Intersectin 1: a versatile actor in the synaptic vesicle cycle

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Abstract

During neurotransmitter release, SVs (synaptic vesicles) fuse at the active zone and are recovered predominantly via clathrin-mediated endocytosis at the presynaptic compartment surrounding the site of release, referred to as the periactive zone. Exo- and endo-cytosis in synapses are tightly temporally and spatially coupled to sustain synaptic transmission. The molecular mechanisms linking these two cellular events, which take place in separate compartments of the nerve terminal, remain largely enigmatic. Several lines of evidence indicate that multiple factors may be involved in exocytic-endocytic coupling including SV integral membrane proteins, SV membrane lipids and the membrane-associated actin cytoskeleton. A number of recent studies also indicate that multimodular adaptor proteins shuttling between the active and periactive zones aid the dynamic assembly of macromolecular protein complexes that execute the exo- and endo-cytic limbs of the SV cycle. Here, we discuss recent evidence implicating the multidomain scaffolding and adaptor protein ITSN1 (intersectin 1) as a central regulator of SV cycling.

A brief overview of the SV (synaptic vesicle) cycle

Neuronal communication relies on the conversion of electrical signals into chemical signals at intercellular contacts termed synapses. Arrival of an action potential elicits the influx of calcium into the presynaptic nerve terminal, resulting in the exocytotic fusion of neurotransmitter-filled SVs with the presynaptic plasma membrane at specialized release sites, termed AZs (active zones). Exocytosed neurotransmitter molecules then traverse the synaptic cleft and stimulate postsynaptic neurotransmitter receptors, mostly ligand-gated ion channels.

To reliably sustain neurotransmission and to compensate for the net insertion of membrane, SVs have to undergo rapid local recycling [1–3] with a half-time of less than 1 min [4,5]. A large body of data including both genetic and acute biochemical perturbations indicates that at least in the long run SV recycling requires the activity of the clathrin-based endocytic machinery. However, at some synapses or under specific physiological conditions, alternative, more rapid pathways of exo/endocytic SV cycling may occur ("kiss-and-run"). After their endocytic itinerary, SVs are refilled with neurotransmitter and trafficked back to the SVC (SV cluster) [5–7]. Conceptually the SV cycle can be subdivided into several molecularly and morphologically distinct steps: (i) docking and priming of SVs; (ii) 

\[ \text{Ca}^{2+} \] -triggered fusion/exocytosis; (iii) clathrin- and dynamin-mediated endocytosis; (iv) reacidification and neurotransmitter reuptake; and (v) translocation to the SVC [2,3]. Exocytosis and endocytosis are tightly temporarily and spatially coupled requiring cross-talk between components driving the individual steps of the SV cycle. Precisely how such coupling is achieved remains largely unclear.

In the present review, we discuss putative coupling mechanisms with a special emphasis on the multidomain adaptor and scaffolding protein ITSN1 (intersectin 1) [8,9]. We speculate that ITSN1 might serve as a connecting adaptor linking the exo- and endo-cytic limbs of the SV cycle.

ITSN: a multifunctional adaptor and scaffolding protein

ITSN1 is conserved throughout evolution with homologues in human, rodents, Xenopus, Drosophila and Caenorhabditis elegans [10,11]. It is also known as Dap160 (dynamin-associated protein of 160 kDa) in Drosophila [12], Ese-1/2 in mouse [13] or ESH1-1/2 in rat [9]. ITSN1 contains multiple EH (eps15 homology) domains and SH3 domains (Src homology 3 domains), which allow the protein to co-assemble with its corresponding binding partners into macromolecular complexes [14], which have been speculated to act as platforms for endocytic membrane traffic and cell signalling in various organisms [5,9,12,15,16]. Mammals express two ITSN genes (ITSN1 and ITSN2), each coding for several alternatively spliced isoforms [17,18]. For both ITSNs, the predominant transcripts are: (i) a ubiquitously

Key words: actin, clathrin, dynamin, endocytosis, intersectin (ITSN), synaptic vesicle.

