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Invited review

Need for speed: Super-resolving the dynamic nanoclustering of syntaxin-1 at exocytic fusion sites

Pranesh Padmanabhan[a,1], Adekunle T. Bademosi[a,1], Ravikiran Kasula[a], Elsa Lauwers[b,c], Patrik Verstreken[b,c], Frédéric A. Meunier[a,*]

[a] Clem Jones Centre for Ageing Dementia Research, Queensland Brain Institute, The University of Queensland, Queensland, Australia
[b] VIB-KU Leuven Center for Brain & Disease Research, 3000 Leuven, Belgium
[c] Department of Neurosciences and Leuven Brain Institute, KU Leuven, 3000 Leuven, Belgium

HIGHLIGHTS

- Syntaxin-1 forms nanoclusters on the plasma membrane.
- Multiple molecular mechanisms regulate syntaxin-1 nanoclustering on the plasma membrane.
- Synaptic activity, phosphoinositides and SNARE complex assembly differentially control syntaxin-1 nanoclusters.
- Super-resolution microscopy has the potential to uncover the design principles governing regulated exocytosis.

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ABSTRACT

Communication between cells relies on regulated exocytosis, a multi-step process that involves the docking, priming and fusion of vesicles with the plasma membrane, culminating in the release of neurotransmitters and hormones. Key proteins and lipids involved in exocytosis are subjected to Brownian movement and constantly switch between distinct motion states which are governed by short-lived molecular interactions. Critical biochemical reactions between exocytic proteins that occur in the confinement of nanodomains underpin the precise sequence of priming steps which leads to the fusion of vesicles. The advent of super-resolution microscopy techniques has provided the means to visualize individual molecules on the plasma membrane with high spatiotemporal resolution in live cells. These techniques are revealing a highly dynamic nature of the nanoscale organization of the exocytic machinery. In this review, we focus on soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) syntaxin-1, which mediates vesicular fusion. Syntaxin-1 is highly mobile at the plasma membrane, and its inherent speed allows fast assembly and disassembly of syntaxin-1 nanoclusters which are associated with exocytosis. We reflect on recent studies which have revealed the mechanisms regulating syntaxin-1 nanoclustering on the plasma membrane and draw inferences on the effect of synaptic activity, phosphoinositides, N-ethylmaleimide-sensitive factor (NSF), α-soluble NSF attachment protein (α-SNAP) and SNARE complex assembly on the dynamic nanoscale organization of syntaxin-1.

1. Overview

Regulated exocytosis is a fundamental biological process that is crucial for numerous cellular functions, including neurotransmission, cell migration and cell differentiation. This process is complex and entails multiple steps: 1) the translocation of secretory vesicles to the plasma membrane, 2) the docking of vesicles to the plasma membrane, 3) the priming step where the vesicle becomes fusion-competent, and

Abbreviations: SNARE, soluble N-ethylmaleimide-sensitive factor attachment receptor; NSF, N-ethylmaleimide-sensitive factor; α-SNAP, α-soluble NSF attachment protein; PC12, pheochromocytoma; NMJ, neuromuscular junction; TIRF, total internal reflection fluorescence; STED, stimulated emission depletion; dSTORM, direct stochastic optical resolution microscopy; PALM, photoactivated localization microscopy; SPT, single particle tracking; sptPALM, single particle tracking photoactivated localization microscopy; uPAINT, universal point accumulation in nanoscale topography; PtdIns(4,5)P_2, phosphatidylinositol (4,5)-biphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol (3,4,5)-biphosphate; FRAP, fluorescence recovery after bleaching; GFP, green fluorescent protein

* Corresponding author.
E-mail address: f.meunier@uq.edu.au (F.A. Meunier).
[1] These authors contributed equally.

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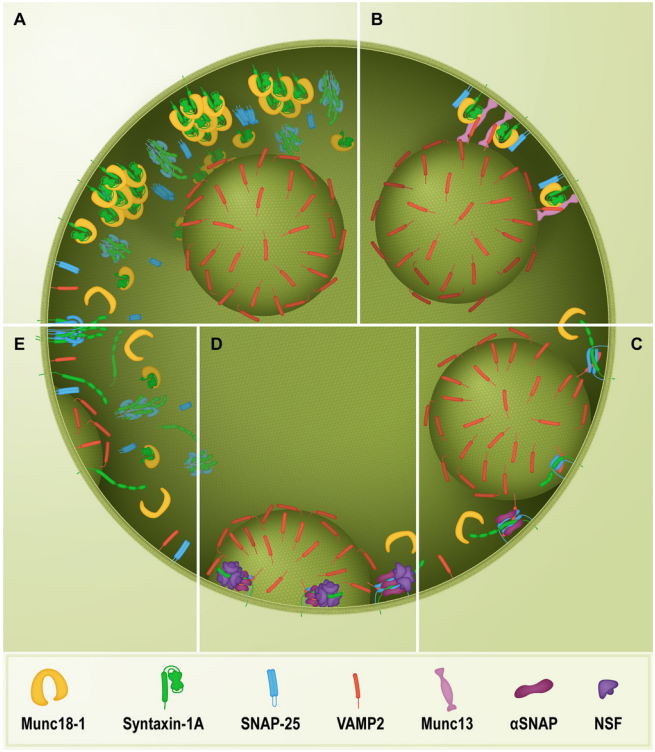

