Munc18a Regulation of SNARE Complex Assembly

Keywords: neuron, exocytosis, membrane fusion, Munc18a, SNAREs, Syntaxin1a, PKC

In the brain, neurons communicate across specialized intercellular junctions called synapses. Arrival of an action potential in a pre-synaptic neuron triggers a calcium (Ca^{2+}) influx, which leads to exocytosis of pre-synaptic vesicles and release of neurotransmitters for uptake via receptors in the post-synaptic neuron. Fusion of synaptic vesicles to the pre-synaptic membrane is a critical step in Ca^{2+}-triggered exocytosis, and is mediated by Soluble NSF Attachment protein Receptors (SNAREs). Three neuronal SNARE proteins, Syntaxin1a, SNAP-25 and VAMP combine to form a four-helix bundle, a stable complex essential for membrane fusion[1] (Figure 1). A neuronal protein, Munc18a, is required for synaptic vesicle fusion and regulates SNARE complex assembly through its interaction with Syntaxin1a. Syntaxin1a contains a conserved N-terminal peptide (aa 1-9) followed by the Habc domain, a linker, and the H3 domain involved in SNARE protein binding (Figure 2). Munc18a binds the N-peptide, Habc domain and H3 domain of Syntaxin1a, rendering the latter inaccessible to its SNARE binding partners, effectively preventing SNARE complex assembly[2,3].

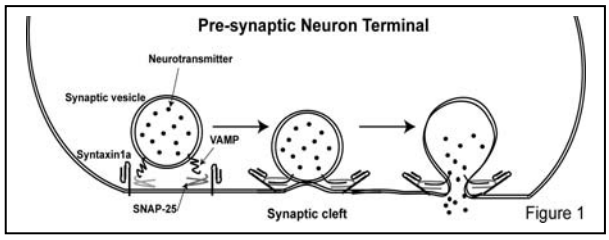

Figure 1

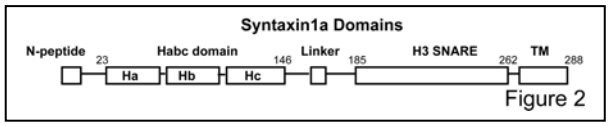

Figure 2

A recent finding shows that Munc18a in complex with Syntaxin1a lacking its N-peptide allows SNARE complex assembly *in vitro* upon addition of SNAP-25 and VAMP[3]. Hence the interaction of the N-peptide of Syntaxin1a with Munc18a plays a key role in the regulation of SNARE complex assembly. This result prompted us to solve the crystal structure (unpublished) of Munc18a bound to Syntaxin1a lacking its N-peptide (Syx^{25-262}) for comparison with the known crystal structure[2] of Munc18a in complex with full-length Sytnaxin1a (Syx^{1-262}). We found only slight conformational differences between the two complexes. Importantly, one such difference is seen in the vicinity of two serines (S306 and S313) on Munc18a that are known phosphorylation sites for protein kinase C (PKC)[4]. PKC phosphorylation of Munc18a enhances pre-synaptic vesicle fusion and neurotransmitter release[5], but the molecular mechanism of this pathway is unclear. I hypothesize that the synergistic actions of Syntaxin1a N-peptide removal and PKC-catalyzed phosphorylation of Munc18a expose the SNARE-binding domain of Syntaxin1a, thus facilitating SNARE complex assembly (Figure 3). The following aims will elucidate the roles of Munc18a phosphorylation and Syntaxin1a N-peptide removal in SNARE complex assembly.

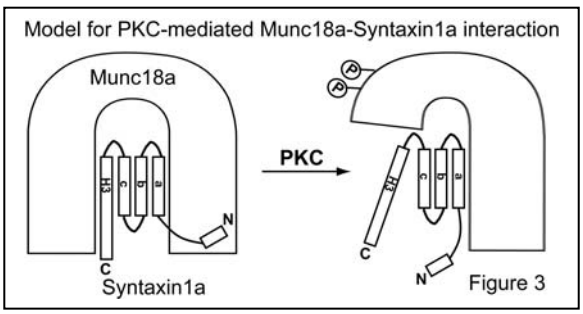

Figure 3

Aim 1*: Determine quantitatively the thermodynamics of the Munc18a-Syntaxin1a interaction.* To test the combined effects of Munc18a phosphorylation and Syntaxin1a N-peptide removal, I will use Isothermal Titration Calorimetry (ITC) to measure the *in vitro* thermodynamics of interactions between PKC-phosphorylated Munc18a and full-length Syntaxin1a (Syx^{1-262}) or Syntaxin1a lacking its N-peptide (Syx^{25-262}). Munc18a will be phosphorylated *in vitro* using PKC, ATP, and Ca^{2+} (to activate PKC). Phosphorylation efficiency will be determined in a separate experiment using γ-^{32}P labeled ATP and subjecting the reaction sample to SDS-PAGE,

