Presynaptic mechanisms: neurotransmitter release, synaptic vesicle cycle

For a basic review and background, Fundamental Neuroscience (Squire et al): Chapter 8.

Other reviews:

- Jahn et al (2003) Membrane Fusion. Cell 112, 519-533.
- Rizo and Sudhof (2002) SNAREs and Munc18 in synaptic vesicle fusion. Nat Review Neurosci 3, 641-653.
- Stevens CF (2003). Neurotransmitter release at central synapses. Neuron 40, 381-388.

Also see: "Cycling of Synaptic Vesicles: How Far? How Fast!" by Thierry Galli, and Volker Haucke *Science's STKE,* 26 June 2001. (A website with animations and figures showing the synaptic vesicle cycle.)
http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2001/88/re1#SEC1

Neurotransmitter (NT) release

At the physiological level, concepts of Bernard Katz dominate historically (based largely on studies of NMJ).

Quantal Hypothesis:

Morphological correlate = synaptic vesicle, which are relatively uniform in size (40-50 nm)
Quantal synaptic transmission corresponds to exocytosis of single or integral number of synaptic vesicles (SVs).
Quantal release gives rise to small postsynaptic signal, known as miniature EPSP/C or IPSP/C.
Quantal release occurs spontaneously, infrequently – but is accelerated and synchronized by action potential invading the terminal.
Single SV contains ~5000 molecules of neurotransmitter.

Mean number of quanta released per impulse = **N** (number of release sites) x **p** (probability of release per release site)

At NMJ, hundreds of quanta can be released from a large number of release sites. (NMJ is an all-or-none synapse, designed to faithfully transmit.)

Current view: central synapses probably have 1 release site (corresponding to 1 active zone), which releases only 1 SV (even when p approaches 1).
However, still contentious: there is evidence for multivesicular release in response to single impulse in certain synapses.

Quantal release via SVs implies membrane fusion, re-endocytosis (membrane recycling in axon terminal)

The rapidity of NT release and the Calcium hypothesis

NT release is highly dependent on calcium -- 4th order

Very rapid: 100-200 microseconds between action potential arrival and NT release

implies that SV ready for release
SV must be "primed" (biochemically fusion-ready) and "docked" (morphologically tethered) at active zone (2-20 SVs are believed to be fusion competent in a CNS synapse)

SV is close to site of influx of calcium (voltage-gated calcium channels, typically N-type and P/Q type)

An enzymatic reaction or cascade (as in phototransduction, or muscle contraction) would be too slow
ATP is not required for SV fusion

Thus it is believed that a preassembled SV-fusion complex (which has high stored energy) awaits for the Ca signal and a conformational change in some proteins triggered by calcium binding results in membrane fusion (or formation of the "fusion pore").

Calcium sensor for SV fusion must be within the Ca microdomain during channel opening
Within the calcium domain calcium reaches very high levels (>100 um) for a very short time.
Local calcium transients in the microdomain are difficult to measure with indicator dyes because no dye is sufficiently fast or sensitive.

Calcium sensor for NT release
should show fast, low affinity and highly cooperative (4th order) calcium binding.