Fig. 1. Schematic of the critical molecular steps involved in vesicle fusion. (A) Vesicle approaching the plasma membrane where syntaxin-1, SNAP-25 and Munc18-1 are organized as nanoclusters. (B) Secretory vesicle in close apposition to the plasma membrane of the cell where the three SNARE proteins and priming regulators such as Munc18-1 and Munc13 mediate SNARE complex formation. (C) The initiation of SNARE complex and fusion pore formation. (D) The inclusion of αSNAP and NSF during vesicle fusion. (E) Disassembly of SNAREs and the release of proteins involved in vesicle fusion.

finally, 4) the fusion step triggered by calcium influx (Fig. 1). The soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins are central players in mediating exocytosis. The plasma membrane-associated SNAREs syntaxin-1 and SNAP-25 interact with the vesicle-associated SNARE synaptobrevin to form trans-SNARE complexes. Zippering of the SNARE complex provides the energy required to initiate vesicle fusion (Jahn et al., 2003; Rizo et al., 2006; Sudhof and Rothman, 2009). Over the past few decades, our understanding of regulated exocytosis has increased substantially with the identification of regulatory proteins and lipids involved in this process. However, the molecular mechanisms which control the events leading to vesicle fusion are not fully understood (Han et al., 2017; Jahn and Fasshauer, 2012). These events are thought to stem from a series of biochemical reactions which facilitate the docking, priming and fusion of vesicles with the plasma membrane. As molecules are subjected to Brownian motion, their availability to perform these reactions at exocytotic sites is restricted in space and time. The spatiotemporal organization of molecules into nanodomains of the plasma membrane is therefore emerging as a fundamental means to generate a confined environment with high local concentrations of the molecules which are necessary for the reactions that mediate the vesicle docking, priming and fusion steps.

Syntaxin-1 steady-state distribution is predominantly on the plasma membrane, and several studies have shown that it forms nanoclusters in this region (Bademosi et al., 2017; Kasula et al., 2016; Milovanovic and Jahn, 2015). Syntaxin-1 constantly switches between freely diffusing (mobile) and clustered (immobile) states at the plasma membrane which facilitate rapid assembly or disassembly of syntaxin-1 nanoclusters in response to stimulation. In this review, we begin by discussing our current understanding of the dynamic organization of syntaxin-1 on the plasma membrane. We describe the recent super-resolution microscopy studies which have investigated how activity, phosphoinositides, N-ethylmaleimide sensitive-factor (NSF), α-soluble NSF attachment protein (α-SNAP) and SNARE complex assembly differentially affect the dynamics and organization of syntaxin-1 in neuronal synapses and neurosecretory cells. We then discuss the mechanisms which are known to regulate syntaxin-1 clustering on the plasma membrane and present insights gained from computational modelling of syntaxin-1 dynamics on the plasma membrane.

2. Dynamic organization of syntaxin-1 on the plasma membrane

Earlier studies using conventional fluorescence microscopy revealed a spatially heterogeneous distribution of syntaxin-1 on the plasma membrane (Lang et al., 2001; Ohara-Imaizumi et al., 2004; Rickman et al., 2004). Using epifluorescence microscopy, Lang et al. (2001) found that syntaxin-1 forms clusters ~ 170 nm in diameter on plasma membrane sheets derived from pheochromocytoma (PC12) cells. Ohara-Imaizumi et al. (2004) subsequently used total internal reflection fluorescence (TIRF) microscopy to demonstrate similar syntaxin-1 clusters (~256 nm in diameter) in pancreatic β cells. Rickman et al. (2004) observed larger syntaxin-1 clusters of diameter ~ 700 nm in chromaffin cells using confocal microscopy. Furthermore, these earlier studies found that syntaxin-1 colocalizes with SNAP-25 in clusters as well as with secretory vesicles in various cells types, suggesting that syntaxin-1 clusters could act as docking and fusion sites for secretory vesicles in neuroendocrine cells (Lang et al., 2001; Ohara-Imaizumi et al., 2004; Rickman et al., 2004). However, more recent super-resolution microscopy studies have provided a much more refined picture of syntaxin-1 organization on the plasma membrane.

