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REVIEW

Putting a brake on synaptic vesicle endocytosis

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Abstract In chemical synapses, action potentials evoke synaptic vesicle fusion with the presynaptic membrane at the active zone to release neurotransmitter. Synaptic vesicle endocytosis (SVE) then follows exocytosis to recapture vesicle proteins and lipid components for recycling and the maintenance of membrane homeostasis. Therefore, SVE plays an essential role during neurotransmission and is one of the most precisely regulated biological processes. Four modes of SVE have been characterized and both positive and negative regulators have been identified. However, our understanding of SVE regulation remains unclear, especially the identity of negative regulators and their mechanisms of action. Here, we review the current knowledge of proteins that function as inhibitors of SVE and their modes of action in different forms of endocytosis. We also propose possible physiological roles of such negative regulation. We believe that a better understanding of SVE regulation, especially the inhibitory mechanisms, will shed light on neurotransmission in health and disease.

Keywords Endocytosis inhibitor · Cain · Cdk5 · Synaptotagmin · Cl^- · Calpain · BDNF · Physiological temperature · Osmotic pressure

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Introduction

During neurotransmission, action potentials induce the opening of Ca^{2+} channels, and the resulting Ca^{2+} transient stimulates synaptic vesicle exocytosis, leading to neurotransmitter release. Sustained neurotransmission requires repeated rounds of vesicle exocytosis and the number of synaptic vesicles on demand can greatly exceed the supply transported from the cell body. To meet the demand for repeated rounds of vesicle exocytosis, nerve terminals have developed efficient local vesicle recycling mechanisms to regenerate synaptic vesicles. Exocytosis-coupled endocytosis retrieves proteins and membranes from the plasma membrane and new vesicles are generated either directly from endocytosis or from endosome intermediates and are filled with neurotransmitters for new rounds of exocytosis.

Both the precision and efficiency of exocytosis-coupled endocytosis are critical for vesicle recycling. So far, four modes of synaptic vesicle endocytosis (SVE) have been reported: clathrin-independent ultrafast endocytosis (50–500 ms), kiss-and-run (<1 s), clathrin-mediated endocytosis (CME, 10–20 s), and activity-dependent bulk endocytosis (ADBE, probably slow) [1]. Different modes of endocytosis co-exist in the same nerve terminal and the preference for particular modes may depend on synapse type [2], stimulation strength, temperature, and synaptic maturation [3]. Distinct and faster endocytosis kinetics occur at physiological temperatures and in more mature synapses. Among these routes of endocytosis, CME is the best characterized mode with dozens of proteins participating in temporally and spatially specific ways.

The regulation of SVE has been extensively studied in the past four decades. Positive regulators such as proteins that regulate $PI(4,5)P_2$ levels [4, 5], voltage-dependent Ca^{2+} channels [6, 7], calcineurin [8], major vesicle proteins

including synaptotagmin 1 (Syt1) [9, 10], synaptophysin [11], and the SNARE complex [12, 13], along with endocytic machinery [14], all function to promote SVE. However, negative regulators are rarely reported and their studies are still limited. In this review, we focus on how the inhibitors may regulate SVE and discuss possible roles of the negative regulation.

Cain and Cdk5 counteract calcineurin

Synaptic vesicle endocytosis is a Ca^{2+}-dependent process. However, the exact role of Ca^{2+} in triggering and regulating different modes of endocytosis remains elusive. Numerous studies have suggested that Ca^{2+} accelerates, does not affect, or inhibits SVE [15]. The inhibitory role of a Ca^{2+} increase was first proposed in the ribbon synapses of bipolar neurons in goldfish retina and later in the small synapses of hippocampal neurons at the single synaptic vesicle level [16, 17]. In the calyx of Held, a prolonged, small, and global Ca^{2+} increase inhibits endocytosis, whereas a transient and large Ca^{2+} increase in the micro/nanodomain triggers and facilitates endocytosis [18]. This finding may reconcile apparent conflicts regarding the Ca^{2+} effect. The Ca^{2+} regulation of SVE has been reviewed in depth by Yamashita and Leitz and Kavalali recently [15, 19]. It is suggested that two proteins, calcineurin and synaptotagmin 1, serve as high-affinity Ca^{2+} sensors during endocytosis [8, 20, 21].