Membrane fusion is a general cell problem –

Energy barrier for fusion of two membranes (requires generating discontinuity of lipid bilayers)
Specificity of membrane fusion (which compartment with which compartment, [vesicle to target membrane]?)
Regulation of fusion (calcium-regulated SV exocytosis is extreme example)
Similar proteins probably involved in different types of membrane fusion
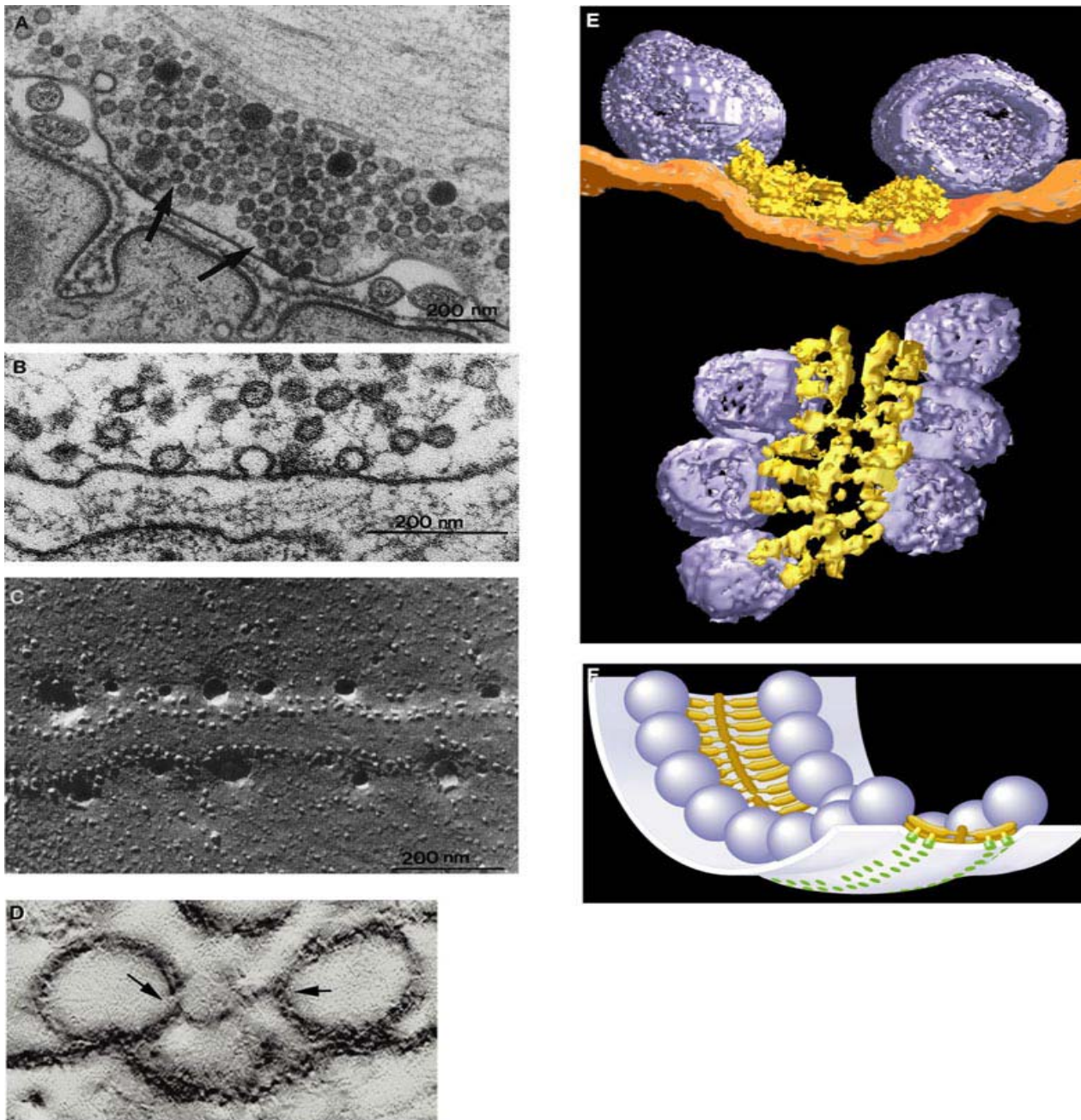
FIGURE 1 Ultrastructural images of exocytosis and active zones. (A–C) Synapses from frog sartorius neuromuscular junctions were quick-frozen milliseconds after stimulation in conditions that enhance transmission. (A) A thin section showing vesicles clustered in the active zone, some docked at the membrane (arrows). (B) Shortly (5 ms) after stimulation, vesicles were seen to fuse with the plasma membrane. (C) After freezing, presynaptic membranes were freeze-fractured and a platinum replica was made of the external face of the cytoplasmic membrane leaflet. Vesicles fuse about 50 nm from rows of intramembranous particles thought to include Ca^{2+} channels. (D–F) The fine structure of the active zone at a frog neuromuscular junction as seen with electron tomography. (D) In a cross-sectional image from tomographic data, two vesicles are docked at the plasma membrane and additional electron-dense elements are seen. When these structures are traced and reconstructed through the volume of the EM section (E), proteins of the active zone (gold) appear to form a regular structure adjacent to the membrane that connects the synaptic vesicles (silver) and plasma membrane (white). Viewed from the cytoplasmic side (E, lower image), proteins are seen to extend from the vesicles and connect in the center. (F) Schematic rendering of an active zone based on tomographic analysis. An ordered structure aligns the vesicles and connects them to the plasma membrane and to one another. Parts A and B from Heuser (1977) ; part C from Heuser *et al.* (1979). Part B reproduced from the *Journal of Cell Biology*, 88, 564—580 (1981). (D–F) After Harlow *et al.*, (2001).

