Amisyn Regulates Exocytosis and Fusion Pore Stability by Both Syntaxin-dependent and Syntaxin-independent Mechanisms*

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Amisyn and tomosyn are related by the possession of a C-terminal vesicle-associated membrane-protein-like domain that allows them to bind to syntaxin 1 and assemble into SNARE complexes. The formation of inactive complexes may sequester syntaxin and allow amisyn and amisyin to act as inhibitors of exocytosis. We aimed to use adrenal chromaffin and PC12 cells to probe this possible mode of action of amisyn and tomosyn in dense core granule exocytosis. Although tomosyn is expressed by adrenal chromaffin and PC12 cells, amisyn expression could not be detected allowing examination of the effect of introduction of amisyn expression onto a neuronal-like background. Overexpression of m-tomosyn1 and expression of amisyn both inhibited Ca2+-induced exocytosis in transfected PC12 cells. Surprisingly, this inhibition was not removed when amisyn and tomosyn constructs were used in which key residues required for efficient binding to syntaxin1 were mutated. The effect of amisyn was further characterized using carbon fiber amperometry in chromaffin cells. Expression of amisyn had no effect on the basic characteristics of the amperometric spikes but reduced the number of spikes elicited. This inhibitory action on the extent of exocytosis was also seen with the amisyn mutant deficient in syntaxin1 binding. In addition, expression of amisyn resulted in an increase in the lifetime of the prespike foot, and this effect was abolished by the mutations. These results show that tomosyn and amisyn can negatively regulate exocytosis independently of syntaxin and also that amisyn can regulate the stability of the fusion pore.

Neurotransmitters are released by regulated exocytosis where calcium triggers the fusion of secretory vesicles with the plasma membrane following vesicle recruitment, docking, and priming (1, 2). It is accepted that a complex of the soluble NSF attachment protein receptor (SNARE), where NSF is N-ethylmaleimide-sensitive factor, proteins play a major role (3, 4). The neuronal SNARE complex (3, 5) contains syntaxin1A (6), SNAP-25 (synaptosome-associated protein of 25 kDa) (7) and vesicle-associated membrane protein (VAMP) (8, 9), which possess SNARE motifs that interact to form a four-helix bundle (10). The inner core of this complex is composed of 16 hydrophobic layers (10). At the zero layer there are ionic interactions between three conserved glutamates from SNAP-25 and syntaxin1A and an arginine from VAMP leading to classification of SNAREs as R-SNAREs or Q-SNAREs (11). The SNAP-25 0 layer glutamates are not essential for membrane fusion (12, 13), but the glutamine in syntaxin1A is required for dissociation of the SNARE complex by NSF and α-SNAP (14). Assembly of the SNARE complex drives docking and fusion, and this process is regulated by several syntaxin-interacting proteins. Syntaxin1 in its closed state (15) binds Munc18-1 (16) and Munc18-1 prevents formation of the SNARE complex. Munc18-1 is not a purely negative regulator as it is essential for neurotransmitter release (17), and it has syntaxin-independent functions (18). Synaptotagmin is believed to act as the Ca2+ sensor for exocytosis (19) through its interactions with SNAREs (20, 21).

Two additional syntaxin1-interacting proteins, tomosyn (22) and amisyn (23), have C-terminal R-SNARE motifs that allow their assembly into SNARE complexes. A rudimentary R-SNARE motif (24) is present in all tomosyn-like proteins (22, 25–29). Overexpression of full-length tomosyn1 (30, 31) or its VAMP-like domain or introduction of a fragment of amisyn into permeabilized cells (23) inhibits exocytosis. These effects have been ascribed to interaction with syntaxin1 and the formation of non-productive SNARE complexes. Using PC12 and adrenal chromaffin cells as model neurosecretory cells (2), we show that they express tomosyn but not amisyn. Expression of amisyn or overexpression of m-tomosyn1 inhibited exocytosis, but surprisingly this was not affected by mutations that disrupt their binding to syntaxin1. Furthermore, expression of amisyn resulted in an increase in the stability of the initial fusion pore. These findings suggest, therefore, that amisyn and tomosyn have distinct regulatory roles in the control of exocytosis.

