

Disrupted Schwann cell–axon interactions in peripheral nerves of mice with altered L1-integrin interactions

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The cell adhesion molecule L1 is important for peripheral nerve development. Mice lacking the 6th Ig domain of L1 (L1-6D mice) lose L1 homophilic binding and RGD dependent L1-integrin binding [Itoh, K., Cheng, L., Kamei, Y., Fushiki, S., Kamiguchi, H., Gutwein, P., Stoeck, A., Arnold, B., Altevogt, P., Lemmon, V., 2004. Brain development in mice lacking L1-L1 homophilic adhesion. *J. Cell Biol.* 165, 145–154]. We examined the ultrastructure of sciatic nerves from L1-6D at postnatal day 7 and 8 weeks. Unmyelinated axons frequently detached at the edge of Schwann cells, and naked axons were observed. Myelin was thinner in L1-6D and abnormal, multiple axons wrapped in a single myelin sheath were routinely observed. Previous work has shown that L1 on axons interacts with a heterophilic binding partner on Schwann cells to facilitate normal peripheral nerve formation. Taken together, it is likely that L1 on axons binds integrins on Schwann cells, resulting in interactions between axons and Schwann cells that are essential for ensheathment and myelination.

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Introduction

The cell adhesion molecule L1 is important in neuronal migration, axon growth, guidance, fasciculation, and synaptic plasticity (Kamiguchi et al., 1998; Haspel and Grumet, 2003). L1 consists of six immunoglobulin (Ig) domains, five fibronectin type III (FN) domains and a highly conserved cytoplasmic tail. It binds to several extracellular ligands, such as phosphacan

and neurocan, integrins, axonin-1/TAG1, and contactin/F11, as well as binding to itself. L1 also binds to neuropilin to form a coreceptor for Semaphorin 3A (Castellani et al., 2000). Interestingly, the sixth Ig domain, which contains RGD sequences (two in mice, one in humans), binds β 1-integrins (Ruppert et al., 1995; Montgomery et al., 1996).

A number of X-linked forms of mental retardation have been linked to mutations in the L1 gene, including X-linked hydrocephalus and MASA syndrome (mental retardation, aphasia, shuffling gait, adducted thumbs) (Fransen et al., 1995; Kenwright et al., 1996). More severe consequences are associated with mutations of the extracellular region, which may disrupt both adhesion and signaling, whereas milder symptoms occur with mutations in the cytoplasmic domain, which may alter either signaling or interactions with the cytoskeleton (Yamasaki et al., 1997; Kamiguchi et al., 1998).

L1 knockout mice (L1KO mice) show hydrocephalus, reduced corticospinal tract, abnormal pyramidal decussation, ventricular dilatation, and hypoplasia of the cerebellar vermis (Dahme et al., 1997; Cohen et al., 1998; Fransen et al., 1998). Peripheral nerves in L1KO mice also show abnormalities with non-myelinating Schwann cells often failing to ensheath small caliber axons appropriately (Dahme et al., 1997; Haney et al., 1999), likely accounting for abnormal nociception in these mice (Thelin et al., 2003). In nerve transplant studies, loss of axonal-L1, but not Schwann cell-L1, reproduced the L1-deficient phenotype (Haney et al., 1999). These data indicate that axonal-L1 binds heterophilically to an unknown ligand on Schwann cells that is essential for normal Schwann cell–axon interactions.

In order to evaluate which portion of the L1 molecule is involved in the defects observed in the L1KO mice, we analyzed a novel knock-in mouse in which the 6th Ig domain of L1 was deleted (L1-6D) (Itoh et al., 2004). This deletion prevents both L1-L1 homophilic binding and L1 binding to RGD-dependent

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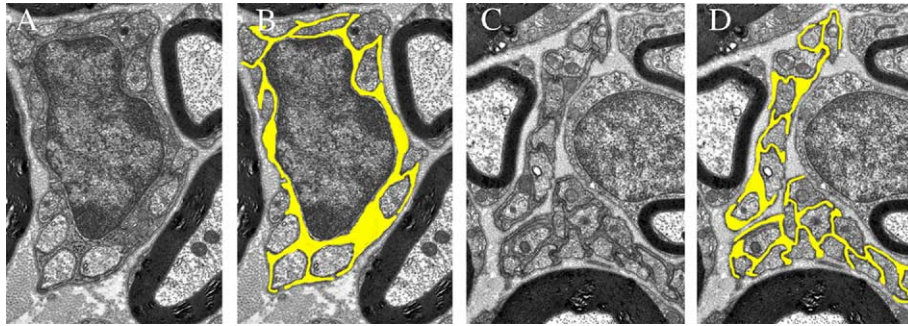