Active Zone:
Where do SVs fuse with surface?

Heuser and Reese – influential EM studies, primarily of NMJ

SVs fuse with active zones (specialized presynaptic membrane, with "fuzzy" electron dense thickening), where SVs are in contact ("docked") with plasma membrane; fusion leads to NT release.
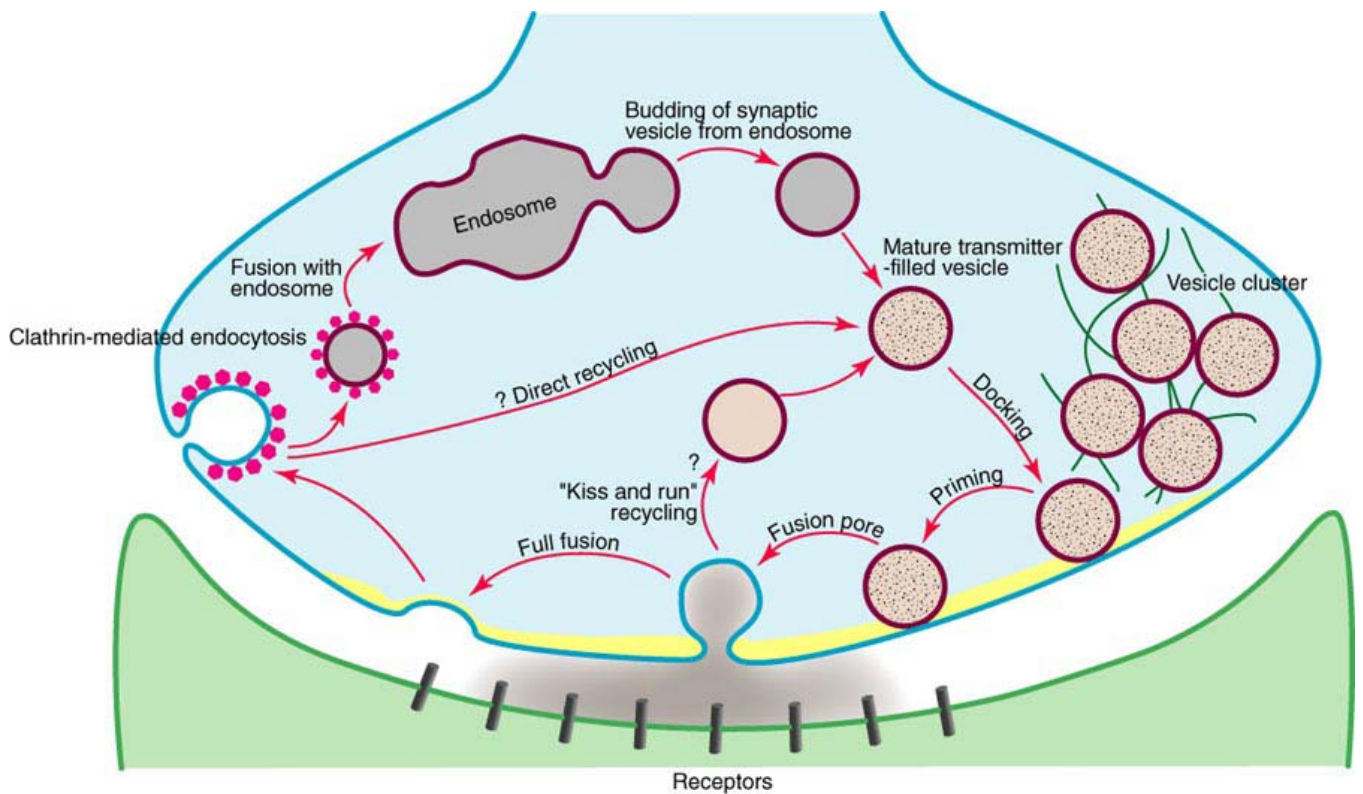
Membrane is re-endocytosed (probably lateral to the active zone) via clathrin-mediated dynamin dependent endocytosis; fuses with endosome; new vesicles bud from endosome to regenerate SVs, which are then recharged with NT via the action of vesicular NT transporters.

Active zone contains a high concentration of voltage gated calcium channels. Any docked SV is likely to be "surrounded" by such calcium channels.

