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Synaptophysin-dependent synaptobrevin-2 trafficking at the presynapse - mechanism and function.

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Abbreviations – ANTH - AP180 N-terminal homology; AP-2 -adaptor protein complex 2; AP180 - adaptor protein 180; CALM - clathrin assembly lymphoid myeloid leukaemia; FRET - Forster resonance energy transfer; NSF - NEM-sensitive factor; SNAP-25 – synaptosomal-associated protein 25kDa; α-SNAP - α-soluble NSF-attachment protein; SNARE - soluble NSF attachment protein receptor; STP – short-term plasticity; SV2A - synaptic vesicle glycoprotein 2A; SV - synaptic vesicle; Syb2 – Synaptobrevin; Syp – synaptophysin; VAMP1 - vesicle-associated membrane protein 1; vGAT – vesicular GABA transporter; vGLUT – vesicular glutamate transporter

Synaptophysin-dependent synaptobrevin-2 trafficking at the presynapse - mechanism and function.

Abstract

Synaptobrevin-2 (Syb2) is a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) that is essential for neurotransmitter release. It is the most numerous protein on a synaptic vesicle (SV) and drives SV fusion via interactions with its cognate SNARE partners on the presynaptic plasma membrane. Synaptophysin (Syp) is the second most abundant protein on SVs, however in contrast to Syb2 it has no obligatory role in neurotransmission. Syp interacts with Syb2 on SVs, and the molecular nature of its interaction with Syb2 and its physiological role has been debated for decades. However, recent studies have revealed that the sole physiological role of Syp at the presynapse is to ensure the efficient retrieval of Syb2 during SV endocytosis. In this review current theories surrounding the role of Syp in Syb2 trafficking will be discussed, in addition to the debate regarding the molecular nature of their interaction. A unifying model is presented that describes how Syp controls Syb2 function as part of an integrated mechanism involving key molecular players such as intersectin-1 and AP180/CALM. Finally, key future questions surrounding the role of Syp-dependent Syb2 trafficking will be posed, with respect to brain function in health and disease.

Introduction

Synaptic transmission is reliant on the fast, efficient and synchronous release of chemical neurotransmitter in response to the action potential-dependent entry of extracellular calcium via voltage-gated channels. In turn, this process is dependent on the fusion of neurotransmitter-containing synaptic vesicles (SVs) with the presynaptic plasma membrane. For SVs to be functionally competent, they must be formed with a specific complement of lipids and proteins in a defined stoichiometry (Takamori *et al.* 2006, Wilhelm *et al.* 2014, Wittig *et al.* 2021). SVs are generated at the presynapse via a series of different endocytosis modes that are triggered by different patterns of neuronal activity in both time and space (Kononenko & Haucke 2015, Chanaday *et al.* 2019). Central to the accurate formation of SVs, are cargo selection mechanisms that ensure essential proteins are incorporated with the correct stoichiometry appropriate for their role. This cargo selection occurs regardless of the endocytosis mode via which SVs are formed and can occur at either the plasma membrane or presynaptic endosomes (Kononenko *et al.* 2014, Watanabe *et al.* 2014, Ivanova *et al.* 2021).

The two most abundant proteins on SVs are synaptobrevin-2 (Syb2, also known as vesicle-associated membrane protein 2) and synaptophysin (Syp). Syb2 is the most numerous, with approximately seventy copies present on SVs (Wittig et al. 2021, Takamori et al. 2006, Wilhelm et al. 2014). It is a single-pass transmembrane SV protein, that is comprised almost exclusively of a cytoplasmic N-terminus, that contains a highly conserved soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) motif (Sudhof *et al.* 1989) (Figure 1). Syb2 is essential for SV fusion and its absence is incompatible with life. This is because Syb2 knockout neurons display an almost complete ablation of calcium-dependent evoked synchronous neurotransmitter release (Schoch *et al.* 2001). Syb2 controls neurotransmitter release via its SNARE motif, which progressively interacts with the respective SNARE motifs of the plasma membrane target-SNAREs proteins syntaxin-1 and synaptosomal-associated protein, 25 kDa (SNAP-25) to drive SV fusion with the plasma membrane (Rizo 2018, Brunger *et al.* 2018). Because of its central role in neurotransmission, the molecular interactions of Syb2 with its cognate SNAREs has been intensively studied. Somewhat surprisingly however, the mechanisms that dictate its clustering, retrieval and copy number on SVs have not had similar levels of scrutiny.

Syp is the second most abundant cargo on the SV, with approximately 30-32 copies present in a 1:2 ratio with Syb2 (Takamori et al. 2006, Wilhelm et al. 2014, Wittig et al. 2021). It contains four transmembrane domain regions and cytoplasmic N- and C-termini, the latter being the major site for protein – protein interactions (Daly & Ziff 2002, Wheeler *et al.* 2002, Felkl & Leube 2008) (Figure 1). In contrast to Syb2, early studies in Syp knockout neurons revealed that it had no essential role on SV fusion (McMahon *et al.* 1996, Eshkind & Leube 1995). Because of the absence of an obligatory role, studies examining Syp function at the presynapse had been sparse, however in the past decade considerable progress have been made in determining its physiological role. Intriguingly, Syp controls the post-fusion trafficking of Syb2, meaning the two most abundant proteins on SVs have intricately linked functions at the presynapse.

This review will summarise the evidence for Syp as an essential mediator of Syb2 trafficking at the presynapse, including interaction and functional studies. It will also discuss other

proposed roles of Syp, in the context of recent studies that revealed that the sole physiological role of Syp is to coordinate the retrieval of Syb2 during SV endocytosis, potentially in concert with other Syb2 trafficking molecules such as intersectin-1 and adaptor protein 180 (AP180). Finally, key unaddressed questions will be posed.

