SV2B Regulates Synaptotagmin 1 by Direct Interaction*

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SV2 proteins are abundant synaptic vesicle proteins expressed in two major (SV2A and SV2B) and one minor (SV2C) isomorf. SV2A and SV2B have been shown to be involved in the regulation of synaptic vesicle exocytosis. Previous studies found that SV2A, but not SV2B, can interact with the cytoplasmic domain of synaptotagmin 1, a Ca²⁺ sensor for synaptic vesicle exocytosis. To determine whether SV2B can interact with full-length synaptotagmin 1, we performed immunoprecipitations from brain protein extracts and found that SV2B interacts strongly with synaptotagmin 1 in a detergent-resistant, Ca²⁺-independent manner. In contrast, an interaction between native SV2A and synaptotagmin 1 was not detectable under these conditions. The SV2B-synaptotagmin 1 complex also contained the synaptic t-SNARE proteins, syntaxin 1 and SNAP-25, suggesting that SV2B may participate in exocytosis by modulating the interaction of synaptotagmin 1 with t-SNARE proteins. Analysis of retinae in SV2B knock-out mice revealed a strong reduction in the level of synaptotagmin 1 in rod photoreceptor synapses, which are unique in that they express only the SV2B isoform. In contrast, other synaptic vesicle proteins were not affected by SV2B knock out, indicating a specific role for SV2B in the regulation of synaptotagmin 1 levels at certain synapses. These experiments suggest that the SV2B-synaptotagmin 1 complex is involved in the regulation of synaptotagmin 1 stability and/or trafficking. This study has demonstrated a new role of SV2B as a regulator of synaptotagmin 1 that is likely mediated by direct interaction of these two synaptic proteins.

SV2 proteins are a family of three homologous integral synaptic vesicle (SV)¹ proteins. SV2 proteins share homology with mammalian ion and sugar transporters (1–4), but transport activity has thus far not been demonstrated. The two most abundant isoforms, SV2A and SV2B, have a wide distribution throughout the brain; SV2C has a more restricted distribution in the brain, mainly in evolutionarily older brain regions (5, 6). The major isoform, SV2A, appears to be found in all synapses in the nervous system with the exception of certain synapses in the retina. Synapses formed by rod photoreceptor cells and rod bipolar cells contain only SV2B, not SV2A or SV2C (7).

Studies using SV2A and SV2B knock-out mice have shown that SV2 proteins regulate Ca²⁺-dependent exocytosis of synaptic vesicles in neurons and of large dense core vesicles in neuroendocrine cells (8–10). SV2A knock-out mice develop severe seizures that eventually lead to death, indicating that SV2 proteins are essential for normal synaptic function. However, the functional role of SV2 proteins in synaptic vesicle exocytosis is still unclear.

SV2 proteins have been shown to form a complex with the synaptic vesicle protein synaptotagmin 1 using a monoclonal antibody that reacts with all SV2 isoforms (11). Synaptotagmin 1 is a Ca²⁺ sensor for synaptic vesicle exocytosis in neurons (reviewed in Refs. 12–14); thus, SV2 proteins may modulate synaptic vesicle exocytosis through direct interaction with synaptotagmin 1. Studies using affinity chromatography have demonstrated a Ca²⁺-dependent interaction between native SV2A and GST-fused cytoplasmic domains of synaptotagmin 1 (15). In contrast, an interaction between SV2B and the cytoplasmic part of synaptotagmin 1 was not detected in this study. Further experiments have demonstrated synaptotagmin 1 interaction with the isolated N terminus of SV2A, but not SV2B. We performed immunoprecipitations from native brain protein and found that full-length synaptotagmin 1 complexes with SV2B in a Ca²⁺-independent manner. This complex was found to contain t-SNARE proteins syntaxin 1 and SNAP-25. In further experiments, we have demonstrated a direct role for SV2B in the regulation of synaptotagmin 1 levels in retinal ribbon synapses.

EXPERIMENTAL PROCEDURES

Animals—SV2B knock-out mice have been described (8) and were maintained as a heterozygous line. Knock-out and wild type control littermates were generated by crossing heterozygous parents. Genotyping by PCR has been previously described (8).

