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Commentary

Molecular chaperones and the regulation of neurotransmitter exocytosis

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Abstract

Regulated neurotransmitter release depends on a precise sequence of events that lead to repeated cycles of exocytosis and endocytosis. These events are mediated by a series of molecular interactions among vesicular, plasma membrane, and cytosolic proteins. An emerging theme has been that molecular chaperones may guide the sequential restructuring of stable or transient protein complexes to promote a temporal and spatial regulation of the endo- and exocytotic machinery and to ensure a vectorial passage through the vesicle cycle. Chaperones, specialized for a few substrates, are ideally suited to participate in regulatory processes that require some molecular dexterity to rearrange conformational or oligomeric protein structures. This article emphasizes the significance of three molecular chaperone systems in regulated neurotransmitter release: the regulation of soluble NSF attachment protein receptor (SNARE) complexes by N-ethylmaleimide-sensitive factor (NSF) and the soluble NSF attachment protein (SNAP), the uncoating of clathrin-coated vesicles by the 70 kDa heat-shock cognate protein (Hsc70), and the regulation of SNARE complex-associated protein interactions by cysteine-string protein and Hsc70.

Keywords: Neurotransmitter release; Exocytosis; Synaptic transmission; *N*-ethylmaleimide-sensitive factor (NSF); Soluble NSF attachment protein (SNAP); Cysteine-string protein (CSP); Auxilin; 70-kDa Heat-shock cognate protein (Hsc70); Chaperone

1. Introduction

Chemical synaptic transmission at synapses is the dominant mode of transferring information from one neuron to another. Quantal packets of neurotransmitter are stored in synaptic vesicles that fuse with the presynaptic membrane to secrete their contents onto the postsynaptic target cell. Temporal fidelity for rapidly changing signals is attained by coupling nerve activity and exocytosis on a sub-millisecond scale such that depolarization-dependent Ca^{2+} influx through Ca^{2+} channels triggers vesicle fusion. Sustained release is ensured by trafficking synaptic vesicles through repeated cycles of exocytosis and endocytosis [1]. The stages leading to exocytosis include loading synaptic vesicles with neurotransmitter, targeting and docking vesicles to release sites, priming, triggering Ca^{2+}-dependent fusion, and membrane fusion. After exocytosis, vesicle membranes and most of their proteins are rapidly recaptured by endocytosis and locally recycled to replenish releasable vesicle pools. A combination of biochemical and genetic approaches by many laboratories have led to the identification of many synaptic proteins and the elaboration of molecular models describing exocytotic and endocytotic mechanisms [2].

Neurotransmitter exocytosis is a complex and tightly regulated process that involves sequential interactions of many synaptic proteins. The key event, vesicular membrane fusion, is apparently mediated by the SNARE or core complex. SNARE proteins are associated with vesicles (v-SNAREs) or plasma membranes (t-SNAREs) and form a stable complex [3,4] that includes synaptobrevin/VAMP [5–7], syntaxin [8], and SNAP-25 [9]. These proteins interact with each other in a parallel 4-helix bundle that is structurally conserved and bridges apposed membranes [10–12]. Parallel protein binding at the N-termini initiates complex formation, and further zippering of coiled-coils pushes the vesicle and the plasma membrane into close contact, presumably driving the process of fusion [13–15]. The fusogenic activity of the SNARE complex has been revealed by studies using recombinant neuronal SNAREs reconstituted in separate phospholipid bilayer vesicles that form *trans*-SNARE complexes linking both bilayers [16].

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Abbreviations: SNARE, soluble NSF attachment protein (SNAP) receptor; NSF, *N*-ethylmaleimide-sensitive factor; Hsc70, 70-kDa heat-shock cognate protein; CSP, cysteine-string protein; VAMP vesicle-associated membrane protein; SNAP-25, synaptosome-associated protein 25 kDa; NEM, *N*-ethylmaleimide; AAA ATPases, ATPases Associated to a variety of Activities; and Hsp70, Hsp90, and Hsp60, 70-kDa, 90-kDa, and 60-kDa heat-shock protein, respectively.

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Assembly of such *trans*-SNARE complexes forms a metastable state at 0–4° from which bilayer fusion occurs after warming to 37° [16–18]. Regulated exocytosis, however, requires further interactions of the core complex with numerous synaptic proteins [19–25].

