Biochimica et Biophysica Acta 1818 (2012) 2994–2999

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

# Effect of juxtamembrane tryptophans on the immersion depth of Synaptobrevin, an integral vesicle membrane protein

M. Sameer Al-Abdul-Wahid [a,1], Colin M. DeMill [b,1], Marzena B. Serwin [b], R. Scott Prosser [a,c], Bryan A. Stewart [b,*]

[a] Department of Chemistry, University of Toronto Mississauga, Mississauga, Ontario, Canada, L5L 1C6
[b] Department of Biology, University of Toronto Mississauga, Mississauga, Ontario, Canada, L5L 1C6
[c] Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada, M5S 1A8

ARTICLE INFO

*Article history:*
Received 26 April 2012
Received in revised form 20 July 2012
Accepted 23 July 2012
Available online 27 July 2012

*Keywords:*
Neuronal-Synaptobrevin
Membrane protein positioning
Paramagnetic contact shifts
$^{19}$F NMR
Solvent-induced isotope shifts
Solution state NMR of membrane proteins

ABSTRACT

Proper positioning of membrane proteins in the host membrane is often critical to successful protein function. While hydrophobic considerations play a dominant role in determining the topology of a protein in the membrane, amphiphilic residues, such as tryptophan, may 'anchor' the protein near the water–membrane interface. The SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) family of membrane proteins mediates intracellular membrane fusion. Correct positioning of the SNAREs is necessary if fusion is to occur. Synaptobrevins are integral vesicle membrane proteins that are well conserved across species. Interestingly, mammalian Synaptobrevins typically contain two adjacent tryptophans near the water-membrane interface whereas the *Drosophila*, neuronal-Synaptobrevin (n-Syb), contains a single tryptophan in this same region. To explore the role of these tryptophan residues in membrane positioning, we prepared a peptide containing residues 75–121 of *D. melanogaster* n-Syb in DPC micelles, biosynthetically labeled with 4-fluorophenylalanine and 5-fluorotryptophan for the examination by $^{19}$F NMR spectroscopy. Mutations of this construct containing zero and two tryptophan residues near the water-membrane interface resulted in changes in the positioning of n-Syb in the micelle. Moreover, the addition of a second tryptophan appears to slow dynamic motions of n-Syb near the micelle–water interface. These data therefore indicate that juxtamembrane tryptophan residues are important determinants of the position of Synaptobrevin in the membrane.



## 1. Introduction

The fusion of cellular membranes, guided by key proteins that mediate membrane disruption and association, presents a fundamental biophysical problem where the repulsive forces between membranes must be overcome [1]. A solution to this problem is achieved with the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) superfamily of membrane proteins [2,3]. These proteins share an evolutionally conserved motif of 60–70 amino acids [4], and are located in both the vesicle and target membranes. During membrane fusion, SNAREs from both target and vesicle membranes combine to form a four-helix complex. A well-characterized example of SNARE mediated fusion is the neuronal exocytosis, where two cellular membrane proteins, syntaxin-1A [5], and SNAP-25 (synaptosomal associated protein of 25 kD) [6] complex with VAMP-1/-2 (vesicle associated membrane protein, also referred to as Synaptobrevin) [7,8]. It is thought that the complex remains partially assembled [9], until calcium binding to syntaptotagmin [10] triggers full assembly. Energy released [4] upon formation of the ternary stable parallel coiled coil [11] SNARE complex is believed to provide the energy necessary for membrane fusion [12–14].

Of particular interest is the VAMP subfamily of SNARE proteins, which have been characterized across a wide range of species, including yeast [15], *Drosophila melanogaster* [16], rat [17,18], *Torpedo californica* [7], and human [19]. VAMPs consist of a variable N-terminal domain, a conserved SNARE domain, a conserved linker (or juxtamembrane) region, a conserved transmembrane domain and a variable intravesicular C-terminal region. While much attention has been focused on studies of the SNARE domain, less has been paid to the linker region which contains highly conserved aromatic and positively charged residues. These residues have been shown to interact with membrane phospholipids [20,21] and may be important for membrane fusion. Moreover, it has been proposed that these interactions play a role in lipid mixing during SNARE complex formation [4,22]. Additionally, several of these residues

*Abbreviations:* wt-TM-n-Syb, residues 75–121 of *Drosophila melanogaster* n-Synaptobrevin, biosynthetically labeled with 5-fluorotryptophan and 4-fluorophenylalanine; AA-TM-n-Syb, W89A/L90A double mutant of wt-TM-n-Syb; WW-TM-n-Syb, L90W mutant of wt-TM-n-Syb

* Corresponding author. Tel.: +1 905 828 5364; fax: +1 905 828 4275.
*E-mail address:* bryan.stewart@utoronto.ca (B.A. Stewart).

[1] These authors contributed equally to the work.

