SV2 regulates neurotransmitter release via multiple mechanisms

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SV2 regulates neurotransmitter release via multiple mechanisms. Am J Physiol Cell Physiol 299: C960–C967, 2010. First published August 11, 2010; doi:10.1152/ajpcell.00259.2010.—Among the proteins that mediate calcium-stimulated transmitter release, the synaptic vesicle protein 2 (SV2) stands out as a unique modulator specific to the neurons and endocrine cells of vertebrates. In synapses, SV2 regulates the expression and trafficking of the calcium sensor protein synaptotagmin, an action consistent with the reduced calcium-mediated exocytosis observed in neurons lacking SV2. Yet SV2 contains amino acid motifs consistent with it performing other actions that could regulate presynaptic functioning and that might underlie the mechanism of drug action. To test the role of these functional motifs, we performed a mutagenic analysis of SV2A and assessed the ability of mutant SV2A proteins to restore normal synaptic transmission in neurons from SV2A/B knockout mice. We report that SV2A-R231Q, harboring a mutation in a canonical transporter motif, restored normal synaptic depression (a measure of release probability and signature deficit of neurons lacking SV2). In contrast, normal synaptic depression was not restored by SV2A-W300A and SV2A-W666A, harboring mutations of conserved tryptophans in the 5th and 10th transmembrane domains. Although they did not rescue normal neurotransmission, SV2A-W300A and SV2A-W666A harboring mutations of conserved tryptophans in the 5th and 10th transmembrane domains. This indicates that tryptophans 300 and 666 support an essential action of SV2 that is unrelated to its role in synaptotagmin expression or trafficking. These results indicate that SV2 performs at least two actions at the synapse that contribute to neurotransmitter release.

THE CALCIUM-REGULATED SECRETION of neurotransmitters is a specialized form of membrane fusion that requires regulatory proteins that are unique to transmitter-containing vesicles. One of these is synaptic vesicle protein 2 (SV2), a membrane glycoprotein expressed exclusively in neurons and endocrine cells. SV2 is the binding site of a class of drugs typified by levetiracetam (5, 17, 26, 29, 31). Levetiracetam is a Food and Drug Administration-approved treatment for epilepsy (reviewed in Ref. 11) that also shows promise in the treatment of anxiety disorders (27, 28, 47), pain (12, 13, 36), dyskinesias (7, 32, 40, 43, 48), and posttraumatic stress disorder (28). Thus SV2 represents a vesicle protein whose action is likely to play an important (and targetable) regulatory action at synapses.

SV2 is essential for normal neurotransmission. Neurons lacking SV2A and SV2B demonstrate reduced neurotransmission and reduced synaptic depression (8–10, 22, 41, 44). These phenotypes reflect reduced release probability due to impaired ability of vesicles to fuse in response to elevated cytoplasmic calcium. This effect occurs after vesicle docking (10) and before formation of the SNAP complex (soluble N-ethylmaleimidesensitive fusion) complex (44), suggesting that SV2 contributes to the priming of vesicles for release. Indeed, the releasable pool of vesicles is decreased in cells cultured from SV2 knockout (KO) mice (8, 10, 41, 44). SV2’s action appears linked to calcium-dependent processes: in hippocampal neurons, the SV2 KO phenotype can be transiently rescued by increased calcium influx (10), whereas, in retinal bipolar neurons, decreased elevated resting cytoplasmic calcium restores wild-type neurotransmission (41).

Loss of SV2 results in a significant decrease in the amount of the calcium sensor synaptotagmin in vesicles (46). The decrease is due to two effects of SV2, an effect on synaptotagmin expression (30, 33, 46) and an effect on synaptotagmin internalization from the plasma membrane (46). SV2’s effect on synaptotagmin internalization depends on tyrosine-based endocytosis motifs in SV2 that are predicted to serve as binding sites for the clathrin adaptor AP2. Mutation of the first endocytosis motif in SV2A (SV2A-Y46A) produces a protein that does not restore normal neurotransmission or synaptotagmin internalization in neurons cultured from SV2A/B KOs. Thus SV2’s role in endocytosis is essential to its function.

