Research Paper

Munc18: A Presynaptic Transmitter Release Site N type (CaV2.2) Calcium Channel Interacting Protein

Allen W. Chan†
Rajesh Khanna†
Qi Li
Elise F. Stanley*

Toronto Western Research Institute, University Health Network, Toronto, Ontario, Canada
†These authors contributed equally to this study.
*Correspondence to: Elise F. Stanley, MPh14-320, Toronto Western Research Institute; 399 Bathurst Street; Toronto, Ontario M5T 2S8 Canada; Tel: 416.603.5131; Fax: 416.603.5745; Email: estanley@uhnres.utoronto.ca

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ABSTRACT

Munc18 is a presynaptic protein that is essential for transmitter release. Recent studies have indicated that this protein is involved in secretory vesicle docking but its binding partners in this role remain a mystery. We demonstrate using the isolated calyx-type presynaptic terminal of the chick ciliary ganglion that staining for Munc18 colocalizes and covaries with that for transmitter release site N type calcium channels (CaV2.2), consistent with elements of a common release site complex. Biochemical analysis demonstrated that the protein coprecipitates with CaV2.2 from lysates of rat or chick brain, including its synaptic, long-splice variant; presynaptic terminal surface membrane proteins, and a cell line coexpressing Munc18 and CaV2.2. Munc18 bound with high affinity to the CaV2.2 I-III intracellular loop, low affinity to the I-II loop but not to other channel intracellular regions. Overexpression of Munc18 in dorsal root ganglion neurons did not affect CaV2.2 current amplitude or fast kinetics but siRNA-knockdown resulted in a negative shift in the steady state inactivation curve, an effect attributed to an indirect action via syntaxin 1. Recombinant Munc18 also coprecipitated strongly with the v-SNARE synaptotagmin, but only weakly with other SNAREs. Thus, the calcium channel may serve as a surface membrane platform anchoring a Munc18-containing bridge to synaptotagmin and the synaptic vesicle.

INTRODUCTION

Munc18-1 (herein Munc18), the prevailing mammalian neural orthologue of c. elegans unc-18, is widely distributed, is present at the active zone and is essential for transmitter release from presynaptic nerve terminals in the worm, drosophila and mouse. It binds tightly to syntaxin 1 with nM affinity and the crystal structure of the complexed proteins has been reported. This binding involves the full span of syntaxin 1, maintaining it in a 'closed' configuration and screening regions known to be involved in SNARE complex formation.

While the high-affinity interaction of Munc18 with syntaxin 1 must reflect an important biological function, this is unlikely to be its only role in neurosecretion. Indeed, Munc18 has been suggested to also be involved in the docking of secretory vesicles, supported both by peptide injection into the squid giant synapse and observations on knockouts and mutants of Unc-18 in c. elegans or Munc18 in mouse anterior pituitary and chromaffin cells. Several studies have concluded that Munc18 is not involved in the initial contact of the vesicle with the membrane but in subsequent prefusion steps that lead to the formation of a stable prefusion attachment.

It might be anticipated that a protein with a critical role in the prefusion steps of exocytosis would interact directly with a number of synaptic proteins. However, Munc18 has a conservative interaction spectrum. In addition to the syntaxin family, the only well characterized presynaptic binding partners are the cytoplasmic proteins Mint and DOC2. Thus, syntaxin 1 remains the only candidate membrane-linking protein for Munc18.

We used high-resolution and quantitative immunocytochemistry at a calyx-type synapse to localize Munc18 at the presynaptic transmitter release face. The protein is located in distinct puncta along the transmitter release face that colocalize with the transmitter release site calcium channel (N-type, CaV2.2) clusters. We have previously demonstrated that these clusters colocalize with RIM and, hence, are located at the transmitter release sites. Quantitative analysis demonstrated that these two staining patterns covary, a relationship that is suggestive of proteins that are components of the

KEY WORDS

vesicle docking, exocytosis, munc18, unc18, CaV2.2, N-type, docking complex, calcium channel, synprint, DRG

NOTE

Supplementary material can be found at: www.landesbioscience.com/supplement/chanCHAN1-1-sup.pdf
same molecular complex, although most of the transmitter release face Munc18 appears to be complexed with syntaxin 1. Further biochemical analysis demonstrated that this link is direct. Thus, the calcium channel itself may serve as a membrane-anchoring site for Munc18 during secretory vesicle docking.