2.1. Nanoscale organization of syntaxin-1 on the plasma membrane

Using stimulated emission depletion (STED) super-resolution microscopy (see Box 1), Lang's group made a major advance in characterizing syntaxin-1 nanoclusters on the plasma membrane sheet derived from PC12 cells (Sieber et al., 2006, 2007). They estimated the average syntaxin-1 clusters to be ~50–60 nm in diameter, with a density of ~20 clusters per μm^2. In a subsequent study, photoactivated localization microscopy (PALM) technique (see Box1) was used to confirm the presence of syntaxin-1 nanoclusters (~50 nm in diameter) in the plasma membrane of fixed PC12 cells (Rickman et al., 2010). More recently, by applying the direct stochastic optical resolution microscopy (dSTORM) technique (see Box1), Bar-On et al. (2012) were able to provide a comprehensive quantification of various parameters of syntaxin-1 clusters in fixed PC12 cells. They found that the average diameter of a syntaxin-1 cluster is ~94 nm, that the average density is ~14 clusters/μm^2 and that these clusters are elliptical-shaped. Notably, they presented evidence for a density gradient within individual syntaxin1 clusters, with the density decreasing from the centre of the cluster to its periphery. Some of the apparent discrepancies in the estimates of cluster size between the earlier studies using conventional microscopy (Lang et al., 2001; Ohara-Imaizumi et al., 2004; Rickman et al., 2004) and the super-resolution microscopy studies (Bar-On et al., 2012; Sieber et al., 2006, 2007) could have stemmed from the diffraction-limited resolution of conventional microscopy.

More recent studies have also reported syntaxin-1 nanoclusters in neuronal synapses. A recent study using dual colour super-resolution imaging demonstrated that syntaxin-1, SNAP25 and Munc18-1 are organized into nanodomains with a diameter < 200 nm in cultured mouse hippocampal neurons (Pertsinidis et al., 2013). Another study using PALM imaging observed co-clustering of syntaxin-1 and Munc18-1 in synapses of cultured rat cortical neurons (Kavanagh et al., 2014). While a STED microscopy-based study demonstrated syntaxin-1 nanoclusters at the neuromuscular junctions (NMJs) of fixed *Drosophila* larvae and reported that these clusters were more abundant and

Box 1
A brief overview of imaging techniques.

Numerous imaging techniques have been used to investigate syntaxin-1 dynamics and organization on the plasma membrane. Here, we briefly describe different imaging techniques discussed in this review (see Maglione and Sigrist (2013); Sydor et al. (2015); and Nicovich et al. (2017) for more detailed reviews of these techniques). **Fluorescence recovery after photobleaching (FRAP)** works by selective bleaching of a fluorescently tagged protein of interest in a small region of the plasma membrane. The recovery of the fluorescence signal in the bleached region is then recorded to assess the mobility of the protein (Halemani et al., 2010; Merklinger et al., 2017; Ribrault et al., 2011; Sieber et al., 2007; Zilly et al., 2011). **Stimulated emission depletion (STED) microscopy** works by overlapping a doughnut-shaped beam with the excitation beam to selectively switch off fluorescent proteins with the exception of those at the centre of the doughnut. This technique allows for the quantification of the size distribution of protein clusters on the plasma membrane (Klar and Hell, 1999; Sieber et al., 2006; Sieber et al., 2007; van den Bogaart et al., 2011). **Single-molecule localization microscopy (SMLM)** techniques stochastically activate a small subset of photoswitchable fluorescent probes at any given time. The centroid of the point spread function of detected fluorescent probes is used to determine the position of the protein of interest with a precision of ~10–50 nm. The SMLM techniques that have been used to study syntaxin-1 are as follows: **Photoactivated localization microscopy (PALM)** uses photo-switchable fluorescent proteins such as mEos2 and Dendra2. PALM allows for the characterization of the size and distribution of protein clusters in fixed and live cells and allows single particle tracking (sptPALM) in live cells (Bademosi et al., 2017, 2018a, 2018b; Manley et al., 2008; Vanhauwaert et al., 2017; Yang et al., 2012). Unlike PALM, **stochastic optical reconstruction microscopy (STORM)** uses photoswitchable organic fluorophores to immunolabel endogenous proteins (Bar-On et al., 2012; Rust et al., 2006). In **universal point accumulation in nanoscale topography (uPAINT),** a low concentration of fluorescently labelled ligand is added to cells, and the protein of interest is tracked when the fluorescently labelled ligand in the extracellular milieu binds to the protein on the membrane (Giannone et al., 2010; Kasula et al., 2016). It is an excellent imaging technique for tracking the dynamics of endogenous membrane proteins.

While super-resolution imaging techniques have started to yield quantitative insights into the organization of syntaxin-1 and other exocytic proteins on the plasma membrane, the potential pitfalls in using these techniques should be avoided to ensure accurate data interpretation. Here, we briefly outline potential sources of artefacts (see Durisic et al. (2014); Endesfelder and Heilemann (2014); Lambert and Waters (2017)) for comprehensive reviews on this topic). It is common practice in imaging studies to transiently overexpress fluorescently tagged protein of interest. Overexpression could alter the cellular localization and even the function of the overexpressed protein (Gibson et al., 2013), and imaging overexpressed fluorescently tagged proteins could lead to an under- or over-estimation of nanocluster size and molecular density within nanoclusters. One possible way to overcome overexpression artefacts is to use genome editing to label endogenous proteins with fluorescent proteins (Xia et al., 2016). The fluorescent tags such as GFP and mEos2 could themselves cause undesired protein clustering and altered protein organization on the plasma membrane (Wang et al., 2014). This limitation could possibly be overcome by validating the findings using different fluorescent tags. Antibodies can be used to localize endogenous proteins. However, due to bivalency, antibodies could lead to cross-linking and artificial clustering of proteins. This could be prevented by developing and using small-sized monovalent nanobodies for super-resolution imaging (Maidorn et al., 2019; Ries et al., 2012). Due to blinking and reactivation of photoactivatable fluorophores, the same fluorophore can appear multiple time during imaging leading to overcounting and artificial clustering (Annibale et al., 2011; Durisic et al., 2014; Endesfelder and Heilemann, 2014). The overcounting could be corrected using appropriate computational tools and appropriate controls (Coltharp et al., 2014). Sample preparations and fixation methods could affect the protein organization on the plasma membrane (Richter et al., 2018; Tanaka et al., 2010; Whelan and Bell, 2015), so careful validation of protocols is essential to avoid fixation artefacts. In summary, reliable super-resolution microscopy studies require paying careful attention to choosing the appropriate fluorescent tags, optimising the sample preparation and fixation protocols, using the right image acquisition parameters, correcting for undercounting and overcounting, and analysing the data with suitable computational tools. It is therefore advisable to use independent super-resolution techniques to quantify nanoclustering parameters of proteins of interest.

