Acta Med. Okayama, 2009
Vol. 63, No. 6, pp. 305–323
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Acta Medica Okayama
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Review

Amphiphysin I and Regulation of Synaptic Vesicle Endocytosis

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Amphiphysin I, known as a major dynamin-binding partner localized on the collars of nascent vesicles, plays a key role in clathrin-mediated endocytosis (CME) of synaptic vesicles. Amphiphysin I mediates the invagination and fission steps of synaptic vesicles by sensing or facilitating membrane curvature and stimulating the GTPase activity of dynamin. Amphiphysin I may form a homodimer by itself or a heterodimer with amphiphysin II *in vivo*. Both amphiphysin I and II function as multilinker proteins in the clathrin-coated complex. Under normal physiological conditions, the functions of amphiphysin I and some other endocytic proteins are known to be regulated by phosphorylation and dephosphorylation. During hyperexcited conditions, the most recent data showed that amphiphysin I is truncated by the ca^{2+}-dependent protease calpain. Overexpression of the truncated form of amphiphysin I inhibited transferrin uptake and synaptic vesicle endocytosis (SVE). This suggests that amphiphysin I may be an important regulator for SVE when massive amounts of Ca^{2+} flow into presynaptic terminals, a phenomenon observed in neurodegenerative disorders such as ischemia/anoxia, epilepsy, stroke, trauma and Alzheimer's disease. This review describes current knowledge regarding the general properties and functions of amphiphysin I as well as the functional regulations such as phosphorylation and proteolysis in nerve terminals.

Key words: amphiphysin I, calpain, SVE, hyperexcitation, seizure

The synaptic vesicle membrane is recovered by synaptic vesicle endocytosis (SVE) after synaptic vesicles are released to the synaptic plasma membrane during synaptic transmission. Several modes of retrieval have been proposed to operate at small synaptic terminals of central neurons. They are classical clathrin-mediated endocytosis (CME), nonclassical "kiss and run", and bulk endocytosis. The classical CME is a slow track in which vesicles fuse completely with the presynaptic plasma membrane, followed by clathrin-mediated recycling of the vesicular components [1]. Nonclassical "kiss and run" is a fast track that may correspond to the transient opening and closing of a fusion pore [2]. Though the relative importance of both has been controversial, recent studies have shown that CME is the major mechanism for maintaining the synaptic vesicle recycling pool for synaptic transmission during repetitive high-frequency electrical stimulation (HFS) and physiological stimulation [3–5]. Bulk endocytosis was induced during continuous and strong stimulations such as those that

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occur with high potassium treatment in central synapses [6, 7]. Ca^{2+}-dependent phosphatase 2B calcineurin activity [8], actin polymerization [9], and F-BAR-containing endocytic protein syndapin [10], but not dynamin I (the major pinchase in synapses) [11], have been reported to be involved in this process. But the detailed molecular mechanism about bulk SVE remains to be defined.

CME has been divided into 4 steps: nucleation, invagination and contraction, fission, and uncoating [12]. Many endocytosis-related proteins are recruited to form complexes on the retrieved pits at each step after neural excitation activates calcium/calmodulin-dependent calcineurin activity. These proteins include clathrin, AP2, AP180, dynamin, amphiphysin, endophillin, synaptojanin, intersectin, syndapin, auxillin, HSC70 ATPase and so on [13]. Amphiphysin I is suggested to be colocalized with dynamin 1 on the collar of the retrieved pits [14]. It senses and facilitates membrane curvature [15] and stimulates the GTPase activity of dynamin in the presence of lipid membrane [16], and thus is involved in the invagination and fission steps of clathrin-mediated SVE. Besides its interaction with dynamin 1, amphiphysin I also interacts with clathrin heavy chain [17], α-appendage of AP2 [18], endophilin [19], synaptojanin [20], Cdk5 activator p35 [21], PLD (phospholipase D) [22] and cain [23].

CME is regulated by the phosphorylation and dephosphorylation of endocytic proteins. When nerve terminals in the brain are stimulated, a group of phosphoproteins (called dephosphins, including dynamin 1, amphiphysins I and II, synaptojanin, epsin, eps15, and AP180) are coordinately dephosphorylated by calcineurin [13]. The switching from the phosphorylated state of the endocytic proteins to the dephosphorylated state after nerve terminal depolarization is essential for triggering the CME. After one round of stimulation, following the synaptic membrane repolarization, all the phosphoproteins will be rephosphorylated before the next round of stimulation. Endocytic protein phosphorylation can regulate a variety of protein-protein and protein-lipid interactions [24–29]. *In vitro* experiments showed that a lot of kinases are involved in the phosphorylation of endocytic proteins in the resting conditions. They include cdk5, cdc2, PKC, CK2, PKA, MAPK, AAK1, GAK, and Src-family kinases [30].

CME may also be regulated by the proteolysis of endocytic protein during hyperexcitation, such as repetitive HFS or high potassium stimulation. It has been found that amphiphysin I is truncated to N-terminal fragments by the Ca^{2+}-dependent protease calpain under high potassium stimulation in mice hippocampal slices. Overexpression of the amphiphysin I truncation inhibited clathrin-mediated transferrin uptake in Cos-7 cells and synaptic vesicle endocytosis in neurons. Calpain-dependent amphiphysin I truncation is involved in HFS-induced postsynaptic short-term depression, which is thought to be an autoprotective mechanism for neurons against neuroexcitotoxicity. Further, calpain-dependent amphiphysin I truncation is also observed in kainate (KA)-induced seizures in FVB/NJ mice. Amphiphysin I cleavage by calpain may play a part in reducing the development of seizures *in vivo* [31].

General Properties of Amphiphysin I and Its Function in SVE

Amphiphysin I is an acidic, hydrophilic protein that is abundant in the nervous system and concentrated in presynaptic terminals; this was originally identified in chicken synaptic vesicle-associated protein in 1992. Chicken amphiphysin I is highly expressed in the chicken central nervous system (including forebrain, cerebellum, hippocampus, olfactory bulb, and spinal cord) and also in the testis [32]. Similarly, human amphiphysin I was later found to be expressed highly in the brain and testis. Its transcripts and antigens can also be found at low levels in the ovary, pituitary, pancreas, and adrenal gland [33–36]. A fraction of brain amphiphysin is found to be firmly associated with synaptic vesicles, but there also appears to be a cytosolic pool. Its cell-type distribution and its subcellular localization suggest that it may participate in mechanisms of regulated exocytosis in synapses and certain endocrine cell types. Human amphiphysin I is also highly overexpressed in some breast tumors and breast cancer cell lines [37]. It was first found as an autoantigen in stiff-man syndrome associated with breast cancer [33]. But the molecular mechanism underlying amphiphysin I's role in these diseases is not yet clear.

Human amphiphysin I is a transcript from chromosome 7p13-p14 [38]. So far, amphiphysin I is known

to have 6 splice variants, one brain amphiphysin I (695 aa), one non-neuronal amphiphysin I (653 aa, with a deletion of amino acids 425–466) [39], and 4 retina-specific amphiphysin Irs [40]. The brain form (695 aa) of amphiphysin I is mainly expressed in the brain, whereas the non-neuronal form (653 aa) of amphiphysin I is predominantly expressed in tissues other than brain. Amphiphysin Ir is specifically expressed in the retina, and it has been shown to be specifically expressed in rat ribbon synapses [41]. The differences among the amino acids sequences of the 6 amphiphysin I splice variants are compared in Fig. 1. An isoform of Amphiphysin I, called amphiphysin II or Bin 1, showed similar structure, function, and subcellular localization with amphiphysin I in the brain. Amphiphysin II has 13 splice variants. Some are expressed in specific tissues, while others are ubiquitously expressed [42]. Some studies have suggested that amphiphysin I may function in SVE by forming a heterodimer with amphiphysin II by their N-terminal coiled-coil domains [36, 43]. It appears that the relationship with amphiphysin I is important for the stability of the brain form of amphiphysin II, because amphiphysin II is nearly absent from amphiphysin I knockout mouse brain even though amphiphysin II mRNA is unchanged [44].

