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LNX1 is a perisynaptic Schwann cell specific E3 ubiquitin ligase that interacts with ErbB2

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Non-myelinating perisynaptic Schwann cells wrap motor axon terminals and are required for both functional and structural integrity of the neuromuscular junction. Several lines of evidence indicate that fine-tuning of neuregulin-1/ErbB signaling is critical for maintaining perisynaptic Schwann cells at synapses and that this control may be achieved by the developmental downregulation of the ErbB2 receptor. Here, we identify a direct interaction between ErbB2 and LNX1, an E3 ubiquitin ligase that can target interacting proteins for degradation through ubiquitination. Immunostaining shows that LNX1 is specifically localized in perisynaptic Schwann cells but not in Schwann cells along the motor axon. Developmentally, levels of LNX1 protein are inversely correlated with the responsiveness of perisynaptic Schwann cells to neuregulin-1. Furthermore, the LNX1 staining disappears upon denervation, whereas ErbB2 reappears in Schwann cells after denervation. Taken together, these data suggest that LNX1 may play a role in regulating neuregulin-1/ErbB signaling in perisynaptic Schwann cells.

Introduction

Perisynaptic glial cells play an important role in synaptic development and function (Araque et al., 1999; Auld and Robitaille, 2003a; Ullian et al., 2004). The glial components of the neuromuscular junction (NMJ) are the non-myelinating perisynaptic Schwann cells (PSCs). These cells cap each nerve terminal and therefore are also called terminal Schwann cells. PSCs are critical for both the structure and function of the NMJ (Auld and Robitaille, 2003b). PSCs have been shown to respond to synaptic activity and, in turn, regulate neurotransmitter release (Castonguay and Robitaille, 2001; Jahromi et al., 1992; Reist and Smith, 1992; Robitaille, 1998). Perturbation of PSC development and maturation in neonatal mice leads to the withdrawal of nerve terminals from synaptic sites (Trachtenberg and Thompson, 1997). Furthermore, selective ablation of PSCs leads to the retraction of nerve terminals in tadpoles and a decrease in presynaptic function in adult frog NMJ, directly demonstrating the importance of PSCs in maintaining the function and integrity of the NMJ (Reddy et al., 2003). PSCs also play an important role after nerve injury by extending processes which guide nerve growth during muscle reinnervation (Son and Thompson, 1995a,b; Son et al., 1996).

Gene knockout studies have shown that many aspects of Schwann cell development are controlled by neuregulin-1 signaling (Adlkofer and Lai, 2000; Garratt et al., 2000a). Neuregulin-1 can function as either a soluble or transmembrane ligand that binds to members of the ErbB family of receptor tyrosine kinases. Developing Schwann cells express both ErbB2 and ErbB3 receptors. However, ErbB2 cannot bind neuregulin-1 directly while ErbB3 has an inactive tyrosine kinase domain, and so they are thought to function as heterodimers in vivo (Alroy and Yarden, 1997; Carraway and Cantley, 1994; Carraway et al., 1994; Guy et al., 1994; Jones et al., 1999; Sliwkowski et al., 1994). In agreement with this, expression of both ErbB2 and ErbB3 is absolutely required for the generation, proliferation and survival of Schwann cell precursors and consequently is essential for the development of all types of Schwann cells (Lee et al., 1995; Lin et al., 2000; Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999). Postnatally, axon-derived neuregulin-1 signaling through ErbB2/B3 is also important in the differentiation of myelinating Schwann cells along motor axons, and the modulation of neuregulin-1/ErbB signaling is thought to regulate myelin sheath thickness (Garratt et al., 2000b; Lee et al., 1995; Lin et al., 2000; Meyer and Birchmeier,

Abbreviations: NMJ, Neuromuscular junction; PSC, Perisynaptic Schwann cell; PBS, Phosphate buffered saline; PDZ, *P*SD-95, *D*lgA, *Z*O-1; α-Btx, α-bungarotoxin; TTX, tetrodotoxin.

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1995; Michailov et al., 2004; Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999; Wolpowitz et al., 2000).

The role of neuregulin-1/ErbB signaling in the differentiation of non-myelinating PSCs at the NMJ is not as well understood, but several lines of evidence indicate that the modulation of neuregulin-1/ErbB signaling is also involved in this process. Firstly, neuregulin-1 promotes survival of PSCs in neonates, while in adults, nerve-derived neuregulin-1 is no longer required for PSC survival (Trachtenberg and Thompson, 1996). Secondly, excessive neuregulin-1 signaling caused by the application of exogenous neuregulin-1 to neonatal muscles causes dramatic sprouting, proliferation and migration of PSCs that eventually lead to the complete loss of functional synapses (Trachtenberg and Thompson, 1997). In mature muscles, however, neuregulin-1 application does not cause PSC migration or synapse loss. Thirdly, ectopic expression of activated ErbB2 receptor in Schwann cells of young adult mice causes sprouting and migration of PSCs similar to that induced by neuregulin-1 application in neonates (Hayworth et al., 2004). Finally, ErbB2 is downregulated in Schwann cells postnatally and, in adults, ErbB2 is not detected in PSCs (Cohen et al., 1992; Grinspan et al., 1996; Moscoso et al., 1995; Trinidad et al., 2000). Together, these data strongly suggest that the fine-tuning of neuregulin-1/ErbB signaling is critical for the development, maturation and/or maintenance of PSCs and that the modulation of ErbB2 receptor levels plays an important role in this process. Currently, the mechanisms which regulate ErbB2 levels in maturing PSCs are not known.

In this study, we report the identification of LNX1 as a binding partner and thus a potential regulator of ErbB2 signaling in PSCs. LNX1 is a RING type E3 ubiquitin ligase which contains, in addition to its RING domain, an NPXY motif and 4 PSD-95, DlgA, ZO-1 (PDZ) domains (Fig. 1A). E3 ubiquitin ligases are a large and

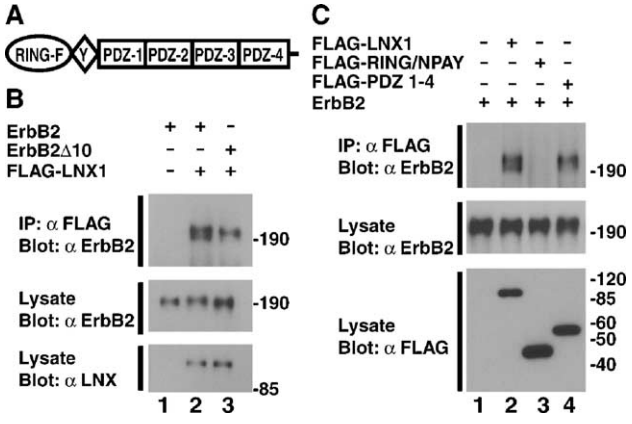

Fig. 1. Interaction of LNX1 and ErbB2. (A) Domain structure of LNX1 protein. RING-F = RING finger domain, Y = NPXY motif, PDZ = PSD-95, DlgA, ZO-1 domain. (B) Co-immunoprecipitation of LNX1 with ErbB2 from transfected COS-7 cells. FLAG epitope tagged LNX1 (FLAG-LNX1) was co-expressed in COS-7 cells with either full length ErbB2, or ErbB2 lacking the C-terminal 10 amino acids (ErbB2Δ10). Immunoprecipitation was performed with anti-FLAG tag antibodies, and cell lysates and immunoprecipitated proteins were subjected to Western blotting for ErbB2 and LNX1. LNX1 interacts specifically with ErbB2 though the interaction is not completely dependent on the ErbB2 C-terminal PDZ binding motif. (C) Co-immunoprecipitation of ErbB2 with deletion constructs of LNX1. COS-7 cells were transfected with ErbB2 and FLAG epitope tagged LNX1 constructs either containing or lacking the PDZ domains (FLAG-PDZ1-4 or FLAG-RING/NPAY respectively). Immunoprecipitation was performed as in panel (B). The LNX1 PDZ domains are both necessary and sufficient for the interaction of LNX1 with ErbB2.

