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PRESYNAPTIC CALCIUM CHANNELS AND $\alpha 3$ -INTEGRINS ARE COMPLEXED WITH SYNAPTIC CLEFT LAMININS, CYTOSKELETAL ELEMENTS AND ACTIVE ZONE COMPONENTS

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Abstract

At chemical synapses, synaptic cleft components interact with elements of the nerve terminal membrane to promote differentiation and regulate function. Laminins containing the $\beta 2$ subunit are key cleft components, and they act in part by binding the pore-forming subunit of a presynaptic voltage-gated calcium channel ($\text{Ca}_v\alpha$) (Nishimune, et al., 2004). Here, we identify $\text{Ca}_v\alpha$ -associated intracellular proteins that may couple channel-anchoring to assembly or stabilization of neurotransmitter release sites called active zones. Using $\text{Ca}_v\alpha$ -antibodies, we isolated a protein complex from Torpedo electric organ synapses, which resemble neuromuscular junctions but are easier to isolate in bulk. We identified 10 components of the complex: six cytoskeletal proteins ($\alpha 2/\beta 2$ spectrins, plectin 1, AHNK/desmoyokin, dystrophin, and myosin 1), two active zone components (bassoon and piccolo), synaptic laminin, and a calcium channel β subunit. Immunocytochemistry confirmed these proteins in electric organ synapses, and PCR analysis revealed their expression by developing mammalian motor neurons. Finally, we show that synaptic laminins also interact with presynaptic integrins containing the $\alpha 3$ subunit. Together with our previous finding that a distinct synaptic laminin interacts with SV2 on nerve terminals (Son, et al., 2000), our results identify three paths by which synaptic cleft laminins can send developmentally important signals to nerve terminals.

Pre- and postsynaptic elements of developing chemical synapses provide signals that organize differentiation of post- and presynaptic structures, respectively. At the skeletal neuromuscular junction (NMJ), one prominent organizer of nerve terminals is laminin 421, a heterotrimer of $\alpha 4$, $\beta 2$ and $\gamma 1$ subunits. Laminin 421 is synthesized by muscle fibers and inserted into the basal lamina that occupies the synaptic cleft between nerve and muscle membranes. In mature muscle, the $\alpha 4$, and $\beta 2$ subunits are highly concentrated in the synaptic cleft, along with the $\alpha 5$ subunit, while the $\gamma 1$ subunit is also present in laminin 211 ($\alpha 2/\beta 1/\gamma 1$), a major component of the basal lamina that coats the remainder of the muscle fiber surface (Patton et al., 1997). In mice with a targeted mutation of the laminin $\beta 2$ (*lamb2*) gene, presynaptic maturation is dramatically disrupted and homozygous mutants die by the time of weaning (Nishimune et al., 2004; Noakes et al., 1995). Mice with a targeted mutation of the *lama4* gene are outwardly healthy, but exhibit disruption of the precise

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apposition between presynaptic neurotransmitter release sites (active zones) and postsynaptic invaginations (junctional folds) (Patton et al., 2001). The more severe phenotype of *lamb2* compared to *lama4* mutants presumably reflects the fact that laminin $\alpha 4$ is present only in laminin 421, whereas laminin $\beta 2$ is present in three distinct laminin heterotrimers, laminins 221, 421 and 521 (Patton et al., 1997); thus, considerable laminin $\beta 2$ is present at synapses in *lama4* mutants.

Together, these results suggest that the laminin $\alpha 4$ and $\beta 2$ chains play distinct roles in the development on the NMJ, and raise the question of what receptors that they interact with on the nerve terminal. We recently showed that one receptor is the voltage-gated calcium channel (VGCC), which was already known to play a central role in neurotransmitter secretion: the VGCC opens in response to membrane depolarization, allowing the influx of calcium ions that trigger neurotransmitter release (Kandel, et al., 2000). In addition, interaction of the pore-forming VGCC α subunit ($\text{Ca}_v\alpha$) with synaptic laminin is required for formation and stabilization of active zones in early postnatal life (Nishimune, et al., 2004).

It remains to be determined, however, how $\text{Ca}_v\alpha$ links to active zones. Here, we address this issue by isolating and identifying intracellular proteins associated with the VGCC in nerve terminals. To this end, we used the Torpedo electric organ. This tissue contains nicotinic cholinergic synapses that are similar to NMJs in many respects, but are far larger and more abundant. Synapses in the electric organ occupy most of one surface of slender electroplaque cells, whereas NMJs occupy only ~0.1% of the surface of large, cylindrical muscle fibers. Consequently, the concentration of synaptic molecules such as postsynaptic acetylcholine receptors (AChRs) is >100X higher in electric than in muscle. Indeed, many key components of NMJs were originally purified from electric organ; these include AChRs, acetylcholinesterase, agrin, rapsyn, and several synaptic vesicle proteins (Hall, 1992). Likewise, use of electric organ allowed us to isolate a VGCC-associated complex in amounts sufficient to identify several of its major components by mass spectroscopy. Our results suggest that the presynaptic $\text{Ca}_v\alpha$ anchors a complex of cytoskeletal and active zone-associated proteins.

Our previous studies demonstrated that the presynaptic $\text{Ca}_v\alpha$ subunit binds directly to laminin $\beta 2$ in the laminin 421 heterotrimer (Nishimune et al., 2004). As noted above, however, the distinct phenotypes of targeted *lama4* and *lamb2* mutants suggest distinct roles for laminins $\beta 2$ and $\alpha 4$, implying the existence of a laminin $\alpha 4$ binding protein on nerve terminals. In the second part of this study, we used electric organ to seek such a protein. We focused on integrins, which are the best-studied laminin receptors in both neural and non-neural tissues, and are known to interact with laminin α subunits (Suzuki, et al., 2005). Among several integrin subunits present at the NMJ (Martin et al., 1995), integrin $\alpha 3$ was of particular interest because it is concentrated near active zones in frog nerve terminals (Cohen, et al., 2000), and might explain the mislocalization of active zones in mouse *lama4* mutants (Patton, et al., 2001). Indeed, we show that laminin 421 interacts with integrin $\alpha 3$ and that this integrin, in turn, interacts intracellularly with at least one component of the VGCC-associated complex. Thus, our results define two paths by which laminin 421 in the synaptic cleft can provide developmentally important signals to motor axons.