0005-2736/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2012.07.018

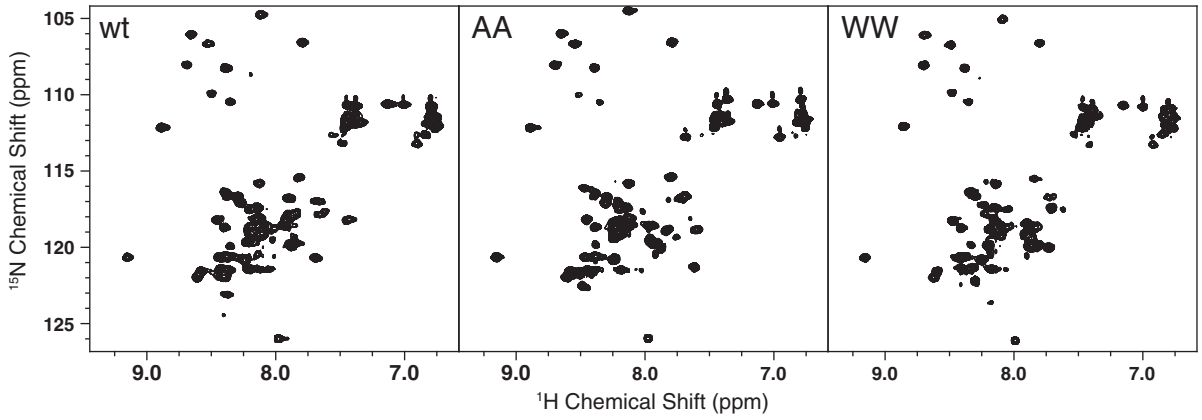


**Fig. 1.** NH-HSQC spectra of wt-TM-n-Syb (left), AA-TM-n-Syb (center), and WW-TM-n-Syb (right). Both the "AA" and "WW" mutants exhibit similar HSQC spectra as the wild type, indicating that the mutations do not grossly alter the structure of n-Syb.

may interact with Syntaxin during the final stage of SNARE complex formation and membrane fusion [23]. Thus, aromatic and charged residues in the linker region may be essential for membrane fusion.

The linker region of mammalian VAMP2 contains two consecutive tryptophan residues (positions 89 and 90) which have been reported to localize at the membrane–water interface [22]. Mutation of these residues to alanine severely inhibits neuronal exocytosis in cultured mouse hippocampal neurons with complete loss of evoked transmitter release and yet an increase in spontaneous fusion [24]. Other works assessing fusion with mutations to this region have produced conflicting results with no effect on fusion of liposomes [25], an increase in fusion of liposomes [26] and decreased exocytosis from PC12 cells [27]. The mechanisms underlying these divergent results have yet to be fully described.

Due to the amphipathic nature of the membrane–water interface, membrane proteins are often anchored to the membrane by distributing tryptophan and tyrosine residues at the interface [28–30]. In fact, the position of Trp residues in a membrane protein can have profound effects on immersion depth and protein orientation [31]. Tryptophan residues have been suggested to serve as "anchors" at the membrane interface [32–34], thus stabilizing transmembrane regions of the protein. This may allow force, generated during SNARE complex assembly, to be transduced to the membrane [4,35,36]. Indeed, De Haro et al. found that mutation of tryptophan residues increased water exposure of this normally buried region of the molecule [37]. In VAMP2, strong anchoring by tryptophan (residue 89), along with presence of a lysine (residue 94) just inside the membrane, may force the transmembrane domain to adopt an oblique 36° angle with respect to the membrane which is thought to promote fusion [38].

Tryptophan's fundamental role in membrane fusion is exemplified through work on viral fusion proteins. Mutation of tryptophan and other aromatic residues in Herpes Simplex Virus fusion proteins reduced both membrane association and fusogenic activity [39]; similarly, mutation of aromatics inhibited membrane fusion of HIV [40], and influenza [41]. In addition, tryptophan has been shown to be important in membrane anchoring and maintenance of secondary structure in Ebola fusion proteins [42]. Interestingly the biophysical challenges that need to be overcome and mechanisms employed are similar for membrane fusion by both viruses and cells. This includes strong anchoring of proteins to membranes that are subsequently forced into close opposition causing first hemi-fusion and then full fusion (see [43] for a review). Therefore, studying the role of tryptophan in membrane anchoring and fusion across various biological models provides insight fundamental to biology.

The VAMP family member responsible for neurotransmitter release in *Drosophila melanogaster* is known as neuronal-Synaptobrevin (n-Syb) [44], and it provides a unique tool to study this mechanism because it possesses only one tryptophan residue (W89) in the linker region [45]. Does the presence of a second tryptophan significantly improve anchoring of the protein in the membrane? Conversely, how does the substitution of tryptophan with another residue affect n-Syb topology? In this work we explore the effect of tryptophan residues on the immersion depth of n-Syb peptides in DPC micelles, using constructs that contain zero (W89A/L90A mutant), one (wild-type), or two (L90W mutant) tryptophan residues.

## 2. Observing n-Syb by $^{19}$F NMR

The $^{1}$H nuclei naturally present in proteins are readily detected by NMR, and for small membrane peptides where the $^{1}$H spectra are well-resolved, immersion depth studies require no modification or isotopic enrichment of the protein [46]. The $^{1}$H spectrum of n-Syb, however, contains significant overlap of peaks and is too complex to be assigned. In such cases, NMR studies typically rely on one of the two methods to improve spectral resolution: isotopic enrichment, or the incorporation of an NMR-active probe moiety. Isotopic enrichment, with $^{15}$N and $^{13}$C, allows multidimensional NMR experiments and the concomitant increase in spectral resolution. Alternatively, the introduction of specific NMR probes, *via* biosynthetic labeling or chemical modification, provides a means of studying specific sites on the protein, reducing extraneous information and obviating the time-consuming assignment of $^{15}$N,$^{13}$C-labeled proteins. Moreover, large proteins labeled thusly may be studied *via* one-dimensional NMR spectroscopy, requiring less spectrometer time than a multidimensional NMR-based approach.

In this work, 5-fluorotryptophan and 4-fluorophenylalanine are introduced biosynthetically into n-Syb and its two mutants, providing two to four $^{19}$F reporter groups. This simple substitution, of a $^{1}$H atom for a $^{19}$F atom, provides a powerful tool to study n-Syb, owing to the high gyromagnetic ratio (94% that of $^{1}$H), absence of $^{19}$F in natural systems, and the high sensitivity of the $^{19}$F chemical shift to changes in local environment. In particular, F88, which is adjacent to the anchoring tryptophan residue in n-Syb, reports directly on the immersion depth of residues in this region.

