In search of the molecular mechanism of intracellular membrane fusion and neurotransmitter release

Richard H Scheller

Along with the honor of receiving the Albert Lasker Basic Medical Research Award, I welcome the opportunity to reflect on a number of discoveries that I have been involved in. My goal is to show how a series of ideas evolved from simple models to more complex hypotheses resulting in the understanding, in molecular detail, of a complex biological process.

It is likely that all scientists or maybe even most people have wondered where thoughts come from, how memory works or how it is that we have feelings and emotions. These questions had fascinated me since my earliest days as a scientist. Specifically, how could I, a molecular biologist and biochemist, contribute to understanding how the brain works? I was lucky enough to stumble upon the perfect situation to begin a career dedicated to understanding these problems. As a postdoc, I had the great fortune to work at Columbia University's College of Physicians and Surgeons with Richard Axel and Eric Kandel. We applied the relatively new techniques of molecular biology to problems in neuroscience working together on the egg-laying behavior of the marine snail *Aplysia californica*. After a number of fun discoveries, I became an alumnus of what should now be called the 'Columbia School of Neuroscience' owing to the prominence of ideas that have originated from this institution.

Research in my laboratory in the Department of Biological Sciences at Stanford University began by continuing our work on neuropeptides in *Aplysia*. Along the way, I met Jack McMahon, a faculty member in the Neuroscience Department at Stanford, and we struck up a collaboration to better understand the development of the neuromuscular junction. McMahon's lab had characterized a protein he named agrin, which has a critical role in organizing the acetylcholine receptors beneath the presynaptic nerve terminal. He had raised an antibody against the protein and determined a portion of the amino acid sequence. To clone the agrin gene, we made an expression cDNA library from neurons of the electric lobe of the marine ray, *Torpedo californica*, the species used to purify agrin.

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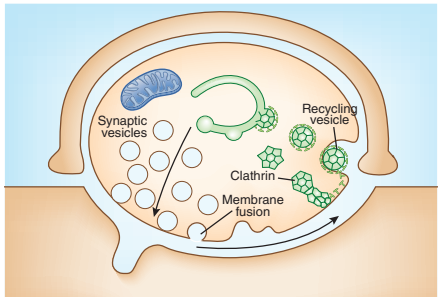

Figure 1 The mechanism of neurotransmitter release and recycling. Vesicles dock at the presynaptic membrane and, upon calcium entry into the terminal, fuse with the presynaptic plasma membrane, releasing neurotransmitter[1].

At the same time, I began thinking about other problems in neuroscience, and for me, the mechanism of neurotransmitter release at the presynaptic nerve terminal was particularly interesting. Owing to the work of many who preceded me, a general outline of the cellular process was quite well understood (**Fig. 1**). Classic electron microscopic studies had established that the transmitter-containing vesicles are stored at a region called the active zone. When the action potential invades the terminal, channels open, allowing calcium to flow into the nerve ending, and this triggers the fusion of the synaptic vesicle membrane with the presynaptic membrane and transmitter release. The membrane then recycles, resulting in new vesicles for another round of release[1].

Essentially nothing was known about the molecular mechanisms that governed this process. I remembered that the synaptic vesicles were studied from marine rays and that the vesicles had been purified from the electric organ of these interesting animals. In fact, a colleague at the University of California–San Francisco, Reg Kelly, had made an antibody against purified vesicles[2]. My idea was that we could screen the expression library that we had made to clone the agrin cDNA with the antibody raised against purified vesicles and that this should result in cDNA clones encoding proteins of the vesicle, some of which must be involved in transmitter release. A postdoc (Bill Trimble), a graduate student (Jim Campenelli)

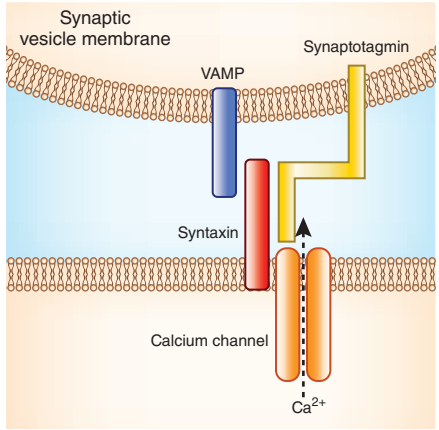

Figure 2 An initial model of the vesicle fusion complex. VAMP-1 and synaptotagmin on the synaptic vesicle membrane interact with syntaxin on the plasma membrane, which associates in turn with the calcium channel[4].