MATERIALS AND METHODS

Plasmids and Antibodies—A plasmid encoding amino acids 4–266 from the cytoplasmic domain of syntaxin 1A fused to glutathione S-transferase (GST) was a gift from Dr. R. Scheller (University of Stanford). The amisyn plasmid was generated by PCR amplification using the BIO-X-ACT polymerase enzyme (Bioline Ltd., London, UK) from human brain cDNA (BD Biosciences). The resulting PCR product was inserted into pcDNA 3.1(−), using the XbaI and EcoRV restriction sites. Note that this plasmid encoded a 210-amino-acid version of amisyn not represented in the human genome. The full-length tomosyn plasmid was generated by PCR using a template encoding the human form of tomosyn (IMAGE clone 5275542). The PCR product was then ligated into pcDNA 3.1(−), using the NheI and BamHI restriction sites. A frameshift mutation derived from the IMAGE clone was corrected by site-directed mutagenesis. All described mutations were introduced using the Stratagene QuikChange system or the four-primer method.

An anti-amisyn rabbit antiserum was custom produced against the peptide sequence NSLRAVLTCSTMSRAISKEIFAPLDEC by Co-
valAb (Cambridge, UK) France using standard procedures. Mouse monoclonal anti-tomosyn was obtained from BD Biosciences.

Preparation of Cytosol and Membrane Extracts—Rat brains were homogenized in buffer A (20 mM Tris-HCl, 1 mM MgCl₂, 2 mM dithiothreitol, pH 8). The sample was then centrifuged at 100,000 × g for 60 min at 4 °C, and the supernatant retained as the cytosolic fraction. The pellet was resuspended in 10 ml of buffer A by homogenization and centrifuged at 100,000 × g for 30 min. The pellet was resuspended in buffer B (10 mM HEPES, 100 mM KCl, 1 mM dithiothreitol, pH 7-8), then centrifuged at 100,000 × g for 30 min and resuspended again in buffer B. 4 °C (v/v) Triton X-100 was slowly added with mixing, incubated at 4 °C for 45 min with frequent mixing. This membrane fraction was centrifuged at 100,000 × g for 30 min. Both fractions were dialyzed overnight and centrifuged by centrifuging at 100,000 × g for 60 min.

Reverse Transcription-PCR Amplification—RNA was isolated from PC12 and chromaffin cells using standard protocol of the RNase kit (Qiagen). The isolated RNA was then converted to cDNA using the ImProm-II Reverse Transcription system (Promega). For the PCR amplification of amisyn the following primers were used: 3′-AGG ATA GGC GAT TTC TCA ACA TGA TTT GGT-5′ and 5′-TGT TCT GTA AGT AGT GCC AAA-3′. For the PCR amplification of a tomosyn fragment the following primers were used: 5′-GGG AGG CCG ATG CCC TAC CTC AGA-3′ and 3′-AGC ACT ACT TCT TAT CTT TGT TGT-5′.

Growth Hormone Assay—Analysis of release of growth hormone used a modification of the growth hormone (GH) release assay (32, 33). PC12 cells were plated at a density of 1 × 10⁶/ml on collagen-coated 24-well trays, cultured in RPMI with 5% fetal bovine serum and 10% horse serum, and left overnight. The following day each well was transfected with 1 μg of growth hormone plasmid along with 1 μg of the test plasmid and 6 μg of Lipofectamine 2000. Cells were left for 4–6 h, after which time the transfection mixture was removed and replaced with 1 ml of fresh culture medium containing 10 mM CDCl₃ to induce expression of GH. Cells were permeabilized 36 h later with KGEP (139 mM K-glutamate, 5 mM EGTA, 20 mM Pipes, 2 mM ATP, and 2 mM MgCl₂) with 5 mM CaCl₂ for 30 min. Both fractions were centrifuged at 100,000 × g, and the test protein (20 ∙ 10⁶/ml) was added to the supernatant. The membrane was incubated with primary antibody 1 h, washed, and incubated in secondary antibody. After multiple washes bound antibody was detected using enhanced chemiluminescence (Amersham Biosciences).

Growth Hormone Binding Assay—Full-length amisyn and tomosyn protein were produced as 35S-labeled proteins by using the Promega TnT quick coupled transcription/translation system according to the manufacturer’s instructions. Glutathione-Sepharose beads were washed in binding buffer (150 mM potassium acetate, 1 mM MgCl₂, 0.5% Tween 20, 20 mM HEPES, pH 7.4) resuspended at 100 ∙ 10⁶/ml for 60 min at 4 °C and incubated in 3% nonfat dried milk phosphate-buffered saline for 45 min. The membrane was incubated with primary antibody 1 h, washed, and incubated in secondary antibody. After multiple washes bound antibody was detected using enhanced chemiluminescence (Amersham Biosciences).

