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

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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 myelin 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 unmyelinated by the final Schwann cell progeny.

A single 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 proteins. Loss of Ln-2 through mutations in the α2 chain causes a complex neuromuscular disease including peripheral dysmyelination. In the most studied dynein and dynactin strains of Ln-2 mice, peripheral nerves contain bundles of unmyelinated 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; Feltre 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 (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 (Lama4−/−) retracted hindlimbs toward the body, with toes clenched (Fig. 1 b). In contrast, normal and heterozygous Lama4+/− littermates extended limbs downward, potentially minimizing fall injuries (Fig. 1 a). Lama4−/− hindlimb retractions often progressed to rigid rearward extension (Fig. 1 c), but ceased upon landing. Forelimbs were unaffected. Similar suspension-induced hindlimb retraction was observed in juvenile Lama2dy2J (dy2J) mice (Fig. 1 d), before permanent contractures (Fig. 1 e). The overlap in dysfunction suggested Lama4−/− 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 Lama4−/− 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, Lama4−/− 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. 1 k; unpublished

Figure 1. Amyelinating peripheral neuropathies in Lama4−/− and dy2J mice. (a–d) Overlapping postural defects. When suspended, wild type (a) mice extend limbs downward, whereas Lama4−/− 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), Lama4−/− (g and h), and dy2J (i and j) sciatic nerves at low (f, g, and i) and high (h and j) magnification. Bundles of unmyelinated axons are present in mutants, but not controls. (k–o) Electron micrographs show most bundles lack intervening Schwann cell processes. Some Lama4−/− 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 \( \text{Lama4}^{-/-} \) were remarkably similar to amyelination described in \( \text{dy} \) and \( \text{dy2J} \) 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 \( \text{dy2J} \) and \( \text{Lama4}^{-/-} \) in the number of amyelinated axons or their distribution in bundles (Table I). In both \( \text{dy2J} \) and \( \text{Lama4}^{-/-} \), bundles contained mixed caliber axons. The number of larger axons (minimum diameter > 2 \( \mu \text{m} \)) in \( \text{Lama4}^{-/-} \) bundles (average ± SEM: 292 ± 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 \( \text{Lama4}^{-/-} \), we found no CNS amyelination in either mutant (Fig. 1, g and i; insets). Thus, independent genetic lesions in \( \text{Ln} \alpha 2 \) and \( \alpha 4 \) produce essentially similar amyelinating peripheral neuropathies.

Similarity between \( \text{Ln} \alpha 2 \)- and \( \alpha 4 \)-deficient neuropathy included origin and progression (Fig. 2). Most axons in wild-

Table I. Amyelination in nerves and roots of \( \text{Ln} \alpha 2 \)- and \( \alpha 4 \)-deficient mice

| Genotype | Wild type | \( \text{Lama4}^{-/-} \) | \( \text{Lama2dy2J} \) |
|----------|-----------|------------------------|------------------------|
| Tibial N. (n) | 3 | 5 | 2 |
| Myelinated axons | 2226 ± 16 | 1697 ± 49* | 1258 ± 16* |
| Amyelinated axons | 0 | 2043 ± 458 | 2380 ± 600 |
| Bundles | 0 | 31 ± 4 | 31.0 ± 1.4 |
| Nonmyelinated bundles | NA | 17 ± 3** | 28.5 ± 0.7** |
| "myelinated" bundles | NA | 14 ± 2** | 2.5 ± 2.1** |
| Axons/bundle (total) | NA | 67 ± 16 | 77 ± 22 |
| Axons/nonmyelinated bundle* | NA | 105 ± 9 | 80 ± 9 |
| Axons/myelinated bundle* | NA | 18 ± 1 | 16 ± 5 |
| V. root (n) | 3 | 4 | 3 |
| Myelinated axons | 760 ± 53 | 698 ± 56** | 32 ± 7** |
| Amyelinated axons | 0 | 21 ± 6** | 502 ± 42** |
| Bundles | 0 | 2 ± 1 | 10 ± 2* |
| Axons/bundle (total) | NA | 11 ± 1** | 49 ± 21** |

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

*Errors represent pooled bundles of all nerves.