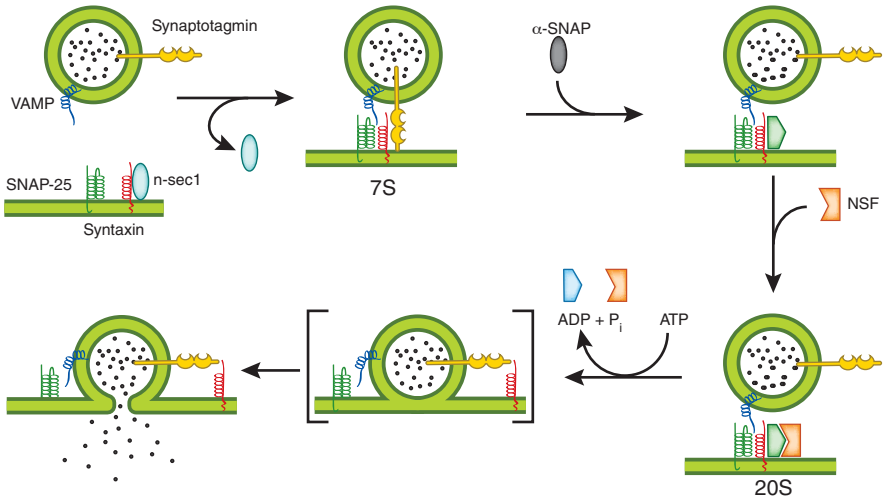

Figure 3 Model of the membrane fusion reaction. A series of assembly and disassembly reactions was proposed to mediate membrane fusion and vesicle targeting specificity[6].

and I did the screen and identified a number of positive clones. Trimble went on to characterize one of the positive clones, which we called vesicle-associated membrane protein-1 (VAMP-1)[3].

Another postdoc, Mark Bennett, and an MD-PhD student, Nicole Calakos, then joined our effort. They immunoprecipitated detergent-solubilized membranes from rat brain with an antibody against synaptotagmin (then called p65), a protein we now know to be the calcium sensor for neurotransmitter release. We isolated a 35-kDa protein and characterized it by determining a portion of the amino acid sequence and cloning the cDNA. We named this protein syntaxin[4]. Interestingly, syntaxin, unlike VAMP-1, was largely localized to the plasma membrane. This was something we were hoping to find because this established a molecular link between the pre- and postsynaptic membranes. An initial model of these interactions is shown in **Figure 2**. At the time, we felt that these molecules and their interactions had to be important in the release process, but we did not know how. We suggested that these proteins formed a scaffold for assembly of the soluble factors α-SNAP and *N*-ethylmaleimide–sensitive factor (NSF) as these molecules were known to be involved in vesicle trafficking and membrane fusion from studies in yeast and mammalian species[4].

Sure enough, James Rothman's group showed that VAMP-1, syntaxin and a third protein, synaptosomal protein of 25 kDa (SNAP-25), bound a column with α-SNAP attached and that the three proteins were released when ATP was hydrolyzed by NSF[5]. At this time, the three proteins, VAMP-1, syntaxin and SNAP-25 became known as SNAP receptor proteins (SNAREs). Rothman proposed the SNARE hypothesis, which postulates that families of SNARE proteins decorate membrane compartments and that the specificity of membrane fusion is achieved by the formation of protein complexes[5]. So, VAMP-1 turned out to be the first vesicle SNARE (v-SNARE), and syntaxin was the first target SNARE (t-SNARE).

This led to a collaboration between Rothman's group and my own lab, which resulted in the model shown in **Figure 3**. The idea was that VAMP-1 and synaptotagmin, another vesicle protein, bind SNAP-25 and syntaxin on the plasma membrane; we called this the 7S complex. After adding α-SNAP, we showed that synaptotagmin was displaced from the complex, and, upon addition of NSF, a larger complex formed, which we called the 20S complex. Upon ATP hydrolysis, not only did NSF and α-SNAP dissociate from the complex, but also the SNARE complex itself disassembled[6].

I knew this could not be the full fusion reaction; calcium triggered transmitter release so rapidly that there was not enough time for ATP hydrolysis and complex disassembly to take place. To reconcile this, I proposed an intermediate step in the process (shown in brackets in **Fig. 3**) that would lead to membrane fusion and transmitter release upon calcium influx. It was also at this time that another soluble factor known to be important in exocytosis in yeast, called sec1, was added to the picture. Our group at Stanford and Thomas Südhof's group at the University of Texas Southwestern showed that neuronal Sec1 (n-sec1) bound syntaxin. We showed biochemically and using crystallographic methods that the conformation of syntaxin bound to n-sec1 was incompatible with SNARE complex formation[7]. We referred to this as the closed conformation of syntaxin and proposed that conformational changes lead to the opening of syntaxin before initiation of the SNARE complex formation.