## 3. Results and discussion

To confirm that our desired mutations did not alter the overall structure of the n-Syb, we collected NH-HSQC spectra of $^{15}$N-labeled samples of each construct. The NH-HSQC is a fast method of 'fingerprinting' the backbone conformation of a sample. If a mutation does not affect the overall structure, the 'fingerprint' will be very similar (*i.e.* only the mutated residues will have large chemical shift changes). On the other hand, any mutations affecting the overall structure of the peptide (*e.g.*

the breakage of a disulfide bond) will cause large changes in the chemical shifts of multiple peaks, resulting in a different 'fingerprint'. The spectra of the wild-type fragment (wt-TM-n-Syb), the W89A/L90A mutant (AA-TM-n-Syb) and the L90W mutant (WW-TM-n-Syb) are very similar (Fig. 1), indicating that the mutations do not affect the overall structure of the protein.

$^{19}$F spectra of wt-TM-n-Syb (Fig. 2) exhibit three peaks: one from the 5-fluorotryptophan at residue 89 (−124.6 ppm) and two from the 4-fluorophenylalanines at residues 77 (−116.1 ppm) and residue 88 (−115.6 ppm). The two 4-fluorophenylalanine residues were assigned using an F77A mutant of wt-TM-n-Syb (data not shown).

### 3.1. Effects of temperature on the immersion depth of F88

$^{19}$F chemical shifts are highly sensitive to changes in local environment. For $^{19}$F atoms in membrane proteins, an increase in the immersion depth in the micelle/bilayer results in a downfield shift of $^{19}$F resonances, [47] due to an increase in the number of nearby aliphatic $^{1}$H atoms. To examine the ability of tryptophan to 'anchor' n-Syb in the micelle, we collected $^{19}$F 1D spectra as a function of temperature (Fig. 2), and consider the temperature dependence of the $^{19}$F chemical shifts. In order to account for the sensitivity of the $^{19}$F chemical shift to temperature (*i.e.* in the absence of changes in immersion depth), we also collected $^{19}$F spectra of free 4-fluorophenylalanine in aqueous solution, and observed a small (<0.1 ppm/ 10 °C) temperature dependence on chemical shift (Figure SI-1). In wt-TM-n-Syb, both F77 and W89 exhibit similar (<0.1 ppm/ 10 °C) small temperature dependences on their chemical shifts, suggesting no change in environment (Figures SI-1 and SI-2). Meanwhile, F88 shows a pronounced downfield shift (−0.2 ppm/10 °C) at lower temperatures (Fig. 2), indicating that F88 is more deeply embedded in the micelle at lower temperatures. Interestingly, at these colder temperatures, downfield shifts are accompanied by an increase in peak linewidth of F88. Such increases are consistent with exchange broadening resulting from sampling two different chemical environments.

Taking into account the proximity of F88 to the micelle–water interface, the increase in the peak linewidth of F88 at lower temperatures suggests that the $^{19}$F atom 'criss-crosses' the micelle–water interface (*e.g.* a 'bobbing'-type motion, Fig. 3). In n-Syb, the amplitude of the motions leading to this exchange must be small, as the adjacent W89 residue is not strongly broadened, nor does W89 exhibit downfield shifts at lower temperatures, indicating that W89 does not undergo a change in local environment. Therefore, if the motions giving rise to the observed F88 dynamics are in fact 'whole-body' motions, the amplitude must be sufficiently small to keep the chemical environment surrounding W89 unchanged. More likely, the motions are localized to the F88 sidechain.

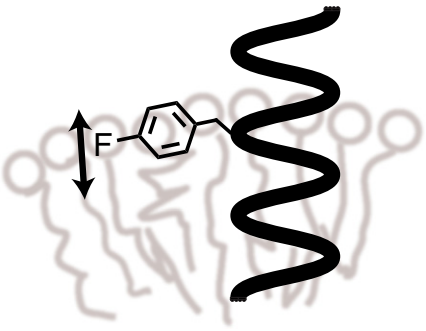


**Fig. 3.** Cartoon schematic of a 4-fluorophenylalanine residue in an alpha helical protein embedded in a detergent micelle (similar to the F88 residue of wt-TM-n-Syb). Translational motion of the $^{19}$F atom, as suggested by the double arrow, would give rise to chemical exchange between water-exposed and micelle-exposed states. Exchange rates on the order of the difference in chemical shift between the two states result in an increase of NMR linewidths, as observed for wt-TM-n-Syb.

To determine the effect of adding or removing tryptophan residues on the dynamics of n-Syb in the vicinity of F88, we compare the temperature dependence of the F88 chemical shift in the "AA" and "WW" mutants to that of the wild-type (Fig. 4). Replacing W89 with an alanine (*i.e.* AA-TM-n-Syb) does not affect the temperature dependence of the F88 chemical shift; both the line broadening (not shown) and downfield shift of F88 are similar to the wild-type construct. The removal of the juxtamembraneous tryptophan does not appear to significantly alter n-Syb dynamics near the micelle–water interface.

Adding a second tryptophan near F88 (*i.e.* WW-TM-n-Syb) changes the chemical shift profile of F88 (Fig. 4). Here, F88 exhibits only small upfield shifts at low temperature, consistent with the normal temperature dependence of $^{19}$F-bearing residues not undergoing a change in local environment. We conclude that the addition of a second tryptophan 'anchors' the position of F88 in the micelle, reducing the motions that give rise to chemical exchange.