Heuser/Reese described a classical SV cycle involving full fusion (loss of SV membrane integrity) and sorting through endosome system. One evidence for this is that SV integral membrane proteins are found in the presynaptic membrane.

Alternative model – "Kiss and run" (Ceccarelli): SV retains integrity after fusion.
More rapidly regenerated without endosomal intermediate.
May release NT more slowly or incompletely.

Evidence for both full fusion and "kiss-and-run" release in central synapses.

Budding of synaptic
vesicle from endosome

Endosome

Fusion with
endosome

Mature transmitter
-filled vesicle

Vesicle cluster

Clathrin-mediated endocytosis

? Direct recycling

Docking

?

"Kiss and run"
recycling

Priming

Fusion pore

Full fusion

Receptors

FIGURE The life cycle of synaptic vesicles. Transmitter-filled vesicles can be observed in clusters in the vicinity of the active zone. Some vesicles are recruited to sites within the active zone in a process called docking. These vesicles are subsequently primed for release. The rise in cytosolic Ca^{2+} that occurs during an action potential triggers the opening of a fusion pore between some of the primed, docked vesicles and the plasma membrane. Transmitter exits through this fusion pore. Three pathways are proposed by which the now empty vesicle can be recovered and returned to the releasable pool. (1) by a direct reclosing of the fusion pore and reformation of the vesicle, often called "kiss and run", (2) by complete fusion (i.e., the flattening of the vesicle onto the membrane surface) followed by clathrin-mediated endocytosis, removal of the clathrin coat, and return of the vesicle to the releasable pool, and (3) by complete fusion and recycling as in the second pathway, but the endocytosed vesicle fuses first with an endosome and mature vesicles are subsequently formed by budding from the endosome. After or during this recycling process, the vesicle must be refilled with transmitter.

How do you measure NT release?

Postsynaptic response –

Especially EPSP or mini-EPSP
Rapid, sensitive
Most common, but most indirect
Most accessible, but can be distorted by other factors

Change in presynaptic membrane capacitance

SV fusion with presynaptic membrane adds surface area, thereby increasing capacitance. This can be measured electrophysiologically.
Very rapid, direct, "quantal" inference of NT release. Measures SV fusion rather than NT release.
However most terminals are too small to patch onto for this kind of measurement.
Exception is Calyx of Held. Capacitance measurements are most often performed on secretory cells that secrete large dense core vesicles / "granules" (eg mast cells).

Dense core vesicles (peptides) versus *small clear vesicles* (small NTs eg glutamate, GABA, Ach)

Amperometry – carbon fiber electrode

Certain neurtransmitters are oxidized on carbon, generating a current that can be measured. Directly measures released NT.
Can have high temporal resolution, but few synapses "accessible" and main NTs not oxidizable. Therefore of limited use.

Vesicular uptake of tracers (e.g. FM dye)

Based on uptake of histochemical (horse radish peroxidase) or fluorescent tracers (e.g. FM1-43 amphipathic styryl dyes; or fluorescent antibodies to SV protein eg synaptotagmin).
Assumes full fusion.
FM dye uptake and subsequent destaining is popular; (but only easy in dissociated culture); gives good temporal and spatial resolution. Can reach quantal sensitivity. Frequently used to measure readily releasable pool dynamics and identify active presynaptic terminals.

How would you approach trying to dissect the "molecular mechanisms of neurotransmitter release"?

Biochemistry:

Purify synaptic vesicles (SVs)
  (Scheller, Sudhof, Jahn, de Camilli, Greengard)

highly abundant in brain, relatively homogeneous in size and composition, amenable to purification and identification and cloning of component proteins (by direct sequencing or by raising monoclonal antibodies)

Purify factors that reconstitute fusion between two membrane compartmnents in vitro
  (Rothman)

Genetic approaches

Isolate mutants that have defective synaptic transmission
e.g. Drosophila, C. elegans
  (Bellen, Schwarz, Jorgensen, etc)
Such mutants are often lethal, but genetic "tricks" can be employed.

e.g.