Calcineurin is a Ca^{2+}- and calmodulin-dependent serine/threonine protein phosphatase [22–24]. It is originally identified as the target of immunosuppressive drugs such as cyclosporin A and FK506, leading to discovery of its important role in T-cell activation [25, 26]. Calcineurin gene is conserved from yeast to mammals. Importantly, it is highly expressed in the nervous system and regulates synaptic function [27], neuroinflammation, and glial signaling [28]. In nerve terminals, SVE is triggered by calcineurin dephosphorylation of a number of essential endocytic proteins, i.e., dynamin, amphiphysin1/2, synaptojanin 1, AP180, epsin, and eps15, upon Ca^{2+} influx [29]. The dephosphorylation event stimulates protein–protein interactions that may target proteins to sites of action and help assemble the endocytic complex.

Both positive and negative endogenous regulators of calcineurin have been identified in the brain. Among its inhibitors, cain 1 (calcineurin inhibitor, or cabin) was first discovered in the mouse T-cell cDNA library by yeast two-hybrid system screening for proteins interacting with calcineurin [30]. In T cells, its binding to calcineurin is dependent on PKC and Ca^{2+} signaling. Overexpression of cain or its C-terminal inhibits calcineurin signaling. Independently, Synder's group also reported the cloning of cain from the rat hippocampus cDNA library and showed that its C-terminal 2078–2173 amino acids interact with calcineurin and non-competitively inhibit calcineurin activity ($K_i = 0.44 \pm 0.07$ μM) in vitro [31]. An additional domain of cain (701–900) was later recognized for its inhibition activity in cain1ΔC mice [32]. Orthologs of cain genes have been identified from zebrafish to mammals with 70% amino-acid sequence similarity [33]. Importantly, cain is highly expressed in rat brain and colocalizes with calcineurin expression [31].

While investigating the neuronal functions of cain, the Synder group found that cain is a component of the endocytic complex, interacting with the SH3 domain of amphiphysin 1 through its PRD domain [34]. Interestingly, dynamin also co-immunoprecipitates with the cain-amphiphysin complex, suggesting that cain does not affect dynamin binding. Overexpression of cain in HEK293 cells leads to a 70% reduction of transferrin uptake, similar to the dynamin K44E mutant. The calcineurin inhibitors cyclosporin A and FK506 also reduce endocytosis to a similar level. These results suggest that cain serves as a potent inhibitor of SVE. It remains to be determined whether the endogenous level of cain inhibits endocytosis, how different modes of SVE are affected in cain-deficient neurons, and whether its inhibition is stimulated by Ca^{2+} and PKC, as shown in T cells.

At least three other calcineurin inhibitors have been identified so far. A 79-kDa protein kinase A anchoring protein (AKAP79) is a noncompetitive inhibitor of calcineurin and colocalizes with calcineurin in rat hippocampal neurons [35]. CHP (calcineurin homologous protein) shares a high degree homology with the regulatory B subunit of calcineurin, and inhibits ~50% of the immunoprecipitated calcineurin activity in CCL39 cells overexpressing CHP [36]. Furthermore, the in vitro activity of purified calcineurin is almost abolished by recombinant CHP. Yeast protein Rcn1p (Calcipressin-like protein) or human homolog DSCR1 (Down syndrome candidate region 1), which is overexpressed in Down syndrome, also binds calcineurin and strongly inhibits its phosphatase activity [37, 38]. It remains to be determined whether these inhibitors regulate SVE as well.

It has been shown that cycles of dephosphorylation and re-phosphorylation of endocytic proteins are essential for SVE [29]. The group of endocytic proteins termed dephosphins include dynamin 1 [39], amphiphysin 1/2 [40, 41], synaptojanin [42, 43], epsin, eps15 [44], and AP180 [45]. They are constitutively phosphorylated in resting nerve terminals, dephosphorylated when stimuli arrive, and rephosphorylated for subsequent rounds of endocytosis. Cdk5 was the first kinase identified to phosphorylate dephosphins and is essential for SVE [46]. Cdk5 is a proline-directed serine/threonine kinase with a structure similar to mitotic Cdks, and is expressed

abundantly in mature presynaptic terminals [47]. Cdk5 has multiple functions, including neuronal migration, transmitter release and endocytosis, and signal transduction [48]. Three synaptic substrates of Cdk5 associated with synaptic vesicle recycling are amphiphysin 1 (in vitro) [49, 50], dynamin 1 (both in vitro and in vivo) [46, 50], and synaptojanin (in vitro) [46].