The sole physiological presynaptic role for Syp is the control of Syb2 retrieval

Syp has no obligatory role in neurotransmitter release

Early studies revealed that Syp and Syb2 were interaction partners, which raised the possibility that Syp controlled Syb2 function in neurotransmitter release (Calakos & Scheller 1994, Edelmann *et al.* 1995, Washbourne *et al.* 1995). This hypothesis was driven by the observation that Syb2 binds to either Syp or the plasma membrane t-SNAREs in a mutually exclusive manner (Edelmann et al. 1995, Siddiqui *et al.* 2007). This therefore suggested that Syp controlled the access of Syb2 to the trans-SNARE complex and thus neurotransmitter release. However this idea was dismissed, mainly due to the observation in that neurotransmission was unaffected in Syp knockout mice (Eshkind & Leube 1995, McMahon et al. 1996). Furthermore, later studies revealed that while these interactions were mutually exclusive, exogenously added t-SNAREs could displace Syb2 from Syp that was resident on SVs (Siddiqui et al. 2007). Therefore Syp has no obligatory role in SV fusion and/or neurotransmitter release, even though it is highly abundant on SVs.

Synaptophysin is essential for efficient Syb2 retrieval

The absence of a neurotransmitter release defect after deletion of the *Syp* gene, hinted that its association with Syb2 may be required for a different aspect of the SV life cycle. The first demonstration of this was obtained from studies that examined activity-dependent Syb2 trafficking in primary cultures of Syp knockout neurons. This work revealed that the absence of Syp resulted in a mislocalisation of endogenous Syb2 from nerve terminals and an accumulation of exogenously-expressed Syb2 at the plasma membrane (Gordon *et al.* 2011). These studies also revealed that the mislocalisation of Syb2 in Syp knockout neurons was due to a defect in the activity-dependent retrieval of Syb2 during SV endocytosis, since the trafficking of exogenous Syb2 tagged with a pH-sensitive version of enhanced green fluorescent protein (pHluorin) was retarded after stimulation terminated (Gordon et al. 2011, Gordon & Cousin 2013).

Early studies suggested Syp may perform a more fundamental role in SV retrieval, since injection of its isolated cytoplasmic C-terminus resulted in SV depletion and an increase in clathrin-coated SVs at the squid giant synapse (Daly *et al.* 2000). Furthermore, the absence of Syp also had a small effect on the global speed of SV endocytosis. In multiple studies, a variety of different genetically-encoded reporters of SV cargo trafficking including synaptic vesicle glycoprotein 2A (SV2A), synaptotagmin-1 and the vesicular glutamate transporter displayed a significant slowing in their retrieval in Syp knockout neurons (Gordon et al. 2011, Gordon & Cousin 2013, Kwon & Chapman 2011, Rajappa *et al.* 2016). Importantly, this slowing did not alter the number of SVs retrieved during an action potential train and was minor compared to the more pronounced effects on Syb2 retrieval (Gordon et al. 2011). Therefore, it was suggested that Syp had a dual role at the presynapse, which was to both accelerate SV endocytosis and specifically facilitate Syb2 retrieval during this event. However, more recent work that examined these potential roles at physiological temperatures

has simplified this model. These experiments revealed that no global kinetic defect in SV endocytosis occurred in Syp knockout neurons when studies were performed at 37 ºC, whereas Syb2 retrieval was still perturbed (Harper *et al.* 2021). Therefore the only physiologically relevant role for Syp at the presynapse is to ensure the accurate retrieval of Syb2 after SV fusion, regardless of the endocytosis mode that is triggered.

Syp and active zone clearance of Syb2

A different, but related, role for Syp has also been proposed, namely the clearance of Syb2 from the active zone (Rajappa et al. 2016). In this model Syp binds to Syb2 immediately after the dissociation of plasma membrane SNARE complexes via the action of NSF (N-ethylmaleimide-sensitive factor) and α-SNAP (soluble NSF-attachment protein). This Syp/Syb2 interaction was proposed to prevent the formation of futile cis-SNARE complexes, facilitating the lateral movement of Syb2 from the active zone to the periactive zone for retrieval by SV endocytosis. This role is not mutually exclusive to the role for Syp in Syb2 retrieval, indeed would be complementary and facilitate the latter.

In support of this model, the clearance of SV cargo from the active zone immediately after fusion is a key factor in the regulation of release probability, with a short-term depression in neurotransmitter release resulting from occupancy of a limited number of release sites (Hua *et al.* 2013, Hosoi *et al.* 2009, Kawasaki *et al.* 2000). In agreement, Syp knockout neurons display detectable defects in short-term plasticity (STP) (McMahon et al. 1996, Janz *et al.* 1999, Kwon & Chapman 2011). Furthermore, the role of Syp in facilitating Syb2 active zone clearance was supported in experiments using an optical correlate of STP in Syp knockout neurons, which demonstrated a reduced ability of exogenously expressed Syb2 to visit the cell surface after an initial stimulus (Rajappa et al. 2016). Unfortunately, Syb2-pHluorin was also used as the reporter of SV fusion, making it difficult to separate true effects on STP from artefacts of increased Syb2 at the plasma membrane from a prior reference stimulus.

Recent experiments have resolved this issue however, by observing the effect of overexpressing Syb2 on SV fusion in Syp knockout neurons. The rationale for these experiments was that if Syp controlled the retrieval of Syb2, overexpression of Syb2 in Syp knockout neurons should rescue the defect (by providing more substrate for an inefficient process). In contrast, if Syp was required to clear Syb2 from active zones, excess Syb2 should exacerbate effects on SV fusion in these neurons. When Syp knockout neurones were challenged with repeated trains of action potentials, a significant depression in SV fusion events were observed, a depression that was corrected by expression of exogenous Syp (Kokotos *et al.* 2019). Importantly, Syb2 overexpression also prevented this rundown, even in the absence of Syp, indicating that the most likely explanation of the depression was inefficient Syb2 retrieval during SV endocytosis. This was confirmed in parallel biochemical experiments, where purified SVs from Syp knockout neurons displayed a depletion of Syb2 after multiple stimulation trains (Kokotos et al. 2019). Therefore, the only identified physiological role of Syp is to ensure the accurate retrieval of Syb2 during SV endocytosis.

What is the molecular mechanism of Syp-dependent Syb2 retrieval?