**METHODS**

Plasmid construction and recombinant protein expression. Full-length rat Munc18-1 (amino acids 1-594) was digested from plasmid pCMV-Munc18-1 (Dr. S. Sugita; Toronto Western Research Institute, Toronto) and sub-cloned into the GST (glutathione S-transferase) vector pGEX-KG (GE Healthcare/Amersham Biosciences, Piscataway, NJ). Chick Munc18-1 (Supplemental Data, Fig. 1) deletion mutants S1 (amino acids 1-341) and S2 (amino acids 342-594) were constructed using PCR followed by sub-cloning into pGEX-6P-2 (GE Healthcare). Constructs encoding the N-terminus (residues 1-95), full-length C-terminus (residues 1691-2336), distal part of the C-terminus (residues 2163-2336) and intracellular loops I-II (residues 357-483), II-III synprint region (residues 726-984) of rat CaV2.2 (Accession Q02294) were engineered into vector pGEX-6P-2 using PCR from plasmid α1B-pMT as a template (Dr. T. Snutch, University of British Columbia). The synprint region of the II-III loop of CaV2.2 in a six histidine (His6) and Xpress-tagged vector was a gift of Dr. Z-H Sheng (National Institutes of Health, Bethesda, MD). A Syntaxin 1A construct in pGEX-4T-3 vector was obtained from Dr. G. Zamponi (University of Calgary). The fidelity of all constructs was confirmed by dideoxy sequencing in both directions (Innobiotech Inc., Toronto, ON). All fusion constructs were transformed into E. coli BL21(DE3) cells (Novagen, Madison, WI). Proteins were purified using standard procedures. Briefly, protein expressed in BL21 cells was harvested by sonication and incubation with glutathione Sepharose beads (GE Healthcare) or Nickel-NTA agarose (Qiagen, Chatsworth, CA) followed by extensive washing. The bound GST- or His6-fusion proteins were eluted from the beads using 10 mM glutathione (Sigma, St. Louis, MO) or 20 mM imidazole (Sigma) respectively. Eluted fusion proteins were concentrated with Centriprep columns (Amicon, Beverly, MA) and checked by SDS-PAGE followed by BioSafe Coomassie blue staining (BioRad, Hercules, CA). For binding experiments (see below), GST was removed by in-solution protease cleavage using either 4 μg/ml thrombin (Pharmacia) or on-bead protease cleavage using 1 unit/100 μg PreScission protease (GE Healthcare/Amersham Biosciences) as per the manufacturer's instructions. Thrombin was scavenged by incubation with p-AminoBenzamidine agarose beads (Sigma) prior to use.

Plasmids encoding calcium channel α1B, β1b, and α2-δ subunits in vector pMT2 for expression in mammalian cells were kindly provided by Dr. T. Snutch (University of British Columbia). Plasmids for Munc18 over expression (pCMV-Munc18;16) and knockdown (pSuperiRNA-Munc18; Oligoengine, Seattle, WA) were provided by Dr. S. Sugita (TWRI).

**Antibodies and reagents.** Polyclonal antibody Ab571 was raised against a peptide from the II-III loop of the chick CaV2.2 channel and has been characterized in detail. Ab-C4569 and Ab-C4570 were raised in rabbits against a unique peptide from the long splice region of rat CaV2.2.20 Two anti-Munc18 antibodies were used, a polyclonal (Affinity Bioreagents) and a monoclonal (BD Transduction Lab, San Diego, CA), both of which gave a single band of ~67 kD on brain tissue Western blot corresponding to native Munc18 (results not shown). However, only the polyclonal antibody could be used for immunostaining.19 An antibody against the membrane marker protein, Na+/K+ pump was from Developmental Studies Hybridoma Bank (Iowa City, IA) while an anti-Xpress mouse monoclonal antibody was from Invitrogen Life Technologies (Carlsbad, CA). All other chemicals were of reagent grade or higher and, unless otherwise stated, obtained from Sigma.

**Chick/rat brain lysate preparation.** Lysate from brains of 20 E15 day chick embryo brains or 10 neonatal (2 to 10 days) Sprague Dawley rats (Charles River, Quebec) and were extracted as described18,20. Protein concentrations were determined with a BioRad protein assay reagent (Hercules, CA).

**Chick/rat brain synaptosome preparation.** Purified synaptosomes were prepared as described earlier.18,20-22

**In vitro protein-binding assay.** For binding studies, the cytoplasmic loops of CaV2.2 and full-length Munc18 proteins were purified as GST- or His6-tagged proteins. Binding reactions were performed in binding buffer (20 mM HEPES, 150 mM NaCl, 2 mM MgCl2, 1 mM dithiothreitol, 0.5% (v/v) Triton X-100, pH 7.4). Glutathione-Sepharose beads (GE Healthcare) saturated with various CaV2.2 cytoplasmic loop constructs or GST vector were incubated with purified untagged Munc18 protein in a total reaction volume of 100 μl. Reactions were incubated end-over-end for 4 h at 4°C, washed three times with a 100-fold excess of binding buffer, and the proteins were eluted in 60 μl of SDS gel buffer and boiled for 5 min, after which 20 μl of each assay was run on SDS-PAGE and analyzed by immunoblotting with Munc18 antibody. To determine binding affinity (Kd), the experiments were performed using a fixed amount of I-II or II-III CaV2.2-GST fusion proteins and increasing