diverse group of proteins which catalyze the final attachment of ubiquitin to substrate proteins and are believed to confer specificity to the ubiquitination process (Pickart, 2004). LNX1 was previously identified as a ligand of Numb, a component of the Notch signaling pathway. This interaction is mediated by the NPXY motif of LNX1 and leads to ubiquitination of Numb by the RING domain of LNX1, thereby targeting Numb to proteasomal degradation (Nie et al., 2002). Here, we identify ErbB2 as a novel ligand of LNX1 and demonstrate direct binding of ErbB2 to the PDZ domains of LNX1 by co-immunoprecipitation. Immunostaining reveals that LNX1 is specifically localized in PSCs at the NMJ but not in Schwann cells along the motor axon. Developmentally, the expression of LNX1 protein in PSCs increases postnatally, reaches peak levels around postnatal day 14–21 and persists in adults, a pattern that inversely correlates with the level of ErbB2 in PSCs. Furthermore, the LNX1 staining disappears upon denervation, consistent with the observation that ErbB2 proteins are upregulated in Schwann cells after denervation (Carroll et al., 1997; Cohen et al., 1992). Taken together, these data suggest that LNX1 may regulate the level of ErbB2 receptors in PSCs, possibly through the ubiquitination of ErbB2, and thus may play an important role in the maturation and maintenance of PSCs at the NMJ.

Results

LNX1 proteins interact with the ErbB2 receptor

Using yeast two-hybrid screening, we identified LNX1 as a potential binding partner of ErbB2 receptors (see Experimental methods). To confirm the interaction of LNX1 and ErbB2 in mammalian cells, we co-expressed FLAG epitope tagged LNX1 and full length ErbB2 in COS-7 cells. ErbB2 could be specifically co-immunoprecipitated with LNX1 using an anti-FLAG antibody (Fig. 1B, lane 2). A specific interaction between ErbB2 and LNX1 was also seen using the reciprocal combination of antibodies (not shown). The ErbB2 receptor contains a typical class II PDZ binding motif (VPV) at its C-terminus. This PDZ binding motif has been shown to interact with Erbin and PICK1, both of which are PDZ domain-containing proteins that are thought to regulate the subcellular localization of ErbB2 receptors (Borg et al., 2000; Huang et al., 2001, 2002; Jaulin-Bastard et al., 2001). To test whether the interaction of LNX1 and ErbB2 is mediated by the PDZ domains of LNX1, we made LNX1 constructs with or without the PDZ domains and tested their interaction with ErbB2 by co-immunoprecipitation. Only the PDZ domain containing region interacts, indicating that the PDZ domains of LNX1 are required for the interaction (Fig. 1C, lane 4). To determine if the C-terminal PDZ binding motif of ErbB2 was also required, we deleted the last 10 amino acids of the ErbB2 receptor and examined its binding to full length LNX1. To our surprise, the interaction of LNX1 and ErbB2 does not completely depend on the C-terminal PDZ binding motif (Fig. 1B, lane 3). These data suggest that the PDZ domains of LNX1 may interact with internal PDZ binding motifs as well as the C-terminal motif on the cytoplasmic domain of ErbB2. Although PDZ domains most commonly bind to C-terminal motifs, their interactions with internal PDZ binding motifs have been well documented (Christopherson et al., 1999; Cuppen et al., 1998; Shieh and Zhu, 1996; Uemura et al., 2004; Xia et al., 1997).

To examine the interaction of LNX1 and ErbB2 in vivo, we generated antibodies against LNX proteins. There are two

members of the LNX gene family, LNX1 and LNX2, which share identical structural motifs and 47% sequence identity at the amino acid level (Rice et al., 2001). We therefore generated and characterized LNX1-specific (LNX1-PDZ3/4) and LNX2-specific (LNX2) antibodies. When tested with transfected COS-7 cells, both of these antibodies are highly specific in immunoblotting and immunostaining (Figs. 2B, C, Supplemental Fig. S1). We also used a previously described antibody (LNX-NPAY/RING) that recognizes both LNX1 and LNX2 (Dho et al., 1998; Fig. 2A).

To demonstrate that LNX1 and ErbB2 interact in vivo, we immunoprecipitated LNX protein from P14 mouse brain lysate. LNX1 cannot be detected in whole brain lysates but is readily detected following immunoprecipitation (Fig. 2D). ErbB2 is specifically co-purified with LNX following immunoprecipitation from brain lysates (Fig. 2D). This indicates that LNX1 and ErbB2 can indeed form a complex in vivo.

LNX1 is exclusively localized to perisynaptic Schwann cells at the NMJ

To examine the potential role of LNX1 in regulating neuregulin-1/ErbB signaling at the NMJ, we examined the expression of

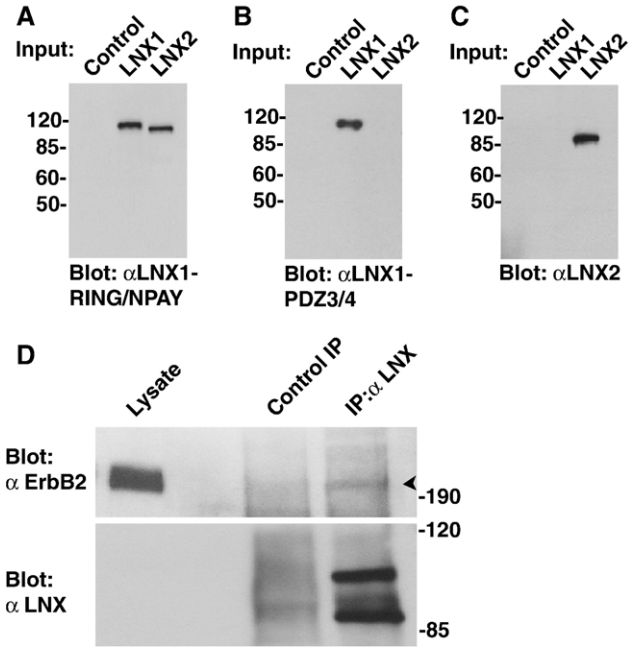

Fig. 2. Characterization of anti-LNX antibodies and interaction of LNX and ErbB2 in vivo. Antibodies raised against LNX1 or LNX2 were tested for specificity and cross-reactivity by Western blotting. (A–C) Lysates from untransfected (Control) and LNX1 or LNX2 transfected COS-7 cells were separated by gel electrophoresis and subjected to immunoblotting with the indicated antibodies. The LNX1-RING/NPAY antibody can recognize both LNX1 and LNX2 (A), while the LNX1-PDZ3/4 antibody is specific for LNX1 (B) and the LNX2 antibody recognizes LNX2 specifically (C). (D) Co-immunoprecipitation of LNX and ErbB2 from tissue. Immunoprecipitation from P14 mouse brain lysates was performed using either anti-LNX serum or preimmune serum as a control. ErbB2 is specifically co-purified with LNX in the anti-LNX immunoprecipitation (arrowhead). Similar results were obtained in three independent experiments. Note that the lower LNX band detected following immunoprecipitation represents a short alternatively spliced isoform of LNX1 that is expressed in the brain as previously reported (Dho et al., 1998).