mutations expressed only in photoreceptors of Drosophila, then screen for blindness and abnormal electroretinogram (ERG)
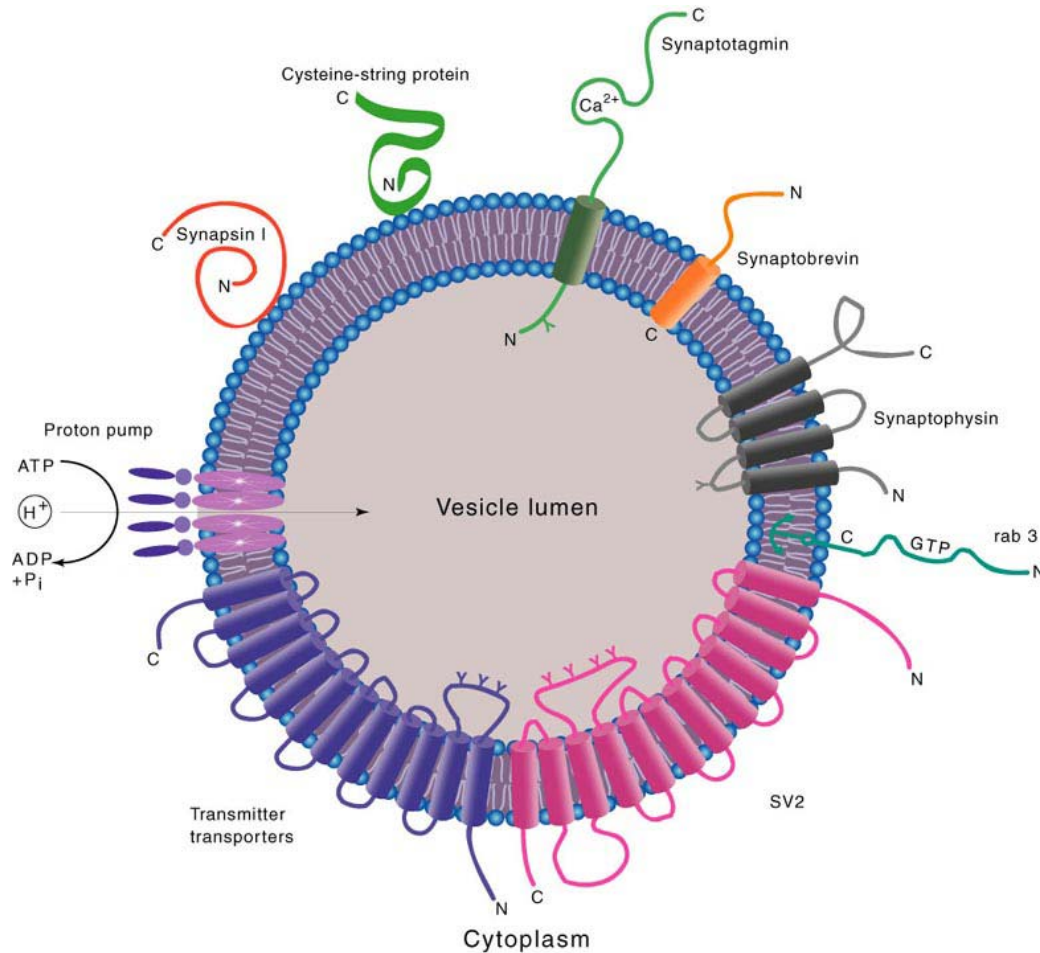
Isolate mutants in yeast that have defective secretion (Sec mutants)
  (Scheckman)

Indirectly came to similar conclusions about fusion

Molecular biology of SV fusion

SVs are "easy" organelle to purify and characterize biochemically
        (Scheller / Sudhof / Jahn / de Camilli / Greengard / Kelly)

FIGURE Schematic representation of the structure and topology of major synaptic vesicle membrane proteins (see also Table 8.1). [How many are there of each?]

First 3 SV proteins cloned were synapsin I, synaptophysin, VAMP1
SV2, Synaptophysin – unknown function
Synapsin may tether SV to actin cytoskeleton
Proton pump acidifies lumen and provides gradient for accumulating NT in lumen
Synaptotagmin can bind calcium and phospholipid
Synaptobrevin (also called VAMP) was found to bind Syntaxin (a presynaptic membrane protein) in affinity chromatography.

What are SNAREs?

SNAREs are central components and core concept in mechanisms of membrane fusion.

Three synaptic proteins: synaptobrevin/VAMP (SV membrane protein), syntaxin and SNAP-25 (presynaptic plasma membrane) form an extremely tight complex that can be isolated intact from brain even in strongly denaturing conditions. This is the core SNARE complex, and it can bring together the SV and presynaptic membranes.