Expression of Tomosyn but Not Amisyn in Chromaffin and PC12 Cells—To characterize the properties of the antiseraum that was raised against the N-terminus of amisyn it was tested on rat brain cytosol and membrane samples (Fig. 1A). The results showed that amisyn is present in cytosol and membrane fractions, confirming previous observations (23). The specificity of the antibody was confirmed with another Western blot with the same samples but this time in the presence of the immunizing peptide, which abolished the appearance of any bands on the blot (Fig. 1A). The possible presence of amisyn in PC12 and chromaffin cells was examined, but no amisyn could be detected in either of these cell types (Fig. 1B). The expression of tomosyn was also examined. Tomosyn1 was mainly a cytosolic protein in brain extracts. Unlike amisyn, tomosyn could be found in both PC12 and chromaffin cells (Fig. 1B). To confirm the lack of expression of amisyn in PC12 and chromaffin cells, reverse transcription-PCR was performed on RNA extracted from these cells. There was no evidence of any PCR products derived from mRNA extracted from PC12 or chromaffin cells, but a PCR product was found in the brain sample and a tomosyn1 product found from both PC12 and chromaffin cells (Fig. 1C).
VAMP-like domain of amisyn has previously been shown to glutamine (R176Q). This mutation introduced into the isolated zero layer in the VAMP-like domain of amisyn was mutated to with syntaxin1A, the arginine in the predicted position of the interactions that could potentially disrupt the interaction of amisyn detected with GST-syntaxin1A was easily experiment. Binding of amisyn to GST-syntaxin (Fig. 2 A). To detect tomosyn the primers to amplify an 300-base-pair fragment (see "Materials and Methods") were used. Duplicate reactions are shown for amisyn PCR for PC12 and chromaffin cells (CC). PCR products were detected for both amisyn and tomosyn in control reactions with brain cDNA.

with the aim of analyzing the effect of mutations introduced into the VAMP-like domain (Fig. 2A) that could potentially reduce the binding to syntaxin. The mutations were chosen based on sequence comparison with VAMP2 and using the layers defined from the structure of the neuronal SNARE complex (10). The binding study was performed using the cytoplasmic domain of syntaxin1A fused to GST and an in vitro transcription/translation system to generate radiolabeled full-length amisyn and tomosyn (Fig. 2B). Less of the radiolabeled amisyn mutants were produced compared with the wild-type protein by the in vitro transcription and translation in this experiment. Binding of amisyn to GST-syntaxin1A was easily detected with ~35% in the bound fraction, whereas no binding to control GST-coated beads was observed. To generate mutations that could potentially disrupt the interaction of amisyn with syntaxin1A, the arginine in the predicted position of the zero layer in the VAMP-like domain of amisyn was mutated to glutamine (R176Q). This mutation introduced into the isolated VAMP-like domain of amisyn has previously been shown to reduce its ability to assemble into a thermostable SNARE complex (23). In addition, mutations were also introduced into the −1 and +1 hydrophobic layer positions to generate a triple mutant (L173A,R176Q,L180A). Both the single and the triple mutations abolished the binding of amisyn to syntaxin1A in vitro (Fig. 2B). Similarly, binding of full-length m-tomosyn1 generated by in vitro transcription and translation to GST-syntaxin1 was examined. Again specific binding of the wild-type protein was detected with ~80% of tomosyn found bound to GST-syntaxin1 and no binding detectable to the GST control. The triple mutations in m-tomosyn1 substantially reduced the binding to GST-syntaxin (Fig. 2C).

