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Coordinate control of axon defasciculation and myelination by laminin-2 and -8

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Schwann cells form basal laminae (BLs) containing laminin-2 (Ln-2; heterotrimer α2β1γ1) and Ln-8 (α4β1γ1). Loss of Ln-2 in humans and mice carrying α2-chain mutations prevents developing Schwann cells from fully defasciculating axons, resulting in partial amytelination. The principal pathogenic mechanism is thought to derive from structural defects in Schwann cell BLs, which Ln-2 scaffolds. However, we found loss of Ln-8 caused partial amytelination in mice without affecting BL structure or Ln-2 levels. Combined Ln-2/Ln-8 deficiency caused nearly complete amytelination, revealing Ln-2 and -8 together have a dominant role in defasciculation, and that Ln-8 promotes myelination without BLs. Transgenic Ln-10 (α5β1γ1) expression also promoted myelination without BL formation. Rather than BL structure, we found Ln-2 and -8 were specifically required for the increased perinatal Schwann cell proliferation that attends myelination. Purified Ln-2 and -8 directly enhanced in vitro Schwann cell proliferation in collaboration with autocrine factors, suggesting Lns control the onset of amytelination by modulating responses to mitogens in vivo.

Introduction

Myelin increases the speed of neural conduction in thin axons. Defects in myelination cause debilitating loss of function in a variety of congenital and acquired neurological disorders. Mechanisms coordinating myelination in the peripheral nervous system are poorly understood, despite descriptions of cellular events (Martin and Webster, 1973; Webster et al., 1973) and the identification of molecular cues to developing Schwann cells (Mirskey et al., 2002). We show that two members of the laminin (Ln) family of glycoproteins act in concert to regulate the onset of myelination in peripheral nerves.

Peripheral myelination is a concerted process in which Schwann cell proliferation, axon defasciculation, and myelination assembly overlap (Webster, 1971; Martin and Webster, 1973; Webster et al., 1973; Stewart et al., 1993). Premyelinating Schwann cells cover fascicles of cotargeted axons. Their proliferation rate initially matches axonal growth, but increases during myelination to supply Schwann cells for individual axons, at perinatal ages in rodents. Progeny invade fascicles after longitudinal division, which increases Schwann cell density along subsets of axons. Invading cells often transiently ensheath several axons, but retract all but one process and myelinate a single axon. Recurrence of these events ultimately reduces fascicles to axons lacking promyelinating signals, which are defasciculated but remain unmethylated by the final Schwann cell progeny. Webster described the progressive defasciculation and myelination of peripheral axons as radial sorting, and proposed that Schwann cell proliferation is intimately involved in the commitment of longitudinal cohorts to defasciculate and ensheath subjacent axons (Webster, 1971; Martin and Webster, 1973; Webster et al., 1973). Although neuregulins have been identified as key signals for Schwann cell proliferation (Garratt et al., 2000), molecular mechanisms that accelerate perinatal proliferation and propel radial sorting are not known.

The one factor known to have specific roles in radial sorting is Ln-2 (merosin), a major component of the Schwann cell surface basal lamina (BL). Lns comprise a family of αβγ heterotrimeric. Loss of Ln-2 through mutations in the α2 chain causes a complex neuromuscular disease including peripheral dysmyelination. In the most studied dyt and dyt2J strains of Ln α2 mutant mice, peripheral nerves contain bundles of unsheathed axons that resemble embryonic fascicles (Bradley and Jenkinson, 1973; Biscoe et al., 1974). This unique pattern of dysmyelination...
presumably represents incomplete radial sorting and has therefore been termed “amyelination.”

Mechanistic hypotheses for amyelination presume endoneurial BLs are necessary for Schwann cell motility and/or differentiation during rapid remodeling (Madrid et al., 1975; Bunge, 1993; Feltrei et al., 2002; Chen and Strickland, 2003). Lns that self-polymerize, including Ln-2, are the key structural component of BLs (Yurchenco et al., 2004), and Ln-2–deficient Schwann cells form patchy, discontinuous BLs (Yurchenco et al., 2004), and Ln-2–deficient nerves; lack of SNAREs (Madrid et al., 1975). However, only spinal roots and cranial nerves are severely amyelinated in dy and dy2J mice; sciatic nerves are partially affected and brachial nerves are nearly normal (Bradley and Jenkinson, 1975; Stirling, 1975; Weinberg et al., 1975). One possibility is that BL structure and Ln have limited roles in radial sorting, only critical in large nerves. Alternatively, loss of Ln-2 may be partially compensated by isoforms containing the α1, α4, and α5 chains. Ln α1 is absent in normal nerves, but is expressed in dy2J sciatic nerves; lack of α1 expression in dy2J spinal roots may account for severe amyelination there (Previtali et al., 2003b). Ln α5 is selectively expressed in roots (Nakagawa et al., 2001), which could interfere with α1-Ln heterotrimer assembly in dy2J. Ln α4 is normally low in mature nerves, but is up-regulated in developing nerves and α2-deficient nerves (Patton et al., 1997, 1999; Nakagawa et al., 2001). Targeted deletion of the Ln γ1 chain causes more widespread peripheral dysmyelination than occurs in dy mice, consistent with roles for multiple isoforms (Chen and Strickland, 2003). Here, we address independent and combined roles of Lns containing the α2, α4, and α5 chains.