Although reduced synaptotagmin in vesicles from SV2 KO mice is consistent with reduced calcium-evoked secretion, the neurotransmission phenotype of neurons lacking SV2 is not identical to that of neurons lacking synaptotagmin (15, 16, 34). This suggests that SV2 may perform additional functions. In considering SV2’s action at the synapse, most researchers have focused on its structural similarity to transporters (3a, 14, 18) and its matrix-like glycosyl moieties (39). This focus has led to the hypotheses that SV2 is a transporter (22) or provides a luminal matrix that concentrates neurotransmitter in the vesicle lumen (37). To test these potential actions, we generated mutations in SV2A at residues predicted to underlie these functions and assessed their ability to rescue release probability in neurons cultured from SV2A/B KO mice.

METHODS

KO mice and neuronal cultures. Primary neuronal cultures from SV2A−/−SV2B−/− double KO mice were generated as previously described (10). The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Washington.

Lentiviral constructs. Lentiviral constructs were made to encode the SV2A protein, as well as SV2A mutations: R231Q, W330A, and W666A. SV2A mutations were made in pIRES2-EGFP (internal ribosomal entry sequence 2-enhanced green fluorescent protein), using a QuikChange PCR strategy (Stratagene). The IRES sequence was removed to produce SV2A fused to EGFP, which would later act as a real-time visual reporter of infection. Constructs were sequenced and subcloned into the Lentiviral transfer vector pRRL-pPT-CMV-X-PRE-SIN, which was graciously donated by Z. Xia, and is de-
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results in neurons from SV2A/B double KO mice is due to secondary or developmental effects, we examined the effect of acute expression of SV2 in autaptic hippocampal neurons cultured from SV2A/B double KOs using Semliki Forest virus-mediated expression. The expression constructs generated included cDNA encoding EGFP, separated from the SV2A cDNA by an IRES, which directed the expression of EGFP as a separate protein. This allowed identification of infected neurons. Neurons were assayed within 18 h of viral infection for synaptic depression, an indicator of release probability and the hallmark deficit of SV2 mutants. Exogenous expression of SV2A plus EGFP resulted in robust synaptic depression, indicating rescue of the SV2A/B double KO phenotype. In contrast, cells expressing EGFP alone exhibited the double KO phenotype, which consists of synaptic facilitation followed by reduced depression (Fig. 1). Thus acute expression of SV2 restores normal synaptic depression, indicating that both the phenotype and rescue are due to the absence or presence of SV2 and not to other, compensatory effects.

We also examined the effects of chronic expression of SV2 using a Lenti virus expression system. For these experiments, we utilized viral vectors encoding SV2-EGFP fusion proteins rather than bicistronic cassettes, as attempts to generate lentivirions containing SV2 and EGFP separated by an IRES sequence resulted in consistently poor infection rates. Moreover, they allowed us to easily monitor the trafficking of the expressed protein. Neurons were infected within 4 days of plating and assayed 10–12 days later. Expression of either SV2A-EGFP or SV2B-EGFP produced punctate localization of the fusion protein (Fig. 2A), consistent with a synaptic localization. To verify this, we compared the location of SV2-EGFP fusion proteins with that of the synaptic vesicle protein synaptophysin. In both cases, we observed colocalization (Fig. 2B), indicating that the fusion proteins were traf-

RESULTS

Both acute and chronic expression of SV2 restores normal neurotransmission to neurons from SV2A/B KO mice. As with any mutation that results in total loss of protein expression, disruption of the SV2 gene may produce compensatory changes that are the direct source of the phenotype observed. To determine whether the reduced release probability observed
ficked to synapses, and thus that the presence of EGFP on the carboxy terminus of SV2A does not disrupt its expression and localization in neurons. In contrast, an EGFP fusion of a related protein, SVOP (synaptic vesicle 2-related protein) (23), did not traffic to synapses.