Experimental Procedures

Reagents

Rabbit antibodies (CP15) to VGCC α subunit and synaptotagmin and monoclonal anti-HNK-1 were prepared and purified as described previously (Striessnig et al., 1990; Sunderland et al., 2000). The CP15 antibody was made to a peptide containing the sequence between residues 1382–1400 of rabbit muscle calcium channel $\alpha 1$ subunit (Cav1.2)

following Striessnig et al. (1990). The sequence is highly conserved across all VGCC α subunit types. The antibody was affinity purified with the same peptide. Sheep antisera were prepared against the sequence RIQPSETERLTDDY from the carboxy-terminal of chicken $\alpha 3$ integrin, and antibodies were affinity-purified from serum following standard procedures (Hockfield et al., 1993). Antibodies to this cytosolic domain are known to cross-react with the $\alpha 3$ integrin subunit from a number of different vertebrate species (DiPersio et al., 1996). Affinity-purified rabbit anti-VGCC β subunit was a gift from Dr. Ruth Westenbroek and Dr. William Catterall (University of Washington) and was made against the highly conserved sequence SNTRSSLAEVQSEIER from residues 338–353 of rat $\text{Ca}_v\beta 2$. The sequence is found in $\beta 1, \beta 2$, & $\beta 4$. Rabbit affinity-purified anti-bassoon/piccolo antibodies were generated to a GST-fusion protein containing a 60 amino acid (residues 1065–1124) fragment from rat bassoon. A nearly identical sequence is found in rat piccolo. The antibodies were affinity purified with a pET fusion protein containing the identical bassoon sequence following standard procedures (Hockfield et al., 1993). These fusion proteins were generated from bassoon clone pGBK-RB25 obtained from Drs. W. Altmann and E. Gundelfinger (Magdeburg, Germany). Rabbit anti-piccolo antibody (Cat. No. 142 002) was purchased from Synaptic Systems (Gottingen, Germany). Anti-Dystrophin mouse monoclonal antibodies (1958B & 1808L) were obtained from Drs. M. Adams, and S. Froehner (University of Washington); rabbit anti-AHNAK/Desmoyokin was a gift from Drs. M. Kouno and J. Takeda (Osaka University, Japan); rabbit anti-Plectin 1 (#9) was from Dr. G. Wiche (University of Vienna, Austria); rabbit anti-Myosin 1 antibody (2652) was from Dr. P. Gillespie (Oregon Health & Science University); rabbit anti- α -spectrin antibody was from Dr. R. Moon (University of Washington) (Giebelhaus et al., 1987). Mouse monoclonal anti-SV2 was made by Buckley and Kelly (Buckley and Kelly, 1985) and mouse monoclonal anti-neurofilament m protein (1592) was purchased from Millipore.

Secondary antibodies and toxins used for fluorescence microscopy were purchased from Invitrogen and are as follows: Alexa 488 and 568 conjugated donkey anti-rabbit IgG, Alexa 488 and 568 conjugated donkey anti-mouse IgG, and Alexa 647 conjugated α -bungarotoxin. HRP conjugated goat anti-rabbit IgG, and HRP conjugated goat anti-mouse IgG used for Western blots were purchased from GE Healthcare.

Isolation and identification of VGCC-associated proteins

The preparation of synaptosomes (“standard preparation”) from electric organ and immunoprecipitation of nerve terminals from this membrane fraction were performed as previously described (Son et al., 2000; Sunderland et al., 2000). The VGCC protein complex was immunoprecipitated from a Triton X-100 extract of electric organ synaptosomes (standard preparation) as described by Sunderland et al. (2000). We performed fifteen separate immunoprecipitations, each using 500 μg of Triton X100-solubilized synaptosomes and 33 ml of goat anti-rabbit immunobeads coated with affinity purified rabbit anti-VGCC (CP15). To coat the immunobeads with primary antibody, each 33 μl of packed beads was incubated at a 10% slurry with 31 μg of CP15. All of the immunoprecipitates were eluted with a total of 80 μl of SDS final sample buffer and applied to one lane of a 2.4–6% SDS-polyacrylamide gel (Hockfield, et al., 1993). The gel was stained with Coomassie Blue, and stained bands were cut out for mass spectroscopic analysis at the Harvard Microchemistry Facility.

Analytical immunoprecipitation of the VGCC protein complex was performed as described by Sunderland et al. (Sunderland et al., 2000), with rabbit antibodies to $\text{Cav}\alpha$ or $\text{Cav}\beta$. Using similar methods, we immunoprecipitated $\alpha 3$ -integrin with sheep anti- $\alpha 3$ integrin antibodies and immunobeads covalently coated with rabbit anti-sheep IgG antibodies (Irvine Scientific). Proteins were separated on 2.4–8%, 2.7–14% or 2.4–14%, SDS-acrylamide gradient gels and Western blots were performed as previously described (Sunderland et al.,

2000). All SDS-PAGE was performed under reducing conditions unless otherwise noted. In order to separate the ~30KD carboxy-terminal fragment of $\alpha 3$ -integrin from sheep IgG light chains, immunoprecipitated integrin was subjected to SDS-PAGE on 11% polyacrylamide gels. As a test of antibody specificity, affinity purified anti- $\alpha 3$ integrin antibody (10–20 μ g) was mixed with an excess of antigenic peptide RIQPSETERLTDDY (500 μ g) in 1 ml of 150 mM NaCl, 10 mM Tris, 10% powdered milk pH 7.4 and applied to a Western blot containing the $\alpha 3$ -integrin chain.