Results

Neuromuscular dysfunction and peripheral neuropathy

When lifted by the tail, Ln α4-deficient mice (Lama4Δ4–) retracted hindlimbs toward the body, with toes clenched (Fig. 1b). In contrast, normal and heterozygous Lama4Δ4/Δ4 littermates extended limbs downward, potentially minimizing fall injuries (Fig. 1a). Lama4Δ4– hindlimb retractions often progressed to rigid rearward extension (Fig. 1c), but ceased upon landing. Forelimbs were unaffected. Similar suspension-induced hindlimb retraction was observed in juvenile Lama262J (dy2J) mice (Fig. 1d), before permanent contractures (Fig. 1e). The overlap in dysfunction suggested Lama4Δ4– might possess an abbreviated form of Ln α2-deficient neuromuscular disease.

Ln α2 and α4 are coexpressed in developing muscles and nerves (Patton et al., 1997). As Lama4Δ4– has no apparent myopathy and limited defects at neuromuscular junctions (Patton et al., 2001), we assessed peripheral myelination (Fig. 1, f–j). Normal nerves are composed of large myelinated axons and thin axons ensheathed by nonmyelinating Schwann cells. In addition to properly myelinated axons, Lama4Δ4– sciatic nerves contained bundles of axons lacking ensheathment. EM confirmed such bundles were largely devoid of Schwann cell processes, and found no solitary naked axons. Premyelinating Schwann cells associated with the large bundles occasionally extended processes between axons or established a promyelinating relationship with a solitary axon (Fig. 1k; unpublished

Figure 1. Amyelinating peripheral neuropathies in Lama4Δ4– and dy2J mice. (a–d) Overlapping postural defects. When suspended, wild type (a) mice extend limbs downward, whereas Lama4Δ4– mice retract and then extend hindlimbs backward (b and c). dy2J mice retract hindlimbs at juvenile ages (d, 4 wk), before the onset of permanent contractures (e, 3 mo). (f–j) Toluidine blue–stained resin sections of adult control (f), Lama4Δ4– (g and h), and dy2J (i and j) sciatic nerves at low (f, g, and i) and high (h and j) magnification. Bundles of unsheathed axons are present in mutants, but not controls. (k–o) Electron micrographs show most bundles lack intervening Schwann cell processes. Some Lama4Δ4– Schwann cells along large bundles establish promyelinating relations with single axons (k, asterisk), but usually myelinate small bundles altogether (l and m; Table I). Some polygonal myelination included intervening Schwann cell processes (m, arrows), possibly from adjacent cells along the nerve. In dy2J, polygonal myelination was rare (o, left), but large rafts of partially defasciculated, unmyelinated, mixed caliber fibers were common (o, right). Bar in f, 38 μm (f, g, and i); 15 μm (h and j); 3 μm (k, l, n, and o); 1.8 μm (m).
data). However, most small bundles were polyaxonally myelinated (Fig. 1, l and m; Table I). Defects in Lama4/H11002 were remarkably similar to amyelination described in dy and dy2J mice (Bradley and Jenkison, 1973; Biscoe et al., 1974; Weinberg et al., 1975; Okada et al., 1977) (Fig. 1, i and j). Indeed, quantitative analysis of the tibial branch revealed no significant differences between dy2J and Lama4/H11002 in the number of amyelinated axons or their distribution in bundles (Table I). In both dy2J and Lama4/H11002, bundles contained mixed caliber axons. The number of larger axons (minimum diameter $\geq 2 \mu m$) in Lama4/H11002 bundles (average $\pm$ SEM; 292 $\pm$ 86/tibial nerve; $n = 4$) was similar to deficits in myelinated axons. Isolated axons were well myelinated and degenerating myelin figures were absent in both mutants. Finally, although we could not rule out limited central nervous system (CNS) defects in Lama4/H11002, we found no CNS amyelination in either mutant (Fig. 1, g and i; insets). Thus, independent genetic lesions in Ln H92512 and /H92514 produce essentially similar amyelinating peripheral neuropathies.

Table I. Amyelination in nerves and roots of Ln $\alpha$2- and $\alpha$4-deficient mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild type</th>
<th>Lama4 $^{-/-}$</th>
<th>Lama2$^{dy2J}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibial N. (n)</td>
<td>3</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Myelinated axons</td>
<td>2226 $\pm$ 16</td>
<td>1697 $\pm$ 49*</td>
<td>1258 $\pm$ 16*</td>
</tr>
<tr>
<td>Amyelinated axons</td>
<td>0</td>
<td>2043 $\pm$ 458</td>
<td>2380 $\pm$ 600</td>
</tr>
<tr>
<td>Bundles</td>
<td>0</td>
<td>31 $\pm$ 4</td>
<td>31.0 $\pm$ 1.4</td>
</tr>
<tr>
<td>Nonmyelinated bundles</td>
<td>NA</td>
<td>17 $\pm$ 3**</td>
<td>28.5 $\pm$ 7**</td>
</tr>
<tr>
<td>&quot;myelinated&quot; bundles</td>
<td>NA</td>
<td>14 $\pm$ 2**</td>
<td>2.5 $\pm$ 2.1**</td>
</tr>
<tr>
<td>Axons/bundle (total)</td>
<td>NA</td>
<td>67 $\pm$ 16</td>
<td>77 $\pm$ 22</td>
</tr>
<tr>
<td>Axons/nonmyelinated bundle*</td>
<td>NA</td>
<td>105 $\pm$ 9</td>
<td>80 $\pm$ 9</td>
</tr>
<tr>
<td>Axons/myelinated bundle*</td>
<td>NA</td>
<td>18 $\pm$ 1</td>
<td>16 $\pm$ 5</td>
</tr>
<tr>
<td>V. root (n)</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Myelinated axons</td>
<td>760 $\pm$ 53</td>
<td>698 $\pm$ 56**</td>
<td>32 $\pm$ 7**</td>
</tr>
<tr>
<td>Amyelinated axons</td>
<td>0</td>
<td>21 $\pm$ 6**</td>
<td>502 $\pm$ 42**</td>
</tr>
<tr>
<td>Bundles</td>
<td>0</td>
<td>2 $\pm$ 1</td>
<td>10 $\pm$ 2</td>
</tr>
<tr>
<td>Axons/bundle (total)</td>
<td>NA</td>
<td>11 $\pm$ 1**</td>
<td>49 $\pm$ 21**</td>
</tr>
</tbody>
</table>