Expression of SV2A-EGFP in neurons from SV2A/B KO mice resulted in synaptic depression similar to that seen in wild-type neurons (Fig. 3). This indicates that the addition of EGFP at the carboxy terminus of SV2A does not disrupt its function. In contrast, expression of SVOP-EGFP did not restore synaptic depression. We also assayed the ability of SV2B-EGFP to rescue synaptic depression. Of the three SV2 isoforms, SV2B is the most divergent (3, 23). It is almost universally coexpressed with SV2A and displays pronounced changes in expression during development (2, 33). In addition, SV2B lacks a synaptotagmin binding site that is present at the amino terminus of SV2A and SV2C (38). Together, these observations suggested that SV2B acts differently than other SV2 isoforms. We found, however, that SV2B-EGFP restored synaptic depression as efficiently as SV2A. This indicates that SV2B can provide full SV2 action at the synapse. It also indicates that the synaptotagmin-binding domain in the amino terminus of SV2A/C is not crucial to SV2’s effects on synaptic transmission. Therefore, the more severe phenotype in SV2A mutants likely reflects its higher expression levels in the central nervous system.

Testing hypotheses of SV2 action by mutational analysis. In addition to its role in the trafficking of synaptotagmin, SV2 has been proposed to function as a transporter and to serve as the anchor of a vesicular glycosyl matrix. To test these hypotheses, we generated a series of SV2 mutants, targeting residues predicted to support each of these proposed actions (Table 1).

To disrupt SV2’s putative transport function, we mutated residues shown to be essential to the transport activity of other major facilitator (MF) proteins. MF transporters contain a signature sequence, DXXGRR/K, in the cytoplasmic loop between transmembrane domains 2 and 3. Within this motif, the aspartate and first arginine are reported to be crucial to transport activity in one or more MF transporters (24, 45). In SV2A, these residues are D227 and R231. Other residues reported to be essential to MF transporter action include aromatic residues, especially tryptophans, in the 10th transmembrane domain (25, 35). In SV2A W666, a tryptophan in the 10th transmembrane domain is conserved across SV2 isoforms. We also targeted a conserved tryptophan in the fifth transmembrane domain, W300, which is the analog of W666 in the first half of the protein and is also conserved across isoforms. Since charged residues in membrane domains often contribute to transport activity, we also mutated conserved charged and polar residues predicted to be in transmembrane domains. These included two acidic residues predicted to be in the first transmembrane domain, D179 and E182, a basic residue in transmembrane domain 11, K694.

To test the hypothesis that the sugar side chains of SV2 constitute a matrix that concentrates neurotransmitter (37), we mutated the three consensus sites for N-linked glycosylation in the predicted loop between membrane domains 7 and 8. Finally, we examined the sequences of SV2A, SV2B, and SV2C for conserved residues. Based on this analysis, we mutated a highly conserved glycine in the fifth transmembrane domain, G303.

All mutant proteins were expressed in cultured hippocampal neurons at significant levels; however, only three of them were localized to synapses, with all others demonstrating diffuse localization concentrated in the cell body (Table 1). This precluded our testing the requirement for several residues implicated in transporter function and SV2’s potential role as
the source of the vesicular matrix. There was no correlation between the location of a mutation and aberrant trafficking. Mutation of residues in transmembrane domains and residues predicted to be in cytoplasmic loops both produced proteins that trafficked and proteins that did not. One of the mutants, D227N (also D227A), deserves special mention because it was identified in analyses of bacterial MF transporters to be universally essential to function (24). Our results suggest that it may play a crucial role in protein folding. Two chemical chaperones, glycerol (1.25 M) and trimethylamine N-oxide (100 mM), which rescue trafficking of cystic fibrosis transmembrane conductance regulator mutants (6), did not improve the trafficking of the D227N mutant when added to the medium at the same time as the virions (data not shown). Therefore,