relatively larger in size (diameter > 80 nm) at active zones (Ullrich et al., 2015), a PALM-based study provided further evidence for syntaxin-1 nanocluster organization at the NMJs of fixed *Drosophila* larvae (Bademosi et al., 2017). The diameter of these clusters estimated in the latter study was relatively large (~100 nm in radius) but decreased significantly following thermogenetic or optogenetic stimulation, suggesting synaptic activity-dependent dynamic reorganization and release of syntaxin-1 molecules from nanoclusters. Using nanobodies specifically targeting syntaxin-1 and SNAP-25, a STED microscopy-based study reported that these two molecules form clusters in the extrasynaptic regions and that the extrasynaptic population of syntaxin-1 relocates to the synapses upon stimulation in hippocampal cultured neurons (Maidorn et al., 2019).

Overall, these studies established the existence of nanometre-sized syntaxin-1 clusters on the plasma membrane. These clusters have different sizes, and it is unclear what causes such variations in morphology. It is likely that a combination of factors, including cell-type specificity, super-resolution imaging techniques, fluorescent probes and analysis tools, could contribute to such variations. More recent research has shed light on the dynamic nature of the syntaxin-1 nanoclusters, which we review next.

2.2. Nanoscale dynamics of syntaxin-1 on the plasma membrane

Using the fluorescence recovery after bleaching (FRAP) technique (see Box 1), Sieber et al. (2007) assessed the mobility of wild-type and mutant forms and deletion constructs of syntaxin-1 in PC12 cells. They found that a weak homophilic interaction between syntaxin-1 molecules involving the SNARE motif is sufficient for syntaxin-1 clustering on the plasma membrane and that there is a dynamic equilibrium between free and clustered subpopulations of syntaxin-1 molecules. Brownian dynamics simulations of self-interacting syntaxin-1 molecules further confirmed these findings (see Section 5). Subsequent studies which tracked individual syntaxin-1 molecules tagged with green fluorescent protein (GFP) using TIRF microscopy provided further evidence for constant exchange between freely diffusing and clustered subpopulations of syntaxin-1 in PC12 cells (Gandasi and Barg, 2014; Knowles et al., 2010). Syntaxin-1 clusters have also been observed to assemble and disassemble at sites of docked secretory vesicles (Barg et al., 2010; Gandasi and Barg, 2014).

Combining FRAP and single particle tracking (SPT) experiments with computational modelling, an elegant study investigated syntaxin-1 mobility in cultured rat spinal neurons (Ribrault et al., 2011). The

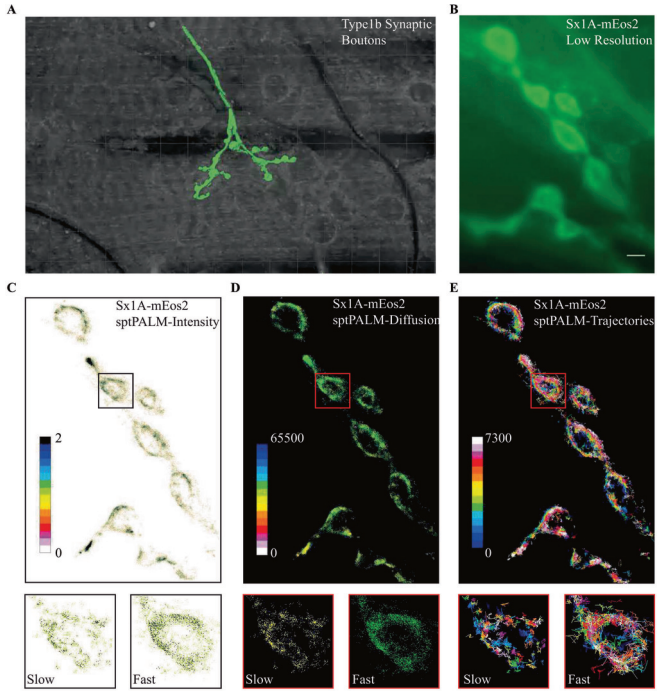

Fig. 2. Single molecule imaging of syntaxin-1A-mEos2 in the NMJs of live *Drosophila* larvae. (A) Type 1b synaptic boutons embedded in muscle 6 of the second abdominal segment of the larvae were used for syntaxin1A-mEos2 imaging, (B) Low-resolution image of NMJs of live *Drosophila* third instar larvae expressing syntaxin-1A-mEos2. (C–E) The average intensity, diffusion coefficient and trajectory maps of syntaxin-1A-mEos2 acquired using the sptPALM technique. The scale bar is 5 µm. Inset: average intensity, diffusion coefficient and trajectory maps showing slow and fast populations of syntaxin-1A-mEos2 on the presynaptic membrane. This figure is reproduced with permission from Bademosi et al. (2017).