Endocytosis and synaptic vesicle recycling is apparently mediated by at least two basic pathways: receptor-mediated endocytosis via coated pits [26], and coupled exo- and endocytosis [27]. A third model suggests that endosome-like intermediates of nerve terminals originate by bulk-uptake of the plasma membrane while vesicle budding takes place in parallel from the plasmalemma and from these internalized membranes [28]. Receptor-mediated endocytosis requires the formation of a clathrin coat surrounding the budding vesicle that is, in part, accomplished by binding of clathrin adaptor proteins to specific receptors in the vesicle membrane patch [29–32]. In turn, clathrin triskelions, three-legged structures composed of three clathrin molecules, are moved to the membrane where they polymerize to form a curved polygonal lattice of hexagons and pentagons that provides the scaffold for a coated pit [33]. After its formation, the coated pit is pinched off the plasma membrane by the cooperative action of amphiphysin and the GTPase dynamin [34–39]. Once internalized, the clathrin coat must be enzymatically removed, since it prevents further fusion of the vesicle [40].

The sequential assembly, rearrangement, and disassembly of a series of protein complexes are important features of current models for vesicle trafficking. Therefore, it is not surprising that molecular chaperones emerge as important factors in the synaptic vesicle cycle. Chaperones are collectively known as proteins that recognize denatured proteins and stabilize partially folded protein intermediates during polypeptide folding, assembly, and disassembly [41–43]. In addition to the classical picture of chaperone action, an emerging theme is that maintaining proper protein intermediate states may be crucial for the normal function of many proteins, especially in signaling pathways that employ sequential interactions of transient protein complexes. Specialized chaperones are ideally suited to participate in regulatory processes that require some molecular dexterity to rearrange conformational or oligomeric structures of protein complexes [44–46]. In the past decade, three synaptic chaperone systems, each specialized for a unique set of substrates, have been identified as critical factors in the synaptic vesicle cycle. Here we discuss the specialized chaperone activities of SNAP/NSF, auxilin/Hsc70, and CSP/Hsc70, and their prominent roles to ensure fast and sustained neurotransmitter release.

2. Regulation of SNARE complexes by the ATPase NSF and its cofactor SNAP

NSF was originally identified as a factor that reconstituted vesicular intra-Golgi transport after inactivation by NEM [47,48]. The sequence of NSF revealed that it was homologous to the yeast protein sec18 [49], previously shown to mediate endoplasmic reticulum (ER) to Golgi transport [50] and endocytotic vesicle fusion [51]. Later, NSF was also implicated in synaptic vesicle exocytosis [3,4]. NSF is conserved from yeast to mammals and contains two AAA domains, the signature module of AAA ATPases, which may act as molecular chaperones [52]. The two homologous domains of NSF each contain an ATP-binding site, and mutations of either site significantly compromise the weak intrinsic ATPase activity [53,54].

NSF is primarily a cytosolic protein that requires SNAP to attach to membranes, and to stimulate its intrinsic ATPase activity [55–58]. SNAP binds to membrane-associated SNAP receptors (SNAREs) including complexed syntaxin, synaptobrevin, and SNAP-25 [3,4], whereas NSF will only interact with complexed SNAP [59]. Binding of NSF to the SNAP–SNARE complex forms the 20S SNARE complex, and subsequent ATP hydrolysis by NSF dissociates the complex into monomers [3,4]. Originally, it was assumed that the energy released by ATP hydrolysis would also drive membrane fusion [3,4]. Temporal constraints of neurotransmitter release and the fact that ATP does not trigger exocytosis prompted a reassessment of this theory [60,61]. Instead, it has been suggested that NSF and SNAP may act as molecular chaperones to regulate the conformation of SNARE complexes by dissociating *cis*-SNARE complexes that are assembled on the same membrane [62]. This raised the possibility that SNAP/NSF may mediate a post-docking pre-fusion priming step and/or a post-fusion step to disassemble *cis*-SNARE complexes. The priming step could allow the formation of fusion-competent *trans*-SNARE complex, while the post-fusion step could prepare the *cis*-SNARE complex for endocytosis [62–65].