Amphiphsyin I or II is a modular protein from N the terminal, and has an α-helix, a BAR domain, a proline-rich domain (PRD), a CLAP domain (clathrin, AP2-binding domain), and a C-terminal SH3 domain (Fig. 2). Each domain plays an important role in amphiphysin I's functions. Amphiphysin I is involved

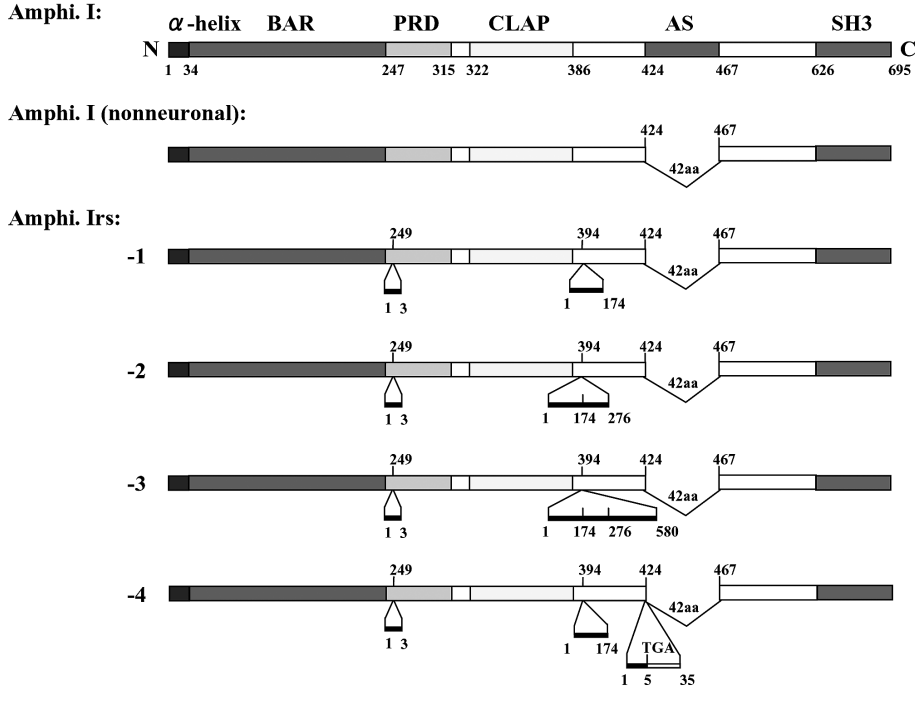

Fig. 1 Amphiphysin I splice variants expressed in human. Amphi. (amphiphysin) I is the canonical human amphiphysin I, which is specifically expressed in brain. It contains 695 aa and comprises α-helix (33 aa in amphi. II), BAR, PRD, CLAP, and SH3 domains from the N- to C-termini. The AS (alternative splicing) region (425–466, 42 aa) is deleted in other variants. Amphiphysin I (nonneuronal) is a nonneuronal variant of amphiphysin I and has the same amino acid sequence as canonical amphiphysin I except for a deletion at the AS region. Amphiphysin Irs are specifically expressed in brain retina. Each of them has 2 insert sequences and one AS deletion. The first insert sequence is 3 aa at 249. The second insert sequence occurs at 394, but the number of inserted aa differs among these variants: amphiphysin Irs-1 is 174 aa, amphiphysin Irs-2 is 276 aa, amphiphysin Irs-3 is 580 aa, and amphiphysin Irs-4 is 174 aa. Amphiphysin Irs-4 has the third insert DNA sequence at 424; it contains 35 aa, but the fifth aa is the stop code TGA, so Amphiphysin Irs-4 protein translation is ended at the stop code and the C-terminal region of the canonical amphiphysin I is not contained in amphiphysin Irs-4.

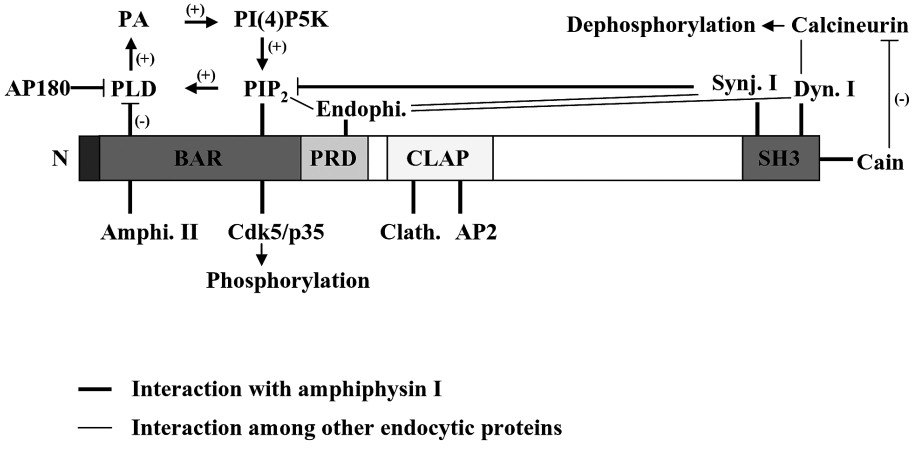

Fig. 2 Interactions between amphiphysin I and other endocytic components during clathrin-mediated SVE. Amphiphysin I is a multilinker protein. It interacts with amphiphysin II (amphi. II), lipid membrane (PI(4, 5)P_2), cdk5/p35 complex, and PLD (phospholipase D) at the N-terminus concluding BAR domain, with endophilin (endophi.) at the PRD domain, with clathrin (clath.) and AP2 at the CLAP domain, and with dynamin 1 (dyn. I), synaptojanin 1 (synj. I), and cain at the C-terminal SH3 domain. There is a positive feedback between PLD and PIP_2: PLD will increase PIP_2 production by hydrolyzing PC to PA. PA activates PI(4)P5K activity, then PIP_2 will be produced from PI(4)P by PI(4)P5K activity, while at the same time PIP_2 activates PLD. Amphiphysin I (1–373 aa) has been reported to bind PLD and to inhibit PLD activity. The interaction between amphiphysin I and PLD will regulate PIP_2 production, at the same time AP180 and synj. I also have an inhibiting effect at PLD activity and thus regulate PIP_2-dependent clathrin complex formation. On the other hand, amphiphysin I can regulate CME by recruiting cain (calcineurin inhibitor). Calcineurin binds dynamin and is recruited to the endocytic zone by amphiphysin I to execute its dephosphorylation effect on endocytic proteins, which is essential for CME induction. But its activity will be downregulated by its inhibitor, cain. This may promote cdk5-dependent rephosphorylation and dissociation of endocytic protein. Besides the association with amphiphysin I, the schematic figure also shows the interactions among other endocytic proteins (thin line).

in the SVE by its interactions with a lot of other endocytic components, including interactions with PIP2 of the plasma membrane, the cdk5/p35 complex, PLD and amphiphysin II at its N-terminal region; interactions with endophilin by the PRD domain; interactions with AP2 and clathrin by the CLAP domain; and interactions with dynamin I, synaptojanin and cain by the C-terminal SH3 domain (Fig. 2). Thus, amphiphysin I not only directly participates in the membrane curvature sensing and bending at the invagination step but also acts as an important multilinker protein that recruits various endocytic proteins and regulators to the clathrin complex. CME is finished by the coordinated work of all these endocytic proteins and regulators. Below we describe the interactions of amphiphysin I (domains from the N terminus to C terminus) with its partners and how these interactions function or regulate SVE.