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Type</th>
<th>Synaptic Location</th>
<th>Rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td>D179N E182Q</td>
<td>Acidic residues in TM1 hypothesized to contribute to cation transport</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>D227N</td>
<td>Acidic residue in loop between TM domains 2-3 that is essential to action of many MF transporters</td>
<td>No</td>
<td></td>
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<tr>
<td>D227A</td>
<td>Acidic residue in loop between TM domains 2-3 that is essential to action of many MF transporters</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>R31Q</td>
<td>Basic residue in loop between TM domains 2-3 that is essential to action of a subset of MF transporters</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>W300A</td>
<td>Hydrophobic residue conserved in TM domain 5 of all SV2 s and in SVOP</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>G303A</td>
<td>Glycine in TM domain 5 conserved in all SV2s, SVOP, and some MFS transporters</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>W666A</td>
<td>Transmembrane residue conserved in all SV2 and in SVOP isoforms</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>K694A</td>
<td>Basic residue in TM domain 11 conserved in all SV2 isoforms and SVOP (the corresponding residue is acidic residue in a related cation transporter)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>N498D N548D N573D</td>
<td>Sites of N-linked glycosylation, predicted to anchor vesicular matrix. These mutants were generated singly and in combination. None trafficked to synapses</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>
arginine 231, a canonical residue in the MF transporter signature motif, is not required for SV2 function. Mutation of either the aspartate (D) or the first arginine (R) in the MF signature sequence DXXGRR/K is associated with loss of}

SV2 folding and/or trafficking appears to be very sensitive to changes in protein composition in a manner that cannot be rescued with chemical chaperones that support proper folding of cystic fibrosis transmembrane conductance regulator.

Fig. 5. SV2A-R231Q, but not W300A or W666A, restores synaptic depression in neurons from SV2 double KO mice. Recordings are from autaptic hippocampal neurons cultured from SV2A/B double KO mice expressing the indicated SV2A-EGFP fusion protein. EPSC amplitudes in response to a 10-Hz stimulus train were normalized to the amplitude of the first response. Average normalized values are graphed with error bars representing the SE. The number of cells recorded from (nos. in parentheses) is shown for each protein. Expression of SV2A-R231Q restored synaptic depression of the same magnitude as observed when WT SV2A was expressed. Neurons expressing either W300A or W666A showed synaptic facilitation, indicative of a reduced release probability.

Fig. 6. SV2A-R231Q, W300A, and W666A restore expression of synaptotagmin 1. A, top: a representative Western blot analysis of conventional cultures of hippocampal neurons expressing the indicated SV2A-EGFP fusion construct or EGFP alone. Blots were probed for SV2A (left) and synaptotagmin 1 (right). Anti-actin labeling was used as loading control. Band net intensity was normalized to the net intensity of actin in the same lane. Bottom: average values normalized to WT in the same blot. The graphs represent data from four to seven independent experiments. Error bars represent SE. All three mutants restored synaptotagmin 1 expression levels to those seen in neurons expressing WT SV2A. B: comparison of synaptotagmin 1 levels at synapses expressing EGFP, SV2A, SV2A-R231Q, SV2AW300A, or SV2A-W666A. Synapses, detected with an anti-synaptophysin antibody, were labeled with a polyclonal antibody directed against synaptophysin 1. Normalized fluorescent labeling intensity was normalized to WT within each experiment. The table lists the normalized averages from three independent cultures. Data were assessed for significant differences by one-way ANOVA. N indicates the total number of images analyzed.
transport activity in sugar and antibiotic transporters (19, 24, 45). Thus we targeted these residues in SV2A to test the hypothesis that SV2 is a transporter. Of the mutants we generated, only the R231Q mutant was properly trafficked to synapses (Fig. 4). Neither of two aspartate 227 mutants was found at synapses (Table 1). Expression of SV2A-R231Q restored synaptic depression to autaptic hippocampal neurons from SV2A/B KO mice. The level of synaptic depression was the same as seen when wild-type SV2A was expressed (Fig. 5). Thus this residue is not crucial to SV2’s action at the synapse.