Immunohistochemistry

Torpedo electric fish were sacrificed as described previously (Carlson et al., 1978). In some cases, small pieces of electric organ (0.5–0.8 cm³) were digested for 2–4 hr at room temperature with collagenase (5 mg/ml Clostridiopeptidase Collagenase AEC 3.4.2.4.3, Invitrogen, in fish ringer) to dissociate nerve terminals from the postsynaptic electrocyte. To label the external surface of nerve terminals with an antibody (anti-SV1 mAb) to the luminal domain of SV2, small pieces of electric organ were incubated for 5 hours at 4°C with this mAb (hybridoma supernatant) diluted 1/7 in fish ringer. After antibody or enzyme treatment tissue pieces were washed extensively with fish ringer at 4°C.

Small pieces of untreated, antibody labeled, or collagenase-digested electric organ were quick frozen in liquid N₂-cooled isopentane and sectioned in a cryostat at 10 μ m. Most pieces were fixed by immersion in 4%paraformaldehyde/0.1M sodium phosphate prior to freezing (2 days at 4°C), occasionally others were fresh frozen and fixed for 20 min with 0.5%paraformaldehyde in 280 mM NaCl, 20 mM Hepes, pH 7.2 (ER buffer), after sectioning. In some cases, sections of immersion-fixed tissue were heated to 90°C for 30 sec in 1%SDS in ER (Nguyen et al., 1992). Following appropriate treatments, sections were washed with ER and incubated with primary antibodies overnight at 4°C and with secondary antibody for 2 hrs at room temp. For these incubations antibodies were dissolved in ER/1% donkey serum, with or without 0.08% Triton X-100.

The electromotor nucleus of the Torpedo electric fish was removed, fixed with 4% paraformaldehyde, quick frozen, and the resulting 10 μ m sections treated with SDS as described above. To suppress autofluorescence, these tissue sections were incubated with .005% Sudan Black B (Sigma -199664) in 70% methanol for 5 min after the application of antibodies (Schnell et al., 1999). The sections were then washed three times (5 min per wash) with PBS before mounting. Images were obtained with a Leica Sp1 confocal microscope.

Motor Neuron Isolation and RT-PCR

Mouse motoneurons from E13 spinal cords were purified essentially as described by Henderson et al. (1996), except that 6 % Nycodenz was used for density gradient separation of motor neurons from other cell types. Total RNA was then extracted from the purified motor neurons using the RNeasy kit (Qiagen) following the manufacturer's instructions. Prior to reverse transcription, RNA was treated with turbo Dnase (Ambion). RT-PCR was performed on RNA samples using Superscript III (Invitrogen). Gene-specific primers (Supplementary Table S1) were used to assess expression of VGCC binding partners.

RESULTS

Identification of cytoskeletal components of the VGCC protein complex

To identify proteins associated with the VGCC in nerve terminals, we prepared synaptosomes from Torpedo electric organ, solubilized proteins with Triton X-100, and immunoprecipitated the VGCC complex from the extract. Torpedo synaptosomes are ideally

suited for this type of analysis, since although the standard synaptosomal preparation contains postsynaptic membrane, in electric organ only nerve terminals contain the VGCC (Sunderland et al., 2000). Furthermore, postsynaptic membrane is not attached to synaptosomal membrane, immuno-purified synaptosomes lack of postsynaptic markers (Sunderland et al., 2000).

Previously we found that the immunoprecipitated VGCC complex contained $\text{Ca}_v\alpha$, and laminin 421, but lacked a synaptic vesicle protein (SV2) and a presynaptic plasma membrane protein (syntaxin) (Sunderland et al., 2000). However, the VGCC complex did contain a number of major proteins not identified in our earlier analysis. To identify, these proteins, the immunoprecipitated VGCC complex was subjected to SDS-PAGE and the gel was stained with Coomassie blue. Approximately 14 bands were visible on the gel; they are labeled A-N in Figure 1. We focused on these bands in the expectation that they would include the major VGCC-associated proteins in synaptosomes. We show below that bands H, I, M and N correspond, respectively, to laminin 421, $\text{Ca}_v\alpha$, and heavy and light chains of the antibody used for immunoprecipitation. Bands A, D–G, J and K were cut out and digested with trypsin; the resulting peptides were separated by liquid chromatography and subjected to mass spectrometry (Eng et al., 1994). Bands B and C were too faint and band L was too near to band M for isolation; their identification by other means is described below.

Sequences of major peptides obtained from proteins A, D–F, J and K are shown in Supplementary Table S2. Given that limited Torpedo genomic or protein sequence is available, identification of proteins from which these peptides were derived depends on evolutionary conservation. Fortunately, conservation sufficed to tentatively identify these proteins as plectin 1, dystrophin, $\alpha 2$ spectrin, $\beta 2$ spectrin, the neurofilament M subunit and myosin 1, respectively. These proteins are components of either the filamentous (plectin 1, neurofilament M, and myosin 1) or the submembraneous cytoskeleton (dystrophin, $\alpha 2$ spectrin and $\beta 2$ spectrin). In contrast, identification of Protein G required *de novo* sequencing by tandem mass spectrometry; in this case, rather than comparing empirical with calculated mass spectra, the actual sequences of the tryptic peptides are determined and compared directly with the protein sequence database. The sequence data on four tryptic peptides from protein G identify it as AHNAK/Desmoyokin (Supplementary Table S3).

As a check on our protein identifications, we compared reported molecular weights of proteins identified by Ms/Ms with those of the bands from which sequence was obtained. As shown in Table 1, reported and empirically determined molecular weights were similar for plectin 1, $\alpha 2$ spectrin, $\beta 2$ spectrin, myosin 1, and neurofilament M subunit. The observed molecular weight of AHNAK/Desmoyokin, 250 kD, is far lower than the molecular weight of the full-length protein, 629 kD, but similar to that of a smaller, ~300 kD form reported recently (Huang et al., 2008).