Antibodies—The following antibodies were used at the indicated dilutions: monoclonal anti-SV2, 1:5000 (Developmental Studies Hybridoma Bank, Iowa City, IA); polyclonal anti-synaptogyrin-1, 1:5000 (16); polyclonal anti-SV2A, SV2B, or SV2C, 1:5000 Western blots and 1:5000 immunostaining (5); goat anti-GST, 1:1000 (Amersham Biosciences); and anti-synaptotagmin ASW30, 1:500 (Stressgen Biotechnologies, Inc., San Diego, CA) (17). The following antibodies were purchased from Synaptic Systems (Göttingen, Germany): polyclonal anti-synaptotagmin-1, 1:1000; monoclonal anti-synaptotagmin 1 (CL41.1 and CL604.1), 1:5000 Western blots and 1:5000 immunostaining; monoclonal anti-SNAP-25 (CL71.1), 1:10,000; polyclonal anti-synapsin, 1:5000; monoclonal anti-synaptophysin (CL72.2), 1:5000; monoclonal anti-synaptotachy- toxin 1 (CL78.2), 1:1000; and monoclonal anti-synaptobrevin/VAMP2 (CL69.1), 1:1000. The following secondary antibodies were used: horseradish peroxidase-conjugated goat anti-rabbit, goat anti-mouse, and rabbit anti-goat, 1:5000 (Zymed Laboratories, Inc., San Francisco, CA); rabbit anti-goat, 1:5000 (Zymed Laboratories, Inc., San Francisco, CA).

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peroxidase-conjugated goat anti-mouse IgG preadsorbed against human, mouse, and rat serum, 1:10,000 (Jackson Immunoresearch Laboratories, Inc., West Grove, PA); Cy3-conjugated goat anti-mouse or anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA); and Alexa 568- or Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes, Eugene, OR), all 1:500.

Immunoprecipitations—Tissue was homogenized 10 strokes, 900 rpm, in a glass teflon homogenizer in 10 volumes of 20 mM HEPES (pH 7.4) with protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of aprotinin, peptatin, and leupeptin). For protein extraction, 1 volume of buffer B (20 mM HEPES, pH 7.4, 0.2 mM NaCl, and 2% Triton X-100) was added and the sample agitated at 4 °C for 30 min. The homogenate was centrifuged at 13,000 rpm, 30 min in a microcentrifuge at 4 °C. The supernatant (total brain extract) was then used for immunoprecipitations. 1 ml of brain extract was mixed with 10–50 μl of antibody on an overhead rotator for 1 h at 4 °C. 20 μl of protein A-Sepharose beads (Amersham Biosciences) (protein G beads for mouse monoclonal antibodies) were added and the reaction incubated overnight at 4 °C on an overhead rotator. Beads were washed three times, 15 min each, with cold wash buffer (20 mM HEPES, pH 7.4, 0.2 mM NaCl).

2× SDS loading buffer was added to the beads and samples incubated for 10 min at 65 °C. Protein was then analyzed by SDS-PAGE and Western blotting. For Ca2+ immuno precipitations, all buffers contained 2 mM EGTA or 150 mM CaCl. For the NaCl wash and all Western blotting, for Ca2+ immunoprecipitations, all buffers were the same molarity as for standard immunoprecipitations, except that wash buffers contained 0.5% NaN3 concentrations. A final 5-min wash in standard wash buffer (200 mM NaCl) equilibrated salt concentration before sample preparation for SDS-PAGE.

GST Fusion Construct Binding Experiment—Fusion proteins containing the cytoplasmic part of syntaxin 1A (amino acids 4–287) fused to GST (18) and full-length GST alone were expressed in Escherichia coli and purified using GST beads. Bead-bound recombinant proteins were incubated overnight at 4 °C with brain extract prepared as described for immunoprecipitations. Buffers used in the binding experiments contained 2 mM EGTA or 150 mM CaCl. Beads were washed in standard buffer and samples prepared for SDS-PAGE.

