MOLECULAR NANOMACHINES OF THE PRESYNAPTIC TERMINAL

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MOLECULAR NANOMACHINES OF THE PRESYNAPTIC TERMINAL

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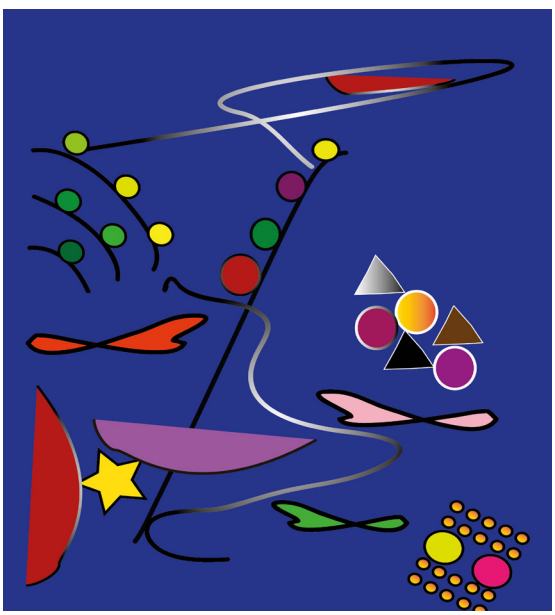

Synaptic Nanomachines during a Midsummer Night's Dream Image by Lucia Tabares

Synaptic transmission is the basis of neuronal communication and is thus the most important element in brain functions, ranging from sensory input to information processing. Changes in synaptic transmission can result in the formation or dissolution of memories, and can equally lead to neurological and psychiatric disorders. The proteins composing the synapse, and their respective functions, are getting increasingly known. One aspect that has become evident in the last years is that most synaptic functions are performed not by single proteins, but by highly organized multi-protein machineries, which interact dynamically to provide responses optimally suited to the needs of the neuronal network. To decipher synaptic and neuronal function, it is essential to understand the organisational, morphological and functional aspects of the molecular nanomachines that operate at the synapse. We discuss these aspects in 11 different chapters, focusing on the structure and function of the active zone, on the functional anatomy of the synaptic vesicle, and on some of the best known soluble protein complexes from the synapse, including those involved in endocytosis and vesicle recycling.

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EDITORIAL
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Editorial: Molecular Nanomachines of the Presynaptic Terminal

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Keywords: synapse, neurotransmitter release, active zone, nanomachine, synaptic organization

The Editorial on the Research Topic

Molecular Nanomachines of the Presynaptic Terminal

Cell biology and neurobiology have started many decades ago as attempts to unravel the complexity of cellular processes, by studying different elements, from membranes and organelles to molecules in isolation. Through ever increasing technological improvements, a huge amount of information has accumulated on the molecular organization of the cell, and on the function of the individual molecules. On the basis of this information, we can now switch the general aim of cell biology from the investigation of single elements to the global understanding of cellular function, to a level that will enable us to predict its behavior.

We are not quite there yet, but we are getting close for one compartment of the cell, which is sufficiently well understood at the level of the individual cellular machineries: the synapse, and especially the presynaptic bouton. The bouton is small, contains a limited subset of proteins and organelles, and has a single major function: neurotransmitter release. The molecules and organelles arrange themselves in complexes, or nanomachines, concerned with a handful of critical functional steps, from synaptic vesicle fusion (exocytosis), to vesicle reformation, retrieval (endocytosis), and refilling with neurotransmitter. This Research Topic presents an overview of the most important nanomachines of the presynaptic bouton. Their function and regulation are presented in a level of detail that surpasses that available for most other cellular structures.

Perhaps the most complex, and the most organized machinery of the presynaptic bouton is the active zone, the electron-dense area where synaptic vesicles dock and fuse. This is the subject for two of our chapters, focusing on scaffolding proteins that are essential for this structure (Gundelfinger et al.), and on its plasticity (Kittel and Heckmann). The active zone is probably also linked to the periactive zone, an area where the recently exocytosed synaptic vesicles may prepare for endocytosis. The organization of the periactive zone is far less well understood than that of the active zone, and is here discussed by Cano and Tabares.

The interaction of the active zone with the synaptic vesicles is one of the factors determining the probability with which the latter release neurotransmitter. This is discussed at length by Körber and Kuner. Albeit most of the molecules involved in neurotransmitter release, and especially the fusion SNARE proteins, have been known for several decades, they can still offer surprises, especially when they present function that are not related to exocytosis. This is presented in detail by Michela Matteoli and colleagues, focusing on the most abundant of the SNAREs, SNAP-25 (Antonucci et al.).

Exocytosis needs to be balanced by endocytosis, to ensure the long-term function of the synapses. The molecules involved in endocytosis have been studied for many years, just as the SNAREs, albeit many points are still open. For example, it is still unclear whether synaptic vesicles maintain their composition after exocytosis, as a patch of molecules in the plasma membrane, or whether their components diffuse and intermix with other plasma membrane molecules. The first

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scenario predicts that one synaptic vesicle will maintain its protein composition, and thereby its identity, for long time periods, not only of a few minutes, but of hours, through many rounds of synaptic vesicle recycling. The second scenario predicts that the synaptic vesicle composition is far more fluid, and will change on a timescale of minutes. The key to this question, which is still open, probably lies within the molecules that interact with the vesicle proteins after exocytosis, and may prevent their diffusion in the plasma membrane. Gordon and Cousin term such molecules intrinsic trafficking partners or iTRAPs. Furthermore, additional nanomachines involved in the fate of the vesicle are discussed by Fassio et al.

After endocytosis, the synaptic vesicle is re-filled with neurotransmitter. This process is far more complex than, for example, SNARE-mediated exocytosis, albeit the regulation of the latter through various proteins, including the active zone components, may be even more complex than neurotransmitter uptake into vesicles. The molecules involved in this process, and their relation to the amount of neurotransmitter loaded into individual vesicles, are presented by Takamori. One hypothesis that was once shunned, but increasingly wins acceptance, is that specific subsets of neurons may contain vesicles loaded with different small transmitters, thereby resulting in more complex signaling than previously thought. A timely review of this subject is given by Ahnert-Hilger et al. (Münster-Wandowski).

None of the synaptic functions discussed so far could be performed without a steady flow of proteins and organelles to and from the synapse, to ensure the replacement of old and damaged components with fresh ones. This subject, which is surprisingly less well-known than many other synaptic aspects, is reviewed by Yagensky et al.

Finally, components of nanomachines can also go wrong, and result in synaptic damage. This is most evident for the amyloid precursor protein, APP, which has been for many years known as the main components of plaques in Alzheimer's Disease. Its physiological and pathophysiological roles are reviewed here by Walter Volknandt and colleagues (Laßek et al.), focusing on the interactions of APP with other nanomachines discussed in this Research Topic.

Overall, this Research Topic therefore provides a timely look at some of the machines composing one of the best-understood compartments of the cell, the synaptic bouton. We hope that the different chapters will spark further quantitative work on the functional assemblies described here, and will also encourage research into nanomachines that are still poorly understood.

AUTHOR CONTRIBUTIONS

Both authors wrote the manuscript together, and approved it for publication.

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REVIEW
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Role of Bassoon and Piccolo in Assembly and Molecular Organization of the Active Zone

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Bassoon and Piccolo are two very large scaffolding proteins of the cytomatrix assembled at the active zone (CAZ) where neurotransmitter is released. They share regions of high sequence similarity distributed along their entire length and seem to share both overlapping and distinct functions in organizing the CAZ. Here, we survey our present knowledge on protein-protein interactions and recent progress in understanding of molecular functions of these two giant proteins. These include roles in the assembly of active zones (AZ), the localization of voltage-gated Ca^{2+} channels (VGCCs) in the vicinity of release sites, synaptic vesicle (SV) priming and in the case of Piccolo, a role in the dynamic assembly of the actin cytoskeleton. Piccolo and Bassoon are also important for the maintenance of presynaptic structure and function, as well as for the assembly of CAZ specializations such as synaptic ribbons. Recent findings suggest that they are also involved in the regulation activity-dependent communication between presynaptic boutons and the neuronal nucleus. Together these observations suggest that Bassoon and Piccolo use their modular structure to organize super-molecular complexes essential for various aspects of presynaptic function.

Keywords: Bassoon, Piccolo, Aczonin, cytomatrix at the active zone, neurotransmitter release, synapto-nuclear signaling, actin dynamics, synaptic vesicle

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INTRODUCTION

Synapses are sophisticated cellular devices designed for the efficient and rapid communication between neurons via the regulated release of neurotransmitter substances from presynaptic boutons and their detection by postsynaptic receptor systems. The release of neurotransmitters is mediated by the recruitment and fusion of synaptic vesicles (SVs) at specialized regions of the presynaptic plasma membrane called active zones (AZ). The molecular and ultra-structural characterization of these release sites revealed that they are composed of a dense cytomatrix assembled at the AZ (CAZ). This matrix is organized by a small number of multi-domain proteins, including Munc13s, Rab3-interacting molecules (RIMs), RIM-binding proteins (RBPs), Liprins-α, ELKS2/CAST as well as the two large scaffolding proteins Bassoon and Piccolo (aka Aczonin; **Figure 1**) (Garner et al., 2000; Schoch and Gundelfinger, 2006; Gundelfinger and Fejtova, 2012; Südhof, 2012; Ackermann et al., 2015). This review will focus on the contributions of Bassoon and Piccolo to the molecular and functional organization of the CAZ.

Bassoon and Piccolo were originally discovered in a screen designed to identify structural components of rat brain synaptic junctions (Cases-Langhoff et al., 1996; Langnaese et al., 1996). Their molecular characterization revealed that they are structurally related multi-domain proteins including ten highly conserved regions, Piccolo-Bassoon homology domains (tom Dieck et al., 1998; Wang et al., 1999; Fenster et al., 2000). Initially these molecules were thought to be vertebrate specific, however,

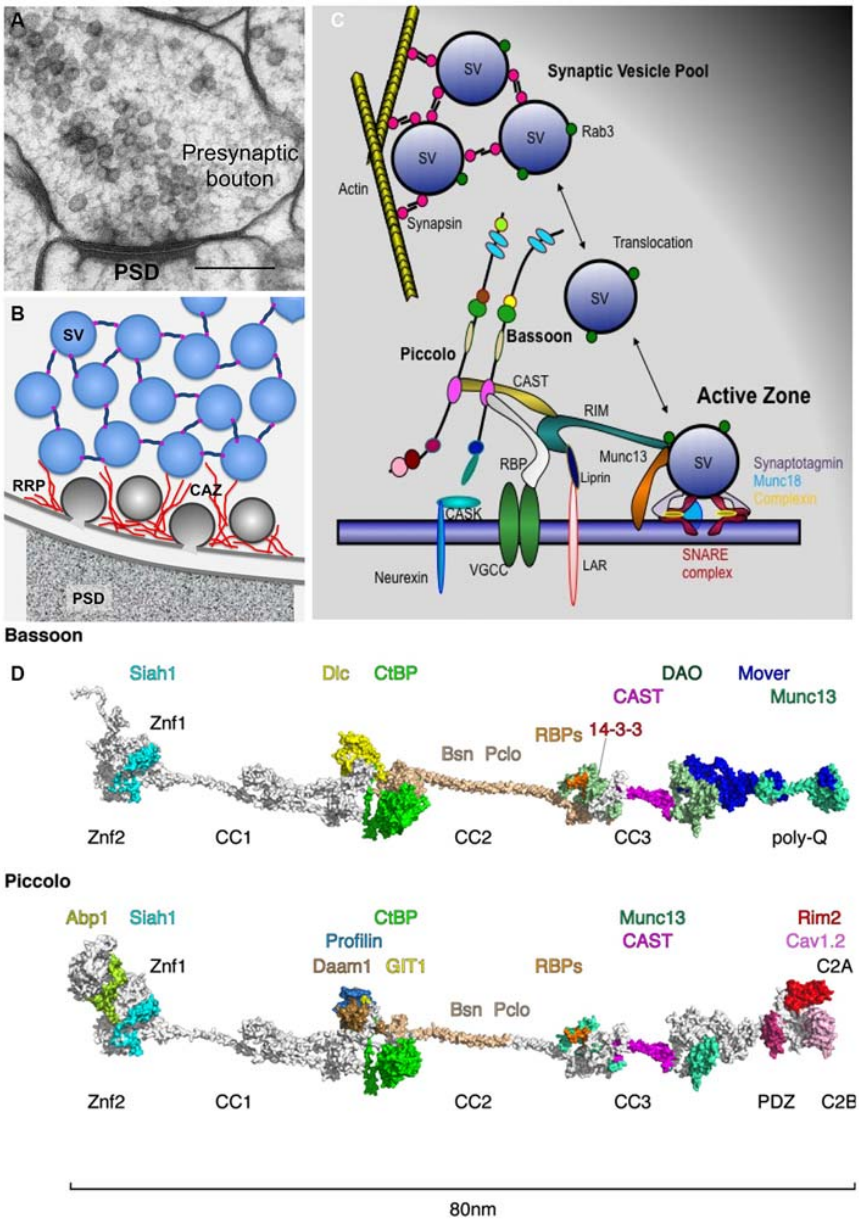

FIGURE 1 | Piccolo and Bassoon at presynaptic active zones (AZ). (A) Cryo-electron micrograph of an excitatory synapse from rat brain (originally published in Rostaing et al., 2006; size bar, 200 nm). **(B)** Schematic organization of SVs within presynaptic boutons with SVs in the reserve pool tethered together via synapsin and those in the docked pool embedded in the CAZ (red filaments). **(C)** Schematic of CAZ molecules directing the clustering, translocation, docking, positional and molecular priming and fusion of SVs. **(D)** *In-silico* modeling of Bassoon and Piccolo structures with docking sites for binding partners color coded. The structures include X-ray and NMR data for Znf [protein data bank (Berman et al., 2000), PDB entry: 1ZBD], PDZ (1UJD), coiled-coil (3QH9 and Gruber and Lupas, 2003) and C2 (1RH8, 5CCG) domains. Piccolo C-terminus has been arranged similar to synaptotagmin (Zhou et al., 2015). Most of the remaining parts contain proline and glycine residues that prevent persistent folding but build compact distorted domains (Quiroz and Chilkoti, 2015), while the homologs coiled-coil helices elongate both proteins to about 80 nm. These domains were modeled *ab initio* using threading (Kelley and Sternberg, 2009) and BLAST routines (Sauder and Dunbrack, 2000) and visualized using PyMOL (https://www.pymol.org). For further details compare **Table 1**. Abbreviations: PSD, postsynaptic density; SV, synaptic vesicle; RRP, release-ready SV pool; CAZ, cytomatrix assembled at the active zone.

structurally more distantly related versions are also found in invertebrates, i.e., Fife and Bruchpilot (Wagh et al., 2006; Sigrist and Schmitz, 2011; Bruckner et al., 2012; Ackermann et al., 2015) that may serve similar functions in the CAZ. In vertebrates, Piccolo and Bassoon are selectively localized to the AZs of central and peripheral synapses as well as peptide/protein-secreting cells of the endocrine system (Cases-Langhoff et al., 1996; tom Dieck et al., 1998, 2005; Brandstatter et al., 1999; Richter et al., 1999; Dick et al., 2001, 2003; Fujimoto et al., 2002; Haeberle et al., 2004; Nishimune et al., 2004; Shibasaki et al., 2004b; Hagiwara et al., 2005; Khimich et al., 2005; Juranek et al., 2006; Siksou et al., 2007; Limbach et al., 2011; Nishimune, 2012).

The identification and molecular characterization of interacting partners revealed that Piccolo and Bassoon use their multi-domain structure to contribute to various features of presynaptic function (**Table 1**, **Figure 1D**). These include assembly of AZ scaffolds, organization of neurotransmitter release machinery, linkage of actin dynamics and endocytosis, maintenance of synapse integrity as well as integration of signaling pathways and synapto-nuclear signaling.

SCAFFOLDING AND ASSEMBLY OF ACTIVE ZONES

The assembly of presynaptic AZs starts at the trans-Golgi network, where components are recruited into precursor vesicles and delivered to nascent synapses (Ahmari et al., 2000; Zhai et al., 2001; Garner et al., 2002; Shapira et al., 2003; Tao-Cheng, 2007; Fairless et al., 2008; Bury and Sabo, 2011; Maas et al., 2012). Although it was initially thought that AZ proteins were pre-assembled on a single precursor vesicle (Ahmari et al., 2000; Zhai et al., 2001; Shapira et al., 2003), more recent studies argue for the existence of at least three types of precursor vesicles that carry complexes between Piccolo-Bassoon-ELKS/CAST, RIM-Neurexin-CASK-voltage-gated Ca^{2+} channels (VGCCs) or Munc13 (Tao-Cheng, 2007; Fairless et al., 2008; Maas et al., 2012). Intriguingly, the assembly of Piccolo-Bassoon-ELKS/CAST transport vesicles (PTV) not only depends on Piccolo and Bassoon, but also on their ability to interact with Golgi-membranes, ELKS/CAST and the membrane fission protein CtBP1/BARS (Dresbach et al., 2003, 2006; Maas et al., 2012). The trafficking and delivery of PTVs to nascent and mature synapses utilizes microtubule-based transport (Fejtova et al., 2009; Bury and Sabo, 2011; Maas et al., 2012) and the activities of anterograde and retrograde motors. Here, attachment to the retrograde motor dynein is mediated by the direct interaction of Bassoon with dynein-light chains (Dlc1/2; Fejtova et al., 2009). Kinesins are also involved and require the adaptor protein Syntabulin, though the linkage to PTVs is currently unknown (Cai et al., 2007).

Once delivered to nascent synapses, the core AZ complex assembles in response to transsynaptic signals from adhesion molecules such as Neurexins and Neuroligins (Waites et al., 2005; Siddiqui and Craig, 2011), occurring within minutes of initial axo-dendritic contact (Vardinon-Friedman et al., 2000; Garner et al., 2002; Lucido et al., 2009). The organization of this complex is determined by a series of protein-protein interactions between Munc13, RIM, Liprin-α, ELKS/CAST, RBP, Bassoon and Piccolo (see Ackermann et al., 2015). From the Bassoon-Piccolo perspective, this can be mediated via domains that promote interactions between Piccolo-Bassoon (coiled-coil region 2, CC2), Bassoon-Piccolo-ELKS/CAST and/or Munc13 (CC3), Piccolo-RIM (C2A domain) or Bassoon-RBPs (**Figure 1**; **Table 1**). These are complemented by interactions between Munc13, RIM, ELKS/CAST, Liprin-α and RBPs (Wang et al., 2009; Gundelfinger and Fejtova, 2012; Südhof, 2012). Together, this scaffold is thought to create a platform for integrating other key features of the CAZ.

Importantly, loss of function studies of Bassoon or Piccolo have not been shown to affect the ultra-structural organization of presynaptic AZs of central synapses (Altrock et al., 2003; Leal-Ortiz et al., 2008; Mukherjee et al., 2010), perhaps due to structural redundancy between these and/or other CAZ proteins. Notable exceptions have been observed at vertebrate sensory synapses. Here, loss of Bassoon disrupts the attachment of synaptic ribbons to the arciform density at retinal photoreceptor terminals and at auditory inner hair cell synapses (Dick et al., 2003; Khimich et al., 2005; tom Dieck et al., 2005). At these synapses, Bassoon appears to utilize its interactions with Ribeye and ELKS/CAST (**Table 1**) to tether ribbons to the AZ (Takao-Rikitsu et al., 2004; tom Dieck et al., 2005; Magupalli et al., 2008). Conversely, loss of the main Piccolo isoform (Piccolino) from mouse photoreceptor cells alters the maturation and ultrastructure of ribbons, i.e., their transition from spherical to ribbon shape structures (Regus-Leidig et al., 2013, 2014).

EXOCYTOSIS AND LOCALIZATION OF VOLTAGE-GATED CA^{2+} CHANNELS

A core function of presynaptic AZs is the regulated release of neurotransmitters, a process which involves the tethering, docking, priming and fusion of SVs with the AZ plasma membrane in a calcium-dependent manner (Südhof, 2012). This requires a delicate interplay between RIMs, Munc13s, SNARE complexes, synaptotagmin and VGCCs. Recent studies on synapses lacking Bassoon indicate a role for this CAZ protein in the recruitment of SVs into vacated release sites as well as the positioning of VGCC near release sites, i.e., positional priming (Frank et al., 2010; Hallermann et al., 2010; Jing et al., 2013; Mendoza Schulz et al., 2014). Regarding the latter, Bassoon, similar to RIMs, has been found to bind RBPs—molecules known to associate with VGCCs (Hibino et al., 2002). In contrast to RIMα-isoforms, which appear to use RBPs to tether various types of VGCCs (e.g., $Ca_V2.2$ or N-, and $Ca_V2.1$ or P/Q-types) to AZs (Han et al., 2011; Gundelfinger and Fejtova, 2012; Kaeser et al., 2012), Bassoon selectively positions P/Q-type channels near SV release sites of hippocampal synapses (Davydova et al., 2014). At present, it is unclear why different protein interactions are used to tether VGCC into the AZs. One explanation is that it allows synapses to have functional diversity and/or plasticity. For example, N- and P/Q-type channels have been linked to immature and mature synapses, respectively (Scholz and Miller, 1995). Theoretically, the loss of Bassoon

TABLE 1 | Interaction partners of Piccolo and Bassoon.

Binding site on Piccolo and/or Bassoon	Interaction partner [Uniprot ID (name)]	Description/potential function	Reference	Cellular processes
Pclo, Q-domain, aa 372–491 (rat)—absent in Bsn	Abp1, Actin-binding protein 1/drebrin-like, [Q9JHL4 (DBNL_RAT)]	Links piccolo to the actin cytoskeleton and to endocytic machinery	Fenster et al. (2003)	Actin cytoskeleton dynamics
Pclo, Znf2, aa 1010–1033 (rat)—not tested for Bsn	Pra1, prenylated rab3A acceptor [O35394 (PRAF1_RAT)]	Interaction with SV via rab3 and VAMP2/synaptobrevin-2	Fenster et al. (2000)	Membrane trafficking
Pclo, Znf1 521–582 Pclo, Znf2 1010–1071 Bsn, Znf1 162–225 Bsn, Znf2 459–523 (rat)	Siah1, seven in absentia homolog 1 [Q920M9 (SIAH1_RAT)]	E3 ubiquitin ligase, ubiquitinates SV proteins, component of the ubiquitin-proteasome system.	Waites et al. (2013)	Protein turn-over/degradation
Bsn aa 1360–1692 (rat)—multiple binding sites, not present in Pclo	Dynein light chains Dlc-1, Dlc-2 [P63170 (DYL1_RAT), Q78P75 (DYL2_RAT)]	Link to dynein motors, (retrograde) transport of Piccolo-Bassoon transport vesicles	Fejtova et al. (2009)	Membrane trafficking
Pclo, aa 2197–2350 (rat)—absent in Bsn	GIT1 [Q9Z272 (GIT1_RAT)]	GTPase-activating protein for ADP ribosylation factor family; regulation of actin cytoskeleton, endocytosis	Kim et al. (2003)	Actin cytoskeleton dynamics, Membrane trafficking
Pclo, aa1980–2553 (rat)—absent in Bsn	Daam1, Dishevelled-associated activator of morphogenesis 1 [(D4ABM3 (D4ABM3_RAT)]	Formin, actin cytoskeleton binding protein	Wagh et al. (2015)	Actin cytoskeleton dynamics
Bsn, aa 1653–2087 (rat) Pclo (homologous region)	CtBP1/BARS [Q9Z2F5 (CTBP1_RAT)], CtBP2/RIBEYE [Q9EQH5 (CTBP2_RAT)]	Anchoring of synaptic ribbons to active zones at ribbon synapses; regulation of gene expression; potential relation to membrane trafficking processes	tom Dieck et al. (2005), Jose et al. (2008), Hübler et al. (2012), and Ivanova et al. (2015)	Synapto-nuclear communication, Membrane trafficking, Scaffolding and Assembly of CAZ core complex
Pclo/Acz, ~aa 2300–2400 PP (mouse) Absent in Bsn	Profilin-2 > profilin-1 [Q9JJV2 (PROF2_MOUSE)]	Actin modulating protein	Wang et al. (1999)	Actin cytoskeleton dynamics
Bsn, CC2 aa 2088–2563 (rat)	Bsn CC2 Pclo CC2	Homo-/heterodimerization region, Golgi-binding domain of Bassoon	Dresbach et al. (2006) and Maas et al. (2012)	Scaffolding and Assembly of CAZ core complex
Pclo/Acz, CC2 aa3094–3218 (mouse)	Bsn	Heterodimerization domain, serves presumably scaffold formation	Wang et al. (2009)	Scaffolding and Assembly of CAZ core complex
Pclo/Acz, CC3 aa 3593–3865 (Mouse)	Munc13 (N-term) [Q62768 (UN13A_RAT)], Potentially via CAST	Priming factor, SV priming	Wang et al. (2009)	Scaffolding and Assembly of CAZ core complex, SV priming
Bsn, CC3 aa 2933–2995 (rat) Bsn, CC3 aa 2873–3077 (mouse) Pclo, CC3 aa 3601–3960 Pclo/Acz, CC3 aa 3657–3715 (mouse)	ERC2/ELKS2/CAST [Q8K3M6 (ERC2_RAT)]	Interaction with CAZ scaffolding proteins. Potentially involved in anchoring synaptic ribbons to the active zone	Takao-Rikitsu et al. (2004), tom Dieck et al. (2005) and Wang et al. (2009)	Scaffolding and Assembly of CAZ core complex

(Continued)

TABLE 1 | (Continued).