Abbreviations used: AD, Alzheimer’s disease; Arp2/3 complex, actin-related protein 2/3 complex; CC, coiled-coil; COP, clathrin-coated pit; Cdc42, cell division cycle 42; Dap160, dynamin-associated protein of 160 kDa; DH, Dbl (disabled) homology; DS, Down’s syndrome; Dyrk1A, dual-specificity tyrosine-phosphorylation-regulated kinase 1A; EH, eps15 homology; EGF, epidermal growth factor; ERLK, endocytic receptor; GEF, guanine-nucleotide-exchange factor; GEF-H超级家族, GEF家族, GEF-H, GEF家族; GTPase; ITSN, intersectin; ITSN1, ITSN2, long isoform; mEJP, miniature excitatory junctional potential; Mbn, minibrain; Mnb, minibrain; NMJ, neuromuscular junction; N-WASP, neuronal Wiskott–Aldrich syndrome associated protein; PH, pleckstrin homology; PH domain; SNAP-25, 25 kDa synaptosome-associated protein; SV, synaptic vesicle; SVC, SV cluster.

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expressed short isoform (ITSN-s) comprising two N-terminal EH domains, a central CC domain and five tandem SH3 domains. The long isoform ITSN1-L bears an additional C-terminal extension harbouring DH, PH and C2 domains. Binding partners are shown above the domain model with arrows indicating the domain they are interacting with. The DH-PH module serves as a GEF for Cdc42. SCAMP, secretory carrier membrane protein; Sos, son of sevenless.

**Figure 1** | Domain structure of ITSN1

The short isoform ITSN1-s comprises two N-terminal EH domains, a central CC domain and five tandem SH3 domains. The long isoform ITSN1-L bears an additional C-terminal extension harbouring DH, PH and C2 domains. Binding partners are shown above the domain model with arrows indicating the domain they are interacting with. The DH-PH module serves as a GEF for Cdc42. SCAMP, secretory carrier membrane protein; Sos, son of sevenless.

**ITSN1 cycles between the SVC and the periactive zone**

Like many other endocytic proteins, ITSN is highly enriched in brain. In *Drosophila* and *C. elegans* ITSN is concentrated within the periactive zone of NMJs [32,34] where it co-localizes with its binding partners dynamin and synaptojanin at sites of SV membrane cycling [12]. In mammalian cells including fibroblasts and primary neurons, ITSN1 is found at plasmalemmal clathrin-coated pits and in internal compartments, probably reflecting the TGN (trans-Golgi network) and/or endosomes [8,14,20,35]. Recent work has also suggested a role for ITSN1 in the development of dendritic spines [36], a process intimately linked to actin polymerization (see further below).

High-resolution ultrastructural studies using immunogold EM (electron microscopy) in lamprey reticulospinal axons show that lamprey ITSN1 predominantly resides within the SVC at rest (Figure 3i) and undergoes activity-dependent cycling between the SV pool and the periactive endocytic zone [22] (Figures 2 and 3ii). A migration cycle between the SVC and sites of endocytosis similar to that of ITSN1 is observed for a number of dephosphins, synaptic proteins that undergo stimulation-dependent dephosphorylation including the ITSN1-binding partners dynamin [22], eps15 [31], epsin [37] and amphiphysin [38]. The mechanistic basis of this migration cycle is unknown, but Ca^{2+}-triggered post-translational modification of presynaptic proteins probably contributes to it. In agreement with this notion, ITSN-L contains a C2 domain, which may interact with Ca^{2+}. Based on its multiple binding partners ITSN would seem to be ideally suited to function as a proteinaceous scaffold for the activity-dependent concentration of endocytic proteins at the periactive zone. According to this model, an ITSN-based macromolecular complex could serve as a scaffold that guides recycling vesicles through the various stages of endocytosis. Consistent with this model, acute biochemical or sustained genetic perturbations of ITSN complex formation with eps15 [31,32] or dynamin and synaptojanin [22] cause profound defects in SV membrane retrieval and synapse development (further discussed below).

ITSN1 as well as a number of other endocytic proteins including dephosphins also directly or indirectly interact with actin or actin regulatory factors (i.e. N-WASP and Cdc42 (cell division cycle 42); see below) [39], suggesting that regulation of actin dynamics is intimately linked to the endocytic limb of the SV cycle. Actin appears to form a
proteinaceous cage around the SVC that may restrict lateral movement of SVs at rest. Synaptic activity not only triggers the migration of ITSN and other endocytic proteins from the SVC to the periactive zone but also induces localized actin rearrangements. These observations suggest that both processes are directly linked, presumably via ITSN1 and other actin-regulatory factors. Hence, ITSN is at the heart of the machinery that compartmentalizes the endocytic limb of the SV cycle.