Native SV2B Interacts with Full-length Synaptotagmin 1—To identify the SV2 isoforms that interact with full-length synaptotagmin 1 in the brain, we performed immunoprecipitations from mouse brain extract using a monoclonal antibody directed against the cytoplasmic portion of synaptotagmin 1 (CL41.1; Synaptic Systems). The immunoprecipitate was probed on a Western blot with isoform-specific polyclonal antibodies against SV2A, SV2B, or SV2C (Fig. 1, IP Stg/CL41.1). SV2B efficiently coprecipitated with the antibody against synaptotagmin 1, whereas SV2A coprecipitation was not detected. A low level of SV2C coprecipitated. Two synaptic vesicle proteins, synaptophysin (Sphy) and synaptogyrin 1, were not present in the precipitate, confirming that other synaptic proteins did not precipitate nonspecifically with the antibody against synaptotagmin 1. An antibody against synaptophysin was used as a negative control in parallel immunoprecipitations (IP Sphy). This control precipitate did not react with the isoform-specific polyclonal SV2A, SV2B, or SV2C antibodies, demonstrating the specificity of the SV2B-synaptotagmin 1 interaction. The observed immunoreactive bands were also not because of reactivity of the primary or secondary antibodies used in the Western blot with the precipitating antibody as confirmed by loading the monoclonal synaptotagmin 1 antibody (Stg1/CL41.1 Ab).

These results indicate that native SV2B binds full-length synaptotagmin 1 in the brain. Although an interaction between SV2A and synaptotagmin 1 was not found, interaction between full-length SV2A and synaptotagmin 1 may have blocked the cytoplasmic epitope of the synaptotagmin 1 antibody used for immunoprecipitation. To address this possibility, immunoprecipitations from total brain extract were performed using a

FIG. 1. SV2B interacts with synaptotagmin 1. Full-length, native synaptotagmin 1 was immunoprecipitated from total brain extract using a monoclonal antibody against synaptotagmin 1 (Stg1/CL41.1). Control immunoprecipitations were conducted in parallel with an antibody against synaptophysin (IP Sphy). Bead-bound protein was analyzed by Western blot using isoform-specific polyclonal antibodies against SV2A/B/C, synaptotagmin 1/2, synaptophysin, and synaptogyrin 1. SV2B was the predominant SV2 isoform that immunoprecipitated with an antibody against synaptotagmin 1. SV2A immunoprecipitation was not detected.

solution. Primary antibodies were applied for 2 days at 4 °C. After rinsing and blocking, fluorescent secondary antibodies were applied for 45 min. Sections were rinsed extensively and mounted with coverslips using a fade-retardant mounting medium (Vectorshield; Vector Labs, Burlingame, CA).

Cell Culture—HEK 293 cells were cultured in Dulbeco’s modified Eagle medium supplemented with 10% fetal calf serum, glutamine, and antibiotics penicillin and streptomycin. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the protocol supplied by the manufacturer.

RESULTS

Native SV2B Interacts with Full-length Synaptotagmin 1—

To identify the SV2 isoforms that interact with full-length synaptotagmin 1 in the brain, we performed immunoprecipitations from mouse brain extract using a monoclonal antibody directed against the cytoplasmic portion of synaptotagmin 1 (CL41.1; Synaptic Systems). The immunoprecipitate was probed on a Western blot with isoform-specific polyclonal antibodies against SV2A, SV2B, or SV2C (Fig. 1, IP Stg/CL41.1). SV2B efficiently coprecipitated with the antibody against synaptotagmin 1, whereas SV2A coprecipitation was not detected. A low level of SV2C coprecipitated. Two synaptic vesicle proteins, synaptophysin (Sphy) and synaptogyrin 1, were not present in the precipitate, confirming that other synaptic proteins did not precipitate nonspecifically with the antibody against synaptotagmin 1. An antibody against synaptophysin was used as a negative control in parallel immunoprecipitations (IP Sphy). This control precipitate did not react with the isoform-specific polyclonal SV2A, SV2B, or SV2C antibodies, demonstrating the specificity of the SV2B-synaptotagmin 1 interaction. The observed immunoreactive bands were also not because of reactivity of the primary or secondary antibodies used in the Western blot with the precipitating antibody as confirmed by loading the monoclonal synaptotagmin 1 antibody (Stg1/CL41.1 Ab).