Binding site on Piccolo and/or Bassoon	Interaction partner [Uniprot ID (name)]	Description/potential function	Reference	Cellular processes
Bsn, Ser2845 (rat)	14–3–3η (and other isoforms) [P68511 (1433F_RAT)]	Phospho-dependent regulation of anchoring of bassoon to CAZ Phosphorylation depends on RSK family	Schröder et al. (2013)	Cellular signaling, Scaffolding and Assembly of CAZ core complex?
Bsn, aa 2869–2899 (rat) Pclo (homologous region)	RIM-BPs (RBP1, RBP2) [Q9JIR0 (RIMB1_RAT); Q9JIR1 (RIMB2_RAT)]	RIM binding proteins, presynaptic scaffold/link to voltage-gated $Ca_V2.1$ Ca^{2+} channels. Specificity for Pclo unclear.	Davydova et al. (2014)	Calcium signaling, Regulation of VGCCs, Regulation of exocytosis
Bsn, aa 2715–3263 (rat) Not tested for direct interaction with Pclo	D-Amino Acid Oxidase, DAO [O35078 (OXDA_RAT)]	Enzyme that metabolizes the NMDA receptor co-agonist D-serine. DAO activity significantly inhibited by interaction with Bsn	Popiolek et al. (2011)	Cellular signaling, Regulation of enzymatic activity
Bsn, aa 3601–3820 (C-term) (mouse)	Munc13 (N-term) [Q62768 (UN13A_RAT)] and RIM [Q99NE5 (RIMS1_MOUSE)]	Interaction with presynaptic scaffolding	Wang et al. (2009)	Scaffolding and Assembly of CAZ core complex, SV priming
Bsn, aa 1886–3938 (C-term) (mouse)	$Ca_V\beta1b$; $Ca_V\beta4$ Q8R3Z5 (CACB1_MOUSE) Q8R0S4 (CACB4_MOUSE)	Interaction and potentially localization of presynaptic voltage-gated $Ca_V2.1$ and $Ca_V2.2$ Ca^{2+} channels	Chen et al. (2011)	Calcium signaling, Regulation of VGCCs
Bsn aa 3263–3938 (rat) Absent in Pclo	Mover [A8WCF8 (TPRGL_RAT)]	SV protein, negative regulator of synaptic release probability	Kremer et al. (2007), Ahmed et al. (2013), and Korber et al. (2015)	Membrane trafficking? Regulation of exocytosis
Pclo, PDZ, aa 3900–4244 (mouse) Absent in Bsn	Rap guanine nucleotide exchange factor 4/GEFII/Epac 2 [Q9EQZ6 (RPGF4_MOUSE)]	cAMP-dependent exocytosis in pancreas β cells	Fujimoto et al. (2002)	Membrane trafficking, Regulation of exocytosis in pancreatic β-cells
Pclo, C2A, aa 4704–5010 (mouse) Absent in Bsn	RIM2, Rab3 interacting molecule C2A domain Q9EQZ7 (RIMS2_MOUSE)	Presynaptic scaffolding protein involved in synaptic vesicle tethering and priming/interaction detected in exocytosis in pancreas β cells	Fujimoto et al. (2002)	Scaffolding and Assembly of CAZ core complex, SV priming?
Pclo, C2A, aa 4704–5010(mouse) Absent in Bsn	Pclo C2A, [Piccolo/Aczonin] [Q9QYX7 (PCLO_MOUSE)]	Site of Piccolo homo-dimerization/ interaction originally detected in pancreas β cells	Fujimoto et al. (2002) and Garcia et al. (2004)	Scaffolding and Assembly of CAZ core complex
Pclo. C2A, aa 4704–5010 and Pclo, C2B, aa 4955–5165 Absent in Bsn	$Ca_V1.2$ [Q01815 (CAC1C_MOUSE)]	L-type voltage gated Ca^{2+} channel/interaction detected in exocytosis in pancreas β cells	Shibasaki et al. (2004a)	Calcium signaling, Regulation of VGCCs

Abbreviations: Bsn, Bassoon; Pclo, Piccolo; C2A, C2B, C2 domains of Piccolo; CC1–3, coiled-coil regions; PDZ, PDZ domain; PP, proline-rich domain; SV, synaptic vesicles; Znf1–2, zinc finger domains; SV, synaptic vesicle; UniProt information: Piccolo rat: Q9JKS6 (PCLO_RAT); Piccolo/Aczonin mouse: Q9QYX7 (PCLO_MOUSE); Bassoon rat: O88778 (BSN_RAT); Bassoon mouse: O88737 (BSN_MOUSE).

and thus P/Q type channels could result in more immature synapses. Based on our present knowledge one can hypothesize that RIMα-isoforms are the key regulators for VGCC recruitment into the AZ, while Bassoon seems to be involved in more subtle subtype-specific regulation of positional priming events at various types of brain synapses (Südhof, 2012; Davydova et al., 2014).

At inner ear hair cell ribbon synapses, Bassoon also contributes to the appropriate localization of L-type VGCC ($Ca_V1.3$). Ca^{2+} influx and $Ca_V1.3$ density is reduced in *Bassoon (Bsn)*-mutant mice (Khimich et al., 2005; Frank et al., 2010). This phenotype can be partly attributed directly to Bassoon function, but is enhanced by the lack of ribbon anchoring due to loss of Bassoon (Jing et al., 2013).

For the vertebrate neuromuscular junction, the C-terminal third of Bassoon has been shown to interact with β1b and β4 VGCC subunits suggesting an involvement of these interactions in the recruitment, localization and regulation of presynaptic calcium channels (Chen et al., 2011; Nishimune et al., 2012). Additional evidence for an interaction between VGCC and Piccolo and/or Bassoon derives from the proteomic analysis of calcium channel complexes both in the CNS (Müller et al., 2010) and at the neuromuscular junction (Carlson et al., 2010).

In contrast to Bassoon, a functional link between Piccolo and the localization of VGCC at brain synapses is less well explored. Here, one point of focus with respect to the regulated neurotransmitter release has been on its C2A domain. Similar to other C2A domains, this region in Piccolo binds calcium (albeit with low affinity) and induces a conformational change in this domain (Gerber et al., 2001; Garcia et al., 2004) that affects its association with phospholipids and its dimerization. However, no effect on synaptic transmission was observed if mutations that disrupt calcium binding were knocked into the C2A domain in the *Piccolo (Pclo)* gene (Mukherjee et al., 2010), leaving open the role of the C2A domain in CNS synapse function. Intriguingly, the Piccolo C2A domain has been reported to regulate the surface levels of dopamine transporters (DAT; Cen et al., 2008), and when over-expressed in transgenic mice to induce depression-like behaviors (Furukawa-Hibi et al., 2010), implying a role for Piccolo in dopaminergic transmission. Consistently, a genome wide association study identified a mutation in the C2A domain of Piccolo in patients with major depressive disorders (Giniatullina et al., 2015), though how this mutation causes depression remains unclear.

Other insights into Piccolo's role in regulated secretion have come from experiments on β-islet cells. Here, the C2A domain was found to bind RIM2 and L-type VGCC ($Ca_V1.2$). Moreover, Piccolo can interact with the cAMP-sensor Epac2 (Rap guanine nucleotide exchange factor 4 or cAMP-GEFII; see **Table 1**) and loss of Piccolo function was found to impair *cAMP-dependent* insulin secretion (Fujimoto et al., 2002; Shibasaki et al., 2004a; Jacobo et al., 2009).

Bassoon and Piccolo seem to have functions in organizing the neurotransmitter release machinery including the tethering and priming of SVs at AZs. Thus both proteins participate in the formation of the CAZ core complex that recruits factors essential for tethering and priming of SVs, such as Munc13s and RIM1/2α-isoforms (Wang et al., 2009; **Table 1**). Upon over-expression, the Piccolo/Aczonin CC3 region is targeted to nerve terminals and impairs SV recycling similar to the over-expression of the RIM1 zinc finger (Znf) domain. In addition the C-terminus of Bassoon can interact with the N-terminal C2A domain in Munc13 (Wang et al., 2009). It is thus conceivable that this complex interplay might contribute to the regulation of RIM-dependent activation of Munc13 priming functions (Deng et al., 2011; Han et al., 2011; Südhof, 2012).

Mover, a SV-associated phospho-protein, was originally identified as a binding partner for Bassoon (Kremer et al., 2007; Ahmed et al., 2013) and recently characterized as a negative regulator of evoked SV exocytosis (Korber et al., 2015). ShRNA-mediated knock-down of the protein in the Calyx of Held leads to an accelerated and enhanced synaptic depression caused by an increased sensitivity of the release apparatus to Ca^{2+}. The role of Bassoon in this context is yet unclear, as a similar synaptic depression phenotypes have not been observed in *Bsn*-mutant mice (Altrock et al., 2003).

Indirect evidence that Bassoon may also be involved in the release of brain-derived neurotrophic factor (BDNF) from large dense-core vesicles comes from the investigation of *Bsn*-mutant mice. Accumulation of high amounts of BDNF, in particular brain regions including hippocampus, cerebral cortex and striatum, has been observed in mutant brains (Ghiglieri et al., 2010; Heyden et al., 2011; Dieni et al., 2012, 2015).

ACTIN DYNAMICS AND ENDOCYTOSIS

While Piccolo and Bassoon share a great deal of structural homology, each also contains unique segments, implying divergent functions. Studies of Piccolo have shown that it is selectively required for activity-induced F-actin assembly within presynaptic boutons and efficient synaptic transmission (Waites et al., 2011). Consistent with this concept, Piccolo, but not Bassoon, contains regions that interact with a variety of actin-associated proteins (**Figure 1D**; **Table 1**). These include Abp1 (Fenster et al., 2003), GIT1 (Kim et al., 2003), Daam1 (Wagh et al., 2015), Profilin2 (Wang et al., 1999; Waites et al., 2011) and Epac2 (Fujimoto et al., 2002). Functionally, these interactions appear to contribute to the regulated delivery and recycling of SVs within nerve terminals. For example, emerging evidence indicate that Profilin2, GIT1 and Daam1 may contribute to the polymerization of linear rather than branched F-actin filaments from AZs (Wagh et al., 2015), thought to be involved in the translocation of SVs from the reserve into the readily releasable pools. In this regard, Piccolo seems to act as a platform for the polymerization of these filaments via a region situated between CC1 and CC2 (Wagh et al., 2015). This region binds to GIT1, a GTPase-activating protein (GAP) for ADP-ribosylation factors (ARFs), involved in the regulation of membrane trafficking (Chavrier and Goud, 1999), as well as PIX1, a focal adhesion kinase, and Paxillin, both of which regulate the assembly of the actin cytoskeleton at focal adhesion site (Kim et al., 2003). This region also binds Daam1, a member of the formin family of molecules that directly polymerize

actin upon activation by RhoA and/or the Wnt/Disheveled signaling complex (Habas et al., 2001; Liu et al., 2008) and Profilin2, an ADP/ATP nucleotide exchange factor for G-actin (Kovar, 2006), that binds at the mouth of formins, allowing for the rapid processive assembly of linear F-actin polymer (Higgs, 2005). Importantly, Daam1 only binds Piccolo and promotes F-actin assembly after activation, implying that Piccolo spatially controls the assembly of these filaments radial out from the AZ.

Intriguingly, two of these Piccolo binding partners, Abp1 and GIT1 are also linked to SV endocytosis (Fenster et al., 2003; Kim et al., 2003). For example, the actin-binding protein, Abp1 has been shown to interact the endocytic protein Dynamin (Kessels et al., 2001), while GIT1 directly associates with Stonin2, an endocytic adaptor protein (Podufall et al., 2014). In flies, SV recycling is impaired at synapses lacking GIT1 and is associated with the displacement of Stonin2/StonedB away from AZ implying that GIT1, and possibly Piccolo in vertebrates, help to couple exo- and endocytosis by creating a bridge between active and endocytic zones (Podufall et al., 2014). Of note, ultrafast endocytosis and bulk endocytosis both require the dynamic assembly of F-actin (Akbergenova and Bykhovskaia, 2009; Nguyen et al., 2012; Watanabe et al., 2013a,b), a process that could be facilitated via complexes between Piccolo, GIT1, Abp1 and other F-actin assembly proteins at endocytic sites.

SYNAPSE MAINTENANCE AND INTEGRITY

In addition to their roles in the structural and functional assembly of presynaptic AZs, Piccolo and Bassoon are involved in the maintenance of SV pools (Mukherjee et al., 2010) and act as regulators of presynaptic proteostasis and the integrity of synapses as observed upon knock-down of the proteins in primary neuronal culture (Waites et al., 2013). This new functionalities were only revealed at synapses lacking both proteins implying that it involves structurally conserved regions between these molecules. In this regard, there seem to be three mechanisms that contribute to the stability and integrity of presynaptic AZs. The first involves the dynamic exchange of synaptic proteins, which occurs on the order of tens of minutes to hours (Minerbi et al., 2009; Ziv and Fisher-Lavie, 2014); the second one the activity-dependent reorganization of AZs, which modulate the efficiency of neurotransmitter release (Lazarevic et al., 2011, 2013); and the third one the turnover/degradation of synaptic proteins, most of which have half-lives of 2–7 days (Cohen et al., 2013). Regarding the latter, Piccolo and Bassoon appear to play fundamental roles in regulating the ubiquitin-proteasome (UPS) and autophagy-lysosomal systems within presynaptic boutons (Waites et al., 2013). Specifically, it was observed that, in boutons lacking these AZ molecules, SV pools and synaptic junctions were lost. Moreover, ubiquitinated proteins, pleomorphic vesicles, and multi-vesicular bodies were observed to accumulate within these degenerating boutons, a process that required the activation of the UPS, lysosomal and ubiquitin systems. Thus far one molecular link between Piccolo and Bassoon and these degradative systems has been identified, i.e., Siah1 (Waites et al., 2013). Siah1 is an E3 ubiquitin ligase involved in the poly-ubiquitination of SV proteins such as synaptophysin and synuclein. Importantly, Siah1 binding to the Znf domains of Bassoon and Piccolo inhibits its activity (Waites et al., 2013). The functional importance of this locally regulated degradative system is unclear. Possibilities include the regulated release of neurotransmitter, the recycling of SVs and/or the removal of misfolded or aging proteins (Waites and Garner, 2011; Ackermann et al., 2015).

PRESYNAPSE-TO-NUCLEUS SIGNALING

Communication between synapses and the soma, in particular the nucleus, of a neuron is essential for neuronal survival as well as for processes of homeostatic and associative plasticity. While multiple synapto-nuclear signaling pathways have been reported for the postsynapse (for review, see Jordan and Kreutz, 2009; Panayotis et al., 2015), much less is understood about presynapse to nucleus communication. Known pathways from the presynapse or the axon to the nucleus include retrograde signaling of neurotrophins via signaling endosomes (Cosker and Segal, 2014) and retrograde signal transduction from sites of injury (Panayotis et al., 2015). One recently discovered signaling pathway involves Bassoon and Piccolo as synaptic key regulators for presynaptic recruitment and release of the transcriptional co-repressor CtBP1 (aka BARS; Ivanova et al., 2015). The interaction between Bassoon or Piccolo and CtBP1 is mediated by the metabolic sensor system NAD^+/NADH and contributes to activity-dependent distribution of CtBP1 between presynapses and the nucleus. High activity shifts the equilibrium between synaptic and nuclear CtBP1 pools to synapses, low activity to the nucleus (Ivanova et al., 2015; Kravchick and Jordan, 2015). CtBP1 (as its paralogue CtBP2) is involved both gene-specific and global repression of gene transcription and has been implicated in processes of development and tumorigenesis (Chinnadurai, 2007). By controlling the synapto-nuclear distribution of CtBPs, Bassoon and Piccolo contribute significantly to the adjustment of activity-regulated gene expression and in turn to processes of long-term implementation of memories.

Of note, the interaction of CtBPs with Bassoon and Piccolo has long been known (tom Dieck et al., 2005; Jose et al., 2008; Hübler et al., 2012). Because of its functional involvement in dynamin-independent fission events, such as budding of vesicles from trans-Golgi membranes, macropinocytosis or fluid-phase endocytosis (Corda et al., 2006; Valente et al., 2013) the interaction may play also a role in membrane trafficking events within the presynapse and from the Golgi to the synapse (e.g., Maas et al., 2012). Moreover, as mentioned above, Bassoon's interaction with an N-terminally extended isoform of CtBP2, i.e., Ribeye (Schmitz et al., 2000), plays an important role in the assembly and anchoring of synaptic ribbons to their AZs in retinal photoreceptors and inner ear hair cells (Khimich et al., 2005; tom Dieck et al., 2005; Magupalli et al., 2008). An important question is how CtBPs act in so many different cellular functions? One simple concept is that it works together with different scaffold proteins such as Bassoon and Piccolo to direct its activity as an NAD/NADH sensor to control vesicular membrane fission, synaptic ribbon assembly and nuclear gene expression.

CONCLUSIONS

The detailed analysis of protein binding partners for Piccolo and Bassoon support the concept that they are fundamentally involved in scaffolding a large number of proteins involved with various aspect of presynaptic function within AZs. State of the art *in silico* analysis of their structure (**Figure 1D**) reveals that individual subdomains of these molecules are likely organized into larger modules, which might facilitate the assembly of supera-molecular complexes devoted to specific AZ functions. For example, the C-terminal halves of both molecules seem to organize proteins involved in the docking, molecular priming and positional priming of SVs. Conversely the central regions of these molecules possess modules that have acquired unique functions, e.g., devoted to the dynamic assembly of actin and endocytosis vs. vesicular transport for Piccolo and Bassoon, respectively. Clearly the relevance of these super-modules needs to be explored further. Finally, although not discussed here, the functional relevance of the large number of posttranslational modifications (phosphorylation, O-glycosylation, ubiquitination; Trinidad et al., 2006; Vosseller et al., 2006; Munton et al., 2007; Chalkley et al., 2009; Lazarevic et al., 2011; Schröder et al., 2013; Ackermann et al., 2015) has yet to be examined, though roles in the regulated rearrangement during learning-related plasticity of AZs are likely (e.g., Kähne et al., 2012).

AUTHOR CONTRIBUTIONS

EDG and CCG have written the article. CR contributed essential discussions and designed **Figure 1D**.

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frontiers in Synaptic Neuroscience

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Synaptic Vesicle Proteins and Active Zone Plasticity

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Neurotransmitter is released from synaptic vesicles at the highly specialized presynaptic active zone (AZ). The complex molecular architecture of AZs mediates the speed, precision and plasticity of synaptic transmission. Importantly, structural and functional properties of AZs vary significantly, even for a given connection. Thus, there appear to be distinct AZ states, which fundamentally influence neuronal communication by controlling the positioning and release of synaptic vesicles. Vice versa, recent evidence has revealed that synaptic vesicle components also modulate organizational states of the AZ. The protein-rich cytomatrix at the active zone (CAZ) provides a structural platform for molecular interactions guiding vesicle exocytosis. Studies in *Drosophila* have now demonstrated that the vesicle proteins Synaptotagmin-1 (Syt1) and Rab3 also regulate glutamate release by shaping differentiation of the CAZ ultrastructure. We review these unexpected findings and discuss mechanistic interpretations of the reciprocal relationship between synaptic vesicles and AZ states, which has heretofore received little attention.

Keywords: synaptotagmin I, Rab3, cytomatrix at the active zone, synaptic transmission and plasticity, synaptic vesicle, active zone, neurotransmitter release

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INTRODUCTION

Chemical synapses are important regulators of neuronal information transfer. At these specialized intercellular contact sites the arrival of an action potential at the presynaptic terminal triggers the release of neurotransmitter onto a postsynaptic cell, where subsequent receptor activation gives rise to signal transduction. Remarkable electrophysiological work by Bernard Katz and colleagues on the quantal nature of neurotransmitter release in the mid-20th century set the basis for interpreting morphological features of the synaptic ultrastructure (Fatt and Katz, 1952; del Castillo and Katz, 1954; Palay, 1956; Couteaux and Pécot-Dechavassine, 1970). This combination of functional and structural studies helped to establish that transmitter is packaged into synaptic vesicles and discharged at a morphological specialization of the presynapse termed the active zone (AZ).

AZs transform a presynaptic action potential into the release of a chemical signal with high spatial and temporal precision. To perform this task, different proteins, some ubiquitously expressed and some highly specialized, are recruited to the AZ. Here, these molecules act in concert to control the final stages of the synaptic vesicle cycle. Vesicles are guided to the AZ membrane, docked and primed in a release-ready state and fused with the plasma membrane upon calcium ion (Ca^{2+}) influx through voltage-gated Ca^{2+} channels (VGCCs). To ensure spatial precision of exocytosis, molecular interactions spanning the synaptic cleft align the AZ membrane exactly opposite the postsynaptic receptor field. The impressive speed and precise timing of neurotransmitter release, in turn, is provided by the coordinated interplay of individual protein-protein interactions occurring at the AZ (Jahn and Fasshauer, 2012).

In addition to the core fusion complex, containing SNARE ("soluble NSF-attachment protein receptor") and SM ("Sec1/Munc18-like") proteins, vesicle components and AZ-specific proteins contribute to these interactions and help to position synaptic vesicles in close proximity to Ca^{2+} channels. The operation of these molecular machines enables presynaptic Ca^{2+} inflow to be followed by a postsynaptic current in less than a millisecond (Geiger and Jonas, 2000).

Current evidence suggests that the central protein complex surrounding VGCCs at *Drosophila* AZs is made up of RIM (Graf et al., 2012; Müller et al., 2012), RIM-BP (RIM-binding protein; Liu et al., 2011), Unc13 (Aravamudan et al., 1999), Liprin-α (Kaufmann et al., 2002; Fouquet et al., 2009), Syd-1 (Owald et al., 2010), Fife (homolog of vertebrate Piccolo; Bruckner et al., 2012) and Brp (Bruchpilot; Kittel et al., 2006b; Wagh et al., 2006). The membrane-proximal N-terminal domain of Brp is homologous to the vertebrate AZ component CAST/ELKS/ERC (CAST hereafter), while its coiled-coil rich C-terminus, which reaches into the cell interior, is related to large cytoskeletal proteins and is not conserved in its shorter ortholog (Wagh et al., 2006; Fouquet et al., 2009). The large vertebrate AZ scaffolding protein Bassoon does not appear to be encoded by the *Drosophila* genome. This has led to the suggestion that by tethering synaptic vesicles to the cytomatrix at the active zone (CAZ; via its C-term) and by clustering AZ VGCCs (via its N-term) Brp incorporates the functions of several vertebrate AZ proteins thereby ensuring efficient excitation-secretion coupling (Kittel et al., 2006a; Hallermann et al., 2010c).

In electron micrographs, the protein complexes assembled at the AZ are depicted as electron-dense material on the presynaptic plasma membrane. This CAZ often includes prominent structures reaching into the cytoplasm, which vary considerably between different synapses in a species- and cell-type specific manner (Zhai and Bellen, 2004). While chemical synapses operate by the same basic principle (Katz, 1970) the ultrastructural diversity emphasizes the non-uniform protein composition and organization of AZs. This observation raises the question how these complex molecular architectures are mechanistically linked to diverse functional adaptations of synaptic neurotransmitter release (Atwood and Karunanithi, 2002).

Here we follow the hypothesis that discrete organizational states can be specified for AZs. At present it is unclear how many such states may exist, what their functional significance is, or whether AZ differences may turn out to be more appropriately described by a continuum. However, for the time being this heuristic mode of inquiry is a useful means to clarify organizational principles underlying an information processing system. The precise spatial arrangement of AZ proteins, i.e., their orientation relative to the membrane and other AZ molecules, their copy number and the stoichiometry of macromolecular complexes, is functionally relevant. We therefore suggest that studying the nanoscopic arrangement of core CAZ components, such as the large filamentous Brp protein, relative to other proteins like VGCCs can help to distinguish and interpret AZ physiology.

PLASTICITY OF AZ STATES

Structural features and functional properties of AZs differ between various neuron types, between individual synapses belonging to the same neuron and at one and the same site over time (Atwood and Karunanithi, 2002). This plasticity of AZ states is both a developmental phenomenon and can also occur in the mature nervous system in response to changes in synaptic activity. Generally speaking, synaptic plasticity can be divided into short-lived and long-term forms.

Functional changes at the AZ feature prominently in short-term synaptic plasticity (Hallermann et al., 2010b; Regehr, 2012). Synaptic transmission can undergo rapid facilitation during ongoing activity through the accumulation of free Ca^{2+} in the presynaptic terminal, thereby raising vesicle release probability (Katz and Miledi, 1968; Schneggenburger and Neher, 2005). Conversely, depression of transmitter release can occur on a short time scale due to an inactivation of VGCCs or a depletion of readily-releasable vesicles (RRVs; Forsythe et al., 1998; Neher, 2015). Thus, in addition to spatio-temporal Ca^{2+} dynamics, kinetics of vesicle recruitment, priming and AZ release site (re)generation play an important role in shaping short-term plasticity (Junge et al., 2004; Hallermann et al., 2010c; Neher, 2010).

The CAZ is a dynamic structure and its molecular reorganization can shape synaptic function on a time scale of minutes (Matz et al., 2010). Such AZ plasticity can be induced by artificial changes of synaptic activity (Wojtowicz et al., 1994; Spangler et al., 2013) and by natural stimuli. Particularly striking examples of CAZ remodeling *in vivo* have been observed in visual systems of flies and vertebrates where light-dark changes affect the CAZ ultrastructure of photoreceptors (Abe and Yamamoto, 1984; Rybak and Meinertzhagen, 1997; Spiwoks-Becker et al., 2004). Recent work in *Drosophila* has shown that light exposure triggers the removal of Brp, RIM-BP and Liprin-α from photoreceptor terminals, whereas VGCCs and Syd-1 remain unaffected by this molecular plasticity (Sugie et al., 2015).

Homeostatic synaptic plasticity describes a particular form of activity-dependent plasticity, which serves to maintain constant transmission strength in response to altered pre- or postsynaptic function (Davis and Müller, 2015). In an evolutionarily conserved homeostatic process, observed for example at end-plates of myasthenia gravis patients, reduced postsynaptic sensitivity is counteracted by upregulated neurotransmitter release to restore action potential-evoked postsynaptic current amplitudes and maintain muscle excitation (Cull-Candy et al., 1980; Wang et al., 2016). At the *Drosophila* neuromuscular junction (NMJ), molecular mechanisms underlying related modifications of AZ states have been studied in considerable detail. Here, a homeostatic enhancement of Ca^{2+} influx through VGCCs is mediated by RIM-BP and plasma membrane insertion of epithelial sodium channels (ENaC; Younger et al., 2013; Müller et al., 2015). The concurrent recruitment of RRVs is guided by RIM as well as RIM-BP and is accompanied by the enlargement of the Brp-positive CAZ (Weyhersmüller et al., 2011; Müller et al., 2015).