Interaction with Membrane, p35 and PLD at N-terminal Region

The N-terminal BAR domain of amphiphysin senses and drives plasma membrane curvature, which is important for the invagination step of clathrin-mediated SVE. The crystal structures of the human and Drosophila amphiphysin II BAR domain reveal a crescent-shaped homodimer with a positively charged concave surface, suggesting that driving and/or sensing curvatures of membranes by BAR domains occurs by the binding of negatively charged membranes to this positively charged surface. The α-helix (1–24) is an N-terminal extension with an amphipathic character that is predicted to undergo a random coil-to-helix transition by binding to the membrane. The α-helix and BAR domain together has been called the N-BAR domain. A crystal structural analysis and molecular dynamic simulations suggest that the membrane curvature is generated by the synergistic action of the N-terminal helices embedded in the lipid bilayer and the charged crescent-shaped dimer acting to remold

membrane curvature [15, 45–49].

There are 2 conserved regions in human Bin 1 BAR and *drosophila* amphiphysin BAR domains with weak potential (compared with those of arfaptin and endophilin or that of the IMD domain of IRSp53) for protein-protein interaction, as detected by a computational approach. Since these sites are in the convex face of the BAR domain, would not interrupt the binding properties of the concave face directly. The protein-protein interaction sites is expected to be important for modulating the membrane-binding activity of the BAR domain by interacting with some other partner proteins [46].

So far, 2 proteins have been reported to interact with the amphiphysin I N-terminal. But they are not likely to be involved in the regulation of BAR domain binding activity. One is p35, an activator of cyclin-dependent kinase (Cdk5). Amphiphysin I is a substrate for Cdk5 [50], a member of the cyclin-dependent protein kinase family, which has been functionally linked to neuronal migration and neurite outgrowth via its action on the actin cytoskeleton. p35, the activating subunit of the Cdk5 kinase complex, has been found to interact with the amphiphysin I N-terminal region (1–306 aa) in rat brain and *in vitro* [21]. This interaction is analogous to the Pcl2-Rvs167 interaction in yeast [51]. The yeast homologue of amphiphysin I, Rvs167, functions in endocytosis and actin dynamics, is phosphorylated by the Cdk5 homologue Pho85, and binds the Pho85 regulatory subunit Pcl2. The interaction between p35 and the amphiphysin I N-terminal region may be important for amphiphysin I or other endocytic protein phosphorylation by Cdk5 in resting conditions, and thus may be an important SVE-regulating pathway.

The other protein that is known to interact with the amphiphysin I N-terminal is phospholipase D (PLD). PLD hydrolyzes the phosphodiester bond of the glycerolipid phosphatidylcholine, resulting in the production of phosphatidic acid (PA) and free choline. PA is widely considered to be the intracellular lipid mediator of many of the biological functions attributed to PLD. In mammalian systems, PLD activity regulates the actin cytoskeleton, vesicle trafficking for secretion and endocytosis, and receptor signaling. PLD is activated by phosphatidylinositol-4, 5-bisphosphate (PI(4, 5)P_2), protein kinase C (PKC), ADP ribosylation ractor (ARF1) and Rho family GTPases. PLD has 2 isoforms, PLD1 and PLD2. PLD1 has been reported to be localized to membranous structures: Golgi, endoplasmic reticulum, endosome, lysosome, and plasma membrane. PLD2 is most often reported to localize to the plasma membrane, but also localizes to the cytosol and submembranous vesicular compartments as well as co-localizing with β-actin [see review 52]. Four nerve-terminal concentrated and clathrin-coat-associated proteins have been related to PLD activity inhibition: synaptojanin [53], AP180 [54], amphiphysin I and amphiphysin II [22]. The inhibition of PLD by synaptojanin was attributed to its ability to dephosphorylate PI(4, 5)P_2, whereas the inhibitory effects of AP180, amphiphysins I and II are the result of direct interaction with PLD. Amphiphysin I and II N-terminal (1–373 aa) is critical for PLD binding and inhibition activity. The inhibition of PLD activity by synaptojanin, AP180 and amphiphysins I and II is thought of as a negative regulation for clathrin coat assembly. Because the PI(4, 5)P_2 component in the plasma membrane is important for clathrin coat assembly [55–60], at the same time it is an activator for PLD activity. PA, produced by the action of PLD, is a potent activator of PI(4)P 5-kinase [61–64] and thereby increases the synthesis of PI(4, 5)P_2, which, in turn, leads to further stimulation of PLD activity. A rapid increase in the concentration of PI(4, 5)P_2 induced by this positive feedback loop would be expected to facilitate clathrin coat assembly. Inhibition of PLD by amphiphysins and AP180, together with the hydrolysis of PI(4, 5)P_2 by synaptojanin, provides a mechanism to break the feedback loop (Fig. 2), which is important for coated vesicle disassembly and its subsequent fusion with plasma membrane.

These observations suggest that, aside from the sensing and/or driving membrane curvature in the invagination step, the conserved N-terminal region/BAR domain of amphiphysin I may also be involved in the phosphorylation of amphiphysin I and other endocytic proteins by cdk5 under resting conditions, as well as in regulating membrane PI(4, 5)P_2 levels and thus coated vesicle disassembly during synaptic activity.

Interaction with Endophilin at PRD Domain

The N-BAR domain is followed by a positively

charged stretch (248–315) that is very rich in proline (32%) and other small amino acids. Because of its high proline content, the proline-rich domain (PRD) harbors potential SH3-binding and proline-directed kinase phosphorylation sites. The sequences of both the N-BAR domain and the PRD domain are highly conserved among different species (for example, 94% of identity between chicken and human). Endophilin 1 has been reported to interact with the amphiphysin I and II PRD domains by its SH3 domain specifically [19]. Endophilin 1, a cytoplasmic Src homology 3 (SH3) domain-containing protein, localizes in brain presynaptic nerve terminals. Endophilin dimerizes through its N-terminus [65] and participates at multiple stages in CME, such as inducing CME initiation as a Ca^{2+} sensor, driving early membrane invagination by its BAR domain and uncoating synaptic vesicles by recruiting synaptojanin in synapses [66–75]. Both its C-terminal SH3 domain and the N-terminus are required for endocytosis. Through its SH3 domain, endophilin binds to PRDs in other endocytic proteins, including synaptojanin and dynamin [72, 73, 76] except amphiphysins. The N-terminal region possesses unique functions affecting lipid membrane curvature [77]. Endophilin is another important N-BAR domain-containing endocytic protein. Previous studies proposed that endophilin binds to tubulate membrane through its BAR domain and its lysophosphatidic acid acyl transferase (LPAAT) activity. By the LPAAT activity, arachidonate transfer to lysophosphatidic acid generated phosphatidic acid in the membranes. This activity is thought to change the bilayer asymmetry in such a way that negative membrane curvature at the neck of a budding vesicle will be stabilized [78]. But recently, the LPAAT activity associated with endophilin proved to be a contaminant of the purification procedure [79]. The proposed locus of activity in endophilin includes the BAR domain, which has no catalytic site but instead senses positive membrane curvature. Amphiphysin and endophilin BAR domains have similar tertiary and quaternary structuresas well as amphipathic properties in N-terminal α-helices, except for the significant difference that endophilin has a short loop, disordered in the middle of a crescent-like crystal, on the concave surface of helix 1. The protrusion coming from the middle of the endophilin BAR domain crystal has been suggested to be another structure-based mechanism that helps to drive membrane curvature by its insertion into the membrane bilayers [80]. Amphiphysin I and endophilin1 have similar properties in binding with lipid membrane *in vitro*, including the diameters of the lipid tubules and the recruitment of dynamin and clathrin coat protein to lipid tubules. But they showed different effects on dynamin 1 GTPase activity *in vitro*; whereas amphiphysin I has a stimulatory effect, endophilin has an inhibitory effect on vesicle formation when added with dynamin and GTP to liposomes [77]. The study using anti-endophilin antibody microinjection into lamprey giant reticulospinal pre-synapse showed that SVE was inhibited at an earlier invagination stage and that synaptic vesicles were trapped by shallow clathrin-coated pits (CCPs) [66]. In contrast, interruption of amphiphysin I by the amphi-SH3 domain showed the inhibition of SVE at a very late invagination stage or fission step, where SVs were trapped by constricted CCPs [81]. Together, these findings suggest that the membrane-binding property of endophilin may be more necessary at an earlier stage of the invagination step of clathrin-mediated SVE compared with amphiphysin I, in which GTPase activity increases during the fisson step. But how these two N-BAR proteins coordinately work and the importance of the interaction between them needs to be explored further.