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**Figure 1.** Munc18 colocalizes with CaV2.2 at the presynaptic nerve terminal transmitter release face. [A] Immunostaining of a calyx synapse with CaV2.2 (Ab571; green) and Munc18 (red). A region of the release face with colocalized CaV2.2 and Munc18 staining (arrowheads) is shown enlarged and rotated 90° clockwise (lower panel). (B) The staining patterns in the release face region were analyzed by the ICA method. CaV2.2 (left, green) or Munc18 (right, red) normalized pixel staining intensities (I/I_{MAX}) plotted against their respective (A-a)/(B-b) values. Both exhibit a strong positive skew, indicative of staining pairs that vary in synchrony (see text). The ICQ value for this terminal was +0.16 (p = 0.16, sign test < 0.001). C, calyx presynaptic terminal; N, ciliary neuron cell body.
molecular weights standards (Invitrogen) as appropriate. Following electrophoresis, proteins were transferred to PVDF membranes (Invitrogen) for immunoblotting or stained with Coomassie blue (BioRad). Western blots were performed using standard procedures. The membranes were blocked for 1 hr in 5% skim milk powder in TBST at room temperature. All antibody incubations were for 1 hr at room temperature. Following incubations with primary antibody and secondary antibody (goat anti-rabbit or anti-mouse IgG HRP; Stressgen; 1:5000), blots were washed extensively in TBST and probed with Enhanced Chemiluminescence reagent (NEN Life Science) before exposure to photographic film. Blots were exposed for a range of durations to ensure the generation of a print in which the film was not saturated. Films were scanned (Canoscan LiDe 30, Canon, Mississauga, Ontario), digitized and quantified using Un-Scan-It gel V6.1 scanning software (Silk Scientific Inc., Orem), limiting our analysis to the linear range.

Cell surface biotinylation of synaptosomes. Biotinylation was performed as described, with modifications. Freshly prepared purified unsolubilized rat brain synaptosomes (100 μg) were incubated with sulfo-NHS-NHS-ss-biotin; 1 mg/ml protein, Pierce Biotechnology Inc., Rockland, IL) for 30 min at 4°C in cold PBS. Excess biotin was quenched with PBS containing 100 mM glycine, washed twice with PBS and the pellet was resuspended in RIPA lysis buffer. The resuspended synaptosomes were triturated 10 times through a 25 gauge needle and centrifuged at 40,000 g for 20 min. Aliquots corresponding to 5% of the total volume of the solubilized material were reserved. The biotinylated proteins were separated from clear solubilize by adsorption onto Streptavidin agarose beads (Novagen) for 2 h at 4°C. Beads were washed three to five times with RIPA buffer, and bound biotinylated proteins were gently eluted off the beads with RIPA buffer containing 2% Triton X-100 and 650 mM NaCl by end-over-end incubation for 1 hr at 30°C. This biotinylated fraction was then subject to immunoprecipitation and immunoblotting with Ab571 as described above.

Preparation of dorsal root ganglion (DRG) neurons. DRG neurons were isolated as described.

Transfection of DRG neurons and human embryonic kidney 293 (tsA201) cells. For biochemical studies, human embryonic kidney 293 (HEK293) tsA201 cells were transiently transfected with cDNAs encoding for calcium channel α1B, β1b and α2-δ subunits and enhanced green fluorescent (eGFP) protein at a 1:1:1:1.5 molar ratio with or without rat Munc18-1 using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Rat Munc18-1 was used since it exhibited >97% identity with chicken Munc18-1 (Supplementary Fig. 1) and homology modeling showed no structural changes. The molar ratio of Munc18-1:channel was 1:1. HEK-293 tsA201 cells were grown in Dulbecco’s modified Eagle’s (DMEM)/F-12 medium supplemented with 10% fetal calf serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml) in an atmosphere of 5% CO2. The cells were lysed 48 hr after transfection. For electrophysiological recordings, adherent DRGs were cotransfected with cDNAs encoding the marker, enhanced green fluorescent protein (pEGFP; Clontech, Palo Alto, California) and wild-type Munc18-1 in pCMV or Munc18siRNA (vector pSuperRI-Na-M unc18) constructs using Lipofectamine 2000 (Supplemental data Fig. 2). Controls were transfected with GFP together with vector alone (vector control). As assessed by GFP fluorescence, the transfection efficiency was ~7.8 ± 1.1 % (N = 380). After transfection, the cells were maintained in a standard incubator...
under 8% CO₂ for 24-30 h at 37°C. Cells were selected for electrophysiological recording by both GFP fluorescence and general cell health.