These results indicate that native SV2B binds full-length synaptotagmin 1 in the brain. Although an interaction between SV2A and synaptotagmin 1 was not found, interaction between full-length SV2A and synaptotagmin 1 may have blocked the cytoplasmic epitope of the synaptotagmin 1 antibody used for immunoprecipitation. To address this possibility, immunoprecipitations from total brain extract were performed using a
monoclonal antibody against the lumenal portion of synaptotagmin 1 (Fig. 2). Surprisingly, probe of the immunoprecipitate on a Western blot revealed that none of the SV2 isoforms coprecipitated with the lumen antibody against synaptotagmin 1 (IP Stg/CL604.1). Probe of the precipitate with a polyclonal antibody against synaptotagmin 1 (Stg) showed that synaptotagmin 1 precipitated efficiently with the lumen antibody. Because synaptotagmin 1 not associated with SV2B precipitated with the lumen antibody, association with SV2B may block precipitation of synaptotagmin 1. This association may obscure the epitope recognized by the lumenal antibody against synaptotagmin 1, suggesting that the lumenal portion of synaptotagmin 1 may participate in complex formation with SV2B or that a conformational change that occurred because of SV2B association concealed this epitope. In addition, immunoprecipitation with a polyclonal antibody raised against an epitope N-terminal to the first C2 domain of synaptotagmin 1 did not coprecipitate SV2B (data not shown), indicating that this area near the transmembrane region of synaptotagmin 1 may also play a role in association with SV2B. These results suggest that several sites of synaptotagmin 1 may form an interface that associates with SV2B.

Interaction between SV2A and synaptotagmin 1 was not detected in immunoprecipitations using antibodies against synaptotagmin 1. Higher affinity for synaptotagmin 1 and competition with SV2A for synaptotagmin 1 binding may explain why SV2B was the only isoform that strongly coprecipitated with synaptotagmin 1. If this were the case, then absence of SV2B could facilitate binding of other SV2 isoforms to synaptotagmin 1. To address this possibility, immunoprecipitations were performed from SV2B knock-out (−/−) and wild type (+/+ ) brain extract using the antibody against a cytoplasmic portion of synaptotagmin 1 (Fig. 3). SV2A coprecipitation with an antibody against synaptotagmin 1 (IP Stg/CL41.1) was not detected in SV2B knock-outs. These data indicate that SV2B does not compete with SV2A for binding to synaptotagmin 1. Protein levels of SV2A were similar in knock-out and wild type inputs, indicating that levels of SV2A were not different in knock-out compared with wild type mice. Strong immunoreactivity of the polyclonal antibody against SV2B to a high molecular weight protein indicates SV2B was present in wild type but not knock-out input. SV2B antibody immunoreactivity to knock-out input (Fig. 3, asterisks) is caused by nonspecific cross-reactivity of the antiserum to an unrelated protein (probably keratin). Levels of synaptotagmin 1 (Stg), synaptophysin (Sphy), and synaptogyrin 1 (Sgyr) were similar in wild type and knock-out inputs, indicating that levels of other synaptic vesicle proteins were not affected by SV2B knock-out.

The Interaction between SV2B and Synaptotagmin 1 Is Ca2⁺-independent—Studies using GST fusion proteins have shown that interaction between the C2B domain of synaptotagmin 1 and SV2A is inhibited by increased Ca2⁺ concentrations (EC₅₀ = ~10 μM) (15). To determine whether the SV2B-synaptotagmin 1 association is Ca2⁺-dependent, immunoprecipitations were performed from brain extract using an antibody against synaptotagmin 1 in the presence of 2 mM EGTA or 150 μM CaCl₂ (Fig. 4). SV2B was efficiently precipitated with the antibody to synaptotagmin 1 under both EGTA and Ca2⁺ conditions (IP Stg/CL41.1). SV2A was not detected in the precipitate under either condition, indicating that possible undetected association with synaptotagmin 1 does not depend upon Ca2⁺ levels. These data indicate that SV2B and synaptotagmin 1 bind in a Ca2⁺-insensitive manner. Binding to the immunoprecipitation antibody could have affected potential Ca2⁺ sensitivity of SV2B interaction with synaptotagmin 1. To address this possibility, immunoprecipitations were performed from total brain extract with polyclonal antibodies against SV2A, SV2B, or SV2C (Fig. 5). Precipitates were probed with a pan-SV2 antibody (20). Immunoreactivity of the pan-SV2 antibody against proteins of distinct molecular weights (M_r SV2A > M_r SV2B) verifies the
isoform specificity of the antibodies used for the immunoprecipitations. Probe of the Western blot with an antibody against synaptotagmin 1 shows that synaptotagmin 1 specifically co-precipitated with the antibody against SV2B, but not with antibodies against SV2A or SV2C. In addition, the precipitation of synaptotagmin 1 was not Ca\textsuperscript{2+}-sensitive. Together, these immunoprecipitation experiments show that SV2B interaction with synaptotagmin 1 is insensitive to Ca\textsuperscript{2+} levels.