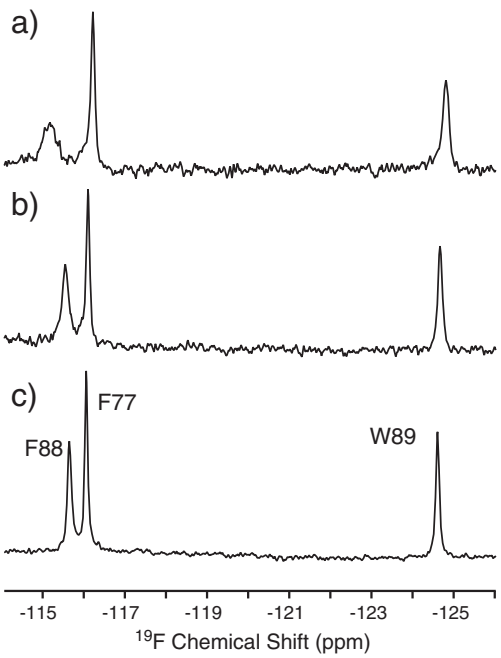


**Fig. 2.** $^{19}$F NMR spectra of wt-TM-n-Syb biosynthetically labeled with 5-fluorotryptophan and 4-fluorophenylalanine at 293 K (a), 310 K (b) and 318 K (c).

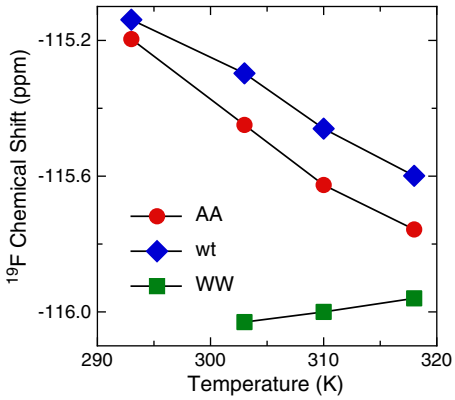


**Fig. 4.** $^{19}$F chemical shift of 4-fluorophenylalanine biosynthetically substituted for F88 of wt-TM-n-Syb (blue diamonds), AA-TM-n-Syb (red circles) and WW-TM-n-Syb (green squares) as a function of sample temperature.

### 3.2. Oxygen-induced paramagnetic contact shifts of F88

Oxygen is a paramagnetic compound; the unpaired electrons of $O_2$ alter the chemical shifts of nearby $^{19}F$ nuclei. The magnitude of this phenomenon, termed the *contact shift*, scales with local [$O_2$]. Owing to the polarity gradient across the micelle, local [$O_2$] is a function of immersion depth in the micelle; at a partial $O_2$ pressure of 20 bar and a temperature of 45 °C, local [$O_2$] spans a factor of seven, from ~15 mM in the bulk water to ~105 mM in micelle core. $O_2$ contact shifts (Fig. 5) thus provide a measure of the relative immersion depth of F88 across the three proteins, with a larger contact shift indicating greater burial in the micelle.

Relative to the wild-type construct, the loss of the tryptophan and introduction of two hydrophobic alanine residues pulls the F88 of AA-TM-n-Syb deeper into the micelle, as evidenced by the larger contact shift (Fig. 5). Conversely, for the wild-type construct, W89 preferentially resides near the micelle–water interface, relegating F88 to a more water-exposed region than in AA-TM-n-Syb, where the observed contact shift is smaller. The addition of a second tryptophan in this region marginally reduces the contact shift of F88, suggesting that the average immersion depth of F88 is not substantially affected by the introduction of a second tryptophan, although this does not preclude the reduction of exchange dynamics by a second tryptophan anchor.

### 3.3. Solvent-induced isotope shifts (SIIS) of F88

The sensitivity of $^{19}F$ chemical shifts to their local environments is such that the substitution of $H_2O$ for $D_2O$ in the solubilizing buffer shifts peaks by as much as 0.25 ppm [48]. The magnitudes of these *solvent-induced isotope shifts* (SIIS) scale with local water concentration and serve as another measure of micelle immersion depth. SIIS values of zero are expected for nuclei located in the micelle center, where water is excluded [47,49]. At the micelle/water interface, where the [$H_2O$] gradient is steep, SIIS values are a function of the immersion depths of $^{19}F$ nuclei in this region [50]. The measurement of SIIS for the three n-Syb constructs (shown in Fig. 6) complements the earlier analysis of $O_2$-contact shifts.

Consistent with the above analysis of the $O_2$-contact shift data, the degree of water exposure at F88 increases with increasing tryptophan content. Comparatively low SIIS values for the F88 residue of AA-TM-n-Syb, are consistent with our assertion that the hydrophobic W89A/L90A substitutions drag F88 into the micelle. In wt-TM-n-Syb, the anchoring of W89 near the micelle–water interface places F88 in a more water exposed region, as evidenced by the greater magnitude of the F88 SIIS in this construct. The addition of a second tryptophan (*i.e.* WW-TM-n-Syb) serves to further push F88 away from the micelle. The magnitudes of the SIIS for all three constructs increase with temperature, indicating a preference for the micelle-bound state at lower temperatures.

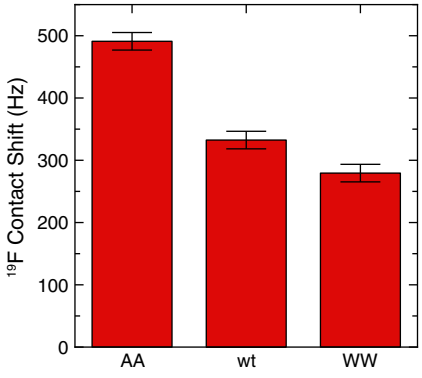


**Fig. 5.** Oxygen-induced contact shifts of 4-fluorophenylalanine biosynthetically substituted for F88 for all three n-Syb constructs, arising from an $O_2$ partial pressure of 20 bar, at 45 °C. Contact shift values are equal to $\delta(O_2)-\delta(He)$.