To be certain the interactions between $\text{Ca}_v\alpha$ and the proteins identified by preparative immunoprecipitation were specific, we repeated the immunoprecipitations at the analytical level using anti- $\text{Ca}_v\alpha$ Ab or a no primary antibody control. The immunoisolated complex was probed with antibodies specific to their mammalian or avian orthologues. Antibodies to plectin 1, AHNAK/Desmoyokin, dystrophin, α -spectrin, and myosin 1 recognized appropriate bands on Western blots (Figure 2 and Table 1). Western blotting also identified proteins H and I as $\text{Ca}_v\alpha$ and a combination of comigrating laminin $\beta 2$ and $\gamma 1$, respectively. These proteins were absent from the control immunoprecipitations, indicating that the interactions are specific. However, we were unable to detect the neurofilament M subunit in these immunoisolates. Its absence here, but presence in preparative immunoprecipitates (Figure 1) is probably due to non-specific trapping by the large amounts of immunobeads

used in the scaled-up procedure. Thus, we suspect that neurofilaments are probably not part of the VGCC protein complex.

The VGCC complex contains the $\text{Ca}_v\beta$ subunit

The pore-forming $\text{Ca}_v\alpha$ subunit of the VGCC is associated with a cytosolic $\text{Ca}_v\beta$ subunit (Catterall & Few, 2008), and AHNAK/Desmoyokin has been reported to interact with a $\text{Ca}_v\beta$ subunit (Hohaus et al., 2002). To determine whether $\text{Ca}_v\beta$ is present in the VGCC complex from Torpedo electric organ, we used an antibody to a highly conserved region of the rat β_2 subunit. This antibody bound to a 52 kD protein in an electric organ synaptosomal fraction (Fig 3a). This molecular weight is similar to the reported molecular weight of the zebra fish $\text{Ca}_v\beta_1$ and $\text{Ca}_v\beta_2$ subunits, 53–68 kD. We were unable to use this antibody to probe immunoblots of the VGCC complex isolated with an antibody to $\text{Ca}_v\alpha$, because of the proximity of the immunoreactive band to the immunoglobulin heavy chain (Bands L and M in Figure 1). As an alternative, we used the $\text{Ca}_v\beta$ antibody to immunoprecipitate a complex from Triton X-100-solubilized electric organ synaptosomes, following the procedures used to isolate the VGCC protein complex with the anti- $\text{Ca}_v\alpha$ antibody. The anti- $\text{Ca}_v\beta$ immunoprecipitate contained $\text{Ca}_v\alpha$ subunit and laminin (Fig 3b), supporting the conclusion that the VGCC complex includes both $\text{Ca}_v\alpha$ and $\text{Ca}_v\beta$ subunits.

The VGCC complex contains active zone components

The VGCC complex contained ill-defined bands of 400–500 kD (Figure 1), which were too diffuse to be excised and submitted for mass spectrometric analysis. We considered the possibility that they were large components of the active zone, such as Piccolo (~500kD) or Bassoon (~400kD) (tom Dieck et al., 1998; Wang et al., 1999; Table 1). To test this idea, we probed the immunoisolated VGCC with an antibody that recognizes both proteins. This antibody recognized bands of ~500 kD and ~400 kD (Fig. 4a & b, black and grey arrowheads, respectively). The ~500 kD protein is also stained with an antibody specific for Piccolo (Fig. 4a & b, black arrowhead), consistent with the reported size of this protein. Conversely, immunoprecipitates generated from Triton X-100 solubilized electric organ synaptosomes with antibodies to Bassoon and Piccolo contained $\text{Ca}_v\alpha$ and laminin (Fig. 4b).

Laminin 421 binds to integrin α_3 on nerve terminals

For reasons detailed in the Introduction, we suspected that $\alpha_3\beta_1$ integrin heterodimers might provide a second link between laminin 421 and active zones. The integrin α_3 subunit has a molecular weight of ~150kD, but is subject to an internal cleavage that results in generation of disulfide-linked amino- and carboxy-terminal fragments of ~120kD and ~30kD respectively (Hynes, 1992). On Western blots of an electric organ synaptosomal extract, an antibody to a peptide in the 30kD carboxy terminal fragment revealed a protein that migrated at ~150 kD under non-reducing conditions and 30 kD under reducing conditions (Figure 5a). Incubation with the antigenic peptide blocked reactivity with both bands. Because α_3 -integrin may be present in multiple cell types, we tested its specific association with presynaptic membranes. We previously demonstrated that nerve terminals in a standard synaptosomal preparation can be immunopurified from contaminating membranes of the postsynaptic cell. The immunopurification utilizes an antibody to the luminal domain of a synaptic vesicle protein SV2 present on the nerve terminal surface (Son et al., 2000; Sunderland et al., 2000). This method demonstrates that α_3 -integrin is present in synaptosomal membranes (Figure 5b).

We immunoprecipitated integrin α_3 from the synaptosome extract and probed immunoblots to seek associated proteins. The 740kD laminin 421 trimer, recognized by the HNK-1 and laminin 111 antibodies, was co-precipitated with integrin α_3 (Figure 5c). The most

parsimonious explanation of this result is that integrin $\alpha 3$ binds to laminin 421. Further analysis showed that α -spectrin was also associated with this complex. The associations were selective in that major nerve terminal and synaptic vesicle proteins, syntaxin and SV2, respectively, were abundant in the extract but absent from the immunoprecipitate (Figure 5c). Likewise, these immunoprecipitates do not contain a 900 kD $\alpha 5$ -containing laminin associated with electric organ nerve terminals (compare + lanes of figure 5b with 5c, grey arrowheads). Thus, a specific synaptic laminin can interact with and bind to an integrin on nerve terminals.