Activity-dependent, long-term synaptic plasticity plays an important role in complex brain functions and represents a likely cellular correlate of memory formation. Mossy fiber synapses in the mammalian hippocampus undergo presynaptically expressed long-term potentiation (LTP), which requires the CAZ constituent RIM1α and the vesicle protein Rab3A (Castillo et al., 1997, 2002). Interactions between RIM1α with Rab3A and VGCCs promote tight Ca^{2+} channel-vesicle coupling (Han et al., 2011; Kaeser et al., 2011) and this process has been suggested to underlie increased transmitter release during mossy fiber LTP (Nicoll and Schmitz, 2005). However, details and direct evidence to support this hypothesis have not yet been presented. In *Drosophila*, odor memory formation is associated with presynaptic plasticity of Kenyon cells, the intrinsic mushroom body neurons (Heisenberg, 2003). Moreover, recent work has uncovered long-term synaptic depression in the context of aversive olfactory learning, consistent with functional modifications of Kenyon cell AZs (Hige et al., 2015). Brp and the vesicle-associated phosphoprotein Synapsin have been implicated in associative learning (Godenschwege et al., 2004; Knapek et al., 2011), though here too, we still lack basic information on the molecular mechanisms underlying memory-related changes of AZ states.

Developmental processes can also target the molecular architecture of AZs giving rise to changes in synaptic strength. For example, at several synapses of the mammalian auditory pathway, developmental changes affect the coupling distance between RRVs and VGCCs at the AZ (Fedchyshyn and Wang, 2005; Wong et al., 2014). Whereas immature AZs display loose coupling, maturation tightens the spatial association of VGCCs with membrane-docked vesicles. By placing the vesicular Ca^{2+} sensor closer to the source of Ca^{2+} influx, this conversion from "microdomain" to "nanodomain" coupling regimes promotes transmission efficiency by increasing neurotransmitter release probability (Eggermann et al., 2011).

As individual synapses mature during the development of the glutamatergic *Drosophila* NMJ, the molecular complexity and Brp content of their AZs increase (Schmid et al., 2008; Fouquet et al., 2009). Brp helps cluster presynaptic VGCCs and, correspondingly, an AZ's neurotransmitter release probability correlates with Brp protein copy number (Kittel et al., 2006b; Ehmann et al., 2014). The developmental incorporation of Brp therefore likely promotes synaptic strength. Interestingly, Brp recruitment is accompanied by changes to the glutamate receptor subunit composition of an AZ's postsynaptic partner. As the AZ grows, GluR-IIA accumulation is reduced and receptor incorporation shifts towards GluR-IIB (Schmid et al., 2008). This transsynaptic relationship appears to be bidirectional, since *brp* mutants possess elevated GluR-IIA levels and *gluR-IIA* mutants display increased release and elevated Brp levels (DiAntonio et al., 1999; Weyhersmüller et al., 2011). Hence, heterogeneous AZ states are matched with the molecular makeup of postsynaptic receptor fields.

The differentiation of this synaptic system operates at multiple levels of organization. Certain AZs participate in spontaneous and evoked neurotransmitter release, while others preferentially support one mode of exocytosis or the other, possibly depending on their maturation state (Melom et al., 2013; Peled et al., 2014). Moreover, a structural and functional gradient develops along a specific larval motoneuron. Distal boutons of the "type Ib" neuron are larger than their proximal counterparts, they possess more AZs and these, in turn, contain more Brp molecules (Ehmann et al., 2014; Paul et al., 2015). Accordingly, action potentials in distal boutons generate larger Ca^{2+} signals and release a greater number of synaptic vesicles in a more synchronized manner (Guerrero et al., 2005; Peled and Isacoff, 2011; Paul et al., 2015).

MOLECULAR MANIPULATIONS OF ACTIVE ZONE STATES

CAZ Proteins

The function of individual CAZ proteins has been deduced mainly by studying mutant alleles. In addition to functional phenotypes, such molecular manipulations may also modify the AZ ultrastructure. These changes are most readily detected at synaptic contacts with prominent CAZ architectures visible in electron micrographs, such as ribbons of vertebrate sensory synapses or the T-bar at the *Drosophila* NMJ.

Brp is an integral component of the *Drosophila* T-bar and is essential for its assembly. At *brp* null mutant AZs VGCCs are mislocalized and T-bars are missing (Kittel et al., 2006b). T-bars appear truncated in C-terminal deletion mutants (Fouquet et al., 2009) and are misshaped when posttranslational modification of Brp is disturbed (Miśkiewicz et al., 2011). In the absence of RIM-BP, which acts in concert with Brp to cluster VGGCs and shape the T-bar, Brp-positive CAZ structures are severely misformed (Liu et al., 2011). Besides these core determinants of T-bar morphology, other CAZ proteins also define the T-bar ultrastructure, reflecting the intricate protein complex of the CAZ. Whereas T-bars appear "overgrown" in mutants of *liprin-α* and *syd-1* (Kaufmann et al., 2002; Owald et al., 2010), *fife* mutant AZs display membrane disruptions and detached T-bar-like structures (Bruckner et al., 2012).

Turning to vertebrate sensory synapses, genetic studies in mouse have uncovered contributions of several CAZ components to ribbon morphology. While Bassoon, a large protein component of the CAZ, is involved in attaching ribbons to the AZ membrane in photoreceptors (Dick et al., 2003) and cochlear inner hair cells (Khimich et al., 2005), Piccolino, a ribbon-specific splice variant of the CAZ protein Piccolo, appears to shape the ribbon ultrastructure (Regus-Leidig et al., 2014). CAST in turn exerts a more subtle influence on the CAZ architecture. In rod photoreceptors of CAST knock-out mice ribbons are shorter despite an apparently intact overall molecular organization (tom Dieck et al., 2012).

Disrupting expression or proper function of a CAZ protein may change the spatial arrangement and operation of other AZ constituents. Analyzing this molecular organization, ideally quantitatively and in combination with physiological and biochemical data, can provide insight to complex protein interactions at the CAZ. With rearrangements taking place

on the nanometer scale, until quite recently, such changes to the molecular architecture have been difficult to detect. The introduction of super-resolution light microscopy to the Neurosciences is beginning to change this situation (Sigrist and Sabatini, 2012; Ehmann et al., 2015).

Since small changes to the physical distance between VGCCs and RRVs have a profound effect on neurotransmitter release (Eggermann et al., 2011) information on the nanoscopic distribution of VGCCs in the AZ membrane is important. In an interesting parallel, super-resolution microscopy has uncovered disarranged VGCC clusters at *brp* and *bassoon* mutant AZs of *Drosophila* and mouse, respectively (Kittel et al., 2006b; Frank et al., 2010; Hallermann et al., 2010c). Combining such modern light microscopy techniques with sophisticated electron microscopy (Indriati et al., 2013) opens new prospects of clarifying structure-function relationships of AZ states by resolving VGCC topographies with respect to synaptic vesicles and the CAZ.

Synaptic Vesicle Proteins

The molecular architecture of the AZ is not only altered by interfering with CAZ components, but can also change when VGCCs (Urbano et al., 2003) or synaptic vesicle proteins are manipulated.

Rab3 is a small synaptic vesicle-associated GTPase involved in vesicle cycling, docking and exocytosis (**Figure 1A**; Südhof, 2004). In a seminal study on Rab3 function in *Drosophila*, Graf et al. reported that Rab3 also controls the protein composition of AZs. At *rab3* mutant NMJs the number of Brp positive AZs drops down to about 30%. At the same time, individual AZs containing Brp are dramatically enlarged, these sites frequently display multiple T-bars and accumulate VGCCs (**Figures 1B–D**; Graf et al., 2009). According to quantitative super-resolution imaging, the number of Brp molecules at the fraction of available sites is increased on average 1.6-fold and correspondingly release probability at these AZs increases with increasing Brp content (Peled and Isacoff, 2011; Ehmann et al., 2014). Interestingly, late expression of *rab3* can rescue the already manifested mutant phenotype, illustrating the dynamic control of Rab3 on the distribution and nucleation of Brp at AZs (Graf et al., 2009).

The vesicular protein Synaptotagmin-1 (Syt1) plays a decisive role as a Ca^{2+} sensor by triggering neurotransmitter secretion on the one hand and clamping vesicle fusion on the other (**Figure 1A**; Brose et al., 1992; DeBello et al., 1993). Recent work at the *Drosophila* NMJ has described a surprising additional influence of Syt1 on structural synaptic differentiation (Paul et al., 2015). Reducing Syt1 protein levels leads to major changes in the morphology of the type Ib motoneuron. Both bouton area and the number of AZs per bouton drop below 50% of wild type values, the Brp count per CAZ increases moderately, and the structure-function gradient is lost (**Figures 1B–D**). Proximal and distal boutons at *syt1* knock-down (syt^{KD}) NMJs have uniform dimensions and they posses comparable numbers of AZs with similar Brp content. In agreement with these observations, focal electrophysiological measurements report indistinguishable

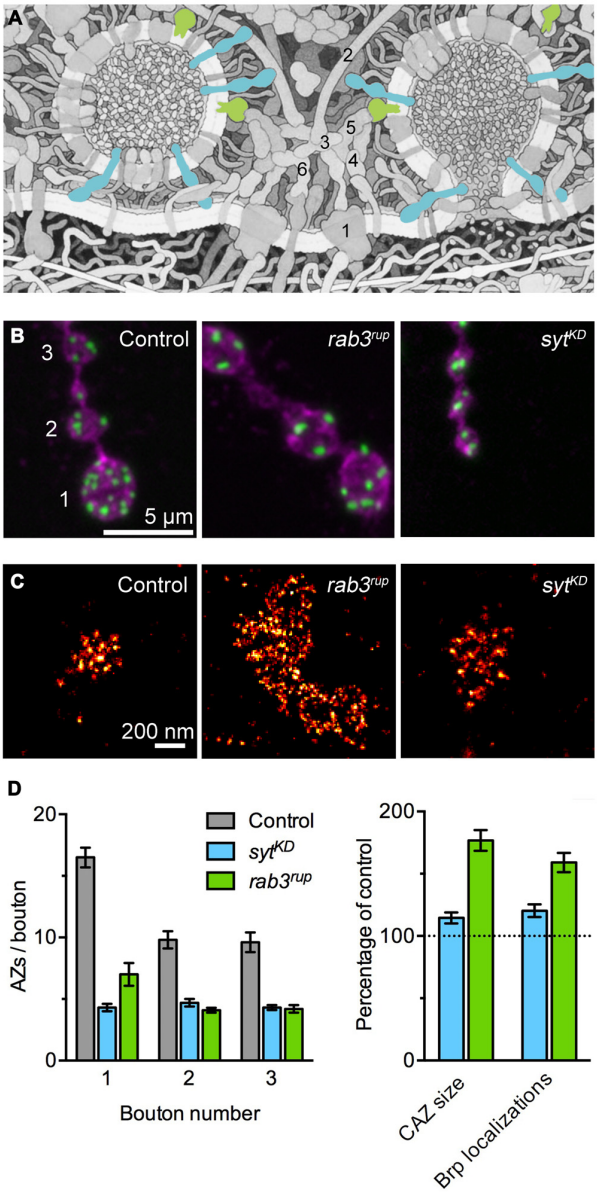

FIGURE 1 | Synaptotagmin-1 (Syt1) and Rab3 shape active zone (AZ) differentiation and ultrastructure. (A) Illustration of the molecular complexity of the AZ. Syt1 (blue) and Rab3 (green) are highlighted. The numbers indicate core AZ proteins mentioned in the text: (1) VGCC; (2) Bassoon; (3) CAST; (4) Munc-13/18; (5) RIM; (6) Liprin-α. Modified from Goodsell (2009; © by The International Union of Biochemistry and Molecular Biology). **(B)** Shown are confocal images of the terminal three boutons along a type Ib axon branch. Staining against the membrane marker HRP (magenta) and Brp (green) illustrates the reduced number of AZs in *rab3* mutant ($rab3^{rup}$) and syt^{KD} motoneurons. Note the small boutons at the syt^{KD} neuromuscular junction (NMJ). Taken from Paul et al. (2015). **(C)** Super-resolution imaging of Brp by *d*STORM (*direct* stochastic optical reconstruction microscopy; Heilemann et al., 2008). Examples of Brp organization at control and syt^{KD} AZs (Paul et al., 2015) and the massively enlarged cytomatrix at the active zone (CAZ) frequently observed at $rab3^{rup}$ NMJs (Graf et al., 2009; Ehmann et al., 2014). **(D)** Quantification of the AZ gradient along type Ib motoneurons (left, related to **B**) and the nanoscopic organization of Brp at the CAZ (right, related to **C**). Summary of data presented in Ehmann et al. (2014) and Paul et al. (2015).

evoked excitatory postsynaptic current amplitudes at proximal and distal locations.

It is recognized that CAZ components can affect the organization of synaptic vesicles at the AZ. For example, filamentous proteins concentrate vesicles in the vicinity of the AZ membrane by tethering them to the CAZ. Such a function is performed by the C-terminal end of Brp to promote rapid vesicle recruitment during high frequency synaptic activity (Hallermann et al., 2010c) and a related role has been ascribed to Bassoon (Hallermann et al., 2010a). It has also been reported that Syt2 participates in positioning vesicles close to VGCCs at the AZ membrane (Young and Neher, 2009). However, to date little attention has been paid to the possibility that vesicle proteins such as Synaptotagmins may influence the structural organization of the AZ (Neher and Penner, 1994).

Work on Syt1 has focused on its role in regulating the final stages of the synaptic vesicle cycle and specifically on its Ca^{2+}-dependent control of exocytosis. In contrast, an additional influence of Syt1 on neuronal differentiation and AZ architecture has remained largely unaddressed. With increasing knowledge of the molecular makeup of the CAZ (**Figure 1A**) and information on its capacity to undergo dynamic rearrangements, further lines of investigation can now be followed up (Graf et al., 2009; Paul et al., 2015). To this end, modern light microscopy methods provide new options for interrogating the involvement of synaptic vesicle proteins in (ultra)structural organization (**Figure 1C**).

OUTLOOK

There is strong evidence that the CAZ is a plastic structure and that its dynamic rearrangement gives rise to different functional properties of AZs. The observations that synaptic vesicle proteins can influence this plasticity are puzzling (Graf et al., 2009; Paul et al., 2015) and suggest a connection between vesicle dynamics and CAZ assembly (Chen et al., 2015). The mechanisms through which Syt1 and Rab3 shape the CAZ are currently not understood. In principle, alterations of CAZ structure and synaptic differentiation may reflect compensatory, homeostatic changes triggered by impaired presynaptic function. However, the structural phenotypes of syt^{KD} and *rab3* mutant NMJs are dissimilar, indicating different pathways, and other manipulations of vesicle release do not parallel the Brp layout seen e.g., in *rab3* mutants (Graf et al., 2009).

Alternatively, the structural abnormalities may be directly linked to the involvement of Syt1 and Rab3 in the synaptic vesicle cycle. Consistent with this notion, a recent study of mutant alleles has demonstrated that normal AZ differentiation depends on a typical vesicle tethering mechanism of Rab3 (Chen et al., 2015). It will be of interest to carry out an analogous mutational analysis of *syt1* to identify protein domains relevant for shaping CAZ ultrastructure and neuronal morphology. Notably, Rab3 is enriched at AZs of the *Drosophila* NMJ. Its punctate clustering requires the presence of Brp and is quite different to the more homogeneous distribution of other synaptic vesicle proteins (Graf et al., 2009). This suggests that Rab3 is associated with a sub-population of vesicles in the vicinity of the CAZ, or with the CAZ itself. Future work will have to clarify how the specific localization pattern of Rab3 is mechanistically connected to its influence on AZ plasticity. An intriguing possibility is that Rab3 and Syt1 exert their structural effects through association with vesicle populations other than neurotransmitter-filled synaptic vesicles. Work in rodent neurons has suggested that preassembled CAZ complexes are transported to developing synapses in so-called Piccolo-Bassoon transport vesicles (PTVs), which include e.g., Piccolo, Bassoon, Munc-13, RIM, CAST and VGCCs (Zhai et al., 2001; Shapira et al., 2003; Maas et al., 2012). Whereas Rab3a is associated with PTVs (Shapira et al., 2003), Syt1 instead appears to be included in synaptic vesicle protein transport vesicles (STVs), which are transported together with PTVs in a coordinated manner (Zhai et al., 2001; Tao-Cheng, 2007; Bury and Sabo, 2011). In *Drosophila*, Brp is co-transported along the axon with RIM-BP (Siebert et al., 2015). However, neither Liprin-α and Syd-1, which precede Brp during AZ assembly (Fouquet et al., 2009), nor Rab3 are associated with this putative precursor complex.

Why have genetic studies of *rab3* and *syt1* in other organisms not reported structural AZ defects similar to those observed in *Drosophila*? One possibility is that the stereotypic morphological layout of the fly NMJ, including its developmental synaptic differentiation, facilitates the quantification of parameters pertaining to neuronal structure and AZ ultrastructure, which are more difficult to measure in other systems. In particular, the T-bar as a prominent marker of the CAZ and the characteristic modular assembly of Brp support analyses of the AZ nanostructure (Kittel et al., 2006b; Graf et al., 2009; Liu et al., 2011; Ehmann et al., 2014; Paul et al., 2015). To clarify whether the structural roles of synaptic vesicle proteins are a peculiarity of the *Drosophila* NMJ or an evolutionarily conserved feature, it will be worthwhile to investigate other synapses with prominent CAZ architectures.

Finally, these new results have important implications for our current understanding of Rab3 and Syt1 functions. Single-synapse resolution is rarely attained in electrophysiological recordings and therefore structural data, e.g., concerning the number of sampled synapses, must be taken into account when extending functional interpretations to the single synapse level. Evidently, we still lack fundamental information on the mechanisms guiding the dynamic organization of AZ states. As we continue filling the gaps old players may be seen in a new light.

AUTHOR CONTRIBUTIONS

RJK and MH conceived and wrote the manuscript.

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The Active and Periactive Zone Organization and the Functional Properties of Small and Large Synapses

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The arrival of an action potential (AP) at a synaptic terminal elicits highly synchronized quanta release. Repetitive APs produce successive synaptic vesicle (SV) fusions that require management of spent SV components in the presynaptic membrane with minimum disturbance of the secretory apparatus. To this end, the synaptic machinery is structured accordingly to the strength and the range of frequencies at which each particular synapse operates. This results in variations in the number and dimension of Active Zones (AZs), amount and distribution of SVs, and probably, in the primary endocytic mechanisms they use. Understanding better how these structural differences determine the functional response in each case has been a matter of long-term interest. Here we review the structural and functional properties of three distinct types of synapses: the neuromuscular junction (NMJ; a giant, highly reliable synapse that must exocytose a large number of quanta with each stimulus to guarantee excitation of the postsynaptic cell), the hippocampal excitatory small synapse (which most often has a single release site and a relatively small pool of vesicles), and the cerebellar mossy fiber-granule cell synapse (which possesses hundreds of release sites and is able to translocate, dock and prime vesicles at high speed). We will focus on how the release apparatus is organized in each case, the relative amount of vesicular membrane that needs to be accommodated within the periAZ upon stimulation, the different mechanisms for retrieving the excess of membrane and finally, how these factors may influence the functioning of the release sites.

Keywords: active zone, neurotransmitter release, endocytosis, periactive zone, release sites

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ORGANIZATION OF THE RELEASE APPARATUS IN SMALL AND LARGE SYNAPSES

Synaptic terminals differ in their strength and short-term plasticity, as well as in the size and spatial organization of the secretory apparatus, mainly in the dimensions, shape, and amount of their Active Zones (AZs), and in the size of their recycling pools of synaptic vesicles (SVs; Atwood and Karunanithi, 2002; Zhai and Bellen, 2004). Most terminals in the central nervous system (CNS) have a small number (1–8) of AZs, and the size of the recycling pool of SVs is not large. For instance, excitatory nerve terminals in area CA1 of the mouse hippocampus have a single AZ, about 10 docked vesicles per AZ, and a recycling pool of

about 200 SVs (Schikorski and Stevens, 1997; Murthy et al., 2001; Rizzoli and Betz, 2005). Large terminals, on the other hand, such the rat cerebellar mossy fiber–granule cell synapse, the calyx of Held and the mouse neuromuscular junction (NMJ), have hundreds of AZs and a large recycling pool of SVs. These structural differences are in accordance with the specific functional roles of each synapse type. When the postsynaptic cell response is determined by the spatial and temporally integrated activity of hundreds of small terminals (**Figure 1A**), the number of quanta released per impulse (quantum content) per terminal is small. Conversely, when the postsynaptic cell receives information from only a few large nerve terminals, the sensitivity and fidelity of the transmission are usually very high. For example, in the cerebellar mossy fiber—granule cell synapse (**Figure 1B**), a burst of action potentials (APs) in a single mossy fiber bouton could be sufficient to generate spikes at the granule cell (Rancz et al., 2007). In this synapse, a large recycling pool of vesicles also contributes to sustaining transmission at high frequency (Saviane and Silver, 2006; Rancz et al., 2007). Finally, when the postsynaptic cell is very large and receives only one input, the size of the presynaptic terminal is also big, as are the number of AZs and the recycling pool of SVs. Typical examples are the NMJ (**Figure 1C**) and the calyx of Held.

Interestingly, even individual neurons can exhibit large differences between neighboring synapses. The hippocampal dentate granule cell mossy fiber, for example, has 11–18 relatively large boutons, each with tens of AZs, and in addition small terminals arising from filopodial extensions of the large ones (Nicoll and Schmitz, 2005).

MEMBRANE LOAD DURING SYNAPTIC ACTIVITY

During synaptic activity, vesicles fused at release sites are translocated to the periAZ. This produces a membrane load in this compartment, the magnitude of which depends on the duration and frequency of the stimulation; nevertheless, its relative impact varies with the AZ organization of each terminal. For example, at the mouse NMJ (**Figure 1C**) from the *levator auris longus* (LAL) muscle, a pure fast muscle, a half-second stimulus train of 50 APs releases about 1700 quanta, the size of readily releasable pool (RRP) of SVs in this terminal (Ruiz et al., 2011). Assuming a mean SV diameter of ∼40 nm, the total membrane load is ∼8.5 μm^2 (1700 SVs × πd^2). However, given the small size of their AZs (0.0054 μm^2 (60 × 90 nm)); Fukunaga et al., 1983; Fukuoka et al., 1987) and the distance between neighboring AZs) ∼0.5 μm; Ruiz et al., 2011), the surface area of each periAZ region (0.5 × 0.5 μm^2 − 0.0054 μm^2 = 0.24 μm^2) increases only by 4.1% when the two primary docked vesicles within each AZ (Nagwaney et al., 2009) fuse.

In another large synapse, the cerebellar mossy fiber bouton, which also has hundreds of release sites (**Figure 1B**), each one hosting ∼7–8 docked vesicles (Xu-Friedman and Regehr, 2004), the mean area of the AZ is about fourfold larger than in mouse motor nerve terminals (0.0216 μm^2), and the distance between neighboring AZs is ∼0.5 μm (Xu-Friedman et al., 2001; Ruiz et al., 2011). Therefore, in this central synapse, if all AZ docked vesicles at rest fuse during phasic nerve activity, the surface area of each periAZ (0.5 × 0.5 μm^2 − 0.0216 μm^2 = 0.2284 μm^2) increases by ∼16.5%.

In contrast, in small central synapses (**Figure 1A**), although the quantal content is much less, a similar number of stimuli may produce a much larger relative increment in the presynaptic membrane surface area. For example, in CA1 excitatory hippocampal presynaptic boutons, which have a mean presynaptic surface area of around 0.2 μm^2 (Schikorski and Stevens, 1997), and an AZ area of ∼0.027 μm^2, if 10 SVs (the mean size of the RRP) fuse with the presynaptic membrane during 20 Hz, 2 s stimulation, the surface increases by ∼0.05 μm^2, which represents a ∼29% increase of the periAZ surface area. Therefore, during high frequency stimulation, if the excess of membrane is not rapidly removed from the AZ and/or the periAZ (Roos and Kelly, 1999) by compensatory endocytosis, or translocated to distant regions for later fission, the relative accumulation of vesicular membrane at the periAZ is larger in small synapses, mainly because their greater number of ready-to-go vesicles per AZ, and their relatively smaller peri-AZ area (**Figure 1D**).

WHEN DOES ENDOCYTOSIS START AND HOW FAST DOES IT GO?

Endocytosis is a complex process that has been studied mainly by ultrastructural analysis, electrical capacitance measurements, and by real-time imaging of fluorescent molecules associated with the membranes. These and other techniques have provided evidence that slow and fast modes of endocytosis exist (**Figure 2**). For example, single SV *clathrin-mediated endocytosis* is a relatively slow process, in the range of tens of seconds (Heuser and Reese, 1973; Granseth et al., 2006; Balaji et al., 2008; Clayton et al., 2008). In the second mode of endocytosis, the so-called *kiss-and-run* mode, after the opening of the fusion pore and releasing of the stored material, the vesicle membrane is rapidly recovered (∼1 s; Ceccarelli et al., 1972; Richards et al., 2000, 2005; Aravanis et al., 2003; Gandhi and Stevens, 2003). Also, the retrieval of a large patch of membrane can be achieved at once by what it is called *bulk endocytosis* (Heuser and Reese, 1973; Miller and Heuser, 1984; Holt et al., 2003; Paillart et al., 2003; Clayton et al., 2007; Wu and Wu, 2007; Hayashi et al., 2008). Finally, an ultrafast mode of endocytosis, only active at physiological temperature, has been described in which membrane patches, corresponding to the area of about 4 SVs, are retrieved within 50–100 ms after stimulation (Watanabe et al., 2013a,b, 2014).