Values represent mean $\pm$ SEM across nerves. *, Different from wild-type value; $P < 0.05$. **, Difference between mutant values; $P < 0.01$.

*aErrors represent pooled bundles of all nerves.

Figure 2. Origin and distribution of amyelination in dy2J and Lama4 $^{-/-}$ mice. Toluidine blue stained sections from wild-type (a–g), dy2J (h–n), and Lama4 $^{-/-}$ (o–u) sciatic nerves at indicated postnatal age, or spinal roots and brachial nerves from 6–9-wk adults. Axon fascicles are sorted by P12 in controls, but persist as amyelinated bundles in mutants. Bundles in year-old mutants are small and surrounded by lightly myelinated fibers (k and l). Spinal roots are severely amyelinated in dy2J (l and m) but not Lama4 $^{-/-}$ (s and t). Brachial nerves contain few amyelinated axons in either mutant (n and u). Bar in o, 14 $\mu m$ (a, h, and o); 8 $\mu m$ (b, i, and p); 16 $\mu m$ (c, d, j, k, q, and r); 20 $\mu m$ (e–g, l–n, and s–u).
type, Lama2+/H11001, and Lama4+/H11002 sciatic nerves were sorted by P5 and myelinated by P12. In dy2J and Lama4+/H11002, axon fascicles persisted throughout postnatal development (Fig. 2, h–j and o–q). The proposition that axon bundles in dy strains arise through incomplete radial sorting (Bradley and Jenkison, 1973) is generally accepted, although lack of efficient methods to identify neonatal mutants prevented developmental studies. We confirm that amyelinated bundles are remnants of embryonic fascicles in both Lama2+/H11002 and Lama4+/H11002 mice. Amyelination has also been thought permanent (Bradley and Jenkison, 1973). However, we found only a few, small axon bundles remaining in year-old dy2J and Lama4+/H11002 sciatic nerves, each surrounded by lightly myelinated axons (Fig. 2, k and r). As axonopathy was not observed among amyelinated axons, bundles likely erode slowly through continued myelination at their edges (Fig. 1 k). Regardless, α2- and α4-deficient neuropathies did not diverge between onset and old age despite considerable remodeling.

Further comparison revealed two significant differences. First, amyelination is especially severe in dy2J spinal roots, but was nearly absent from roots in Lama4+/H11002 (Fig. 2, l, m, s, and t; Table I). Roots are less affected than distal nerves in Lama4+/H11002. We infer amyelination primarily reflects relative dependence on Ln isoforms, rather than nerve diameter or proximity to spinal origin. Sorting in roots depends strongly on Ln-2 and weakly on Ln-8; distal nerves rely more equally on both. Otherwise, amyelination was distributed similarly in Lama4+/H11002 and dy2J; forelimb and intercostal nerves were less affected than hindlimb (Fig. 2, n and u; unpublished data).

Second, polyaxonal myelination (Fig. 1, l, m, and o; Table I) was common in Lama4+/H11002 but rare in dy2J, consistent with previous observations in dy (Okada et al., 1977). Most...
small bundles (≤25 axons) in Lama4<sup>-/-</sup> tibial nerves were “myelinated.” Conversely, dy2J nerves contained many islands of ensheathed-but-not-myelinated axons, which were rare in Lama4<sup>-/-</sup> (Fig. 1 a, right). Superficially similar to Remak bundles, islands were less condensed, more numerous, and included mixed caliber axons. They appear to be abnormal transitional structures, intermediate between amelinated bundles and properly myelinated axons, which preferentially appear in dy2J nerves when fascicles contain few axons. Thus, although Ln-2 and -8 both promote axon sorting, they have decidedly unequal roles in roots, and dissimilar roles in the transition from premyelinating to myelinating Schwann cell phenotype.

**Redundancy and compensation in the BL**

To ask if Ln-2 and -8 independently incorporate into endoneurial BLs, we stained cryostat sections of normal and mutant sciatic nerves with Ln chain–specific antibodies. In normal nerves, Ln α4 was coconcentrated with the α2, β1, and γ1 chains at ab-axonal Schwann cell surfaces; none were detected at axonal surfaces (Fig. 3, d, e, m, and n; unpublished data). Detection of α4 varied considerably with antibody, but endoneurial BLs stained weakly compared with perineurium, as shown previously (Patton et al., 1997; Nakagawa et al., 2001). Loss of α4 did not affect staining for α2 at any postnatal age (Fig. 3 u; unpublished data). Similarly, α4 staining was not decreased in dy2J nerve; indeed, levels increased relative to controls, confirming previous results (Patton et al., 1997, 1999; Nakagawa et al., 2001) with additional reagents. In addition, staining for entactin, perlecan, agrin, and collagen IV was unaffected in either mutant (Fig. 3, g–i, p–r, and y–a'; unpublished data). Thus, amelination-inducing mutations in α2 and α4 do not act by inhibiting expression of their counterpart specifically, or disrupting the molecular composition of endoneurial BLs generally. We found no morphological or histological defects in double-heterozygous Lama2<sup>α2/β1</sup>;Lama4<sup>-/-</sup> offspring (not depicted). Therefore, Ln-2 and Ln-8 each contribute a distinct activity necessary to complete radial sorting.