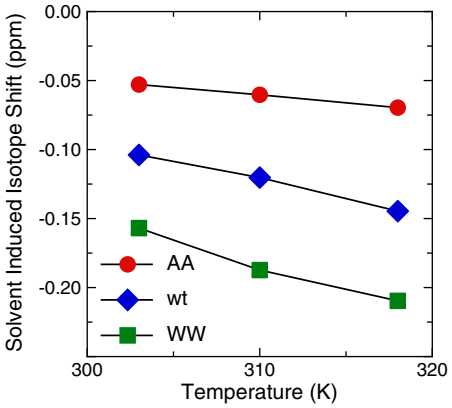


**Fig. 6.** $^{19}F$ solvent induced isotope shifts (SIIS) of 4-fluorophenylalanine biosynthetically substituted for F88 of wt-TM-n-Syb (blue diamonds), AA-TM-n-Syb (red circles) and WW-TM-n-Syb (green squares) as a function of sample temperature. SIIS values are equal to $\delta(D_2O)-\delta(H_2O)$; SIIS values of zero indicate no exposure to water, while increasingly negative values suggest greater exposure. Samples contained 1 mM sodium fluoride, which was used as a chemical shift reference.

## 4. Conclusions

A $^{19}F$ NMR analysis reveals the important role of tryptophan residues on membrane anchoring and dynamics of neuronal-Synaptobrevin. Mutation of the interfacial tryptophan residue (W89) in n-Syb allows hydrophobic considerations to dictate n-Syb immersion depth, resulting in F88 being pulled deeper into the micelle. The presence of an interfacial tryptophan residue anchors n-Syb such that F88 is raised out of the micelle, into a water-exposed region.

$^{19}F$ chemical shifts and linewidths suggest that AA-TM-n-Syb and wt-TM-n-Syb undergo some form of chemical exchange at lower temperatures. We envisage this as a "bobbing" type motion; as F88 is expected to reside near the micelle/water interface, local motions of n-Syb may result in F88 bobbing back and forth between two regions with differing degrees of water exposure. We observed the elimination of this chemical exchange upon the addition of a second tryptophan residue near F88, which we attribute to the increased anchoring effect of the two adjacent tryptophan residues.

We know from previous work that proteins of the Synaptobrevin family are critical for vesicle fusion in general, and neural synaptic transmission in particular. For example, the loss of Synaptobrevin, through genetic techniques, or cleavage of the protein by Tetanus toxin, profoundly impairs synaptic vesicle release [51,52]. Aromatic and charged residues are highly conserved in the juxtamembrane region of Synaptobrevins and may play a role in vesicle fusion.

The role of tryptophan as an anchor to precisely position a protein in a membrane has been demonstrated [20,21]. A functional role for tryptophan has also been shown since elimination of tryptophan residues from viral fusion proteins inhibits viral fusion [39–41]. In a physiological study of VAMP2, the mutation of tryptophan in the linker region to alanine, has been shown to negatively affect neurotransmitter release in cultured hippocampal cells [24], and exocytosis from PC12 cells [27]. It has been hypothesized that strong anchoring of the protein in the membrane by tryptophan is necessary to efficiently transfer energy during SNARE complex to complete membrane fusion [12–14]. However, to our knowledge, no atomic level structural studies of tryptophan to alanine mutations in the Synaptobrevin family have been performed to support this hypothesis. Thus, our work on n-Syb clearly demonstrates tryptophan's role in anchoring and positioning the protein in the membrane and supports the hypothesis

that the deleterious physiological affects observed in different systems may result from decreased anchoring.

While highly conserved, the amino acid sequence of the juxtamembrane region of n-Syb differs from VAMP2 (n-Syb:84-KLKR KFWLQNLK-94; VAMP2:84-KLKRKYWWKNLK-94). The fact that n-Syb has only one tryptophan residue presented us with the opportunity to test the effect of either the addition or removal of one tryptophan on the native structure. The decision to produce a double alanine mutant (*i.e.* AA-TM-n-Syb) rather than a single W89A mutation was made to allow comparison to previous work on double alanine mutants of VAMP2 [24,25,27]. Although mutation of L90 for the less hydrophobic alanine residue may have decreased the immersion depth of F88 in the micelle, we expect this decrease to be minor, without any significant impact on the findings of this study. Interestingly, adding one tryptophan did increase peptide anchoring to the micelle, which is consistent with the anchoring hypothesis. At present we cannot answer the question of why *Drosophila* n-Syb has only one tryptophan while mammalian VAMP2 has two, because there are other differences between the two proteins that we have not yet accounted for. Our WW-TM-n-Syb construct is simply n-Syb with an additional tryptophan and differs from VAMP2 in the SNARE, linker and transmembrane domains. An interesting area to explore is the relationship of these VAMP/Synaptobrevin family members to other species-specific differences in membrane composition or accessory protein structure. For example, mammalian membranes are high in phosphatidylcholine with a substantial amount of the negatively charged phospholipid phosphatidylserine, while dipteran membranes are predominantly composed of phosphatidylethanolamine with very little phosphatidylserine [53]. Answering these questions using different experimental systems, across a variety of model organisms, will help determine the fundamental role that aromatic and charged residues play in membrane fusion.