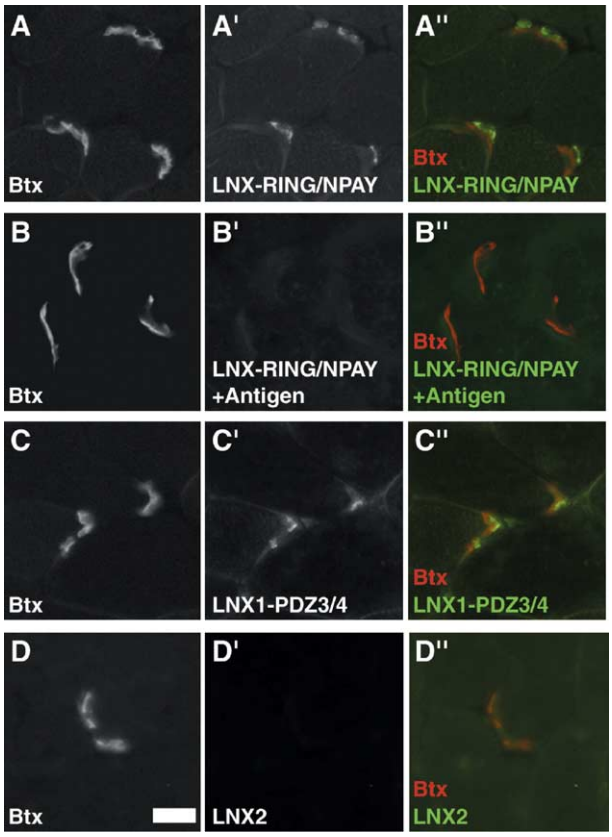

Fig. 3. Localization of LNX1 at the NMJ. Immunofluorescent staining of cross sections of adult mouse tibialis anterior muscle. Rhodamine-conjugated α-bungarotoxin (Btx) was used to stain acetylcholine receptors at the NMJ (A–D). LNX1 protein is detected at the NMJ using both the LNX1-RING/NPAY (A′) and LNX1-PDZ3/4 antibodies (C′). Staining is blocked by preincubation of the LNX1-RING/NPAY antibody with the antigen against which it was raised (B′). LNX2 is not detected at the NMJ (D′). Merged images are shown in panels A″–D″ with α-bungarotoxin in red and anti-LNX staining in green. Scale bar = 20 μm.

LNX1 protein in the peripheral nervous system. To determine the localization of LNX proteins in the neuromuscular system, we co-stained cross sections of adult mouse muscles with anti-LNX antibodies and α-bungarotoxin (α-Btx) which specifically binds nicotinic acetylcholine receptors, thus marking the NMJ. As shown in Fig. 3(A–A″, C–C″), antibodies that recognize LNX1 stain the NMJ brightly with little staining along the muscle membrane outside the synapse. The staining is specific as it can be blocked by preincubation of the antibodies with antigens (Figs. 3B–B″). In contrast, the antibody specific to LNX2 did not show any staining of the NMJ (Figs. 3D–D″).

The NMJ is a tripartite synapse consisting of the presynaptic nerve terminal, the postsynaptic complex and a glial component, the PSCs. Close examination of the staining pattern of LNX1 showed that it did not precisely match the pattern of α-Btx staining for acetylcholine receptors (Figs. 3A″, C″). Indeed, the LNX1 staining is often adjacent to but just outside of the acetylcholine receptor staining relative to the muscle fiber, suggesting that it may be associated with either the presynaptic nerve terminal or the PSCs. To resolve this, we examined LNX1 localization on thick longitudinal muscle sections, in which the three components of the NMJ are readily distinguishable with antibody or α-Btx staining. By examining NMJs that are formed on the sides of muscle fibers,

we get a side view of the NMJ (Figs. 4A and B). The LNX staining is separate from, but adjacent to, both the postsynaptic acetylcholine receptors and the presynaptic marker synaptophysin. LNX is closely associated with DAPI-stained cell nuclei and is primarily localized to the side of the cell that faces the NMJ. When NMJs on the top of muscle fibers are examined en face, LNX1 staining is shown to be restricted to the same area as the acetylcholine receptor staining, but the two patterns never precisely match (Figs. 4C–C″). Unlike α-Btx staining, which marks the pretzel-like pattern of acetylcholine receptors at the postsynaptic site, the LNX1 staining appears to be confined to the cytoplasmic region of a few cells at the NMJ (asterisk in Figs. 4C–C″). This strongly suggests that LNX1 is expressed in PSCs. To verify that these were indeed Schwann cells, we co-stained for the Schwann cell marker S100, which is expressed in all Schwann cells. We found that the LNX1-stained cells were also S100 positive (Arrows in Figs. 4D–D″). The strongest LNX1 staining appears to be in the cell body of the PSCs and is largely excluded from the nucleus.

Staining of the NMJ indicates that LNX1 is expressed in PSCs capping the nerve terminals (arrows) but not in myelinating Schwann cells associated with motor axons approaching the junction (Figs. 4E–E″, arrowheads). To further examine the expression of LNX1 in other types of Schwann cells, we stained sections of the sciatic nerve, which contains mostly myelinating Schwann cells, and sections of a postganglionic sympathetic axon, which contains predominantly non-myelinating Schwann cells (Little and Heath, 1994). Although large numbers of Schwann cells are present in these nerves, as evidenced by staining for S100 which is expressed in all Schwann cells, no LNX1 staining was observed in either of these nerves (Figs. 4F–G). These data strongly indicate that LNX1 is exclusively expressed in PSCs at the NMJ.