**Patch-clamp recording.** Electrophysiological techniques as applied to the DRG neurons have been described in detail. Traces were filtered digitally at 2 kHz for analysis and display. Whole-cell calcium currents were isolated using the following solutions. The external (bath) medium was (in mM): NaCl 135, CaCl₂ 2, MgCl₂ 0.8, α-glucose 5, 4-aminopyridine 2, tetrodotoxin 0.001, HEPES-Na 10, tetraethylammonium-Cl 20, Ni(ClO₄)₂ 0.1; with an osmolarity of 285.0 mosm/l and pH 7.4. The patch electrode intracellular solution was: Cs-glucuronate 120, CsCl 10, EGTA(Cs) 10, MgCl₂ 1, HEPES(Cs) 10, tetraethylammonium-Cl 20, MgATP 2; osmolarity 295 mosm/l, pH 7.4. All experiments were carried out at room temperature (22–24°C).

**Voltage protocols and data analysis.** Cells were held at a holding potential of -80 mV. Immediately after seal rupture -80 to +80 mV ramp depolarizations were given until recording current stability was established. Current to voltage relations were assessed using a standard, incrementing 10 mV step depolarizing pulses at 2 sec intervals. Leak currents were subtracted on line using a P/6 procedure. For steady state inactivation, pulses were applied at 10 sec intervals and the P/6 leak protocol was carried out immediately after the test pulse. The holding potential was stepped in 10 mV increments for the intervening period between test pulses. Current amplitude was measured at the end of the voltage pulse. Data was analyzed using ClampFit 9.0 (pClamp suite, Axon Instruments) and Origin 7.0 software (Microcal, Northampton, Mass., USA).

**Chick ciliary ganglion calyx synapse preparation.** This has been described in detail. After trituration of the ganglia the cells/terminal preparation was plated at 37°C in a standard cell incubator for 45 minutes.

**Immunostaining, microscopy and iterative deconvolution deblurring.** This has been described in detail. Staining with two rabbit polyclonal antibodies was as described using the pretty-poly method. Microscopy techniques were as described. Regions of interest (ROIs) were identified by eye from the sampled and neighboring optical sections.

**Intensity correlation analysis (ICA)/intensity correlation quotient (ICQ).** This analysis has been described in detail. Basically, for the ICA we calculated the function \((Ai-a)(Bi-b)\), where \(a\) and \(b\) are the means of each pixel staining pair intensity values \(Ai\) and \(Bi\). \(Ai\) or \(Bi\) was graphed in separate scatter plots against their respective \((Ai-a)(Bi-b)\) value. Distributions that skew to the right reflect dependent staining patterns (where the two pixel staining intensity values vary in synchrony), ones that are symmetrical about the axis indicate random staining, while those that skew to the left reflect segregated staining Note that the analysis can be carried out for each stain separately so that a dependence of stain A on B but a lack of dependence of B on A can be identified and, further, that the plots permit detection of complex or mixed staining relations. The intensity correlation quotient (ICQ) reflects the ratio of the number of positive \((Ai-a)(Bi-b)\) values to the total number of pixels in the ROI, corrected to a -0.5 (segregated staining) to +0.5 (dependent staining) range by subtracting 0.5. The ICQ provides a single value indication that can be used for statistical comparison. Typically, with N≤6 ROIs, a mean ICQ value of -0.05 to +0.05 indicates random staining, +0.05 to +0.1, indicates a moderate covariance and >+1.0 a strong covariance. ICA/ICQ analysis was carried out by means of an automated graphic plugin (Morris and Stanley, unpublished see 18) for the public domain image analysis software ImageJ (Wayne Rasband; Research Services Branch, National Institutes of Health, Bethesda MD).

**Image presentation.** Images were cut, background was subtracted (using an area outside the cell), overlays were created and brightness/contrast was adjusted with ImageJ and PowerPoint software. No nonlinear or partial image adjustments were made.

**Statistical Analysis.** Student’s t-test was used, except where stated. Values are either tested for two means between conditions (P) or for one mean for a difference from 0 (P = 0). Each calyx ICQ value reflects the mean of two determinations from image planes at least 400 nm apart (two z axis increments). If this was not possible a single image plane was used. N values refer to the number of calyx terminals examined. The normal approximation to the sign test was used for statistical tests of ICQ values for single experiment, as in Figure 1 (see ref. 19), One-way ANOVAs were used to determine significance between the GFP, Munc18, Munc18 siRNA electrophysiology data.