Despite the information provided by the different techniques, the mode of vesicle recycling in each synapse type is still controversial. For instance, the *kiss-and-run* mode has been described at the NMJ (Ceccarelli et al., 1973), at the calyx of Held (He et al., 2006), and at hippocampal neurons in culture (Harata et al., 2006). Nevertheless, in small hippocampal boutons, many authors have reported that endocytosis starts with a delay of a few seconds upon stimulation and proceeds slowly. For example,

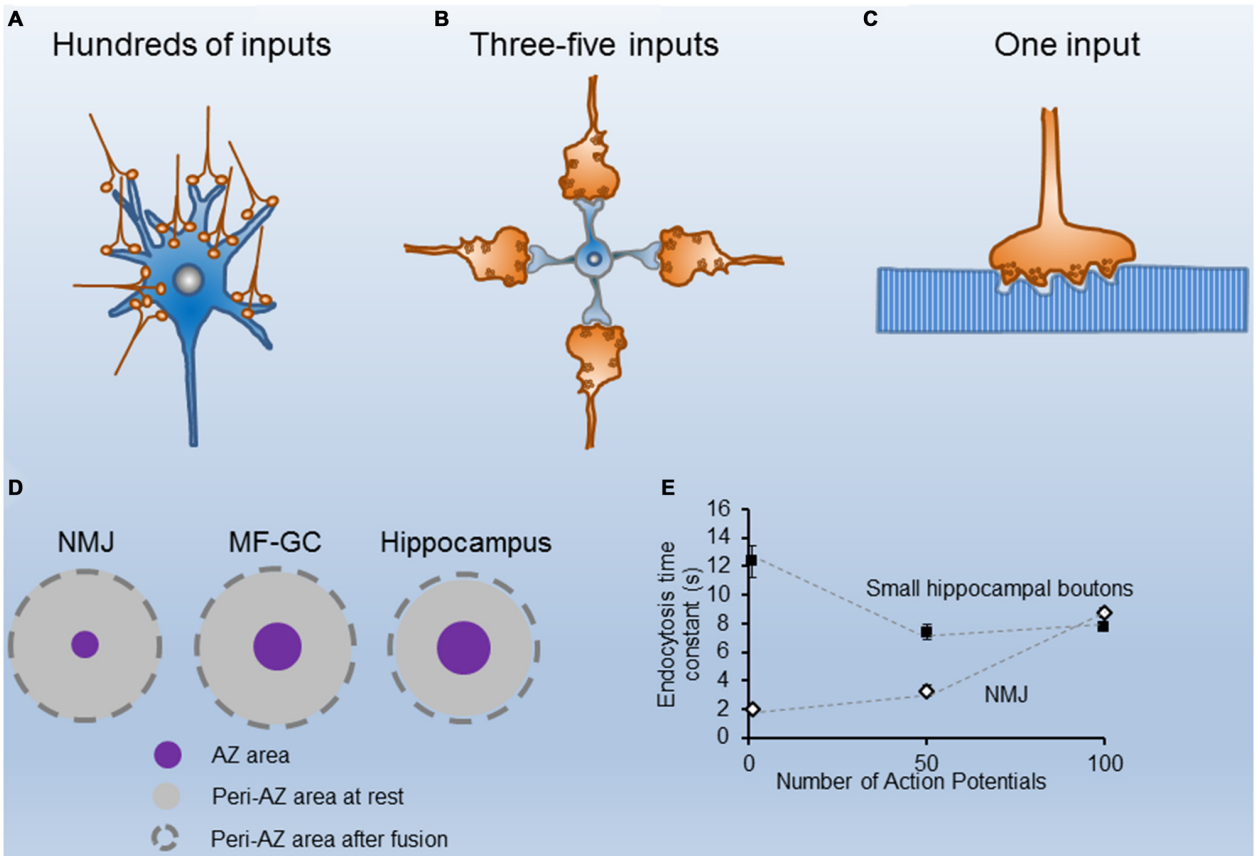

FIGURE 1 | Structural and functional properties of different nerve terminals. (A) Cartoon of CA1 excitatory hippocampal boutons making contacts with a postsynaptic neuron. **(B)** Sketch of four cerebellar mossy fibers contacting a granule cell. **(C)** Illustration of a single motor nerve terminal innervating a muscle fiber. **(D)** Scaled representation of the mean sizes of single Active Zones (AZs; purple circles) and their corresponding peri-AZs (gray circles) in three synapse types (the neuromuscular junction (NMJ), the cerebellar mossy fiber-granule cell, and the hippocampus), at rest (gray circles), and after fusion of all synaptic vesicle (SVs) docked at each AZ (broken line circles). Note the similarity in the peri-AZ areas among different synapses. **(E)** Time constant of fluorescence recovery (tau) vs. number of stimuli (1, 50 and 100 AP) in mouse motor nerve terminals (white symbols adapted from Tabares et al., 2007; Gaffield et al., 2009), and in small hippocampal presynaptic boutons from neurons in culture, black symbols adapted from Armbruster et al. (2013).

at room temperature, the time constant of endocytosis was estimated to be 14–16 s after 1 AP, and the same up to 100 nerve impulses (**Figure 1E**; Sankaranarayanan and Ryan, 2001; Mueller et al., 2004; Granseth et al., 2006; Balaji and Ryan, 2007; Balaji et al., 2008). At the adult mouse NMJ, however, the endocytosis time constant, measured *ex vivo*, was reported to be threefold faster (4–5 s after 50 APs; **Figure 1E**; Tabares et al., 2007; Cano et al., 2012, 2013). When these measurements were done at physiological temperature, the difference persisted between these two synapses. For example, after a single AP the time constant of endocytosis is <2 s at the NMJ (**Figure 1E**; Gaffield et al., 2009), and between 6–15 s at hippocampal small boutons (**Figure 1E**; Balaji et al., 2008; Armbruster et al., 2013).

Besides the difference in the prevalent mode of endocytosis in distinct synapses types, differences in the modulation of membrane recycling are also probable. Calcium is a major modulator of endocytosis in small and large synapses. However, depending on the spatiotemporal profile of the calcium increment at release sites, which in turn, depends on the density of calcium channels, the activity of kinases and phosphatases, and the amount and distribution of the different calcium buffers, the outcome may vary. Even more, the modulation process is a very dynamic process in the same synapse. In small hippocampal boutons, the endocytosis kinetics is accelerated when stimulus strength increases from 1 to 25 APs and then progressively slows for stimulus >25–100 APs (Armbruster et al., 2013). One of the mechanisms by which calcium could accelerate endocytosis is the calcineurin-dependent dephosphorylation of the proteins implicated in endocytosis, known as dephosphins, which include dynamin, synaptojanin, amphiphysin, AP-2, AP-180, among others (Marks and McMahon, 1998; Cousin and Robinson, 2000). For example, the amount of dynamin dephosphorylated determines, in turn, the interaction of dynamin with other proteins of the endocytic machine (Koch et al., 2011; Armbruster et al., 2013; Herman and Rosenmund, 2013; Wu et al., 2014). Therefore, the endocytosis speed in different terminals could be regulated not only by the amount of calcium influx

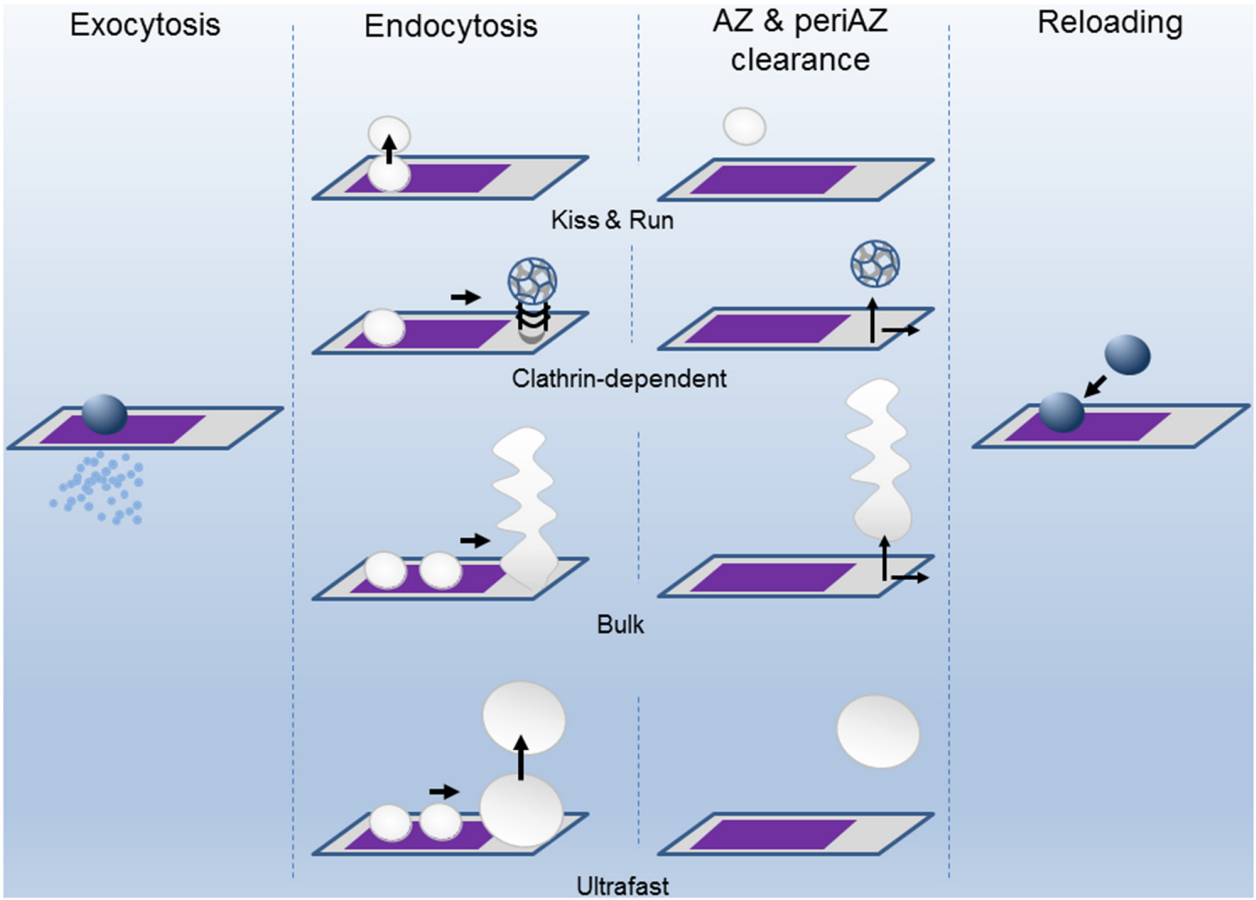

FIGURE 2 | The successive states of the Release Sites (AZ) and the Peri-AZ during synaptic activity. Upon calcium entry during the action potential (AP), vesicles fuse at the AZ (purple area) and release neurotransmitter into the synaptic cleft by exocytosis. The SV membrane components are recovered by endocytosis through different pathways: kiss-and-run, clathrin-dependent, bulk, and ultrafast modes. The clearing of the spent vesicular material from AZ and periAZ areas is critical for the subsequent reloading of new vesicles. Clearance can take place at the AZ, at the periAZ (gray area), or outside the periAZ. For simplicity, the shapes of the AZ and periAZ are shown as rectangles and are not drawn to scaled. The widths of the arrows are proportional to the speeds of the process.

during each AP but also by the expression level, and spatial distribution, of the endocytic molecular components in each synapse type.

"*Hot spots*" of endocytic proteins near sites of exocytosis have been described in large synapses, for example, in *Drosophila* (Estes et al., 1996; González-Gaitán and Jäckle, 1997; Roos and Kelly, 1998), snake (Teng et al., 1999) and mouse (Gaffield et al., 2009) NMJs. In central synapses, an enrichment of endocytic proteins at the edges of the AZs is also probable, as suggested by the observation of ultrafast endocytosis in this location at hippocampal boutons (Watanabe et al., 2013b).

The mechanism by which calcium slows endocytosis after prolonged stimulation remains unclear. It could be that calcium increases the rate of endocytosis during stimulation until the capacity of the endocytic machinery becomes insufficient (Sankaranarayanan and Ryan, 2000). At rest, the endocytic machinery is abundant (Roos and Kelly, 1999), but after several rounds of activity, the consumption of the endocytic proteins may slow the process.

The existence of a "*clathrin-coated ready-to-go pool of vesicles*" at rest has been suggested at the frog NMJ (Miller and Heuser, 1984). The origin of these stranded protein spots is, however, not clear. Do they come from fused vesicles that never lost their identity or, alternatively, result after protein intermixing and sorting? A degree of intermixing between fresh and old vesicle proteins has been proposed to occur in hippocampal synapses during phasic stimulation given that stranded, and newly incorporated vesicle proteins are both internalized during compensatory endocytosis (Fernandez-Alfonso et al., 2006; Wienisch and Klingauf, 2006); the longer the stimulus duration, the greater the intermixing. However, it is also possible that endocytosis of both new and stranded protein patches occur in parallel without previous intermixing (Opazo and Rizzoli, 2010). The development of new tools will provide a deeper understanding of the vesicle membrane components dynamics during phasic and sustained stimulation.

RELEASE SITE REUSE

During ongoing synaptic transmission, release sites are repeatedly used. However, has the release site a refractory period after use? Knowing the number of release sites a presynaptic terminal has and the amount of quanta released during a train of stimulation, it is possible to estimate the mean minimum number of times a site is used and the time interval between uses. For example, at the mouse NMJ, a train of 100 APs at 100 Hz, produces the fusion of about 3200 SVs, which represents the size of the whole RRP of vesicles (1700) plus about 1500 more (Ruiz et al., 2011). If evoked release occurs only within the limits of an AZ, each one hosting two release sites (Nagwaney et al., 2009), and all release sites are used at least once, the "*mean reuse index*" is, in this example, 1.88, resulting from dividing cumulative release by the total number of release sites (3200/1700). In this case, 88% of sites release, clear out of vesicular components, dock, prime, and release again in less than 1 s. In the calyx of Held, a similar time of re-usage has been estimated during the first second of stimulation at 100 Hz (Neher, 2010). At higher frequencies of stimulation, the process could be even faster. Such a rapid clearance of the excess of membrane at the release site could be achieved either by endocytosis *in situ* (kiss-and-run) or by moving the vesicular components to the periAZ from where they are later recycled (**Figure 2**). However, when the membrane load is too large, for example, during sustained high-frequency stimulation, the system becomes less efficient. An excessive membrane accumulation at the periAZ may interfere with the lateral movement of the fused membrane from the AZ to the periAZ, even before depletion of SVs occurs, contributing to short-term depression (Neher, 2010; Hua et al., 2013). Remarkably, translocation, docking, and priming of vesicles during the plateau phase that follows short-term depression can also occur very fast during sustained stimulation. For example, in the cerebellar mossy fiber terminal, this process has been suggested to take 12 ms (Saviane and Silver, 2006), similar to the release site recharging time in some ribbon synapses (Griesinger et al., 2005). These observations suggest that, within a given synapse, not all the release sites has the same capability of being reused at high rates of sustained stimulation. The basis of this heterogeneity is not clear. It could be only apparent if some vesicles fuse outside the "well structured" release sites and this speed up the process (Zenisek et al., 2000; Neher, 2010). Nevertheless, it could also be due to molecular differences in the molecular components involved in docking/priming of the vesicles, to spatial variation in the probability of "*in situ*" endocytosis, and even to disparities in the velocity at which distinct periAZs translocate vesicular components to neighbor regions before endocytosis.

SUMMARY

The structural and functional properties of presynaptic terminals are principal determinants of the successful transmission of information in the nervous system. Nerve terminals differ not only in size but also in the number, shape and dimensions of their AZs and periAZs, as well as in the magnitude of the recycling pool of SVs. Large terminals have hundreds of AZs and release a large number of quanta in response to stimulation. Small terminals possess one or few AZs and release a low number of quanta. The greater exocytic response in large terminals does not imply a higher load of vesicular components at the periAZs, quite the contrary, the distribution of this material in a larger number of units probably facilitates the management of the membrane excess until endocytosis takes place. The preferred mode of endocytosis used under each regime of activity in each synapse type is still controversial, but it seems to be fast after brief stimulation trains, at least at physiological temperature. With sustained repetitive activation, however, endocytosis becomes slower, probably due, among other factors, to the saturation of the endocytosis mechanisms and the subsequent accumulation of vesicular membrane at the periAZ. Remarkably, many presynaptic terminals can sustain a small and almost constant amount of activity (plateau) upon prolonged high-frequency stimulation, apparently supported by a subpopulation of release sites that can operate faster than others. If the basis of such heterogeneity is at the level of the AZ proteins or the periAZs organization remains to be determined.

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RC and LT conceived and wrote the manuscript.

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frontiers in Synaptic Neuroscience

REVIEW
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The Amyloid Precursor Protein—A Novel Player within the Molecular Array of Presynaptic Nanomachines

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More than 20 years ago the amyloid precursor protein (APP) was identified as the precursor protein of the Aβ peptide, the main component of senile plaques in brains affected by Alzheimer's disease (AD). The pathophysiology of AD, characterized by a massive loss of synapses, cognitive decline, and behavioral changes was in principle attributed to the accumulation of Aβ. Within the last decades, much effort has gone into understanding the molecular basis of the progression of AD. However, little is known about the actual physiological function of APPs. Allocating APP to the proteome of the structurally and functionally dynamic presynaptic active zone (PAZ) highlights APP as a hitherto unknown player within the setting of the presynapse. The molecular array of presynaptic nanomachines comprising the life cycle of synaptic vesicles, exo- and endocytosis, cytoskeletal rearrangements, and mitochondrial activity provides a balance between structural and functional maintenance and diversity. The generation of genetically designed mouse models further deciphered APP as an essential player in synapse formation and plasticity. Deletion of APP causes an age-dependent phenotype: while younger mice revealed almost no physiological impairments, this condition was changed in the elderly mice. Interestingly, the proteomic composition of neurotransmitter release sites already revealed substantial changes at young age. These changes point to a network that incorporates APP into a cluster of nanomachines. Currently, the underlying mechanism of how APP acts within these machines is still elusive. Within the scope of this review, we shall construct a network of APP interaction partners within the PAZ. Furthermore, we intend to outline how deletion of APP affects this network during space and time leading to impairments in learning and memory. These alterations may provide a molecular link to the pathogenesis of AD and the physiological function of APP in the central nervous system.

Keywords: Alzheimer's disease, amyloid precursor protein, calcium homeostasis, mitochondria, presynaptic active zone

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Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; APLP2, amyloid precursor like protein 2; APP, amyloid precursor protein; CAST, CAZ-associated structural protein; CAZ, cytomatrix of the active zone; CoQ, coenzyme Q; CoQH2, reduced CoQ; LTCC, L-type calcium channels; Munc, mammalian uncoordinated; NSF, N-ethylmaleimide sensitive fusion protein; PPI, protein-protein interaction network; PAZ, presynaptic active zone; RIM, Rab-interacting molecule; RIM-BP, rab-interacting binding protein; ROS, reactive oxygen species; SNARE, soluble NSF-attachment receptor proteins; SNAP25, synaptosomal associated protein 25; VAMP2, vesicle associated membrane protein2/synaptobrevin2; VDCC, voltage dependent calcium channels.

APP—NETWORKING WITHIN PRESYNAPTIC NANOMACHINES (INTRODUCTION)

The idea of nanotechnology helping to redesign everything at the atomic level goes back to the American engineer Eric Drexler (Drexler, 1987). More than 20 years ago, he published his still controversially discussed book "Engines of Creation" almost at the same time as scientists discovered a protein that fits perfectly in the conceptual idea of nanotechnology. The protein, named amyloid precursor protein (APP), was originally discovered as precursor of amyloid beta (Aβ), the main component of senile plaques and hallmark of Alzheimer's disease (AD; Glenner and Wong, 1984; Kang et al., 1987). Nanomachines are autonomic miniature machines that can work on their own. However, under distinct circumstances they demand on helping hands called assemblers. The vision of autonomic assemblers performing every physical and chemical application, that can have a lasting positive but also negative effect on people's life, would change the way of scientific thinking in a sustainable manner (Drexler, 1987). If we transpose this image to APP, our protein of interest, we can observe similar properties in regulating synaptic development and degeneration. Enzymatic processing, ligand-binding, and dimerization of APP can induce the development and maintenance of neuronal circuits but also their degeneration. Aβ, mainly associated with the pathogenesis of AD can also protect neurons from neurotoxicity. Furthermore, elimination of synapses initiated by Aβ already occurs during the development of neuronal circuits (Kamenetz et al., 2003; Hsieh et al., 2006; Abrahamsson et al., 2007; Wasling et al., 2009). This sensitive balance between physiological benefit and pathophysiological hallmarks is reflected by many processes taking place within a synapse.

Regarding the synapse at the macroscopic level, there are two highly complex nanomachines named pre- and postsynaptic terminal. However, on a microscopic level, both termini can be further subdivided into more restricted nanomachines with specific tasks and assemblers that regulate and control their functions (e. g., neurotransmitter release, signal transduction and reorganization). Within this review, we will focus on the presynaptic terminal with special emphasis on the neurotransmitter release site and its constituent the APP.

Allocating APP to the proteome of the structurally and functionally dynamic presynaptic active zone (PAZ) identified APP as a hitherto unknown player within the setting of the presynaptic nanomachines (Laßek et al., 2013). The molecular array of presynaptic nanomachines comprising the life cycle of synaptic vesicles, exo- and endocytosis, cytoskeletal rearrangements, and mitochondrial activity provides a balance between structural and functional maintenance and diversity (Südhof, 2012; Laßek et al., 2014b, 2015; Weingarten et al., 2014, 2015). The generation of genetically designed mouse models further deciphered APP as an essential player in synapse formation and plasticity (Heber et al., 2000; Wang et al., 2005, 2009b; Ring et al., 2007; Weyer et al., 2011; Hick et al., 2015). Deletion of APP causes an age-dependent phenotype: while younger mice revealed almost no physiological impairments, this condition was changed in the elderly mice (Phinney et al., 1999; Priller et al., 2006; Ring et al., 2007). Interestingly, substantial changes of the proteomic composition of neurotransmitter release sites are already detectable in younger mice (Laßek et al., 2014a). Since APP plays an essential role during the development of neuronal circuits, it was suggested that the amyloid precursor like protein APLP2 compensates for the loss of APP (Weyer et al., 2011; Hick et al., 2015). Therefore, it is tempting to speculate that APP itself can act as a nanomachine with its assembler APLP2. The deletion of the assembler does not account for a severe phenotype. However, at the protein level the abundance of the nanomachine APP becomes increased (Laßek et al., 2014a). Reversely, deletion of the APP can only be compensated by the assembler up to a certain time. Like an electronic device that still works after removing from its charger until the battery has discharged.

Within the presynapse many clusters of nanomachines could be identified. These machines are involved in numerous physiological processes that have to be regulated, coordinated and modified. In the following sections APP will be embedded into selected presynaptic clusters of nanomachines (**Figure 1**).

APP AND SYNAPTIC VESICLE EXOCYTOSIS—ASSEMBLERS AT WORK

Synaptic transmission requires a coordinated network decoding an action potential into a chemical signal at the PAZ. Therefore, consecutive steps including the recruitment of Ca^{2+}-channels, exocytosis and endocytosis are processed by nanomachines and its assemblers. The recruitment of Ca^{2+}-channels as nanomachines is performed by a protein complex comprising the assemblers RIM, Munc13, RIM-BP, α-liprin and CAST-proteins. These assemblers belong to the so called "active zone key proteins", also including bassoon, ELKS and CASK proteins. APP as an assembler is functionally integrated within this conserved network of active zone key players (**Figure 1**). The interaction with bassoon regulates the recruitment of ERC protein 2 (ELKS), RIM, and Munc13 that are essential for docking and priming of synaptic vesicles (Südhof and Rizo, 2011; Südhof, 2012, 2013b).

Exocytosis of synaptic vesicles is mediated by SNARE-complex formation whereby the integral synaptic vesicle protein VAMP2 forms a complex with the presynaptic plasma membrane proteins syntaxin-1 and SNAP25. The SNARE complex is a nanomachine that is characterized by a quadruple α-helix bundle with VAMP2 and syntaxin-1 contributing one α-helix each and SNAP25 contributing two α-helixes. Before the SNARE complex can be formed, syntaxin-1 has to change its conformation from closed (due to binding to the assembler Munc-18); to open (supported by the assembler Munc13). In the open conformation, Munc-18 is still associated with syntaxin-1 supporting the interaction between SNARE motifs derived from syntaxin-1 and SNAP25 (Südhof, 2013b). The resulting donor-complex further binds to VAMP2 assembling to a Munc-18-SNARE complex, with Munc-18 being supposed

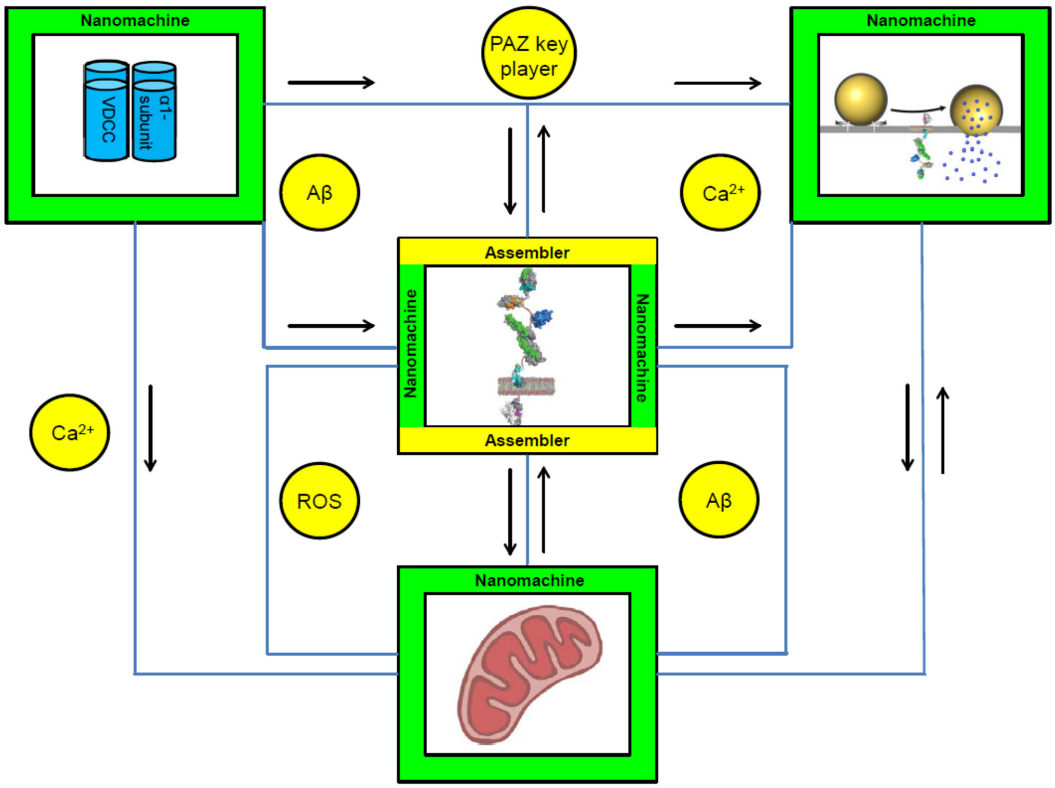

FIGURE 1 | Conceptual circuit integrating nanomachines and assemblers within the presynaptic active zone (PAZ). APP is embedded into the circuit of nanomachines within the PAZ. It can act within different physiological processes at the presynaptic terminal including synaptic vesicle exocytosis, Ca^{2+}-homeostasis and mitochondrial function. A well balanced proportion between the machines and their assemblers is the prerequisite for the development and maintenance of the neuronal circuits. Nanomachines are visualized as green squares; assemblers as yellow circles. APP can act as a nanomachine and as an assembler—the square is therefore colored in green and yellow. Black arrows represent the interactions between individual nanomachines. Connecting lines are highlighted in blue. Depicted are: α-subunit of voltage dependent Ca^{2+}-channels (VDCC); exocytosis of SV, with APP embedded into the presynaptic plasma membrane; illustration of APP; mitochondria. Abbreviations: Aβ, amyloid-beta; Ca^{2+}, calcium ion; PAZ, presynaptic active zone; ROS, reactive oxygen species.

to mediate interactions between the four-helix bundle and the plasma membrane necessary for membrane fusion (Deák et al., 2009). Furthermore, Munc-18 does not detach during the SNARE complex assembly/disassembly cycle, emphasizing its essential role in exocytosis (Südhof, 2013a). APP plays a crucial role in synaptic vesicle exocytosis and further emphasizes the compensatory role of APLP2 in APP-KO mice. Moreover, the interaction of APP with presynaptic proteins involved in the regulation of exocytosis and the putative function in tuning this process is important to unravel the physiology of APP within the CNS (Fanutza et al., 2015).