LNX1 expression in PSCs is developmentally regulated

ErbB2 is developmentally downregulated in Schwann cells, and, by adulthood, ErbB2 protein is undetectable in PSCs

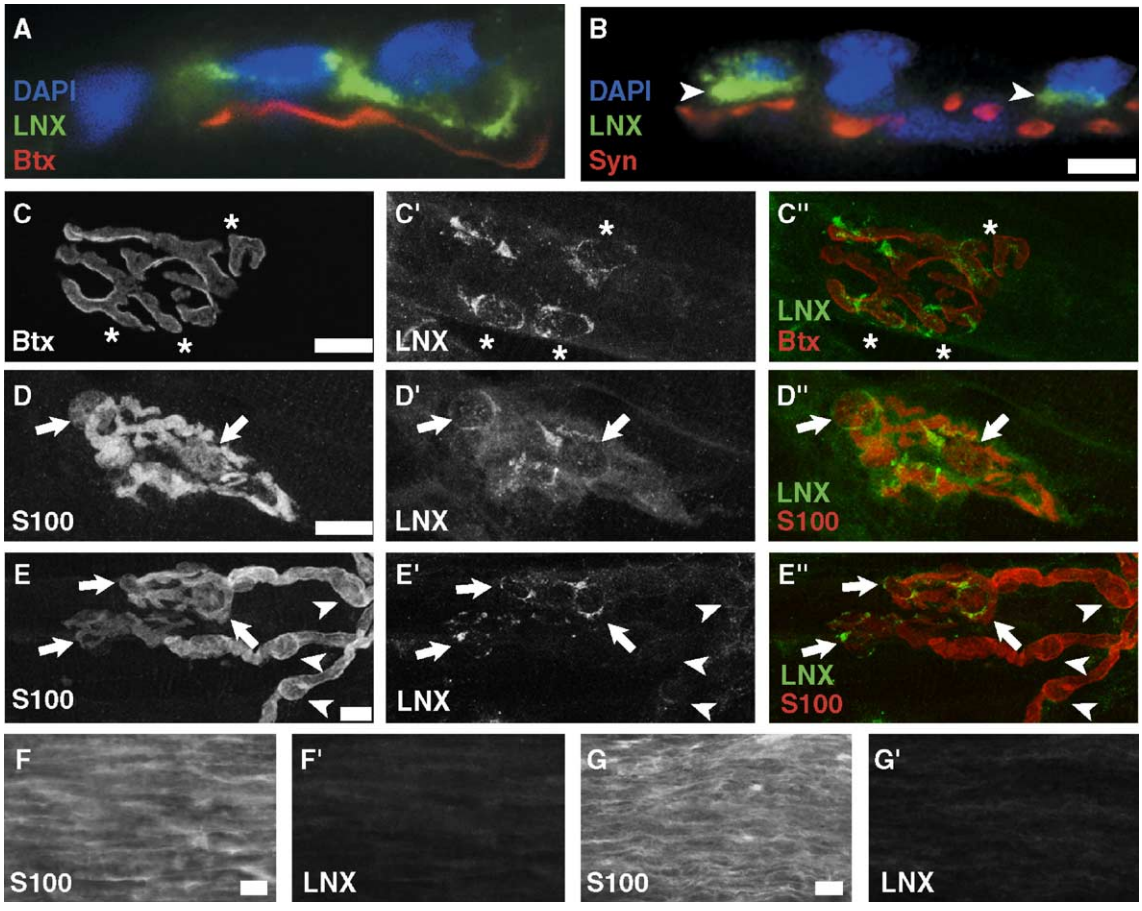

Fig. 4. LNX1 expression is restricted to perisynaptic Schwann cells. (A–E) Staining for LNX1 in longitudinal sections of P19 sternomastoid muscle. Sections were stained with the LNX1 specific LNX-PDZ3/4 antibody (C′–E′ and green in merged images A, B, C″–E″). Co-staining was for acetylcholine receptors with α-bungarotoxin (A, C), the presynaptic marker synaptophysin (B) or for the Schwann cell marker S100 (D and E; red in the merged images). In side views of the NMJ (A, B), LNX1 staining is clearly separated from both the postsynaptic and presynaptic markers. LNX1 is associated with perisynaptic Schwann cells whose cell nuclei were stained with DAPI. LNX1 is localized predominantly in the cytoplasm on the junctional side of these nuclei, directly adjacent to the nerve terminal (arrowhead). In en face views of the NMJ (C–E), LNX1 staining is confined to several S100 positive perisynaptic Schwann cells at each NMJ (asterisk in C–C″ and arrows in D–D″ and E–E″). Note that the PSC cell bodies stain less intensely for S100 than the Schwann cell processes that wrap around the nerve terminal. No LNX1 is detected in myelinating Schwann cells along the motor axon (arrowheads in panels E–E″). (F, G) Staining for LNX1 in longitudinal sections of P21 peripheral nerves. Sciatic nerve (F, F′) or postganglionic sympathetic nerve (G, G′) was co-stained for the Schwann cell marker S100 (F, G) and LNX1 using the anti-LNX-PDZ3/4 antibody (F′, G′). No LNX1 staining was observed in Schwann cells in either nerve. Scale bar = 10 μm.

(Moscoso et al., 1995; Trinidad et al., 2000). This downregulation coincides with the maturation of PSCs and with the establishment of the independence of PSCs from neuregulin-1/ErbB signaling. Because excessive activation of ErbB signaling by neuregulin-1 leads to the destabilization of PSCs in young rats but not in adults, the downregulation of ErbB2 in PSCs may be an important part of the development and/or maturation of PSCs. Since LNX1 is an E3 ubiquitin ligase that interacts with ErbB2 and is exclusively expressed in the PSCs at the NMJ, it could potentially play a role in the regulation of ErbB2 receptor levels by targeting ErbB2 to a protein degradation pathway. To test whether the expression profile of LNX1 is consistent with a role in the regulation of ErbB2 signaling during the development and maturation of PSCs, we examined the expression of LNX1 at the NMJ of various developmental stages. In muscle cross sections, staining for LNX1 was not detected at E13 (not shown) but is present by embryonic day 16 (E16). At this stage, the staining is dim and loosely associated with the NMJ (Figs. 5A–A″). Staining intensity increases postnatally and reaches a peak around P14–21 and persists into adulthood (Figs. 5B–F″), a pattern that inversely correlates with the level of ErbB2 in PSCs. Furthermore, the staining of LNX1 becomes progressively more closely associated with the NMJ postnatally, reminiscent of maturation and capping of the NMJ by PSCs (Figs. 5B–F″). These data are consistent with the idea that LNX1 may play a role in the postnatal modulation of ErbB2 levels in PSCs.

LNX1 expression in PSCs is downregulated following denervation

Schwann cells in adults respond to denervation of muscle fibers by undergoing molecular and morphological changes, resulting in the growth of processes that play an important role in guiding nerve growth during muscle reinnervation (Son et al., 1996). One such molecular change is the upregulation of ErbB2 in Schwann cells after axotomy (Carroll et al., 1997; Cohen et al., 1992). If LNX1 plays a role in the dynamic regulation of ErbB2 in PSCs, we might expect that the expression of LNX1 in PSCs would also change after denervation. To test this, we denervated hindlimb muscles in 8-week-old mice by transection of the sciatic nerve on one side of the body and compared the expression of LNX1 in denervated and non-denervated muscles three days later. The presynaptic marker protein synaptophysin was not detected at NMJs from the denervated muscle, indicating that the denervation was successful (Figs. 6D–D″). As expected, Schwann cells were still present at denervated NMJs as indicated by S100 staining (Figs. 6E–E″). However, staining for LNX1 is completely absent in denervated muscle, indicating that LNX1 expression is dramatically downregulated after denervation (Figs. 6F–F″). This is again consistent with a role for LNX1 in dynamically regulating the level of ErbB2 in PSCs.