Next, we asked if myelination achieved in dy2J and Lama4<sup>-/-</sup> reflects partial compensation between Ln-2 and -8, by generating double mutant dy2J;α4null mice (see Materials and methods). dy2J/α4null mice had normal birthweight, fed actively, and responded to stimuli, but were dyskinetic by P14. Adults had splayed stance and strong tremor, moved haltingly, retracted all limbs when suspended, were 50% smaller than normal littermates, and rarely survived 3 mo (Fig. 4, a–f). Peripheral nerves were translucent, rather than the opaque white of myelinated regions of the nervous system. Histological sections showed nearly all forelimb and hindlimb axons were concentrated in large amelinated bundles (Fig. 4, g and h), dramatically exceeding defects in the parent dy2J and Lama4<sup>-/-</sup> strains. Premyelinating Schwann cells surrounded bundles, and a few isolated axons were well myelinated, demonstrating neural crest migration and proliferation of Schwann cell precursors was not prevented. CNS myelination appeared unimpaired (Fig. 4 h, inset), showing potential metabolic disorders in these mice do not, per se, prevent formation of myelin. Thus, Ln-2 and -8 act in concert to play a dominant role in the myelination of distal nerve, and each only partially compensates the other’s deficiency.

Remarkably, however, spinal roots in dy2J/α4null mice were completely sorted. Compared with dy2J, the additional
Figure 5. Roles for Ln α5, not α1, in dy2J myelination. (a–c) Amyelination in Lna2/a4-DKO mice (P11 shown) includes spinal roots as well as distal nerve, revealing dy2J variant Ln-2 is required for sorting in dy2J/a4null roots. (d–l) Ln α1 is not detected in wild-type (d) or dy2J (e) sciatic nerves at juvenile ages (P8 shown), or adult dy3K (f). (g–l) In adult dy2J/a4null nerve, Ln α1 is detected on myelinated fibers but not amylinated bundles (g–i), and Ln α5 is restricted to perineurium and capillaries. (k and l) In dy2J/a4null/TgA5 nerve, Ln α5 is present in endoneurial BL, and radial sorting is greatly increased. BSs are present on myelinating (m) but not promyelinating (n) cells in dy2J/a4null/TgA5. Labels as in Fig. 4; NF, neurofilaments (green); my, myelin autofluorescence (blue in i). d–n; tibial branch, medial sciatic nerve. Bar in j, 25 μm in a–c; 45 μm in d; 30 μm in e, f, i, and k; 18 μm in g; 25 μm in h and i; 15 μm in l; 0.38 μm in m and n.

loss of Ln α4 strongly enhanced radial sorting in dy2J/a4null roots (Fig. 4, i and j; compare with Fig. 2, l and m). Paradoxically, Ln-8 promotes radial sorting in distal dy2J nerves but inhibits sorting in dy2J roots. To explain disparate results in root and distal nerve, we sought differences in Ln receptors and components of the endoneurial matrix that could modulate Schwann cell responses to Ln-2 or -8. We found no reliable differences in receptor components integrin α3, α6, β1, and β1D subunits, α-dystroglycan, β-dystroglycan, or β-sarcoglycan, between normal, dy2J, or Lama4−/− mice (unpublished data), extending results in dy2J and dy3K (Nakagawa et al., 2001; Previtali et al., 2003a,b). Two matrix differences between sciatic nerves and roots are reported; both are Ln α chains. Ln α1, which is absent in normal endoneurium, is expressed in dy2J sciatic nerves, but not their roots (Previtali et al., 2003b). In contrast, Ln α5 is expressed in normal and α2-deficient roots, but not distal nerve (Nakagawa et al., 2001).

Several aspects of Ln α1 expression were inconsistent with roles in radial sorting. First, α1 expression and myelination were poorly correlated in adult dy2J (6–9 wk). α1 was undetectable on many myelinated fibers (Fig. 3 k), and was absent from any brachial or sciatic fibers in 4 of 8 dy2J mice. Several α1 antibodies, which strongly stained CNS pial surfaces included as controls, gave similar results. Second, we were unable to detect α1 in dy2J nerves before P14, when radial sorting has largely ended even in mutants (Fig. 5, d and e; unpublished data). Third, α1 was absent from partially myelinated nerves in Lama4−/− (Fig. 3 t) and α2-null dy3K mice (Fig. 5 f). Fourth, levels of α1 were highest in severely amylinated dy2J/tg4null nerves (Fig. 5, g–i), indicating endogenous α1 expression is insufficient for radial sorting. As Ln-1 (α1β1γ1) is elsewhere implicated in BL assembly (Yurchenco et al., 2004), the heterogeneous expression of Ln α1 we observed in dy2J may account for the variable integrity of endoneurial BSs present (but not often acknowledged) in this strain. In dy2J/a4null nerves, α1 was predominantly associated with myelinated fibers (Fig. 5, h and i), many of which contained well-formed BSs (Fig. 4 k). Pre- and promyelinating dy2J/a4null Schwann cells lacked BSs (Fig. 4 k). Thus, Ln α1 may not promote radial sorting because α2-deficient Schwann cells express it after myelination.