**RESULTS**

Localization of Munc18 and CaV2.2 at the presynaptic transmitter release face. Our first objective was to test if Munc18 is colocalized with CaV2.2 clusters at the presynaptic transmitter release face. We used the calyx-type presynaptic terminal at the chick ciliary ganglion synapse to localize these proteins. This nerve terminal has a combination of advantages that make it unique for this analysis: first, virtually all the presynaptic calcium channels are CaV2.2. Second, the synapse can be fully isolated on a cover slip and imaged with high resolution (~200 nm) deconvolution microscopy. Finally, the terminal is sufficiently large to permit not only a clear distinction of the release face, Schwann cell face and intracellular regions but also to distinguish active zone from non active zone areas.

CaV2.2 was localized with an antibody against the II-III intracellular loop, Ab571 while Munc18-1 was identified by a commercially available polyclonal antibody (see Methods). Since both antibodies were rabbit polyclonals, we used the pretty-poly staining method for costing.

We observed an overlap in the staining patterns for Munc18 and CaV2.2, although areas stained with either dye alone were also evident (Fig. 1A). Colocalization is hampered at the sub-cellular level by complex staining patterns even with well-established protein binding partners and quantitative analysis methods are generally required. We used the previously described Intensity Correlation Analysis (ICA) method. This method tests if the two stains covary, as they should if the two proteins are parts of the same complex, This test also generates an Intensity Correlation Quotient (ICQ), which ranges from -0.5 to +0.5 and can be used for statistical comparison, ICA plots exhibited an obvious positive skew, suggesting a strong stain covariance (Fig. 1B). The mean ICQ value was 0.092 ± 0.012 (p= 0 <0.01, N = 7), indicating a moderate covariance and was consistent with two proteins that are components of a common complex. We have previously reported that staining for a control membrane protein, the Na+/K⁺ pump, does not covary with that for release face CaV2.2.

**Biochemical evidence for a Munc18-1/CaV2.2 channel complex.** We next used standard coimmunoprecipitation to test if CaV2.2 and Munc18 can associate in the same complex. Precipitation of CaV2.2 with Ab571 or (N = 19) or Munc18 (N = 15) from chick brain lysate reliably recovered the opposite protein (Fig. 2A). Similar results were obtained from whole rat brain lysate (N=7, data not shown; Ab571 is equally effective and selective against rat CaV2.2, Supplementary...
Immunoprecipitation of the biotinylated proteins with anti-CaV2.2 biotin was omitted from the protocol (Fig. 3, upper panel, lane 2). Membrane fraction (Fig. 3, upper panel, lanes 5 and 8) but not when for Munc18. CaV2.2 was detected in the biotinylated synaptosomal immunoblotting while coimmunoprecipitation was used to search with streptavidin-agarose, gently eluted and immunoprecipitated with solubilized in RIPA buffer. The biotinylated fraction was recovered. Excess biotin was quenched; the synaptosomes were washed and resolubilized prior to standard immuno precipitation with Ab571. The resulting blots were probed with anti-CaV2.2 (Ab571; upper panel) or Munc18 (monoclonal; lower panel). Samples representing 5% of the lysate (L), the entire Ab571-immunoprecipitated biotinylated fraction (B) and 5% of the Ab571-unbound biotinylated fraction (U) were resolved by SDS-PAGE. CaV2.2 was detected at the cell surface only in the presence of biotin [a, lanes 5 and 8]. Munc18 coprecipitated with surface-expressed CaV2.2 in the presence of 1 or 2 mM biotin (b, lanes 5 and 8).

The above biochemical tests indicate that CaV2.2 and Munc18 can be a part of a common complex and provides compelling evidence for the presence of this complex in presynaptic terminals. However, in order to link the immunocytochemical covariance data to the biochemical coprecipitation we felt it necessary to test if this complex is present on the nerve terminal surface membrane, as required for the transmitter release sites. For this we carried out a biotinylation assay. Inspection of the amino acid sequence of CaV2.2 revealed the presence of 30 extracellular lysine residues. Untreated synaptosomes were incubated with the biotinylation reagent sulfo-NHS-SS-biotin. Excess biotin was quenched; the synaptosomes were washed and solubilized in RIPA buffer. The biotinylated fraction was recovered with streptavidin agarose, gently eluted and immunoprecipitated with Ab571 as above. Surface expressed CaV2.2 channels were assessed by immunoblotting while coimmunoprecipitation was used to search for Munc18. CaV2.2 was detected in the biotinylated synaptosomal membrane fraction (Fig. 3, upper panel, lanes 5 and 8) but not when biotin was omitted from the protocol (Fig. 3, upper panel, lane 2). Immunoprecipitation of the biotinylated proteins with anti-CaV2.2 coprecipitated Munc18 (Fig. 3 lower panel, lanes 5 and 8). Hence, surface expressed CaV2.2 was associated with Munc18 at the synaptosomal membrane.