One consequence of denervation is the loss of neurotransmission at the NMJ. Since PSCs are known to be responsive to neurotransmitter release (Auld and Robitaille, 2003b), the loss of neurotransmission might trigger downregulation of LNX1 in PSCs. To test whether LNX1 expression in PSCs is regulated by neurotransmitter release, we performed a nerve block experiment. Tetrodotoxin was applied to the sciatic nerve on one side of the body for 7 days to block all evoked activity at the NMJ. LNX1 staining was still present in PSC under these conditions (Figs. 6G, H). Thus, the downregulation of LNX1 following denervation is unlikely to be a consequence of the loss of evoked neural activity.

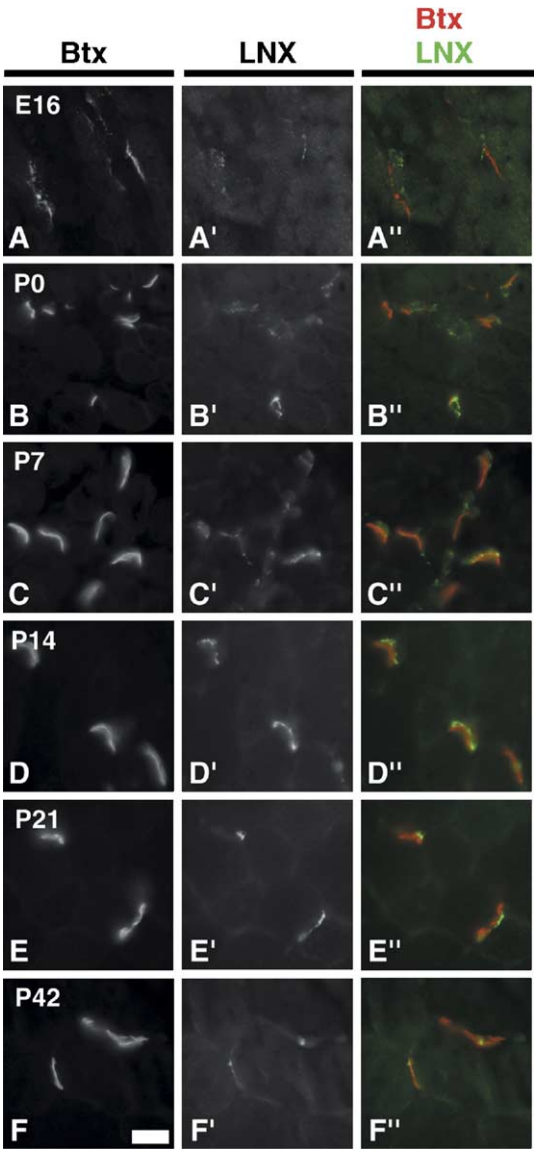

Fig. 5. Developmental regulation of LNX1 expression in perisynaptic Schwann cells during maturation of the NMJ. Cross sections of mouse tibialis anterior muscle at the indicated developmental stages were stained with the LNX-RING/NPAY antibody (A′–F′, green in merged images A″–F″) and α-bungarotoxin to mark acetylcholine receptors (A–F, red in merged images A″–F″). LNX1 staining at the NMJ is detected as early as embryonic day 16 (E16), increases postnatally to peak levels around postnatal day 14–21 and then persists in adults (P42). Scale bar = 20 μm.

Application of neuregulin to neonatal muscle causes PSCs to extend processes in a manner that is similar to, though more dramatic than, the morphological changes observed in PSCs following denervation in adult animals (Trachtenberg and Thompson, 1997). We therefore examined LNX1 expression in PSCs after exogenous neuregulin application in neonatal mice. S100 staining revealed that PSCs had extended processes away from the NMJ in neuregulin-treated muscle but not in vehicle-treated control muscle (Figs. 7A, B). LNX1 staining was present in both the cytoplasmic region as well as in the processes of these cells (Fig. 7B). In neuregulin-treated muscle, we also observed S100 positive cells in extra-junctional regions that represent PSCs that have migrated away from the NMJ as reported by Trachtenberg and Thompson

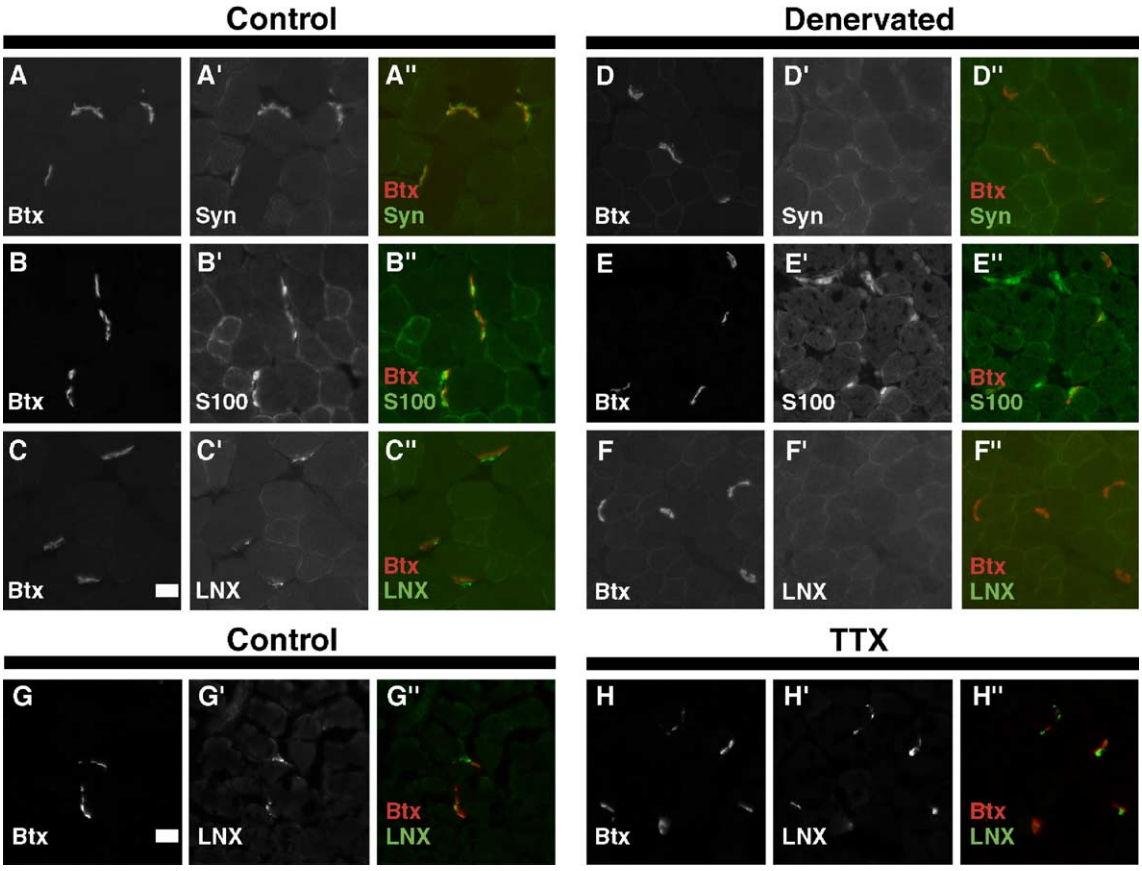

Fig. 6. Downregulation of LNX1 expression after denervation of the NMJ. Cross sections of control (A–C) or denervated (D–F) tibialis anterior muscle were stained with α-bungarotoxin to identify neuromuscular junctions (A–F, red in A″–F″). Sections were co-stained for the presynaptic marker synaptophysin (A′, D′, green in A″, D″), the Schwann cell marker S100 (B′, E′, green in B″, E″) and for LNX1 using the LNX-RING/NPAY antibody (C′, F′, green in C″, F″). Schwann cells are still present at denervated NMJs, but LNX1 has been downregulated and is not detected in perisynaptic Schwann cells 3 days following denervation. (G, H) Cross sections of control muscle (G) or muscle where evoked neural activity had been blocked by application of tetrodotoxin to the sciatic nerve (H) were co-stained for LNX1 (G′, H′, green in G″, H″) and α-bungarotoxin (G, H, red in G″, H″). LNX1 expression in PSCs was unaffected by nerve block. Scale bar = 20 μm.