Next, we asked if Ln α5 fosters radial sorting in dy2J/a4null nerve roots. Lama5−/− mice die as embryos (Miner et al., 1998), before radial sorting. Therefore, we used a broadly expressed α5 transgene (TgA5) (Kikkawa et al., 2002) to ask if ectopic α5 expression would promote sorting and myelination in the distal portions of dy2J/a4null nerves. Although TgA5 did not increase levels of Ln α5 in wild-type or dy2J endoneurial BSs (unpublished data), possibly due to competition from endogenous α2 and α4 chains for heterotrimer assembly, α5 was readily detected in dy2J/a4null/TgA5 nerves (Fig. 5 k). α5 colocalized with the β1 and γ1 chains, and β2 was ab-
Cell and molecular mechanisms

Amyelination has been thought to derive in large measure from disruption of Schwann cell BLs (Madrid et al., 1975; Bunge et al., 1986; Eldridge et al., 1989; Feltri et al., 2002). However, amyelination in Lama4−/− was not accompanied by disruption of endoneurial BLs, on either myelinating or premyelinating Schwann cells (Fig. 3 d; unpublished data). Moreover, Schwann cells in dy2J/a4null roots ensheathed and myelinated all axons despite lacking even a trace of endoneurial BLs (Fig. 4 l). Promyelinating Schwann cells in dy2J/a4null/τgA5 nerves also lacked BLs (Fig. 5 n). Thus, BLs are neither sufficient (in Lama4−/−) nor necessary (in dy2J/a4null) for Schwann cells to complete radial sorting.

Therefore, we considered potential signaling roles for Lns. Several lines of research suggest signaling through Ln receptors might promote Schwann cell proliferation during myelination. Radial sorting is closely coupled to Schwann cell mitosis (Bradley and Ashbury, 1970; Webster et al., 1973), amyelinated regions of Ln α2 mutant nerves have fewer Schwann cells than normal (Bray and Aguayo, 1975; Okada et al., 1976), and Ln-1 promotes Schwann cell proliferation in vitro (Porter et al., 1987). First, we asked if Schwann cell deficits correlate specifically with amyelination, or with loss of Ln-2 or BL structure. We found Schwann cell deficits accompanied amyelination in Lama4−/−, and large deficits accompanied severe amyelination in dy2J/a4nulls (Fig. 6 a). In transverse sections of Lama4−/− tibial nerve, deficits in myelinated axons (24 ± 2%; calculated from Table 1) and Schwann cells (30 ± 13%; Fig. 6 a) were proportional. In teased Lama4−/− nerve preparations, myelinated fibers had normal numbers of Schwann cells (Fig. 6, b and c; controls, 12 ± 4 nuclei/field at 1000×; Lama4−/−, 14 ± 5). Thus, Schwann cell deficits specifically accrue from amyelinated axons.

Second, we asked if Schwann cell deficits temporally correlate with radial sorting. Sciatic nerves in Lna2/a4-DKO, dy2J/a4null, and normal littersmates had statistically similar numbers of Schwann cells at E17 (Fig. 6 d). Severe deficits in Lna2/a4-DKO nerves accrued almost entirely between E17.5 and c; controls, 12 ± 4 nuclei/field at 1000×; Lama4−/−, 14 ± 5). Thus, Schwann cell deficits specifically accrue from amyelinated axons.
(14.7 ± 8.1%) and P3.5 (73 ± 6.9%) (Fig. 6 k). Moreover, deficits occurred through inadequate proliferation rather than cell death (Fig. 6, e–l). Proliferating cells were identified with antibody to Ki67 (Lalor et al., 1987), and necrotic cells by TUNEL assay. At P3.5, 40% of normal (littermate control) endoneurial cells were Ki67-positive, whereas <20% were labeled in Ln α2/α4-DKO pups (compare with values in Fig. 6, k and l). Fewer than 1% of nuclei in normal and mutant nerves were TUNEL stained at any perinatal age (Fig. 6, g and j; unpublished data). Thus, Ln-2 and -8 are specifically required for the perinatal increase in Schwann cell proliferation that coincides with radial sorting. They appear dispensable for the proliferation of immature Schwann cells covering fascicles, consistent with EM observations.

To ask if Ln-2 and -8 promote proliferation directly, we cultured primary Schwann cells on substrates containing purified isoforms (Fig. 7, a–d). Populations plated at moderate densities on Ln-1, -2, and -8 expanded at similar rates, doubling the rate on uncoated surfaces. When Ln concentration or cell density were limiting, proliferation was significantly faster on Ln-8 than on Ln-1 or -2. These data extend previous studies with Ln-1 (Porter et al., 1987) to suggest that Ln-2 and -8 promote Schwann cell proliferation in concert with autocrine growth factors. The results are consistent with the early hypothesis that increasing cell density activates Schwann cells to invade fascicles and ensheath axons (Martin and Webster, 1973).

Lastly, we used adhesion assays to ask if Schwann cells interact with Ln-2 and -8 through distinct or similar receptors (Fig. 7, e–o). Adhesion to purified Ln-1, -2, and -8, but not poly-lysine, was blocked by EDTA, consistent with a role for integrin receptors. Antiserum raised against Ln-8 blocked adhesion to Ln-8 but not Ln-2, indicating that binding to Ln-8 relies on distinct epitopes. Finally, adhesion to Ln-8 but not Ln-2 was inhibited by function-blocking antibody to integrin α6. The simplest interpretation of these data is that Ln-2 and -8 regulate Schwann cells through distinct integrin subtypes. Consistent with this notion, Schwann cell–specific disruption of the integrin β1 gene (Feltri et al., 2002) produced the pattern of amylination (partial in sciatic nerve; absent in roots) characteristic to Lamα4−/−, and distinct from that in dy2J. The combined results suggest Ln-8 (and not Ln-2) promotes radial sorting through integrin α6β1.