Tan et al. reported the essential role of Cdk5 for SVE [46]. They discovered that Cdk5 phosphorylates dynamin I on Ser 774 and Ser 778 in vitro and these sites are phosphorylated in vivo by mass spectrometry and phospho-specific antibodies. In the presence of roscovitine, a well-characterized Cdk5 antagonist, they found decreased dynamin re-phosphorylation in intact synaptosomes after one round of endocytosis. Dominant-negative Cdk5 blocks dynamin phosphorylation in the neurites of differentiated B104 rat neuroblastoma cells. FM2-10 uptake in synaptosomes showed little effect of roscovitine on the first round of endocytosis, but it significantly decreased the second round of endocytosis with stimulation by 30 mM KCl. EM analysis showed depleted synaptic vesicles and a ruffled plasma membrane. Therefore, Cdk5 is required for SVE.

Independently, Tomizawa et al. reported that Cdk5 is a negative regulator of endocytosis using mouse hippocampus neurons deficient in p35 (a Cdk5 activator) [50]. They first used wild-type primary neurons to measure FM1-43 uptake after 30 s of stimulation at 20 Hz. The FM dye uptake increased in the presence of two Cdk5 antagonists, olomoucine, or roscovitine. Endocytosis kinetics was measured by FM uptake after different delays (30–90 s after depolarization) and olomoucine-treated terminals showed accelerated SVE. In p35-deficient neurons, olomoucine no longer had any effects on FM1-43 uptake, and SVE was accelerated compared to that of the wild type. They went on to identify amphiphysin and dynamin 1 as Cdk5 substrates both in vitro and in vivo (using p35-knockout mice). Phosphorylation interrupted their interaction and the amphiphysin binding of β-adaptin in vitro. Altogether, they showed that Cdk5 activity inhibits the endocytosis process, consistent with the view of the constitutive action of protein phosphorylation in the synapse.

These seemingly contradictory conclusions could be explained by the dephosphorylation–re-phosphorylation model proposed by Nguyen et al. [51]. During depolarization, Cdk5 inhibits endocytosis by interacting with calcineurin. When cells are repolarized, Cdk5 is then required for the re-phosphorylation of dephosphins to prepare for the next round of endocytosis. Actually, Tan et al. also reported a mildly accelerated, albeit statistically insignificant, first round of endocytosis (~12%) [51]. This could be due to the different modes of endocytosis evoked by different stimuli (30 mM KCl vs. 20 Hz, 30 s).

Synaptotagmins regulate endocytosis

In addition to calmodulin/calcineurin, Syt1 also serves as a Ca^{2+} sensor for coupling endocytosis with exocytosis. Syt1, primarily known as the Ca^{2+} sensor for synchronized release, is the best characterized member of the Syt family which contains 17 members in mammalian cells [52, 53]. Syt family proteins are type I membrane proteins, which share the conserved cytosolic repeats C_2A and C_2B that are homologous with the Ca^{2+}-binding C_2 domain of phospholipid-dependent protein kinase [54]. Interestingly, the C_2B domain of Syt1 binds to AP-2 with high affinity (1.2×10^{-9} M) [9, 55] as well as to endocytic protein stonin 2 [56], indicating that Syt1 also plays an important role in endocytosis. Furthermore, photoinactivation of Syt1 after exocytosis leads to impaired endocytosis in *Drosophila* [10]. Mouse knockout (KO) studies using the synaptopHluorin method have revealed that depletion of Syt1 significantly impairs endocytosis [57]. Mutational analysis uncouples Syt1 endocytic function from its exocytic function, and either the C_2A or the C_2B domain acts as the Ca^{2+} sensor in endocytosis [58]. These findings support the idea that Syt1 is a positive regulator of SVE.

Syt4 also functions both in stimulation-coupled exocytosis and endocytosis. Syt4 and Syt11 harbor an aspartate-to-serine substitution in a Ca^{2+} coordination site of the C_2A domain and fail to bind Ca^{2+} biochemically. Syt4 is mainly expressed in brain and neuroendocrine tissues at a relatively low level but is induced by activity [59]. Zhang et al. investigated the exocytic and endocytic functions of Syt4 in posterior pituitary nerve terminals, which are peptidergic [60]. They found Syt4 on both dense-core vesicles and microvesicles by vesicle immunoprecipitation and immuno-EM. Membrane capacitance analysis revealed that Syt4 KO neuron terminals had a reduced Ca^{2+} current density and abnormal exocytosis, while a degree of compensatory endocytosis similar to wild-type neurons was found. Furthermore, Syt4 KO exhibited similar time constants for both slow and fast endocytosis ($\tau = 1.4$–2.2 s for slow, 0.1–0.2 s for fast component). However, Syt4 KO had a reduced slow-to-fast component ratio, indicating that Syt4 depletion favors fast endocytosis. It would be interesting to understand the molecular nature of the slow and fast components and how Syt4 interacts with the endocytic machinery.