Core SNARE complex

Crystal structure of core complex (see colored ribbons in diagram below) shows 4 helix bundle in such orientation that "zippering" of the bundle would bring together membrane-spanning segments of synaptobrevin/VAMP (red) and syntaxin (yellow). Since these proteins are in vesicle (synaptobrevin/VAMP – hence v-SNARE) and in target plasma membrane (syntaxin; hence t-SNARE), the bundling of the core complex should juxtapose these two membranes. A current model is that formation of this tight complex provides the energy that "drives" membrane fusion. Accessory proteins control this reaction, by binding to SNARE components (e.g. nSec1 and Munc13, which bind syntaxin). Synaptotagmin also binds to SNAREs. These proteins likely prevent full core complex formation; the "loose" SNARE complex is "cocked". Calcium triggers via the calcium sensor (prob synaptotagmin) the formation of the tight SNARE complex.
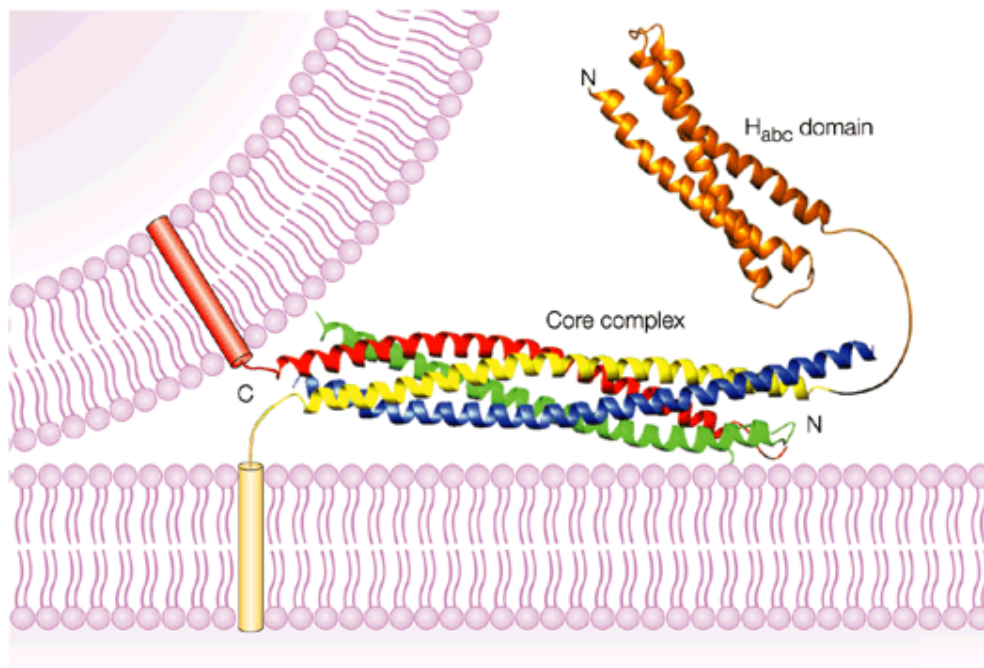
Note that after membrane fusion, the core SNARE complex, which forms *in trans* between vesicle and plasma membrane, ends up *in cis* in the plasma membrane! Thus the tight SNARE complex has to be split apart and recycled to their original membranes (e.g. VAMP to SVs via endocytosis) in order to maintain the synaptic vesicle cycle and NT release. This unfolding is achieved by an ATPase called NSF, which was originally purified as a factor required for ER-Golgi trafficking in vitro. Temp sensitive mutants in NSF in Drosophila result in delayed paralysis upon shift to restrictive temperature.

The convergence of biochemical, genetic and pharmacological approaches in the understanding of SV exocytosis

Biochemical analysis of SVs and membrane fusion identified the same proteins/genes as forward genetic screens for defective synaptic transmission (e.g. the SNARE proteins syntaxin, synaptobrevin, NSF).

Secretion (constitutive) in yeast and SV exocytosis use similar principles and require homologous genes/proteins (e.g. SNARE proteins, interacting proteins of SNAREs, NSF, small GTPases (Rabs) etc – ie membrane fusion is a fundamental and conserved process.

Clostridial neurotoxins (e.g. tetanus toxin) inactive NT release – they act as specific proteases that cleave individual SNARE proteins at the synapse.