Within the molecular nanomachinery of SNARE-complex formation, α-synuclein is an indispensable assembler. The role of α-synuclein during SNARE complex formation is the implementation of VAMP2 into the SNARE donor complex (Burré et al., 2010). Therefore, the interaction between synaptophysin and VAMP2 has to be repealed before complete vesicle fusion can take place (Valtorta et al., 2004) putatively mediated by α-synuclein. At protein level, deletion of α-synuclein does not alter the abundance of SNARE proteins, but diminishes the ability of SNARE complex formation. In terms of nanotechnology, loss of the assembler accounts for severe impairments of the nanomachine. Interestingly, α-synuclein and APP are both assemblers within this highly complex SNARE-machinery, but they act differently. While α-synuclein mediates SNARE-complex formation, APP might execute a regulatory function during docking and priming of synaptic vesicles. After neurotransmitter release, the assembler NSF mediates the hydrolysis of ATP and triggers the disassembling of the SNARE nanomachine (Littleton et al., 2001).

APP AND CALCIUM—PARTNERS IN CRIME

During the mid-1980's when APP was discovered as precursor protein of Aβ, another crucial player within the pathogenesis of AD was identified—calcium (Khachaturian, 1987; Landfield, 1987; Landfield et al., 1989; Small et al., 2009). APP and calcium share some interesting features that interconnect them as partners in crime. Moreover, they fit perfectly into the conceptual

idea of nanomachines and assemblers. They can operate as neurotrophic assemblers supporting proper physiological function of the presynaptic terminal, but they also trigger neurodegeneration.

Ca^{2+}-influx through voltage-dependent channels within the presynaptic terminal is essential for neurotransmitter release (Mattson, 2007). The complex protein machinery (Südhof, 2012) that ensures the presence of voltage dependent calcium channels (VDCC) at active zones after the arrival of action potentials comprises a varying set of assemblers (**Figure 1**). These assemblers have to act in a consecutive manner to provide a rapid increase in intracellular Ca^{2+}. However, the elevation of intracellular Ca^{2+}-levels is only transiently and residual amounts of Ca^{2+} have to be removed quickly to avoid adverse effects on neurons (Mattson, 2007; Bezprozvanny and Mattson, 2008; Small et al., 2009).

Disruption of Ca^{2+}-homeostasis can account for neuronal dysfunction and neurodegeneration. In this context, Aβ oligomers have been discussed to induce increase intracellular Ca^{2+}-levels, thereby altering synaptic signaling and changing the activity of neighboring neurons. Since neuronal activity is under the control of a neuronal network, systematic failure within this network account for alterations within the network circuit (Small, 2008; Small et al., 2009). The vicious cycle describing the maintenance of the neuronal signaling within the network is based on synaptic compensation (scaling). Aβ oligomers induces elevated Ca^{2+}-influx, that accounts for synaptic dysfunction, followed by synaptic scaling and consequently increased excitability with in turn leads to elevated intracellular Ca^{2+}. At the end, healthy neurons degenerate due to Ca^{2+}-dysregulation and synaptic dysfunction (Small, 2008). Interestingly, the abundance of VDCC, in particular L-type calcium channels (LTCC), is also increased during aging and in AD (Thibault and Landfield, 1996; Thibault et al., 2001).

APP is involved in the recruitment of VDCC (**Figure 1**) and the regulation of their abundance at hippocampal neurotransmitter release sites. The hippocampus, a brain region of interest regarding learning and memory consolidation, is highly susceptible for excitotoxicity and neurodegeneration. Both can be induced and triggered by long-lasting elevated intracellular Ca^{2+}-levels. Moreover, diminished endocytosis of VDCC in the absence of APP accounts for a dysregulation of the balance between inhibitory and excitatory neurons. GABAergic hippocampal neurons revealed increased activity due to an increased abundance of VDCC at presynaptic terminals (Yang et al., 2009). Moreover, LTP deficits in APP double and single mutant mice were rescued by application of $GABA_A$ receptor inhibitor picrotoxin (Fitzjohn et al., 2000; Weyer et al., 2011). This molecular interpretation of changes in calcium homeostasis and subsequent alterations in synaptic plasticity was manifested by electrophysiological network analysis. In this context, it was proposed that APP deletion induces an altered neuronal excitation-inhibition ratio (Korte et al., 2012). Within the hippocampus memory formation is based on a variety of synchronized network oscillations that are regularly synchronized between the CA1 and CA3 region (Korte et al., 2012). As inhibitory interneurons play an essential role in synchronizing these oscillations they can affect a large population of pyramidal neurons, inhibit specific input pathways and guarantee for a high background-to-noise ratio (Mann and Paulsen, 2007). This might explain the reported alteration in the excitation-inhibition ratio (Korte et al., 2012). Similarly, alterations during the pathogenesis of AD such as changes in personality, sleep disturbance and changes in awareness could be traced back to a dysregulation of the glutamate-GABA metabolism (Robinson, 2000; Doert et al., 2015). GABA can be produced by the so called GABA shunt, a bypass mechanism that skips the intra-mitochondrial α-ketoglutarate-dehydrogenase (Mamelak, 2012). This mechanism becomes operative when the metabolism of glutamate is enhanced probably due to reduced glutamine synthase activity. Elevated GABA levels in transgenic APP mutant mice, carrying the Swedish and London mutation for APP, (Doert et al., 2015) reflect a situation for the glutamate-GABA ratio similar to that observed for APP knockout mice (Weyer et al., 2011; Korte et al., 2012). Both phenotypes indicate a critical role of APP in memory formation and consolidation as well as behavioral aspects that are severely affected in in the respective mutants and in AD patients (Ring et al., 2007; Doert et al., 2015).

APP AND MITOCHONDRIA—NANOMACHINES AND ASSEMBLERS TWO-IN-ONE

Within the presynaptic terminal, mitochondria are essential nanomachines, providing energy supply and calcium buffering for a large variety of physiological functions. However, mitochondrial dysfunctions are in focus of playing an important role in the pathology of AD.

Considering the pros and cons of nanomachines at work as described above for APP and Aβ, we can outline a similar feature for mitochondria that can be physiological but also pathophysiological. Both subjects can function as nanomachine and assembler depending on the perspective. Mitochondria produce reactive oxygen species (ROS) that are assemblers (**Figure 1**). The amount of ROS is in general regulated by oxygen donor concentration and enzymes of the electron transport chain. Oxygen level and donor concentration trigger the amount of ROS in a linear manner. ROS, produced as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) at complex I and complex III of the electron transport chain need to be critically regulated (Leuner et al., 2012a). Within the mitochondrial matrix ROS production depends critically on the proton gradient (Δp), the $NADH/NAD^+$ and $CoQH_2/CoQ$ ratios and the local O_2 concentration. Superoxide anions are mainly produced at complex I. Under physiological conditions, the production of $O_2^{\bullet -}$ at complex III is insignificant as compared to the production rates by complex I (Murphy, 2009). Therefore, the outcome "oxidative stress" is on the one hand initiated by the mitochondria themselves, but at the same time affects its producer as first objective (Harper et al., 2004; Korge et al., 2008; Poyton et al., 2009; Müller et al., 2010). Besides ROS, dysfunction

of the entire respiratory system, associated by a decrease in mitochondrial membrane potential and reduced levels of ATP, account for impairments in mitochondrial function and trigger the early onset of neurodegeneration (Rhein et al., 2009; Wang et al., 2009a; Leuner et al., 2012a). APP and Aβ have been directly associated with mitochondrial function (Leuner et al., 2010, 2012a; Eckert et al., 2012) and in this context, it would be of great interest to study the effect of APP deletion on mitochondrial function at neurotransmitter release sites.

Neuronal communication is based on reliable functioning of synaptic vesicle exo- and endocytosis. Already slight alterations can disrupt this procedure. In terms of our topic nanomachines and its assemblers, APP and mitochondria both provide a clear picture of a classical nanomachine and their proteolytic and/or metabolic products are assemblers (**Figure 1**). The balance between physiological benefit and pathophysiological effect is fragile. Similar to the sensitive Ca^{2+}-homeostasis, imbalance between ROS and/or Aβ causes mitochondrial dysfunction, membrane protein and membrane lipid modifications (Eckert et al., 2003). Generation of ROS occurs along the electron transport chain at complex I and complex III. Recently, Leuner et al. (2012b) identified complex I in conjunction with elevated ROS levels as starting point for mitochondrial dysfunctions and onset of the amyloidogenic cascade in AD. ROS can trigger the production of Aβ via enhancing the enzymatic activity of BACE1 and γ-secretase. Interestingly, Aβ oligomers, as well as Aβ fibrils can account for a decrease in mitochondrial membrane potential and ATP levels. This phenotype has already been detected in young transgenic mice prior to Aβ plaque formation (Leuner et al., 2012a,b). Conversely, patients with mitochondrial disorders like mitochondrial encephalopathy, lactic acidosis, stroke-like episodes (MELAS) show cognitive impairments, behavioral decline and AD-like plaque formation in the absence of familiar AD evidence (Kaido et al., 1996).

It is widely accepted that changes in the interplay between APP, Aβ and mitochondrial function are likely to correlate with the onset of neurodegenerative diseases (Eckert et al., 2012; Leuner et al., 2012a). Therefore, novel therapeutic strategies that account for mitochondrial protection will be a promising approach for AD treatment or prevention.

CONCLUSION AND OUTLOOK

Assemblers and nanomachines execute different but synergistic functions within the PAZ. By communicating with individual nanomachines, assemblers can control, navigate and trigger physiological functions at the presynaptic terminal. Their operations are based on an appropriate balance accompanied by a variety of interaction- and combination possibilities (e. g., SNARE complex formation and Ca^{2+}-signaling). If this balance is dysregulated (e. g., increase in Aβ, ROS or calcium) and extend physiological compensation mechanisms, pathophysiological hallmarks (senile plaque formation, mitochondrial dysfunction and neurodegeneration) will increase alarmingly.

One important player within this conceptual idea of nanomachines is APP. Within the molecular array of presynaptic nanomachines APP can equally act as an individual nanomachine and assembler at once. It participates in crucial steps like synaptic vesicle exocytosis and Ca^{2+}-homeostasis and has a major impact on proper mitochondrial function. All these interplays depend on a highly coordinated proteinaceous network. Unravelling how APP is embedded into individual networks within neurotransmitter release sites and how disturbances of the network may account for neurodegenerative diseases needs to be addressed in future studies. Until now, data derived from APP mutant mice (transgenic Alzheimer mouse models or knockout mouse models) clearly point to an essential role of APP in synaptic development, function and plasticity. Changes at the behavioral level (e. g., cognitive decline, impairments in LTP and memory) are characteristic for all APP mutants and reveal a time depended development.

One possibility to address the question how APP is embedded into the presynaptic nanomaschine is provided by bioinformatic tools. The mathematical field of graph theory allows the integration of all possible physical and functional relations between proteins into a single model. This model is called a protein-protein interaction network (PPI). By analyzing the topological properties of such a PPI network the key molecular players are identified and classified. These properties are used to assign a topological role to a protein of interest. Whether a protein is a highly connected hub or a less connected linker between functional modules is indicated by its topological properties in the PPI network and adds another layer of information.

The pure topological model can be augmented with additional information using, e.g., gene ontology terms and protein localizations. The understanding of the functional composition of the PAZ is facilitated by applying community detection methods. These methods detect highly connected clusters of proteins. The highly connected clusters of proteins are assumed to act within the same biological processes. By analyzing the biological functions represented by the individual communities a global picture of interconnected functions and pathways arises.

If one takes all these individual approaches together, one can design a molecular network of nanomachines and their assemblers to gain further insights into the complex physiology of presynaptic functions. Since APP affects this network during space and time, leading to the development and the loss of synapses, it is essential to understand how APP acts within these individual networks. Currently, it is not known which physiological function APP is executing but it is obvious that APP plays an indispensable role in proper synaptic function—including development and degeneration.

AUTHOR CONTRIBUTIONS

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REVIEW
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Presynaptic Molecular Determinants of Quantal Size

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The quantal hypothesis for the release of neurotransmitters at the chemical synapse has gained wide acceptance since it was first worked out at the motor endplate in frog skeletal muscle in the 1950's. Considering the morphological identification of synaptic vesicles (SVs) at the nerve terminals that appeared to be homogeneous in size, the hypothesis proposed that signal transduction at synapses is mediated by the release of neurotransmitters packed in SVs that are individually uniform in size; the amount of transmitter in a synaptic vesicle is called a quantum. Although quantal size—the amplitude of the postsynaptic response elicited by the release of neurotransmitters from a single vesicle—clearly depends on the number and sensitivity of the postsynaptic receptors, accumulating evidence has also indicated that the amount of neurotransmitters stored in SVs can be altered by various presynaptic factors. Here, I provide an overview of the concepts and underlying presynaptic molecular underpinnings that may regulate quantal size.

Keywords: synaptic vesicle, quantal size, VGLUT, VGAT, V-ATPase

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INTRODUCTION

Synaptic transmission requires the release of neurotransmitters from presynaptic terminals. Since the pioneering work by Katz and colleagues in the frog neuromuscular junction and the morphological identification of synaptic vesicles (SVs) of apparently homogeneous size at presynaptic terminals by electron microscopy, neurotransmitters have been believed to be packed in SVs and released from them as discrete and uniform "quanta". However, emerging evidence has suggested that quantal response, which is a postsynaptic current elicited by the fusion of a single vesicle, in the mammalian central nervous system (CNS) exhibits a certain degree of variation. Because postsynaptic receptors at mammalian glutamatergic synapses are not usually saturated by release of neurotransmitters from a single SV, possible sources of the variations in quanta are presynaptic. In fact, detailed investigations at central glutamatergic synapses called the calyx of Held have revealed that the vesicular glutamate concentration is a plausible source of quantal variations (Wu et al., 2007). In this review, I will introduce our current molecular knowledge and the possible presynaptic determinants responsible for the regulation of quantal size.

MINIMAL MOLECULAR COMPLEXES FOR THE FORMATION OF QUANTUM IN SVs

Neurotransmitters are usually synthesized in the presynaptic cytoplasm and are concentrated into SVs by the activity of vesicular transporters specific for the respective neurotransmitters. All known vesicular transporters responsible for neurotransmitter uptake into SVs utilize

a proton electrochemical gradient ($\Delta\mu H^+$) generated by the vacuolar-type H^+ ATPase (V-ATPase). The V-ATPase consists of at least 13 subunits with a total molecular weight of ~800 kDa and represents the largest molecular complex on SVs. The V-ATPase is divided into two functionally distinct portions. The V_1 part consists of one large transmembrane protein (a1 subunit) in association with several globular A and B subunits and catalyzes ATP hydrolysis, releasing energy for proton transfer. The V_0 part forms a ring-like structure in the membrane and provides a proton permeation pathway. By transferring protons into the SV lumen, the V-ATPase generates both a pH gradient (ΔpH) and a membrane potential (inside positive voltage referred to $\Delta\Psi$) across the SV membrane. The vesicular transporters for classical neurotransmitters utilize ΔpH, $\Delta\Psi$, or both, depending on the neurotransmitter type. Five classes of vesicular transporters have been cloned and molecularly characterized so far, including two vesicular monoamine transporters (VMAT1, VMAT2), vesicular acetylcholine transporter (VAChT), three vesicular glutamate transporters (VGLUT1–3), vesicular GABA/glycine transporter (VGAT; also referred to as vesicular inhibitory amino acid transporter, VIAAT; Edwards, 2007), and vesicular nucleotide transporter (VNUT; Sawada et al., 2008). Biochemical assays using isolated vesicles in the presence of compounds that selectively dissipate either ΔpH or $\Delta\Psi$ revealed energy requirements for their uptake. The uptake of cationic transmitters such as acetylcholine and biogenic amines largely depends on ΔpH, whereas that of anionic transmitters such as glutamate predominantly depends on $\Delta\Psi$. The uptake of zwitterionic and therefore neutral transmitters such as GABA and glycine depends on both ΔpH and $\Delta\Psi$. Despite extensive studies on the bioenergetics of the transport process, the details of the mechanisms of transport, especially how H^+ differentially drives transport of different neurotransmitters, remain enigmatic. In addition to neurotransmitter transporters and the V-ATPase, potential modulators that may alter the driving force for neurotransmitter uptake also play a role, including ion channels/transporters on SVs (**Figure 1**). In this review, I will introduce key components that underlie the formation and regulation of quanta with a special focus on glutamate and GABA, the major excitatory and inhibitory neurotransmitters in the mammalian CNS, respectively.

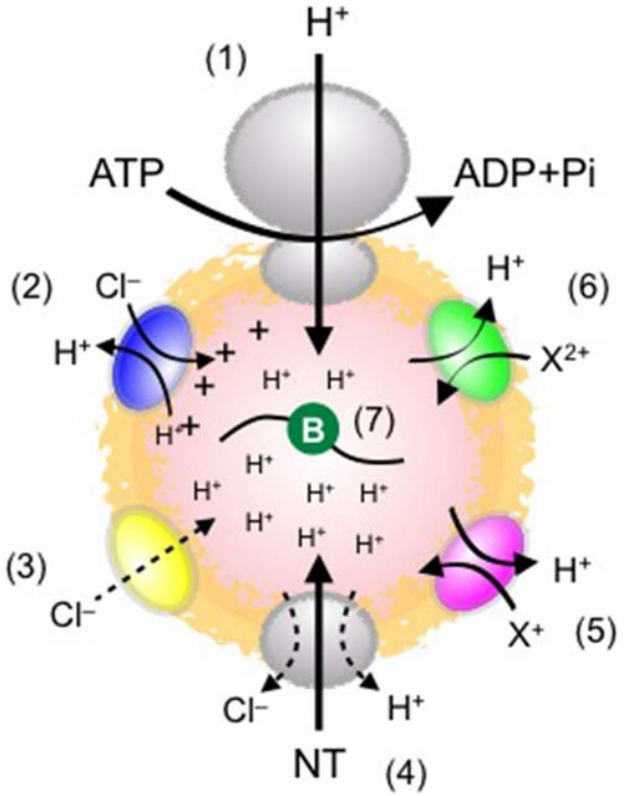

FIGURE 1 | Schematic diagram of possible elements influencing $\Delta\mu H^+$ and quantal size. (1) Vacuolar-type H^+ ATPase (V-ATPase), which drives neurotransmitter transport into SVs. ATP concentrations affect the proton-transporting activity. (2) ClC-type Cl^- channel family. Some ClC isoforms operate as a $2Cl^-/H^+$ exchanger. This stoichiometry seems to alkalize the vesicle lumen, but may also dissipate $\Delta\Psi$, thereby facilitating ΔpH. (3) A putative Cl^- channel. This provides a shunting current for H^+, leading to acidification. The molecular identity of the Cl^- conductance has yet to be determined. (4) Vesicular neurotransmitter transporters. Their expression levels affect quantal size. How transporters are activated by H^+ and how Cl^- modulates the transport remain obscure. (5) Cation/H^+ exchanger. This activity potentially dissipates ΔpH and facilitates $\Delta\Psi$, leading to an increase in $\Delta\Psi$-driven glutamate transport. (6) Divalent cation/H^+ exchanger. This activity plays essentially the same role as (5). The molecular identity and physiological relevance of the system remain unknown. (7) Luminal buffers (symbolized by green circle). The buffering capacity of the lumen conferred by intrinsic proteins, lipids, and the intravesicular solution affects the formation of ΔpH (presumably also $\Delta\Psi$), thereby influencing quantal size.

EXPRESSION LEVEL OF THE TRANSPORTERS

Intuitively, we expect that expression of transporter proteins on a SV will affect the rate of transport rather than the amount of transmitters at equilibrium. However, overexpression of vesicular transporters in primary cultured neurons and *in vivo* in fruit fly embryos as well as other model systems such as PC12 cells results in an increase in neurotransmitter content in individual vesicles (Pothos et al., 2000; Daniels et al., 2004; Wojcik et al., 2004; Wilson et al., 2005; **Table 1**). In some cases, enlargement of vesicles is associated with an increase in quantal size (Daniels et al., 2004), although it is unknown whether this can be explained simply by swelling of the vesicles due to alterations in osmolality or if insertion of phospholipids into the vesicles is also involved. In contrast to this view, analyses of VGLUT heterozygous mice revealed somewhat conflicting results. Suppression of VGLUT1 in hippocampal neurons derived from $VGLUT1^{+/-}$ mice does not produce a significant reduction in miniature excitatory postsynaptic currents (mEPSC; Fremeau et al., 2004; Wojcik et al., 2004), whereas $VGLUT2^{+/-}$ neurons that originate from the striatum exhibit reduced mEPSCs (Moechars et al., 2006). Nevertheless, because each SV contains ~10 copies of VGLUT, a robust change in expression levels may be necessary to induce detectable changes in quantal size (Takamori et al., 2006).

TABLE 1 | Gene manipulations of vesicular transporters/channels influence quantal size.

Gene	Manipulation	Preparation	Quantal size	Reference
VGLUT1	Overexpression	Mouse hippocampal culture	Glu ↑	Wilson et al. (2005) and Wojcik et al. (2004)
	Heterozygous	Mouse hippocampal culture	Glu →	Wojcik et al. (2004) and Fremeau et al. (2004)
	Knockout	Mouse hippocampal culture	Glu ↓	Wojcik et al. (2004)
	Knockout	Mouse hippocampal culture	Glu →	Fremeau et al. (2004)
VGLUT2	Heterozygous	Mouse striatal culture	Glu ↓	Moechars et al. (2006)
	Knockout	Mouse striatal culture	Glu ↓	Moechars et al. (2006)
DVGLUT*	Mutants	Neuromuscular junction	Glu → or zero	Daniels et al. (2006)
	Overexpression#	Neuromuscular junction	Glu ↑	Daniels et al. (2004)
VGAT	Heterozygous	Mouse striatal culture	GABA →	Wojcik et al. (2006)
	Knockout	Mouse striatal culture	GABA ↓	Wojcik et al. (2006)
VMAT2	Overexpression#	Rat ventral midbrain culture	Dopamine ↑	Pothos et al. (2000)
ClC-3	Knockout	Acute hippocampal slice	Glu →, GABA →	Stobrawa et al. (2001)
	Knockout	Acute hippocampal slice	GABA ↓	Riazanski et al. (2011)
	Knockout#	Hippocampal culture	Glu ↑	Guzman et al. (2014)

**DVGLUT stands for drosophila VGLUT. Glu stands for glutamate. #Enlargement of vesicles were observed by electron microscopy.*

Is the expression of vesicular neurotransmitter transporters regulated in physiological or pathological conditions? Several lines of evidence have suggested that this is the case. First, *VGLUT1* and *VGLUT2* were originally identified as genes that are up-regulated upon stimulation in neurons and endocrine cell lines (Ni et al., 1994; Aihara et al., 2000). Second, VGLUT1 expression is developmentally up-regulated, which seems to be accompanied by an increase in mEPSCs (De Gois et al., 2005; Yamashita et al., 2009). Third, expression of VGLUT1, VGLUT2, and VGAT in cultured hippocampal neurons is altered by manipulations that change neural activities, i.e., exposure to tetrodotoxin and antagonists for the respective neurotransmitter receptors (De Gois et al., 2005). Fourth, VGLUT3 expression in some brain regions is very transient, resulting in biphasic expression profiles (Gras et al., 2005). Finally, in addition to the total expression levels, the extent of VGLUT sorting to the plasma membrane may regulate the vesicular level of VGLUT proteins, which oscillate during light/dark cycles (Darna et al., 2009). Collectively, these observations suggest that temporal regulation of expression of vesicular transporters for neurotransmitters scales and shapes synaptic transmission and thus network activity in the brain.