authors showed that syntaxin-1 rapidly exchanged between synaptic and extra-synaptic regions and that syntaxin-1 mobility was significantly lower in the former regions. Importantly, syntaxin-1 exhibited relatively more frequent pause events at these synaptic sites, and perturbations of the interactions between syntaxin-1 and its partners altered syntaxin-1 mobility and the frequency of pause events. The authors used a reaction-diffusion kinetics-based model to estimate kinetic parameters of the interactions between syntaxin-1 and its partners (see Section 5). The model predicted that, due to region-specific variations in kinetic rate constants, the fraction of bound syntaxin-1 molecules was higher in synaptic regions than in extrasynaptic regions. Further, the deletion of the syntaxin-1 SNARE domain and the enzymatic cleavage of SNAP-25 decreased the values of affinity constants, leading to increased syntaxin-1 mobility and reduced frequency of pausing events. Recently, similar mobility patterns of syntaxin-1 were observed in synaptic and extrasynaptic regions of cultured rat hippocampal neurons (Schneider et al., 2015). Together these studies demonstrate that the interactions between syntaxin-1 and its partners affect syntaxin-1 mobility in synapses.

3. Stimulation differentially regulates the dynamic organization of syntaxin-1 in neurosecretory cells and neurons

Although the same molecular players appear to regulate exocytosis in different cells, the kinetics of exocytosis vary widely across cell types. For instance, the characteristic timescale of calcium-triggered synaptic vesicle exocytosis in neuronal synapses is ~0.1–1 ms, whereas that of calcium-triggered secretory vesicle exocytosis in PC12 cells is ~8–160 s (Martin, 2003). One of the main morphological differences between nerve terminals and neurosecretory cells is the abundance of pre-docked vesicles. Whereas presynaptic active zones are filled with docked vesicles, there is a paucity of pre-docked vesicles in PC12 cells (Li et al., 2018). It is therefore conceivable that, in the presynaptic membrane, the exocytic molecular machinery and fusion sites are pre-assembled to cater for fast neurotransmitter release, whereas, in neurosecretory cells, the exocytic machinery needs to be assembled to generate *de novo* fusion sites in response to stimulation.

3.1. Syntaxin-1 dynamics in neurosecretory cells

As discussed earlier, syntaxin-1 clustering on the plasma membrane is critical for secretory vesicle docking and priming in PC12 cells. However, whether activity-dependent secretory vesicle release affects the nanocluster organization of syntaxin-1 is not well understood. To address this, Kasula et al. (2016) imaged syntaxin-1-GFP using universal point accumulation in nanoscale topography (uPAINT) and Munc18-1-mEos2 using sptPALM in PC12 cells engineered to knock down Munc18-1/2 (DKD-PC12 cells). First, using dual colour super-resolution imaging, the authors demonstrated a partial overlap between syntaxin-1-GFP and Munc18-1-mEos2 nanodomains in fixed DKD-PC12 cells. Interestingly, upon acute stimulation with barium, which triggers exocytosis, the mobility of Munc18-1-mEos2 increased in live DKD-PC12 cells. Although barium stimulation did not affect syntaxin-1-GFP mobility in DKD-PC12 cells, syntaxin-1-GFP mobility decreased significantly in DKD-PC12 cells in which wild-type Munc18-1 was re-expressed. These results suggest that syntaxin-1 is recruited to and Munc18-1 is released from nanoclusters in an activity-dependent manner, as the extent of mobility reflects the fraction of molecules trapped in clusters. The domain 3a of Munc18-1 is essential for priming vesicle prior to fusion (Martin et al., 2013). Intriguingly, there was no activity-dependent change in syntaxin-1-GFP mobility in DKD-PC12 cells expressing a priming-deficient Munc18-1 mutant lacking 17 residues of domain 3a hinge-loop, suggesting that the Munc18-1 hinge loop is involved in the release of Munc18-1 and the trapping of syntaxin-1 in nanoclusters. Furthermore, upon expression of botulinum neurotoxin type E, which prevents SNARE complex formation, the activity-dependent change in the mobility of syntaxin-1-GFP was blocked. Together, these results suggest that a conformational change in the Munc18-1 hinge loop controls the activity-dependent nanoscale reorganization of syntaxin-1 and Munc18-1 and SNARE complex formation in PC12 cells.