Munc18 binds directly to CaV2.2 intracellular regions. It is well established that Munc18 binds with high affinity to syntaxin 1. In addition, it has been shown that syntaxin 1 can bind to the ‘synprint’ region of the calcium channel intracellular II-III loop.32 Thus, we anticipated that Munc18 links to CaV2.2 via a syntaxin 1 bridge and, hence, that Munc18 would not bind directly to CaV2.2. Our first test of this hypothesis was to express CaV2.2 with or without Munc18 in HEK293 tsa201 cells, a simple cell expression system that lacks syntaxin 1,33 and test for coimmunoprecipitation. Two days after transfection the cells were lysed, immunoprecipitated with Ab571 and immunoblotted for Munc18-1. Munc18-1 coprecipitated with CaV2.2 only when both proteins were present (Fig. 4). No coprecipitation was detected in HEK293 tsa201 cell lysate expressing vector or CaV2.2 channel without Munc18-1.

The HEK293 cell result suggests that these two proteins can bind directly to each other. To explore this interaction we carried out in vitro pull-down assays using recombinant proteins encoding cytoplasmic loops of CaV2.2 and full length Munc18. Fusion proteins of GST-tagged cytoplasmic loops of CaV2.2 were allowed to bind with purified untagged Munc18 proteins in the vitro complexes were then recovered by incubation with glutathione sepharose, washed extensively and analyzed by immunoblotting. Munc18 bound to the I-II and II-III loops of CaV2.2 in vitro (Fig. 5A, lanes 4 and 5, respectively) but not to beads alone or GST protein (Fig. 5A, lanes 2 and 3, respectively). Incubation of GST-tagged Munc18 with Xpress-tagged CaV2.2 II-III loop also demonstrated binding between the two...
proteins (Fig. 5B). In contrast to binding to loops I-II and II-III, no binding was observed between Munc18 and the N-terminus, III-IV loop or the C-terminus of CaV2.2 (results not shown). Attempts to dissect the Munc18 binding region with protein constructs comprising the syntaxin 1 binding and nonbinding regions failed as neither bound to CaV2.2 or its intracellular loops (data not shown), presumably because Munc18 tertiary structure was lost (see ref. 9).

We measured the strength of binding to the CaV2.2 I-II and II-III loops in vitro using an affinity assay. Munc18 bound to CaV2.2 I-II loop with a binding affinity ($K_D$) of $-12.0 \pm 0.5 \mu M$ ($N = 3$) and to loop II-III with a $K_D$ of $-13.8 \pm 4.0 \mu M$ ($N = 3$; Fig. 5C). As a control, we measured the $K_D$ of interaction between syntaxin 1A (Syn 1A) and Munc18. The $K_D$ value was found to be $-48 nM$ (Fig. 5C, inset), consistent with the previously reported value of $-50 nM$. Thus, Munc18 can bind directly to CaV2.2 via a high affinity (II-III loop) and a lower affinity (I-II loop) binding site.

The effect of Munc18 on CaV2.2 function. We carried out patch clamp recording from isolated chick dorsal root ganglion (DRG) neurons to test if Munc18 affects calcium channel behavior in a primary neuron. Virtually all the calcium current in these cells is CaV2.2, as assessed by $\alpha$-conotoxin GVIA block.24 We examined the effect of Munc18 on CaV2.2 function by both over-expression and siRNA knockdown,25 identifying positive cells with GFP. Successful knockdown was confirmed by immunocytochemistry in a double-blind assay (Supplemental Data Fig. 2). Neither transfection condition affected the pulse-evoked Ca$^+$ current amplitude, voltage dependence or activation time constant (Fig. 6). However, Munc18 knockdown caused a $-9$ mV hyperpolarizing shift in the steady-state inactivation profile (Fig. 7) while over expression had no effect.

Association of Munc18 with synaptic markers. We also explored whether Munc18 associates with other presynaptic markers. In order to avoid Munc18 already complexed with other synaptic proteins we used a pull-down strategy using GST-Munc18. In addition, we tested for low-affinity or nonspecific binding by exposing the precipitated complexes to high salt. Thus, GST-Munc18 immobilized on precipitation beads was incubated with chick brain lysate. The sample was split and each half was exposed to normal (150 mM) or high (1150 mM) salt prior to washing and recovering bound proteins in denaturing buffer. The proteins were separated by SDS-PAGE and immunoblotted with various antibodies. GST-Munc18 was not dissociated from the bead by high salt treatment. We did not recover untagged Munc18, indicating that the protein does not dimerize and confirming that any coprecipitated proteins must be associated with the fusion protein and are not captured indirectly via native Munc18 (Fig. 8). CaV2.2 was also recovered, consistent with the results above. Further, the finding that the channel was not displaced by electrophoretic competition in high salt supports high affinity binding. Syntaxin 1 coprecipitated with the channel but was shed in high salt, suggesting a relatively weak association. Similar results were obtained for SNAP-25 ($N = 2$, data not shown). Since CaV2.2 was retained in high salt, this observation provides additional evidence that Munc18 does not require syntaxin 1 to bind to the channel. We also tested two secretory vesicle