Although a chaperone role for NSF and SNAP first appeared highly speculative, it has been consistently supported by a series of arguments. Similar to many chaperones, NSF function requires ATP hydrolysis [41,54]. Furthermore, NSF has a low ATPase activity that is stimulated by binding to the SNAP–SNARE complex [58], similar to ATP-dependent chaperones whose weak intrinsic ATPase activity is substantially stimulated by binding to substrate proteins or co-chaperones [42,66]. Chaperones regulate the assembly/disassembly of multi-protein complexes by inducing a conformational change in their substrate proteins [41,42,66]. Consistently, NSF drives the disassembly of the SNARE complex [3,4], induces a conformational change in syntaxin [67], and is presumably capable of large conformational motions that may drive SNARE complex disassembly [68,69]. Furthermore, electron micrographs of NSF show a cylindrical shape reminiscent of molecular chaperones [13]. Recent studies provided surprising evidence that SNAPs and NSF also interact with glutamate receptors at the postsynaptic membrane, suggesting that SNAP and NSF may act as molecular chaperones not only on SNAREs but also on other proteins [70–76].

A role for NSF and SNAP in neurotransmitter exocytosis was originally indicated by their binding to complexed synaptobrevin, syntaxin, and SNAP-25, since these are proteolytic targets for potent inhibitors of neurotransmission, the botulinum neurotoxins [77,78]. Direct evidence implicating SNAP in exocytosis came from studies employing squid, *Drosophila*, and secretory cell cultures. Presynaptic injection of recombinant SNAP into the squid giant synapse enhanced transmitter release, while injection of peptides that mimic sites of SNAP protein interaction inhibited release. This inhibition was accompanied by an accumulation of docked vesicles supporting a post-docking pre-fusion role. Furthermore, peptide injection also reduced the number of cytoplasmic vesicles normally surrounding active fusion sites, indicating a requirement for SNAP in replenishing this vesicle pool [79]. Similar presynaptic injections of α-SNAP into crayfish neuromuscular junctions also indicated a role in maintaining a fusion competent vesicle pool [80]. Consistently, studies in chromaffin cells suggest that α-SNAP may recruit vesicles into the readily releasable vesicle pool [81–84]. During membrane capacitance measurements, the readily releasable pool appears as a fast exocytotic burst followed by a slow release component [85]. Injection of α-SNAP into chromaffin cells significantly increased both the exocytotic burst and the slow component [82]. Expression of dominant-negative α-SNAP inhibited release in chromaffin cells without affecting the kinetics of single release events, consistent with a role in priming but not in vesicle fusion [86].

Studies manipulating NSF in a variety of secretion systems have revealed defects highly reminiscent of those obtained with SNAP, suggesting that both proteins act cooperatively in exocytosis to replenish a readily releasable vesicle pool by priming vesicles. Presynaptic injection of inhibitory NSF peptides into the giant squid synapse reduced nerve-evoked neurotransmitter release in an activity-dependent manner, increased the number of docked vesicles, and reduced the number of cytoplasmic vesicles, suggesting a post-docking pre-fusion role for NSF in exocytosis [87]. In *Drosophila*, temperature-sensitive paralytic mutations of the *comatose* locus disrupt dNSF1 [88], causing an activity-dependent loss of nerve-evoked release [89–92], and increasing the number of docked vesicles supporting a role in vesicle priming [89]. Most importantly, excess accumulation of the 7S SNARE complex is observed in *comatose* mutants, confirming that NSF is required to disassemble the SNARE complex [91,93]. NEM dialysis of chromaffin cells consistently blocked granule fusion in an activity-dependent manner, suggesting that inhibition of NSF disrupts replenishment of a readily releasable vesicle pool [82]. Interestingly, inhibition of NSF slowed the kinetics of evoked release in both squid and *Drosophila* synapses [87,89]. Nevertheless, it is unlikely that the slower kinetics are caused by a primary fusion defect, since a similar effect is observed in *Drosophila* dynamin mutants that block endocytosis and progressively reduce exocytosis [89]. Thus, a defect in an early step of endocytosis may slow down exocytosis in both systems.