Syt11, an abundant protein in brain, was first identified by the Südhof group in 1997 [61]. It is a candidate gene for susceptibility to schizophrenia and a risk locus for Parkinson's disease [62–67]. The first evidence implicating Syt11 in endocytosis was found in macrophage phagocytosis by Arango Duque et al. [68, 69]. Syt11 knock down (KD) increased both cytokine release and phagocytosis but with compromised phagosome protein

recruitment and microbicidal activity. We characterized the neuronal function of Syt11 and found that it inhibits endocytosis without affecting evoked exocytosis [70]. Membrane capacitance measurements revealed accelerated exo-endocytosis in the somata of Syt11 KD dorsal root ganglion (DRG) neurons. Furthermore, 45% of the KD neurons had excessive membrane retrieval, indicating unbalanced endocytosis coupling to exocytosis. Importantly, FM uptake and discharge both increased in Syt11 KD hippocampal terminals, while FM discharge kinetics (reflecting exocytosis) remained unaffected, indicating that Syt11 inhibits SVE as well.

Both CME and bulk endocytosis are accelerated in Syt11-KD DRG neurons. Transferrin uptake increases and the application of MDC, an inhibitor of CME, inhibits the slow component of exo-endocytosis in Syt11-KD neurons (KD, $\tau = 5.78 \pm 1.23$ s; KD + MDC, $\tau = 12.32 \pm 1.69$ s), leaving the fast component unaffected (KD, $\tau = 1.44 \pm 0.48$ s; KD + MDC, $\tau = 1.39 \pm 0.40$ s). The fast component likely reflects bulk endocytosis, since the capacitance shift reveals an increased frequency of bulk-like events. Single-cell EM after HRP uptake shows increased clathrin-coated pits and bulk-like structures on the plasma membrane of Syt11-KD neurons, while the sizes of endocytic vesicles and endosomes remain unchanged, suggesting that Syt11 inhibits the initial stage of endocytosis. Domain analysis indicated that the transmembrane domain and AP-2-binding sites (KKAA in the C_2B domain) are critical for Syt11 to specifically inhibit CME. The C_2A domain is specific for bulk endocytosis. On the other hand, C_2B is important for both events. These data suggest that Syt11 inhibits different pathways by interacting with different endocytic machinery. We propose that Syt11, together with positive regulators, functions to ensure precision during vesicle retrieval. Dissection of the molecular mechanisms of Syt11 inhibition in SVE is needed.

Syt1 and Syt11 are abundantly expressed in most brain regions. Although Syt4 has a relatively low expression level, it acts as an immediate–early gene upon neuronal depolarization; it is also upregulated by seizures and psychoactive drugs, and is induced by neuronal activity [59, 71–74]. It has been reported that different Syt members are co-expressed in neurons, bind the same interacting proteins, and form hetero-oligomers to modulate neurotransmission [75–77]. Furthermore, Syt1 and Syt4 form hetero-oligomers in *Drosophila* and changes in their expression ratio modulate the efficiency of excitation-secretion coupling [78]. It is plausible that Syt members with distinct Ca^{2+}-binding affinities also work together to fine-tune endocytosis.

Calpains cleave endocytic proteins during neural hyperexcitation

Calpains are a family of evolutionarily conserved Ca^{2+}-dependent cysteine proteases that function in cell adhesion, migration, proliferation, and cell death [79, 80]. The calpain system constitutes an irreversible and long-lasting post-translational regulatory mechanism which modulates the function and/or localization of target proteins through partial proteolysis. Calpain-1 and calpain-2, also known as μ- and m-calpain (activated by micro- and millimolar Ca^{2+}), are the major isoforms in the brain. They are known to regulate synaptic plasticity and neuronal survival/degeneration. Their involvement in the endocytosis pathway was first suggested by their localization on coated vesicles purified from bovine brain and they bind to the vesicle membranes but not the coats in a Ca^{2+}-dependent manner [81]. Furthermore, activation of endogenous calpains or addition of exogenous calpains to purified coated vesicles cleaves a subset of coat components such as clathrin light chain, tubulins, and adaptins.