**Discussion**

We establish a dominant role for Lns in peripheral myelination, identify which of the four isoforms (Ln 1, 2, 8, and 10) expressed by Schwann cells are primarily involved, and clarify mechanisms by which they act. Ln-2 and Ln-8 act in concert to increase rates of Schwann cell proliferation at the onset of radial sorting, such that combined Ln-2/Ln-8 deficiency prevents radial sorting altogether. Ln-2 and -8 also regulate the onset of myelin formation by postmitotic Schwann cells, but through distinct effects on axonal ensheathment. At both steps, their combined activities foster solitary relationships between myelinating Schwann cells and axons (Fig. 8).

**Functions for Lns in nerve development**

Mechanisms underlying the transition from immature Schwann cells surrounding axon fascicles to myelinating and nonmyelinating Schwann cells ensheathing individual axons are not known. Martin and Webster (1973) observed that involution of axons along fascicle edges is immediately preceded by Schwann cell mitosis and an increase in cell density along those axons, and proposed that Schwann cell proliferation plays a primary role in the onset of radial sorting. Indeed, developing Schwann cell populations appear to expand in two
General nervous systems may be conserved. controlling the onset of myelination in the central and peripheral nervous systems. Additionally, interactions between integrin and neuregulin signaling pathways regulate the myelinating activity of oligodendrocytes as well (Colognato et al., 2002). Thus, mechanisms controlling the onset of myelination in the central and peripheral nervous systems may be conserved.

Interestingly, disruption of floxed Ln-γ1 alleles occurred unexpectedly in the Schwann cell lineage at E17, through Cre expression off a CaM kinase II (CaMKII) promoter (Chen and Strickland, 2003). This coincides with the onset of Ln-dependent proliferation we identify, CaMKII-Cre expression could begin in Ln-dependent progeny. Defects in CaMKII-Cre/Ln-γ1-deficient nerves would be consistent with additional roles for Ln-2 and -8 in the invasion of fascicles and/or ensheathment of axons, as suggested previously (Chen and Strickland, 2003). Similarly, β1 integrins could preferentially mediate these later roles (Feltri et al., 2002).

Our results suggest Ln-2 and -8 coordinate axonal ensheathment by promyelinating Schwann cells. Lama4−/− Schwann cells prematurely myelinated bundles of axons, before completing their separation. dy2/J Schwann cells lingered between ensheathing multiple axons and myelinating single axons. In principle, both activating and inhibitory mechanisms could underlie these results. For example, Ln-8 may promote the formation of multiple ensheathing Schwann cell processes, or simply retard myelin formation; loss of either function could produce polyaxonal myelination. Similarly, Ln-2 may promote myelin formation, and/or limit the formation of axon-ensheathing processes. Regardless, the results provide an initial insight into the mystery of how each Schwann cell manages to myelinate a single axon.

Molecular mechanisms

Ln-2 and -8 do not fully compensate each other’s loss in vivo, and have distinct binding and proliferative activities for Schwann cells in vitro, suggesting distinct receptors mediate their actions. Schwann cells express several Ln-binding integrins and dystroglycan (Previtali et al., 2001). Early steps of myelination depend greatly on β1-integrins (Fernandez-Valle et al., 1994; Feltri et al., 2002), and not dystroglycan (Saito et al., 2003). Amyelination caused by loss of integrin β1 (partial in distal nerves; nearly absent in roots) now appears to largely phenocopy loss of Ln-8 rather than Ln-2. The major β1-integrin in developing Schwann cells is integrin α6β1. As blocking antibody to integrin α6 inhibited Schwann cell binding to Ln-8 and not Ln-2 (Fig. 7), the simplest interpretation at present is that integrin α6β1 primarily mediates the effects of Ln-8 in radial sorting. Ln-2 engages additional receptors, as adding its loss to α4-deficiency (i.e., in dy2/Jα4null and Lama4/α4-DKO) produces amyelination far exceeding that in β1-integrin−deficient nerves. Testing in vivo roles for integrin α6β4, expressed by perinatal Schwann cells, may require tissue-specific mutations, as mice lacking these subunits die at birth (Dowling et al., 1996; Georges-Labouesse et al., 1996).

Endoneurial BLs

The idea that Schwann cell BLs are necessary for the proper defasciculation and myelination of axons in developing nerves...
(Madrid et al., 1975) has endured for nearly 30 years (Bunge, 1993; Feltri et al., 2002; Chen and Strickland, 2003). In muscle, disruption of myofiber BLs likely initiates α2-deficient myodegeneration (Moll et al., 2001; Durbeej and Campbell, 2002). However, our results show no correlation in nerves between radial sorting and endoneurial BL integrity. First, α4-deficiency has no ultimate effect on endoneurial BL structure or composition, but causes the same degree of sciatic myelinization as α2-deficiency. Second, Ln-8 promotes considerable sorting and myelination without BLs. For example, radial sorting and myelination in dy2J brachial nerves is nearly normal, develops without BLs, and is almost entirely dependent on Ln-8 (Xu et al., 1994). Amyelination in In this context, it is worth reconsidering the receptors regulates Schwann cell activation during myelination. (Web- ster and Billings, 1972).