Interconnecting exo- and endocytosis
Numerous studies have indicated a role for ITSN in CME (clathrin-mediated endocytosis) including overexpression studies in cultured cells [13,35,40] as well as genetic and acute perturbation experiments in a variety of models. Stable complex formation between ITSN/Dap160 and eps15 (EHS-1 in C. elegans) has been shown to be essential for SV membrane retrieval and synapse development in Drosophila and C. elegans [31,32]. Expression levels of ITSN and eps15/EHS-1 at NMJs are interdependent and the phenotypes observed on loss of either protein are nearly identical, indicating a close functional relationship between eps15 and ITSN/Dap160 [31,32]. Drosophila dap160 mutants display severe defects in synaptic membrane recycling including a reduced number of SVs, aberrant large vacuolar structures, and an accumulation of endocytic intermediates at active and periactive zones paired with a corresponding synaptic depression during prolonged stimulation. These changes are accompanied by reduced levels of key endocytic proteins such as dynamin, synaptojanin, endophilin, eps15, AP180 and synapsin [15,16,31]. Similarly, C. elegans itsn-1/ehs-1-null mutants display increased numbers of large irregular vacuoles and an accumulation of membrane-associated vesicles at endocytic hot spots [32]. Thus functional ITSN constitutes a prerequisite to organize and concentrate the endocytic machinery at the periactive zone (Figure 3i). Particular attention has been paid to the function of the SH3 domains of ITSN. Acute perturbation of ITSN1 SH3 domain interactions by microinjection of specific antibodies or the ITSN1 SH3C domain in lamprey reticulospinal synapses inhibits SV recycling at the level of constricted CCPs (clathrin-coated pits). These results suggest that ITSN1 regulates endocytic vesicle fission, perhaps by affecting the recruitment of dynamin to the endocytic zone [22] (Figure 3iii). Stage-specific in vitro reconstitution assays revealed that the SH3A domain of ITSN acts at early steps of clathrin-mediated vesicle formation, whereas SH3 domains B–E preferentially inhibit later stages [40]. Interestingly, the SH3A domain is subject to neuron-specific alternative splicing, which increases the affinity of the neuronal splice variant for dynamin and synaptojanin [41].

Several studies have implicated ITSN in exocytosis [8,9]. Based on immunoprecipitation experiments, ITSN1 forms a stable complex with the Q-SNARE (SNARE is soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) SNAP-25, a key component of the SV fusion
**Figure 3** | Putative functional roles of ITSN1 at multiple steps of the SV cycle

Model of a synapse depicting the SV cycle. At rest, ITSN1 resides in the SVC (i) from where it migrates to the periactive zone after Ca^2+ influx. Within the periactive zone, ITSN1 forms a complex with eps15 (ii), presumably assisting clathrin coat formation during initial stages of SV endocytosis. After completion of the coat, forming SVs are pinched off from the plasma membrane by the large GTPase dynamin whose subcellular localization appears to be regulated by ITSN1 (iii). Uncoating of newly formed SVs and their migration to the SVC are coupled to actin dynamics, a process potentially regulated by ITSN1 complex formation with actin regulatory proteins such as N-WASP and Cdc42 (iv).

machinery [9]. In chromaffin and PC12 cells, ITSN1 is concentrated at exocytic hot spots and constitutes a functional element of the exocytic machinery [8]. In agreement with an exocytic function of ITSN, spontaneous release measured as mEJPs (miniature excitatory junctional potentials) in NMJs of third instar larvae from *dap160* mutant flies is significantly altered [15]. Moreover, unusually large mEJP amplitudes have been observed in *dap160* mutants. A similar increase in mEJP frequencies has been described for eps15 mutant flies [31]. Exocytic defects have also been noted in *Itsn1*-knockout mice [42]. It is therefore tempting to speculate that a macromolecular scaffold containing ITSN1 and eps15 directly or indirectly regulates SV release, albeit the precise underlying mechanisms remain to be elucidated.