A direct inhibitory role of calpain in SVE during neuronal hyperexcitation was first reported by Wu et al. in 2007 [82]. In hippocampal slices exposed to high K^+ or neurotoxic levels of glutamate, amphiphysin 1 was partially truncated by calpains into three small fragments and both μ- and m-calpain co-fractionated with synaptosomes. In vitro experiments have identified cleavage sites at positions 333, 377, and 392. Interestingly, amphiphysin binding of liposomes or dynamin I inhibits calpain activity, while a calpain-truncated form (1–392) tubulates liposomes like full-length amphiphysin but fails to form ring structures with dynamin I in vitro. These results suggest that the truncated form fails to bind dynamin I, which was expected, since it lacks an SH3 domain. Overexpression of this truncated form in Cos-7 cells or rat hippocampal neurons both inhibited endocytosis, as revealed by transferrin and FM 4–64 uptake. Using the calpain inhibitor ALLM, amphiphysin has been shown to be cleaved by calpain only under high-frequency stimulation, and calpain activity is responsible for the frequency-dependent depression in hippocampal slices. Importantly, a kainate-induced seizure model revealed that amphiphysin cleavage and ALLM attenuated the preconditioning effect of kainate, suggesting that calpain modification inhibits excessive neurotransmitter release.

Rudinskiy et al. reported that calpain hydrolyzes the α- and β2-subunits of the AP-2 complex, which inhibits CME [83]. The α- and β2-adaptins are cleaved by calpain in brain tissue in the presence of Ca^{2+}, and this is prevented by ALLN. This kind of proteolysis also exists in cultured striatal neurons upon glutamate stimulation and is inhibited by the NMDA receptor antagonist MK801 or the calpain 2

inhibitor Z-LLY-FMK, but not the calpain 1 inhibitor PD 151,746. The calpain cleavage sites have been mapped to the hinge region of both adaptins and such cleavages have been predicted to disrupt clathrin and clathrin accessory factor binding to the AP-2 complex. As expected, overexpression of the β2-adaptin cleavage mutant leads to reduced clathrin recruitment to the plasma membrane. Glutamate stimulation elicits a calpain-dependent decrease of transferrin uptake in primary cultured striatal and cortical neurons, while dextran uptake and LDH release remain unaffected. However, neurons overexpressing the β2-adaptin-truncated mutants are more sensitive to glutamatergic excitotoxicity. In two models that are known to activate endogenous calpain, the ischemia–reperfusion injury rat model, and the postmortem human Alzheimer's disease brain, both α- and β2-adaptins are cleaved. Other clathrin adaptors, AP180, epsin 1, and CALM, are also cleaved upon in vitro exposure to calpain 2 and Ca^{2+}. Therefore, calpain may be involved in neurodegeneration by inhibiting CME via cleavage of multiple endocytic proteins, and modulate neuronal sensitization to glutamatergic excitotoxicity.

Among other target proteins of calpains, p35 [84], calcineurin [85], and GSK3β [86], all play critical roles in SVE and interestingly, they are all activated by calpain under neurotoxic conditions. These modifications promote apoptosis and excitotoxic neuronal death, and may also be involved in neurodegenerative diseases. It remains unknown whether these modifications affect SVE.

Extracellular signals that inhibit synaptic vesicle endocytosis

It had been assumed that SVE was only regulated in presynaptic sites, but Micheva et al. reported retrograde up-regulation by postsynaptic NO diffusion to the presynaptic site, leading to a cGMP-dependent increase of presynaptic phosphatidylinositol 4,5-biphosphate [87]. In addition, von Gersdorff's group reported inhibitory regulation of fast endocytosis by GABA-mediated chloride influx in the reciprocal synapse of goldfish bipolar cells [88]. The bipolar cell terminal releases glutamate, followed by GABAergic feedback on presynaptic GABA receptors. They found that in acutely dissociated terminals, capacitance measurement displayed a double exponential decay with a τ_{fast} of 1.6 s and a τ_{slow} of >10 s. Surprisingly, only τ_{slow} was recorded in retinal slices. $GABA_A$ and $GABA_C$ receptor antagonists, AMPA and NMDA receptor antagonists, and removal of extracellular chloride all reverse the inhibition of fast endocytosis in slices. Furthermore, elevating the internal chloride concentration in acutely dissociated terminals abolished the fast component of SVE. Much larger methanesulfonate or gluconate anions had no effect. To eliminate possible contamination of asynchronous exocytosis on slowed capacitance decay, Ca^{2+} was buffered with EGTA. No effect on the chloride inhibition was found. Therefore, the authors proposed a 'second messenger' role of chloride to modulate cellular processes near the presynaptic membrane. They hypothesized that chloride acts via intracellular chloride channels on the membrane of synaptic vesicles, changing the internal surface charges which may affect protein–lipid interactions, or chloride-dependent molecules such as the molecular motor, kinases, and enzymes.