Interaction between Clathrin and AP2 at the CLAP Domain

Clathrin triskelia and adaptor protein complexes (APs) are the major coat proteins involved in CME. The formation of a cage structure from clathrin helps to invaginate the membrane and shape vesicles. AP2 was shown to bind and cluster transmembrane proteins destined for internalization and to promote clathrin polymerization on the plasma membrane. The CLAP domain (322–386) in the middle of amphiphysin I is shown to interact with the clathrin heavy chain and the α-appendage of AP2 directly. The interaction between clathrin and AP2 has been reported to be essential for receptor-mediated transferrin endocytosis by overexpression of amphiphysin I fragments that contain both clathrin heavy-chain- and AP2-binding domains in CHO cells [82]. Mutation of residues 323 to 326 (FFED) abolishes α-adaptin binding without affecting clathrin heavy-chain binding. Binding to the

clathrin heavy chain is conferred by residues 347 to 386 [82]. The three-dimensional structure of the α-adaptin appendage domain has been determined and a model has been proposed to account for its remarkable ability to mediate binding not only to amphiphysin 1 but also to Eps15 and epsin in clathrin coats via a single interaction interface [83, 84]. Mutational analysis identified 2 motifs (LLDLD and WDLW) in amphiphysin II that are important for binding to the clathrin heavy chain. The affinity of the recombinant clathrin-binding domain of amphiphysin II for native clathrin triskelia was estimated to be 1 nM [85]. The mutation of either LLDLD or WDLW motifs in amphiphysin I weakens clathrin heavy-chain binding, and the double mutation abolishes clathrin heavy-chain binding but has no effect on α-adaptin binding. As expected, the combination of the mutations in α-adaptin and the clathrin heavy-chain binding sites abolished amphiphysin I interaction with both α-adaptin and the clathrin heavy chain [82].

Interaction with Dynamin 1

Dynamin plays an important function in the fission of endocytic buds from the plasma membrane. An essential role for dynamin in all forms of SVE has been strongly suggested by studies with dynasore, which rapidly inhibits the GTPase activity of dynamin with high specificity and potently inhibits synaptic vesicle recycling in rodent hippocampal neurons [86]. Mammals express three dynamins with different expression patterns. Dynamin 1 is expressed exclusively in the brain, whereas dynamin 2 is ubiquitously expressed and dynamin 3 is expressed selectively in brain and testis. In the neurons, dynamin 1 is present at overwhelmingly high levels compared to dynamin 2 or 3. However, surprisingly, the nervous-system-specific dynamin 1, and by far the major dynamin in neurons, is largely dispensable for the endocytic recycling of SVs in dynamin 1 knockout mice cortical neurons [87]. Dynamin 1 becomes essential only when an intense stimulus imposes a heavy load on endocytosis and only as long as the stimulus persists. The defects in SVE could be rescued with WT dynamin 1 or dynamin 3 overexpression in dynamin 1 KO cortical neurons, which suggests that dynamin 3 may compensate for dynamin 1's function in the absence of dynamin 1. Nevertheless, lines of proof showed that dynamin 1 play a key role in clathrin-mediated SVE. The role of dynamin in endocytosis was established when the Drosophila gene shibire was cloned in 1991 and was found to be homologous to dynamin [88, 89]. Drosophila expressing the temperature-sensitive shibire gene product exhibit rapid and reversible paralysis at a nonpermissive temperature. Examination of the nerve terminals of the shibire flies at the nonpermissive temperature revealed an absence of synaptic vesicles and the accumulation of clathrin-coated pits at the plasma membrane. This led to speculation that dynamin plays a role in CME [90]. *In vitro*, dynamin spontaneously self-assembled into rings and stacks of interconnected rings, comparable in dimension to the 'collars' observed at the necks of invaginated coated pits that accumulate at synaptic terminals in shibire flies [91]. In GTPγS (a nonhydrolized form of GTP)-treated nerve terminals, elongated tubular invaginations of plasmalemma were found to be decorated by dynamin-formed transverse electron-dense rings [92]. Ultrastructural analysis of GTPγS-injected nerve terminals also showed dynamin is localized on invaginated clathrin-coated pits and forms a ring structure on the neck [93]. All these *in vitro* and *in vivo* studies indicate an important role for dynamin at the fission step by the assembly of dynamin into rings around the neck of clathrin-coated pits, and a concerted conformational change is expected to pinch off the coated vesicles. Further, fluorescence microscopic and electron microscopic analyses of dynamin 1 KO neurons revealed a dramatic accumulation of clathrin-coated pits in inhibitory synapses, suggesting that clathrin-mediated SVE is seriously inhibited at the fission step in dynamin 1 KO synapses [11].

Both amphiphysin I and II have SH3 domains at the C-terminal that recognize the PSRPNR sequence within dynamin's PRD domain [94]. *In vitro*, the amphiphysin I-SH3 domain bound the dynamin I-PRD domain with high affinity (KD~10 nM) [95]. Confocal immunofluorescence revealed that COS-7 cells transfected with the amphiphysin SH3 domain showed a potent blockade in receptor-mediated endocytosis. When COS-7 cells were cotransfected with both dynamin and the amphiphysin I SH3 domain, transferrin uptake was efficiently rescued. Importantly, the SH3 domains of Grb2, phospholipase Cγ and spectrin all failed to exert any effect on endocytosis [96]. Microinjection of amphiphysin's SH3 domain or of a

dynamin peptide containing the SH3 binding site inhibited synaptic vesicle endocytosis at the stage of invaginated clathrin-coated pits, which resulted in an activity-dependent distortion of the synaptic architecture and a depression of transmitter release [81]. These findings demonstrate that SH3-mediated interactions are required for dynamin function and support an essential role of CME in synaptic vesicle recycling.

Amphiphysin I plays a critical role in CME by recruiting dynamin to the nascent vesicle neck and stimulating dynamin's GTPase activity. Microinjection of the amphiphysin SH3 domain induced trapped CCPs, and no dynamin ring structure could be found on the vesicle neck [81], suggesting that dynamin recruitment to the neck is inhibited by the interruption of interaction between amphiphysin SH3 and the dynamin PRD domain. Microtubules [97, 98], growth factor receptor-bound protein 2 (Grb2) [98] and anionic phospholipids (small liposomes) [99] have been shown to stimulate the GTPase activity of dynamin, and each of these substrates promotes dynamin assembly [100]. Similarly, amphiphysin I could form a ring structure with dynamin under the same physiological buffer conditions. In this way, dynamin molecules are polymerized in the rings and are in close enough proximity for the GED domain of one molecule to make contact with the GTPase domain of a neighboring molecule, resulting in the stimulation of dynamin GTPase activity [101, 16]. Liposome size strongly influences amphiphysin's effect on dynamin GTPase activity. In the presence of large liposomes (1779.57 ± 461.7 nm in diameter), dynamin GTPase activity is dramatically enhanced by the addition of amphiphysin. On the other hand, when the liposomes are small (80.77 ± 10.8 nm in diameter), the addition of amphiphysin I led to a drastic decrease in GTPase activity. This suggests that amphiphysin I can sense plasma membrane curvature and thus can regulate dynamin GTPase activity or the assembly/disassembly of amphiphysin complex with dynamin. At the same time, the lipid component also influences the stimulatory effect of amphiphysin on dynamin GTPase activity. A higher concentration of PI(4, 5)P_2 or phosphatidylserine (PS) to liposome could increase the stimulatory effect more strongly. In addition, both the BAR and SH3 domains are required for the stimulation of dynamin GTPase activity and ring structure formation with dynamin. The deletion of central regions of the amphiphysin molecule Amph Δ248–601, containing the BAR and SH3 domains, strikingly enhanced the effect of amphiphysin I on dynamin GTPase activity. This is thought of as intramolecular interaction between the PRD domain and SH3 domain; this interaction blocks the binding of amphiphysin with the dynamin PRD domain by the SH3 domain [101, 102].