REGULATION OF THE PROTON ELECTROCHEMICAL GRADIENT

ATP

Although little is known about regulation of V-ATPase activity *in vivo*, several factors affect the activity, and therefore, neurotransmitter uptake. First, cytoplasmic ATP concentrations may be a key factor in regulation. In isolated SVs, glutamate uptake reaches a plateau with 2 mM ATP, which also requires Mg^{2+} in low mM concentrations (Naito and Ueda, 1985). The cytoplasmic ATP concentration in the presynaptic terminals of rat hippocampal cultured neurons is ∼2 mM in resting conditions. During neural activity, as cytoplasmic ATP is rapidly consumed mainly by the process of endocytosis, ATP concentrations decrease to ∼1 mM during repetitive stimulations (Rangaraju et al., 2014). Therefore, the physiological concentration range of ATP may impact the activity of V-ATPase, which in turn alters the kinetics and/or extent of glutamate refilling of SVs. Furthermore, in some pathological conditions such as anoxia, hypoglycemia, and ischemia, dramatic ATP breakdown may occur, leading to reductions in V-ATPase activity and neurotransmitter refilling (Santos et al., 1996).

Cl^- Channel

V-ATPase is an electrogenic pump, and therefore, the pump does not transport much H^+ unless counter-ion movements across the SV membrane are present that abolish the voltage due to the H^+ flux (Moriyama and Nelson, 1987). Studies with biochemical assays using acridine orange as a pH gradient indicator have shown that membrane-permeable Cl^- confers a shunting current for the H^+ movement, which inhibits the formation of $\Delta\Psi$, and in turn, promotes the pH gradient (Maycox et al., 1988; Cidon and Sihra, 1989; Tabb et al., 1992). Accordingly, with high extravesicular (cytosolic) Cl^-, uptake of cationic neurotransmitters is maximal because ΔpH is large, whereas glutamate uptake, which is driven primarily by $\Delta\Psi$, is minimal (Hell et al., 1990). Interestingly, glutamate uptake in the absence of Cl^-, where $\Delta\Psi$ is maximal, does not show the highest activity (Naito and Ueda, 1985), leading to the hypothesis that Cl^- allosterically activates VGLUTs (Hartinger and Jahn, 1993; Wolosker et al., 1996; see also below); however, contributions of ΔpH or low luminal pH have also been suggested (Tabb et al., 1992; Bellocchio et al., 2000; Schenck et al., 2009). Consistent with biochemical assays with isolated SVs in which the highest glutamate transport is observed in the presence of a Cl^- concentration in the range of ∼10 mM, efficient glutamate refilling of SVs

assessed in the calyx of Held synapses requires 5–30 mM cytosolic Cl^- (Hori and Takahashi, 2012). GABA uptake also exhibits biphasic Cl^- dependance, albeit to a lesser extent (Hell et al., 1990). Although GABAergic SVs also contain Cl^- channel activity (Takamori et al., 2000), the contributions of the channel activity to GABA transport have not been clarified.

The molecular identity of the putative Cl^- channel on SVs has not been firmly established. One member of the ClC chloride channel family, ClC-3, is localized in SVs, and the SV fraction derived from ClC-$3^{-/-}$ mice shows a reduced Cl^--induced acidification, suggesting that ClC-3 is the Cl^- channel on SVs (Stobrawa et al., 2001). In addition, however, both glutamate-induced acidification and glutamate transport activity are dramatically reduced in ClC-3-deficient SV fractions (Note that if ClC-3 were the Cl^- channel on SVs, the absence of ClC-3 would result in a reduction in ΔpH, leading an increase in glutamate transport). These phenotypes are associated with a reduction in VGLUT1 protein expression, which may be due to the severe neurodegeneration of the ClC-$3^{-/-}$ brain, including total loss of the hippocampus. Contrary to these biochemical experiments, electrochemical assessments of mEPSCs (and also miniature inhibitory postsynaptic currents, mIPSCs) in acute hippocampal slices from ClC-$3^{-/-}$ mice did not show significant changes compared to wild-type preparations. More recently, the contribution of ClC-3 to glutamatergic neurotransmission was challenged by using cultured hippocampal neurons established from postnatal day 1 mice to avoid alterations in neuronal functions due to neurodegeneration (Guzman et al., 2014). In this preparation, both mEPSCs and evoked EPSCs were increased in ClC-$3^{-/-}$ neurons, supporting the proposal that ClC-3 contributes to SV acidification. Loss of ClC-3 produces additional phenotypes including an increased release probability and an enlargement of SVs, suggesting multiple roles for ClC-3 in presynaptic physiology. Furthermore, contrary to the original report by Stobrawa et al. (2001), ClC-3 may also contribute to GABA transport into SVs. mIPSCs recorded from hippocampal CA1 pyramidal neurons from ClC-$3^{-/-}$ mice have lower amplitudes than those from wild-type mice (Riazanski et al., 2011). Further studies will be necessary to clarify these contradictory results (see **Table 1**).

An alternative candidate for the Cl^- channel on glutamatergic SVs is VGLUTs themselves. When SV fractions derived from VGLUT1$^{-/-}$ mice were examined in an acidification assay, a drastic reduction in Cl^--induced acidification was observed both in 3- and 8-week-old mice. In contrast, acidification of SVs derived from 3-week-old ClC-$3^{-/-}$ mice is not significantly different from that of SVs derived from wild-type mice, indicating that the acidification deficit observed in adult ClC-$3^{-/-}$ mice is due to the massive neurodegeneration of the VGLUT1-expressing region (Schenck et al., 2009). The Cl^--induced acidification can also be functionally reconstituted in proteoliposomes only when recombinant VGLUT1 protein is included (Schenck et al., 2009; Preobraschenski et al., 2014). An unanswered question is whether one of the two proteins or both is responsible for the Cl^- permeation pathway in SVs.

Cation/H^+ Exchanger

A proteomic study of purified SVs identified a Na^+/H^+ exchanger (Grønborg et al., 2010), and addition of a high concentration of Na^+ as well as K^+ decreases ΔpH and increases $\Delta\Psi$ as intravesicular H^+ is exchanged by K^+ or Na^+, providing favorable conditions for glutamate transport (Goh et al., 2011). Furthermore, replacing cytoplasmic K^+ with a non-permeable cation attenuates the quantal size recorded from the calyx of Held synapses. Also, increasing the presynaptic Na^+ concentration facilitates the mEPSC amplitude (Huang and Trussell, 2014), indicating physiological significance for the cation/H^+ exchange in regulating quantal size. In the case of Na^+, presynaptic hyperpolarization-activated cyclic nucleotide-gated (HCN) channels may play an important role in controlling the presynaptic Na^+ concentration. Intriguingly, VGLUTs themselves may confer Na^+/H^+ exchanger activity when reconstituted in liposomes, although direct evidence for the movement of monovalent cations through VGLUTs is still lacking (Preobraschenski et al., 2014).

Ca^{2+}/H^+ Antiport

In analogy to the role of monovalent cations in the regulation of $\Delta\mu H^+$, the existence of a putative Ca^{2+}/H^+ antiport system in SVs was demonstrated by using isolated SVs (Gonçalves et al., 1998). Addition of Ca^{2+} ($\sim$600 μM) causes a slight reduction in acidification as monitored by acridine orange, indicating H^+ efflux associated with Ca^{2+} influx (Gonçalves et al., 1999). In an independent experiment, ATP-dependent $^{45}Ca^{2+}$ transport into SVs was also demonstrated. Other divalent cations such as Zn^{2+} and Cd^{2+} also reduce acidification, albeit with higher efficiency. Because both Zn^{2+} and Cd^{2+} attenuate ATP-dependent Ca^{2+} uptake in a concentration-dependent manner, a common transporter may share the substrates (Gonçalves et al., 1999). Because glutamate uptake is predominantly driven by $\Delta\Psi$, the Ca^{2+}/H^+ antiport was expected to increase glutamate uptake by converting ΔpH to $\Delta\Psi$. However, the effect of Ca^{2+} on glutamate uptake depends on external Cl^- concentrations, indicating a complex interplay among ΔpH, $\Delta\Psi$, and Cl^--dependent activation of VGLUTs (Gonçalves et al., 2001). Interestingly, Ca^{2+}-induced "de-acidification" is decreased when the Ca^{2+} sensor protein, synaptotagmin 1, is knocked down in PC12 cells (Cordeiro et al., 2013). The precise mechanisms and physiological relevance in SVs are poorly understood.

Luminal Buffers

Because SVs are recycled at presynaptic terminals, and newly endocytosed SVs may engulf extracellular solution, the ionic composition as well as the buffering strength of the extracellular solution may impact the formation of $\Delta\mu H^+$. In cultured hippocampal neurons expressing synaptopHluorin (a luminal pH sensor), application of 100 mM Tris buffer strongly inhibits re-acidification of newly endocytosed SVs (Ertunc et al., 2007), although the effect on the $\Delta\Psi$ component remains unclear.

Even with lower buffer concentrations, i.e., 15 mM, short-term depression is facilitated, indicating that neurotransmitter refilling is suppressed when a solution with a high buffering capacity is incorporated into SVs during recycling. This was clearly observed when evoked IPSCs were recorded from CA1 pyramidal neurons in an acute hippocampal slice preparation in the presence of 50 mM Tris. Thus, the buffering strength of the extracellular solution critically affects ΔpH (and probably also $\Delta\Psi$), leading to alteration of quantal size.

Another source of luminal H^+ buffer may be intrinsic membrane proteins and lipids that cover the luminal surface of SVs. A recent study estimated that the SV lumen in cultured hippocampal neurons confers relatively high buffering capacity (56 mM/pH) that requires ~1200 H^+ influx into a SV to establish a ΔpH of 1.8 (Egashira et al., 2015). Because neurotransmitter content and the intravesicular solution (which may be equivalent to the extracellular bath solution) should not contribute much to the luminal buffering capacity, the luminal domains of the intrinsic membrane proteins are likely the acceptor for protons, in good agreement with the extremely high protein density in SVs (Takamori et al., 2006). Although activity-dependent or homeostatic alterations in expression levels of vesicle proteins have often been demonstrated (De Gois et al., 2005; Wilson et al., 2005), possible contributions of these changes in protein levels to regulation of the V-ATPase activity and the quantal size have not been considered.

VESICULAR SYNERGY

Identification of the proton-driven neurotransmitter transporters and the characterization of their localizations in the nervous system have revealed that some of them are co-expressed in the same presynaptic terminals and even on the same vesicles. In particular, VGLUT3 is expressed in subsets of neurons that had not been considered to be glutamatergic neurons, including GABAergic interneurons in the hippocampus, amacrine cells in the retina, and cholinergic neurons in the striatum (Fremeau et al., 2002; Gras et al., 2002). Furthermore, VGLUT2 is also present in a subpopulation of striatum dopaminergic neurons as well as cultured dopaminergic neurons and contributes to co-release of dopamine and glutamate from the same neurons (Dal Bo et al., 2004, 2008).

In addition to enabling co-release of glutamate with other transmitters, evidence is emerging that suggests that the expression of VGLUTs facilitates the transport of other cationic transmitters. For instance, vesicular transport of glutamate results in increased acetylcholine transport in vesicles isolated from the striatum, and this facilitation is diminished in VGLUT3$^{-/-}$ mice (Gras et al., 2008). Moreover, analysis of the hippocampus and prelimbic cortex from VGLUT2$^{-/-}$ mice demonstrated that glutamate transport into VMAT2-carrying SVs stimulates transport of serotonin (Hnasko et al., 2010). Such synergistic effects of glutamate transport on the uptake of other neurotransmitters were named "vesicular synergy" (see El Mestikawy et al., 2011). Vesicular synergy was also shown for GABA uptake in the presence of glutamate in isolated vesicles (Zander et al., 2010), but such "vesicular synergy" in neurons co-releasing glutamate and GABA turned out to be unlikely, at least in some experimental conditions (Case et al., 2014; Zimmermann et al., 2015). Nevertheless, these distinct synergistic effects of glutamate co-transport on refilling of other transmitters may involve VGLUT-dependent acidification of SVs. In the presence of VGLUTs, both glutamate transport and Cl^- influx through VGLUT facilitates vesicle acidification (Maycox et al., 1988; Schenck et al., 2009). Transport of cationic transmitters may be more sensitive to acidification than that of GABA, because transport of cationic transmitters is primarily driven by ΔpH. Conversely, transport of cationic transmitters may prevent efficient glutamate transport into the same SVs. Because transport of cationic transmitters involves net efflux of one positive charge with the proposed stoichiometry of the substrate of H^+ = 1:2 (Nguyen et al., 1998), consequent reduction of $\Delta\Psi$ may attenuate glutamate transport.

ALLOSTERIC REGULATION OF THE TRANSPORTER ACTIVITY

In addition to the observation that Cl^- ions affect the proton electrochemical gradient and in turn influence neurotransmitter uptake into SVs, Cl^- may directly modulate transporter activity. This concept was originally suggested from biochemical data showing that DIDS, a non-selective Cl^- channel blocker, inhibits Cl^--induced acidification and glutamate-induced acidification with different IC_{50} values (Hartinger and Jahn, 1993), leading to the hypothesis that DIDS may directly bind to VGLUT and inhibit its activity. The hypothesis could explain why glutamate uptake is low in the absence of external Cl^- where $\Delta\Psi$, the primary driving force for the transport, is maximal. Furthermore, $\Delta\Psi$-driven glutamate transport by reconstituted VGLUTs requires a Cl^- concentration of several mM, suggesting that VGLUTs are the Cl^--activated glutamate uniporter (Juge et al., 2010), although reconstitution of VGLUT with a proton pump suggested a contradictory conclusion (Schenck et al., 2009; Preobraschenski et al., 2014). A similar approach investigating the role of VGAT demonstrated that although GABA transport also requires Cl^-, VGAT operates as a GABA/Cl^- co-transporter that is solely driven by $\Delta\Psi$ (Juge et al., 2009).

CYTOSOLIC FACTORS

Neurotransmitter Concentrations

Biochemical transport assays using SV fractions purified from native brains indicated that the transport process obeys Michaelis-Menten kinetics describing authentic enzymatic reactions (Naito and Ueda, 1985; Maycox et al., 1988). With this analogy, the velocity and extent of transport critically depends on the substrate concentrations, where greater and faster transport occurs with higher concentrations. Furthermore, like other protein-mediated transport pathways, neurotransmitter uptake into SVs is substrate saturable. In the case of glutamate uptake into isolated SVs, K_m was measured to be ~1 mM (Naito and Ueda, 1985; Maycox et al., 1988), meaning that the transport system becomes saturated with >2 mM cytoplasmic glutamate. Heterologous

expression of VGLUT1-3 in non-glutamate-releasing cells confers similar, if not completely identical, affinity for glutamate uptake into isolated vesicles (Kaneko and Fujiyama, 2002; Takamori, 2006). The dependance of glutamate uptake on cytoplasmic glutamate concentrations was further confirmed in the calyx of Held synapses, where presynaptic cytoplasmic glutamate concentrations can be manipulated with a glass pipet at the presynaptic terminals. Interestingly, Ishikawa et al. (2002) loaded the presynaptic terminal with 100 mM glutamate and detected a dramatic increase in average mEPSC amplitudes by >50%, confirming that the concentration of cytoplasmic glutamate affects vesicular glutamate content. Of note, because a glutamate concentration of 100 mM may be extremely supra-physiological and may saturate the VGLUT-mediated glutamate transport, a portion of the increase in mEPSC amplitudes may be due to passive glutamate influx through VGLUT or other non-specific pathways.

From where is the neurotransmitter glutamate derived and how is it regulated? Because SVs contain large amounts of neurotransmitters, the mechanism for replenishing released transmitters at high rates of firing must be present at the presynaptic terminals. Classically, the glutamate-glutamine cycle is believed to serve as the major source of the neurotransmitter glutamate (for a review, see Chaudhry et al., 2002). In this cycle, glutamate released from the presynaptic terminals is taken up by the plasma membrane glutamate transporters that are mainly located on the surface of surrounding glial cells. Glutamate is then converted to glutamine by glutaminase. Glutamine is released by the system N transporter (SN1) and then is transported into the presynaptic terminal by the system A transporter (SAT1). Finally, glutamine is converted to glutamate by phosphate-activated glutaminase (PAG also referred as GLS1) at the presynaptic terminal. Although alterations in any step in the cycle would potentially affect the concentration of presynaptic glutamate, the rate-limiting steps have not been determined. However, despite the long-standing notion that the conversion of glutamine to glutamate is the major source of the neurotransmitter glutamate, surprisingly subtle deficits on basal glutamate transmission are seen in GLS1 knockout mice. Although the amplitude of evoked EPSCs decays more rapidly during a long train stimulation, mEPSC amplitude and duration are quite normal in GLS1-null neurons, indicating that the conversion of glutamine to glutamate in the presynaptic terminal is not an indispensable source of the neurotransmitter glutamate (Masson et al., 2006). Alternatively, α-ketoglutarate has recently been proposed as a precursor of glutamate. In fact, isolated SVs from rat brains can synthesize glutamate from α-ketoglutarate by aminotransferase in which L-aspartic acid specifically acts as an amino group donor. Therefore, enzymes involved in this pathway could potentially affect glutamate levels in the presynaptic cytosol (Takeda et al., 2012).

The synthetic pathway of GABA clearly involves the conversion of glutamic acid to GABA and is mediated by two glutamic acid decarboxylases (GAD65 and GAD67), because only trace amounts of GABA are detected in GAD65/GAD67 double knockout mouse brain (Ji et al., 1999). Consistent with this, administration of glutamate or glutamine, both of which are precursors of GABA, in cultured hippocampal neurons effectively prevents rundown of GABA transmission during recordings. Furthermore, exposure of cultured hippocampal neurons to excess concentrations of glutamine facilitates IPSCs, indicating that cytosolic GABA concentrations control vesicular GABA content (Wang et al., 2013). Recently, another non-canonical GABA synthesis pathway was discovered in midbrain dopaminergic neurons (Kim et al., 2015). This type of neuron co-releases dopamine and GABA, but GABA release does not require GABA synthesis by GAD65 and GAD67. Instead, these neurons use aldehyde dehydrogenase (ALDH1a1) to produce GABA from putrescine, which is an evolutionarily conserved pathway for the production of GABA that is also present in plants, *Xenopus*, and mammalian cells (see Kim et al., 2015). Interestingly, provided that mutations in ALDH1a1 have been linked to alcoholism in humans (Liu et al., 2011), deletion of ALDH1a1 in mice not only attenuates GABA co-release but also causes behavioral effects including increased EtOH consumption and preference of EtOH over daily water in mice, implying that diminished GABA co-release due to an insufficient GABA supply is linked to alcoholism.

Trimeric G Proteins

Experiments using isolated SVs demonstrated that glutamate uptake is inhibited by the G-protein activator GMP-P(NH)P (Pahner et al., 2003). Kinetic experiments suggested that the inhibitor decreases both V_{max} and K_m of glutamate uptake. Analyses of isolated SVs derived from mice lacking $G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_q$, or $G\alpha_{11}$ have demonstrated that $G\alpha_{o2}$ is responsible for inhibition of glutamate uptake (Winter et al., 2005). SVs lacking $G\alpha_{o2}$ exhibit normal Cl^--induced acidification, but have lost their Cl^- dependency for glutamate transport activities, indicating that $G\alpha_{o2}$ may modulate allosteric activation of VGLUTs by Cl^-. In the case of monoaminergic vesicles, the intravesicular monoamine concentration in the lumen may trigger G-protein activation (Höltje et al., 2003), providing an inhibitory feedback loop that down-regulates transporter activity when the vesicle is full. Currently, whether the same mechanism exists in glutamatergic vesicles is not clear, and if so, what types of signals, i.e., glutamate itself, protons, or other unknown factors, transfer such a signal from the lumen to the cytoplasmic side is also unknown.

Other Factors

Besides the G proteins described above, we know little about endogenous cytoplasmic proteins that could regulate neurotransmitter uptake into SVs. Ueda and colleagues purified cytosolic proteins from bovine brains. These proteins inhibit uptake of both glutamate and GABA and were named inhibitory protein factor (IPF) αβγ (Ozkan et al., 1997). IPF appears

to contain the amino acid sequence of the α subunit of the cytoskeletal protein fodrin, but fodrin itself does inhibit glutamate transport (Tamura et al., 2001). The molecular identity and physiological relevance of the function of IPFs remain to be elucidated.

CONCLUDING REMARKS

Over the past several decades, presynaptic molecular components that regulate the quantal size have emerged. Because the amount of neurotransmitters in single vesicles may influence both spontaneous and evoked synaptic transmission, changes in quantal size may have a strong impact on brain functions including cognition, learning and memory, and behavior. Despite intensive research, we know little about the mechanisms and the rate-limiting steps underlying the formation of quanta. In particular, the mechanisms of how the vesicular neurotransmitter transporters operate and utilize the proton electrochemical gradient, as well as how Cl^- and other ions directly or indirectly modulate the transport activities have been enigmatic and controversial. One problem that has hampered the complete understanding of the transport systems is the lack of techniques to quantitatively manipulate the H^+ electrochemical gradient that drives the transport. Recent technical developments, including a chimeric protein named pHoenix that allows light-driven H^+ pumping (Rost et al., 2015) and the optimal pH probe for SV pH measurement (Egashira et al., 2015), may help to solve some of the issues discussed in this review.

AUTHOR CONTRIBUTIONS

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frontiers in Synaptic Neuroscience

REVIEW
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Molecular Machines Regulating the Release Probability of Synaptic Vesicles at the Active Zone

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The fusion of synaptic vesicles (SVs) with the plasma membrane of the active zone (AZ) upon arrival of an action potential (AP) at the presynaptic compartment is a tightly regulated probabilistic process crucial for information transfer. The probability of a SV to release its transmitter content in response to an AP, termed release probability (P_r), is highly diverse both at the level of entire synapses and individual SVs at a given synapse. Differences in P_r exist between different types of synapses, between synapses of the same type, synapses originating from the same axon and even between different SV subpopulations within the same presynaptic terminal. The P_r of SVs at the AZ is set by a complex interplay of different presynaptic properties including the availability of release-ready SVs, the location of the SVs relative to the voltage-gated calcium channels (VGCCs) at the AZ, the magnitude of calcium influx upon arrival of the AP, the buffering of calcium ions as well as the identity and sensitivity of the calcium sensor. These properties are not only interconnected, but can also be regulated dynamically to match the requirements of activity patterns mediated by the synapse. Here, we review recent advances in identifying molecules and molecular machines taking part in the determination of vesicular P_r at the AZ.

Keywords: release probability, synaptic vesicles, active zone, calyx of Held, short-term synaptic plasticity, calcium channels

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INTRODUCTION

Information transfer in the nervous system relies on the precisely timed release of neurotransmitter from the presynaptic compartment once an action potential (AP) arrives. The arrival of the AP causes the depolarization of the presynaptic plasma membrane which in turn leads to the opening of VGCCs, resulting in a calcium influx into the presynaptic compartment during the repolarization phase of the AP. The calcium ions entering the cell subsequently interact with a calcium sensor located at the synaptic vesicles (SVs) and trigger the fusion of one or more SVs with the presynaptic plasma membrane, thereby releasing neurotransmitter into the synaptic cleft (Lisman et al., 2007; Rizzoli, 2014). Importantly, this basic mechanism is a highly probabilistic, yet tightly regulated, process. In a typical forebrain synapse, only ~15% of APs arriving at the presynaptic terminal cause fusion of a SV (Branco and Staras, 2009; Borst, 2010). Thus, regulating P_r of a given synapse is a powerful mechanism to adjust information transfer to specific requirements of the circuit, as it provides the basis for information coding by synchronous activity of a population of neurons (low P_r), as well as for the faithful transmission of information on a one-to-one basis (high P_r). Differences in P_r do not only occur between different types of synapses in functionally different circuits, but also among presynaptic compartments of the same axon,

leading to a situation in which the presynaptic compartments of a given neuron vary in their P_r, depending on the postsynaptic partner and the functional state of the particular synapse (Atwood and Karunanithi, 2002). Additionally, P_r is not even homogeneous for SVs of the same presynaptic terminal (Neher, 2015).

Therefore, we would like to address the following questions: how is P_r regulated at the presynaptic compartment and what mechanisms cause differences in the P_r between single SVs of the same presynaptic termial? We review recent advances in identifying the molecular machines involved in the various mechanisms controlling the P_r of a presynaptic SV at central mammalian synapses. This includes the availability of release-ready SVs, the process of SV fusion, calcium influx and calcium handling. Moreover, we will discuss changes of these parameters that may occur during ongoing synaptic activity.

THE READILY RELEASABLE POOL

The basis for every form of synaptic neurotransmitter release is the availability of SVs that are competent for immediate fusion on the time scale of microseconds, once an AP arrives at the presynapse. However, only a small fraction of the SVs present at a presynaptic compartment possess fusion competence. SVs associated with an active zone (AZ) can be divided, according to the classic model, into three different functional pools: the readily releasable pool (RRP), the recycling pool and the reserve pool (Rizzoli and Betz, 2005; Denker and Rizzoli, 2010). Here, we focus on the SVs of the RRP that are thought to be in close contact with the plasma membrane at the site of SV fusion and ready to immediately release transmitter upon arrival of an AP (Rizzoli and Betz, 2005; Denker and Rizzoli, 2010). However, the RRP is not a uniform population of SVs and depending on the synapse, can be further subdivided into a fast and a slow releasing subpopulation (Wu and Borst, 1999; Sakaba and Neher, 2001; Neher, 2015). Furthermore, it is controversial whether all three forms of synaptic release (synchronous, asynchronous and spontaneous release, for a recent review, see Kaeser and Regehr, 2014) originate from the same pool of readily releasable SVs, or whether there is a distinct population of SVs that exclusively supplies spontaneous release (Kavalali, 2015; Schneggenburger and Rosenmund, 2015, respectively). Here, we will focus on mechanisms that maintain the RRP available for AP-induced release. The size of the RRP, defined as the number of SVs in close contact with the plasma membrane and immediately available for release, is determined by various molecular machines of the presynaptic compartment. SVs that are part of the RRP need to be transported to the AZ, anchored there and subsequently rendered fusion competent. The latter two steps are known as docking and priming, respectively. Proteins interfering with either of these steps will alter the number of release-ready SVs and thereby the P_r of individual SVs. Non-docked and primed SVs need to undergo molecular steps in order to become fusion-competent and thereby join the RRP. Moreover, the P_r of the entire synapse will be changed, because the size of the RRP, and thus the reservoir of SVs that can potentially be released, is altered.