Hugh Pelham next posed the question of whether the SNARE complex formed a helical bundle in a parallel or antiparallel fashion. A parallel formation might result in the actual fusion of the membranes, whereas an antiparallel organization would suggest that the complex was more important in docking the vesicle at the acceptor membrane (**Fig. 4**). Using fluorescence resonance energy transfer studies, an MD-PhD student, Richard Lin, showed a parallel organization[8], which was also seen at higher resolution in the crystal structure obtained by Reinhard Jahn and Axel Brunger[9]. This resulted in the general model of membrane fusion that we now know to be correct (**Fig. 5a**)[8]. The SNARE pairing drives membrane fusion, α-SNAP and NSF dissociate, and then the SNARE complex disassembles to allow recycling.

At this point, though, the model had very little experimental support. In my lab, a graduate student, Yu Chen, and a postdoc, Suzie Scales, decided to investigate the model in PC12 cells that had been 'cracked open'. One could add back cytosol and ATP in a priming step and trigger release of radioactive norepinephrine with the addition of calcium[10]. But how could one manipulate exocytosis if the endogenous SNAREs were present in this system? In the intervening years, it had been shown that the clostridial and botulinum neurotoxins cleaved the SNARE proteins and that this was their mechanism of action. By cleaving one of the four coils of the SNARE complex with

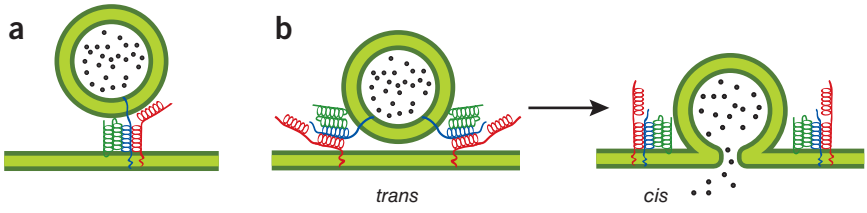

Figure 4 The two possible engagements of SNARE coils suggested different functions. (**a**) An antiparallel arrangement suggested a role for the complex in vesicle docking. (**b**) A parallel arrangement suggested a role in membrane fusion. In this orientation the *trans*-SNARE complex would bring opposing membranes together and mediate the fusion. The result of the fusion reaction is a *cis*-SNARE complex.

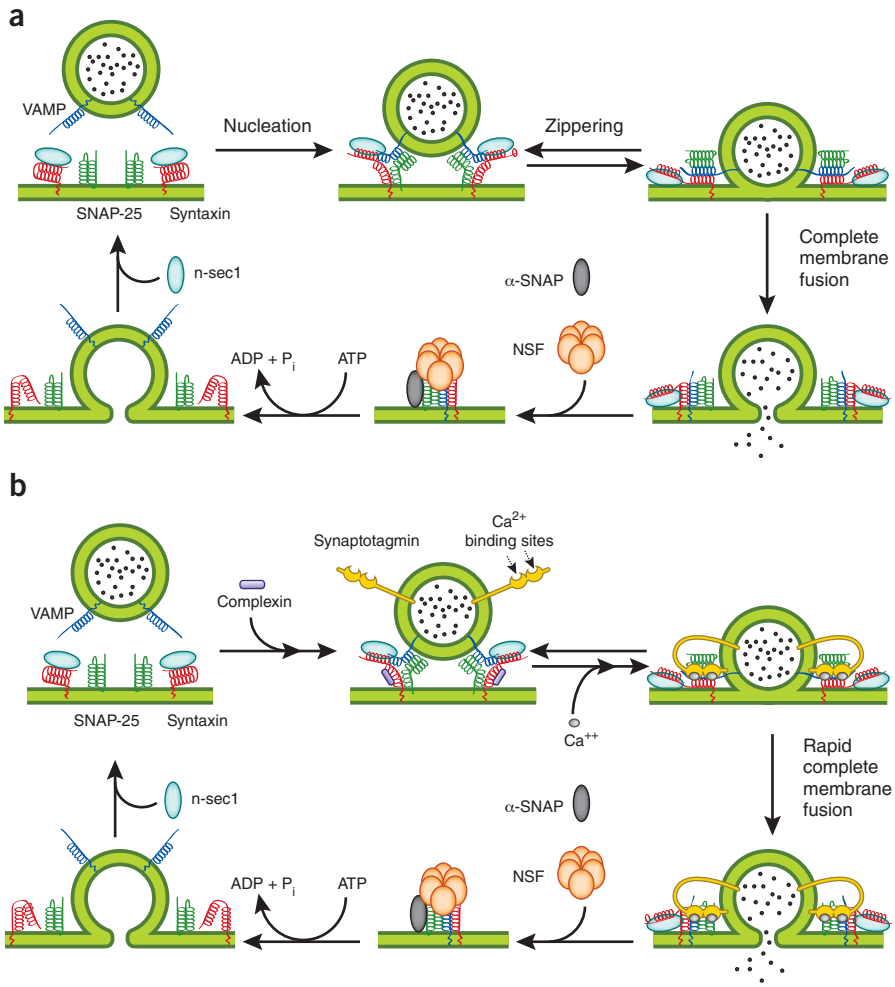

Figure 5 A later model of the vesicle fusion complex. (**a**) The general mechanism of membrane fusion mediated by SNARE proteins[8]. (**b**) Regulatory proteins synaptotagmin and complexin regulate calcium sensing in rapid neurotransmitter release[12].