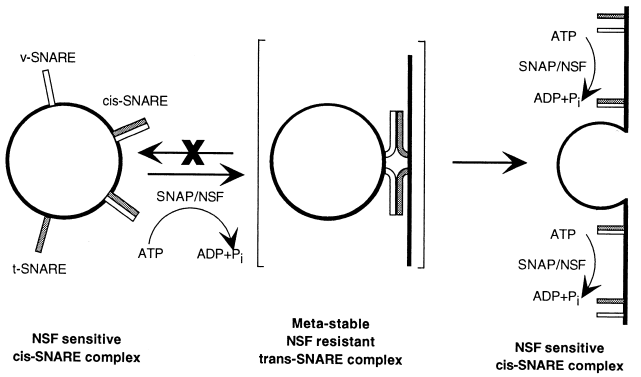

Fig. 1. A multi-step model of SNAP and NSF function in the synaptic vesicle cycle. SNAP in cooperation with the NSF catalyzes the disassembly of *cis*-SNARE complexes (residing on the same membrane) by recruiting NSF to SNAP–SNARE complexes and subsequently stimulating NSF-mediated ATP hydrolysis. After docking, the disassembly of *cis*-SNAREs may facilitate transition of vesicles into a releasable pool and prime the formation of meta-stable, fusion-competent *trans*-SNARE complexes. These, however, are functionally resistant to SNAP/NSF activity. Once the vesicle has fused with the membrane, collapsing the vesicle membrane into the plasma membrane, *cis*-SNARE complexes are formed that are again sensitive to SNAP/NSF disassembly activity. The disruption of *cis*-SNARE complexes formed after fusion may occur at an early step of endocytosis to avoid excessive vesicle association of t-SNAREs or at a subsequent step of vesicle recycling. Modified after Weber and colleagues [95].

The physiological evidence that SNAP and NSF mediate a post-docking pre-fusion priming step in regulated exocytosis to replenish the readily releasable vesicle pool is compelling. In addition, biochemical studies show that *cis*-complexes containing NSF, SNAP, and SNAREs will form and dissociate on the surface of cytoplasmic undocked vesicles [94]. Studies with reconstituted membranes demonstrate that SNAP and NSF disassemble recombinant *cis*-SNARE complexes on liposomes while *trans*-SNARE complexes become functionally resistant to NSF and remain fusogenic, supporting the idea that NSF and α-SNAP disrupt *cis*-SNARE complexes to prime the formation of *trans*-SNARE complexes [95]. An interesting feature of this model (Fig. 1) is that the overall process is vectorial since *trans*-SNARE complex formation is an essentially irreversible step. What accounts for the functional resistance of *trans*-SNARE complexes to NSF degradation and how this maintains a readily releasable vesicle pool without massive spontaneous fusion, however, remain to be elucidated.

3. Uncoating of clathrin-coated vesicles by Hsc70 and auxilin

The classical example of a molecular chaperone acting at nerve terminals is the uncoating of clathrin-coated vesicles during synaptic vesicle recycling. Clathrin is released from

coated vesicles as triskelions [96,97]. While clathrin triskelions spontaneously reassemble into cages resembling coats of coated vesicles [98], the disassembly of a clathrin coat is enzyme-mediated and requires an input of energy [99]. The clathrin-uncoating ATPase catalyzes the strictly ATP-dependent release of clathrin from coated vesicles [100] such that the hydrolysis of three ATP molecules is required for the release of one clathrin triskelion [101]. Subsequently, the clathrin-uncoating ATPase has been identified as Hsc70 [102], a member of the 70-kDa family of stress-induced heat-shock proteins (Hsp70). As the name implies, this protein family originally received attention because of their induced expression during the cellular response to heat and other stress factors [103–105].

Hsp70 chaperones participate in numerous processes essential to cell survival under both stressed and normal conditions [66]. They are specifically involved in signal transduction [106], apoptosis [107], progression of the cell cycle [108], circadian rhythms [109], neurodegeneration [110–113], and intracellular vesicle trafficking [114]. Such versatility is intriguing and raises the question of how Hsp70 chaperone activity is regulated to specifically accomplish each of these diverse functions.

To understand how Hsp70 chaperones work, one has to appreciate that the reversible binding and release of substrates are tightly coupled to a cycle of ATP hydrolysis and conformational change. Substrates have a low affinity for Hsp70-ATP but a high affinity for Hsp70-ADP, primarily due to high or low off-rates [66]. Furthermore, the stages of the ATPase cycle are regulated by a number of co-factors. The family of "J-domain containing proteins" (here we use J-proteins) is needed to stimulate the weak intrinsic ATPase activity of Hsp70 [44–46,115]. Further co-factors, such as BAG-1 and Hip, facilitate or prevent nucleotide release [107,116]. DnaJ proteins, containing at least four distinct domains including the 70 amino acid long J-domain, represent the prototypical members of the J-protein family. However, a large subclass of the J-protein family contains only the J-domain [44, 46], raising the question as to why certain proteins possess only the J-domain and not the remaining domains of DnaJ proteins? One intriguing possibility is that, unlike DnaJ proteins which interact with a wide range of targets, these J-proteins act as specialized co-chaperones that recruit Hsp70 to a unique target. This strategy could promote a spatial and temporal regulation for sequential reactions by increasing the local concentration of an Hsp70 chaperone in the vicinity of a particular substrate.