3.2. Syntaxin-1 dynamics in neuronal synapses

Syntaxin-1 is organized as nanoclusters in the NMJs of *Drosophila* larvae (Bademosi et al., 2017; Ullrich et al., 2015). To understand how activity-dependent neurotransmitter release alters the nanocluster organization of syntaxin-1 in the presynaptic membrane, Bademosi et al. (2017, 2018a, 2018b) developed a new protocol to image syntaxin-1-mEos2 in the NMJs of fixed and live third instar *Drosophila* larvae (Fig. 2). Both thermogenetic and optogenetic stimulation raised the ongoing presynaptic activity, increased the mobility of syntaxin-1-mEos2, and decreased the cluster size and number of syntaxin-1 molecules in nanoclusters. These observations suggest that syntaxin-1 molecules are released from the pre-existing nanoclusters during activity-dependent neurotransmitter release at NMJs, and raise the question of what causes syntaxin-1 to pre-exist in nanoclusters at the basal activity levels.

It is known that motor nerve terminals contain a large number of docked and primed synaptic vesicles (Couteaux and Pecot-Dechavassine, 1970) and that syntaxin-1 clusters at the vesicle docking sites (Barg et al., 2010; Gandasi and Barg, 2014). One possibility therefore is that these docked and primed synaptic vesicles trap syntaxin-1 into nanoclusters at basal activity levels. By expressing the light chain tetanus toxin to prevent SNARE complex formation, Bademosi et al. (2017) observed an increase in syntaxin-1 mobility and a decrease

in the size of syntaxin-1 nanoclusters at the NMJs, akin to activity-dependent changes in syntaxin-1 dynamics and organization. Furthermore, by using a *Drosophila* NSF mutant (comatose), the authors showed that syntaxin-1 mobility decreased by preventing SNARE complex disassembly.

Phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P_3) is required for syntaxin-1 clustering at the NMJs (Khuong et al., 2013). Bademosi et al. (2017) found that PtdIns(3,4,5)P_3 binding-defective mutant syntaxin-1A^{KARRAA}-mEos2 had higher mobility compared to the wild-type syntaxin-1-mEos2 at NMJs, and thermogenetic stimulation did not alter the mobility of syntaxin-1A^{KARRAA}-mEos2. These observations provide evidence that PtdIns(3,4,5)P_3 promote syntaxin-1 clustering, that syntaxin-1 nanoclusters contain pre-assembled SNARE complexes, and that presynaptic activity leads to SNARE complex disassembly and the release of syntaxin-1 from nanoclusters at NMJs of *Drosophila* larvae.

4. Factors that regulate syntaxin-1 clustering on the plasma membrane

Numerous other factors have been shown to control syntaxin-1 clustering on the plasma membrane (Milovanovic and Jahn, 2015). Earlier studies identified cholesterol as a critical regulator of syntaxin-1 cluster stability, as cholesterol depletion from the plasma membrane significantly reduced syntaxin-1 clustering in the plasma membrane sheet derived from PC12 cells and pancreatic β cells (Lang et al., 2001; Ohara-Imaizumi et al., 2004). A recent study found that cholesterol controls the stability of many different protein assemblies on the plasma membrane (Saka et al., 2014). It is therefore unclear whether cholesterol has a direct or an indirect effect on syntaxin-1 clustering. Milovanovic et al. (2015) found that changes in cholesterol levels affect the membrane thickness and thereby affect local hydrophobic mismatch in the membrane. Hydrophobic mismatch, which occurs when the length of the transmembrane domain region of a membrane protein and the hydrophobic thickness of the membrane are different, affects protein conformation, folding, activity and clustering (Killian, 1998). Cholesterol-dependent changes in the hydrophobic mismatch in the local membrane could be one possible mechanism by which cholesterol regulates syntaxin-1 clustering. Interestingly, the transmembrane domain of syntaxin-1 is slightly shorter than that of syntaxin-4. The authors further showed that this difference is sufficient to segregate the two proteins into different clusters.

Apart from cholesterol, protein-protein interactions play a critical role in regulating syntaxin-1 clustering on the plasma membrane (Sieber et al., 2006, 2007). Although the transmembrane domain region of syntaxin-1 alone can form clusters on the plasma membrane (Sieber et al., 2006), the homophilic protein-protein interactions involving the SNARE motif are required to trap syntaxin-1 molecules into nanoclusters (Sieber et al., 2007). Merklinger et al. (2017) recently proposed that the transmembrane domain promotes loose clustering of syntaxin-1, whereas the SNARE motif of the cytoplasmic domain facilitates the tight packing of molecules within the clusters.