As discussed above, the sole role for Syp at the presynapse is to ensure the accurate retrieval of Syb2 during SV endocytosis. However, what is the molecular mechanism that underpins

this role? This discussion should be considered in the context of the requirements for Syb2 trafficking post SV fusion. During SV fusion, Syb2 transitions from being constituent of a trans-SNARE complex (where it resides on SVs) to a cis-SNARE complex in the presynaptic plasma membrane. This cis-SNARE complex then has to be cleared from release sites to allow new SVs to fuse, and be disassembled to free Syb2 from its t-SNARE partners. It would also be advantageous for Syb2 to be prevented from reforming futile cis-SNARE complexes, in addition to being clustered for retrieval via compensatory endocytosis.

What controls the Syp/Syb2 interaction?

The mutually exclusive interaction of Syp with Syb2 and the SNARE complex suggest that Syp may capture Syb2 after its NSF/α-SNAP-dependent dissociation to restrict the assembly of cis-SNARE complexes. Convincing evidence for this role is still absent however, with a series of biochemical and imaging studies producing what appear to be contradictory results. For example, chemical cross-linking studies revealed that the Syp/Syb2 interaction is upregulated following membrane fusion (Khvotchev & Sudhof 2004), whereas immunoprecipitation studies suggest the opposite (Reisinger *et al.* 2004). In agreement with the latter study, Forster resonance energy transfer (FRET) analysis of exogenously expressed Syp and Syb2 in cultured neurons suggest the complex is dissociated following incubation with the spider toxin α-latrotoxin, which triggers SV fusion (Pennuto *et al.* 2002). Interestingly calcium is required, though not sufficient, for this dissociation in intact nerve terminals (Reisinger et al. 2004, Daly & Ziff 2002, Prekeris & Terrian 1997, Chapman *et al.* 1995). However, the studies outlined above are limited both in terms of their poor temporal resolution (immunoprecipitation or cross-linking studies) or potential for overexpression artefacts (FRET studies), therefore it is still unclear when and where Syp and Syb2 associate in the plasma membrane and whether this association restricts the entry of Syb2 into cis-SNARE complexes (unlike trans-SNARE complexes).

What is the molecular nature of their interaction?

Considering the number of studies that have been performed investigating the Syp-Syb2 interaction, it is surprising that relatively little definitive information regarding the molecular nature of their binding interfaces is present. Early biochemical studies suggested that they may interact via their transmembrane domains. For example, Syp was unable to be isolated from SV extracts by the cytoplasmic domain of Syb2 (Becher *et al.* 1999). Furthermore, cleavage of Syb2 at its SNARE motif by clostridial neurotoxins, resulting in production of N- and C-terminal fragments, revealed Syp bound to the Syb2 C-terminus (containing the transmembrane domain), with no binding at the N-terminus. A recombinant form of this C-terminus, which retained the C-terminal half of the SNARE motif, also extracted Syp from SV lysates (Yelamanchili *et al.* 2005). The requirement for the Syb2 transmembrane domain in Syp binding has also been suggested by both yeast two-hybrid experiments (Felkl & Leube 2008) and immunoprecipitation studies from a heterologous expression system, where the interaction is retained using a mutant version of Syp that lacks a C-terminus (Bonanomi *et al.* 2007).

However, there is also biochemical evidence for an interaction between the cytoplasmic domains of Syp and Syb2. For example, the Syp/Syb2 complex can be dissociated by high ionic strength buffers (Edelmann et al. 1995), suggesting their association occurs via non-

hydrophobic interactions. Furthermore, a peptide corresponding to the first 32 amino acids of the Syb2 N-terminus is sufficient to dissociate the Syp/Syb2 complex (Washbourne et al. 1995), again hinting at a cytosolic interaction. The most definitive evidence thus far comes from recent work revealing that the Syp C-terminus contains a cryptic interaction site for Syb2 (Harper et al. 2021). In these studies recombinant full length Syp C-terminus could not extract Syb2 from nerve terminal lysates. However, Syb2 could be extracted by a Syp mutant which had the extreme C-terminus removed (encompassing approximately 40 % of the Syp C-terminus). Further truncations revealed the Syb2 interaction site was located in the first 26 amino acids of the Syp C-terminus (Harper et al. 2021). It was also discovered that the truncated Syp mutant C-terminus strongly interacts with both full length Syb2 and its cytoplasmic N-terminal domain. Further removal of the SNARE motif from the N-terminus abrogated Syp binding, suggesting that the Syp C-terminus binds to the Syb2 SNARE motif (Harper et al. 2021).

A key question remains from this model however, how does Syb2 gain access to the Syp C-terminal cryptic binding site? Potential clues came from the discovery that an excess of the extreme Syp C-terminus peptide permitted Syb2 binding to the full-length Syp C-terminus, but had no effect on Syb2 binding to the truncated form of Syp (Harper et al. 2021). This suggests that the Syp C-terminus does not occlude Syb2 binding via intramolecular interactions (since this peptide would have blocked binding to the truncated form), but rather frees the full length version by associating with similar sequences as part of the larger Syp/Syb2 complex.

Functional evidence for Syp – Syb2 interactions

Is there functional evidence that supports any of the biochemical interaction studies discussed above? A number of overexpression experiments in both heterologous and neuronal culture systems support the hypothesis that Syp/Syb2 interact via their cytoplasmic domains. For example, overexpression of Syb2 in wild-type cultured neurons results in its mislocalisation from nerve terminals (Pennuto *et al.* 2003) and its redistribution to the plasma membrane (Gordon *et al.* 2016). Co-expression of Syp with Syb2 redistributed the latter to nerve terminals and SVs (Gordon et al. 2016, Pennuto et al. 2003). Interestingly, the nerve terminal localisation of a Syb2 mutant that had its transmembrane domain replaced by that of the related vesicle-associated membrane protein 1 (VAMP1) was fully rescued by Syp expression. In contrast, a mutant containing the transmembrane domain of Syb2 and the cytoplasmic domain of VAMP1 was not (Pennuto et al. 2003). This suggests that the N-terminal cytoplasmic domain of Syb2 must bind Syp to enable its accurate trafficking. In a different overexpression experiment, this time in a heterologous expression system, Syb2 mislocalisation to the plasma membrane was rescued by full-length Syp, but not a C-terminal truncated version (Bonanomi et al. 2007). This again suggested that the cytoplasmic C-terminus of Syp is essential for efficient Syb2 trafficking.