Auxilin is one such specialized co-chaperone that specifically recruits Hsc70 to clathrin coats through its J-domain and clathrin-binding domain [117–119]. Auxilin was originally identified as a minor assembly protein that bound to clathrin triskelions and induced clathrin assembly into regular baskets [120]. Further analysis showed that auxilin acts as an essential cofactor of Hsc70 to dissociate clathrin coats by binding to assembled clathrin lattices and subsequently recruiting Hsc70 in the presence of ATP [119].

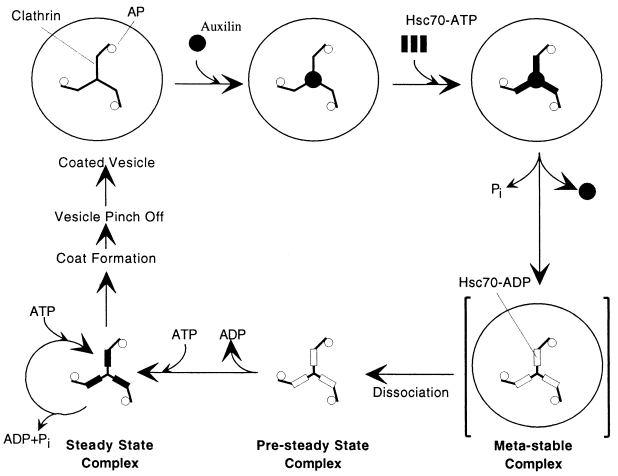

Fig. 2. A model of auxilin and Hsc70 function in synaptic vesicle recycling. Auxilin initiates uncoating by recruiting Hsc70-ATP to clathrin-coated vesicles. Stimulation of Hsc70-mediated ATP hydrolysis by auxilin rapidly forms a meta-stable complex of Hsc70-ADP and a clathrin triskelion associated with the clathrin lattice. Dissociation of triskelions from the clathrin lattice is driven by conversion of the meta-stable complex to a pre-steady-state complex. Following nucleotide exchange this pre-steady-state complex is then transformed to a steady-state complex that dissociates very slowly. The steady-state complex may chaperone triskelions to prevent inappropriate sequestration of clathrin. Subsequently, Hsc70 may also prime clathrin for coat formation. Modified after Refs. 119 and 128.

Since other DnaJ homologues cannot substitute for auxilin [121, 122], the clathrin-binding domain of auxilin is apparently crucial to support uncoating by Hsc70. While auxilin shares many properties with other J-proteins such as stimulating ATP hydrolysis and ATP-dependent polymerization of Hsc70 [117,119,123,124], it also shows two unique differences. Auxilin strongly binds to Hsc70 in the presence of ATP and induces polymerization stoichiometrically, while other J-proteins induce polymerization catalytically [123].

A unique feature of auxilin is that it specifically presents clathrin as a substrate to Hsc70 (Fig. 2). Clathrin uncoating is initiated by auxilin first binding to assembled clathrin triskelions, and then catalytically inducing Hsc70-ATP binding to the auxilin–clathrin complex [117,119,120,125,126]. Stimulation of ATP hydrolysis then forms a meta-stable complex comprised of Hsc70-ADP and clathrin baskets, which converts to a pre-steady-state clathrin–Hsc70-ADP complex that releases clathrin triskelions from the basket [123,126–128]. In the presence of ATP, a steady-state complex forms containing clathrin, Hsc70-ATP, and assembly proteins that ties up Hsc70, preventing further uncoating [128]. This molecular model accords well with the biphasic time course of clathrin uncoating [125,129–131].

The role of Hsc70 in synaptic vesicle recycling, however, does not appear to be restricted to the uncoating reaction *per se* (Fig. 2). Recent evidence suggests that Hsc70 may also chaperone clathrin triskelions and assembly proteins in a classical way to keep both depolymerized in the cytosol, preventing abnormal sequestration of clathrin.