To investigate the association of integrin $\alpha 3$ with VGCCs, we probed integrin $\alpha 3$ -immunoprecipitated material with antibodies to $\text{Ca}_v\alpha$ and probed $\text{Ca}_v\alpha$ -immunoprecipitated material with antibodies to integrin $\alpha 3$. Unexpectedly, $\text{Ca}_v\alpha$'s were readily detectable in the integrin $\alpha 3$ precipitate but integrin $\alpha 3$ was undetectable in the $\text{Ca}_v\alpha$ precipitate (Figure 5b and data not shown). We cannot explain this lack of reciprocity. Perhaps the anti- $\text{Ca}_v\alpha$ antibody binds a region with which integrins interact, and therefore precipitates only $\text{Ca}_v\alpha$ molecules that are not associated with integrins. Alternatively, only a small subpopulation of $\text{Ca}_v\alpha$ molecules might be associated with integrin $\alpha 3$.

Association of laminin- and VGCC-associated proteins with nerve terminals

We used double- and triple-label immunohistochemistry to ask whether the proteins that co-precipitate with $\text{Ca}_v\alpha$ and laminin 421 are localized to electric organ nerve terminals. We visualized nerve terminals with an antibody to synaptic vesicle proteins (SV2 or synaptotagmin) and stained the postsynaptic membrane with α -bungarotoxin, which binds AChRs. As shown in Figure 6, $\alpha 3$ -integrin, AHNAK/Desmoyokin, myosin 1 and plectin-1 were all present at synaptic sites in electric organ, and appeared to be associated with nerve terminals. Previous studies have shown that spectrin is also present at this site (Walker et al., 1985).

Because pre- and postsynaptic membranes are closely apposed, we used a second method to determine whether the presumed VGCC- and laminin-associated proteins were associated with nerve terminals rather than electrocytes. Collagenase is known to sever the linkages between pre- and postsynaptic membranes in electric organ and the NMJ (Hartzell et al., 1976; Kriebel et al., 1996). We therefore treated pieces of electric organ with collagenase prior to freezing and section, leading to sporadic dissociation of pre- and postsynaptic cells. This method was critical for localization of dystrophin, which is abundant in the postsynaptic membrane (see Supplementary Figure S1), but was also useful for other antigens. By focusing on regions in which the AChR-rich postsynaptic membrane had become separated from the SV2-rich nerve terminal, we were able to confirm that $\alpha 3$ -integrin, plectin-1, AHNAK/Desmoyokin and myosin 1 are associated with nerve terminals (Figure 7).

To further test the idea that nerve terminals as well as postsynaptic structures contain dystrophin, we immunostained the electromotor nucleus, which contains the somata of the electromotor neurons whose axons innervate electroplaques. These neurons make large amounts of presynaptic proteins to supply their extensive arbor of nerve terminals. Somata of electromotor neurons were rich in the synaptic vesicle protein, synaptotagmin, and in dystrophin (Supplementary Figure S2).

Expression of VGCC-binding partners in mouse motor neurons

Finally, we asked whether the proteins we had identified in electric organ nerve terminals are also expressed by mammalian motor neurons during the period when NMJs are forming. However, available antibodies stained developing NMJs poorly. We therefore purified

motor neurons from embryonic mouse spinal cord, extracted RNA, and probed for expression of VGCC-binding partners. Using gene-specific primers (Table S3), we detected messenger RNAs for AHNAK/Desmoyokin, plectin 1, myosin 1, α 2-spectrin, β 2-spectrin, dystrophin, and α 3-integrin (Fig. 8).

DISCUSSION

Previous studies at the NMJ showed that β 2 containing laminins, which are deposited into the synaptic cleft by muscle fibers, promote presynaptic differentiation (Martin et al., 1995; Noakes et al., 1995; Porter et al., 1995; Son et al., 1999) in part by interactions with VGCCs on motor nerve terminals (Nishimune et al., 2004; Sunderland et al., 2000). These results raise new questions, of which we address two here: how does the VGCC convey signals to the interior of the motor nerve terminal, and what other nerve terminal components interact with synaptic laminins?

To address the first question, we isolated and identified proteins complexed with VGCCs in Torpedo electric organ nerve terminals. We hoped thereby to gain insight into what kinds of messages the VGCC delivers to the presynaptic cytosol. In addition to the $\text{Ca}_v\alpha$ and $\text{Ca}_v\beta$ subunits and Laminin 421 (α 4/ β 2/ γ 1), the complex contained three sets of proteins (Figure 9). The first set includes α 2 spectrin, β 2 spectrin, AHNAK/desmoyokin, and dystrophin, which are components of the submembraneous cytoskeleton. Dimeric α/β spectrins are known components of the submembraneous cytoskeleton in axons as well as nerve terminals (Davis & Bennett, 1983); their loss leads to nerve terminal retraction at the *Drosophila* NMJ (Pielage, et al., 2005). AHNAK/desmoyokin provides a link between the VGCC and the actin cytoskeleton in cardiac cells (Hohaus et al., 2002). In MDCK cells an epithelial cell line, AHNAK/desmoyokin, appears to regulate the cortical actin cytoskeleton, affecting the cell membrane cytoarchitecture (Benaud et al., 2004).

Our finding of presynaptic dystrophin may seem at odds with previous work, but we believe it is not. Dystrophin is well known to be associated with the postsynaptic membrane of skeletal muscle as well as the electric organ electrocyte (e.g. Sealock et al., 1991). We also find a strong postsynaptic localization for dystrophin (Figure S1). In electric organ the postsynaptic immuno-staining is so strong that a weaker presynaptic signal is overwhelmed and not apparent. Only by separating the pre and postsynaptic membranes by collagenase digestion can the presynaptic signal also be seen. Presynaptic localization has been reported previously in motor nerve terminals (Huard et al. 1991) and photoreceptor nerve terminals (Ueda et al, 1997).

A second group of VGCC-associated proteins, plectin 1 and myosin 1, are components of the filamentous cytoskeleton. The association of myosin 1 with the VGCC is intriguing in light of the demonstration of a dynamic presynaptic actin scaffold that undergoes rounds of polymerization and depolymerization when nerve terminals are stimulated (Sankaranarayanan et al., 2003). Myosin 1 could reversibly couple the VGCC complex to dynamic actin filaments. Nonmuscle Myosin II is thought to be important for synaptic vesicle mobility in the nerve terminal (Seabrooke et al., 2010).