Brain-derived neurotrophic factor (BDNF) has also been reported recently to slow the endocytosis process [89]. It is an important neurotrophin with multiple physiological functions in neuronal development, differentiation, synapse formation and stabilization, neurotransmission, and synaptic plasticity [90, 91]. BDNF is predominantly synthesized in neurons and is preferentially localized in dendrites rather than axons, but it can be released at both sites via a regulated pathway in a slow mode [92–94].

Cousin's lab reported that BDNF inhibits activity-dependent bulk endocytosis (ADBE) in cerebellar neurons [89]. They had previously shown that GSK3 phosphorylation of dynamin Ser-774 is required for ADBE [95]. Since BDNF inhibits GSK3 activity via the phosphatidylinositol 3-kinase (PI3K) pathway, they reasoned that BDNF might inhibit ADBE. They first confirmed that BDNF inhibits GSK3 via the PI3K pathway and dynamin phosphorylation of Ser-774 in primary cultures of cerebellar granule neurons. In experiments using the uptake of large fluorescent dextrans (40 kDa), BDNF had no effect on ADBE after a high-frequency train of action potentials (80 Hz, 10 s), but induced a robust inhibition after a second round of stimulation. This inhibition was relieved by PI3K or GSK3 antagonists, as expected. Electron microscopy (EM) analysis after HRP uptake revealed that endosome-like structures (from ADBE), but not small vesicles (from CME), were reduced by BDNF after the second round of exocytosis. However, they did not find an exocytic effect due to synaptopHluorin dynamics or FM2-10 discharge kinetics in their system. In search of the physiological function of this inhibition, they found that BDNF relieved the synaptic depression after a second high-frequency train of action potentials at Purkinje cell synapse in cerebellar slices. This relief was reversed by GSK3 and PI3K antagonists and was not due to an increase of release probability. Therefore, the authors proposed that BDNF released from postsynaptic sites increases neurotransmission by inhibiting ADBE.

Another report on BDNF inhibition of endocytosis came from Wu's lab [96]. They used rat brainstem slices to examine the presynaptic functions of BDNF at the calyx of Held. Both BDNF and its receptor TrkB colocalized with vGlut1, indicating their presence in the glutamatergic terminal of the calyx. By measuring the membrane capacitance and recording I_{Ca} charge (QI_{Ca}) and excitatory postsynaptic

currents, they found that BDNF inhibited Ca^{2+} channel activation and vesicle release probability. It also reduced the vesicle retrieval $Rate_{decay}$ of both the slow mode of endocytosis (20 ms depolarization, $\tau = 15.6 \pm 1.1$ s, $Rate_{decay} = 27 \pm 3.7$ fF/s) and the fast mode of endocytosis (10×20 ms depolarization at 10 Hz, $\tau = 2.05 \pm 0.1$ s, $Rate_{decay} = 65 \pm 7$ fF/s), while QI_{Ca} was unaffected. By assessing endocytosis in P8-P10 and P13-14 calyces, they demonstrated that BDNF inhibited the rapid and slow modes of endocytosis in a Ca^{2+}-independent manner in both pre-hearing and post-hearing calyces. The authors proposed that BDNF may activate phospholipase Cγ, another downstream effector of TrkB, to activate Ca^{2+}/calmodulin-dependent kinases which may regulate Ca^{2+} channels. The difference in BDNF effects on exocytosis and endocytosis in these two reports may stem from the different synapse types and stimulation regimes.