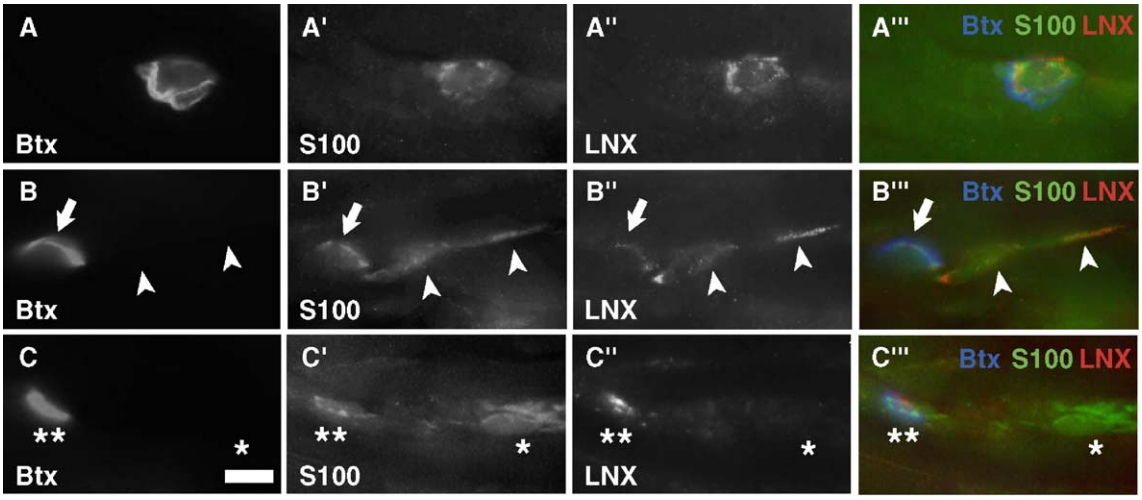

Fig. 7. Expression of LNX1 in PSCs following exogenous neuregulin application. Soleus muscles in neonatal mice were treated for 5 days with vehicle control (A–A‴) or neuregulin (B–B‴, C–C‴). Longitudinal sections were prepared and stained with α-bungarotoxin to identify neuromuscular junctions, anti-S100 to mark Schwann cells and with the anti-LNX-PDZ3/4 antibody. S100 staining shows that PSCs have extended processes away from the NMJ in neuregulin-treated (B′, B‴ arrowheads) but not in control (A′, A‴) muscle. LNX1 is present in both the cytoplasmic region (B″, B‴ arrows) and the processes (B″, B‴ arrowheads) of neuregulin-treated PSCs that remain in contact with the NMJ. However, LNX1 staining is not observed in PSCs that have migrated away from the endplate region (C–C‴ asterisk). The nearest NMJ is shown for reference in panels (C–C‴) (double asterisk). Scale bar = 10 μm.

(1997). LNX1 staining was absent or greatly diminished in these migrating cells (Fig. 7C). These results provide further evidence that LNX1 expression is limited to PSCs that are associated with the NMJ.

Discussion

Perisynaptic glial cells ensheath synapses throughout the peripheral and central nervous system, yet the full importance of glial cells in synaptic development and function has only been recognized recently. It is now clear that most synapses are tripartite structures consisting of presynaptic nerve terminals, postsynaptic cells and perisynaptic glial cells and that there is extensive signaling and communication among the three synaptic components (Araque et al., 1999; Auld and Robitaille, 2003a,b; Fields and Stevens-Graham, 2002; Ullian et al., 2004). In the past few years, an increasing body of evidence suggests that glial cells not only play a supportive role in synaptic maintenance, but also have more active roles during synapse formation and in the modulation of synaptic transmission (Castonguay and Robitaille, 2001; Jahromi et al., 1992; Reist and Smith, 1992; Robitaille, 1998; Ullian et al., 2001).

At the NMJ, each synapse is capped by 2–4 PSCs with their processes wrapping around every nerve terminal. This tight association of PSCs with the nerve terminal is critical for both the structural stability and the function of the NMJ (Auld and Robitaille, 2003b). The non-myelinating PSCs are distinct from the myelinating Schwann cells along the motor axon in both their function and cell biology (Jessen and Mirsky, 2002). Little is known about the differentiation and maintenance of PSCs, and it is not clear whether PSCs represent a default state for Schwann cell precursors that do not enter the myelination pathway or whether their differentiation is triggered by inductive signals from either the nerve terminal and/or the muscle fiber. While many factors involved in the myelination process have been identified, few perisynaptic glial cell specific genes have been identified (Jessen and Mirsky, 2002). The exclusive localization of LNX1 in developing PSCs at the NMJ indicates the existence of molecular pathways which are unique to PSCs. In addition, LNX1 expression is lost when the nerve degenerates following denervation. This supports the notion that the nerve plays an instructive role in determining some of the unique properties of PSCs.

Following denervation, PSCs undergo a variety of biochemical changes including alterations in the levels of several cytoskeletal and signaling proteins that may play a role in regulating PSC morphology (Auld and Robitaille, 2003b). Some of these changes, such as the upregulation of GFAP and the p75 neurotrophin receptor, appear to be a consequence of the loss of neurotransmitter release since they are also observed when neurotransmission is blocked (Georgiou et al., 1994; Hassan et al., 1994). Changes in the levels of other proteins are independent of neurotransmission and might be triggered by the loss of nerve contact or the products of axonal degeneration (Auld and Robitaille, 2003b; Hassan et al., 1994). Our results show that blocking action potentials in the nerve with tetanus toxin did not alter LNX1 expression in PSC, indicating that the loss of evoked neurotransmission is unlikely to play a role in the downregulation of LNX1 following denervation. While the role of spontaneous activity has yet to be examined, this result suggests that the downregulation of LNX1 may be a consequence of the loss of the nerve terminal rather than alterations in neurotransmission. The question of whether LNX1 downregulation is a primary response to the loss of the nerve terminal or a secondary effect of other biochemical changes that occur in the PSCs following denervation remains open.