Altogether our data support the idea that tryptophan residues near the membrane–water interface are important determinants of Synaptobrevin peptide depth within the micelle environment. Our future work will address the physiological importance of these results by introducing these mutants into *Drosophila* synapses using transgenic technology.

## 5. Materials and methods

The cDNA clone for n-Syb in a pOT2 vector (isoform PE; GH4664; chloramphenicol resistance) was obtained from the Drosophila Genomics Resource Center. Primers (Sigma-Aldrich) incorporating NdeI and BamHI restriction sites were used to PCR amplify the coding sequence for the 47 residue peptides (Residues 75–121, SQFEQQAGKLK RKFWLQNLKMMIIMGVIGLVVVGIIANKLGLIGGEQ) encoding a portion of the SNARE domain, the entire linker and transmembrane domains and a portion of the intravesicular domain. The PCR product was purified in 3.5% low melting temperature gel (NuSieve GTG Agarose, Mandel). pET15b vector (Novagen; ampicillin resistance) was digested with NdeI and BamHI, CIP treated and gel purified. The vector was combined with the PCR product in low temperature gel, ligated at room temperature overnight and transformed into DH5α competent cells (New England Biolabs). Site directed mutagenesis was employed to produce the following clones: L90W (WW-TM-n-Syb), W89A L90A (AA-TM-n-Syb), and F77A, which were subsequently confirmed by sequencing (ACGT Corp, Toronto). Clones were transformed into BL-21 (DE3) competent cells (Novagen) along with a vector (Magic, kanamycin resistance) incorporating coding for rare tRNA (gift from Dr. W.S. Trimble, University of Toronto). The final 68 residue peptide incorporated a 6xHis-tag and thrombin cleavage site (MGSSHHHHHHSSGLVPRGS HMSQFEQQAGKLKRKFWLQNLKMMIIMGVIGLVVVGIIANKLGLIGGEQ).

Protein expression in M9 media and subsequent purification was performed based on an established method for membrane proteins [54], and is detailed in the supporting information. 5-fluorotryptophan and 4-fluorophenylalanine-labeled samples were produced by glyphosate-induced auxotrophy of the production of aromatic amino acids [55]. Briefly, as cell cultures reached an OD of 1.0, glyphosate (1 g/L) was added to stop the production of Phe, Trp, and Tyr residues by *E. coli*. Unlabelled tyrosine (37.5 mg/L) was also added at this time. After waiting for 1 h to ensure depletion of any remaining Trp and Phe residues, 5-fluorotryptophan (36 mg/L) and 4-fluorophenylalanine (36 mg/L) were added to the cell culture, and protein expression was immediately induced using IPTG. After 6 h, cells were collected by centrifugation and stored at −20 °C. Protein purification (supporting information) was conducted using a denaturing protocol based on that described by Sanders and co-workers [56].

NMR experiments were performed on a 600 MHz Varian Inova spectrometer equipped with a triple resonance cold probe capable of tuning to $^{19}F$ (564 MHz), or a triple resonance room temperature probe also capable of tuning to $^{19}F$. Typical HSQC spectra were acquired with 64 scans per increment and 160 increments, and processed with the NMRPIPE processing suite. $^{19}F$ 1D spectra were typically acquired in 1 h, with 2048 scans and an interscan delay of 1.3 s. $^{19}F$ spectra were processed using both VNMRJ (Agilent) and MestReNova (Mestrec). A fifth order polynomial baseline correction was applied to remove $^{19}F$ signals arising from the fluorinated materials in the probe assembly.

$O_2$-PRE values were obtained using a 5 mm sapphire NMR tube (Saphikon, NH), with a home-built Swage-lok fitting. Samples were pre-equilibrated at an $O_2$ partial pressure of 20 bar for at least two days prior to measuring relaxation rates to ensure a constant [$O_2$] during the experiments. Relaxation rates under diamagnetic conditions were acquired on samples pressurized with He gas to a pressure of 20 bar.

Solvent-induced isotope shifts were obtained as the difference in chemical shift for samples pure (99%) $D_2O$ (Cambridge Isotopes Laboratories, Andover, MA) and samples in 10% $D_2O$/90% $H_2O$ (*i.e.* $SIIS = \delta_{100\%\ D2O} - \delta_{10\%\ D2O/90\%\ H2O}$). Sodium fluoride (1 mM) was added to all samples as a chemical shift reference. In a separate experiment, the precise chemical shifts of NaF in 100% $D_2O$ and in 10% $D_2O$/90% $H_2O$ were simultaneously measured using a coaxial NMR tube.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.07.018.

## References

[1] L.V. Chernomordik, M.M. Kozlov, Protein-lipid interplay in fusion and fission of biological membranes, Annu. Rev. Biochem. 72 (2003) 175–207.
[2] A.T. Brunger, Structure and function of SNARE and SNARE-interacting proteins, Q. Rev. Biophys. 38 (2005) 1–47.
[3] R. Jahn, R.H. Scheller, SNAREs—engines for membrane fusion, Nat. Rev. Mol. Cell Biol. 7 (2006) 631–643.
[4] R.B. Sutton, D. Fasshauer, R. Jahn, A.T. Brunger, Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature 395 (1998) 347–353.
[5] M.K. Bennett, N. Calakos, R.H. Scheller, Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones, Science 257 (1992) 255–259.
[6] G.A. Oyler, G.A. Higgins, R.A. Hart, E. Battenberg, M. Billingsley, F.E. Bloom, M.C. Wilson, The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations, J. Cell Biol. 109 (1989) 3039–3052.
[7] W.S. Trimble, D.M. Cowan, R.H. Scheller, VAMP-1: a synaptic vesicle-associated integral membrane protein, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 4538–4542.
[8] L.A. Elferink, W. Trimble, R. Scheller, Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system, J. Biol. Chem. 264 (1989).
[9] S.Y. Hua, M.P. Charlton, Activity-dependent changes in partial VAMP complexes during neurotransmitter release, Nat. Neurosci. 2 (1999) 1078–1083.
[10] Z.P. Pang, O.H. Shin, A.C. Meyer, C. Rosenmund, T.C. Südhof, A gain-of-function mutation in synaptotagmin-1 reveals a critical role of Ca2+-dependent soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex binding in synaptic exocytosis, J. Neurosci. 26 (2006) 12556–12565.
[11] M.A. Poirier, W. Xiao, J.C. Macosko, C. Chan, Y.K. Shin, M.K. Bennett, The synaptic SNARE complex is a parallel four-stranded helical bundle, Nat. Struct. Biol. 5 (1998) 765–769.