Multiple lines of evidence suggest that phosphoinositides play a critical role in syntaxin-1 clustering, with phosphatidylinositol (4,5)-biphosphate (PtdIns(4,5)P_2) being concentrated at the docking sites of secretory vesicles (Aoyagi et al., 2005; Laux et al., 2000). Syntaxin-1 and PtdIns(4,5)P_2 are known to associate with each other in different model systems (Murray and Tamm, 2009; Murray and Tamm, 2011; van den Bogaart et al., 2011). The electrostatic interaction between PtdIns(4,5)P_2 and syntaxin-1 is sufficient for syntaxin-1 clustering in PC12 cells (van den Bogaart et al., 2011). Micromolar concentrations of calcium ions have also been shown to increase syntaxin-1 cluster size in these cells (Milovanovic et al., 2016; Zilly et al., 2011). Milovanovic et al. (2016) suggested that calcium could act as a charge bridge linking multiple complexes of syntaxin-1 and PtdIns(4,5)P_2 to promote syntaxin-1 clustering. More recently the electrostatic interaction between PtdIns(3,4,5)P_3 and syntaxin-1 was shown to be necessary for syntaxin-1 clustering and neurotransmitter release at the NMJs of *Drosophila* larvae. Consistent with these findings, Bademosi et al. (2017) found that the cluster size of PtdIns(3,4,5)P_3 binding-defective mutant syntaxin-1A^{KARRAA}-mEos2 was smaller than the wild-type syntaxin-1A-mEos2 in the NMJs of *Drosophila* larvae and did not change with thermogenetic stimulation.

Propofol is one of the most commonly used general anesthetics in humans. A recent study found that a clinically relevant concentration of propofol restricts the mobility of syntaxin-1 and increases nanoclustering of syntaxin-1 molecules on the plasma membrane of PC12 cells and NMJs of *Drosophila* larvae (Bademosi et al., 2018b). Interestingly, preventing the interaction between syntaxin-1 and SNAP-25 and SNARE assembly blocked this effect, suggesting that propofol can interfere with the building up of a release site and render cells fusion incompetent. Whether propofol-induced syntaxin1A confinement to nanoclusters is a cause or a consequence of impaired neurotransmission remains to be investigated. This study also demonstrates that single-molecule imaging could be used for screening small molecules, as recently proposed (Beghin et al., 2017).

Overall, the studies discussed above show that cholesterol, protein-protein interactions, electrostatic protein-lipid interactions, hydrophobic mismatch, SNARE complex assembly and perhaps additional as yet unknown factors collectively modulate the dynamic organization of syntaxin-1 on the plasma membrane. Future work will focus on understanding how these multiple mechanisms act in concert to regulate syntaxin-1 clustering and the exocytic machinery.

5. Computational models of syntaxin-1 dynamics on the plasma membrane

Computational models have helped to advance our understanding of the mechanisms underlying syntaxin-1 clustering and to interpret experiments investigating syntaxin-1 dynamics on the plasma membrane (Bademosi et al., 2017; Bar-On et al., 2012; Ribrault et al., 2011; Sieber et al., 2007; Ullrich et al., 2015; van den Bogaart et al., 2011). Sieber et al. (2007) constructed a model in which weak attraction and a cluster size-dependent increase in repulsion between molecules regulate syntaxin-1 clustering on the plasma membrane. In this model, the relative strengths of the attractive and repulsive forces determine the fractions of syntaxin-1 molecules that are freely diffusing or trapped in clusters. The model predictions quantitatively described FRAP-based measurements of reaction-diffusion kinetics of syntaxin-1 in live PC12 cells. The model predicted that syntaxin-1 clusters contain an average of 75 molecules and about 16% of total syntaxin-1 molecules is freely diffusing on the plasma membrane. However, recent SPT experiments have reported a much larger percentage of free syntaxin-1 molecules at neuronal synapses (Ribrault et al., 2011; Schneider et al., 2015), at the NMJs (Bademosi et al., 2017) and in PC12 cells (Kasula et al., 2016). The observed differences in the percentage of free syntaxin-1 molecules could stem from the different imaging techniques and experimental conditions used across studies. Ullrich et al. (2015) considered a similar model where syntaxin-1 dynamics is governed by attractive and repulsive forces between these molecules. One prediction was that altering the interaction energy of these forces on the order of $\sim$1 K_BT, where K_B is the Boltzmann constant and T is the temperature, could drive the plasma membrane from a state with no clusters to a state where all syntaxin-1 molecules are clustered. The model captured the distribution of syntaxin-1 cluster size in the NMJs of fixed *Drosophila* larvae measured using STED and suggested that the observed differences in the size distribution of syntaxin-1 clusters between active zones and other regions could be due to subtle region-specific differences in the interaction energies between syntaxin-1 molecules. Using Monte Carlo-based simulations, Bar-On et al. (2012) investigated the influence of syntaxin-1 clustering on *cis*-SNARE complex formation. This model predicted that syntaxin-1 clustering reduces the extent of the *cis*-SNARE