**The SV2B-Synaptotagmin 1 1 Complex Associates with t-SNARE—**The presynaptic plasma membrane t-SNARE proteins, syntaxin 1 and SNAP-25, and the synaptic vesicle protein synaptobrevin/VAMP2 form a very stable core complex essential to synaptic vesicle fusion with the plasma membrane (21). Synaptotagmin 1, an important Ca\textsuperscript{2+} sensor for synaptic vesicle exocytosis, has been implicated in regulation of the fusion reaction through interaction with these t-SNARE proteins (22–27). To determine whether the SV2B-synaptotagmin 1 complex can bind to the t-SNARE protein syntaxin 1, we performed affinity chromatography using brain extract. The cytoplasmic portion of syntaxin 1A was fused to GST and immobilized with glutathione beads (clone Syn1A11 (24)). Native brain protein was incubated with these beads under 2 mM EGTA or 150 mM CaCl\textsubscript{2} conditions. Bead-bound protein was analyzed by Western blot using antibodies against synaptotagmin 1, SV2A, and SV2B (Fig. 6, top). Similar to published data (24, 25, 28), synaptotagmin 1 bound the GST-fused cytoplasmic domain of syntaxin 1A in elevated Ca\textsuperscript{2+}, but not in EGTA. SV2B was not detected in the precipitate that contained synaptotagmin 1, indicating that synaptotagmin 1 does not simultaneously complex with SV2B and the cytoplasmic part of syntaxin 1A. Probe with an antibody against GST (Fig. 6, bottom) indicates that an equivalent amount of GST-syntaxin was used for precipitations under EGTA and calcium conditions. Asterisks indicate nonspecific binding of the GST antibody and immunoreactivity to recombinant breakdown products.
immunoprecipitate under both EGTA and Ca\(^{2+}\) conditions, which indicates syntaxin 1 associates with the SV2B-synaptotagmin 1 complex. In contrast to the experiments with GST-syntaxin 1A, immunoprecipitation with an antibody against synaptotagmin 1 (Fig. 7, bottom) shows that there was not a significant increase in synaptotagmin 1 association with syntaxin 1 in 150 μM CaCl\(_2\) compared with 2 mM EGTA. These data are consistent with published data that found native synaptotagmin 1 and syntaxin 1 associate in a Ca\(^{2+}\)-independent complex (29, 30). In contrast to the affinity chromatography experiments, these experiments with native brain proteins show that synaptotagmin 1 associates simultaneously with both SV2B and syntaxin 1. Most likely, a difference in conformation between the GST-fused recombinant syntaxin 1 and native syntaxin 1 explains this discrepancy. Synaptotagmin 1 association with GST-fused syntaxin 1 may have blocked SVB interaction with synaptotagmin 1.

One possible function of SV2B in exocytosis may be regulation of the efficiency of synaptotagmin 1 association with t-SNARE proteins. To examine this possibility, immunoprecipitations were performed with a monoclonal antibody against synaptotagmin 1 in extracts prepared from SV2B knock-outs. Protein in the precipitate was analyzed with Western blotting (Fig. 8). The relative amounts of syntaxin 1 and SNAP-25 that coprecipitated with the antibody against synaptotagmin 1 were not significantly different from wild type. These results show that SV2B does not regulate the efficiency of complex formation between synaptotagmin 1 and the t-SNARE proteins. However, these experiments do not eliminate the possibility that SV2B could regulate some functional aspect of synaptotagmin 1 association with the t-SNAREs.

To study the biochemical properties of the SV2B and t-SNARE association with synaptotagmin 1, immunoprecipitations were performed from brain extract with an antibody against synaptotagmin 1, with the precipitate washed with buffers of varying ionic strength (Fig. 9). The level of bound SV2B decreased when the bead-bound precipitate (IP Stg1/CL41.1) was washed with 1 M NaCl, and SV2B was not detected in the precipitate treated with a 1.5-M NaCl wash. Syntaxin 1 and SNAP-25 levels in the same precipitate were decreased by a 1 M NaCl wash, and levels of these proteins were below detection in samples washed with 1.5 M NaCl. Synaptotagmin 1 association with SV2B and t-SNAREs was disrupted by similar salt molarities, consistent with previous studies showing that native synaptotagmin 1 association with t-SNAREs was disrupted in 0.6 M NaCl (29). Qualitative comparison of the relative amounts of SV2B precipitated and the amount of SV2B in the supernatant reveals that the majority of SV2B in brain extract was bound to synaptotagmin 1. In contrast, a small fraction of total syntaxin 1 was bound to synaptotagmin 1.