Further evidence for a role for the Syp C-terminus in Syb2 trafficking came from molecular replacement studies in Syp knockout neurons. In these experiments, the effect of expressing Syp mutants with sequential C-terminal truncations on Syb2-pHluorin retrieval was examined (Harper et al. 2021). Both full-length Syp and Syp containing C-terminal truncations including the cryptic Syb2 interaction motif, fully rescued Syb2-pHluorin retrieval. In

contrast, truncations in which the cryptic interaction motif was removed did not rescue, indicating an essential requirement for this region in Syb2 retrieval (Harper et al. 2021).

Mutations in the *SYP* gene found in individuals with X-linked intellectual disability have provided further evidence of both a role for the Syp C-terminus in Syb2 retrieval and access to the cryptic interaction site. One specific mutation, a frame shift in the cytoplasmic C-terminus, which retains the cryptic interaction site but has the final 40 % replaced by a completely different sequence, failed to rescue Syb2 retrieval when expressed in Syp knockout neurons (Gordon & Cousin 2013). This suggested that Syb2 could not gain access to the cryptic site, due to substitution of an alien sequence at the extreme C-terminus. Therefore, evidence is accumulating that the cryptic interaction site of the cytoplasmic domains of Syp with the SNARE motif of Syb2 is essential for accurate trafficking of the latter.

Role of other Syb2 interactors in its trafficking

N-terminal interactors - α-synuclein and intersectin-1

In addition to the essential role of Syp in Syb2 retrieval, other molecules interact with Syb2 to control its trafficking. The extreme N-terminus of Syb2 appears to be an important interface in this regard. For example, peptides corresponding to this sequence, or Fab fragments directed against it, disrupted replenishment of the readily releasable pool and neurotransmitter release when injected into large atypical nerve terminals (Wadel *et al.* 2007, Japel *et al.* 2020). Furthermore, a Syb2 mutant lacking this region displayed a mislocalisation to the plasma membrane when expressed in neuronal cultures in which endogenous Syb2 was depleted. These neurons also displayed a small but significant decrease in their ability to sustain SV fusion events during action potential trains (Japel et al. 2020). Therefore it appears that the extreme N-terminus of Syb2 has interactions that control its trafficking and also neurotransmitter release.

These roles outlined above are unlikely to be due to interactions with Syp, since this region does not bind the isolated C-terminus of Syp in interaction assays (Harper et al. 2021). Instead, this region appears to mediate its effect via the endocytosis protein intersectin-1. Evidence for this is as follows; intersectin-1 co-immunopreciptiates Syb2 from neuronal lysates, with inhibition of NSF-induced SNARE disassembly increasing the interaction. This suggested that intersectin-1 associated with intact SNARE complexes, rather than Syb2 alone (Japel et al. 2020). In agreement, Syp was absent from these immunoprecipitates, most likely due to its inability to bind Syb2 as part of the SNARE complex. Furthermore, an increase in surface stranded Syb2, but not other SV cargoes, was observed in intersectin-1 knockout neurons, with no additive effect of expressing Syb2 with its extreme N-terminus deleted (Japel et al. 2020). This surface stranding is likely to result from the defective clearance of Syb2 from the active zone, and not a defect in Syb2 retrieval, since both SV endocytosis and Syb2-pHluorin retrieval were unaffected in intersectin-1 knockout neurons (Japel et al. 2020). Therefore intersectin-1 may act to clear assembled cis-SNARE complexes from the active zone, before their disassembly via NSF/α-SNAP.

This same N-terminal region of Syb2 can also interact with the presynaptic protein α-synuclein, with this interaction facilitating SNARE complex assembly (Burre *et al.* 2010).

However, this interaction is unlikely to control the trafficking of Syb2, since α-synuclein dissociates from SVs during fusion (Fortin *et al.* 2005). Therefore α-synuclein interacts with the extreme N-terminus of Syb2 to catalyse SNARE complex assembly, whereas intersectin-1 captures assembled cis-SNARE complexes via the same region to clear them from the active zone.

SNARE motif interactors – AP180/CALM

As discussed above, the cryptic interaction site on Syp interacts with the SNARE motif of Syb2, most likely the C-terminal half (Yelamanchili et al. 2005). However, Syp is not the only endocytosis protein that binds to this key region on Syb2. Syb2 is unusual in that it does not contain canonical motifs that are recognised by classical endocytic adaptor protein complexes such as adaptor protein complex 2 (AP-2); however, its SNARE motif can be bound by the monomeric adaptor protein AP180 and the related clathrin assembly lymphoid myeloid leukaemia protein (CALM) (Miller *et al.* 2011). Both AP180 and CALM have the ability to assemble clathrin lattices and AP-2 (Morgan *et al.* 1999, Morgan *et al.* 2000, Ford *et al.* 2001) thus have the potential to be a molecular bridge that facilitates Syb2 retrieval during SV endocytosis.

Evidence for a key role of AP180 / CALM in Syb2 trafficking came from a number of knockout / knockdown studies in different organisms such as *C. elegans* (Nonet *et al.* 1999), *Drosophila* (Bao *et al.* 2005) and mouse (Koo *et al.* 2011). In all cases, Syb2 was mislocalised from nerve terminals in mutant axons lacking either AP180 or its orthologues. In addition, Syb2 displayed increased surface stranding at the plasma membrane and inaccurate retrieval during SV endocytosis in AP180 knockout neurons, with knockdown of CALM exacerbating this phenotype (Koo *et al.* 2015). This mislocalisation and surface stranding is likely to be a result of inefficient clustering of Syb2 at the periactive zone. This is because live imaging studies revealed that exogenously expressed Syb2 rapidly diffuses within the nerve terminal before this movement becomes restricted near this region (Gimber *et al.* 2015). This restriction is dependent on AP180 / CALM, since either Syb2 with a mutated SNARE motif unable to form AP180 / CALM interactions, or Syb2 expressed in AP180 knockout neurons display a higher mobility in nerve terminals. Super-resolution imaging confirmed that this restriction occurred at the periactive zone (Gimber et al. 2015).