**Effect of Expression of Amisyn or Overexpression of m-Tomosyn1 on Exocytosis in PC12 Cells and Requirement for Syntaxin Binding**—It has been assumed that the inhibitory effect of tomosyn1 on exocytosis results from its interaction with syntaxin and formation of non-functional SNARE complexes. This has not, however, been tested experimentally nor has the effect of full-length amisyn on exocytosis been examined. With the availability of mutations in amisyn or m-tomosyn1 that abrogated their interactions with syntaxin1A, we were able to examine the effect of full-length constructs of these two proteins with or without the mutations on exocytosis. To examine the effect on exocytosis, use was made of a well characterized GH assay, which involves co-transfecting PC12 cells with the plasmid of interest and a plasmid encoding human GH (32, 33). As GH is not expressed by PC12 cells, this allows the assay of exocytosis specifically from transfected cells. The PC12 cells were transfected with control plasmid or to express amisyn, or the syntaxin binding-defective mutants. The mutants were expressed to similar levels as the wild-type protein (Fig. 3A). To bypass any effects of transfected constructs on receptors or channels, the cells were permeabilized with digitonin and challenged with 0 or 10 μM Ca2+ after which released and retained GH were assayed, and released GH was expressed as a percentage of total GH. There are examples where the overexpression of proteins in PC12 cells disrupts GH storage leading to its constitutive secretion and reduced cellular levels (36) or accumulation in the secretory pathway and severalfold increases in cellular levels (37) of GH. No significant effect of any of the amisyn constructs on total cellular GH levels was observed (Fig. 3C). In addition, immunofluorescence with anti-GH indicated that the GH in both the control and amisyn-expressing cells had a punctate localization throughout the cell consistent with its accumulation in secretory granules (Fig. 3E). Expression of wild-type amisyn resulted in inhibition of both basal and stimulated exocytosis (Fig. 3A). Expression of amisyn constructs with syntaxin binding mutations did not prevent the inhibitory effect, and in fact it was reproducibly seen that the R176Q and the triple amisyn mutant were more inhibitory than wild-type amisyn (Fig. 3A) suggesting that the inhibitory effect of amisyn on exocytosis does not require its ability to bind to syntaxin1.

Overexpression of tomosyn has previously been shown to inhibit exocytosis in PC12 and adrenal chromaffin cells (30, 31). We set out to examine whether the inhibition by m-tomosyn1 was dependent on its interaction with syntaxin1 by expressing the triple mutant that is impaired in syntaxin binding and testing its effect in the GH assay. The full-length m-tomosyn1 construct resulted in a small increase in total cellular GH levels, but no effect of the triple mutant was observed (Fig. 3D), and GH had the expected punctate localization throughout the cell as seen with its accumulation in secretory granules (Fig. 3E). As shown in Fig. 3B, full-length wild-type m-tomosyn1 inhibited basal and stimulated exocytosis, and as seen for amisyn the triple mutant inhibited exocytosis to a greater extent than wild-type protein.