Interaction with Synaptojanin 1

Synaptojanin 1 is the only other brain protein that binds the SH3 domain of amphiphysin with an important function in synaptic vesicle endocytosis. Synaptojanin 1 has 2 distinct phosphatase domains: a Sac 1-like inositol phosphatase domain near the amino terminus, and a central inositol 5-phosphatase domain. A third important region is the C-terminal proline-rich domain (PRD) that interacts with endocytic proteins, including endophilin and amphiphysin. The amino-terminal Sac 1-like phosphatase hydrolyzes PI(3, 5)P_2, PI(4)P, or PI(3)P to PI [103], whereas the central 5-phosphatase hydrolyzes PI(4, 5)P_2 to PI(4)P [104]. A recent study revealed that synaptojanin 1 (145-kDa), the predominant isoform expressed in the brain, was rapidly recruited as a "burst," together with endophilin, at a late stage of CCP formation [105]. In contrast, the nonneuronal ubiquitously expressed 170-kDa isoform of synaptojanin was present at all stages of CCP formation, which is important for clathrin coat maturation in nonneuronal cells. These results raise the possibility that dynamic phosphoinositide metabolism may occur throughout the lifetime of a CCP. Genetic ablation of synaptojanin 1 in mice [106], worms [107], and flies [73] causes depressed synaptic transmission after prolonged stimulation, decreased synaptic vesicle numbers, and accumulation of clathrin-coated vesicles, suggesting that synaptojanin 1 regulates the uncoating and remobilization of vesicles during CME. In the nerve terminal, synaptojanin 1 forms 2 separate complexes by distinct sites in its PRD domain at the same time: 1 is interaction with endophilin by site PKRPPPPR, and the other is interactions with amphiphysin I, II by 2 sites, PIRPSR and PTIPPR [108, 109]. Though amphiphysins I and II have been reported as partners of synaptojanin, no further studies have shown the significance of the interaction in clathrin-dependent

endocytosis. In contrast, endophilin as the major partner of synaptojanin showed an important role in SVE. Manipulations of endophilin's SH3 domain caused an accumulation of deeply invaginated pits and clathrin-coated vesicles at the lamprey giant synapse [66], suggesting that endophilin plays an important role in recruiting synaptojanin and dynamin I to the clathrin coat for endophilin during SVE.

Amphiphysin I is Involved in Regulating Calcineurin Activity

Following exocytosis, synaptic vesicle components are recovered by endocytosis. Ca^{2+} influx into the nerve terminal is not only the trigger for exocytosis but is also important for clathrin coat nucleation in SVE. Ca^{2+}/calmodulin-dependent phosphatase 2B calcineurin plays an essential role in endocytosis by dephosphorylating the dephosphins: dynamin 1, synaptojanin, epsin 1, eps 15, and AP180 except amphiphysins I and II. The dephosphorylation of all these dephosphins is essential for endocytosis [13]. Calcineurin binds dynamin 1 at the dynamin 1 C-terminal PRD domain. The calcineurin-dynamin 1 interaction is calcium-dependent, with an EC50 for calcium in the range of 0.1–0.4 μM. Disruption of the calcineurin-dynamin 1 interaction inhibits CME. Thus, the calcium-dependent formation of the calcineurin-dynamin 1 complex provides a calcium-sensing mechanism that facilitates endocytosis. Amphiphysin I is also combined with this calcium sensor complex, the addition of amphSH3 peptide, which corresponds to the binding sites with dynamin PRD domain. This causes both dynamin 1 and calcineurin to dissociate from the GST-amphiphysin 1 column, showing that amphiphysin I plays an anchoring role for the calcineurin-dynamin 1 complex to clathrin-coated pits through the interaction between amphiphysin I and dynamin 1 [110]. On the other hand, amphiphysin I also recruits cain (calcineurin inhibitor) by its SH3 domain binding to the cain PRD domain. Cain is a 240-kDa protein that binds to calcineurin and inhibits calcineurin activity. Cain overexpression in HEK293 cells blocks transferrin-induced endocytosis, suggesting it has a regulatory role in CME. Co-immunoprecipitation experiments showed that cain binds to calcineurin and amphiphysin I simultaneously. These data suggest that amphiphysin I has a two-sided effect on calcineurin's dephosphorylation action. Upon calcium influx into the nerve terminal, amphiphysin I recruits the calcineurin (active)-dynamin 1 complex to the plasma membrane or partly formed clathrin coat, delivering calcineurin's dephosphorylation effect to the complex of endocytic proteins. Once the endocytic coat complex is fully assembled, cain, another partner associated with amphiphysin 1, promotes the rephosphorylation and dissociation of endocytic proteins by inactivating calcineurin [23].

A Potential Role in Actin Function

The dynamic polymerization of actin has a central role in several processes that reshape the plasma membrane. These processes include the protrusion of lamellipodia and filopodia during cell migration, and different forms of endocytic internalization (phagocytosis, macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis) [111]. Neurons express only the β and γ isoforms of actin, lacking the α isoform popular with skeletal muscle. Actin is ubiquitously expressed in neurons. Interestingly, it is highly concentrated at synapses. Both light microscopy and ultrastructural studies indicate that, at mammalian CNS synapses, actin is most highly enriched in postsynaptic dendritic spines. It is also indeed enriched in presynaptic nerve terminals relative to the adjacent axon segment. In developing synapses, the rapid turnover of actin facilitates axon outgrowth, de novo synapse formation, molecular scaffolding, and synaptic plasticity. Actin has many potential roles in nerve endings, and its effects have been classified into 4 broad categories: a barrier effect, in which polymerized actin is distributed under plasma membrane and between synaptic vesicles, which can impede vesicle fusion; a carrier effect, in which the highest concentration of actin reportedly often appeared to surround the core of synaptic vesicle clusters, forming a corral to restrain vesicles from fusion with plasma membrane too; a recycler effect, in which active polymerization of actin could facilitate endocytic recycling of fused vesicles by constriction or propulsion forces; and a scaffold effect, in which actin might act as a scaffold to sequester regulatory molecules at or near the vesicle pools [112]. The functional links between actin cytoskeleton and CME have been identified. Actin polymerization has been

involved in earlier and later stages of SVE in lamprey reticulospinal synapses. At stimulated synapses, specific disruption of actin polymerization with latrunculin and swinholide (2 actin polymerization inhibitors) induced a selective increase in unconstructed clathrin-coated pits and, in the case of swinholide, an additional increase in the size of plasma membrane evaginations. These results indicate that actin polymerization participates initially in the maturation of CCPs during the early stages of synaptic vesicle recycling [113]. Ultrastructural observations of stimulated lamprey synapses in the presence of F-actin-stabilizing reagents, phalloidin, and an N-ethylmaleimide-inhibited subfragment of myosin I (NEM-S1) caused tethered vesicles or accumulated aggregates of vesicles, suggesting that actin disassembly is involved in transporting vesicles back to the synaptic vesicle pool [114].