![Figure 5. Munc18 binds directly to CaV2.2 cytoplasmic loops.](image-url)
v-SNARES, VAMP (synaptobrevin) and synaptotagmin 1 (STG). Recovery of the former was poor, even in the low salt sample and was eliminated by high salt, suggesting a weak and possibly nonspecific interaction. However, the recovery of synaptotagmin 1 was robust in control salt and was resistant to electrostatic competition, suggesting high affinity binding.

**DISCUSSION**

Previous functional studies using both peptide injection and protein knockout models have established that Munc18 is an essential transmitter release-associated protein. This study provides evidence for the direct binding of this protein to intracellular loops of the presynaptic calcium channel. The finding that this protein is also in a biochemical complex with synaptotagmin 1, a secretory vesicle v-SNARE, provides a new mechanism whereby Munc18 may contribute to the molecular bridge between the release site and the secretory vesicle.

A presynaptic transmitter release face Munc18/CaV2.2 complex. We present several lines of evidence that point to a prominent Munc18-CaV2.2 complex at the presynaptic terminal. First, we demonstrate that immunostaining for the two proteins both colocalizes, as assessed by the simple dye-overlay method, and, more significantly, covary at the presynaptic transmitter release face. Staining covariance is strong evidence that the two proteins are parts of a common molecular complex or subcellular organelle while much of the Munc18 that is not associated with the channel at the transmitter release face is likely complexed to syntaxin 1.

Biochemical analysis supported and expanded on the immunocytochemistry findings. Immunoprecipitation of the channel with three CaV2.2-specific antibodies directed at two different regions of the channel, the II-III loop while much of the Munc18 that is not associated with the channel at the transmitter release face is likely complexed to syntaxin 1.
Munc18 (Fig. 2; Supplemental data Fig. 3B). The latter finding is of particular significance since the long splice variant has been localized to presynaptic transmitter release sites.\textsuperscript{20,36} Similar results were obtained with purified rat brain synaptosome lysate and chick ciliary ganglion lysate, providing further support for the presence of this complex at the presynaptic terminal while biotinylation experiments demonstrated the association of Munc18 with membrane-inserted CaV2.2. Further, Munc18 fusion protein pulled down CaV2.2 from chick brain lysate (Fig. 8, top panel, lanes 4, 5) and this pull-down was resistant to high salt dissociation, consistent with a high-affinity interaction. Syntaxin 1 was also captured with the fusion protein but binding to this cardinal Munc18 partner was disrupted by high salt.

The imaging and coimmunoprecipitation experiments provide evidence for the presence of Munc18 and CaV2.2 in a common biological molecular entity associated with the transmitter release sites. However, they do not address the question as to whether these two proteins interact directly with each other. Syntaxin 1 is an obvious potential bridging partner since it binds both to Munc18 and to the CaV2.2 II-III intracellular loop.\textsuperscript{32} Indeed, we originally set out to test for such a link. To our surprise, Munc18 bound directly to both the I-II and the II-III loop in the absence of syntaxin 1. The high affinity of the latter, -14 nM, essentially precludes nonspecific binding and supports a biologically significant interaction. Indeed, this affinity was about 4 fold higher than that for syntaxin 1 to the same II-III loop. We presume that these two proteins bind to nonidentical regions of the loop since pretreatment of the peptide with syntaxin 1 failed to occlude Munc18 binding (data not shown).

Munc18 and Modulation of CaV2.2. A few previous reports have explored physiological interactions between Munc18 and the calcium channel. The most direct demonstration was the finding that although injection of a (Squid)-UNC18 peptide into the squid giant synapse presynaptic terminal led to a pronounced inhibition of transmitter release, calcium influx was unaffected.\textsuperscript{12} When Munc18 was coexpressed with CaV2.2 in a cell line the protein induced a 9 protein like, voltage sensitive inhibition of the calcium channels,\textsuperscript{37} an effect that was not explored here. However, no study has carried out a systematic analysis of possible CaV2.2 current modulation by Munc18.