**Effect of Expression of Amisyn on the Number of Release Events and Exocytosis Dynamics in Adrenal Chromaffin Cells**—The growth hormone data show that expression of amisyn inhibits exocytosis, but to determine what effects, if any it has on the kinetics of vesicular release, carbon fiber amperometry was used in assays on adrenal chromaffin cells. This approach also has the advantage that it measures release of endogenous catecholamine from secretory granules with a half-life of ~15 days (38), and so the effects of expression cannot be because of effects on granule biogenesis. Previous work on both PC12 and adrenal chromaffin cells has failed to find any effect of overexpression of tomosyn on the kinetics of single release events and only an inhibitory effect on the extent of release.
This has not, however, been examined for amisyn. In these experiments, the cells were transfected to express amisyn and also EGFP as a marker for the small percentage of transfected cells and exocytosis assayed using a well characterized approach (39–42). The cells were permeabilized and stimulated individually by pressure ejection onto the cells of a buffer containing digitonin and 10 mM Ca^{2+}. The protocol used allows recording from control and transfected cells in the same dishes and direct comparison of the data from each group of cells for each construct tested. Example traces from control and amisyn-transfected cells are illustrated in Fig. 4A. A significant number of amisyn-expressing cells failed to respond to stimulation. There are, however, usually failures also in control cells, which could have many different explanations. We, therefore, took into account for quantification only those cells in which there was some response so that we were certain that the cell was healthy and that the recording conditions were appropriate. This will inevitably mean that we underestimate any inhibitory effect on release probability. Nevertheless, from the analysis of responding cells, it was observed that the number of spikes recorded per chromaffin cell was significantly reduced in cells transfected to express amisyn (Fig. 4B) indicating an inhibition of the extent of exocytosis by amisyn. To determine whether early or late release events were preferentially affected, a plot of cumulative spike numbers was produced, and this revealed that the number of spikes was reduced over the whole time-course of the experiment (Fig. 4B). Expression of amisyn did not affect the average integrated charge per spike or the quantal size (Fig. 4B). We also examined the frequency distribution of spike size for each cell population, and no obvious changes were seen. In addition, amisyn expression did not have any obvious affect on any of the kinetics of vesicular release based on analysis of individual amperometric spike parameters (data not shown) unlike expression of certain other proteins involved in exocytosis (39–43). To determine whether the effect of amisyn expression on spike number required syntaxin1 binding by amisyn, the effects of expression of the triple mutant impaired in syntaxin1 binding was examined. The triple mutant significantly reduced the number of spikes elicited by stimulation (Fig. 5). It should be noted that the inhibitory effect of the triple mutant on the number of release events was actually an underestimate, as non-responding cells were not taken into account in this analysis. As seen for the wild-type amisyn, expression of the triple mutant did not affect the mean spike height or quantal size (Fig. 5B) or the frequency distribution of spike heights. No obvious effects of expressing the triple mutant were seen on any of the kinetic parameters of the amperometric spikes.
The release of catecholamine during amperometric spikes is often preceded by a so-called foot signal suggested to be because of release through an initial fusion pore (44–46). Examples of these from our recordings are shown in Fig. 6A. To address the question of whether the foot properties are affected by amisyn expression, analyses of the feet from our data sets were performed. Foot signals were detected before 12–19% of the amperometric spikes. No significant change was seen in the probability of the spikes being preceded by a foot signal but the analysis revealed that expression of amisyn caused a significant increase in the mean foot charge (Fig. 6B). This increase was not due to an effect on the foot amplitude but to an increase in mean foot duration (Fig. 6B). The robustness of this effect on the foot parameters is illustrated by the fact that we originally tested an N-terminally extended amisyn sequence based on that first described in the literature (23), and this resulted in the exact same changes in foot parameters.2 To determine whether the effect of amisyn expression on foot properties required syntaxin1 binding by amisyn, the effects of expression of the triple mutant impaired in syntaxin1 binding was examined. This triple mutant had no discernible effect of any of the foot parameters.

**DISCUSSION**

In this study we have examined the role of amisyn and tomosyn in regulated exocytosis of dense core granules in PC12 and adrenal chromaffin cells, two well characterized neurosecretory model cell types that have been widely studied because of the similarities of their exocytotic machinery to that in the synapse (2). The absence of endogenous amisyn in these cell types provided the opportunity to examine the consequences of introducing amisyn into the cells that express the neuronal SNARE proteins and the neuronal isoforms of many of the SNARE-associated proteins involved in neurotransmitter release. Importantly, the effect of full-length amisyn was examined rather than just a C-terminal fragment as previously described (23). This analysis was combined with examination of the effects of overexpression of m-tomosyn1. It is well established that tomosyn has an inhibitory role in exocytosis based on studies on the endogenous protein (47, 48) and also from overexpression studies (30, 31). Amisyn has orthologues in only mammals, amphibians, and fish, and to date no genetic studies have addressed its function. We have so far been unable to detect its expression in a specific non-neuronal cell type, and a knock-down in its expression in neurons would be an important future study. We have confirmed previous findings that overexpression of m-tomosyn1 inhibits exocytosis but show that this is not dependent on the ability of tomosyn to bind to syntaxin. We have now also demonstrated that expression of full-length amisyn in PC12 or chromaffin cells reduces the number of exocytotic events (a reduction in release probability) and also regulates fusion pore dynamics via distinct syntaxin-dependent and syntaxin-independent mechanisms.