[12] R.C. Lin, R.H. Scheller, Structural organization of the synaptic exocytosis core complex, Neuron 19 (1997) 1087–1094.
[13] J.A. McNew, T. Weber, D.M. Engelman, T.H. Söllner, J.E. Rothman, The length of the flexible SNAREpin juxtamembrane region is a critical determinant of SNARE-dependent fusion, Mol. Cell 4 (1999) 415–421.
[14] J.A. McNew, T. Weber, F. Parlati, R.J. Johnston, T.J. Melia, T.H. Söllner, J.E. Rothman, Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors, J. Cell Biol. 150 (2000) 105–117.
[15] J.E. Gerst, L. Rodgers, M. Riggs, M. Wigler, SNC1, a yeast homolog of the synaptic vesicle-associated membrane protein/synaptobrevin gene family: genetic interactions with the RAS and CAP genes, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 4338–4342.
[16] T. Sudhof, M. Baumert, M. Perin, R. Jahn, A synaptic vesicle membrane protein is conserved from mammals to drosophila, Neuron 2 (1989) 1475–1481.
[17] M. Baumert, P.R. Maycox, F. Navone, P. De Camilli, R. Jahn, Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain, EMBO J. 8 (1989) 379–384.
[18] W.S. Trimble, T.S. Gray, L.A. Elferink, M.C. Wilson, R.H. Scheller, Distinct patterns of expression of two VAMP genes within the rat brain, J. Neurosci. 10 (1990) 1380–1387.
[19] B.T. Archer, T. Ozçelik, R. Jahn, U. Francke, T.C. Sudhof, Structures and chromosomal localizations of two human genes encoding synaptobrevins 1 and 2, J. Biol. Chem. 265 (1990) 17267–17273.
[20] E.K. Esbjörner, C.E.B. Caesar, B. Albinsson, P. Lincoln, B. Nordén, Tryptophan orientation in model lipid membranes, Biochem. Biophys. Res. Commun. 361 (2007) 645–650.
[21] J. Killian, G. von Heijne, How proteins adapt to a membrane–water interface, Trends Biochem. Sci. 25 (2000) 429–434.
[22] D.-H. Kweon, C.S. Kim, Y.-K. Shin, Insertion of the membrane-proximal region of the neuronal SNARE coiled coil into the membrane, J. Biol. Chem. 278 (2003) 12367–12373.
[23] A. Stein, G. Weber, M. Wahl, R. Jahn, Helical extension of the neuronal SNARE complex into the membrane, Nature 460 (2009) 525–528.
[24] A. Maximov, J. Tang, X. Yang, Z.P. Pang, T.C. Sudhof, Complexin controls the force transfer from SNARE complexes to membranes in fusion, Science 323 (2009) 516–521.
[25] T.J. Siddiqui, O. Vites, A. Stein, R. Heintzmann, R. Jahn, D. Fasshauer, Determinants of synaptobrevin regulation in membranes, Mol. Biol. Cell 18 (2007) 2037–2046.
[26] D. Kweon, C. Kim, Y. Shin, Regulation of neuronal SNARE assembly by the membrane, Nat. Struct. Biol. 10 (2003) 440–447.
[27] S. Quetglas, C. Iborra, N. Sasakawa, L. De Haro, K. Kumakura, K. Sato, C. Leveque, M. Seagar, Calmodulin and lipid binding to synaptobrevin regulates calcium-dependent exocytosis, EMBO J. 21 (2002) 3970–3979.
[28] G. von Heijne, Membrane proteins: from sequence to structure, Annu. Rev. Biophys. Biomol. Struct. 23 (1994) 167–192.
[29] W.-M. Yau, W.C. Wimley, K. Gawrisch, S.H. White, The preference of tryptophan for membrane interfaces, Biochemistry 37 (1998) 14713–14718.
[30] C. Landolt-Marticorena, K. Williams, C. Deber, R. Reithmeier, Non-random distribution of amino acids in the transmembrane segments of human type I single span membrane proteins, J. Mol. Biol. 229 (1993) 602–608.
[31] M.R. de Planque, B.B. Bonev, J.A. Demmers, D.V. Greathouse, R.E. Koeppe 2nd, F. Separovic, A. Watts, J.A. Killian, Interfacial anchor properties of tryptophan residues in transmembrane peptides can dominate over hydrophobic matching effects in peptide-lipid interactions, Biochemistry 42 (2003) 5341–5348.
[32] A.N. Ridder, S. Morein, J.G. Stam, A. Kuhn, B. de Kruijff, J.A. Killian, Analysis of the role of interfacial tryptophan residues in controlling the topology of membrane proteins, Biochemistry 39 (2000) 6521–6528.
[33] K. Kachel, E. Asuncion-Punzalan, E. London, Anchoring of tryptophan and tyrosine analogs at the hydrocarbon-polar boundary in model membrane vesicles: parallax analysis of fluorescence quenching induced by nitroxide-labeled phospholipids, Biochemistry 34 (1995) 15475–15479.
[34] G.S. Laco, Y. Pommier, Role of a tryptophan anchor in human topoisomerase I structure, function and inhibition, Biochem. J. 411 (2008) 523–530.