The SV2B-Synaptotagmin 1 Interaction Does Not Require Other Synaptic Proteins—SV2B and synaptotagmin 1 may bind each other either directly or indirectly via a bridging protein. AP2 has been shown to bind to synaptotagmin 1 (31), and a peptide derived from the N terminus of SV2A has been shown to enhance the association between AP2 and synaptotagmin 1 (32). Although this peptide sequence is not conserved in the SV2B sequence, SV2B may indirectly associate with synaptotagmin 1 via an adaptor such as AP2. To characterize the protein requirements for synaptotagmin 1 association with SV2B, brain extract was enriched for synaptic vesicles (LP2) via differential centrifugation and peripheral membrane proteins were removed by washing with alkaline carbonate buffer. Immunoprecipitations from the LP2 fraction were performed with an antibody against SV2B, and precipitates were analyzed on a Western blot (Fig. 10, top). Synaptotagmin 1 coprecipitated with the antibody against SV2B from synaptic vesicles prepared under alkaline conditions, demonstrating that high

![Figure 8](http://www.jbc.org/)
FIG. 9. SV2B and t-SNAREs are removed from synaptotagmin 1 with washes of similar ionic strength. Immunoprecipitations were performed in total brain extract using a monoclonal antibody against synaptotagmin 1 (IP Stg1/CL41.1), and precipitates were washed with buffers containing increasing concentrations of NaCl. The remaining immunoprecipitate was probed on a Western blot using antibodies against SV2B, syntaxin 1 (Stx), and SNAP-25. SV2B, syntaxin 1, and SNAP-25 were removed from the synaptotagmin 1 immunoprecipitate with 1.5 M NaCl. Levels of precipitated synaptotagmin 1 were not affected by the salt washes (data not shown).

FIG. 10. Native SV2B and synaptotagmin 1 do not require a peripheral protein for interaction. Top, brain extract was enriched for synaptic vesicles (LP2) via differential centrifugation and washed with 100 mM carbonate buffer (pH 11.3) (+) or normal buffer (20 mM HEPES, pH 7.4) (−). Immunoprecipitations with an antibody against SV2B were subsequently performed from extracts prepared from the washed LP2 fractions. High pH removal of peripheral membrane proteins (+) does not block synaptotagmin 1 immunoprecipitation with an antibody against SV2B. Bottom, removal of synapsin 1 indicates that alkaline treatment was effective. Supernatants from the immunoprecipitations were probed on a Western blot with synapsin antibodies. Synapsin 1 was not present in the immunoprecipitation supernatant from the carbonate-treated LP2 fraction.

pH removal of peripheral proteins did not eliminate the association of synaptotagmin 1 with SV2B. The amount of SV2B precipitated, and the proportion of synaptotagmin 1 protein levels compared with SV2B levels in the precipitate, were similar to control conditions, showing that high pH treatment did not affect immunoprecipitation efficiency. These results demonstrate that SV2B and synaptotagmin 1 do not require a peripheral protein for association. To determine the efficiency of peripheral protein removal, supernatants of the immunoprecipitations were probed for the presence of the peripheral vesicle-associated protein synapsin 1 (Fig. 10, bottom). Synapsin 1 levels were strongly reduced in carbonate-treated immunoprecipitation supernatants (+) compared with control supernatants (−), indicating that peripherally associated proteins were efficiently removed from the synaptic vesicles.

Although these experiments suggest that peripheral proteins are not required for the interaction between SV2B and synaptotagmin 1, it is possible that the interaction is mediated by another integral vesicle protein. To test whether the two proteins interact directly, we expressed recombinant full-length SV2B and synaptotagmin 1 in HEK 293 cells. Immunoprecipitations with an antibody against SV2B were performed from cotransfected cell extract (Fig. 11). Precipitates were tested for the presence of synaptotagmin 1 and SV2 on a Western blot. Synaptotagmin 1 was efficiently precipitated with the antibody against SV2B. These data demonstrate that SV2B and synaptotagmin 1 can interact directly and do not require other presynaptic proteins for this association.