Therefore, it seems likely that signaling through Ln receptors regulates Schwann cell activation during myelination. In this context, it is worth reconsidering the dy2J isoform of Ln-2 (Xu et al., 1994). Myelinization in dy2J/α4null and Lna2/ α4-DKO sciatic nerves were similarly severe, revealing that Ln-2(α2β3) is nearly inactive. Yet, the amino-terminal Lama2(α2β3) mutation specifically impairs the ability of Ln-2(α2β3) to poly- merize and scaffold BLs, and does not prevent binding to cell surface receptors (Colognato and Yurchenco, 1999), which seems to argue strongly for the importance of BL structure. To reconcile these results with the above view that BLs are not required for sorting, we suggest that short-arm interactions between Ln-2 heterotrimers are critical for Ln-2 to activate its Schwann cell receptors, possibly through receptor aggregation. Further, we speculate that transgenic expression of Ln α5 promotes radial sorting without BL assembly by stabilizing short-arm interactions with Ln-2(α2β3) and restoring the activation of Ln-2 receptors. Ln-8, which lacks an α-chain short arm, may paradoxically promote the severe myelinization in dy2J roots by diluting Ln-10/Ln-2(α2β3) interactions.

### Materials and methods

#### Animals

Use was by National Institutes of Health guidelines and approved by Oregon Health and Science University’s Institutional Animal Care and Use Committee. Mutants received accessible food and water and were killed at terminal stages whenever possible. C57BL/6 and Lama2(α2β3) mice were from The Jackson Laboratory. Mice null for Lna2 (Lama2(α4β3)) and Lama4 were described previously (Miyagoe et al., 1997; Patton et al., 2001); both were backcrossed five generations to C57BL/6. Mutant alleles of Lna2 and Lama4 on chromosome 10 were linked by mating heterozy- gous males (Lama2(β3/β3);Lama4(β3/β3)) or Lama4(β3/β3) to wild-type females and screening offspring for possession of both mutant α2 and α4 alleles (which required paternal recombination). Lama2 in combination with Lama4(β3/β3) in 3 of 171 offspring, and with Lama4(β3/β3) in 2 of 125 offspring, in agreement with reported genetic distance (Miner et al., 1997). Linkage was confirmed by mating with wild types. Mutants were born at Mendelian frequencies in all founder lines. dy2J/α4null and α2/ α4null/DKO mutants did not differ significantly between lines, or from F1 mutants from inter-line crosses in which sites of recombination remain het- erozygous, and their data were pooled. Ln α2 and α4 were undetectable in Lna2/α4/DKO tissues, as in single mutants (Miyagoe et al., 1997; Pat- ton et al., 2001). A previously described full-length mouse Ln α5 cDNA transgene (Kikkawa et al., 2002) prevents embryonic lethality and rescues all known defects in Lama2(α4) mice.

Genotypes were identified by PCR off tail-tip DNA, using the following sense (−) and antisense (+) primers. Lama4−/−: 5′-GCCAGGCCTG- CCAAGTGC3′; AS: 5′-CAACAAATTGTTCACTGGGCCT3′. Lama4−/−: 5′-AGGTACCCTCCCCACCCAC3′; AS: 5′-CTGAAAGCCGTCGCT- CAGACTG-3′, in PGK promoter. Lama2−/−: 5′-CCAGATGCCTAAAGGTAAT- TTG3′; AS: 5′-CCCTCTCCATTTCTAAAG3′. Lama2−/−: 5′-CCIT- CATGGT5′-TGCAAGGCTCCAGCCGAGG. Lam4−/−: 5′-TCTCCTGCTGCACTTACCT3′; AS: 5′-AGGCTCATA- GAGTCCTGG-3′. dy2J/α4null/DKO mutants did not differ significantly between lines, or from F1 mutants from inter-line crosses in which sites of recombination remain heterozygous, and their data were pooled. Ln α2 and α4 were undetectable in Lna2/α4/DKO tissues, as in single mutants (Miyagoe et al., 1997; Pat- ton et al., 2001). A previously described full-length mouse Ln α5 cDNA transgene (Kikkawa et al., 2002) prevents embryonic lethality and rescues all known defects in Lama2(α4) mice.

#### Antibodies

Rat mAbS 198 and 200 to Ln α1 were from Lydia Sorokin (Sorokin et al., 1992; Lund University, Lund, Sweden). Rabbit antibodies to Ln α1 and α2 (Rambukkana et al., 1997) were from Peter Yurchenco (Robert Wood Johnson Medical School, Piscataway, NJ); anti-α1 was generated against recombinant carboxy-terminal EHS Ln-1, affinity purified against E3 fragment, and cross-adsorbed against E8 fragment. mAbs to α2 (4H8-2; Biogene), β1 (MAB1928; CHEMICON International), and γ1 (MAB1914; CHEMICON Interna- tional) were purchased. Rabbit and guinea pig antibodies to Ln α4, α5, and β2 are described elsewhere (Miner et al., 1997). A Pab to purified human Ln-8 (Fig. 8), and mAb 1G5 raised to an α4 LG1 domain fusion product were generated in lam-α4 mice; each labels Ln-8(α4β3) in all animals (Fig. 5 a) and all α4-rich BLs in normal mice, but no BLs in Lama2(α2β3). Rabbit antibodies to integrin β1D and agrin were gifts from Eva Engvall (Burn- ham Institute, La Jolla, CA) and David Glass (corporate license from Regeneron Pharmaceuticals), respectively. Other purchased antibodies: MAB1946 (entactin), MAB1948 (perlecan), AB1920 (integrin α3), and MAB1992 (integrin α6) from CHEMICON International; GoH3 (integrin α6; Beckman Coulter), CD29 (integrin β1; BD Biosciences); VIA4-1 (α-dys- troglican; Upstate Biotechnology); β-dystroglycan and β-sarcoglycan (No- voCastra); neurofilament (Zymed; Developmental Studies Hybridoma Bank repository, Ames, Iowa); S100 (Neomarkers); Kim7 (Vector Laboratories); and Alexa 488-- (Molecular Probes, Inc.), Cy3, and Cy5-conjugated (Jackson ImmunoResearch Laboratories) secondary antibodies.