[35] K. Hu, J. Carroll, C. Rickman, B. Davletov, Action of complexin on SNARE complex, J. Biol. Chem. 277 (2002) 41652–41656.
[36] R. Jahn, T.C. Sudhof, Membrane fusion and exocytosis, Annu. Rev. Biochem. 68 (1999) 863–911.
[37] L. de Haro, G. Ferracci, S. Opi, C. Iborra, S. Quetglas, R. Miquelis, C. Leveque, M. Seagar, Ca2+/calmodulin transfers the membrane-proximal lipid-binding domain of the v-SNARE synaptobrevin from cis to trans bilayers, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 1578–1583.
[38] M. Bowen, A. Brunger, Conformation of the synaptobrevin transmembrane domain, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 8378–8383.
[39] S. Galdiero, A. Falanga, M. Vitiello, L. Raiola, R. Fattorusso, H. Browne, C. Pedone, C. Isernia, M. Galdiero, Analysis of a membrane interacting region of herpes simplex virus type 1 glycoprotein H, J. Biol. Chem. 283 (2008) 29993–30009.
[40] K. Salzwedel, J. West, E. Hunter, A conserved tryptophan-rich motif in the membrane-proximal region of the human immunodeficiency virus type 1 gp41 ectodomain is important for Env-mediated fusion and virus infectivity, J. Virol. 73 (1999) 2469–2480.
[41] A. Lai, L. Tamm, Locking the kink in the influenza hemagglutinin fusion domain structure, J. Biol. Chem. 282 (2007) 23946–23956.
[42] M. Freitas, L. Gaspar, M. Lorenzoni, F. Almeida, L. Tinoco, M. Almeida, L. Maia, L. Degrève, A. Valente, J. Silva, Structure of the Ebola fusion peptide in a membrane-mimetic environment and the interaction with lipid rafts, J. Biol. Chem. 282 (2007) 27306–27314.
[43] J. White, S. Delos, M. Brecher, K. Schornberg, Structures and mechanisms of viral membrane fusion proteins: multiple variations on a common theme, Crit. Rev. Biochem. Mol. 43 (2008) 189–219.
[44] D.L. Dertcher, A. Ueda, B.A. Stewart, R.W. Burgess, Y. Kidokoro, T.L. Schwarz, Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the Drosophila gene neuronal-synaptobrevin, J. Neurosci. 18 (1998) 2028–2039.
[45] A. DiAntonio, R.W. Burgess, A.C. Chin, D.L. Deitcher, R.H. Scheller, T.L. Schwarz, Identification and characterization of Drosophila genes for synaptic vesicle proteins, J. Neurosci. 13 (1993) 4924–4935.
[46] M. Respondek, T. Madl, C. Goebl, R. Golser, K. Zangger, Mapping the orientation of helices in micelle-bound peptides by paramagnetic relaxation waves, J. Am. Chem. Soc. 129 (2007) 5228–5234.
[47] W.E. Hull, B.D. Sykes, Fluorine-19 nuclear magnetic-resonance study of fluorotyrosine alkaline-phosphatase — influence of zinc on protein structure and a conformational change induced by phosphate binding, Biochemistry 15 (1976) 1535–1546.
[48] J.T. Gerig, Fluorine NMR of proteins, Prog. Nucl. Magn. Reson. Spectrosc. 26 (1994) 293–370.
[49] D.S. Hagen, J.H. Weiner, B.D. Sykes, Investigation of solvent accessibility of the fluorotyrosyl residues of M13 coat protein in deoxycholate micelles and phospholipid-vesicles, Biochemistry 18 (1979) 2007–2012.
[50] P.E. Hansen, H.D. Dettman, B.D. Sykes, Solvent-induced deuterium-isotope effects on F-19 chemical-shifts of some substituted fluorobenzenes — formation of inclusion complexes, J. Magn. Reson. 62 (1985) 487–496.
[51] G. Schiavo, F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B.R. DasGupta, C. Montecucco, Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin, Nature 359 (1992) 832–835.
[52] S.T. Sweeney, K. Broadie, J. Keane, H. Niemann, C.J. O'Kane, Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects, Neuron 14 (1995) 341–351.
[53] A. Luukkonen, M. Brummer-Korvenkontio, O. Renkonen, Lipids of cultured mosquito cells (Aedes albopictus). Comparison with cultured mammalian fibroblasts (BHK 21 cells), Biochim. Biophys. Acta 326 (1973) 256–261.
[54] K. Oxenoid, H. Kim, J. Jacob, F. Sonnichsen, C.R. Sanders, NMR assignments for a helical 40 kDa membrane protein in 100 kDa micelles, J. Am. Chem. Soc. 126 (2004) 5048–5049.
[55] J.L. Kitevski-Leblanc, F. Evanics, R.S. Prosser, Approaches for the measurement of solvent exposure in proteins by F-19 NMR, J. Biomol. NMR 45 (2009) 255–264.
[56] C. Tian, M.D. Karra, C.D. Ellis, J. Jacob, K. Oxenoid, F. Sönnichsen, C.R. Sanders, Membrane protein preparation for TROSY NMR screening, Methods Enzymol. 394 (2005) 321–334.