Amphiphysin I and dynamin I are colocalized on the nascent vesicle neck. The interaction is essential for the clathrin-mediated SVE. So far, though, there is no direct proof showing amphiphysin is involved in actin function in synaptic vesicle endocytosis. Several studies have suggested that amphiphysin has a potential role in actin polymerization. First, amphiphysin homologs in yeast Rvs 167p and Rvs 161p are known to be physically linked to actin cytoskeleton, or actin-binding protein Abp1p, positioning the Rvs 167p in the heart of the actin cytoskeleton. Genetic and functional analyses of rvs mutant showed a loss of actin cables and the de-localization of actin spots under adverse conditions, defects concerns disorders in endocytosis, bud-site selection and mating. These phenotypes are close to those described for the actin mutants [115]. Second, amphiphysin I colocalizes with dynamin I in developing neurons at all developmental stages and that a pool of both proteins is colocalized with actin patches at the leading edges of growth cones. When amphiphysin I was knocked out with amphiphysin I antisense oligonucleotides, growth cones collapsed and both neurite outgrowth and axon formation were severely inhibited. These data suggest a conserved role of the amphiphysin protein family in the dynamics of the cortical cell cytoskeleton [116]. Third, in Sertoli cells, amphiphysin I is enriched in actin-rich structures, including ruffles, phagocytic cups, and phagosomes after PS-liposome or PS-coated bead stimulation. Knocking out amphiphysin 1 by RNA interference in the cells resulted in reductions in ruffle formation, actin polymerization, and phagocytosis. Phagocytosis was also drastically decreased in amph 1 (−/−) Sertoli cells. In addition, PI(4, 5)P_2-induced actin polymerization was decreased in the knockout testis cytosol. The addition of recombinant amphiphysin 1 to the cytosol restored the polymerization process. Ruffle formation in small interfering RNA-treated cells was recovered by the expression of constitutively active Rac 1, suggesting that amphiphysin 1 functions upstream of the Rac 1 signal pathway [117]. These findings support that amphiphysin 1 is important in the regulation of actin dynamics and that it is required for phagocytosis. The molecular mechanism underlying amphiphysin's role in actin polymerization and its ability to further amphiphysin's effect on SVE remains unclear.

Regulation of the Functions of Amphiphysin I by Phosphorylation

Amphiphysin I also undergoes constitutive phosphorylation and stimulation-dependent dephosphorylation. Dephosphorylation of amphiphysin I requires extracellular Ca^{2+} and is unaffected by pretreatment of synaptosomes with tetanus toxin. Thus, Ca^{2+} influx, but not synaptic vesicle exocytosis, is required for dephosphorylation. Dephosphorylation of amphiphysin I is inhibited by cyclosporine A and FK-506, 2 drugs that specifically block calcineurin activity, but not by okadaic acid, which blocks protein phosphatases 1 and 2B. This suggests that calcineurin is amphiphysin I *in vivo* phosphatase [118]. All the dephosphins have been reported to share the same dephosphatase calcineurin, but each of them may have more than one kinase to keep itself in phosphorylated form during resting conditions. All *in vivo* amphI phosphosites have been identified by ^{32}P tracking in synaptosomes [119]. There are 13 of these sites: serines 250, 252, 262, 268, 272, 276, 285, 293, 496, 514, 539, and 626 and Thr-310 (Fig. 3). These were distributed into two clusters, one around the PRD domain and the other around the C-terminal SH3 domain. Hierarchical phosphorylation of Ser-262 preceded phosphorylation of Ser-268, -272, -276, and -285. Offline HPLC separation and two-dimensional tryptic mapping of ^{32}P-labeled amphiphysin I revealed that Thr-310, Ser-293, Ser-285, Ser-272,

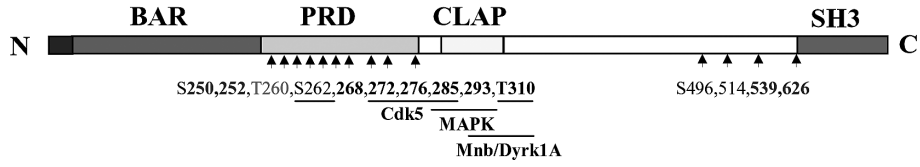

Fig. 3 *In vivo* phosphosites of amphiphysin I. There are 13 phosphosites (S250, 252, 262, 268, 272, 276, 285, 293, 496, 514, 539, 626, and T310) found in rat brain. T260 (red) was found in GABA antagonist PTZ-induced seizure. The phosphosites of S268, 272, 276, 285, 293, T310 (black bold) are most dynamically turning over, and the phosphosites of S250, 252, 539, 626 (blue bold) are most constitutively phosphorylated during the synaptic activity. Cdk5, MAPK, and Mnb/Dyrk1A are potential *in vivo* kinases; they phosphorylated amphiphysin I at the sites shown in the figure *in vitro*.

Ser-276, and Ser-268 contained the highest ^{32}P incorporation and were the most stimulus-sensitive. Individually, Thr-310 and Ser-293 were the most abundant phosphosites. Amphiphysin I's *in vivo* phosphorylase activity is not known. But it is suggested that at least one proline-directed protein kinase and one nonproline-directed kinase are involved in amphiphysin I phosphorylation *in vivo*. Four phosphosites predicted for nonproline-directed kinases, Ser-626, -250, -252, and -539, contained low amounts of ^{32}P and were not depolarization-responsive. The results reveal 2 sets of amphiphysin I phosphosites that either dynamically turn over or are constitutively phosphorylated in nerve terminals. Two other amphiphysin I splicing variants were also found to be phosphorylated under the same conditions, but none of the phosphosites in amphiphysin I isoform were dynamically labeled with ^{32}P, and all of the sites are constitutively phosphorylated. Therefore, all 3 isoforms of amphiphysin I are phosphorylated in nerve terminals, but only amphiphysin I dynamically incorporates ^{32}P radiation or exhibits depolarization-induced dephosphorylation. This indicates that among the 3 isoforms it is the major variant most likely to be involved in SVE [119]. In addition to the 13 *in vivo* phosphosites in amphiphysin I induced under physiological conditions, another *in vivo* phosphosite (T260) was found during seizures triggered by the GABA antagonist pentylenetetrazole (PTZ) [120]. Under such conditions, activation of the c-jun N-terminal kinase (JNK) pathway was detected in hippocampal extracts. Phosphorylated amphiphysin I was identified with the cross-activity of specific phospho-MKK4 antibody, which was raised against the phosphorylation peptide of the upstream JNK kinase MKK4. The protein kinases for amphiphysin I phosphosite T260 remain to be identified. The phosphorylation found specifically during PTZ-induced synaptic hyperexcitation suggests a potential role for amphiphysin I in synaptic function under neuronal hyperexcitation.

The protein kinase or kinases that phosphorylate amphiphysin I in nerve terminals *in vivo* are not known, but amphiphysin I is a substrate for at least 4 protein kinases *in vitro*. Three proline-directed protein kinases phosphorylate amphiphysin I: Cdk5/p35 (Ser-262, Ser-272, Ser-276, Ser-285, and Thr-310) [50], mitogen-activated protein kinase (MAPK) (Ser-285 and Ser-293) [121], and Mnb/Dyrk1A (Minibrain kinase/dual-specificity tyrosine phosphorylation-regulated kinase) (Ser-293 with minor sites including Thr-310, and Ser-295) [122]. CK2 phosphorylates amphI *in vitro* on Thr-350 and Thr-387 [123]. The phosphorylation at these sites is important for regulating the interactions of amphiphysin I with its endocytic partners, clathrin, AP2, endophilin and lipid membrane, but its interaction with dynamin is not. Compared with the other 3 potential amphiphysin I kinases (MAPK, Dyrk1A/minibrain kinase, and CK2), Cdk5 is more likely important amphiphysin I *in vivo* kinase, because the 5 Cdk5-dependent phosphosites in amphiphysin I identified *in vitro* are all consistent with those identified *in vivo*. Four of these phosphosites (except S262) could highly incorporate ^{32}P and are the most stimulus-sensitive in the nerve terminal. In addition, amphiphysin I has been detected in the Cdk5/p25 complex in bovine brain [124], and the Cdk5-activating subunit p35 has been reported to bind the N terminus of amphiphysin I in rat brain [21]. In some experiments, 2 Cdk5 inhibitors, roscovitine and Ro31-8220, could not inhibit amphiphysin I rephosphorylation completely upon recovery from a depolarization stimulus [125, 126], and the phosphorylated

form of amphihysin I still exists in synaptosomes from p35-deficient mice [50]. These data indicate that Cdk5 is not only a kinase for amphiphysin I *in vivo*, but that some other kinases could compensate for Cdk5's role in the phosphorylation of amphiphysin I when cdk5 activity is blocked.