Fig. 1. Schwann cells compactly ensheath multiple unmyelinated axons in control sciatic nerve: representative images are shown in A and C. In B and D, the Schwann cell processes separating small axons are highlighted in yellow. Scale bar = 2 μ m.

integrins. Feltri et al. (2002) reported that Schwann cell-specific disruption of β 1-integrins causes impaired Schwann cell–axon interactions. They suggested that β 1 integrins on the abaxonal surface of the Schwann cells associates with laminin in the process of axon ensheathment and myelination. L1-6D mice are missing the RGD sequence that is responsible for L1 binding to RGD-dependent integrins and provide an opportunity to study how integrins on the adaxonal surface of Schwann cells interact with an RGD expressing protein on axons. We investigated sciatic nerves in L1-6D mice and found that unmyelinated axons were associated with increased numbers of free Schwann cell processes, and naked axons were found in the endoneurium of L1-6D mice. The diameter of myelin wrapping was thinner and abnormal collections of myelinated axons, showing multiple axons wrapped in a single myelin sheath were found. Because deletion of the 6th Ig domain of L1 removes a β 1-integrin binding site, our observations indicate that L1 on axons binds to β 1-integrins on Schwann cells, giving an essential signal that ensures proper ensheathment and myelination.

Results

L1-6D mice show altered unmyelinated bundles of axons in sciatic nerve at 8 weeks

The ultrastructure of unmyelinated axons associated with Schwann cells in an “axon bundle” (Peters et al., 1976) was

compared in sciatic nerves from 8-week-old L1-6D and control mice. Unmyelinated Schwann cells in control sciatic nerve ensheathed individual axons compactly in separate cytoplasmic processes (Fig. 1). In L1-6D sciatic nerve at 8 weeks (Fig. 2), many unmyelinated axons were not surrounded or were only partially surrounded by Schwann cell processes. At the edge of axon bundles, many unmyelinated axons lacked Schwann cell ensheathment and were present as naked axons in the endoneurium. Unensheathed axons lacked intact basal lamina on their surfaces. Many Schwann cell processes lacked tight adhesion to unmyelinated axonal membranes and extended free processes in the endoneurium. The size of axon bundles as well as the number of individual axons ensheathed by a single Schwann cell was variable compared to wild type (see below).

L1-6D mice show altered myelination in sciatic nerve at 8 weeks

Examination of the myelin in the adult animals did not reveal obvious abnormalities in the L1-6D mice. Myelin membranes were tightly packed and periaxonal space was appropriately maintained. However, measurements of several thousand axons and their myelin sheath revealed myelin thickness in larger axons were decreased compared to wild type mice (see below). One type of myelin abnormality was observed; however, aberrant Schwann cell–axon units, showing a single Schwann cell with a myelin sheath around bundles of several axons (more than 20 axons), were observed only in L1-6D sciatic nerves (Fig. 3A). At least one was seen in each L1-6D animal, so while they are not common, they are

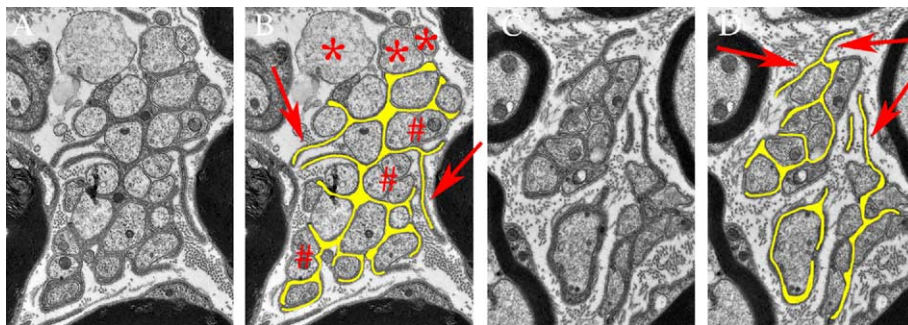


Fig. 2. Abnormal unmyelinated axon bundles in sciatic nerve of L1-6D mice: representative images are shown in A and C. In B and D, the Schwann cell processes separating small axons are highlighted in yellow. The processes of Schwann cells were often detached from axons at the edge of the unmyelinated bundles of axons (red arrows). Some detached axons and naked axons (red asterisks) are still associated with basal lamina but lose tight interactions with Schwann cell membranes. Multiple axons within one Schwann cell pockets are indicated with #. Scale bar = 2 μ m.