Figure 2. Origin and distribution of amyelination in \( \text{dy2J} \) and \( \text{Lama4}^{-/-} \) mice. Toluidine blue stained sections from wild-type (a–g), \( \text{dy2J} \) (h–n), and \( \text{Lama4}^{-/-} \) (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 r). Spinal roots are severely amyelinated in \( \text{dy2J} \) (l and m) but not \( \text{Lama4}^{-/-} \) (s and t). Brachial nerves contain few amyelinated axons in either mutant (n and u). Bar in o, 14 \( \mu \text{m} \) (a, h, and o); 8 \( \mu \text{m} \) (b, i, and p); 16 \( \mu \text{m} \) (c, d, j, k, q, and r); 20 \( \mu \text{m} \) (e–g, l–n, and s–u).
type, Lama2<sup>+/H11001</sup>, and Lama4<sup>+/H11001</sup> sciatic nerves were sorted by P5 and myelinated by P12. In dy<sup>2J</sup> and Lama4<sup>+/H11001</sup>, 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 Jenkinson, 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 Lama<sup>+/H11001</sup> and Lama<sup>+/H11001</sup> mice. Amyelination has also been thought permanent (Bradley and Jenkinson, 1973). However, we found only a few, small axon bundles remaining in year-old dy<sup>2J</sup> and Lama4<sup>+/H11001</sup> 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 dy<sup>2J</sup> spinal roots, but was nearly absent from roots in Lama4<sup>+/H11001</sup>. Roots are less affected than distal nerves in Lama4<sup>+/H11001</sup>. 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<sup>+/H11001</sup> and dy<sup>2J</sup>; 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 Lama<sup>+/H11001</sup> but rare in dy<sup>2J</sup>, consistent with previous observations in dy (Okada et al., 1977). Most
small bundles (≤25 axons) in Lama4−/− tibial nerves were “myelinated.” Conversely, dy2J nerves contained many islands of ensheathed-but-not-myelinated axons, which were rare in Lama4−/− (Fig. 1 o, 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 amylinated 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, amylination-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−/−;Lama4−/− 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−/− 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 littersmates, and rarely survived 3 mo (Fig. 4, a–f). 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
loss of Ln-α4 strongly enhanced radial sorting in dy2J/α4null 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 amyelinated dy2J/α4null 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 BLs present (but not often acknowledged) in this strain. In dy2J/α4null nerves, α1 was predominantly associated with myelinated fibers (Fig. 5, h and i), many of which contained well-formed BLs (Fig. 4 k). Pre- and promyelinating dy2J/α4null Schwann cells lacked BLs (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/α4null nerve roots. Lama5−/− mice die as embryos (Miner et al., 1996), 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/α4null nerves. Although TgA5 did not increase levels of Ln-α5 in wild-type or dy2J endoneurial BLs (unpublished data), possibly due to competition from endogenous α2 and α4 chains for heterotrimer assembly, α5 was readily detected in dy2J/α4null/TgA5 nerves (Fig. 5 f). α5 colocalized with the β1 and γ1 chains, and β2 was ab-
sent (not depicted), indicating ectopic expression of Ln-10 (α5β1γ1). Ln-10 expression was accompanied by suppression of tremor and dyskinesia, and a marked increase in sorting and myelination in distal nerves (Fig. 5, k and l; compare with Fig. 4 g). The results implicate Ln-10 in the sorting and myelination of axons in dy2/J/a4null roots, but do not establish whether Ln-10 acts autonomously or depends on the truncated isoform of Ln-2 produced from the dy2/J allele of Lama2. To completely eliminate Ln-2 and Ln-8, we generated Ln α2/α4 double-knockout mice (Lna2/α4-DKO; see Materials and methods). All Lna2/α4-DKO pups appeared normal at birth and suckled effectively, but died by P13 (n = 20), preventing comparison with mature dy2/J/a4nulls. Nevertheless, a nearly complete absence of sorting in Lna2/α4-DKO spinal roots as well as distal nerves at P11 (Fig. 5, a–c) suggests strongly that Ln-10 promotes defasciculation and myelination via collaboration with dy2J-variant Ln-2.