Furthermore, Hsc70 may also have the potential to prime clathrin triskelions, forming new clathrin-coated pits [128]. An important caveat for the role of Hsc70 in synaptic vesicle recycling is that despite the numerous evidence obtained by *in vitro* studies, there are no studies available testing the *in vivo* significance of Hsc70 or auxilin at nerve terminals. Thus, it remains to be seen whether Hsc70 and auxilin are truly essential components in synaptic vesicle recycling.

4. Do cysteine-string protein and Hsc70 cooperatively mediate a late step of regulated vesicle fusion?

The vesicle-associated CSP represents a second member of the J-protein family found at nerve terminals [132–135]. CSP was originally detected in neuronal cells of *Drosophila* [136], and subsequent studies showed that CSP is expressed on synaptic vesicles in neurons as well as on secretory vesicles in endocrine, neuroendocrine, and exocrine cells [133]. CSP is conserved from invertebrates to humans and features three conserved domains: an N-terminal J-domain, a "linker domain," and a centrally located cysteine-string domain. The J-domain of CSP is evolutionarily conserved down to the bacterial DnaJ proteins, which suggests a possible interaction of CSP with proteins of the Hsp70 family [44,45]. Indeed, CSP forms a transient complex with bovine Hsc70 and Hsp70 *in vitro,* and stimulates their intrinsic ATPase activity [137,138].

The signature domain of CSP is the unique cysteine-string motif that contains 14 cysteines over a span of 24 amino acids in vertebrate CSP. These cysteines are mostly palmitoylated [139], but complete chemical depalmitoylation does not displace CSP from membranes [140,141]. Mutational analysis suggests that the lipidated cysteine residues are required to initiate vesicle membrane targeting but not to maintain membrane association [141]. This is presumably accomplished by the hydrophobic nature of the cysteine-string ensuring membrane association in the absence of lipidation [142]. Beyond membrane targeting, the lipidated cysteine-string domain has been suggested to act as a "fusion promoting agent" by switching the association of the lipidated cysteines from the vesicular to the plasma membrane [143]. Although this idea is intriguing, it is unlikely to be correct since there is no evidence for repeated cycles of CSP lipidation [144]. The third conserved domain of CSP, the unique linker domain, is sandwiched between the cysteine string and the J-domain at the N-terminus. Although its molecular function is unknown, it appears to be critical for CSP function in stimulated insulin secretion but not for the activation of Hsc70 ATPase activity [145].

The significance of CSP in neurotransmitter release became apparent by genetic studies in *Drosophila.* The deletion of the entire *csp* gene in *Drosophila* is semi-lethal—only 4% of the expected flies develop to adulthood. Adult survivors progressively exhibit uncoordinated motor behavior, ending in paralysis that correlates with a loss of synaptic transmission [146]. Recordings from mutant neuromuscular junctions revealed that nerve-evoked neurotransmitter release is reduced by 50% at 22° and completely abolished above 29° [147]. The loss of evoked neurotransmitter release in *csp* mutants is counteracted by increasing extracellular Ca^{2+} levels or by accumulation of residual Ca^{2+} during repetitive stimulation, suggesting that CSP primarily increases the Ca^{2+} sensitivity of the exocytotic machinery [148]. The thermo-intolerant loss of CSP function in fly deletion mutants parallels temperature-sensitive defects of gene deletions in bacterial DnaJ proteins [149], suggesting that target proteins of CSP action must be destabilized in the absence of CSP, consistent with the idea that CSP chaperones exocytotic signaling pathways. The *in vitro* interaction of CSP with Hsc70 originally implied a potential role of CSP in vesicle recycling [1], since the only known function of Hsc70 at the synaptic terminal has been the uncoating of clathrin-coated vesicles. However, subsequent studies using FM1–43 dye uptake and release assays to monitor the dynamics of endocytosis and exocytosis in *Drosophila csp* mutants excluded any defects of synaptic vesicle recycling that could cause the loss of neurotransmitter release at restrictive temperatures [150].