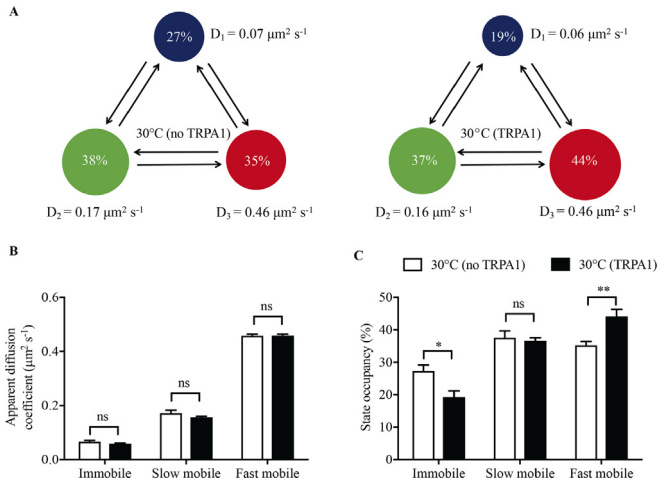

Fig. 3. Hidden Markov modelling reveals distinct diffusive states of syntaxin-1A-mEos2 molecules at the NMJs of live *Drosophila* larvae. (A) A three-state hidden Markov model and the parameters inferred by variational Bayes single-particle tracking analysis of trajectories from NMJs of live *Drosophila* larvae at 30 °C without dTRPA1 expression (left) and with dTRPA1 expression (right). Circles represent the different diffusive states. (B–C) The apparent diffusion coefficients (B) and the state occupancies (C) inferred by analysing trajectories from NMJs without (white bars) and with (black bars) the expression of dTRPA1 at 30 °C. This figure is adapted with permission from Bademosi et al. (2017).

complex formation. Coarse-grained molecular dynamics simulations have also been used to examine the interactions between syntaxin-1 and PtdIns(4,5)P_2 in nanodomains (van den Bogaart et al., 2011) and the mechanism of fusion pore formation (Sharma and Lindau, 2018).

Computational models have helped to extract the kinetic parameters of molecular interactions from SPT experiments. As discussed in section 2, Ribrault et al. (2011) revealed that freely diffusing syntaxin-1 displayed intermittent short and long pause events in cultured rat spinal neurons. They developed a three-state model, with one state representing the freely diffusing syntaxin-1 molecules and the other two states representing syntaxin-1 molecules bound to their partners. The binding and unbinding reaction rate constants determined the transitions between these three states. By constraining the model with FRAP and SPT experiments, the authors estimated the kinetic reaction rate constants and concluded that the interactions between syntaxin-1 and its partners underlie the diverse syntaxin-1 motion patterns observed in their experiments. Recently, Bademosi et al. (2017) employed a three-state hidden Markov model, with each state distinguished by its diffusion coefficient, to analyse the single particle trajectories of syntaxin-1 in the NMJs of live *Drosophila* larvae (Fig. 3). Individual syntaxin-1 molecules stochastically switched between at least three diffusive states: a slow diffusive state with a diffusion coefficient of $\sim 0.07\ \mu m^2\ s^{-1}$, an intermediate state with a diffusion coefficient of $\sim 0.17\ \mu m^2\ s^{-1}$ and a fast-diffusive state with a diffusion coefficient of $\sim 0.46\ \mu m^2\ s^{-1}$ (Fig. 3). Syntaxin-1 molecules trapped in clusters would be expected to have a lower diffusion coefficient than freely diffusing molecules. The observed diffusion-state switching behaviour is therefore suggestive of dynamic exchange between free and clustered states of syntaxin-1 at NMJs. The authors then investigated how synaptic activity affects syntaxin-1 state occupancy, which reflects the amount of time molecules spend in the respective diffusive state. Raising synaptic activity by thermogenetic neuronal stimulation substantially increased syntaxin-1 state occupancy in the fast-diffusive state and decreased its occupancy in the slow diffusive state (Fig. 3). These results suggest that syntaxin-1 molecules are released from clusters during stimulation of neurotransmitter release at the NMJs of *Drosophila* larvae, and that neuronal activity modulates the exchange rates between free and clustered subpopulations of syntaxin-1.

It is apparent from the work described above that computational models can play a vital role in shaping our understanding of the working principles of the exocytic machinery. Future modelling efforts will focus on identifying properties of the molecular interaction network that could potentially link syntaxin-1 clustering, SNARE complex formation and regulated exocytosis.

6. Concluding remarks

Complex formation between SNAREs on the plasma membrane and the vesicle membrane is critical for exocytosis. Although key proteins regulating SNARE complex assembly and exocytosis have been identified (Jahn and Fasshauer, 2012), a conceptual framework describing the temporal sequence of molecular interactions involved in vesicle docking, priming and fusion leading to exocytosis is still lacking. As discussed in this review, super-resolution imaging techniques have provided critical insights into the nanoscale organization and dynamics of syntaxin-1 in the plasma membrane of neurosecretory cells and neurons. It is becoming clear that activity alters the dynamics and molecular composition of syntaxin-1 nanoclusters on the plasma membrane (Bademosi et al., 2017; Kasula et al., 2016). Future work will focus on elucidating the functional link between the dynamics and molecular composition of individual syntaxin-1 nanoclusters and vesicle docking, priming and fusion events. Other SNAREs and regulatory proteins are also known to form nanoclusters in the plasma membrane. By applying super-resolution imaging approaches to study their dynamic nanoscale organization and vesicle dynamics (Joensuu et al., 2016, 2017), a more comprehensive picture of the dynamics of the exocytic molecular machinery will be developed, thereby revealing the principles that govern regulated exocytosis.

Author contributions

PP, ATB, RK, EL, PV, and FAM wrote the paper.

Conflicts of interest

The authors declare no conflict of interest.

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