**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 dy2/J/a4null roots ensheathed and myelinated all axons despite lacking even a trace of endoneurial BLs (Fig. 4 l). Promyelinating Schwann cells in dy2/J/a4null/TgA5 nerves also lacked BLs (Fig. 5 n). Thus, BLs are neither sufficient (in Lama4−/−) nor necessary (in dy2/J/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 Asbury, 1970; Webster et al., 1973), amylinated 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 amylination in Lama4−/−, and large deficits accompanied severe amylination in dy2/J/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 amylinated axons.

Second, we asked if Schwann cell deficits temporally correlate with radial sorting. Sciatic nerves in Lna2/α4-DKO, dy2/J/a4null, and normal littersmates had statistically similar numbers of Schwann cells at E17 (Fig. 6 d). Severe deficits in Lna2/α4-DKO nerves accrued almost entirely between E17.5 and E18 (not depicted), indicating ectopic expression of Ln-10 (α5β1γ1). Ln-10 expression was accompanied by suppression of tremor and dyskinesia, and a marked increase in sorting and myelination in distal nerves (Fig. 5, k and l; compare with Fig. 4 g). The results implicate Ln-10 in the sorting and myelination of axons in dy2/J/a4null roots, but do not establish whether Ln-10 acts autonomously or depends on the truncated isoform of Ln-2 produced from the dy2/J allele of Lama2. To completely eliminate Ln-2 and Ln-8, we generated Ln α2/α4 double-knockout mice (Lna2/α4-DKO; see Materials and methods). All Lna2/α4-DKO pups appeared normal at birth and suckled effectively, but died by P13 (n = 20), preventing comparison with mature dy2/J/a4nulls. Nevertheless, a nearly complete absence of sorting in Lna2/α4-DKO spinal roots as well as distal nerves at P11 (Fig. 5, a–c) suggests strongly that Ln-10 promotes defasciculation and myelination via collaboration with dy2J-variant Ln-2.

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(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 amylolysis (partial in sciatic nerve; absent in roots) characteristic to Lama4−/−, 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
In Ln general nervous systems may be conserved. Signaling pathways regulate the myelinating activity of oligodendrocytes and Schwann cells. Interestingly, interactions between integrin and neuregulin significantly affect Schwann cell proliferation rates and stimulate progeny to envelop axon-fascicles. Ln-2 promotes onset of myelination and/or inhibits 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 B1-integrins (Fernandez-Valle et al., 1994; Feltri et al., 2002), and not dystroglycan (Saito et al., 2003). Amyelination caused by loss of integrin B1 (partial in distal nerves; nearly absent in roots) now appears to largely phenocopy loss of Ln-8 rather than Ln-2. The major B1-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 dy2J/α4null and Lna4/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., 1994; Feltri 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 α4-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 ultrastructural effect on endoneurial BL structure or composition, but causes the same degree of sciatic amylination 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 (dy2J/a4null brachial nerves are severely amylinated). The inability of Ln-8 to promote BL assembly is consistent with Ln-4 lacking amino-terminal domains required for heterotrimer polymerization (Yurchenco et al., 2004). Third, all axons in dy2J/a4null spinal roots are sorted and myelinated without endoneurial BL formation. Fourth, transgenic Ln-α5 promotes myelination in dy2J/a4null sciatic nerves without forming BLs on the pre- and promyelinating Schwann cells involved in radial sorting. This last result is curious as α5-Lns are expected to promote BL formation, but is consistent with observations in α2-deficient spinal roots, which contain α5 but lack BLs (Madrid et al., 1975; Weinberg et al., 1975; Nakagawa et al., 2001). In sum, endoneurial BLs are neither necessary to achieve complete radial sorting nor sufficient to prevent amylination. That BL integrity is irrelevant to the initial myelination of axons brings mammalian myelination into line with amphibians, in which Schwann cells acquire BLs after myelination (Webster 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). Amyelination in dy2J/a4null and Lna2/a4-DKO sciatic nerves were similarly severe, revealing that Ln-2<sup>-/-</sup> (<sup>2</sup>) is nearly inactive. Yet, the amino-terminal Lama2<sup>-/-</sup> mutation specifically impairs the ability of Ln-2<sup>-/-</sup> to polymerize 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<sup>-/-</sup> and restoring the activation of Ln-2 receptors. Ln-8, which lacks an α-chain short arm, may paradoxically promote the severe amylination in dy2J roots by diluting Ln-10/Ln-2<sup>-/-</sup> 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<sup>-/-</sup> mice were from The Jackson Laboratory. Mice null for Lma2 (Lama2<sup>-/-</sup>) and Lma4 were described previously (Miyagoe et al., 1997; Patton et al., 2001); both were backcrossed five generations to C57BL/6. Mutant alleles of Lma2 and Lma4 on chromosome 10 were linked by mating heterozygous males (Lma2<sup>+/+;</sup>Lma4<sup>+/+;</sup>) or Lma2<sup>+/+;</sup>Lma4<sup>+/+;</sup> with wild-type females and screening offspring for possession of both mutant α2 and α4 alleles (which required external recombinase). Lma4<sup>+/-</sup> were recombined with Lma2<sup>+/+;</sup> in 3 of 171 offspring, and with Lma2<sup>-/-</sup> 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/a4null and α2/α4nullDKO 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. In α2 and α4 were undetectable in Lna2/a4-DKO tissues, as in single mutants (Miyagoe et al., 1997; Patton 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 Lama5<sup>-/-</sup> mice.