followed by protein staining and autoradiography. Combining Syx^{1-262} or Syx^{25-262} with non-phosphorylated Munc18a will serve as an experimental control. If phosphorylation is inefficient, three different Munc18a phosphomimetic mutants will be used for ITC: $Munc18a^{S306E}$, $Munc18a^{S313E}$ and the double mutant $Munc18a^{S306E/S313E}$. Munc18a mutants will be generated using site-directed mutagenesis. The single phosphomimetic Munc18a mutants binding either form of Syntaxin1a will indicate the relative contributions of the individual phosphorylation sites to complex stability. Based on my hypothesis, the combination of Syx^{25-262} and the PKC-phosphorylated Munc18a (or $Munc18a^{S306E/S313E}$) should have the weakest affinity, as this interaction is predicted to confer the SNARE-binding conformation of Syntaxin1a, with fewer residues in contact with Munc18a (Figure 3).

Aim 2: ***Determine whether phosphorylation of Munc18a and removal of Syntaxin1a N-peptide facilitate SNARE complex assembly.*** Syntaxin1a, SNAP-25 and VAMP assemble into a highly stable SNARE complex *in vitro*, which is SDS-resistant and dissociates only upon boiling[6]. I will take advantage of this property to test the effect of Munc18a phosphorylation on *in vitro* SNARE complex assembly. Each Syntaxin1a (Syx^{1-262} or Syx^{25-262}) will be pre-incubated with PKC-phosphorylated Munc18a or with the different phosphomimetic Munc18a mutants described above. Munc18a will be used in excess to eliminate any free Syntaxin1a. SNAP-25 and VAMP will then be added and the samples will be subjected to SDS-PAGE, with and without boiling. The components of the boiled samples should run as separate bands on an SDS gel. Non-boiled samples in which SNARE complex formation occurs will run as two bands corresponding to Munc18a and the intact SNARE complex. According to my hypothesis, loss of Syntaxin1a N-peptide or phosphorylation of Munc18a should facilitate SNARE complex assembly. A more quantitative fluorescence-based assay will be used to measure the rate of SNARE complex formation. Upon addition of SNAP-25 and fluorescently-labeled VAMP, the phosphorylated Munc18a-Syx^{25-262} combination is expected to yield the fastest SNARE complex assembly rate.

Aim 3: ***Determine whether phosphorylated Munc18a exposes the SNARE binding domain in Syntaxin1a lacking its N-peptide.*** Given my hypothesis, I expect phosphorylation of Munc18a to induce a conformational change such that the H3 domain of Syx^{25-262} no longer interacts with Munc18a, but is instead exposed for SNARE protein binding (Figure 3). I will obtain high-resolution atomic structures of the PKC-phosphorylated Munc18a-Syx^{25-262} complex, and/or of any Munc18a phosphomimetic mutants in complex with Syx^{25-262} to confirm or refute the hypothesis. Initial crystal growth conditions will be similar to those that yielded the crystals used to solve the wt Munc18a-Syx^{1-262} structure[2]. If necessary, a more general crystallization screen will be carried out. Crystal diffraction will be screened at the Stanford Synchrotron Radiation Laboratory (SSRL). The structures will be computed via molecular replacement using the structure of the Munc18a-Syx^{1-262} complex.

Studying the molecular machinery responsible for the exquisite regulation of synaptic neurotransmission is essential to our understanding of memory and learning. My experience with X-ray crystallography as an undergraduate has cultivated a deep interest and enthusiasm to study protein structure and function in graduate school. Ultimately, I hope to solve relevant problems investigating the structural basis of biology at the molecular and cellular levels.

Statement of Originality: I certify this proposal represents my own original work.

References: 1. Sutton, R et al. *Nature*, 395: 347-353 (1998) **2.** Misura, K et al. *Nature*, 404: 355-362 (2000) **3.** Burkhardt, P et al. In submission. **4.** Fujita, Y et al. *Journal of Biological Chemistry*, 271(13): 7265-7268 (1996) **5.** Majewski, H et al. *Progress in Neurobiology*, 55: 463-475 (1998) **6.** Hayashi, T et al. *EMBO J*, 13(21): 5051-5061 (1994)