The genetic studies on *Drosophila* CSP are complemented by studies using a variety of model systems for regulated exocytosis. Presynaptic injection of anti-CSP antibodies into *Xenopus* neuromuscular junctions inhibited nerve-evoked neurotransmission, confirming a similar role for CSP in vertebrates [151]. Studies using "slow secretion systems" provide accumulating evidence that CSP is likely to modulate a late step of exocytosis. Overexpression of bovine CSP in neuroendocrine PC12 cells enhanced dopamine release from permeabilized cells, while overexpression of CSP or CSP antibody injections in insulin-secreting cell lines derived from pancreatic β-cells decreased insulin release [145,152]. Moreover, reduction of CSP levels by the expression of anti-sense mRNA reduced stimulated insulin release in intact and in permeabilized β-cell lines [153]. Although these findings appear paradoxical and require further investigation, the deleterious effects of CSP suppression or overexpression persisted in permeabilized cells of both systems, suggesting a direct role of CSP in exocytosis. This idea was further strengthened by overexpression of CSP in adrenal chromaffin cells, which not only reduced exocytosis, but more significantly, slowed the kinetics of single granule release events [86]. For comparison, overexpression of dominant-negative α-SNAP, which parallels CSP in its role as cofactor, inhibited exocytosis but did not affect the kinetics of single fusion events. Together, these results suggest that CSP, unlike α-SNAP, plays a key role at the level of the machinery mediating or regulating the fusion pore [86].

Originally, CSP was hypothesized to physically link synaptic vesicles and presynaptic Ca^{2+} channels and to promote neurotransmitter release by increasing Ca^{2+} channel

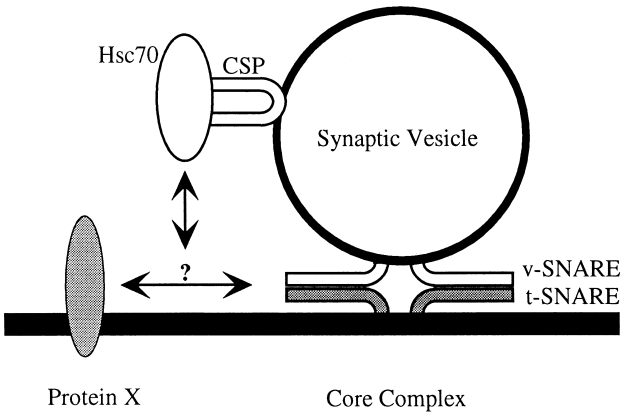

Fig. 3. A hypothetical model of CSP and Hsc70 function in synaptic vesicle exocytosis. CSP is likely to recruit Hsc70 to synaptic vesicles mediating a late step of exocytosis. CSP interacts with the v-SNARE synaptobrevin and the t-SNARE syntaxin, suggesting that CSP may regulate interactions of proteins (protein X) associated with the SNARE complex. Although the synprint site of presynaptic calcium channels can interact with CSP *in vitro*, it is an unlikely candidate to mediate a step at the level of the machinery mediating or regulating the fusion pore as proposed for CSP.

activity at nerve terminals [154]. This idea has been supported by the co-expression of CSP mRNA with an RNA fraction of *Torpedo* electric lobe in frog oocytes, which modulated ectopically expressed N-type Ca^{2+} channel currents [155]. Although several studies failed to demonstrate binding of CSP to native Ca^{2+} channels [156–158], CSP has been found to bind the regulatory "synprint site" in the cytoplasmic loop of presynaptic Ca^{2+} channels [156, 159]. The synprint site mediates modulatory interactions of multiple synaptic proteins with Ca^{2+} channels including syntaxin, synaptotagmin, and SNAP-25 [24]. Since CSP is apparently an effective competitor of the syntaxin–synprint site interaction *in vitro*, it has been suggested that CSP may dissociate syntaxin from Ca^{2+} channels and thereby indirectly promote Ca^{2+} channel activity [159].

The hypothesis that CSP primarily modulates presynaptic calcium channels is not supported by a recent calcium imaging study of *Drosophila* mutants, which suggests that the loss of neurotransmitter release in *csp* mutants is primarily caused by a defect of Ca^{2+}-regulated exocytosis [148] and not by inactivation of presynaptic Ca^{2+} channels, as previously suggested [160]. These results accord with other studies that found no evidence for regulation of presynaptic Ca^{2+} channels by CSP in PC12 cells, pancreatic insulin secreting cells, and peptidergic synapses of *csp* mutant *Drosophila* [145,152,153,161,162]. The role of CSP at nerve terminals is apparently more widespread than previously assumed. In addition to its direct role in exocytosis, CSP appears to stabilize depolarization Ca^{2+} entry and Ca^{2+} clearance, as indicated by increased evoked cytosolic calcium levels and by increased calcium resting levels at high temperatures in mutant *Drosophila* terminals lacking CSP [148].