Finally, the complex contains two large proteins, Bassoon and Piccolo, which are components of the “cytomatrix” associated with active zones (Schoch & Gundelfinger, 2006). They are thought to be the largest elements of the active zone scaffold, interacting with a number of the other active zone components as well as with synaptic vesicle proteins. The binding of Piccolo or Bassoon to a component of the presynaptic plasma membrane might well anchor the active zone apparatus.

Unlike electric organ synaptosomes, detergent-solubilized rat brain synaptosomes yield an immunoprecipitated presynaptic $\text{Ca}_v2.2$ complex without piccolo or bassoon (Khanna et al., 2007). This finding may reflect a difference in the active zone structure at CNS synapses compared to those of neuromuscular and electric organ. The CNS active zone has been described as a “web-like...meshwork of filaments,” whereas the frog neuromuscular active zone is a linear “array-like complex” of elements at the “midline of the presynaptic ridge” (Schoch and Gundelfinger, 2006). In any case, at the NMJ there appears to be a functional link between $\text{Ca}_v\alpha$ and the active zone (Nishimune et al., 2004). The data we present here suggest a structural link as well.

We do not yet know which of the cytosolic VGCC-associated proteins are directly bound to the channel and which are indirectly connected. However, the known properties of several members of the complex suggest possible direct interactions. A functional SH3 domain is present in α -Spectrin (De Matteis & Morrow, 2000; Maximov et al., 1999; Mayer, 2000), and presynaptic $\text{Ca}_v\alpha$ s ($\text{Ca}_v2.1$, $\text{Ca}_v2.2$, and DOE-4 from electric organ) contain potential proline-rich recognition sequences for SH3 domains. These proline-rich recognition sequences in $\text{Ca}_v2.1$ are functional (Maximov et al., 1999). AHNAK/desmoyokin binds the $\text{Ca}_v\beta$ subunit associated with cardiac $\text{Ca}_v1.2$ (Hohaus, et al., 2002; Komuro et al., 2004) and might bind $\text{Ca}_v\beta$ subunits in nerve terminals. The carboxy-terminal region of Piccolo contains two C2 domains, C2A and C2B (Wang et al., 1999). A related C2B domain in the synaptic vesicle protein synaptotagmin binds $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ (Catterall & Few, 2008). Indeed, Piccolo is known to bind $\text{Cav}1.2$ through its C2 domains in pancreatic β cells (Shibasaki, et al., 2004). In contrast, dystrophin, myosin 1 and plectin 1 might associate with $\text{Ca}_v\alpha$ via an intermediary. For example, plectin 1 binds spectrins (Frappier et al., 1992; Wiche, 1998).

These interactions suggest that the presynaptic VGCC might promote presynaptic differentiation by acting as an anchor for the cytoskeleton of the nerve terminal as well as for the cytomatrix of the active zone. Interactions with synaptic laminins could immobilize the VGCCs, which would in turn stabilize cytoskeletal components at the inner surface of the presynaptic membrane. In parallel, by anchoring active zone components, the VGCC could localize the neurotransmitter release apparatus. Previous studies have shown that the VGCC can also interact with synaptic vesicle proteins such as synaptotagmin via a so-called symprint site (Catterall & Few, 2008); our immunoisolation protocol stripped the presynaptic membrane of vesicles, so it is unsurprising that we did not detect these interactions. Thus, synaptic laminins could promote formation or stabilization of presynaptic specializations by a simple, passive mechanism, involving immobilization of an anchoring or nucleating center rather than by signal transduction. Additional mechanisms might act in parallel. For example, interactions with laminins might prevent of VGCCs from interacting with components of the endocytotic machinery (Watanabe et al., 2010), thereby leading to increased levels of VGCCs at active zones.

As to our second question, regarding what other nerve terminal components interact with synaptic laminins, we find that laminin 421 binds not only $\text{Ca}_v\alpha$, but also $\alpha3$ -integrin. The $\alpha3$ -integrin, always paired with the $\beta1$ chain (Hynes, 2002), is known to bind the laminin $\alpha4$ LG1-3 modules of the G domain (Suzuki et al., 2005). This interaction is distinct from that of $\text{Ca}_v\alpha$ which binds an LRE sequence on the carboxy-terminal domain of the laminin $\beta2$ chain (Nishimune et al., 2004). Disruption of the $\text{Ca}_v\alpha$ -laminin $\beta2$ interaction blocks formation and stabilization of active zones, whereas loss of laminin $\alpha4$ disrupts the precise apposition of active zones with junctional folds (Patton et al., 2001). Thus, laminin 421 may use $\text{Ca}_v\alpha$ and $\alpha3$ integrin in conjunction to pattern active zones.

In previous studies, we found that an $\alpha 5$ -containing trimer, laminin 521, is localized along with laminin 421 in the mammalian synaptic cleft (Patton et al., 1997), that loss of laminin $\alpha 5$ blocks synaptic maturation (Nishume, et al., 2008), and that an $\alpha 5$ -containing laminin is complexed with the synaptic vesicle protein SV2 on nerve terminals in electric organ (Son et al., 2000). This $\alpha 5$ -laminin/SV2 protein complex is molecularly distinct from the laminin 421 complexes described here. SV2 and the 900 kD $\alpha 5$ -laminin are not co-immunoprecipitated with $\text{Ca}_v\alpha$ (Sunderland et al., 2000) or $\alpha 3$ -intergin (Fig. 5) from TX100-solubilized electric organ synaptosomes. Similarly, the laminin 421 is absent from the immuno-isolated $\alpha 5$ -laminin/SV2 protein complex (Son et al., 2000).