#### Lns

Mouse Ln-1 and human Ln-2 were from CHEMICON International. Human Ln-8 was purified from T98G cell–conditioned media using non-denaturing methods. Protein precipitated by 40% ammonium sulfate was bound to a DEA-Sepharose Fast-Flow FPLC column (Amersham Biosciences) in imidazole (10 mM; pH 7.0) and eluted with a 0.05–1.0 M NaCl gradient. Pooled fractions containing Ln-8 (0.4–0.5 M NaCl) by dot-blot assay were dialyzed (5 mM phosphate and 50 mM NaCl, pH 7.3) and repurified by DEA-Sepharose. The second DEA pool was concentrated (Aquadite; Calbiochem) and size-fractionated by FPLC through Superose 6 HR. The final pool contained a single major protein complex at 680 kDa on silver-stained SDS-PAGE nonreducing gels (Fig. 5 a). In this material, immunoreactivity for Ln α4, β1, and γ1 chains comigrated, and α1, α2, α5, and β2 were not detectable.

#### Histology

For resin sections, killed animals were perfused with 3% (wt/vol) PFA, 1% (vol/vol) glutaraldehyde, in PBS; nerves were incubated overnight at 4°C.
in 4% PFA, 4% glutaraldehyde in 0.1 M cacodylate; 1-mm pieces were post-fixed 1 h in 1% OsO4, dehydrated through ethanol, and embedded in Epon. Semithin sections (0.5 μm) were stained with toluidine blue (1% in alcohol) and imaged by digital color photomicroscopy. Ultrathin sections (90 nm) stained with uranyl acetate were imaged by transmission EM. Quantitation of myelination patterns was performed on photographic montages or transverse sections of the peripheral nerve. Myelinated nerve fibers were counted from semithin sections; nonmyelinated axons were counted from ultrathin sections on Formvar-coated hole grids photographed at 2,000–10,000×.

Immunohistochemistry was done as described previously (Miner et al., 1997), using 8–10-μm cryostat sections cut from OCT-embedded un-fixed nerve tissue snap frozen in −150°C 2-ethylbutane, or teased sciatric nerves prepared by gently spreading 1–2-mm segments on subbed slides. In α4 and β2 epitopes required denaturation (Miner et al., 1997). In brief, sections were incubated overnight with antibodies diluted in PBS containing 5% (wt/vol) BSA, washed in PBS, and bound antibodies detected with species-specific, fluorescent second antibodies (1 h). Teased fibers were processed for 15 min with 2% PFA, cleared with 0.1 M glycine, and stained in PBS with 5% BSA and 0.5% Triton X-100. Hoechst 33258 (Molecular Probes, Inc.) was added to mounting medium to visualize nuclei. TUNEL staining was performed according to kit directions (Roche; product 1684795). Myelin was visible by intrinsic fluorescence [Ex365 nm/Em450 nm]. Images were made at ambient temperature and planapo 60× (1.4 NA) oil-immersion lenses on BX microscopes (Olympus) using either a DCF500 camera and image capture software (Leica) or an FV300 confocal scan head [Olympus]. Multiply stained images were colored and superimposed in Photoshop 6.0. Quantitation of Schwann cell nuclei was performed strictly on transverse sections of PFA-fixed medial sciatric nerves frozen in situ (the thigh). All nerve nuclei (Hoechst, total; Ki67, proliferating; TUNEL, apoptotic) were counted in digital images taken at 400× without background subtraction.

Cell culture

Proliferation assays used Schwann cells [P2–98% S100-positive] freshly prepared from desheathed E13 chick sciatic nerves. (Patton et al., 1998). Plastic 96-wells were coated with poly-lysine (0.1 mg/ml, 1 h; Sigma-Aldrich), then Ln-1, -2, or -8 in PBS (8 h at 4°C; concentrations in Fig. 7). Cells were plated at indicated densities in 0.2 ml DME and 10% FCS, and incubated at 37°C. Population levels were measured 3 d later, by replate with 0.1 ml of 0.05% trypsin, 10 mM EDTA, and duplicate hemocytometer readings. Experiments included triplicate wells for each condition. In control experiments, fed by 50% medium replacement at d 3, cells remained adherent to all substrates at d 5, when TUNEL assay (Roche) labeled for 30 min in DME with or without anti-Ln antibodies. Cells were col-lected at expected 23,000 cells/cm2 for 2 h at 37°C. After washing with PBS, bound cells were fixed (3% PFA in PBS) and cover-slipped in medium containing Hoechst. Cells were counted at 400× with phase and fluorescence optics and an eyepiece reticule. Averages were calculated from four contiguous image fields, nearly spanning each spot. Reported values show mean ± SEM for three assays.

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