botulinum neurotoxin E and washing away the fragment, we inhibited membrane fusion and transmitter release. By adding back the coil, we showed that release was rescued. Now we had a way to study the relationship between SNARE complex formation, calcium dependence and norepinephrine release. We could also add back mutant coils to see how these mutants affected the release process. From these studies, we concluded that SNARE complex formation drove the fusion reaction, that the full formation of the complex only occurred in the presence of calcium and that the energy of complex formation was used to drive the fusion reaction[11]. In a series of elegant studies, Südhof and his group unraveled the mechanism of calcium sensing and the roles of complexin and synaptotagmin. These discoveries are discussed in his accompanying paper[12] (**Fig. 5b**). Using the cracked PC12 cell system, we were also able to show that the central part of the SNARE helical bundle is required for complex dissociation by NSF[13] and that three SNARE complexes are required to mediate a fusion event[14].

After the initial isolation of VAMP-1, syntaxin and SNAP-25, it became clear that these molecules were the founding members of protein families. Mark Bennett, Jesse Hay, Raj Advani, Jason Bock and others in my group characterized over 30 different SNARE proteins from mammalian cells[15–17]. These proteins were broadly but differentially expressed in different cell types. With immunoelectron microscopy expert Judith Klumperman, we showed that these SNAREs distinctly localize to specific compartments of the secretory pathway. We were again able to use the PC12 system to show that only specific sets of SNAREs could form complexes that resulted in vesicle fusion[18]. This work established that the specific localization and pairing of the SNARE proteins contributes to organizing and maintaining the discrete membrane compartments in cells (**Fig. 6**). Work from Rothman's lab showed that specific SNARE pairs support fusion in a reconstituted system, confirming the SNARE hypothesis, although perhaps not exactly as originally proposed[19].

Finally, the genetic approach taken in yeast to study membrane trafficking converged on the same set of proteins discovered in the studies described above. This was not clear initially because VAMP-1 and syntaxin are duplicated genes in yeast, and they were not found in the original screen. Thus, the membrane fusion machinery described above is conserved in all eukaryotic organisms[20]. Now that we know this is the case, it seems obvious that the nervous

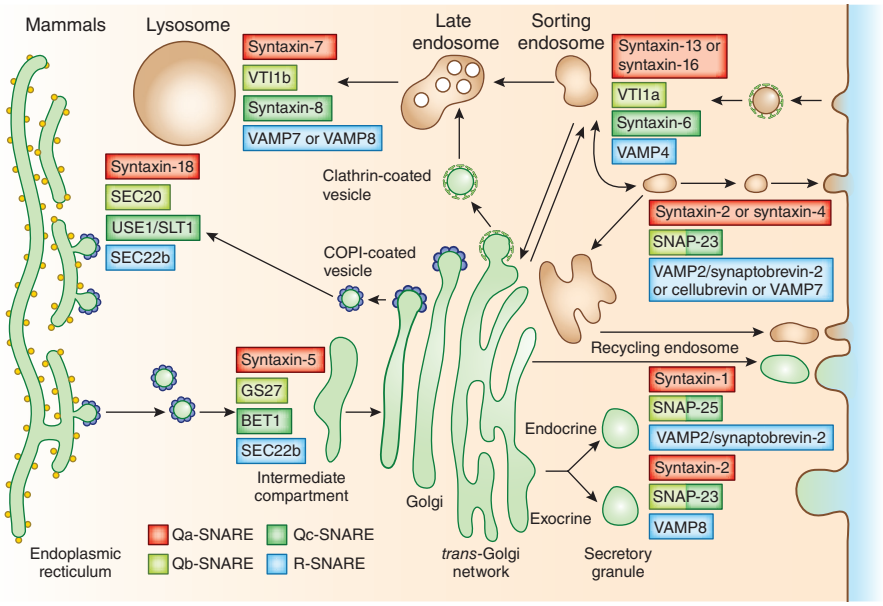

Figure 6 Specific sets of SNARE proteins mediate intracellular membrane trafficking in all cells. Each of the four SNAREs required to mediate membrane fusion at various positions in the secretory pathway are indicated.

system would use this evolutionarily ancient mechanism of membrane fusion with superimposed regulatory proteins to mediate neurotransmitter release.

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COMPETING FINANCIAL INTERESTS

The author declares competing financial interests: details accompany the online version of the paper.

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