We tested for Munc18 modulation of CaV2.2 using an acutely isolated native chick neuron. Over-expression of the protein had no effect on any parameter. Munc18 knockdown also had no effect except for a hyperpolarizing shift in the steady-state inactivation profile. These two findings suggest that the cell normally has an excess of the protein. Previous studies have demonstrated that the CaV2.2 inactivation profile is subject to modulation via a variety of pathways,\textsuperscript{18,39} notably by interaction with syntaxin 1.\textsuperscript{1,37,40-42} Thus, a possible explanation for the siRNA results is that Munc18 knockdown results in an increase in free syntaxin 1 which, in turn, inhibits the channels. This is consistent with expression of Munc18 with CaV2.2 in the absence of syntaxin 1\textsuperscript{15} and also with recent studies that report the inhibitory effect of syntaxin 1 could be counteracted by simultaneous expression of Munc18.\textsuperscript{37,44}

Is Munc18 a vesicle scaffold protein? Although synaptic transmission is absent in Munc18 knockout mice the ultrastructure of the presynaptic terminals is in essence, normal\textsuperscript{13} and the reason for the blocked transmission remains a mystery. Our finding that Munc18 binds directly to the presynaptic Ca channel identifies a new protein interaction that may contribute to this puzzle. One possibility is that Munc18 serves a scaffolding role to link the channel to a third protein binding partner and that this interaction is key for transmitter release. We considered the idea that Munc18 sequesters syntaxin 1 to the channel and, hence, to the release site. While syntaxin 1 is critical for transmitter release we and others have noted that it colocalizes only poorly with the active zone.\textsuperscript{19,46} It has been shown that syntaxin 1 is correctly targeted to nerve terminals in worm and mouse mutants and that SNARE complexes can form.\textsuperscript{4,47} Further, overexpression of syntaxin 1 does not relieve the transmitter release block in Unc-18 mutants\textsuperscript{4} but it is not known if delivery of syntaxin 1 to the release site itself is normal.
A role for uncle-18/Munc18 in secretory vesicle docking is highlighted in recent studies in the worm and mouse. Of particular interest in the c. elegans study was the finding that while transmitter release is grossly reduced (~20%), the slope of its Ca\textsuperscript{2+} dependence is normal. This argues against a defect in the Ca\textsuperscript{2+} sensing apparatus of the transmitter release site. However, the finding is consistent with the idea that the vesicle and its sensor are located at an increased distance from the channel, so that less of the admitted Ca\textsuperscript{2+} ions reach their target. In the mouse study, the authors propose that Munc18 plays a role in the stabilization of synaptic vesicle docking after the formation of the initial contact. It was hypothesized that this stable link is anchored to the surface membrane platform via syntaxin 1. However, this idea seems contrary to the finding that syntaxin 1 cleftage with botulinum C toxin has no effect on the number of morphologically docked secretory vesicles and the finding that Munc18 inhibits VAMP-syntaxin 1 interactions. The high-affinity binding to CaV2.2 reported here provides an attractive alternative anchoring platform. We provide additional support for this hypothesis using the Munc18 fusion protein pull-down assay from brain lysate. Munc18 coprecipitated synaptotagmin 1 as well as CaV2.2 and both of these links were resistant to high salt, indicating high affinity binding (Fig. 8). The association with synaptotagmin 1 is of particular interest. We can be confident that this pull-down was not due to the bulk capture of synaptic vesicle proteins since the second v-SNARE, VAMP was readily shed with the high salt treatment and we also failed to capture a second vesicle protein synapsin 2A (data not shown). Thus, our results are consistent with a CaV2.2-Munc18-synaptotagmin link from the surface membrane to the secretory vesicle as part of stable docking link that might contribute to the dramatic loss of synaptic transmission in the Munc18 knockout models.

The sequence for chicken Munc18-1 has been deposited in the GenBank database (Accession no. AY509605). We thank Drs. Steven Owens for discussions, M. Khanna (University of Toronto) for helpful comments with recombiant protein purification, and Dr. C. Q. Munro (Center for Computational Biology and Bioinformatics, Indiana University School of Medicine) for help with homology modeling of chicken Munc18. We are grateful to Drs. S. Sugita, G. Zampori, Z-H. Sheng and T. Snutch for various plasmids, as indicated. This study was supported by CIHR award MOP57716, a Canada Research Chair (E.F.S.) and a CIHR Canada Doctoral Graduate Scholarship to AW.C. Some of these data have been presented in abstract form: Q. Li, R. Khanna, L. Sun, T.R. Bugg, E.F. Stanley. Munc18-1 association with the CaV2.2 (N-type) calcium channel. Program No. 96A.16. 2005 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2005 Online and A.W. Chan, R. Khanna, Q. Li, L. Sun, E.F. Stanley. A novel Munc18-1 interaction with the [N-type] CaV2.2 calcium channel. Biophys. J. 90, January, 2006.

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