Artificial lipid vesicles containing appropriate SNARE proteins can fuse in vitro.
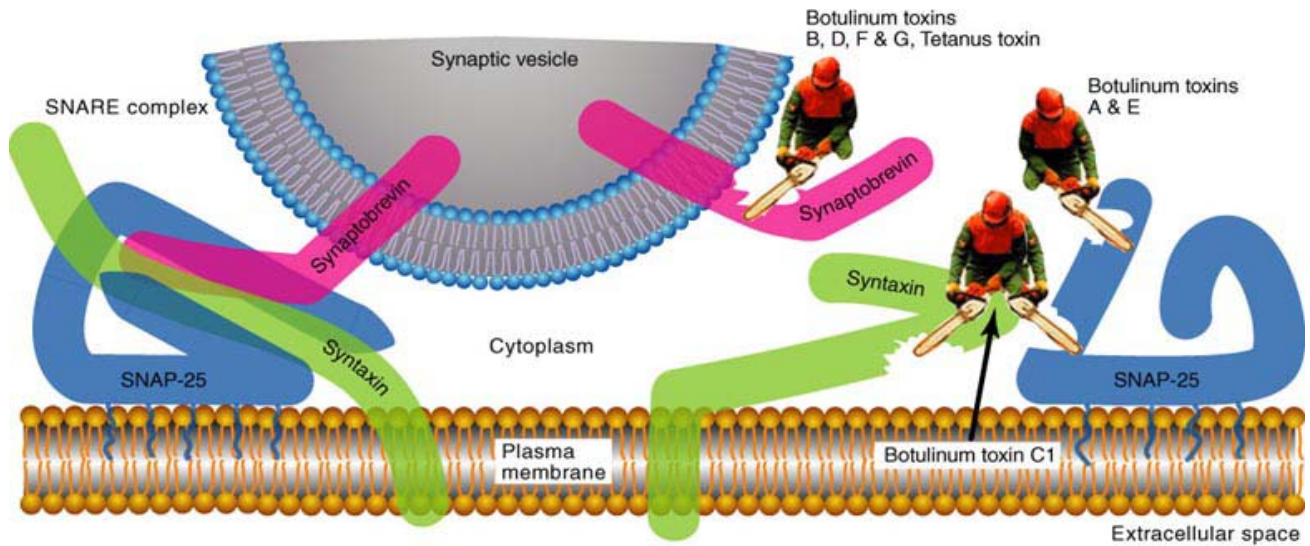
FIGURE 6 SNARE proteins and the action of clostridial neurotoxins. The SNARE complex shown at the left brings the vesicle and plasma membranes into close proximity and likely represents one of the last steps in vesicle fusion. Vesicular VAMP, also called synaptobrevin, binds with syntaxin and SNAP-25 that are anchored to the plasma membrane. Tetanus toxin and the botulinum toxins, proteases that cleave specific SNARE proteins as shown, can block transmitter release.
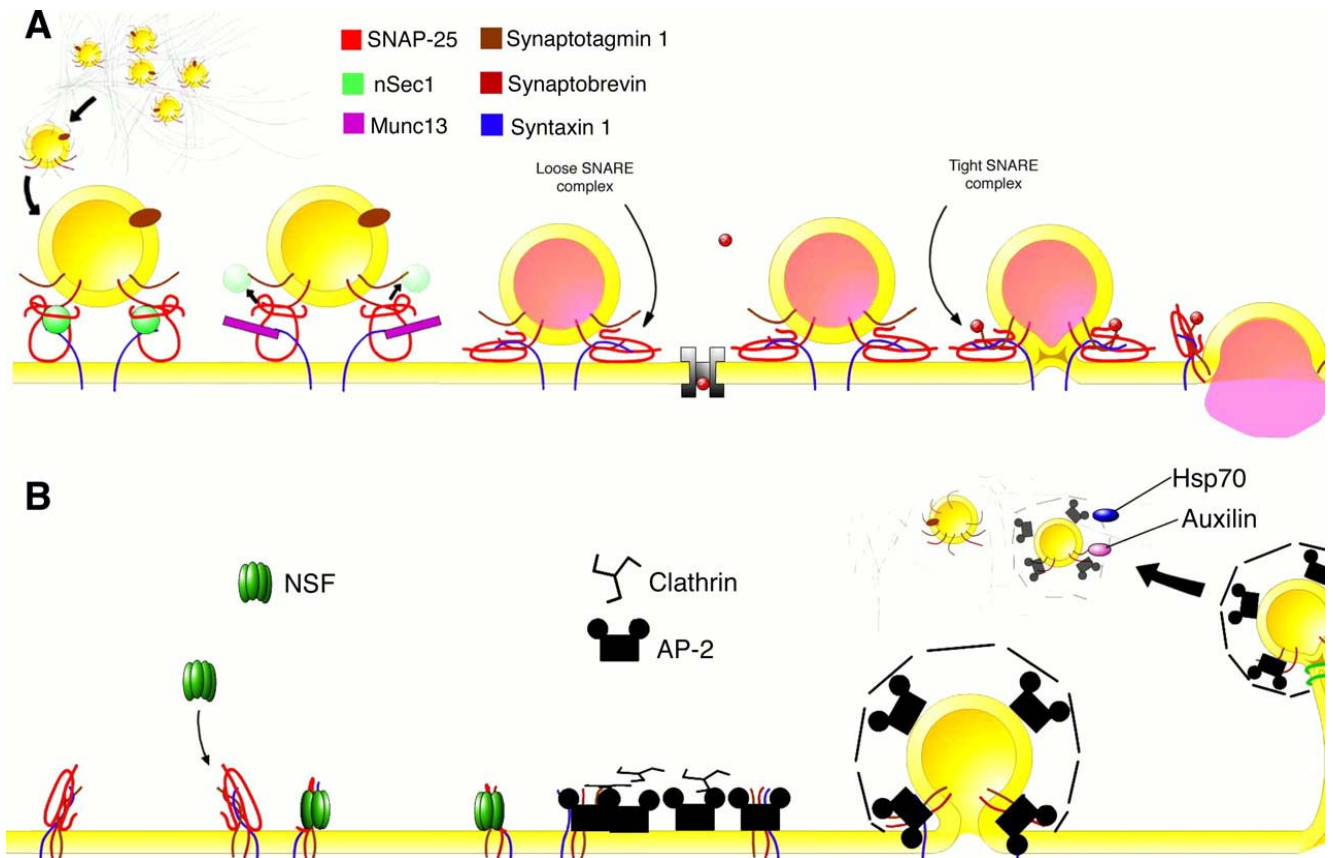
Figure. A Model for the molecular steps in the classical ("slow") synaptic vesicle cycle