Tryptophans in the 5th and 10th transmembrane domains are essential to SV2 function. The SV2s contain tryptophan residues in the 5th and 10th transmembrane domains that are conserved across isoforms. Hydrophobic residues in these membrane domains are crucial to the action of the glucose transporter GLUT-1 (25) and to human organic anion transporter activity (35), where they are proposed to line the pore of the transport channel. When expressed in neurons cultured from SV2A/B KO mice, both SV2A-W300A and SV2A W666A trafficked to synaptic terminals (Fig. 4). Neurons expressing SV2A-W300A demonstrated reduced synaptic depression (Fig. 5), suggesting that W300 contributes to SV2 action. An even greater effect was seen when W666 was mutated. Neurons expressing SV2A-W666A had a more severe phenotype than neurons expressing EGFP, indicating that this residue is essential for SV2-mediated modulation of synaptic transmission.

Mutation of tryptophans 300 or 666 does not affect synaptic tagging expression or trafficking. The crucial role of SV2 in maintaining the vesicular content synaptotagmin can explain the reduced calcium-stimulated exocytosis in SV2 KO. Thus the question arises whether this is the only action of SV2. If so, we would expect that the W300A or W666A SV2A mutants would not rescue synaptotagmin expression and/or trafficking. To test this, we compared expression levels and internalization of SV2 and synaptotagmin in neurons expressing SV2A-WT, SV2A-R231Q, SV2A-W300A, and SV2A-W666A. The expression of SV2 and synaptotagmin was measured by Western blot analyses of hippocampal neurons grown in conventional cultures (Fig. 6A). The results revealed that the SV2A mutants were expressed at ~80%, the level of wild-type SV2A, although the difference was not significant (P > 0.05, one-way ANOVA). Synaptotagmin I levels were not decreased in neurons expressing either wild-type or mutant SV2A. In cultures expressing EGFP, however, synaptotagmin was significantly decreased (P < 0.05).

Because Western blots measure total protein expression in both neurons and astrocytes, we also measured synaptotagmin levels at synapses using immunocytochemistry. Synapses were identified with an antibody against the vesicle protein synaptophysin (Fig. 6B), and the intensity of anti-synaptotagmin labeling quantified in those regions. When assessed in this way, all SV2A constructs significantly increased synaptotagmin 1 expression compared with neurons expressing just EGFP (P < 0.00001, one-way ANOVA). Synaptotagmin expression in neurons expressing mutant SV2As had ~20% less synaptotagmin at synapses than neurons expressing wild-type SV2A, a difference that was significant (P < 0.01). There was, however, no difference in the amount of synaptotagmin expressed in mutants that did rescue synaptic depression (SV2A-R231Q) compared with those that did not (W300A and W666A). Thus the ~20% decrease in synaptotagmin cannot account for the failure of SV2A-W300A and SV2A-W666A to rescue synaptic depression. Thus we have identified mutations that fail to rescue synaptic release probability that do not alter synaptotagmin expression or turnover.

To determine whether W300 and W666 play a role in SV2 or synaptotagmin trafficking, we assessed protein internalization by measuring the proportion of total protein that was biotinylated after treatment with surface biotinylating reagent. As reported previously (46), neurons expressing EGFP had significantly more surface synaptotagmin. Expression of all SV2s, both wild-type and mutant, resulted in a significant decrease in the proportion of biotinylated synaptotagmin. Expression of all SV2s, both wild-type and mutant, resulted in a significant decrease in the proportion of biotinylated synaptotagmin (Fig. 7). Therefore, the two SV2A mutants that did not restore synaptic depression did restore internalization of synaptotagmin. These findings are consistent with W300 and W666 contributing to an action other than regulation of vesicle synaptotagmin levels.