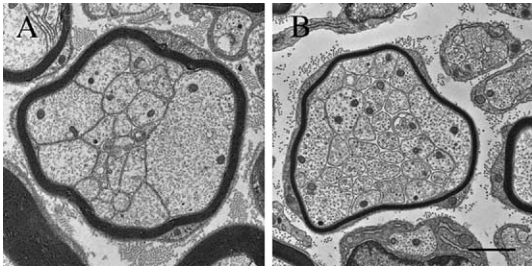


Fig. 3. Aberrant Schwann cell–axon units observed in L1-6D sciatic nerve: a single Schwann cell elaborates a myelin sheath around a bundle of several axons (more than 20 axons) in sciatic nerves from adult (A) and at postnatal day 7 (B) L1-6D mice. The diameter of axons in a bundle varies. Scale bar = 1.2 μ m.

evident. The axons myelinated in a group by a single Schwann cell varied broadly in size.

L1-6D mice show altered development of unmyelinated and myelinated axons in sciatic nerve at P7

Preliminary studies were conducted at P0, P7, and P14 and showed similar patterns of abnormalities to adult L1-6D mice. Subsequent studies focused on P7. Unmyelinated Schwann cells in control sciatic nerve at P7 ensheathed individual axons compactly (Fig. 4A). In L1-6D sciatic nerve at P7, many unmyelinated axons were not surrounded by Schwann cell processes and remained free in the endoneurium (Figs. 4B and C). Many Schwann cell processes failed to have tight adhesion to unmyelinated axonal membranes and the size of axons in each unmyelinated axon bundle varied. Aberrantly myelinated axons, showing a single Schwann cell elaborating a myelin sheath around a bundle of several axons (more than 20 axons), were also observed in L1-6D sciatic nerves at P7 (Fig. 3B).

Morphometric analyses of unmyelinated axons and Schwann cells in adult mice

Unmyelinated axons were examined across the entire cross-sectional area of nerves in control and L1-6D mice. Unmyelinated axons ensheathed by a single Schwann cell showed a wider variation in L1-6D when compared to control mice. The mean number of unmyelinated axons ensheathed by a single Schwann cell in control sciatic nerves was 7.9 ± 0.4 (mean \pm SEM) and 9.4 ± 0.5 in L1-6D (significantly different at $P < 0.02$) with the L1-6D mice having a preponderance of Schwann cells ensheathing greater than 20 axons when compared to control (Figs. 5A and B).

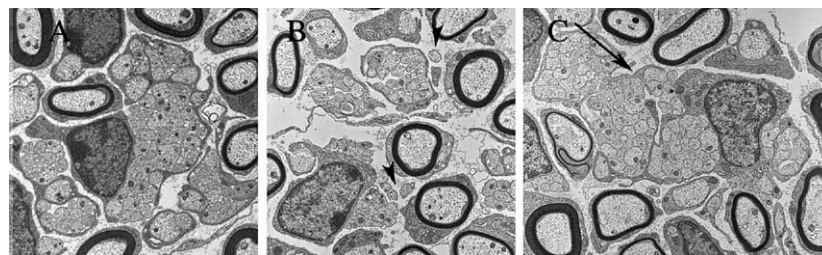


Fig. 4. Schwann cell ensheathment at P7. (A) A Schwann cell at P7 begins to ensheath individual axons compactly with cytoplasmic processes. The axons are similar in size and have a regular arrangement of neurofilaments. (B and C) Abnormal ensheathment at P7 in L1-6D sciatic nerve. Many unmyelinated axons are not compactly surrounded by Schwann cell processes and some naked axons remain free in the endoneurium (arrowhead). The number and size of axons ensheathed by a Schwann cell vary from one to more than 70 (arrow). Scale bar = 2 μ m.

Interestingly, there was also an increased proportion of Schwann cells ensheathing a small number of axons (<5) when compared to control. The number of free Schwann cell processes in the endoneurium in L1-6D was significantly greater in L1-6D mice ($0.81/\text{Schwann cell}$) as compared to control mice ($0.14/\text{Schwann cell}$) ($P < 0.0001$). The mean circumference of unmyelinated axons in L1-6D and control mice was not different (data not shown).

Morphometric analyses of myelinated axons in adult mice

We analyzed the entire cross-sectional area of nerves from 3 control and 3 L1-6D mice. Between 450 and 820 axons per animal were measured. A measure of myelin thickness, the g-ratio, was determined and for the control mice was found to be 0.654 ± 0.012 (mean \pm SD). The g-ratio for the L1-6D mice was 0.697 ± 0.004 . These values were significantly different at the $P < 0.0001$ level, indicating the myelin is thinner in L1-6D mice than control mice.