Synaptotagmin 1 Levels Are Reduced in Synapses Lacking SV2B—Our recent studies found that the ribbon synapses of rod photoreceptor cells and of rod bipolar neurons in the retina contain only the SV2B isoform (7). To determine the effect of SV2B knock out on synaptotagmin 1 protein levels at these unique synapses, we analyzed synaptotagmin 1 immunoreactivity in retinae from SV2B knock-outs and wild type littermate controls (Fig. 12A). Staining of wild type retinae with antibodies against synaptotagmin 1 reveals a strong signal in the outer plexiform layer (OPL) (Fig. 12A, top panels). This signal corresponds mainly to rod photoreceptor synaptic terminals because the majority (>97%) of photoreceptor cells in the mouse retina are rods (33, 34). In contrast, synaptotagmin 1 immunoreactivity in the OPL of retinae from SV2B knock-out animals is strongly reduced. Similar results were obtained with several different synaptotagmin 1 antibodies directed against different epitopes. Some isolated synaptic terminals in the OPL of the retina from SV2B knock-out mice still show strong synaptotagmin 1 immunoreactivity. These correspond to cone photoreceptor terminals that contain the SV2A isoform as demonstrated by double labeling of retina sections from SV2B knock-out mice using antibodies against synaptotagmin 1 and SV2A (Fig. 12B). Similarly, retinae from SV2B knock-out mice show normal levels of synaptotagmin 1 immunoreactivity in the inner plexiform layer, corresponding to conventional synapses that contain SV2A. It is not possible to clearly evaluate the synaptotagmin 1 signal derived from the rod bipolar cells because it overlaps with the intense labeling of conventional synapses in the inner plexiform layer. Staining of adjacent retina sections
with SV2B. In our experiments we could not detect an interaction between native SV2A and synaptotagmin 1. However, SV2A association with synaptotagmin 1 may not have been detected due to constraints of the procedure. The isolated N terminus of SV2A, 59 residues longer than that of SV2B, has been shown to bind synaptotagmin 1 (15). The longer N terminus of native SV2A may block the epitope recognized by the synaptotagmin 1 antibody used for the immunoprecipitation in our experiments and thus produce negative results. Alternatively, association with synaptotagmin 1 may block the N-terminal epitope recognized by the SV2A antibody used for immunoprecipitation.

Interaction between synaptotagmin 1 and the t-SNAREs has been shown to be important for Ca\(^{2+}\)-triggered exocytosis in neurons and neuroendocrine cells (22–27). Immunoprecipitations showed that native syntaxin 1 and SNAP-25 associate with SV2B and synaptotagmin 1. The interaction between synaptotagmin 1 and the t-SNAREs was not affected by SV2B knock out, indicating that SV2B does not regulate this association. However, SV2B could regulate the conformation or other aspects of the synaptotagmin 1-t-SNARE complex.

Analysis of the distribution of presynaptic proteins in SV2B knock-out retinae revealed strongly reduced synaptotagmin 1 immunoreactivity in the outer plexiform layer, the synaptic layer that contains rod photoreceptor terminals. Similar results were obtained using three different synaptotagmin 1 antibodies, indicating that the reduction in synaptotagmin 1 immunoreactivity in knock-out terminals was not likely caused by block or modification of an epitope. The reduction of synaptotagmin 1 immunoreactivity in the SV2B knock-out was most likely due to a specific reduction in synaptotagmin 1 protein levels. Deletion of SV2B may have affected synaptotagmin 1 gene transcription or translation, but synaptic vesicle proteins have thus far not been shown to participate in those processes. The direct interaction of SV2B with synaptotagmin 1 that we characterized in brain extract suggests the SV2B knock-out phenotype reflects a post-translational effect of SV2B on synaptotagmin 1 protein levels.