In the slow track, vesicles are recruited from the reserve pool that corresponds to synaptic vesicles attached to the actin cytoskeleton via synapsin. These vesicles then translocate to the vicinity of the plasma membrane. They undergo priming through an unknown mechanism in which nSec1 and Munc13, two syntaxin-binding proteins, could participate. This step could lead to the formation of a complex between a synaptic vesicle SNARE (synaptobrevin) and SNAP-25, a plasma membrane target SNARE. Formation of loose SNARE complexes between synaptobrevin, SNAP-25 and syntaxin 1 reduces the distance between the synaptic vesicle and the plasma membrane. At this point, the synaptic vesicle would be docked to the plasma membrane. Entry of calcium could trigger a conformational change in synaptotagmin 1 that would allow further zippering of the SNARE complex into a tight state, which would lead to lipid bilayer fusion. The synaptic vesicle membranes would then disintegrate into the plasma membrane. The SNARE complexes, which have been inserted into the plasma membrane, are dissociated by the action of SNAPs (not shown for simplicity) and NSF. Synaptic vesicle proteins are then reinternalized by a clathrin- and dynamin-dependent mechanism at the plasma membrane or at the tip of endosome-like invaginations of the plasma membrane. Dynamin then catalyzes the fission of the emanating vesicle from the membrane. Free clathrin-coated vesicles are subsequently uncoated by hsp70, auxilin, and, possibly, synaptojanin. Finally, the proton pump restores the electrochemical gradient, allowing for the uptake of neurotransmitter into the vesicle. This last step leads to a reformed synaptic vesicle that can reenter the cycle.

Synaptotagmin as calcium sensor

<u>Primary article for presentation:</u>
Yoshihara and Littleton. Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. Neuron. 2002 Dec 5;36(5):897-908.

<u>Reviews and Background</u>
Stevens CF, Neurotransmitter release at central synapses. Neuron. 2003 Oct 9;40(2):381-8. Review.

Yoshihara et al. Is synaptotagmin the calcium sensor? Curr Opin Neurobiol. 2003 Jun;13(3):315-23. Review.

Koh and Bellen. Synaptotagmin I, a Ca2+ sensor for neurotransmitter release. Trends Neurosci. 2003 Aug;26(8):413-22. Review.