Supplementary Discussion

Role of endophilin and amphiphysin

The three best candidates for the phospho-binding partner of dynamin in SVE were amphiphysin I/II, endophilin I and syndapin I, all of which associate with dynamin I. Our study suggests that only the dynamin I-syndapin I interaction is phospho-regulated in vivo in the context of SVE at the synapse (our discussion is restricted to dynamin phosphorylation, not that of its partners). Firstly, only the interaction of full-length syndapin I, rather than endophilin I or amphiphysin I/II, is regulated by in vivo dynamin I phosphorylation. Secondly, phosphorylation of Ser-774 predominates in nerve terminals and this site exclusively regulates syndapin I binding. Thirdly, a peptide that blocks dynamin-syndapin binding, but not dynamin-endophilin, blocks SVE in synaptosomes. Fourthly, point mutations in Ser-774 reduce syndapin I binding and SVE in neurons, but are without effect on endophilin I. We previously excluded amphiphysin I/II as a candidate for dephosphorylation-stimulated recruitment to dynamin I\(^1\) and have now extended these observations to directly demonstrate that its association with dynamin I is not controlled by dynamin I phosphorylation. This does not undermine the importance of amphiphysin in the endocytic machine, but rather rules out any stimulus-dependent interaction with dynamin I.

Pull-downs with full-length protein vs isolated domains

Our main findings concerning dephosphorylation-dependent protein-protein interactions with dynamin I differ from previous reports due to our use of full-length or native proteins, rather than relying solely on isolated recombinant protein domains. Phosphorylation of dynamin I in vitro by Cdk5 was reported to reduce its interaction with either amphiphysin I/ GST-AmphI-SH3 domain\(^7\) or GST-EndoI-SH3 domain\(^8\). The former interaction with amphiphysin conflicts with several other reports (refs 1, 8, as well as the present study). We cannot reproduce their observation with full-length native or recombinant domains of these proteins, nor with pseudo-phosphorylation or endogenous phosphorylation and cannot account for the discrepancy. The latter report of a phospho-dependent interaction with GST-EndoI-SH3 domain with GST-DynI-PRD\(^8\) is consistent with our in vitro observations using GST-DynI-PRD phospho-mimetic mutants which inhibited binding to both syndapin I and endophilin I in vitro. However, we found that the GST-SH3 domains of endophilin I and syndapin I alone showed almost no selectivity of interaction with in vivo phosphorylated dynamin I. Using endogenous and full-length recombinant syndapin I and endophilin I, we
found that in vivo phosphorylation of dynamin I only inhibits binding to syndapin I, and not endophilin I. Therefore this apparent discrepancy probably lies in the reliance on isolated SH3 domains which exhibit increased target affinity. Protein-protein interaction studies using isolated domains of a protein may produce misleading findings compared with the full-length protein.

Role of Ser-774

Additional data suggests that endophilin I is not a phospho-regulated dynamin I partner in SVE. Firstly, endophilin I binding was regulated only by a single phosphorylation site, Ser-778 in vitro, and not at all in vivo. In intact nerve terminals, phospho-dynamin I presents as two major forms; singly phosphorylated at Ser-774 or doubly phosphorylated at Ser-774 plus Ser-778. Although we found that the doubly phosphorylated form predominates 3:1, synaptosomal Ser-774 is twice as abundant as Ser-778. We previously reported that the form of dynamin I singly phosphorylated on Ser-778 is extremely low in abundance (approximately 100-fold less than that singly phosphorylated on Ser-774) and essentially does not occur in isolation of phospho-Ser-774. Therefore we propose that phospho-Ser-774 may be the more significant form of phospho-dynamin I that contributes to specific regulation of the interaction with syndapin I. Interestingly, this site is relatively conserved in both dynamins I and III, while the equivalent amino acid to Ser-774 is absent in dynamin II. One intriguing possibility is that dynamin II may be a candidate phosphorylation-regulated binding partner for endophilin I in neurons, in addition to synaptojanin. Secondly, we found a close correlation between syndapin I binding to the two phosphorylation sites and maximal inhibition of SVE. The effect on SVE of inhibition of Ser-778 alone was partial and implies that dual dephosphorylation is required.

An apparent conflict between experiments in synaptosomes and cultured neurons.

One observation relating to the use of phosphorylation mimetics might, at first sight, suggest a conflict between the functional experiments in synaptosomes vs CGNs. The DynI<sub>769-784</sub>EE peptide did not block SVE in synaptosomes, but DynI<sup>dme</sup>-GFP inhibited SVE in transfected neurons. This probably reflects the nature of the two functional assays and differences between the delivery systems employed. The DynI<sub>769-784</sub>EE peptide was present for a short time in synaptosomes during a single stimulation of SV recycling. The experiment focussed on the events triggering the initial endocytic event. In contrast, the positive effect of DynI<sup>dme</sup>-GFP in transfected neurons was likely due to the ability of DynI<sup>dme</sup>-GFP to integrate into the endogenous pool of dynamin I tetramers over time during multiple cycles of SVE at the
synapse, as proposed for the classical DynF<sup>K44A</sup> mutant. Due to the repetitive recycling nature of exocytosis and endocytosis the Glu mutations may inhibit SVE for different reasons to the Ala mutants. The latter may produce constitutive binding of syndapin I, reducing its overall availability for SVE, whereas the Glu mutant may fail to recruit syndapin I, leading to inappropriate priming for subsequent rounds of SVE. Both cases would result in SVE arrest in neurons (rather than synaptosomes), since syndapin I could not be released (Ala) or recruited (Glu).

**Supplementary Reference List**


8. Solomaha, E., Szeto, F.L., Yousef, M.A., & Palfrey, H.C. Kinetics of SH3 domain association with the proline rich domain of dynamins: specificity, occlusion and the effects of