As stated above, AP180 / CALM bind to the SNARE motif of Syb2. They do so via their N-terminal ANTH (AP180 N-terminal homology) domains (Miller et al. 2011, Koo et al. 2011). The ANTH domain can simultaneously bind both plasma membrane PIP_2 and the SNARE motif, proving a mechanism for its recruitment (Miller et al. 2011). The interaction site for the ANTH domains of both AP180 / CALM is located within the N-terminal half of region of the SNARE motif, which was revealed a series of *in vitro* interaction assays (Koo et al. 2011). When this interaction was perturbed in primary neurons by point mutations in either the Syb2 SNARE motif or the ANTH domain of AP180, exogenously expressed syb2 was mislocalised to the plasma membrane (Koo et al. 2011, Koo et al. 2015). Therefore AP180 / CALM are also essential for the accurate retrieval of Syb2 during SV endocytosis.

Working model of post-fusion Syb2 trafficking

Over the past decade, a series of studies have begun to uncover what appears to be a highly intricate and coordinated transport of Syb2 from post-fusion, cis-SNARE complexes in the active zone plasma membrane to newly formed SVs. Here we present a working model for Syb2 trafficking post SV fusion (Figure 2).

1) *Active zone clearance* – Immediately after SV fusion, the cis-SNARE complex is located to release sites in the active zone. Intersectin-1 binds to Syb2 in this cis-SNARE complex to facilitate its clearance from the active zone. Whether this is an active or passive process is still unclear, however since intersectin-1 only binds Syb2 present in assembled SNARE complexes, it suggests it performs an active role. However definitive evidence of facilitated clearance remains to be demonstrated.
2) *Dissociation of the cis-SNARE complex* – After clearance from the active zone, NSF breaks apart the cis-SNARE complex after its recruitment via α-SNAP. The assumption is that this occurs at the periactive zone, however definitive evidence of this location is still absent. The action of NSF liberates Syb2, the t-SNAREs and intersectin-1.
3) *Syb2 capture by AP180 / CALM* – Syb2 is captured by AP180 / CALM on its liberation from the cis-SNARE complex. This capture occurs via an interaction with the N-terminal region of its SNARE motif and the ANTH domain of AP180 / CALM.
4) Syp stabilises the Syb2/AP180 complex – A conformational change within the Syp C-terminus reveals its cryptic interaction motif. This then permits interactions with the C-terminal region of the SNARE motif, stabilising this tripartite complex. Transmembrane interactions between Syp and Syb2 may also play a role in stabilising this complex.
5) Recruitment of clathrin / AP-2 – Both Syp and AP180 / CALM have AP-2 interaction motifs that facilitate clustering of endocytosis proteins at the peractive zone. Syp recruits AP-2 via its C-terminal tyrosine based motifs that are upstream from the cryptic interaction site, whereas AP180 recruits AP-2 via its C-terminus. Clathrin lattice assembly is also facilitated by AP180 / CALM, and is accelerated its association with AP-2.
6) The Syb2/AP180/Syp complex is ready for retrieval by SV endocytosis.

Perspectives

The working model presented above requires rigorous testing through targeted molecular replacement studies and advanced imaging protocols. Regardless, a series of key questions remain regarding the role of Syp in the trafficking and retrieval of Syb2.

SV stoichiometry

The average number of copies of Syb2 and Syp on a SV has been invariant across a number of different studies (Takamori et al. 2006, Wittig et al. 2021, Wilhelm et al. 2014). This raises the question, does the interaction between Syp and Syb2 also define their stoichiometry on SVs? Single particle electron microscopy analysis of the native Syp/Syb2 complex isolated from SVs, revealed that the proteins interact in a 6:12 (Syp:Syb2) ratio. Within this complex, Syp forms a hexameric core (Thomas *et al.* 1988, Arthur & Stowell 2007) that interacts with six dimers of Syb2 (Adams *et al.* 2015). This suggests that the established stoichiometry on SVs is a direct result of the interaction itself. In support, the relative number

of Syp and Syb2 molecules in nerve terminals has functional consequences. For example, the mislocalisation of Syb2 that results from its overexpression in wild-type neurons can be corrected by providing approximately 50% less exogenous Syp (Pennuto et al. 2003, Gordon et al. 2016), in agreement with the 6:12 ratio observed on SVs. Therefore, Syp may be a key determinant of the copy number of Syb2 on SV, by virtue of stoichiometry of their biochemical interaction.

When discussing SV cargo stoichiometry, it should be noted that most studies have concentrated on excitatory neurons. When SVs from inhibitory neurons are profiled, some interesting observations occur. For example, co-localisation studies in cortical brain sections using antibodies directed against the vesicular transporters for either glutamate (vGLUT1) or GABA (vGAT) revealed a two-fold enrichment of Syp in vGLUT1-positive puncta compared to vGAT (Bragina *et al.* 2007), with a parallel enrichment in Syb2 at vGLUT1 puncta (Bragina *et al.* 2010). Immuno-isolated glutamatergic SVs also displayed an enrichment in Syb2 when compared to GABAergic SVs (Bragina et al. 2010), whereas a near two-fold enrichment of Syp in glutamatergic SVs was revealed in a parallel study (Gronborg *et al.* 2010). Therefore, it appears that Syp and Syb2 may be disproportionally represented in glutamatergic SVs, however both are still present in inhibitory SVs. The functional consequences of this excitatory / inhibitory bias will be of great interest to explore.

The one exception to the established 1:2 Syp/Syb2 ratio is a study where specific SV cargo on purified SVs were subjected to quantification with fluorescent antibodies. In this work, Syp was found to be more numerous on SVs than Syb2 (Mutch *et al.* 2011). However, the number of Syb2 molecules per SV was estimated to be approximately 10, a seven-fold reduction in comparison to other biochemical studies (Takamori et al. 2006, Wittig et al. 2021, Wilhelm et al. 2014). This suggests there was limited antibody accessibility to Syb2 complexes (and potentially complexes from other SV cargoes) and should be interpreted with caution. A true resolution of the protein complement at individual SVs is still lacking due to technical limitations, however it is highly likely that there will be considerable heterogeneity. It remains to be determined whether Syb2 and Syp co-vary in number at single SVs, which may provide compelling evidence for a role of Syp in determining Syb2 SV copy number.