Discussion

L1-6D mice, a knock-in mouse in which the 6th Ig domain of L1 was deleted, have lost L1-L1 homophilic binding and L1 binding to RGD-dependent integrins but not L1 interactions with neurocan or neuropilin (Itoh et al., 2004). L1-6D on the 129 Sv genetic background showed relatively normal development of central nervous system including axonal trajectories of the corticospinal and thalamocortical tracts. However, in peripheral nerves, abnormal Schwann cell–axon interactions were observed both in myelinated axons and unmyelinated axon bundles. Many unmyelinated axons were only partially surrounded by Schwann cell processes, lacking appropriate Schwann cell ensheathment, while other axons were naked in the endoneurium. Our observations suggest that the Schwann cell processes lost tight adhesion to unmyelinated axonal membranes and extended free processes in the endoneurium.

In peripheral nerves of L1-KO, unmyelinated axons at P60 showed abnormal Schwann cell ensheathment: many unmyelinated axons were not surrounded by Schwann cell processes (Dahme et al., 1997; Haney et al., 1999). These changes are similar to those observed in adult L1-6D unmyelinated axons in sciatic nerve. In L1-6D mice, aberrant Schwann cell–axon interactions were observed during development, i.e., at P7 and P14, which suggests that the nerve phenotype in L1-6D is due to a developmental abnormality. In the L1 knock-outs, there were about 50% fewer unmyelinated axons associated with Schwann cells, while the L1-6D mice have a small but significant increase in the measure. It has

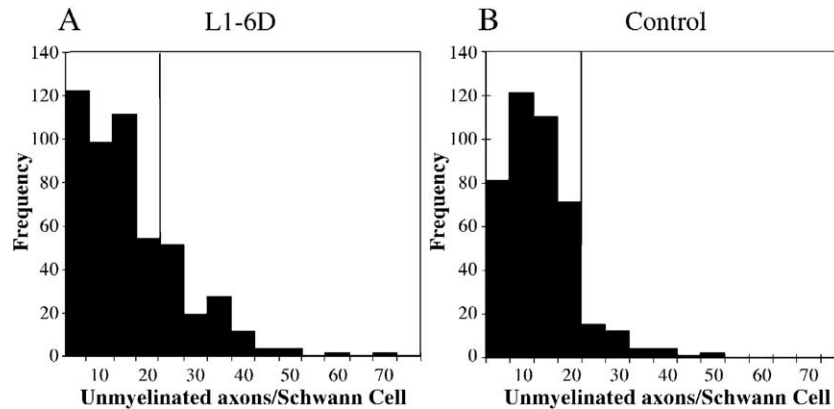


Fig. 5. Morphometry of non-myelinating Schwann cells: the number of axons ensheathed by a single Schwann cell varies in L1-6D (A) sciatic nerve compared to control (B).

been reported that L1 can enhance the survival of neurons (Haney et al., 1999; Loers et al., 2005) so it is plausible that the difference we observed between the L1-6D mice and the L1KO mice is that the presence of mutant L1 in the L1-6D mice supports the survival of the unmyelinated axons.

Interestingly, in some myelinated axons of L1-6D sciatic nerve, multiple axons were surrounded by a single Schwann cell myelin sheath. A similar phenotype of aberrant myelinated axons was observed in transgenic mice expressing a dominant-negative antagonist of POU transcription factor SCIP (delta SCIP), specifically targeted to developing Schwann cells (Weinstein et al., 1995). Mice that express delta SCIP exhibit precocious differentiation of Schwann cells, judging from overexpression of myelin-specific gene products at P0, widespread hypermyelination and premature myelination, and myelination of multiaxon bundles. Endogenous SCIP expression is transient; the protein is detectable in immature Schwann cells (during their last division) and in immature promyelinating but not in mature, myelinating Schwann cells, which express an end-stage myelin-specific gene at high level. SCIP expression is thus required for maintenance of an immature Schwann cell phenotype, and loss of SCIP expression is in turn required for full Schwann cell differentiation. The authors suggested that abnormal multiaxon bundles might arise from the premature differentiation of dividing, immature Schwann cells. It might be argued that differentiation is disrupted at an earlier developmental stage in L1-6D myelinated axons, since both hypomyelination in larger axons and aberrant myelination of multiaxon bundles were observed.