Amisyn and tomosyn have in common a similar VAMP-like

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2 J. R. L. Constable, M. E. Graham, A. Morgan, and R. D. Burgoyne, unpublished observations.
or R-SNARE domain that allows them to bind to syntaxin and assemble into stable SNARE complexes (23, 24). Unlike VAMP, amisyn and tomosyn do not possess trans-membrane domains and therefore are unable to assemble into functional complexes. It has previously been assumed that both of these proteins would inhibit exocytosis through their interaction with syntaxin by inhibiting the formation of productive SNARE complexes. We now show, however, that mutations that abrogate this interaction do not reduce the inhibitory effect of amisyn and m-tomosyn1 on exocytosis. It has been shown in an earlier study that introduction of high concentrations of the recombinant VAMP-like domain of amisyn into permeabilized PC12 cells inhibited exocytosis (23); the effect of full-length amisyn was not examined. The inhibitory effect of the C-terminal amisyn fragment was reduced but not abolished by mutations in the zero layer glutamine. Our data revealed that mutations that substantially reduced syntaxin binding by m-tomosyn1 and abolished binding by amisyn if anything increased rather than reduced the inhibitory effect of tomosyn1 and amisyn on exocytosis. In PC12 cells, expressing the triple mutants of these proteins, evoked exocytosis was almost abolished. This would be consistent with an increased availability of non-syntaxin-bound amisyn and tomosyn in cells expressing the mutated forms allowing them to exert an increased inhibitory effect. Similarly, the inhibition of the number of exocytotic events by expression of amisyn observed using amperometry in chromaffin cells was not prevented by introduction of the triple mutations into amisyn. Overall these findings suggest that amisyn and tomosyn must be able to exert an inhibitory control on exocytosis that is independent of syntaxin and implies that an alternative protein-protein interaction must be involved. One issue is whether the effects of exogenous amisyn are because of a dominant negative-type inhibition of endogenous tomosyn. The data show that, as far as probability of release is concerned, amisyn expression and tomosyn overexpression had the same effects, and this would not be consistent with a dominant negative role. The other effect of amisyn on the foot signals could conceivably be because of interference with tomosyn function, although overexpression of tomosyn did not have this effect. Apart from the VAMP-like domain, amisyn and the tomosyns are not homologous, and in tomosyn the VAMP-like domain encompasses less than 10% of the total protein. The N terminus contains a series of WD40 repeats throughout their N-terminal region (22, 24) that are highly conserved in position and sequence across species from plants and fungi to man (24). This suggests that there must be important functional interactions made by the N terminus of the tomosyns. The much smaller N-terminal region of amisyn is distinct from that of tomosyn and, interestingly, possesses 32% identity with a mammalian Sec3 homologue over residues 4–199 (23). In yeast, Sec3 is a subunit of the exocyst complex involved in vesicle targeting and docking at the plasma membrane (49). No known binding partners for amisyn and tomosyn other than syntaxin1 are currently known.

Our amperometric analysis of the effects of amisyn expression in chromaffin cells indicate that a major effect was to reduce the number of exocytotic events without affecting the kinetics of the main release process indicated by there being no
change in the amperometric spike parameters. An additional role for amisyn was discovered from analysis of the foot signal preceding the amperometric spike that is believed to represent a low level of catecholamine release through an initial fusion pore (44, 45). The effect that we observed was likely to be because of an effect on the fusion pore itself rather than resulting from a change in the overall catecholamine content of the granules as the mean charge of the spike (quantal size) was unaffected by expression of amisyn. Manipulation of the expression of synaptotagmin (20, 50), SNAP-25 (51) or syntaxin 1 (52) has been shown to modify the foot signal in two distinct ways either by changing the conductance through the pore or its stability. Expression of syntaxin1 with certain mutations in its transmembrane domain modified the conductance (mean amplitude of the foot signal) consistent with syntaxin1 forming part of the fusion pore structure. Our findings of an increase in the duration and mean overall current in the foot signal without a change in mean amplitude suggests that amisyn expression, in contrast, resulted in an increased stability of the fusion pore prior to its expansion and full release of vesicle contents. It might be assumed that an increase in the stability of the fusion pore should increase the number of amperometric spikes observed. This is not necessarily the case unless many fusion events do not normally go beyond the initial fusion pore step. Our data are in fact consistent with other studies in the literature. Overexpression of synaptotagmin I, for example, increases the stability of the fusion pore but did not increase the frequency of spikes (50). An effect on the fusion pore would also not necessarily lead to an effect on the kinetics of the spike, which we found to be unaffected by amisyn expression. The foot parameters and the spike parameters seem to be independent of one another, and this has been also been demonstrated by others in the literature for example in SNAP-25 knock-out mice where the foot duration is reduced, but the parameters of the main spike are unaffected (51). Analysis of the triple mutation in the amisyn VAMP-like domain suggesting that the effect of amisyn on the fusion pore (foot parameters) is dependent on its interaction with syntaxin1A consistent with the role of syntaxin as a component of the fusion pore. Two previous studies have examined the effect of overexpression of tomosyn1 on the foot signal either in PC12 (31) or in chromaffin cells (30) and did not observe any difference from controls. This suggests that the effect of amisyn on fusion pore stability may be related to its distinct N-terminal domain that would have a functional effect once the protein is bound to syntaxin1A via its R-SNARE motif. This scenario would predict that amisyn would have to interact with syntaxin1A close to the site of action of functional SNARE complexes or become associated into oligomeric structures containing VAMP at the site of membrane fusion.