**ITSN as a regulator of actin dynamics**

Although actin clearly is enriched at nerve terminals, its precise function remains to be controversially debated [43,44]. A close link between SV cycling and actin dynamics is suggested by the fact that many proteins regulating SV exo/endo-cytosis are directly or indirectly linked to the actin cytoskeleton. Prominent examples include dynamin-binding SH3 domain-containing proteins such as amphiphysin, endophilin, syndapin, Tuba, Abp1, SNX9 and ITSN1 [2,45]. The neuronal isoform of ITSN1 (ITSN1L) via its C-terminal DH–PH domains has been shown to function as a GEF (guanine-nucleotide-exchange factor) for Cdc42 [24,46]. The resulting Cdc42 stimulation then accelerates actin assembly via N-WASP and the Arp2/3 complex (actin-related protein 2/3 complex). Additionally, N-WASP directly binds to and competes with dynamin for the SH3 domains of ITSN1 and up-regulates its GEF activity, thereby potentially facilitating the generation of Cdc42-GTP and thus activation of N-WASP [24,46]. Hence, ITSN1 appears to be directly involved in actin polymerization, perhaps in conjunction with its activity during late stages of endocytic vesicle fission and/or migration of newly formed SVs back to the SVC (Figure 3iv).

A role for ITSN1L-mediated actin polymerization at late stages of SV endocytosis is compatible with observations of CCP dynamics by TIRF (total internal reflection fluorescence) microscopy. These studies revealed that dynamin-mediated fission of late CCPs is closely accompanied by and followed by recruitment of actin, N-WASP and Arp2/3 [47,48]. Genetic data in *Drosophila* [16,31] as well as acute perturbation experiments at lamprey reticulospinal synapses [22] also support a role for ITSN1/Dap160 in actin dynamics. Its biochemical properties and the phenotypic changes seen after manipulation of ITSN function *in vivo* thus position ITSN1 at the interface between SV membrane trafficking and actin dynamics [39].

**ITSN and disease**

The ITSN1 gene localizes to the DS (Down’s syndrome) critical region on the long (q) arm of chromosome 21 [49]. Correspondingly, ITSN1 protein levels are up-regulated in DS brains, suggesting a possible involvement of ITSN1 in the disease [21,42,50]. Consistent with this hypothesis, abnormalities in the endocytic pathway have been reported to contribute to the pathological processes of DS and early AD (Alzheimer’s disease) [51]. A phenotypic hallmark of both disorders is the accumulation of enlarged early endosomes in neurons of young DS and pre-AD brains [42]. Intriguingly, *Itsn1*-knockout mice display a number of endocytic and vesicle trafficking defects including moderately impaired SV endocytosis and aberrantly large neuronal endosomes [42] reminiscent of the defects observed in DS neurons. As mentioned above, similar although more severe defects are observed in *dap160* mutant flies [15,16]. Moreover, *dap160* mutants display alterations in synapse morphology, suggestive of developmental defects [15,16], which may in addition to the endocytic defects contribute to the pathological states caused by ITSN1 mutants in mammals. Whether alterations in the expression level of ITSN1 are truly causatively involved in the genesis of DS remains to be determined. If so, it is likely that other regulatory factors contribute to the disease process. A particularly interesting example of such putative regulators is Dyrk1A.
(dual-specificity Yak1-related kinase 1A), the mammalian homologue of the *Drosophila* kinase Mnb (minibrain) [52]. Like ITSN1, Dyrk1A is localized to the DS critical region on chromosome 21 [52] and its expression is elevated in individuals suffering from DS [53]. Mnb/Dyrk1A appears to regulate SV recycling and synaptic transmission by phosphorylating ITSN1-binding partners such as dynamin 1 [54] and synaptotagmin 1 [49], thereby regulating complex formation between key endocytic proteins [55]. Whether ITSN1 and Mnb/Dyrk1A physically and/or functionally interact and whether this has a role in DS remain to be determined.

Taken together, ITSN1 serves as a large scaffolding adaptor interconnecting diverse cellular processes in nerve terminals ranging from SV recycling to the regulation of actin dynamics and cell signalling. ITSN1-mediated spatiotemporal control of vesicle cycling might not only be crucial for maintaining the SV cycle but higher brain functions such as learning and memory might also depend on a balanced interplay between ITSN1 and its interaction partners. Future studies are needed to address the precise roles of ITSN1 and its complexes at various steps of the SV cycle as well as its involvement in different nervous system disorders.

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