Cdk5 co-phosphorylates amphiphysin I and dynamin I *in vitro* [50]. Amphiphysin I can copolymerize with dynamin I into ring structures and has a stimulatory effect on dynamin I's GTPase activity *in vitro*; this effect is highly dependent on liposome size [16]. Cdk5-dependent phosphorylation regulates the interaction between amphiphysin I and dynamin I. The phosphorylation of each protein reduces the copolymerization into a ring formation. Moreover, the phosphorylation of both proteins completely disrupts the copolymerization into a ring formation. Further, the dynamin I GTPase activity-dependent vesicle formation from liposome is also inhibited by the phosphorylation of both proteins [50]. On the other hand, cdk5-dependent phosphorylation also regulates the interaction of amphiphysin I with the lipid membrane [28]. It is interesting that cdk5-dependent phosphorylation of amphiphysin I was enhanced in the presence of liposome *in vitro*, and that the phosphorylation was markedly decreased by mutation of either Ser276 or Ser285 of amphiphysin I to alanine (S276A and S285A). Furthermore, mutation of both sites (S276 and 285A) completely eliminated the enhanced phosphorylation. Functional studies indicated that binding of amphiphysin I to lipid membrane was attenuated by Cdk5-dependent phosphorylation of wild-type amphiphysin I, but not of the S276, 285A form. Endocytosis was increased in rat PC12 cells expressing amphiphysin I S276, 285A in comparison with the wild type. These results suggest that Ser276 and Ser285 are regulatory Cdk5 phosphosites of amphiphysin I in the lipid-bound state. Phosphorylation at these 2 sites alters binding of amphiphysin I to lipid membranes, and may be an important r aspect of SVE regulation.

Amphiphysin I is Truncated by Calpain during Hyperexcitation and the Regulation in Synaptic Vesicle Endocytosis

Overloading of Ca^{2+} in presynaptic terminals is observed in a host of neuronal diseases, such as ischemia/anoxia, epilepsy, stroke, trauma, and Alzheimer's disease. In these cases, overactivation of Ca^{2+}-dependent neutral protease calpain has been reported to induce neuronal damages by selective proteolysis of some target proteins, including cytoskeletal proteins, membrane proteins, and cytosolic proteins [see reviews 127–129]. The most recent work showed that amphiphysin I is truncated into 3 fragments detected by anti-amphiphysin I-N-terminal antibody in mice hippocampal slices during the hyperexcitation induced by high potassium stimulation, repetitive high-frequency stimulation and kainate-induced seizures [31]. Time-dependent cleavages of amphiphysin I and α-spectrin, a physiological substrate of calpain, in mice hippocampal slices after high potassium stimulation showed similar time-dependent protein degradation patterns. In addition, the truncations were inhibited by pretreatment with calpain inhibitors ALLM and ALLN. These data suggest that calpain is overactivated in the nerve terminal upon high potassium stimulation, and that calpain is an *in vivo* protease for amphiphysin I. Consistently, *in vitro*, m-calpain cleaved amphiphysin I into 3 fragments corresponding to the truncations induced by high potassium stimulation in hippocampal slices. The nine cleavage sites in amphiphysin I for m-calpain *in vitro* have been identified with mass spectrometry (MS) analysis; they are 333, 377, 392, 454, 478, 527, 531, 593 and 609. Three major cleavage sites, 333, 377 and 392, are around the middle CLAP domain, and the other sites are in the C terminus (Fig. 4A). Amphiphysin I N-terminal recombinant proteins (1-333, 1-377 and 1-392) showed the same electrophoretic mobility to calpain-cleaved amphiphysin I fragments *in vitro*. No C-terminal fragments were detected with specific anti-amphiphysin I-C-terminal antibody *in vivo* or *in vitro* after calpain cleaved amphiphysin I. This suggests that amphiphysin I is truncated by calpain to N-terminal fragments at multiple cleavage sites and loses its C terminus during hyperexcitation (Fig. 4A).

Calpain has 2 isozymes (μ-calpain and m-calpain) in the brain. μ-Calpain is activated at a micromolar Ca^{2+}, whereas m-calpain is activated at millimolar Ca^{2+}. The relative abundance and localization of mRNAs for μ- and m-calpain in the brain were studied by using a combination of reverse transcriptase-polymerase chain reaction and *in situ* hybridization [130]. Expression of m-calpain mRNA was found to be 15-fold higher

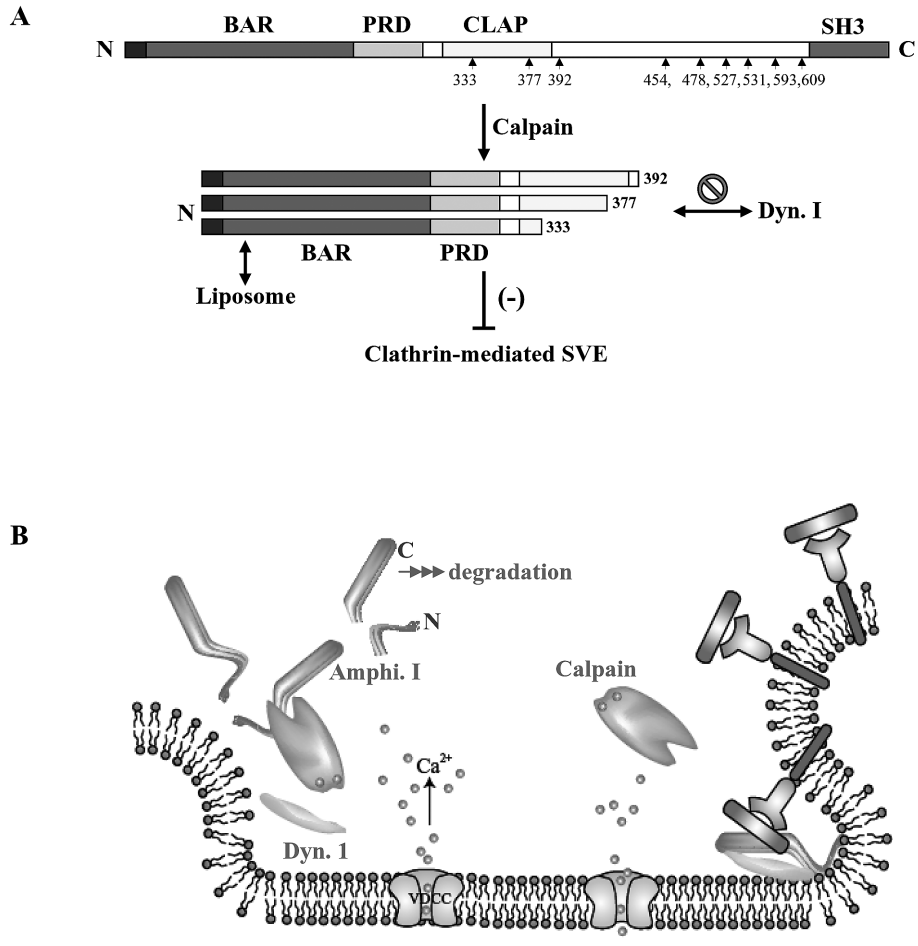

Fig. 4 Amphiphysin I (amphi. I) is truncated by calpain during neural hyperexcitation. **A**, amphi. I is cleaved by calpain to three N-terminal fragments (1–392, 1–377 and 1–333) during neural hyperexcitation at nine sites shown in the figure. These three truncations contain the N-terminal BAR domain but do not have the C-terminal SH3 domain after calpain proteolysis, thus blocking the association with dynamin 1 (dyn. I), although amphiphysin I can still bind with liposome by the BAR domain *in vitro*. The defect in recruiting dyn. I to the clathrin complex is suggested to inhibit clathrin-mediated SVE; **B**, calpain-dependent truncation of amphiphysin I is regulated by interaction with other endocytic components, such as lipid membrane and dyn. I. When nerve terminals are overloaded with Ca^{2+}, calpain will be overactivated. The overactivated calpain only proteolyzes free amphiphysin I, including that in the phosphorylated or dephosphorylated form, but not that in the clathrin complex-binding form.