Physical factors that affect synaptic vesicle endocytosis

Endocytosis is highly sensitive to temperature. Low temperatures suppress low-density lipoprotein uptake in cultured human fibroblasts (4 vs. 37 °C) [97], fluid-phase endocytosis in the mouse fibroblast L929 cell line (4 vs. 37 °C) [98], and SR101 uptake in snake motor boutons (7 vs. 35 °C) [99–101]. Studies of SVE have demonstrated that physiological temperatures (PT, 35–37 °C vs. room temperature) accelerate the endocytosis rate, the rate of readily releasable pool refilling (in hippocampal neurons), turnover of the entire releasable pool during sustained synaptic activity (in hippocampal neurons), the amplitudes of excitatory postsynaptic currents (EPSCs) and miniature EPSCs (in hippocampal neurons and the calyx of Held), and vesicle reacidification ($Q_{10} = 5.5$, in hippocampal neurons) [102–106]. However, the size of the readily releasable pool remains unaffected [102, 105]. Recently, Delvendahl et al. calculated the Q_{10} temperature coefficient for dynamin- and actin-dependent ultrafast endocytosis to be 3.3 ± 1.9 in hippocampal mossy fiber boutons and 3.8 ± 1.3 in cerebellar mossy fiber boutons, using low-noise time-resolved membrane capacitance measurements [107]. The inhibitory mechanisms of low temperatures may be related to blockade of vesicle fission [97], decreased Ca^{2+} current ($Q_{10} = 1.6 \pm 0.1$) and slower Ca^{2+} current activation and deactivation ($Q_{10} \approx 7$, near -10 mV) [108], the absence of a kinetically fast component ($\tau = 1$–2 s) [109], and the modulation of dephosphorylation of dynamin 1 [110]. Other biochemical reactions may also be regulated by temperature. Taken together, PT speeds up endocytosis and increases the vesicular supply, reducing the degree and time course of synaptic depression during sustained neurotransmission [102, 105]. On the other hand, brain cooling has been found to block neurotransmission and has been extensively explored for potential therapeutic treatment of epilepsy, stroke, neonatal encephalopathy, and other neurological diseases [111–114].

Osmolarity is another physical factor that affects endocytosis in general. Rauch et al. reported that when external pressure is decreased by a factor of 0.54, a sudden inhibition of bulk-flow endocytosis occurs in K562 cells (a human erythroleukemia cell line) [115]. This suppression is alleviated by the addition of phosphatidylserine, which can generate phospholipid asymmetry and act as a budding force for endocytosis. In bipolar neurons of the goldfish retina, Heidelberger et al. discovered that a small increase in hydrostatic pressure (≥20 mOsm) dramatically impairs a slow component of endocytosis ($\tau_{slow} = 20.9 \pm 6.9$ s, most likely CME) but does not affect the fast component ($\tau_{fast} = 3.3 \pm 1.0$ s, kiss-and-run-like) [116]. When the hydrostatic pressure is withdrawn, an even slower form of endocytosis ($\tau = 38.9 \pm 5.3$ s) restores the resting membrane capacitance to baseline. The relative hyperosmosis may lead to high membrane tension, which controls cell shape and motility by inhibiting endocytosis and stimulating exocytosis [117, 118].

Possible roles of SVE inhibition

The physiological and pathological functions of these inhibitory mechanisms during SVE are largely unknown. We propose five possibilities as follows. First, the negative regulators may provide additional layers of regulation by fine-tuning the SVE initiation and termination both temporally and spatially. For example, high intracellular Ca^{2+} may inhibit SVE at the active zone when exocytosis occurs [119]. Cdk5 and cain may help to refine the activity of calcineurin. Second, the inhibitors may serve as checkpoints to ensure more precise regulation of endocytosis. In Syt11-KD DRG neurons, the *C*m overshoot suggests the presence of an imbalanced membrane retrieval after exocytosis [70]. The mechanisms of precise protein retrieval may also involve inhibitors. Third, the inhibitors may regulate the rate of specific modes of SVE in particular synapses or neuron types to relieve synaptic depression. BDNF specifically inhibits ADBE in cerebellar granule cells and relieves synaptic depression and enhances synaptic transmission in Purkinje synapses during high-intensity firing [89]. However, BDNF inhibits both slow and rapid endocytosis in the calyx of Held [96]. High osmolarity induced by physical or physiological factors inhibits the slow mode of endocytosis in bipolar neurons of the goldfish retina [116]. In posterior pituitary neurons, Syt4 KO prefers the fast mode [60]. Chloride influx inhibits fast endocytosis in GABAergic goldfish bipolar neurons [88]. Fourth, inhibited endocytosis kinetics may help to better distribute limited resources such as available endocytic machinery and energy resources in