In conclusion, at least three receptors on nerve terminals may be involved in the synaptic organizing effects of laminins on nerve terminals (Figure 9). These receptors act in conjunction with other receptors, including FGF receptor 2b, which transduces signals provided by muscle-derived FGF 7, 10 and 22, and still-unidentified binding proteins that transduce signals provided by synaptic collagens (Fox et al., 2007). This complexity is formidable, but must be addressed in that developmental regulation is unlikely to be less complicated at synapses in the brain. Analysis may be facilitated by the simplicity and accessibility of the NMJ compared to central synapses. For example, our results to date suggest the testable hypothesis that the presynaptic laminin 421 protein complex might help build and locate the sites of vesicle release (exocytosis), whereas the SV2/ $\alpha 5$ -laminin complex might help define sites of vesicle retrieval (endocytosis).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

The abbreviations used

VGCC	voltage-gated calcium channel
NMJ	neuromuscular junction
AchR	acetylcholine receptor
lamb2	the laminin $\beta 2$ chain
lama4	the laminin $\alpha 4$ chain
$\text{Ca}_v\alpha$	the α subunit of the voltage-gated calcium channel
$\text{Ca}_v\beta$	the β subunit of the voltage-gated calcium channel
Laminin 221	laminin containing the $\alpha 2$, $\beta 2$ & $\gamma 1$ chains
laminin 421	laminin containing the $\alpha 4$, $\beta 2$, and $\gamma 1$ chains
laminin 521	laminin containing the $\alpha 5$, $\beta 2$, and $\gamma 1$ chains

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Figure 1. The VGCC protein complex isolated by preparative immunoprecipitation and components separated by SDS-PAGE

The immunoprecipitate was prepared from TX-100 solubilized electric organ synaptosomes with an anti- $\text{Ca}_v\alpha$ antibody (CP15) and subjected to SDS-PAGE on a 2.7–6% polyacrylamide gradient gel stained for protein with Coomassie Blue. The visualized proteins are labeled A–N. Bands M and N correspond to the IgG heavy (M) and light (N) chains, respectively, of the immunoprecipitating antibody. The remaining proteins were identified by MS/MS and/or Western blotting.

Figure 2. Components of the Immunoprecipitated VGCC protein complex identified by Western blots

Electric organ synaptosomes (s lanes) solubilized in Triton X100 were incubated with immunobeads coated with an anti-Ca_Vα antibody (+ lanes) (CP15) or a secondary antibody only (– lanes), and the resulting immunoprecipitates subjected to Western blot. The blots were probed with antibodies to Plectin 1, Dystrophin, α-spectrin, AHNAK/Desmoyokin, laminin β2 and γ1 chains, Ca_Vα, and myosin 1. The laminin chains, which co-migrate on the gel, were detected with antibody to the HNK-1 carbohydrate. Specificity of this antibody for laminins is documented in Sunderland et al. 2000. Letter in parentheses correspond to bands in Figure 1.

Figure 3. Immunoprecipitation of the VGCC protein complex by anti-Ca_vβ antibodies
a) Electric organ synaptosomes were subjected to Western blot and probed with an anti-Ca_vβ antibody. **b)** Electric organ synaptosomes (s lanes) solubilized in Triton X100 were incubated with immunobeads coated with an anti-Ca_vβ antibody (+ lanes) or a secondary antibody only (- lanes), and the resulting immunoprecipitates subjected to Western blot. The blots were probed with antibodies to the Ca_vα subunit or HNK1, which identifies the laminin β2 and γ1 chains (see Sunderland et al., 2000). On this 2.7–14% acrylamide gel, the laminin essentially chains migrate together.

Figure 4. The VGCC protein complex contains Bassoon and Piccolo

TX-100 solubilized electric organ synaptosomes (s lanes) were subjected to immunoprecipitation with anti- $\text{Ca}_v\alpha$ (a, + lanes), anti-Bassoon/Piccolo antibody (b, + lanes), or secondary antibody only (– lanes). The resulting immunoprecipitates were subjected to Western blot probed with an antibody that recognizes both Bassoon and Piccolo (Bass/Pic), an anti-Piccolo antibody, an anti- $\text{Ca}_v\alpha$ antibody, or an anti-HNK-1 antibody specific for laminins (Sunderland et al., 2000). The black arrowhead indicates the protein identified by the anti-Piccolo antibody. The $\text{Ca}_v\alpha$ migrates as two bands at ~190 kD (arrow) as seen previously and ~180 kD (*). The 180 kD protein is probably due to the α subunit being nicked by a protease during the preparation of the synaptosomes. The synaptosomes used for the immunoprecipitations in a) are the same as those shown in lane “s” of b).

Figure 5. Laminin $\alpha 4 \beta 2 \gamma 1$ is associated with $\alpha 3$ -Integrin in electric organ nerve terminals

a) In electric organ synaptosomes an antibody prepared to the 14 residue carboxy-terminal region of the $\alpha 3$ -integrin chain identifies a ~150 kD protein under non-reducing conditions and a ~30 kD protein after reduction. A peptide containing the 14 residue antigenic sequence blocks the reactivity of the anti- $\alpha 3$ -integrin antibody on both the ~150 kD protein and ~30 kD fragment. **b)** Starting with the standard preparation, synaptosomes (s lanes) were immuno-purified away from membranes of the postsynaptic cell as previously demonstrated (Son et al., 2000; Sunderland et al., 2000). This immunoprecipitation was done with a mouse monoclonal antibody to a luminal domain (SV1) of the synaptic vesicle protein SV2 present on the nerve terminal surface. The control immunoprecipitation contained a monoclonal antibody of the same isotype (IgG1) with an unknown specificity. The immunopurified synaptosomes contain $\alpha 3$ -integrin along with two laminins at 900 kD (grey arrowhead) and 740 kD (arrow) shown to be an $\alpha 5$ -containing laminin and $\alpha 4 \beta 2 \gamma 1$ laminin, respectively (Son et al., 2000; Sunderland et al., 2000). **c)** Electric organ synaptosomes (the standard preparation) were applied directly to a Western blot (s lanes), or solubilized in TX100 and immunoprecipitated with immunobeads containing an anti- $\alpha 3$ -integrin antibody (+ lanes) or a

secondary antibody only (– lanes). Lanes were stained with anti- $\alpha 3$ -integrin ($\alpha 3$ I), anti-laminin 1 (Ln1), anti-HNK1, anti- α spectrin, anti- $\text{Ca}_v\alpha$, anti-syntaxin, and anti-SV2 antibodies. The Western blots for detecting laminin were prepared from non-reducing SDS-PAGE so that laminin would migrate as a trimer. Torpedo $\alpha 4\beta 2\gamma 1$ migrates as 740 kD protein which stains with anti-laminin 1 and HNK-1 antibodies (Sunderland et al., 2000).