Apart from those changes attributable to a loss of neurotransmission, the signaling mechanisms that trigger other biochemical/morphological changes in PSCs following denervation have not been elucidated. Application of exogenous neuregulin to neonatal muscle mimics some of the morphological changes seen in PSCs following axotomy and in addition can cause migration of PSCs away from synaptic sites (Trachtenberg and Thompson, 1997). We observed that following neuregulin application LNX1 expression in PSCs that remained at synapses was unaltered while LNX1 was not present in PSCs that had migrated away from the NMJ. The fact that LNX1 is not seen in migrating cells suggests that LNX1 expression in PSCs may depend on their close association with the NMJ and further underscores the specificity and tight regulation of LNX1 expression in PSCs.

Targeted protein degradation through ubiquitination is now recognized as a widespread regulatory mechanism in biology. Attachment of polyubiquitin targets proteins for degradation by the proteasome, whereas monoubiquitination of transmembrane proteins can trigger endocytosis from the cell surface, which may be followed by lysosomal degradation (Hicke and Dunn, 2003). Recent studies have demonstrated that ubiquitination is one of the key mechanisms controlling the level of receptor tyrosine kinases in diverse cellular processes (Marmor and Yarden, 2004; Sweeney and Carraway, 2004). The process of ubiquitination is catalyzed by the sequential actions of three enzymes termed E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase). The E3 ubiquitin ligases are a large and diverse group of proteins which catalyze the final attachment of ubiquitin to the substrate protein. E3 ubiquitin ligases are also believed to confer specificity to the ubiquitination process, often by directly binding the target proteins (Pickart, 2004). LNX1 is a RING domain containing E3 ubiquitin ligase, originally identified as a ligand for Numb (Dho et al., 1998). However, little is known about the cellular localization of LNX1 protein or its biological function in vivo. Our data, using two independently generated antibodies, represent the first in vivo localization of LNX1 protein and show that, in the peripheral nervous system, LNX1 is exclusively expressed in PSCs at the NMJ. While the ubiquitination system has been shown to play a role in presynaptic differentiation of nerve terminals at the NMJ (DiAntonio et al., 2001; Liao et al., 2004), LNX1 is the first component of the ubiquitination pathway to be specifically identified in PSCs, to our knowledge.

Neuregulin-1/ErbB signaling has been shown to play a role in the postsynaptic differentiation of both the NMJ and central synapses (Buonanno and Fischbach, 2001; Fischbach and Rosen, 1997). In addition, the importance of neuregulin-1/ErbB signaling in the early development of Schwann cell precursors and in the differentiation of myelinating Schwann cells is well established (Adlkofer and Lai, 2000; Garratt et al., 2000a). By contrast, the role of neuregulin-1/ErbB signaling in the differentiation and maintenance of PSCs is not fully understood. Excessive activation of ErbB signaling in PSCs of young rats, by the application of exogenous neuregulin-1 to muscles, causes dramatic alterations of PSCs (Trachtenberg and Thompson, 1997). They extend processes, proliferate and migrate away from synapses. These changes lead to the withdrawal of nerve terminals from synaptic sites and eventual

loss of the NMJ. The response of PSCs to exogenous neuregulin-1 in adults is much reduced and does not lead to the proliferation or migration of PSCs nor synaptic loss (Trachtenberg and Thompson, 1997). In addition, ErbB2 is downregulated during PSC development and ErbB2 protein is undetectable in adult PSCs, though ErbB3 is still present (Moscoso et al., 1995; Trinidad et al., 2000). These observations suggest that the attenuation of neuregulin-1/ErbB signaling is a maturation process critical for the stability of PSCs at the NMJ and that this process may be a result of ErbB2 downregulation in PSCs. Currently, the mechanisms by which ErbB2 levels are regulated in maturing PSCs are not known. The identification of LNX1 as a binding partner of ErbB2 in vivo provides a potential mechanism for post-translational regulation of ErbB2 levels through ubiquitination-dependent pathways in PSCs. Consistent with this proposed function, LNX1 expression increases during PSC maturation and persists in adults, a pattern that is inversely related to the level of ErbB2 in PSCs as well as their responsiveness to neuregulin-1. In addition, following denervation, when PSCs become more responsive to neuregulin-1, LNX1 expression disappears. Based on the fact that LNX1 interacts with ErbB2 and that its temporal profile of expression both in development and following denervation inversely correlates with the level of ErbB2 in PSCs, we propose that LNX1 may regulate neuregulin-1/ErbB signaling in PSCs by targeting ErbB2 for ubiquitination and thus play an important role in the development and/or maturation of PSCs.

Fine-tuning of neuregulin-1/ErbB signaling in PSCs may be required for several processes which occur during PSC maturation including the maintenance of PSCs at synapses, the prevention of PSC proliferation and the maintenance of PSCs in a non-myelinating status. The exclusive expression of LNX1 in PSCs and its interaction with ErbB2 may help us to understand these and other aspects of perisynaptic glial cell development. To this end, the generation and analysis of LNX1 knockout mice will be extremely informative. Currently, we know little about the molecular mechanisms unique to PSCs. This is partly because of the lack of molecular markers for PSC and also because of the small size of the PSC population compared to other cell types associated with muscle fibers. The identification of LNX1 as a PSC specific protein may provide useful tools for genetic manipulations specifically in PSCs, such as transgene expression and targeted gene ablation. Furthermore, genetic labeling of PSCs might allow enrichment of PSCs for biochemical studies.

Experimental methods

Yeast two-hybrid screening

Both neuregulin-1 and ErbB2 contain similar consensus Type II PDZ binding motifs at their carboxyl termini. To identify proteins which might regulate neuregulin-1/ErbB signaling, constructs encoding carboxyl terminal fragments of both proteins were cloned into the pGBKT7 plasmid (Clontech) and transformed into the *Saccharomyces cerevisiae* AH109 reporter strain using a modified lithium-acetate protocol (Vojtek et al., 1993). The bait-bearing strain was subsequently co-transformed with a spinal cord cDNA library. Selection for His3 reporter gene activation was performed on selection agar plates without histidine, leucine and tryptophane (SD-LWH), and colonies appearing after 4–5 days at 30°C were restreaked on plates which also lacked adenine. Library plasmids were recovered from Ade2 reporter gene positive clones following the Matchmaker 3 protocol (Clontech) and their inserts sequenced. Several PDZ domain containing proteins, including PICK1 and LNX1, were identified using this approach and were further tested for interaction with ErbB2 by co-immunoprecipitation (see below).

cDNA libraries

Mouse postnatal day 1 spinal cord cDNAs were synthesized and cloned into the HybriZAP 2.1 vector according to the manufacturer's instructions (Stratagene, La Jolla, CA). Briefly, double-stranded cDNAs were synthesized with either oligo-dT primers or random primers. Oligo-dT and random primed cDNAs were cloned directionally into *Eco*RI/*Xho*I sites of the HybriZAP 2.1 vector. Libraries with a primary titer of 6×10^6 (dT primed) and 3.5×10^6 (random primed) and average insert sizes of 1.7 and 1.5 kb (dT and random primed respectively) were obtained. Libraries were amplified, and phagemid DNA was excised and purified.

Cell culture and transfection

COS-7 cells were cultured in DMEM high glucose media (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and penicillin/streptomycin. For transfection, cells were plated without antibiotics in 35-mm tissue culture dishes and grown until 50% confluent. Cells were transfected with a total of 1 μg DNA and 2 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per well. 36–48 h after transfection, cells were washed once with phosphate-buffered saline (PBS) and harvested.