Biochemical and genetic studies provide compelling arguments that CSP acts as a typical J-protein and functions as a presynaptic co-chaperone in neurotransmission. CSP binds and activates the ATPase activity of Hsp70 and Hsc70 through its J-domain, which is apparently essential and sufficient for ATPase stimulation [137,138,145]. CSP binding to Hsp70/Hsc70 is specific as no binding occurs to Hsp60, Hsp90, or NSF [137,163]. CSP acts as a classical chaperone, preventing the aggregation of denatured model substrate proteins *in vitro* [164]. CSP and Hsc70 act synergistically to prevent the aggregation of denatured proteins *in vitro* [138]. Deletion of the *csp* gene in *Drosophila* causes a primary defect in exocytosis that is thermo-intolerant such that exocytosis progressively deteriorates further at higher temperatures [146–148]. CSP co-immunoprecipitates with the synaptic vesicle protein synaptobrevin/VAMP [156], which lacks a secondary structure and thus exhibits the features of an unfolded protein [165].

The co-chaperone features of CSP and the analysis of CSP function in various model systems suggest that CSP may coordinate sequential protein–protein interactions to serve multiple functions, most prominently to mediate a late step in exocytosis but also to stabilize the machinery of Ca^{2+} entry and Ca^{2+} clearance. The *in vitro* studies describing the CSP/Hsc70 interaction, together with the *in vivo* analysis of *Drosophila csp* mutations excluding a major function of CSP in synaptic vesicle recycling, suggest an additional and novel role of Hsc70 in exocytosis. This idea is supported by loss of function mutations in *Drosophila* Hsc70, which impair neurotransmitter release in a way highly reminiscent of *csp* null mutations [166]. Thus far, possible substrates of CSP include synaptobrevin [156], syntaxin [159,167], and N- and P/Q-type Ca^{2+} channels [156,159]. Although the *in vivo* significance of most of these CSP–substrate interactions remains to be established, the *in vitro* and *in vivo* interaction of CSP with syntaxin and the copurification of CSP with synaptobrevin are compatible with a role for CSP in regulating SNARE complex-associated protein interactions (Fig. 3).

5. Concluding remarks

Molecular chaperones have come a long way in overcoming their original reputation as general household folding machinery, to take center stage as critical factors of signaling pathways. The fast and high fidelity coupling of nerve signaling and neurotransmitter exocytosis requires that the participating components are rapidly recycled and that they sustain an optimal conformation despite repeated use. The numerous protein–protein interactions that are involved in this process apparently require a sequential transition through several states of transient protein complexes or protein conformations. Molecular chaperones fill the role of supervising these specific transitions by binding to a unique number of substrates, as exemplified by the SNAP/NSF, auxilin/Hsc70, and CSP/Hsc70 chaperone machinery.

A rather interesting feature of all these systems is that the enzymatically active component (ATPase) is cytoplasmic, and, by itself, lacks any specificity for a particular signaling pathway. The temporal and spatial specificity of either system is uniquely specified by its participating cofactors and is accomplished by their unique protein binding specificities and/or membrane localizations. This intricate design allows Hsc70 to specifically mediate at least two stages of the synaptic vesicle cycle. Recruitment by auxilin accomplishes vesicle uncoating, while recruitment by CSP may facilitate a late step of exocytosis. Similarly, NSF apparently gains specificity by using a combination of SNAPs to mediate SNARE complex transitions and glutamate receptor exposure on postsynaptic membranes. Future work will be necessary to provide a better understanding of how these and potentially other chaperones ensure a smooth passage through the synaptic vesicle cycle. Without doubt, the focus of attention will be on the role of the co-factors, which are likely destined to be at the center of a higher order regulation mechanism. The first evidence for such mechanisms has been obtained for CSP that is up-regulated in rats during long-lasting LiCl exposure, which is used to treat manic depression in humans [167]. Knowing how chaperones pull the strings behind the scenes will be crucial to expanding our current knowledge about the molecular machinery underlying regulated neurotransmitter release.

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