Genotypes were identified by PCR off tail-tip DNA, using the following sense-[S] and antisense-[AS] primers. Lama4<sup>+/+</sup>; 5'-GGCGAGGCTG-CCAGTGTGC-3'; AS: 5'-CAAAAATGTTGCAACTGGGTCT-3'. Lama4<sup>-/-</sup>; S: 5'-AGCCTACCCCTTCACCCAC-3'; AS: 5'-GCTAAAAAGCAGCCTGC-3'. Lama5<sup>+/+</sup>; 5'-CCAGATGCTCTGATGTG-3'; AS: 5'-CTTTGCTTTGCTGATGTG-3'. Lama5<sup>-/-</sup>; 5'-CTTCAATCCCTGATGTG-3'; AS: 5'-AGGCTCATG-3'. Lama2<sup>+/+</sup>; 5'-CCAGATGCTCTGATGTG-3'; AS: 5'-CTTTGCTTTGCTGATGTG-3'. Lama2<sup>-/-</sup>; 5'-CTTCAATCCCTGATGTG-3'; AS: 5'-AGGCTCATG-3'. Lama4<sup>+/+</sup>; 5'-CCAGATGCTCTGATGTG-3'; AS: 5'-CTTTGCTTTGCTGATGTG-3'.

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 Institute, Shrewsbury, NJ). Anti-α1 was generated against EHS Ln-1, affinity purified against E3 fragment, and cross-absorbed against E8 fragment. mAbs to α2 (4H8-2; Biquene), β1 (MAB1928; CHEMICON International), and γ1 (MAB1914; CHEMICON International) 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 TG5 raised to an α4 LG1-domain fusion protein were generated in Lna4<sup>-/-</sup> mice; each labels Ln-8<sup>-/-</sup> (Figs. 5 a) and all α4-rich BLs in normal mice, but no BLs in Lma4<sup>-/-</sup>. Rabbit antibodies to integrin β1D and agrin were gifts from Eva Engvall (Barn-

### 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 of transverse sections of the experimental nerve. Myelinated fibers were counted from semithin 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 unfixed 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). Stained fibers were counted 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 with PlanApo 60×/1.4 NA oil-immersion lenses on BX microscopes (Olympus) using either a DC 5250 camera and IMAC image software (Leica) or an FV300 confocal scan head (Olympus). Multiply stained images were colorized 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) not residing in perineural sheaths (distinguished by integrin α6 counterstaining and morphology) were counted in digital images taken at 400× without background subtraction.

Cell culture

Proliferation assays used Schwann cells (92–98% 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 L-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 were incubated at 37°C. Population levels were measured 3 d later, by release 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 reached confluence by d 7. Myelination patterns were recorded 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 reached confluence by d 7.

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