Co-immunoprecipitation

For immunoprecipitation, LNX1 mouse cDNAs were expressed in COS-7 cells with an N-terminal FLAG epitope tag, while rat ErbB2 cDNAs were expressed without any tag. Transfected cells were harvested in ice-cold lysis buffer containing PBS with 1% Nonidet P-40, 0.1% deoxycholate (Sigma, St. Louis, MO) and *Complete* protease inhibitor (Roche, Indianapolis, IN). After homogenization in a dounce homogenizer (100 strokes), the lysate was incubated on ice for 1 h and then centrifuged at 14,000 rpm for 30 min. The supernatant was transferred to a new tube, and immunoprecipitation was performed by adding 30 μl of anti FLAG M2 antibody coupled to agarose beads (Sigma, St. Louis, MO). The lysate and beads were incubated for 3–6 h with gentle mixing at 4°C. Samples were centrifuged at $1000 \times g$ for 2 min, and the supernatant was removed. Samples were washed six times for 10 min each with lysis buffer. Proteins were eluted from agarose beads by addition of loading buffer, boiled for 5 min and analyzed by Western blotting with appropriate antibodies. In mapping the interaction of ErbB2 on LNX1, FLAG tagged constructs encoding the RING-NPAY region (amino acids 1–277) or the PDZ1–PDZ4 region (amino acids 268–728) of LNX1 were cotransfected with ErbB2 and immunoprecipitated as above. For immunoprecipitation from tissue, brain lysates were obtained by homogenizing 1 g of P14 mouse forebrain in 10 ml of IP buffer (20 mM Tris–Cl pH 7.5, 100 mM NaCl, 1% Nonidet P-40, 0.1% deoxycholate and *Complete* protease inhibitor). Lysate was prepared as described above for transfected cells. Lysate was pre-cleared by adding 100 μl Protein A agarose beads (Invitrogen, Carlsbad, CA), mixing for 20 min and

centrifuging at 1000×*g* to remove Protein A beads. 10 μl of guinea pig anti-LNX1/2 PDZ3/4 serum or control pre-immune serum was then added, and the lysate was gently mixed at 4°C for 2 h. Immunoprecipitation was performed by adding 80 μl Protein A agarose beads and mixing at 4°C for 2 h. Proteins were eluted as described above.

Antibodies

The mouse monoclonal ErbB2 antibodies were from Transduction Laboratories (Lexington, KY; catalog number E19420) and Labvision/Neomarkers (Fremont, CA; catalog number MS-730). The rabbit polyclonal anti-synaptophysin antibody was from Zymed Laboratories Inc. (South San Francisco, CA; catalog number 18–0130). The rabbit polyclonal S100 antibody was from DAKO (Carpinteria, CA; catalog number Z 0311). The rabbit polyclonal LNX antibody (LNX1-RING/NPAY) has been described previously (Dho et al., 1998). The guinea pig polyclonal LNX1 antibody (LNX1-PDZ3/4) was raised against a hexahistidine tagged fusion protein of PDZ domains 3 and 4 of LNX1 p80 isoform (amino acids 498–728) and affinity purified. The guinea pig polyclonal LNX antibody (LNX1/2-PDZ3/4) was raised against the same PDZ3/4 fusion protein and recognizes both LNX1 and 2. The rabbit polyclonal LNX2 antibody was raised against a peptide corresponding to amino acids 129–149 of LNX2 and affinity purified. FITC- and Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Rhodamine- and Alexa-647-conjugated α-bungarotoxin was from Molecular Probes (catalog numbers T-1175 and B-35450).

Antibody characterization

COS-7 cells were transfected with either LNX1 or LNX2 expression constructs. To test antibody specificity, Western blotting was performed on cell lysates prepared from transfected or untransfected control cells. Similarly, immunostaining was performed on transfected COS-7 cells grown on poly-D-lysine-coated glass coverslips. Cells were fixed on the coverslips for 15 min in 4% paraformaldehyde/PBS and washed 3 times in PBS. Coverslips were then blocked with 2% BSA, 5% normal goat serum and 0.2% Triton in PBS for 30 min and incubated with primary antibody 3–4 h in blocking solution minus Triton. Coverslips were then washed 3 times for 5 min in PBS, incubated with the appropriate secondary antibody for 2 h, washed again and mounted in 90% glycerol with 0.1% *p*-phenylenediamine for imaging.

Immunohistochemistry

For muscle cross sections, mice were deeply anesthetized, perfused with Ringers solution and the tibialis anterior was dissected out. The tissue was embedded in OCT compound (TissueTek, Torrance, CA), frozen in 2-methylbutane chilled in liquid nitrogen and sectioned at 10 μm on a Leica CM 1850 cryostat. Prior to antibody staining sections were fixed on the slides for 10 min in 4% paraformaldehyde/PBS and washed 4–5 times in PBS. Sections were then blocked with 2% BSA, 5% normal goat serum and 0.2% Triton in PBS for 1 h and incubated with primary antibody overnight in blocking solution minus Triton. Sections were then washed 6 times for 5 min in PBS, incubated with the appropriate secondary antibodies for 3–4 h, washed again and mounted in 90% glycerol with 0.1% *p*-phenylenediamine. Stained sections were imaged on a Zeiss Axioskop 2 fluorescent microscope using a 20× or 40× objective.

For longitudinal sections, mice were perfused with Ringers solution followed by 4% paraformaldehyde in PBS. Sternomastoid muscles were removed and soaked sequentially in 15% and 30% sucrose/PBS for several hours prior to being embedded in OCT compound as above. 40-μm cryostat sections were then cut and mounted on slides. Antibody staining was as described above with the following modifications: 1% Triton was used in both blocking and antibody solutions, washes were 6 times 30 min and secondary antibody was incubated overnight. Confocal images of longitudinal sections were acquired on a Nikon PCM 2000 confocal microscope using a 63× objective.

Denervation and tetrodotoxin (TTX) cuff application

For denervation experiments, 6- to 8-week-old mice were anesthetized with ketamine/xylazine by intraperitoneal injection, and the sciatic nerve was cut on one side of the body. Tibialis anterior muscle was removed after 3 days and processed for immunostaining. Muscle from the non-denervated side was taken and processed in parallel as a control. For nerve block experiments, TTX cuffs were applied to the sciatic nerve on the left side of 8-week-old mice as described in Wang et al. (2005). Nerve block was maintained for 7 days. Mice were checked for loss of ability to spread their toes to confirm that nerve block had developed and was maintained. Sections were prepared and stained as described above. Staining of adjacent sections for the presynaptic marker protein synaptophysin was performed to check that NMJs had not been inadvertently denervated during cuff application.

Neuregulin application

Recombinant human β1 neuregulin-1 extracellular domain was purchased from R&D systems (Minneapolis, MN, USA; catalog number 377-HB) and dissolved in PBS at a concentration of 0.5 μg/μl. 5 μg of neuregulin (or 10 μl PBS as a control) was administered daily by subcutaneous injections into one hindlimb of ICR mice pups from P6 to P10. Mice were killed on P11, and the soleus muscle was taken, sectioned longitudinally and stained as described above for adult sternomastoid muscle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2005.07.015.

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