Integrins have been implicated in axon–Schwann cell interactions and myelination for some time (Feltri et al., 1994; Fernandez-Valle et al., 1994). Schwann cell-specific disruption of $\beta 1$ integrin causes a severe neuropathy with impaired radial sorting of axons (Feltri et al., 2002). Transverse sections of mutant sciatic nerve at P28 showed few myelinated axons and most axons were tightly packed in large bundles without ensheathment by Schwann cells. The myelination defect in these mice is clearly more profound than what we observed in the L1-6D mice. However, in the mice with Schwann cells lacking $\beta 1$ integrins, there were many similarities to the L1-6D mice regarding the non-myelinating Schwann cells. In developing $\beta 1$ integrin mutant nerve, Schwann cells ensheathed groups of up to 8–9 large caliber axons, and bundles of unsorted, mixed caliber axons and Schwann cell processes contained a larger volume of electron-dense cytoplasm and expanded in many directions, suggesting that both

impaired initial interactions with axons, as well as subsequent retraction of processes, may contribute to bundle formation. Finally, the authors suggested that $\beta 1$ integrin links laminin in the basal lamina to the cytoskeleton in order for Schwann cells to ensheath axons, and alteration of this linkage contributes to the peripheral neuropathy.

Abnormal Schwann cell sorting in unmyelinated axon bundles was also observed in L1-6D sciatic nerve. It was previously shown in L1-KO mice that axonal L1 interacts with an unknown heterophilic ligand on Schwann cells to mediate early axon–Schwann cell interactions (Haney et al., 1999). Supporting this idea are studies that show that the isoform of L1 expressed by Schwann cells is relatively non-adhesive in homophilic binding assays (De Angelis et al., 2001; Jacob et al., 2002). Consequently, even though Schwann cells express L1 at low levels, this L1 cannot participate in L1–L1 interactions bridging Schwann cells and axons. The L1-6D mice, in addition to having lost L1–L1 binding between axons for example, have also lost interactions between L1- and RGD-dependent integrins (Itoh et al., 2004). Taken together with the results of Feltri et al. (2002), it is highly likely that L1 on axons interacts with $\beta 1$ integrins on Schwann cells (Chernousov and Carey, 2003) early in development so that axon–Schwann cell interactions proceed properly to organize ensheathment and myelination.

In summary, the peripheral nerve phenotype in L1-6D exhibited abnormal ensheathment of unmyelinated axons by Schwann cell processes, a small but statistically significant hypomyelination and rare but routinely observed aberrant Schwann cell–axon units resulting in myelinated multiaxon bundles. Based on comparisons with L1-KO mice and mice lacking $\beta 1$ integrins in Schwann cells, we propose that L1 on axons binds to integrins on Schwann cells and provides critical information to Schwann cells inducing correct radial sorting and ensheathment.

Experimental methods

Animals

Production of a mouse line in which 6th Ig domain of L1 was deleted (L1-6D) has been described (Itoh et al., 2004). In that paper, lymphocytes from the L1-6D mice were analyzed for L1 expression using mAbs to define domains using FACS analysis and showed a loss of the expression of a 6th Ig domain epitope but retained other important regions of L1 protein. For control mice,

we used mice carrying the targeting loxP sites but still expressing the 6th Ig domain of L1.

Electron microscopy

L1-6D and control mice at postnatal day 0 (P0), P7, P14 and 8 weeks-old young adult were perfused transcardially with 1% glutaraldehyde and 0.5% paraformaldehyde in 100 mM phosphate buffer. Central segments of sciatic nerves were post-fixed with osmium tetroxide for 3 h. The tissue was then processed for electron microscopy by standard procedures and embedded in epoxy resin. Ultrathin sections (80 nm thick), stained with lead citrate and uranyl acetate, were observed under an electron microscope (JEOL).

Morphometry

Electron micrographs across entire cross-sectional area of sciatic nerve, ignoring regions covered by the grid, were analyzed with a computer-assisted image analysis system (Metamorph, Universal Imaging Co.) for 3 L1-6D mice and 3 control mice at P7 and 8 weeks of age. EM negatives were scanned at 600 DPI prior to analysis. Peters et al. (1976) refer to groups of unmyelinated axons (typically 10–20) ensheathed by a single Schwann cell as “axon bundles”. We have used this element as a unit to study and determined the number of unmyelinated axons ensheathed by a single Schwann cell, the perimeter and area of each axon bundle, the number of free Schwann cell processes per axon bundle and the perimeter and area of the axons in the bundles. For myelinated axons, axon diameter and axon/myelin sheath diameter were measured to calculate the g-ratio.

Statistics

Statview 4.5 software and a G4 Macintosh computer were used to analyze the data. ANOVA and Fisher’s PLSD were used with the level of significance set at 0.05.

Images

Photoshop 7 software and a G4 Macintosh computer were used to prepare the figures. All scanned EM images were processed with the “sharpen edges” filter for publication.

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