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

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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 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 mice. 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.

Key words: paralysis; axon sorting; Cre-loxP; extracellular matrix

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To investigate the function of laminin γ1 in development and in adults in vivo, 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 (neo8) gene for selection of the transformed cells. This neo8 gene was flanked by fRT sites that would allow its removal by the flp recombinase because in some cases the presence of the neo8 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 neo8 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 (CaMKIIα/CaMKIIα, 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

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**Figure 3. Reduced expression of laminin α2, β1, and γ1 chains in the sciatic nerve of CaMKIIα/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 P0 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/CreflAMγ1 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 dy1/dy1 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).

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 P0 promoter, which is Schwann cell specific (Feltri et al., 1999, 2002), to drive Cre expression. The nerves in P0/CreflAMγ1 mice showed a similar defect in axon sorting and myelination as the CaMKII/CreflAMγ1 mice (Fig. 5, M and N). This result shows that the phenotype in the CaMKII/CreflAMγ1 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 4. CaMKII/CreflAMγ1 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.
mutant nerves showed large bundles of unsorted axons co-mixed 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 5. Nerves from CaMKII/Cre:RAMγ1 and P0/Cre:ILAMγ1 mice have defective axonal sorting and myelination. (A–H) Transverse sections of sciatic nerve from control (A, C, E, and G) and CaMKII/Cre:ILAMγ1 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 (B, 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 P0/Cre:ILAMγ1 mutant mice at P28 (N, control in M) showed a similar phenotype to those from CaMKII/Cre:ILAMγ1 mice. Arrows, unsorted axonal bundles; arrowheads, myelinated axons.

Figure 6. Schwann cells in CaMKII/Cre:ILAMγ1 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.
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 P0 (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 premelination 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,
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
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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 reinnervate 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 independent of axons 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 (Grümppe 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 Avrl–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 Th111I 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 (alleles designated ILAM1).

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/fLAM1 and P0/CaMKII-Cre/fLAM1 mice

Homozygous ILAM1/mice were mated with either CaMKIIα-Cre transgenic mice line R1ag5 (# a gift from A. Morozov and E.R. Kandel, Columbia University, New York, NY; Dragatis and Zeilin, 2000) or with mP0/TOTACre transgenic mice (a gift from L. Feltl and W. Brabetz, San Francisco). The upstream Cre site was inserted into a HindIII site 84 bp upstream of exon 1 (in intron 2). The downstream Cre site was inserted into a HindIII site 347 bp downstream of exon 2 (in intron 2). The whole construct was sequenced, and showed correct insertion of the cassette and Cre sites into laminin γ1 gene fragment (alleles designated ILAM1 and ILAM2). Cre (+/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 (ILAM1+/Cre:CaMKII-Cre/+ or ILAM1+/-:P0/CaMKII-Cre/+ were mated with homozygous ILAM1 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 anesthetized 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

Mice at 8–10 wk or 8–10 d old were injected intra-peripherally with atrpine (0.6 mg/kg of body weight) and anesthetized deeply with 2.5% avertin (0.02 ml/kg of body weight). The sciatic nerve was exposed, the upper thigh and gluteus maximus muscles were removed, and fixed in 4% PFA in 0.1 M phosphate buffer. After washing, the sections were dehydrated, critical-point dried with liquid CO2, and mounted on aluminum stubs. Photographs were taken using an AxioVision System.
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 uncushered side was cut at a similar place, and crystals of fluoro- ruby ( 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 uncushered side and a percentage was obtained. The differences in percentage of labeled motoneurons in the crushed side compared with the uncushered 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.

References

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