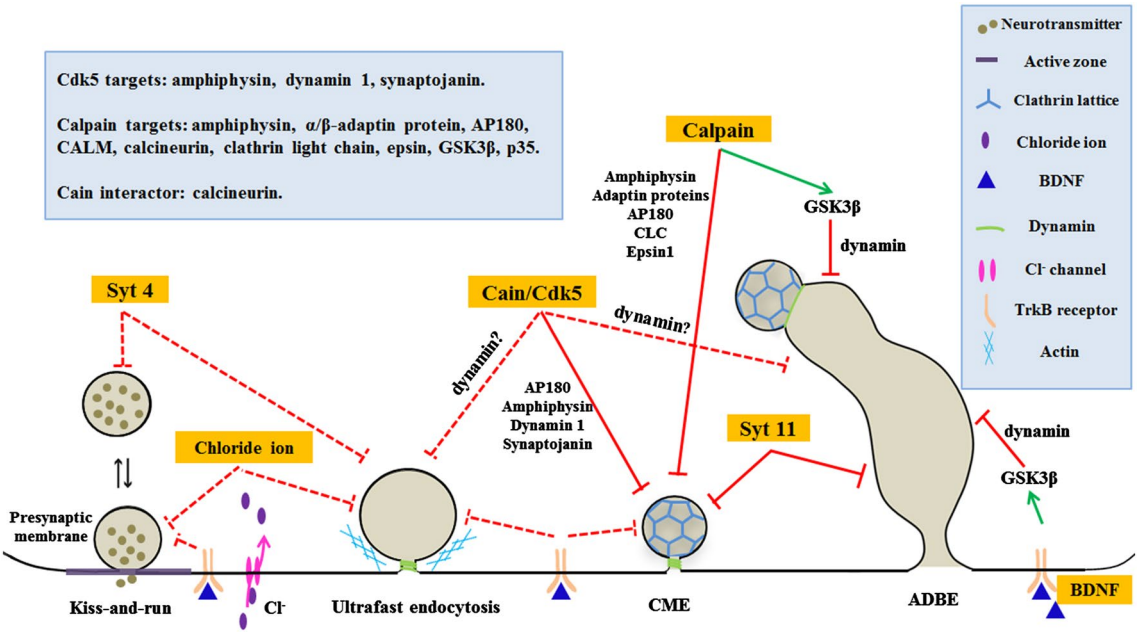

Fig. 1 Negative regulators in different modes of SVE. Inhibitors are shown in *yellow boxes; red solid lines* indicate known inhibitory pathways; *red dotted lines* indicate speculative pathways based on endocytic kinetics (for Syt4 and Cl^- that inhibit fast endocytosis pathways and BDNF which inhibits both fast and slow endocytosis) or protein targets (for cain and cdk5 that inhibit calcineurin dephosphorylation of dynamin); and *green arrows* indicate positive regulation. Endocytic targets or interactors with inhibitors are shown in the *upper left box*

nerve terminals [120]. Multiple steps in the synaptic vesicle cycle, including SVE, rely on ATP [121, 122]. It would be important to use the resources efficiently to keep the terminal active during sustained neurotransmission. Fifth, certain pathological conditions may activate specific inhibitory mechanisms for neuroprotection or neurodegeneration. For example, calpains cleave the endocytic machinery during neural hyperexcitation, which may lead to attenuation of seizures and be involved in neurodegeneration [82, 83].

Conclusions

Our understanding of the inhibitory mechanisms of SVE is still very limited (Fig. 1). The molecular mechanisms of the reported negative regulators, e.g., their interaction with endocytic machinery, when, where, and in which mode of SVE they function, remain largely elusive. There could be more candidates for negative regulation. For example, knockdown of inositol 5-phosphatase SHIP2 (a regulator of PI(3,4,5)P3-dependent signaling) in COS-7 cells shortens the lifetime of clathrin-coated pits [123], indicating an inhibitory role in the early stage of CME. While SHIP2 is mainly expressed in neural stem cells in the adult brain [124], other phosphatases, or kinases downregulating $PI(3,4,5)P_3$ and/or $PI(4,5)P_2$ levels may also inhibit SVE. As most studies have been carried out at room temperature, it is important to understand the inhibitory mechanisms at physiological temperatures when distinct and faster endocytosis occurs. Synapse maturation also leads to a higher capacity of endocytosis [125–127]. The developmental regulation of inhibitory mechanism is another interesting topic. Besides the essential physiological role endocytosis plays in the synapse, it is also tightly associated with multiple forms of neurodegenerative disease [128]. Comprehensive investigation of the mechanisms of SVE inhibition would not only benefit our understanding of how the synapse functions, but also improve our knowledge of neurodegenerative processes and provide potential therapeutic targets.

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