Access to the cryptic Syp binding site

One of the intriguing aspects of the Syp/Syb2 interaction is the revelation Syp contains a cryptic interaction site for Syb2 that is only revealed when the distal C-terminus is removed. The role of the extreme C-terminus is not to occlude Syb2 binding, since when it is present in excess quantities it facilitates the interaction (Harper et al. 2021). It is therefore likely that interactions within the extreme C-terminus are essential to unmask this cryptic binding site. In support, a human frame-shift mutation that replaces this region with a nonsense sequence fails to rescue Syb2 retrieval in Syp knockout neurons (Gordon & Cousin 2013). The only known interaction in this region is with the ubiquitin ligases Siah-1 and Siah-2, which is proposed to facilitate the degradation of Syp via the ubiquitin system (Wheeler et al. 2002). It would be of considerable interest to investigate other interaction partners for this region. Intriguingly, the addition of adult cytosol to isolated SVs increases the amount of Syp/Syb2 complexes, suggesting a cytosolic protein may regulate the interaction (Becher et al. 1999).

It also possible that Syp oligomers have a different conformation on SVs in comparison to those at the plasma membrane. This may relate to the high cholesterol content of SVs, since increased cholesterol concentrations favour Syb2 binding to Syp (Mitter *et al.* 2003, Adams et al. 2015). It may also be the case that the plasma membrane Syb2 retrieval complex is different to the Syp/Syb2 complex present on SVs. For example, Syb2 transitions via a series of interactions with t-SNAREs, intersectin-1 and AP180 / CALM as it is freed from cis-SNARE complexes, cleared from the active zone and clustered at the periactive zone. Therefore, the cryptic interaction site may only be revealed at the plasma membrane, where Syp undergoes a series of conformational transitions to facilitate Syb2 retrieval. In contrast the Syp/Syb2 complex on SV may be stabilised via other interactions (such as between their transmembrane domains (Becher et al. 1999, Yelamanchili et al. 2005, Bonanomi et al. 2007, Felkl & Leube 2008)).

Is the Syp / Syb2 interaction more complex than discussed above?
The potential for the Syp/Syb2 interaction to be different in discrete membrane compartments is supported by a recent large scale mass spectrometry screen investigating protein-protein interactions on SVs revealed by cross-linking (Wittig et al. 2021). This study suggested that there is a complex series of interactions between abundant SV proteins, with both Syp and Syb2 being core constituents of specific sub-complexes. Intriguingly, a large number of potential interactions were observed between the intra-lumenal domains of Syp and Syb2, in addition to other proteins such as SV2A, synaptoporin and synaptogyrin-1 (Wittig et al. 2021). This provides first evidence that Syp/Syb2 complexes share distinct interactions dependent on their location within the nerve terminal.

On a wider point, this potentially complex network of interactions between SV proteins suggests that they may perform key roles in the efficiency and specificity of cargo retrieval. For example, shRNA-mediated knockdown of vGLUT1 results in disrupted retrieval of a specific subset of SV proteins (Pan *et al.* 2015). Furthermore, the SV protein SV2A controls the trafficking of the principal calcium sensor for neurotransmitter release, Synaptotagmin-1 (Yao *et al.* 2010, Kaempf *et al.* 2015, Zhang *et al.* 2015, Harper *et al.* 2020). This trafficking is phosphorylation-dependent and bears significant similarities to the control of Syb2 trafficking by Syp (Zhang et al. 2015). Because of this, both Syp and SV2A were termed intrinsic trafficking partners (Gordon & Cousin 2016). It will be of considerable interest to observe whether more SV proteins are added to this functional grouping in future.

Does Syp-dependent Syb2 trafficking affect neurotransmitter release?
As stated earlier, there are approximately 70 copies of Syb2 on a SV (Takamori et al. 2006, Wilhelm et al. 2014). However only 1-3 molecules of Syb2 are required for a SV fusion event (Mohrmann *et al.* 2010, van den Bogaart *et al.* 2010, Sinha *et al.* 2011). This high level of redundancy suggests that neurotransmission should still proceed, even if Syb2 trafficking is disrupted. However, this is not the case. For example, $Syb2^{+/-}$ mice display a significant reduction in basal neurotransmission, a defect that is phenocopied in AP180 knockout mice (which have 40 % less Syb2 on SVs (Koo et al. 2015)). Furthermore, these neurotransmission defects are exacerbated when these mice lines are crossed (Koo et al. 2015). Hippocampal slices from $Syb2^{+/-}$ mice also display reduced release probability and primary cultures have diminished release rates, when examined using the vital dye FM1-43 (Orock *et al.* 2020).

Finally, primary cultures of Syb2$^{+/-}$ neurons display reduced vGLUT1-pHluorin responses during action potential stimulation, however this is negligible when normalised to the total SV pool (Chanaday & Kavalali 2021). More direct experiments have shown that reducing the level of Syb2 can have disproportionate effects on SV fusion. For example, a decrease in the level of Syb2 by approximately 50% (via titration of tetanus toxin) resulted a reduction of miniature excitatory postsynaptic current frequency to only 10 % of control (Bao *et al.* 2018). Therefore, it appears that maintaining a high copy number of Syb2 molecules on SVs is essential to sustain neurotransmitter release.

Considering this requirement for Syb2 copy numbers on SVs, would defective Syp function result in defects in neurotransmission? Early studies in Syp knockout mice confirmed that the presence of Syp is not obligatory for baseline neurotransmission (Eshkind & Leube 1995, McMahon et al. 1996). This lack of effect is likely due to the large surplus of Syb2 in the nerve terminal that would compensate for the absence of Syp. However, this is not the case during bursts of neuronal activity, where repeated recycling of SVs results in the short-term depletion of Syb2 in Syp knockout neurons. Under such conditions, Syp knockout neurons display defects in STP, which can be restored by expression of exogenous Syp (Kwon & Chapman 2011, Kokotos et al. 2019). Importantly, this STP defect can also be fully corrected by expression of exogenous Syb2 (Kokotos et al. 2019), confirming a key role for Syp in the targeting of Syb2 to SVs.