RECRUITMENT OF SVs TO THE RRP

The first step in the mechanism of SV recruitment to the RRP is the delivery of SVs to the site of release, a process that has been studied intensively at the calyx of Held synapse in the auditory brainstem. There, the polymerization of actin has been identified as one of the main factors for the recruitment of SVs to the RRP, especially to the so-called fast-releasing subpool that consists of the SVs immediately available to fusion upon AP-arrival (Sakaba and Neher, 2003b; Lee et al., 2012, 2013). In addition to actin, myosin light chain kinase (MLCK), another cytoskeleton-associated protein, was found to regulate recruitment of SVs to the RRP. At the calyx of Held, inhibition of MLCK resulted in an increase in RRP size with a specific augmentation of its fast-releasing subpool (Srinivasan et al., 2008). On the other hand, activation of MLCK and thus phosphorylation of MLC triggers the contraction of actomyosin in the presynaptic compartment (Luo, 2002) and reduces the number of SVs in the RRP in hypoglossal motorneurons (García-Morales et al., 2015). MLCK in turn is negatively regulated by Rho-associated kinase (ROCK) and basal ROCK activity is necessary to maintain the RRP (González-Forero et al., 2012). A third link between the recruitment of SVs to the RRP and the actin cytoskeleton comes from a recent study demonstrating that the endocytic scaffolding protein intersectin 1 and its interaction with Cdc42, a GTPase involved in actin remodeling, are necessary for the replenishment of SVs to the RRP (Sakaba et al., 2013). Thus, the actin cytoskeleton and its regulators have a profound effect on the recruitment of SVs to the RRP.

Beside this cytoskeletal machinery, two enzymes play opposing roles in the regulation of the RRP size: cyclin-dependent kinase 5 (CDK5) and the calcium activated phosphatase calcineurin (CaN). Whereas CDK5 activity limits the number of SVs in the RRP, activation of CaN, possibly in response to ongoing synaptic activity, recruits SVs to the RRP and increases its size (Kim and Ryan, 2010; Marra et al., 2012). Relatively little is known about the target proteins that are modified in their phosphorylation state by these two enzymes. CDK5 has been shown to phosphorylate synapsin I which leads to increased SV clustering distant to the AZ, and thus outside the RRP. This clustering is supposed to be mediated by an increased binding of phosphorylated synapsin I to F-actin (Verstegen et al., 2014). Specific targets for CaN in regulating RRP size have not been reported till date, as CaN is mostly known for triggering bulk endocytosis by dephosphorylation of dynamin (Clayton et al., 2009). However, both, CDK5 and CaN have been proposed to regulate the phosphorylation of N-type VGCCs ($Ca_V2.2$; Su et al., 2012; Kim and Ryan, 2013). Pharmacological blockade or knock-down of CDK5 resulted in an increase in calcium influx through N-type VGCCs and a concomitant increase in SV exocytosis, whereas block or knock-down of CaN had the opposite effect (Kim and Ryan, 2013). Contradictory to this mechanism, it has been reported, using

CDK5 phosphorylation-insensitive N-type channel mutants, that phosphorylation of the N-type VGCC increases calcium influx and number of SVs at the AZ, possibly via a direct interaction with the presynaptic scaffolding protein RIM1 (Rab 3 interacting molecule 1; Su et al., 2012). Under physiological conditions the regulation of the balance between CDK5 and CaN is currently not known, although CaN activation and a subsequent increase in RRP size have been reported to depend on postsynaptic N-Methyl-D-aspartate (NMDA) receptor activation and retrograde signaling via NO (Ratnayaka et al., 2012). Moreover, the size of the RRP is determined directly by the influx of calcium through VGCCs during synaptic activity, as higher calcium influx and a larger local calcium domain lead to a rise in calcium concentration sufficient for SV fusion at larger distances from the VGCC (Thanawala and Regehr, 2013).

Finally, size and replenishment of the RRP are dynamically regulated by the level of synaptic activity. In the auditory brainstem, unilateral hearing loss results in a reduction in RRP size at the calyx of Held (Grande et al., 2014), while constant noise exposure induced a reversible increase in RRP size at the endbulb of Held (Ngodup et al., 2015). Of note, an earlier study on $Ca_v1.3$-KO mice, which are deaf due to non-functional glutamate release from inner hair cells, did not reveal changes in RRP size (Erazo-Fischer et al., 2007).

DOCKING AND PRIMING OF SVs AT THE AZ

Once SVs have been recruited to the AZ, they need to be anchored and rendered fusion competent. This process depends on several different molecular machines. The most obvious one is the SNARE complex, as its formation is a prerequisite for all forms of membrane fusion (see separate section below, reviewed by Jahn and Fasshauer, 2012; Südhof, 2013). Additionally, scaffolding proteins of the cytomatrix of the AZ and proteins present on the SVs take part in this process (Takamori et al., 2006; Südhof, 2012).

The scaffolding proteins of the AZ are thought to ensure the correct structural assembly of the release site, including the docking of SVs to the plasma membrane of the AZ. Recent studies using electron tomography have shown that SVs in the vicinity of AZ are connected to the plasma membrane via short filamentous structures called tethers (Siksou et al., 2007; Fernández-Busnadiego et al., 2010). The number of these short tethers as well as the quantity and distribution of SVs close to the AZ are altered in RIM1α-knock-out synapses, suggesting a role for RIM1α in adhering/docking SVs to the AZ (Fernández-Busnadiego et al., 2013). Functional evidence that RIM proteins are crucial for RRP maintenance comes from different preparations. At the calyx of Held and in hippocampal, bouton-type synapses, simultaneous knock-out of RIM1 and RIM2 leads to an almost complete loss of SVs close to the AZ. This in turn caused a severe impairment of synaptic transmission (Han et al., 2011; Kaeser et al., 2011). However, both RIM isoforms seem to be redundant, at least to a certain degree (Kaeser et al., 2012; Han et al., 2015). The mechanism by which RIM assists in the docking of SVs to the AZ and thereby recruits them to the RRP involves the interaction with another AZ protein: Munc13. In the absence of RIM, Munc13 forms an autoinhibitory homodimer that is relieved by the interaction with RIM (Deng et al., 2011). RIM, Munc13 and the SV protein Rab3 can then form a tripartite complex (Dulubova et al., 2005) that links SVs to the cytomatrix of the AZ and thereby positions the SV in close proximity to the presynaptic plasma membrane. Munc13 is absolutely crucial for SV fusion, as the simultaneous genetic removal of the isoforms Munc13-1 and Munc13-2 prevents every form of neurotransmitter release (Varoqueaux et al., 2002). Synapses devoid of Munc13-1/2 are characterized by the loss of SVs docked to the AZ and thus of the RRP (Siksou et al., 2009; Imig et al., 2014). However, SVs tended to accumulate close to the AZ (∼10 nm) in the absence of Munc13-1/2, indicating that SVs are recruited to the AZ but do not docked to the plasma membrane (Imig et al., 2014). Moreover, synapses express multiple Munc13 isoforms simultaneously that may execute different functions in the recruitment of SVs to the RRP. At the calyx of Held, overexpression of a dominant negative form of Munc13-1, the major isoform at this synapse, results in a severe reduction in RRP size, whereas simultaneous deletion of Munc13-2 and Munc13-3 had no effect on RRP size, but slowed down the replenishment of the RRP after intense activity (Chen et al., 2013). Munc13 has also been shown to prime SVs and thereby render them fusion competent. This involves the interaction of Munc13 with the calcium binding protein calmodulin (CaM; Junge et al., 2004). Rendering the Munc13-1 binding site for CaM nonfunctional impairs the recruitment of SVs to the AZ and their incorporation into the RRP (Lipstein et al., 2013). In addition to the CaM-dependent replenishment of docked and primed SVs in the RRP, Munc13 has also been proposed to be necessary for a molecular process of unknown nature called "superpriming". This reaction converts primed SVs of the RRP into SVs ready for immediate fusion within microseconds after the arrival of the AP, independent of CaM or actin (Lee et al., 2013; Ishiyama et al., 2014).

In addition to RIM and Munc13, other AZ proteins such as CAPS1 and CAPS2 have been implicated in docking and priming SVs of the RRP. The combined loss of both, CAPS1 and CAPS2, results in a strong reduction in SVs present at the AZ (Jockusch et al., 2007; Imig et al., 2014), but unlike Munc13-1/2 knock-out synapses, CAPS1/2 knock-out synapses do not accumulate SVs close to the AZ. This suggests that CAPS1/2 are not only required for docking and priming of SVs, essential steps in the generation of the RRP, but also for the recruitment of SVs to the AZ (Imig et al., 2014). Furthermore, the presynaptic scaffolding protein liprin-α2 has been shown to regulate the RRP size. Knock-down of liprin-α2 in cultured hippocampal neurons led to a severe reduction in the number of docked SVs at the AZ and structural remodeling of the AZ, including shortening and reduction of the amount of RIM present. Therefore, the diminished number of docked SVs upon the loss of liprin-α2 could be secondary to the reduction in RIM at the AZ (Spangler et al., 2013). Interestingly, mSyd1A (mouse Synapse-Defective-1A)

that interacts with liprin-α2 and with Munc18 (see below) has also been shown to be involved in SV docking (Wentzel et al., 2013).

In addition to these AZ components, other proteins present on SVs have been implicated in the docking and priming of SVs at the AZ. An obvious candidate for the involvement in SV docking at the AZ is Rab3, as it interacts with both, the major scaffolding protein RIM and SVs (Wang et al., 1997). However, knock-out of all four Rab3 isoforms (Rab3A-D) did not result in a loss of docked SVs but in a profound reduction in P_r. This suggests that Rab3 is involved in priming rather than docking of SVs (Schlüter et al., 2004). More specifically, Rab3 seems to play a role in superpriming since it increases the calcium sensitivity of docked and primed SVs, making it more likely that they get released in response to an AP (Schlüter et al., 2006). Rab3 may therefore be part of the same molecular machine as Munc13 (Lee et al., 2013). Another SV protein that has been implicated in the regulation of the RRP size is SV2. Genetic deletion of the SV2A isoform has been reported to reduce RRP size (Custer et al., 2006), whereas another study reported a normal RRP size and attributed the observed reduction in P_r to a deficit in the responsiveness of primed SVs to calcium influx (Chang and Südhof, 2009). Along similar lines, Mover, a newly described SV protein that modulates P_r in a subset of central synapses, may be involved in the superpriming of SVs, as its knock-down increases P_r at the calyx of Held, without altering RRP size (Körber et al., 2015).

REPLENISHMENT OF THE RRP

Upon the arrival of an AP, fusion competent SVs of the RRP are released at the AZ. To cope with ongoing synaptic activity, the RRP needs to be constantly replenished with newly recruited, docked and primed SVs. Therefore, molecular interactions that interfere with SV replenishment will impair the maintenance of the RRP and thus decrease the P_r of the synapse during sustained activity. Deficits in RRP replenishment often only become evident during high frequency synaptic activity and are, accordingly, particularly prominent at synapses that participate in high frequency signaling under physiological conditions, like the calyx of Held or the cerebellar mossy fiber terminals. A variety of presynaptic components have been implicated in RRP replenishment: presynaptic scaffolding proteins, ATP and the spread of the presynaptic calcium domain upon calcium channel opening.

Bassoon, a large multi-domain presynaptic scaffolding protein has been shown to play a crucial role in the refilling of the RRP at high frequency-firing synapses such as the cerebellar mossy fiber to granule cell synapse and the endbulb of Held in the auditory brainstem (Hallermann et al., 2010; Mendoza Schulz et al., 2014, respectively). Interestingly, in synapses with lower physiological activity, like those connecting hippocampal neurons, the loss of bassoon had no effect on RRP replenishment (Altrock et al., 2003; Mukherjee et al., 2010). Furthermore, loss of bassoon also lacked a functional phenotype in hippocampal neurons when the closely related protein piccolo was knocked-out simultaneously, even though the number of SVs per synaptic terminal was severely reduced (Mukherjee et al., 2010). Piccolo in turn has been proposed to modulate the dynamics of synapsin 1 (Leal-Ortiz et al., 2008). However, knock-out of all three synapsin genes at the calyx of Held resulted in a general reduction of SVs in the presynaptic compartment but not in the RRP (Vasileva et al., 2012), similar to what has been seen at hippocampal bassoon/piccolo double knock-out synapses (Mukherjee et al., 2010). Nevertheless, the lack of all three synapsins led to a slowing of RRP replenishment at the calyx of Held during high frequency signaling (Vasileva et al., 2012). Interestingly, knock-down of piccolo in hippocampal cultured neurons led to a reduced stability of F-actin and thereby to altered synapsin dynamics, linking piccolo to the regulation of SV availability at the AZ (Waites et al., 2011). Of note, a recent imaging study of dissociated calyces of Held suggested that SVs newly recruited to the AZ are not immediately used to replenish the RRP. Instead, SVs have to reside at the AZ for a certain amount of time before they are rendered fusion competent and used to replenish the RRP during ongoing activity (Midorikawa and Sakaba, 2015).

Another important regulator of RRP replenishment is calcium. It has been shown that the increase in presynaptic calcium concentration during high frequency synaptic activity speeds-up the refilling of the RRP at the calyx of Held (Wang and Kaczmarek, 1998). This effect is presumed to be mediated by binding of calcium to CaM. CaM has been shown to be involved in replenishing the fast-releasing subpool of the RRP responsible for maintaining high frequency synaptic transmission (Sakaba and Neher, 2001; Hosoi et al., 2007). This mechanism may predominantly depend on the interaction between CaM and the priming factor Munc13 (Lee et al., 2012; Lipstein et al., 2013). However, CaM also participates in various forms of SV endocytosis (Wu et al., 2009; see below) and has therefore been suggested to link SV endocytosis to replenishment of the RRP (Yao and Sakaba, 2012). Interestingly, the CaM-dependent priming of SVs can be regulated by presynaptic $GABA_B$ receptors ($GABA_B$Rs) at the calyx of Held. Activation of $GABA_B$Rs resulted in a decrease in cAMP that counteracted the activating effect of elevated presynaptic calcium concentration on SV priming (Sakaba and Neher, 2003a). Moreover, replenishment of the RRP has been shown to be strongly dependent on locally produced ATP. ATP maintains the SV cycle and ATP deprivation leads to severe impairments in neurotransmitter release during ongoing activity (Rangaraju et al., 2014; Pathak et al., 2015). Interestingly, ATP also binds to synapsins that are involved in the regulation of RRP size. Deficiency in ATP-binding of synapsins has been reported to lead to a larger initial RRP size, but a slower replenishment (Orlando et al., 2014; Shulman et al., 2015). Besides these proteins and intracellular molecules, an additional factor that controls RRP replenishment is temperature, as raising the temperature increases the SV replenishment rate at the calyx of Held, although temperature has no effect on RRP size and the initial P_r of SVs (Kushmerick et al., 2006).

Thus, the establishment and maintenance of the RRP is a highly regulated process involving a large number of molecular machines, ranging from the actin cytoskeleton to the generation of ATP, that are highly interconnected and act together in order to provide the synapse with enough SVs to cope with the demands of synaptic activity. Interference with any of the different molecular machines leads to an impairment in SV recruitment to the RRP and thereby, to a decrease in synaptic P_r.

THE SNARE COMPLEX AND ASSOCIATED PROTEINS

The SNARE complex is the molecular machine that provides the energy for SV fusion with the plasma membrane (Jahn and Fasshauer, 2012; Südhof, 2013). Besides this, the SNARE complex is also implicated in the docking and priming of SVs to the AZ, and the loss of either of the t-SNAREs syntaxin 1 and SNAP25 results in the loss of SVs that are in close contact with the presynaptic plasma membrane. The situation is a bit more difficult for the v-SNARE synaptobrevin (a.k.a. VAMP2), since the analysis of synapses devoid of synaptobrevin is hampered by the compensatory upregulation of VAMP1 which can, at least partially, take over the function of synaptobrevin (Imig et al., 2014). Nevertheless, acute disruption of synaptobrevin function by tetanus toxin blocked SV release completely at the calyx of Held (Sakaba et al., 2005). Moreover, the proline-rich domain of synaptobrevin has been shown to be involved in the recruitment of SVs into the fast-releasing subpool of the RRP, possibly by positional priming (Wadel et al., 2007). Positional priming is a mechanism that is thought to be responsible for the positioning of docked SVs in close proximity to VGCCs. This ensures a short distance between the SV and the source of calcium (coupling distance) and thereby the rapid release of SVs in response to VGCC opening (see below). Of note, syntaxin 1 has been proposed to not only play a role in the docking and priming of SVs to the RRP (Arancillo et al., 2013), but also to speed-up SV fusion when present in its open conformation (Acuna et al., 2014). However, during the process of SV fusion, the SNARE complex not only needs to be formed, but the so called four-helix bundle needs to be positioned at the right distance from the plasma membrane in order to render the SNARE complex functional (Zhou et al., 2013).

Apart from the three canonical SNARE complex proteins syntaxin 1, synaptobrevin and SNAP25, a number of other proteins have been implicated in the fusion of SVs through their interactions with the SNARE complex. One of these proteins is complexin, whose role in SV release has been discussed controversially in recent years. Complexin has been proposed to either facilitate release or act as a fusion clamp (Brose, 2008; Südhof, 2013). Apart from this highly debated role in SV fusion, complexins (isoforms 1 and 2) have a distinct role in priming SVs and thereby rendering them release-ready (Yang et al., 2013; Chang et al., 2015), a function probably exerted by its C-terminus (Kaeser-Woo et al., 2012). The functional role of complexins in SV priming has been suggested to be the stabilization of newly primed SVs, which would correspond to a fusion clamp mechanism (Chang et al., 2015). However, this function may depend on the type of synapse studied, the examination method used and the developmental history of the neuron (Yang et al., 2013). Complexins have also been implicated to be involved in determining the size of the RRP (Kaeser-Woo et al., 2012; Yang et al., 2013), although this may not apply to all synapses (Chang et al., 2015). Thus, although the exact mechanisms by which complexins act are still unclear, it appears that complexins are involved in the stabilization of the primed state of the SV thereby preventing untimely fusion and thus regulating synaptic P_r by maintaining the RRP.

Another protein interacting with the SNARE complex that is crucial for SV fusion is Munc18-1. The genetic deletion of Munc18-1 leads to the total arrest of all forms of neurotransmitter release although the ultrastructure of the synapses appears normal (Verhage et al., 2000). Munc18-1 binds to syntaxin1 in its closed conformation and is thought to stay bound to the assembled SNARE complex where it assists in the zippering process in a synaptobrevin-dependent manner (Shen et al., 2015). But in order to form, the SNARE complex requires the interaction of the Munc18-1/syntaxin1 complex with additional factors, e.g., Munc13 (Rizo and Rosenmund, 2008; Ma et al., 2013; Südhof, 2013; Kavanagh et al., 2014). However, Munc18-1 critically influences the P_r of a synapse, as the amount of Munc18-1 present at a synapse scales with its strength (Toonen et al., 2006; Cijsouw et al., 2014), presumably by determining the number of SNARE complexes that can be formed and thus the number of release-ready SVs (Toonen et al., 2006).

Munc18-1 is assisted in the formation of the SNARE complex by Munc13 (Ma et al., 2013), that, besides its role in SV docking and priming described above, has additional effects on the SNARE complex once formed. Munc13 contains a C1-domain that binds to diacylglycerol (DAG) produced by activation of phospholipase C (PLC). DAG binding has no effect on the basal docking and priming activity of Munc13 and therefore on the RRP size, but activation of the C1-domain leads to an increase in vesicular P_r by reducing the energy barrier for SV fusion (Basu et al., 2007). Modulation of the energy barrier for SV fusion has been recently shown to be a potent way to regulate the P_r of SVs in general and is not restricted to Munc13, but has also been shown for complexins (Schotten et al., 2015).

Precisely timed, synchronous release of SVs of the RRP upon arrival of an AP at the presynaptic terminal is dependent on synaptogmin (isoforms 1, 2 or 9, depending on the brain region; Xu et al., 2007), the principal calcium sensor for synchronous release. The exact mechanisms of synaptotagmin action are still under debate, although they have been extensively studied in recent years (Chapman, 2008; Südhof, 2013; Zhou et al., 2015). Synaptotagmin possesses two calcium binding C2-domains. Calcium binding to the C2B-domain has been shown to be crucial for synchronous SV fusion (Nishiki and Augustine, 2004) whereas the C2A-domain has only an accessory role in this process (Shin et al., 2009). Additionally, the C2B-domain interacts directly with the lipids of the presynaptic

plasma membrane and the SNARE complex (Rickman et al., 2004; Xue et al., 2008, respectively), which provides additional support for the fusion reaction. In particular, the interaction between the C2B-domain and the plasma membrane has been shown to determine the speed of calcium-induced SV fusion (Evans et al., 2015). Knock-out of synaptotagmin leads to the loss of calcium evoked synchronous release but at the same time to an increase in spontaneous SV fusion (Geppert et al., 1994). Thus, although the P_r of the synapse is decreased in terms of AP-induced synaptic transmission, the P_r of single SVs in synaptotagmin knock-out synapses is actually increased, as they fuse spontaneously. The loss of synchronous release and the concomitant increase in spontaneous fusion can be attributed to discrete parts of the C2-domain. Expression of synaptotagmin 2 lacking the entire C2B-domain in the calyx of Held of synaptotagmin 2 knock-out mice has no effect on synaptic release. However, a synaptotagmin 2 version that contains a C2B-domain that is unable to bind calcium, decreases the rate of spontaneous release although synchronous release is still impaired. This partial rescue is presumably mediated by the basic amino acid residues of the C2B-domain that bind to the assembled SNARE complex (Kochubey and Schneggenburger, 2011). However, ablation of basic amino acid residues of the C2B-domain that interact with the plasma membrane has no effect on the spontaneous release rate, but reduces synchronous release by impairing the positional priming of SVs (Young and Neher, 2009). Interestingly, the fusion of SVs seems also to depend on the composition of the plasma membrane at the site of fusion. Both synaptotagmin and syntaxin 1 interact with the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate that clusters syntaxin 1 and increases the calcium sensitivity of the synaptotagmin C2B-domain (van den Bogaart et al., 2011, 2012; Honigmann et al., 2013; Rizzoli, 2014). Thus, the principal calcium sensor synaptotagmin is a key player in determining the P_r of a SV, as it integrates multiple steps of the release process and thereby sets the P_r according to the current microenvironmental conditions of the SV.

Another component of the AZ that regulates P_r is the G-protein-coupled receptor kinase-interacting protein 1 (GIT1) that has been implicated in the recycling of SVs (Podufall et al., 2014). However, knock-out of GIT1 at the calyx of Held results in an increase in P_r through a yet unknown mechanism that does not involve the size of the RRP or the calcium influx into the terminal. Interestingly, the knock-out of the closely related GIT2 had no effect on synaptic transmission, and simultaneous knock-out of both GIT isoforms phenocopied the GIT1 knock-out, arguing against an involvement of GIT2 in regulation of P_r (Montesinos et al., 2015).

Thus, the complicated molecular machinery of the SNARE complex and its associated proteins play a crucial role in regulating the P_r of the synapse in multiple ways: (1) the assembly of SNARE complex determines the number of SVs available for fusion; (2) binding of additional proteins (e.g., Munc13) regulates the energy barrier of SV fusion; (3) SNARE complex components are involved in positional priming of SVs; and (4) binding of synaptotagmin to calcium, the plasma membrane and the SNARE complex regulates SV fusion.

SV PROTEINS REGULATING P_r

In addition to the SNARE complex protein synaptobrevin and the SV proteins involved in docking and priming of SVs (e.g., Rab3, see above), a number of SV proteins have been implicated in regulating P_r, with the vesicular glutamate transporter VgluT representing the most prominent example. VGluT exists in three isoforms (VGluT1–3) with distinct expression patterns in the mammalian brain (Edwards, 2007). Synapses expressing VGluT1 show a relatively low P_r, whereas synapses expressing VGluT2 have a high P_r (Fremeau et al., 2004). This regulation of P_r is directly mediated by the transporter itself, as domain swapping between VGluT1 and VGluT2 can transpose the P_r setting properties. The low P_r of VGluT1 carrying SVs may be due the ability of VGluT1 but not VGluT2 to bind to endophilin A1 and inhibit an endophilin-mediated enhancement of SV fusion (Weston et al., 2011). Furthermore, it has been shown that the filling state of neurotransmitter in the SV, which correlates with the number of transporter proteins on the SV, regulates P_r (Herman et al., 2014). The filling state of the SV in turn is not only regulated by the neurotransmitter transporters, but also involves the establishment of a proton gradient across the SV membrane by the v-ATPase (Edwards, 2007) and the activity of a K^+/H^+-exchanger to make this electrochemical gradient useable for the neurotransmitter loading (Goh et al., 2011).

In addition to VGluTs, Mover, a recently discovered vertebrate-specific SV protein (Kremer et al., 2007; Ahmed et al., 2013), has been implicated in the regulation of P_r (Körber et al., 2015). Mover binds to the AZ scaffolding protein bassoon and the acute knock-down of Mover at the calyx of Held leads to an increase in P_r by increasing the calcium sensitivity of release. Interestingly, the knock-down of CaM, to which Mover can bind, has also been shown to affect P_r, although the knock-down of CaM rather yields a decrease in P_r (Pang et al., 2010).

Thus, components of the molecular machinery of the SV are not only involved in docking, priming and the generation of the fusion machine, but also regulate vesicular P_r by setting the filling state of the SV or changing the calcium-dependence of release.