Figure 6. Immunocytochemical localization of VGCC- associated proteins at the electric organ synapse

Longitudinal ($\alpha 3$ I, AH, Myo) or cross-sections (PI) of electric organ were stained for $\alpha 3$ -Integrin ($\alpha 3$ I), AHNK/Desmoyokin (AH), myosin 1 (Myo), or plectin (PI). Sections were counterstained with anti-SV2 to label nerve terminals (exSV2 or SV2) and bungartoxin to label AchRs (AR). Colored panels show merged images. Arrows or arrowheads indicate nerve terminals. The SV2 staining in panel exSV2 is on the surface of the nerve terminal; unfixed electric organ was incubated in the anti-SV2 mAb and then washed before being fixed, sectioned, and stained for integrin. In all other panels sections were stained with anti-SV2 mAb after sectioning. Scale bars are 2 μ m.

Figure 7. Presynaptic localization of VGCC-associated proteins seen with collagenase-digested electric organ

Electric organ was digested with collagenase to allow pre- and postsynaptic membranes to separate, then fixed and cryo-sectioned. Sections were stained with antibodies to $\alpha 3$ -integrin ($\alpha 3$ I), plectin1 (Pl), AHNAK/Desmoyokin (AH), dystrophin (Dys), or myosin 1 (Myo). Sections were counterstained with antibodies to synaptic vesicles (anti-SV2 mAb – SV or anti-synaptotagmin Ab - Sy) and bungarotoxin to label AChRs (AR). Panels on right show merged images. Arrowheads indicate examples of co-localization between a VGCC associated protein and a presynaptic marker in dissociated nerve terminals. Scale bars are 3 μ m.

VGCC-binding partners expression in E13 MNs

B = RT-PCR control

Lane	Sample	Size
M	Marker	100 bp Ladder
1	GAPDH	125
2	AHNAK	147
3	Plectin	146
4	MyoC	147
5	SPNA2	145
6	SPNB2	150
7	Dystrophin	134
8	ITGA3	108

Figure 8. E13 mouse motor neurons express VGCC binding partners

Motor neurons were purified from E13 spinal cords and immediately processed for RNA extraction. Gene-specific primers were used to detect VGCC binding partner's transcripts after cDNA synthesis (A) or after RNase-treatment to control for genomic carry-over (B).

Figure 9. Known and potential associations between the components of the VGCC protein complex

The figure shows a $\text{Ca}_v\alpha$ protein complex containing all the cytoskeletal and active zone proteins described here. We have shown that the $\text{Ca}_v\alpha$, synaptic laminin, Piccolo and bassoon are all in one complex. However, the other cytoskeletal proteins might be associated with $\text{Ca}_v\alpha$ in one complex or several. For example, $\text{Ca}_v\alpha$ might be bound to spectrin in one complex and AHNK/Desmoyokin in another. See text for additional details.

Table 1

Proteins of the VGCC complex Identified by MS/MS and/or Western Blot.

Protein Band from SDS-PAGE	Identified protein	Known MW (KD)	Observed SDS-PAGE MW (KD) for VGCC protein complex component	Western Blot MW of VGCC complex (KD)
A	Plectin 1	534 ¹	540	500
B	Piccolo	454 ²	ND	470
C	Bassoon	396 ³	ND	430
D	Dystrophin	400/427 ⁴	400	380
E	Spectrin α 2	285 ⁵	280	280
F	Spectrin β 2	271 ⁶	260	ND
G	AHNAK/Desmoyokin	629 ⁷	250	250
H	Laminin β 2 & γ 1	~220 ⁸	200 ⁹	200 ⁹
I	Ca ν α	265/190 ¹⁰	190	190
J	NFm	145–150/95.6 ¹¹	144	Absent
K	Myosin 1	119 ¹²	110	109
L	Ca ν β	53–68 ¹³	ND	52

¹ Calculated from the rat plectin 1 protein sequence (Accession NP_958791).² Calculated from zebra fish piccolo protein sequence (Accession XP_001921217)³ Calculated from zebra fish bassoon protein sequence (Accession XP_001920788)⁴ SDS-PAGE MW for Torpedo Dystrophin from Royuela et al., 2001 /Calculated from Human Dystrophin sequence (Accession AAA53189).⁵ Calculated from the rat α 2 spectrin protein sequence (Accession NP_741984).⁶ Calculated from the rat β 2 spectrin protein sequence (Accession NP_062040).⁷ Calculated from the human AHNAK protein sequence (Accession NP_001611).⁸ From SDS-PAGE/western blots of Sunderland et al. (2000).⁹ The laminin chains migrate slightly faster in these low percentage (2.7–6% or 8%) gradient gels used here.¹⁰ The electric fish Ca ν α sequence (Accession P56698) predicts a 265 KD protein, but we always find a protein that migrates at 190 KD (Sunderland et al., 2000). The cytosolic C-terminal of Ca ν α is known to be proteolytically processed (Dolphin, 2009).¹¹ The rat NFm protein sequence (Accession NP_058725) codes for a protein of 95.6 kD, but the protein migrates on SDS-PAGE as a protein of 145–150 kD (Napolitano et al., 1987)¹² Calculated from the Xenopus myosin 1 protein sequence (NP_001082518.1).¹³ Calculated from Zebra fish β 1- β 2 protein sequences (NP_001002652 and NP_001077037).