceps (Fine Science tool) three times, for 20 s each. The crush site was marked with India ink, and the skin was sutured (Akassoglou et al., 2000).

Motoneuron tracing and histological analysis

1 or 28 d after sciatic nerve crush, mice were anesthetized deeply. The sciatic nerve on the crushed side was cut 7 mm distal to the lesion, the sciatic nerve on the uncrushed side was cut at a similar place, and crystals of fluororuby (Molecular Probes) were applied to the cut ends. Then, the nerves were inserted into a small polyethylene tube with one end sealed to prevent leakage of the dye to the surrounding muscles. The wound was sutured. 3 d later, the mice were perfused with PBS followed by 4% PFA. Spinal cords from L1 to S2 were removed and postfixed in 30% sucrose in 4% PFA overnight. Serial cryo-sections were prepared at 30 μm, dried, coverslipped with DPX, and examined using fluorescence microscopy (excitation 556 nm; emission 580 nm). All the labeled neurons were within this region and were counted on each section on both sides. Six control and six mutant mice were analyzed. In each animal, the number of labeled motoneurons on the crushed side was compared with the uncrushed side and a percentage was obtained. The differences in percentage of labeled motoneurons in the crushed side compared with the uncrushed side in control and mutant sciatic nerves were analyzed by the Mann-Whitney U test.

Imaging analysis

Tissue sections after staining were examined with a microscope (model Axioskop 2; Carl Zeiss MicroImaging, Inc.) and photographed using an AxioVision System (Carl Zeiss MicroImaging, Inc.). The electron microscope micrographs and Western blot films were digitized using a scanner (Microtek). The images were processed using Adobe Photoshop 7.0 and figures were prepared using Microsoft PowerPoint.

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