Does Syp dysfunction result in human disease?
Mutations in the *SYP* gene have been identified in individuals with X-linked intellectual disability, epilepsy and more recently frontal temporal dementia (Tarpey *et al.* 2009, Harper *et al.* 2017, Prota *et al.* 2021). This suggests that defective Syb2 trafficking may underlie some of these conditions. In agreement, these mutants failed to restore Syb2 retrieval when expressed in Syp knockout neurons (Harper et al. 2017, Gordon & Cousin 2013). Importantly, some of these mutations were predicted to result in nonsense-mediated decay. Since both of these individuals carrying these mutations were male and *Syp* is located on the X-chromosome, they were effectively Syp null (Tarpey et al. 2009). Thus the absence of Syp is compatible with life in humans, but even though its absence results in neurodevelopmental disorders.

The finding that mammals (and humans) can survive without functional Syp raises interesting questions about how defects in Syb2 trafficking are translated into altered brain function in these organisms. As stated earlier, Syp knockout neurons display no overt defects in neurotransmission, however they do display defects in STP due to inefficient Syb2 retrieval. Therefore, defects in neurotransmission will only occur when neurons fire with particular patterns of activity. This means that for the majority of neurons at any one time, neurotransmission will be normal. This may explain why humans that are effectively Syp null can survive, while presenting with neurodevelopmental disorders, since circuits that are highly active during synapse / brain development will be disproportionately impacted, resulting in altered connectivity and function.

Why is Syp needed at all?

Finally, a more fundamental question to address is, why is Syp needed for brain function? For example, Syp is only expressed in vertebrates, suggesting lower organisms can survive and function in its absence. Even in humans, the absence of Syp is compatible with life, since as discussed above, males with nonsense mutations in their one copy of the X-linked *SYP* gene can survive, albeit with X-linked intellectual disability (Tarpey et al. 2009). In further support of a redundant role for Syp, the mislocalisation of Syb2 observed when AP180 and/or CALM is removed in mammalian systems is very similar to that seen in invertebrates (Koo et al. 2011, Koo et al. 2015). However, the specific mistargeting of Syb2 in AP180/CALM null systems appears to be selective for mammals, since removal of the *lap* gene in *Drosophila* resulted in the mislocalisation of several presynaptic proteins (Bao et al. 2005, Vanlandingham *et al.* 2014).

The answer to the requirement for Syp in vertebrate neurotransmission may lie in the complexity of the mammalian brain, which fires across a wide range of frequencies and with and an array of different activity patterns. Such complex activity most likely will require an additional level of control of Syb2 trafficking and targeting to SVs. Therefore, Syp may be required to sustain communication in highly active circuits by maintaining optimal Syb2 trafficking during bursts of intense neuronal activity. In molecular terms, Syp is likely to provide stability to AP180-driven Syb2 retrieval, in situations where neurons and circuits experience short-term periods of high activity. This evolutionary refinement, suggests that Syp acts as a triple-lock to AP-2 and AP180/CALM-dependent Syb2 retrieval to ensure high level function is maintained at the cell, circuit and brain level.

Conclusions

Studies over the past ten years have transformed the understanding of Syp function in neurons, revealing a key role in the control of Syb2 trafficking and in doing so preserving neurotransmission across and range of activity patterns. However these studies have also posed a series of new questions at both the molecular and systems level, regarding the role of Syp in both health and disease. The rapid acceleration in proteomic, systems and bioinformatics technology should ensure that many of these conundrums are resolved within the next decade.

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Conflict of interests

The author declares no conflict of interests. Michael A. Cousin is a handling editor for The Journal of Neurochemistry.

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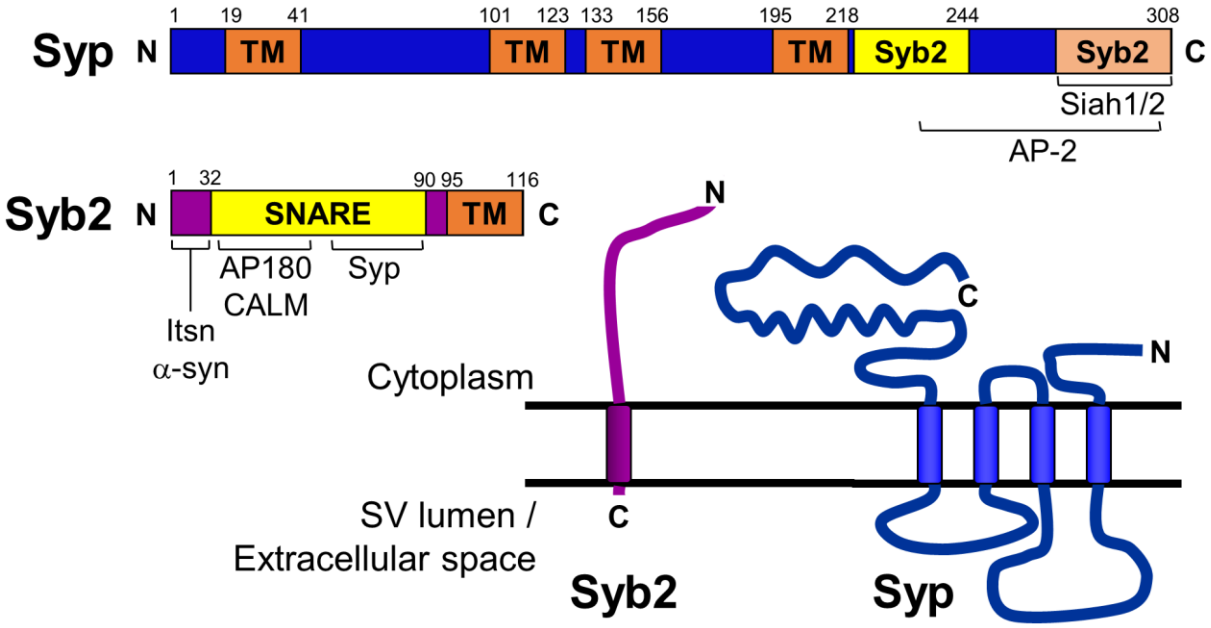

Figure 1 – *Structure and interactions of Syp and Syb2.* The primary structure of both synaptophysin (Syp) and synaptobrevin-2 (Syb2) are displayed in bar format. Transmembrane domains (TM, orange), the N- and C-termini and amino acid numbers for both proteins are indicated. **Syp** – the C-terminus contains a cryptic Syb2 binding site (yellow) with Syb2 access controlled by a region in the extreme C-terminus (light orange). Interaction sites for both adaptor protein complex-2 (AP-2) and seven in absentia homolog (Siah1/2) are displayed. **Syb2** – the extreme N-terminus (amino acids 1-32) binds either α-synuclein (α-syn) or intersectin-1 (Itsn, only as part of an assembled SNARE complex). The N-terminal region of the SNARE motif (yellow) binds adaptor protein 180 / clathrin assembly lymphoid myeloid leukaemia (AP180/CALM), whereas the C-terminal region binds Syp. An image illustrating their membrane orientation is also displayed.