**DISCUSSION**

Of the synapse-specific proteins, SV2 is unique in having no clear homolog in invertebrates. It is also, at present, the only vesicle protein known to be a drug target. Because of these features, understanding SV2’s contribution to the unique features of synaptic transmission is of special importance to developing therapies targeted at synaptic functioning. The data presented here indicate that SV2 is likely to perform at least two actions at the synapse, both of which directly impact synaptic release probability.
Previously published studies of mice lacking SV2 revealed that it is essential for normal levels of calcium-stimulated neurotransmission. A potential confound in interpreting analyses of mouse mutants is the possibility that phenotypic changes are due to indirect developmental effects or compensatory changes. Our finding that acute, Semiliki Forest virus-mediated expression of SV2 rescues synaptic depression in neurons lacking SV2 indicates that the KO phenotype reflects the loss of SV2, and not ancillary changes that occur in its absence. Thus SV2 performs an action that contributes directly to synaptic release probability.

In previously published work, we showed that SV2 interacts with clathrin adaptor proteins and regulates the expression and trafficking of the calcium sensor protein synaptotagmin. Significantly reduced vesicular synaptotagmin is consistent with the reduced ability of calcium to trigger transmitter release. Thus it was possible that this constituted the sole action of SV2. In these studies, we undertook further mutational analysis of SV2 to test other proposed actions, specifically to test the hypotheses that SV2 acts as a transporter or the scaffold for a vesicular matrix. Mutations of residues implicated in these functions were made in SV2A, and the ability of the mutant to rescue synaptic depression in neurons from SV2A/B KOgs tested. We found that the majority of single amino acid changes we made disrupted SV2 trafficking to synapses. Mutation of any of the glycosylation consensus sites in SV2’s large luminal domain resulted in a protein that did not traffic to synapses, indicating that glycosylation plays an essential role in SV2 trafficking. SV2’s sensitivity to other single amino acid substitutions suggests that precise folding or protein interactions are also required for SV2’s exit from the endoplasmic reticulum/Golgi. Of particular interest is our finding that mutation of aspartate 227 results in a protein that is not trafficked to synapses. This residue is part of the major facilitator transporter motif in SV2 and has been proposed to be the essential residue in this motif (19). Our findings suggest that a primary function of this residue may be establishment of protein topology in the membrane. Similarly, acidic residues in the first transmembrane domain that were hypothesized to support SV2 transport of cations (23) appear to be essential for SV2 folding, as mutation of them resulted in a protein that appeared trapped in the endoplasmic reticulum.

Yet despite the sensitivity to single amino acid changes, attachment of EGFP to SV2’s carboxy terminus did not block its trafficking or function at the synapse. We note, however, that addition of EGFP does affect trafficking of some mutants. One of the mutants we generated (K694A) did not traffic when expressed with EGFP at the carboxy terminus (Table 1), but was properly trafficked and rescued normal neurotransmission, when expressed as a fusion protein with EGFP at the amino terminus (8).

A major finding of this work is that typtophans in membrane domains 5 and 10 are essential to SV2 action, but not for the expression or trafficking of synaptotagmin. Neither synaptotagmin expression nor internalization was affected in neurons expressing SV2A-W300A and SV2A-666A. Yet neither of these mutant SV2 proteins rescued normal synaptic depression. Thus SV2 appears to have at least two actions in the synapse. The finding that disruption of a canonical MF transporter motif in SV2 does not impair its ability to support neurotransmission suggests that it does not act as a transporter. On the other hand, two conserved tryptophans that contribute to transport activity in some MF transporters are required for SV2’s ability to function properly in the synapse. Given the variable effects of mutating residues implicated in transporter function, it still remains unclear whether SV2 acts as a transporter. If it does, W300 and W666 are likely to be crucial to transport activity.

Because SV2A is the binding site of a promising class of new drugs, determining how it acts and how it is regulated at the synapse will have important implications for further drug development. Both decreasing (22, 42) and increasing (10) cytoplasmic calcium can reverse the neurotransmission deficit in neurons from SV2 KO mice. Thus SV2 has been hypothesized to regulate cytoplasmic calcium (22, 42), or to stabilize a priming step produced by calcium (10). On the other hand, loss of SV2 results in reduced exocytosis in the absence of changes in cytoplasmic calcium (21, 44), consistent with an action that is independent of calcium. The mutations reported here provide the basis for testing these hypotheses of SV2 function as well as for future structural analyses of SV2 and drugs that modify its action.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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