The findings of this study show that amisyn and tomosyn can exert distinct regulatory effects though different mechanisms that lead to a reduction in the number of release events (a reduction in release probability) or lead to more subtle changes
in fusion-pore dynamics. They raise the possibility that changes in the expression of amisyn in particular neuronal cell types could result in long term changes in fusion pore dynamics and thereby (53) contribute to changes in synaptic function.

REFERENCES
1. Jahn, R., and Sudhof, T. C. (1999) *Annu. Rev. Biochem.* 68, 863–911
2. Burgoyne, R. D., and Morgan, A. (2003) *Physiol. Rev.* 83, 561–632
3. Sollner, T., Whiteheart, S. W., Brüning-Schneider, H., Gerosmanos, S., Tempst, P., and Rothman, J. E. (1993) *Nature* 362, 318–324
4. Sollner, T., Whiteheart, S. W., Scheller, R., and Rothman, J. E. (1993) *Cell* 75, 409–418
5. Chen, Y. A., and Scheller, R. H. (2001) *Nat. Rev. Mol. Cell Biol.* 2, 98–106
6. Bennett, M. K., Calakos, N., and Scheller, R. H. (1992) *Science* 257, 255–259
7. Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E., and Wilson, M. C. (1989) *J. Cell Biol.* 109, 3039–3052
8. Trimble, W. S., Cowan, D. M., and Scheller, R. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 4538–4542
9. Baumann, M., Maycox, P. R., Navone, F., De Camilli, P., and Jahn, R. (1989) *EMBO J.* 8, 379–384
10. Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1999) *Nature* 395, 347–353
11. Fasshauer, D., Sutton, R. B., Brunger, A. L., and Jahn, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 15781–15786
12. Graham, M. E., Washbourne, P., Wilson, M. C., and Burgoyne, R. D. (2001) *J. Cell Sci.* 114, 4497–4505
13. Wei, S., Xu, T., Ashery, U., Kollnewe, A., Matti, U., Antonin, W., Retig, J., and Neher, E. (2000) *EMBO J.* 19, 1279–1289
14. Seales, S. J., Yoo, B. Y., and Scheller, R. H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 14262–14267
15. Dulubova, I., Sugita, S., Hill, S., Hasaka, M., Fernandez, I., Sudhof, T. C., and Rizo, J. (1999) *EMBO J.* 18, 4372–4382
16. Misura, K. M. S., Scheller, R. H., and Weis, W. I. (2000) *Nature* 404, 355–362
17. Verhage, M., Maia, A. S., Plomp, J. J., Brusseau, A. B., Heeroma, J. H., Vermeer, H., Toonen, R. F., Hammer, R. E., van den Berg, T. K., Missler, M., Geuze, H. J., and Sudhof, T. C. (2000) *Science* 287, 864–869
18. Ciuf, L. F., Barry, J. W., Burgoyne, R. D., and Morgan, A. (2001) *J. Biol. Chem.* 276, 470–482
19. Fernandez-Chacon, R., Konigstorfer, A., Gerber, S. H., Garcia, J., Matsos, M. F., Stevens, C. F., Brose, N., Rizo, J., Rosenmund, C., and Sudhof, T. C. (2002) *Nature* 410, 41–49
20. Bai, J., Wang, C.-T., Richards, D. A., Jackson, M. B., and Chapman, E. R. (2004) *Neuron* 41, 929–942
21. Rickman, C., Archer, D. A., Meunier, F. A., Craxton, M., Fukuda, M., Burgoyne, R. D., and Davletov, B. (2004) *J. Biol. Chem.* 279, 12574–12579
22. Fujita, Y., Shirai, H., Sakisaka, T., Asakura, T., Ohyu, T., Ketani, H., Yokoyama, S., Nishikawa, H., Matsuzuka, Y., Mizoguchi, A., Scheller, R. H., and Takai, Y. (1998) *Neuron* 20, 905–915