Reduction in synaptotagmin 1 immunoreactivity in neumo-muscular synapses from Drosophila has previously been shown in “stoned” mutants (35, 36). Mislocalization of synaptotagmin 1 and a strong reduction of synaptotagmin 1 protein levels was shown to be due to defective endocytosis of synaptotagmin 1. Levels of other presynaptic proteins were not affected in the stoned mutant synapses, suggesting a unique recycling pathway for synaptotagmin 1. Here we have shown that SV2B knock-out mice have a specific reduction of synaptotagmin 1 immunoreactivity in synaptic terminals that normally express only the SV2B isoform. The similar reduction of synaptotagmin 1 protein levels in stoned mutants and SV2B knock-outs suggests that absence of SV2B may result in aberrant trafficking and subsequent degradation of synaptotagmin 1. Our data suggest that SV2B may be required for proper trafficking of synaptotagmin 1 at the rod photoreceptor synapse.

Studies have demonstrated that synaptotagmin 1 is trafficked separately from other integral synaptic vesicle proteins. In non-neuronal cells, recombinant synaptotagmin 1 is transported to the plasma membrane but not internalized. In contrast, other integral synaptic vesicle proteins, such as synaptophysin, were internalized (37). The localization of synaptotagmin 1 indicated that the plasma membrane is the default target for synaptotagmin 1 in non-neuronal cells. In contrast, synaptotagmin 1 is endocytosed from the plasma membrane into small synaptic vesicles in neurons or into small vesicle-like structures in neuroendocrine cells (38, 39). The reduction in levels of synaptotagmin 1 protein in rod photoreceptor synapses in
SV2B knock-out mice suggests SV2B may participate in the endocytosis of synaptotagmin 1. SV2B knock out may disrupt retrieval of synaptotagmin 1 from the plasma membrane, resulting in mislocalization of synaptotagmin 1 at the plasma membrane and subsequent degradation.

Previous studies in 2-week-old SV2A knock-outs and newborn SV2A/B double knock-outs have shown that protein levels of synaptotagmin 1 in the brain are not affected by SV2 knock out (8, 9). These data suggest that SV2A and SV2B do not regulate synaptotagmin 1 protein levels in central synapses. The specific effect of SV2B knock out on synaptotagmin 1 protein levels in rod photoreceptors, which are tonically active primary sensory neurons, suggests that rate of synaptic activity may play a role in manifestation of the phenotype. Disruption of efficient endocytosis of synaptotagmin 1 in conventional synapses may result in the mislocalization of a small proportion of synaptotagmin 1 to the plasma membrane. However, disruption of the efficiency of synaptotagmin 1 endocytosis in synapses that have rapid vesicle cycling may result in the rapid accumulation of a large proportion of synaptotagmin 1 at the plasma membrane and subsequent degradation of the mislocalized protein.

Alternatively, reduction of synaptotagmin 1 protein levels in photoreceptor synapses, but not in conventional synapses of SV2 knock outs, may be explained by a difference in synaptic vesicle endocytosis. Recent studies have demonstrated that synaptic vesicle trafficking in photoreceptor terminals differs from that of conventional synapses or other types of ribbon synapses (40, 41). Rapid endocytosis of synaptic vesicles in photoreceptor terminals was shown to differ from the fast “kiss-and-run” endocytosis in conventional synapses and from bulk endocytosis used in ribbon synapses of bipolar neurons (42–44). Different forms of endocytosis in the different cell types may explain why the SV2B knock-out phenotype manifests specifically in photoreceptor terminals. SV2B may act as a cofactor for efficient synaptotagmin 1 recycling in the type of endocytosis that occurs in photoreceptor terminals, whereas other forms of endocytosis in conventional synapses may be less dependent upon SV2B for synaptotagmin recycling.

A recent study (40) revealed that photoreceptor terminals have a very small vesicular reserve pool (<15%) that does not participate in the classical synaptic vesicle cycle (45, 46), in contrast to classical synapses where the majority of vesicles are in the reserve pool. The large proportion of vesicles in the reserve pool of classical synapses suggests that the majority of synaptotagmin 1 is retained in the vesicular reserve pool; thus, a reduction in the efficiency of synaptotagmin 1 endocytosis would not significantly affect levels of synaptotagmin 1 at these synapses because the majority of synaptotagmin 1 does not participate in vesicle cycling.

Previous studies have proposed a role for SV2 as a vesicular transporter. Our studies suggest a new role for SV2B in the regulation of synaptotagmin 1 stability or trafficking in certain synapses. Future studies will analyze the role of SV2B in regulation of synaptotagmin 1 trafficking and stability in different synapses.

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SV2B Regulates Synaptotagmin 1 by Direct Interaction
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