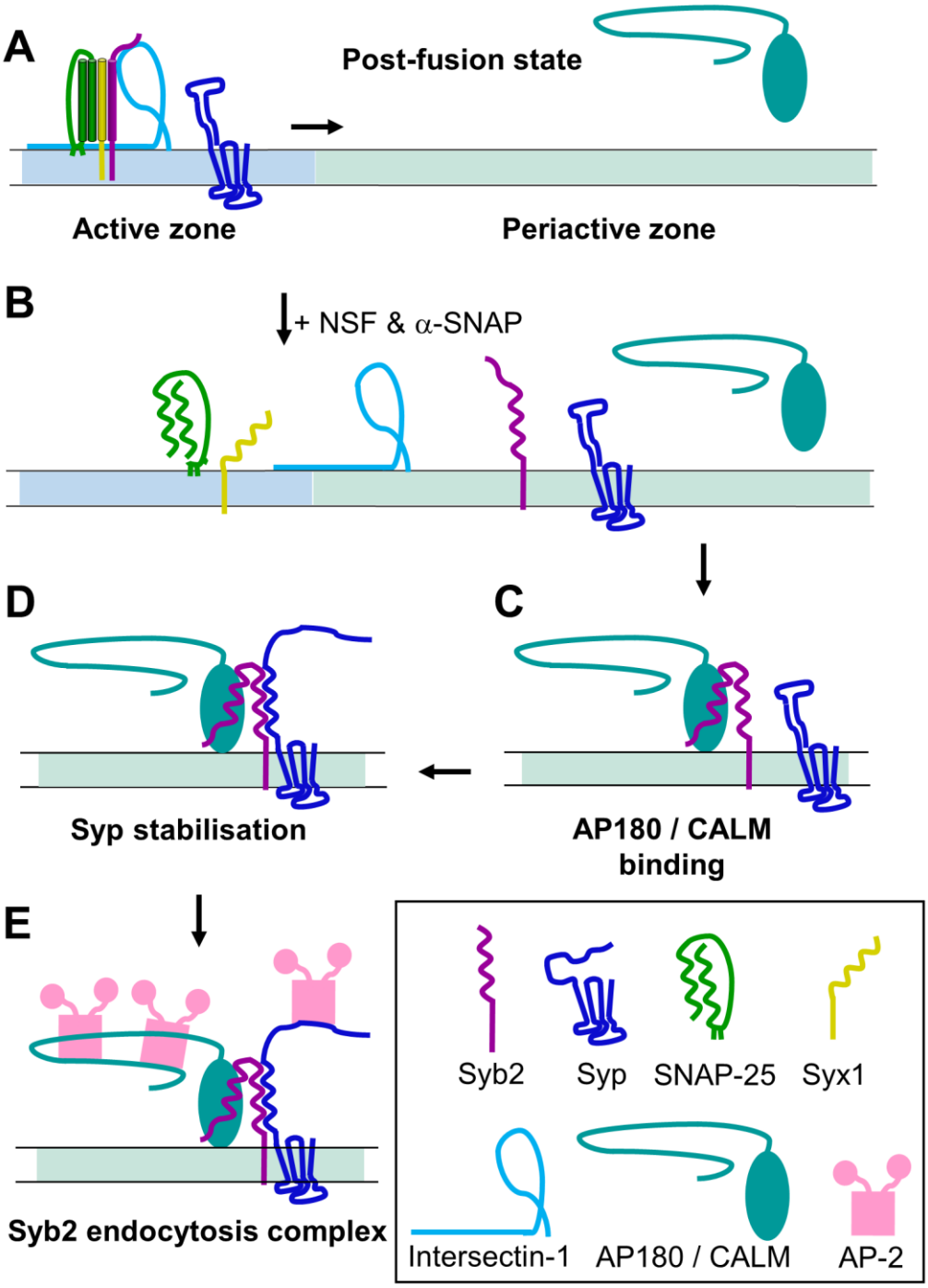

Figure 2 – *Working model of Syp-dependent Syb2 trafficking during SV recycling*. (**A**) After SV fusion, synaptobrevin-2 (Syb2, purple) is part of a cis-SNARE complex with syntaxin-1 (yellow) and synaptosomal-associated protein-25kDa (SNAP-25, green) at the presynaptic active zone (light blue). Intersectin-1 (light blue) binds to the extreme N-terminus of Syb2 and facilitates clearance of the cis-SNARE complex from the active zone to the periactive zone (light green). (**B**) The cis-SNARE complex is disassembled via the action of NEM-sensitive factor (NSF) and α-soluble NSF-attachment protein (α-SNAP), liberating Syb2, syntaxin-1 and SNAP-25. Intersectin-1 is also released via this action. During this process synaptophysin (Syp, dark blue) is inaccessible to newly liberated Syb2, due to the presence its extreme C-terminus. (**C**) Adaptor protein 180 (AP180, light green) is recruited to the plasma membrane via its AP180 N-terminal homology (ANTH) domain binding to locally

produced phosphatidylinositol (4,5) bisphosphate. AP180 then binds the N-terminal region of the SNARE motif of Syb2. (**D**) Syp stabilises the Syb2/AP180 complex by binding to the C-terminal region of the Syb2 SNARE motif. How the cryptic C-terminal interaction site is revealed is still unclear, but involves regulation via the extreme Syp C-terminus. (**E**) Adaptor protein complex-2 (AP-2) is recruited by the C-termini of both AP180 and Syp to establish the Syb2 complex as an endocytosis complex. AP180 and AP-2 assemble clathrin at this stage. The inset box provides a key for the different proteins discussed.