23. Scales, S. J., Hesser, B. A., Masuda, E. S., and Scheller, R. H. (2002) *J. Biol. Chem.* 277, 29271–29279
29. Lehman, K., Rossi, G., Adamo, J. E., and Brennwald, P. (1999) *J. Cell Biol.* **146**, 125–140
30. Yizhar, O., Matti, U., Melamed, R., Hagalili, Y., Bruns, D., Rettig, J., and Ashery, U. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 2578–2583
31. Hatsuzawa, K., Lang, T., Faschauer, D., Bruns, D., and Jahn, R. (2003) *J. Biol. Chem.* **278**, 31159–31166
32. Wick, P. F., Senter, R. A., Parsels, L. A., Uhler, M. D., and Holz, R. W. (1993) *J. Biol. Chem.* **268**, 19983–19989
33. Graham, M. E., Fisher, R. J., and Burgoyne, R. D. (2000) *Biochimie* **82**, 469–479
34. Burgoyne, R. D. (1992) in *Neuromethods, Volume 20: Intracellular Messengers* (Boulton, A., Baker, G., and Taylor, C., eds) pp. 433–470, Humana Press Inc., New Jersey
35. Jankowski, J. A., Schroeder, T. J., Holz, R. W., and Wightman, R. M. (1992) *J. Biol. Chem.* **267**, 18329–18335
36. Schütz, O. M., Khvotchev, M., Jahn, R., and Südhof, T. C. (2002) *J. Biol. Chem.* **277**, 40919–40928
37. Khvotchev, M. V., Ren, M., Takamori, S., Jahn, R., and Südhof, T. C. (2003) *J. Neurosci.* **23**, 10531–10539
38. Corcoran, J. J., Wilson, S. F., and Kirshner, N. (1984) *J. Biol. Chem.* **259**, 6208–6214
39. Fisher, R. J., Pevner, J., and Burgoyne, R. D. (2001) *Science* **291**, 875–878
40. Barclay, J. W., Craig, T. J., Fisher, R. J., Ciufa, L. P., Evans, G. J. O., Morgan, A., and Burgoyne, R. D. (2003) *J. Biol. Chem.* **278**, 10538–10545
41. Graham, M. E., Barclay, J. W., and Burgoyne, R. D. (2004) *J. Biol. Chem.* **279**, 32751–32760
42. Graham, M. E., and Burgoyne, R. D. (2000) *J. Neurosci.* **20**, 1281–1289
43. Archer, D. A., Graham, M. E., and Burgoyne, R. D. (2002) *J. Biol. Chem.* **277**, 18249–18252
44. Chow, R. H., von Ruden, L., and Neher, E. (1992) *Nature* **356**, 60–63
45. Alvarez de Toledo, G., Fernandez-Chacon, R., and Fernandez, J. M. (1993) *Nature* **363**, 554–558
46. Schroeder, T. J., Borges, R., Finnegan, J. M., Piel, J., Amatore, C., and Wightman, R. M. (1996) *Biophys. J.* **70**, 1061–1068
47. Holgado, A. M., Gracheva, E., Nonet, M., Hadwiger, G., and Richmond, J. E. (2004) *West Coast Worm Meeting, abstract 77*
48. Sakiyama, T., Baba, T., Tanaka, S., Izumi, G., Yasumi, M., and Takai, Y. (2004) *J. Cell Biol.* **166**, 17–25
49. Terbush, D. R., Maurice, T., Roth, D., and Novick, P. (1997) *EMBO J.* **16**, 6483–6494
50. Wang, C.-T., Grishanin, R., Earles, C. A., Chang, P. Y., Martin, T. F. J., Chapman, E. R., and Jackson, M. B. (2001) *Science* **294**, 1111–1115
51. Sorensen, J. B., Nagy, G., Varoqueaux, F., Nehring, R. B., Irose, N., Wilson, M. C., and Neher, E. (2003) *Cell* **114**, 75–86
52. Han, X., Wang, C.-T., Bai, J., Chapman, E. R., and Jackson, M. B. (2004) *Science* **304**, 289–292
53. Choi, S., Klingauf, J., and Tsien, R. W. (2003) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **358**, 695–705