than that of μ-calpain in whole-brain homogenates. μ-Calpain messages were observed to be diffused throughout the entire brain in both neurons and glia, whereas messages for m-calpain were localized to distinct neuronal populations, including all cornu ammonis regions of the hippocampus, cortical pyramidal neurons, and cerebellar Purkinje cells. Subcellular localization for both isozymes in hippocampal neurons is not clear. Both μ- and m-calpain were detected in the membrane and cytosolic fractions of purified synaptosomes (LP2 and LS2 fractions) [31]. *In vitro*, μ-calpain can also induce amphiphysin I cleavages to the same 3 major truncated forms as m-calpain, in addition to a new truncated form between the 52- and 47-kDa forms. Considering the expression levels in neurons, the Ca^{2+} requirement for activity and the amphiphysin I truncation pattern, m-calpain is more like amphiphysin I *in vivo* protease upon neuronal hyperexcitation.

What will regulate calpain-dependent amphiphysin I cleavage: interaction with other partners or amphiphysin I's phosphorylation conditions? *In vitro* evidence

showed that the interaction with liposome or dynamin blocked amphiphysin I cleavage upon calpain proteolysis, but amphiphysin I phosphorylation status did not influence the cleavage directly. In the presence of calcineurin inhibitor FK506, the level of amphiphysin I cleavage was increased upon high potassium stimulation in mice hippocampal slices. These results suggest that the association of amphiphysin I with synaptic membranes and dynamin may be crucial for the regulation of its cleavage by calpain. They also suggest that phosphorylation of amphiphysin I does not directly influence the cleavage of amphiphysin I by calpain but rather inhibits the formation of a complex with endocytic proteins and synaptic vesicle membranes, resulting in the induction of amphiphysin I cleavage by calpain during hyperexcitation (Fig. 4B) [31].

Functional analysis showed that the calpain-induced truncated form of amphiphysin I (1-392) is capable of tubulation with liposomes but does not form ring structures with dynamin I in the solution or on lipid tubules *in vitro*. Overexpression of the truncated form in Cos-7 cells and in hippocampal primary culture neurons showed significant inhibition of transferrin uptake and synaptic vesicle endocytosis. By now, this picture emerges: When neuronal hyperexcitability is transferred to the nerve terminal, a massive influx of Ca^{2+} reaches the nerve terminal, and then amphiphysin I and the other endocytic dephosphins are dephosphorylated by Ca^{2+}-dependent phosphatase calcineurin and recruited to form a clathrin complex. On the other hand, at the same time, overloading of Ca^{2+} in presynapses overactivates calpain activities. Free amphiphysin I, including the phosphorylated and dephosphorylated forms, which are not yet recruited to the complex, is quickly truncated to BAR domain-containing N-terminal fragments by calpain (C-terminal SH3 domain is degraded). The truncations block the interaction of amphiphysin I with dynamin I, a key molecule in the fission of clathrin-coated buds from presynaptic membranes by its GTPase activity. In addition, the N-terminal fragments of amphiphysin I still retained the ability to bind to liposome membranes, suggesting a dominant-negative effect of amphiphysin I cleavage, which will inhibit wild-type amphiphysin I from interacting with synaptic plasma membrane and thus will also inhibit the recruitment of its partners. By these 2 inhibitory effects, calpain-dependent amphiphysin I truncations inhibit continuous clathrin-mediated SVE and thus may fail to sustain synaptic transmission during hyperexcitation [31].

But what is the physiological significance of calpain-dependent amhiphysin I truncations during neural hyperexcitation observed in neural diseases such as ischemia/anoxia, epilepsy, stroke, and Alzheimer's disease? Repetitive high-frequency electrical stimulation (HFS) or continuous high potassium stimulation-induced postsynaptic short-term depression has been thought to be a neuronal auto-protective mechanism against neuroexcitotoxicity during neural hyperexcitation. Clathrin-mediated SVE, a major pathway to maintain synaptic vesicle recycling during HFS, is inhibited during HFS-induced short-term depression, suggesting a role for inhibited presynaptic vesicle endocytosis in the induction of postsynaptic short-term depression. Calpain-dependent amphiphysin I truncation was observed during HFS-induced fEPSP (field excitatory postsynaptic potential) depression at synapses from Schaffer collaterals onto CA1 pyramidal neurons in mice hippocampal slices. ALLM perfusion inhibited the HFS-induced fEPSP depression, but had no effect on the fEPSP slope when slices were stimulated with LFS (low-frequency stimulation). These findings suggest that calpain-dependent amphiphysin I truncations may be involved in HFS-induced short-term depression, the auto-protective mechanism during neural hyperexcitation, by inhibiting clathrin-mediated SVE. Further, calpain-dependent amphiphysin I truncations are also observed in low- and high-dose kainate (KA)-induced seizures in FVB/NJ mice. Preconditioning with low-dose KA reduced the development of seizures induced by high-dose KA. However, pretreatment with ALLM attenuated the preconditioning effect. Taken together, the *in vivo* and *in vitro* studies showed that hyperexcitation-induced amphiphysin I cleavage plays a role in protecting neurons from neuroexcitotoxicity during hyperexcitation [31].

Summary

Acute interruption of amphiphysin I function in neurons showed that amphiphysin I plays an essential role in clathrin-mediated SVE by acting as a multifunctional adaptor connecting various endocytic proteins and endocytic machinery regulators to each other

or to the synaptic plasma membrane, including clathrin, AP2, endophillin, synaptojanin, dynamin I, cdk5 /p35 complex, PLD, calcineurin-dynamin complex, and cain. All these components are working coordinately to sustain synaptic vesicle recycling during synaptic transmission. But these initial pieces of evidence for a role of amphiphysin I in synaptic vesicle recycling and endocytosis came from cell-based assays employing overexpression of dominant-negative constructs. Is amphiphysin I really essential for synaptic vesicle recycling and endocytosis in the context of a whole animal? This is examined in *amphiphysin 1* (*AMPH1*) gene knockout mice [44]. Unexpectedly, no apparent defects were found in nerve terminals of amphiphysin I knockout mice during resting conditions. Only under stimulated conditions, amphiphysin I deficiency induced incomplete inhibition of synaptic vesicle endocytosis, though amphiphysin I knockout brain cytosol showed defects in recruiting major endocytic proteins (clathrin, AP2 and synaptojanin) to liposomes as well as showing associations between dynamin PRD domain 1 and both clathrin and AP2 *in vitro*. This suggests that amphiphysin I's function in clathrin-mediated SVE may be partly compensated by other endocytic proteins when in the absence of amphiphysin I *in vivo*, but this effect may depend on the absence of amphiphysin I protein. It is interesting that amphiphysin I knockout mice showed increased susceptibility to seizures, leading to reduced viability [44]; this suggests that amphiphysin I, as an important endocytic protein, is involved in the prevention mechanism from neural hyperexcitation. And amphi–physin I's neural-protective role may be executed by calpain-dependent amphiphysin I truncation-induced SVE inhibition during hyperexcitation [31]. Amphiphysin I knockout mice also showed learning deficits, suggesting a critical role for amphiphysin in higher brain function [44]. Taken together, the results indicate that amphiphysin I has a positive role in sustaining synaptic transmission not only under physiological conditions, but also under physiopathological conditions (hyperexcitation) by regulating clathrin-mediated SVE.

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