ASYNCHRONOUS RELEASE OF NEUROTRANSMITTER

The release of neurotransmitter from docked and primed SVs can occur in different modes; spontaneous, synchronous and asynchronous. The P_r of a synapse is normally defined as the probability of a SV to be released synchronously, immediately after the arrival of an AP at the presynapse. Nevertheless, some synapses show prominent asynchronous, AP-triggered but delayed, neurotransmitter release (for a recent review, see Kaeser and Regehr, 2014). Thus, there is a probability that a given SV will not be released immediately after the arrival of the AP, but asynchronously, with a potentially different P_r. The specific mechanisms and molecular machines involved in this form of release are poorly understood. VAMP4 has been proposed to substitute for synaptobrevin in the SNARE complex of SVs that are destined to asynchronous release (Raingo et al., 2012) and synapsin II has been suggested to be

involved in asynchronous SV release at GABAergic synapses (Medrihan et al., 2013). Furthermore, there is accumulating evidence that synchronous and asynchronous release are triggered by different calcium sensors. Whereas synchronous release depends on synaptotagmin 1, 2 or 9 (Xu et al., 2007), the identity of the calcium sensor for asynchronous release is still under debate. Two proteins have been proposed to trigger asynchronous release: synaptotagmin 7 and Doc2 (Yao et al., 2011; Bacaj et al., 2013, respectively). Doc2 has been originally shown to be involved in spontaneous neurotransmitter release (Groffen et al., 2010; Pang et al., 2011). However, a recent controversial study proposed Doc2 as a sensor for asynchronous release, as Doc2 knock-down in hippocampal cultured neurons resulted in a shift towards synchronous release upon intense stimulation (Yao et al., 2011). Synaptotagmin 1 knock-out synapses on the other hand, show prominent asynchronous release that can be completely abolished by simultaneous knock-down of synaptotagmin 7, suggesting that synaptotagmin 7 acts as the calcium sensor for asynchronous release (Bacaj et al., 2013). Also, synaptotagmin 7 has been proposed recently to act as a calcium sensor for SV replenishment in synapses of cultured hippocampal neurons (Liu C. et al., 2014), a process known to be at least partially calcium-dependent (e.g., Wang and Kaczmarek, 1998). However, this effect could not be confirmed at synapses between rod bipolar and AII amacrine cells in the retina, where synaptotagmin 7 again acts as a sensor for asynchronous release, but has no effect on SV replenishment (Luo et al., 2015). Of note, asynchronous release does not only rely on a specialized calcium sensor, but also on additional components of the AZ. A recent study at the calyx of Held showed that the knock-out of complexin 1 results in a profound increase in asynchronous release which goes along with a reduction in both synchronous and spontaneous release (Chang et al., 2015). Moreover, the protein adapter complex AP-3, which is thought to be involved in the regeneration of SVs from synaptic endosomes (Voglmaier et al., 2006), has been shown to contribute to asynchronous release at hippocampal mossy fiber synapses (Evstratova et al., 2014). Taken together, asynchronous release constitutes another, as of now poorly understood, layer of P_r regulation that depends on several molecular machines of the presynaptic terminal. Nevertheless, it seems to rely on a specialized fusion machinery, in particular a separate calcium sensor, most likely synaptotagmin 7 (**Figure 1**).

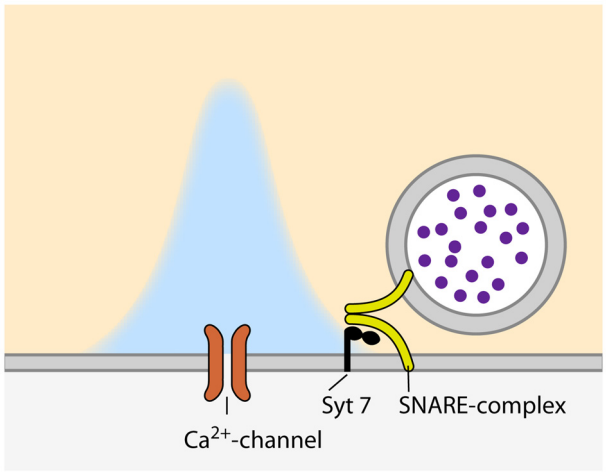

FIGURE 1 | Configuration of synaptic vesicles (SVs) and voltage-gated calcium channels (VGCCs) during asynchronous release. Please note the larger distance between the VGCC and calcium sensor as compared to synchronous relase (**Figure 2**).

THE REGULATION OF P_r BY CALCIUM

In addition to the availability of primed SVs at the site of release, the regulation of calcium influx into the presynaptic compartment is key to define vesicular P_r. Calcium influx occurs via VGCCs precisely located within the AZs. The VGCCs are organized in clusters and their number per cluster correlates with the P_r of the release site (Holderith et al., 2012; Sheng et al., 2012; Nakamura et al., 2015; but see Scimemi and Diamond, 2012). The arrival of an AP opens the VGCCs and leads to the influx of calcium ions into the presynaptic terminal, where they very briefly build-up a transient domain of elevated calcium concentration high enough to trigger synaptotagmin-dependent release of SVs (**Figure 2**). The effective calcium concentration at the SV-bound calcium sensor directly controls the P_r of a given SV, as it decides whether this SV is released in response to the arrival of an AP and the opening the VGCCs. Endogenous buffers, passive diffusion and the active extrusion mechanisms lead to a rapid break down of the calcium domain, so that the fusion of SVs is confined to the vicinity of the VGCC cluster (e.g., Borst and Sakmann, 1996; Eggermann et al., 2012; Neher and Taschenberger, 2013; Babai et al., 2014). How the SVs are arranged with respect to the VGCC cluster is still a matter of debate and may vary according to the synapse type and developmental stage. In fast-spiking GABAergic interneurons of the hippocampus, SVs are thought be placed 10–20 nm away from the VGCCs (Bucurenciu et al., 2008; Arai and Jonas, 2014),

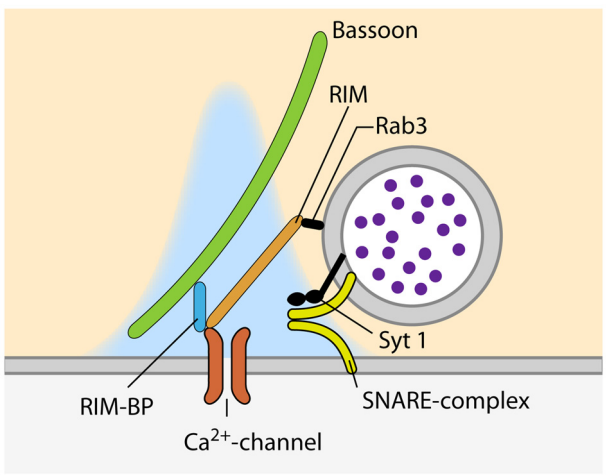

FIGURE 2 | Proteins involved in the recruitment of VGCC and SVs to sites of SV fusion during synchronous release.

whereas the coupling distance is ~75 nm at hippocampal mossy fiber to CA3 synapses (Vyleta and Jonas, 2014). At the calyx of Held, varying values have been estimated for mature synapses: 5–25 nm (Chen et al., 2015), 15–30 nm (Nakamura et al., 2015) and >30 nm (Keller et al., 2015). This coupling distance is subject to developmental regulation and has been shown to decrease at the calyx of Held (Fedchyshyn and Wang, 2005; Kochubey et al., 2009; Leão and von Gersdorff, 2009; Nakamura et al., 2015) and the parallel fiber to Purkinje cell synapse in the cerebellum (Baur et al., 2015), possibly by a septin 5-dependent mechanism (Yang et al., 2010). Different coupling distances can also lead to differences in short-term plasticity (STP) during ongoing synaptic activity due to differences in calcium buffering (e.g., Eggermann and Jonas, 2012; Pan and Ryan, 2012; Vyleta and Jonas, 2014). Hence, calcium channels need to be placed in a specific arrangement within a narrow distance range relative to the SVs to ensure precise setting of P_r.

RECRUITMENT OF CALCIUM CHANNELS TO THE AZ

At the site of SV fusion, the AZ cytomatrix represents a molecular machine that consists mostly of large scaffolding proteins, ensuring that SVs and VGCCs are located at the optimal distance for timed SV fusion (Südhof, 2012, 2013). One of the key molecules involved in the coupling between SVs and calcium channels is RIM, as it binds to SVs via Rab3, and directly to calcium channels of the P/Q-($Ca_v2.1$) and N-type ($Ca_v2.2$) but not L-type ($Ca_v1.1$–3; **Figure 2**; Wang et al., 1997; Kaeser et al., 2011, respectively). Simultaneous genetic deletion of RIM1 and 2 leads to a profound loss of VGCCs at the AZ of cultured hippocampal and the calyx of Held, which greatly decreases the P_r of the synapses (Kaeser et al., 2011; Han et al., 2011, respectively). The importance of different RIM isoforms seems to vary between synapses. RIM1 and 2 have been shown to be functionally redundant at the calyx of Held as the deletion of either of the isoforms had no effect on VGCC recruitment to the AZ (Han et al., 2015). However, at cerebellar granule cell terminals, loss of the RIM1α isoform leads to reduced calcium influx due to reduced channel abundance (Kintscher et al., 2013). Interestingly, RIM abundance at the AZ is regulated via its recruitment by liprin-α (Spangler et al., 2013) as well as by its SUMOylation, which is required for VGCC recruitment but dispensable for SV binding (Girach et al., 2013). Furthermore, RIM1 is regulated in an activity dependent manner. Prolonged silencing of neuronal activity in hippocampal cultured neurons, strongly increasing P_r (Murthy et al., 2001), results in an upregulation of RIM (Lazarevic et al., 2011). This observation supports the idea that the amount of RIM present at the synapse sets its P_r, probably by determining the number of VGCCs at the AZ. Besides RIM, the RIM-binding protein (RIM-BP) that binds to RIM as well as to VGCCs, has been proposed to be involved in VGCC clustering at the AZ (Wang et al., 2000; Hibino et al., 2002; **Figure 2**). Moreover, RIM-BPs bind to the presynaptic scaffolding protein bassoon and a loss of this interaction has been shown to lead to a change in VGCC composition, with P/Q-type channels being lost from the AZ and replaced by N-type channels. The presence of RIM-BPs is thus necessary for the specific recruitment of P/Q-type channels to the SV release sites (Davydova et al., 2014). The simultaneous loss of both isoforms of RIM-BP (RIM-BP1 and 2) results in a reduction in EPSC amplitude, both in hippocampal cultures and the calyx of Held, but does not change the kinetic properties and amplitude of presynaptic calcium currents. Instead, the loss of RIM-BPs causes an increased variability of SV fusion in response to an AP, due to defects in the coupling of VGCCs and SVs (Acuna et al., 2015). In addition to the scaffolding proteins of the cytomatrix, a recent study reported an increase in coupling distance at the calyx of Held of complexin 1 knock-out mice (Chang et al., 2015). Interestingly, the abundance of VGCCs at the AZ is not only controlled by interactions with components of the cytomatrix of the AZ, but also by the VGCC α2δ-subunit. The expression level of this subunit directly sets the number of P/Q-type channels at the presynaptic compartment, thereby determining the number of SVs fusing upon AP-arrival and therefore, defining P_r (Hoppa et al., 2012).

DIRECT MODULATION OF CALCIUM INFLUX THROUGH VGCCs

The calcium influx into the presynaptic compartment through VGCCs is one of the key parameters determining P_r (Ermolyuk et al., 2012) and therefore not only regulated by the means of channel abundance, but also by direct modulation of the calcium flux through the channel. The presynaptic scaffolding proteins ELKS1 and 2 (Südhof, 2012) are two of the proteins that directly affect calcium flux without affecting VGCC abundance. Simultaneous genetic ablation of both ELKS isoforms in hippocampal neurons results in a strongly decreased P_r at inhibitory terminals which is due to a decrease in AP-induced calcium influx (Liu H. et al., 2014). Another presynaptic protein that regulates the functional properties of VGCCs is Munc13. Simultaneous knock-down of Munc13-1 and 2 in hippocampal cultured neurons decreases the amount of AP-induced calcium influx. This is mediated by the C2B-domain of Munc13, which regulates the kinetic properties, such as inactivation and refractory period, of the VGCCs and therefore has a strong influence on the P_r during ongoing synaptic activity (Calloway et al., 2015). Thus, proteins of the presynaptic cytomatrix of the AZ are not only recruiting VGCCs to the AZ, but are also involved in regulating their functional properties and thereby the P_r of the synapse.

Additionally, calcium influx through VGCCs is regulated in a calcium-dependent manner: depending on the recent history of synaptic activity, calcium influx can either be facilitated or inhibited. This VGCC modulation is directly dependent on the interaction of VGCCs, at least of the P/Q-type, with CaM and other calcium binding proteins of the presynaptic compartment (reviewed by Catterall and Few, 2008; Ben-Johny and Yue, 2014). Calcium-dependent CaM binding to the VGCCs C-terminus initially facilitates calcium influx and then promotes channel inactivation, thereby inhibiting calcium influx during prolonged depolarization (Lee et al., 1999, 2000; DeMaria et al., 2001). However, CaM has recently been shown to also bind to the

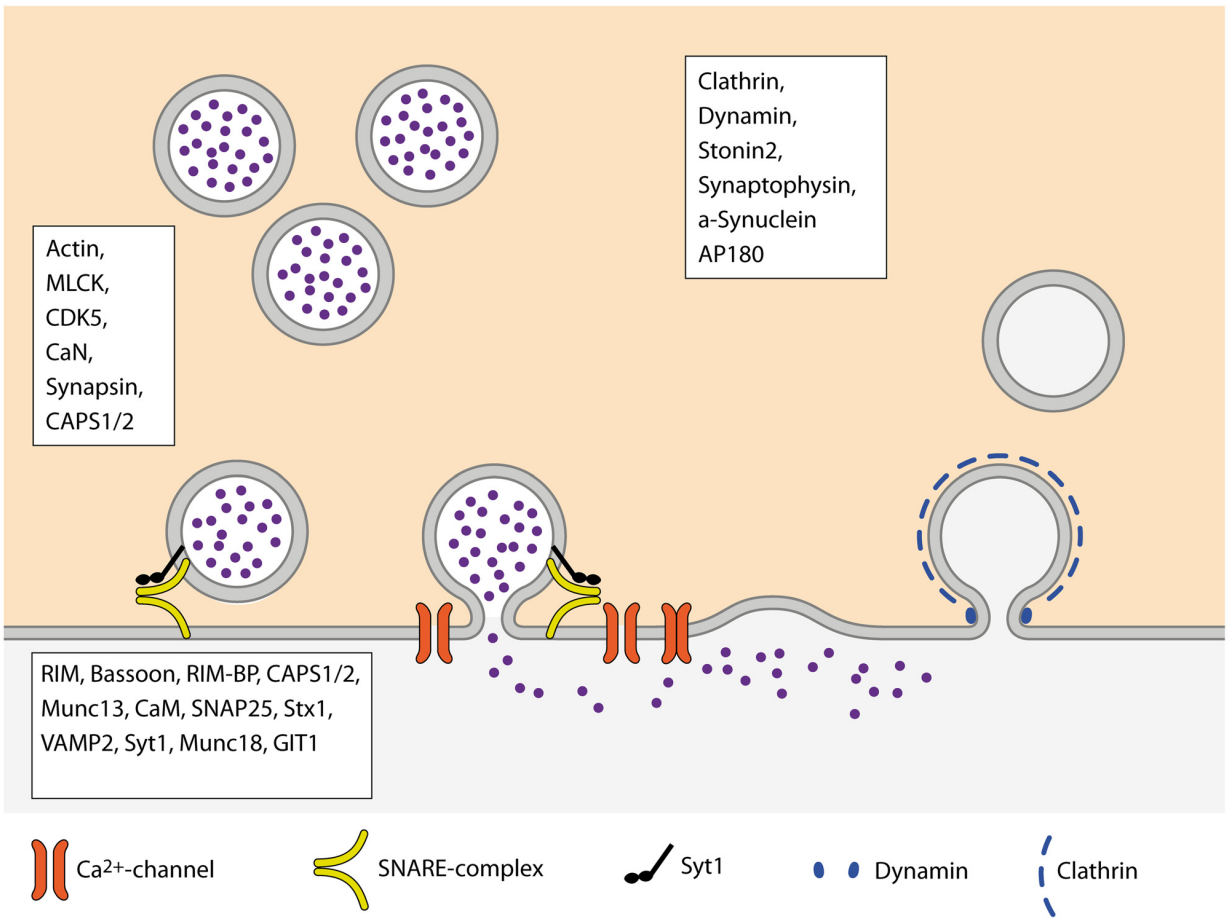

FIGURE 3 | Schematic overview of the SV cycle. Boxes name proteins of the respective steps of the cycle that are involved in the regulation of P_r.

P/Q-type VGCC in the non-activated, calcium-free form, causing robust facilitation of the calcium current. This is released by subsequent calcium binding to CaM, resulting in an apparent VGCC inhibition (Adams et al., 2014). Furthermore, other presynaptic calcium-binding protein have been shown to interact with VGCCs and to modulate their gating properties, and in turn, calcium influx and P_r. CaBP-1, NCS-1 and VLIP-2 all modulate calcium-dependent facilitation and inactivation of the P/Q-type VGCC, albeit at different extents (Lee et al., 2002; Tsujimoto et al., 2002; Lautermilch et al., 2005, respectively). This calcium/CaM-dependent modulation of calcium influx through VGCCs has profound impact on STP and thus information transfer at a variety of synapses (Catterall et al., 2013).

Apart from proteins of the AZ, VGCCs are modulated by a variety of extracellular signals. Activation of presynaptic $GABA_B$Rs for example results in a profound decrease in calcium influx at various synpses due to direct interactions of activated G-proteins with the VGCCs (Dittman and Regehr, 1996; Kajikawa et al., 2001; Sakaba and Neher, 2003a; Wang et al., 2013; Kupferschmidt and Lovinger, 2015). Inhibition of VGCCs by direct interaction with G-proteins upon activation of G-protein-coupled receptors (GPCRs) provides a general mechanism to regulate presynaptic calcium influx during ongoing synaptic activity. The activation of presynaptic A1 adenosine receptors (e.g., Dittman and Regehr, 1996; Wong et al., 2006), α2-noradrenergic receptors (e.g., Leão and Von Gersdorff, 2002) cannabinoid receptors (e.g., Kushmerick et al., 2004) and metabotropic glutamate receptors (e.g., von Gersdorff et al., 1997; Renden et al., 2005; Kupferschmidt and Lovinger, 2015) results in VGCC inhibition, and thereby reduced calcium influx and P_r, too. Furthermore, VGCCs are also regulated by the neurotrophin BDNF (brain-derived neurotrophic factor), which inhibits the activation of VGCCs at the calyx of Held (Baydyuk et al., 2015). Of note, BDNF has also been proposed to regulate the developmental switch in VGCC type at Purkinje cell output synapses (Miki et al., 2013).

Interestingly, VGCCs can additionally be modulated by direct modification. At least N-type VGCCs are regulated by balanced phosphorylation/dephosphorylation mediated by CDK5 and CaN (Su et al., 2012; Kim and Ryan, 2013). And moreover, the α2δ subunit of the VGCC has been shown to set-up VGCC for triggering SV fusion by binding extracellular metal ions (Hoppa et al., 2012). Thus, the calcium flux through VGCC is controlled by intracellular factors, like AZ proteins, as well as

by extracellular signals such as neuromodulators that act via presynaptically expressed GPCRs.

INDIRECT MODULATION OF CALCIUM INFLUX THROUGH VGCCs

Calcium influx through VGCCs, and therefore P_r, is not only regulated by direct modulation of VGCC function as described above, but is also dependent on the resting membrane potential (RMP) of the presynaptic plasma membrane, which has a large effect on the activation of the VGCCs. The RMP of the presynaptic terminal is determined by the expression of specialized ion channels that collaborate to set the optimal RMP according to the current functional needs of the synapse. However, as direct measurements of ion channel function are only possible at few synapses in the mammalian CNS, most of our knowledge on this topic arises from the calyx of Held, although it may also apply to other synapses. At the calyx of Held, the RMP is set and regulated by the potassium channel subunit $K_v7.5$ (Huang and Trussell, 2011), the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel (Cuttle et al., 2001; Kim et al., 2007), the Na^+/K^+-ATPase (Kim et al., 2007) and a persistent sodium channel of currently unknown origin (Huang and Trussell, 2008). Shifting the RMP to more depolarized potentials by either blocking $K_v7.5$ or activating HCN channels results in the facilitation of VGCC opening and thereby to increases in calcium influx and SV release upon arrival of an AP. A hyperpolarizing shift in the RMP can occur after prolonged high-frequency firing at the calyx of Held, due to the activation of the Na^+/K^+-ATPase containing the α3 subunit. Interestingly, this hyperpolarization is counteracted by the activation of HCN channels (Kim et al., 2007). Along the same lines, the RMP of the calyx of Held can be regulated by the activation of presynaptic glycine receptors (GlyRs). Since the chloride concentration in the calyx of Held is relatively high, activation of GlyRs depolarizes the presynaptic terminal and therefore facilitates VGCC opening and increases and P_r (Turecek and Trussell, 2001; Price and Trussell, 2006). Regulation of the presynaptic RMP hence sets the P_r of the synapse by regulating the efficacy of calcium influx into the presynaptic compartment.

Moreover, the calcium influx into the presynaptic terminal and the P_r can be regulated by the modification of the AP-width: at the calyx of Held, the AP-width narrows during synaptic maturation, concomitant with a decrease in P_r (Taschenberger et al., 2002). This mechanism depends on the modulation of presynaptic voltage-gated potassium channels (K_vs). At hippocampal cultured synapses, the modulation of AP-width can counteract changes in presynaptic VGCC abundance to keep P_r in these synapses constant. This mechanism depends on $K_v3.1$ and K_v1 (Hoppa et al., 2014), both of which have also been shown to be present at the calyx of Held terminal (Ishikawa et al., 2003). K_vs are regulated by ongoing synaptic activity which allows them to adjust the P_r according to the current status of the circuit. K_vs have been shown to undergo activity-dependent facilitation during high frequency firing at the calyx of Held, in order to constrain the duration of the presynaptic AP and thus calcium influx and SV release (Yang et al., 2014). However, the mechanisms by which these regulations of K_v-function occur are largely unknown. A recent study has shown that the membrane-derived lipid arachidonic acid can act as a retrograde messenger to broaden the AP by modulation of K_v-function at the hippocampal mossy fiber to CA3 synapse (Carta et al., 2014). Taken together, in addition to direct regulation of VGCC abundance or function, indirect effects originating from either the RMP or the AP-width also constitute alternative, powerful ways of regulating P_r.

REGULATION OF P_r DURING SHORT-TERM PLASTICITY

The P_r is not only regulated by the molecular machines involved in the recruitment, docking and priming of SVs as well as the positioning and regulation of VGCCs, but also by changes in the presynaptic environment during ongoing activity. Stretches of high synaptic activity induce STP in many mammalian CNS synapses. STP can occur, depending on the synapse and the rate of activity, either as short-term facilitation (STF) or short-term depression (STD). STP subsides within seconds and is an important factor in information processing, as e.g., synapses undergoing STF are more likely to transmit information later in a sequence of presynaptic activity, thereby acting as a high-pass filter, whereas synapses showing STD have a higher initial P_r and act as a low-pass filter by having a higher chance to pass-on the information at the beginning of a sequence of synaptic events. STF is generally thought to depend on the build-up of residual calcium in the presynaptic terminal resulting in a spread of the transient calcium domain and an increase in calcium concentration sufficiently high to trigger neurtransmitter release at SVs that would normally not reach the calcium concentration threshold for fusion. Additionally, calcium-dependent modulation of VGCCs has profound effects on STF, as facilitaion of calcium influx can contibute to the build-up of such residual calcium levels (Catterall and Few, 2008; Catterall et al., 2013). A recent study reported STF at synapses between Purkinje cells that relies primarily on calcium dependent fascilitation of calcium influx (Díaz-Rojas et al., 2015). However, different mechanisms of STF have been shown to operate at certain synapses, e.g., at the cerebellar parallel fiber to Purkinje cell synapse, additional release sites are recruited during STF (Brachtendorf et al., 2015). Moreover, a recent study reported STF to be absent from synaptotagmin 7-KO mice, suggesting that synaptotagmin 7 acts as a calcium sensor specific for STF (Jackman et al., 2016). STD on the other hand has been suggested to be caused by a depletion of the RRP that cannot be balanced by the replenishment machinery (for review, see Zucker and Regehr, 2002).

The mechanisms by which changes in P_r regulate STP are diverse. In essence, every step interfering with the molecular machines described so far can cause alterations in STP. Therefore, a large variety of presynaptic proteins have been implicated in the regulation of STP and the P_r of both, a SV to

fuse and the synapse as a whole to transmit information. For example: (1) alterations in the rate of SNARE complex formation, correlated to the availability of syntaxin 1, have a profound effect on STD in hippocampal cultured neurons (Arancillo et al., 2013); (2) the slowing of recruitment of SVs to the AZ in the absence of synapsins also accelerates STD (Vasileva et al., 2012).

The availability of release-ready SVs in general is one of the main factors determining STP and thus synaptic P_r during ongoing synaptic activity. Therefore, impairing of SV endocytosis leads in general to severe effects on STP, mostly to strong STD. Effective endocytosis and thereby reformation of SVs depends on proteins of a variety of different molecular machines. It involves e.g., SV proteins as synaptophysin and α-synuclein (Kwon and Chapman, 2011; Vargas et al., 2014, respectively), but also proteins of the endocytic core machinery such as stonin 2, clathrin, AP180 or dynamin (Hua et al., 2013; López-Murcia et al., 2014; Kaempf et al., 2015; Koo et al., 2015, respectively). The detailed mechanisms by which SV endocytosis is coupled to exocytosis and STP have recently been reviewed elsewhere (Haucke et al., 2011).

Recently, the view that replenishment of SVs to the site of release is the limiting factor during STD, has been challenged. Studies at the calyx of Held revealed that the block of endocytosis has an immediate effect on SV fusion that is too fast to be explained by the impairment of SV regeneration (Hosoi et al., 2009). Therefore, it has been proposed that the rate-limiting step in SV release under ongoing activity is the clearance of the release site of proteins deposited there by the previously fusing SV (Hosoi et al., 2009). This release site clearance model assumes a fixed number of release sites of which most, but not all, are occupied by a primed SV at the beginning of a sequence of activity. Upon the arrival of APs, more and more of the primed SVs fuse with the plasma membrane and clog the release sites with their protein content. In order to refill the release sites with newly primed SVs, this "debris" first needs to be removed to clear the release site. This clearance then is the rate-limiting step during ongoing SV release and hence has a strong impact of the regulation of P_r (Neher, 2010).

POST-TETANIC POTENTIATION

A special case of STP is post-tetanic potentiation (PTP) that can be observed at many synapses in the CNS. PTP is induced by prolonged intense stimulation, typically 100 Hz for 5–10 s, and lasts for tens of seconds to minutes (Zucker and Regehr, 2002). It combines an increase in RRP size and in P_r (Habets and Borst, 2005; Korogod et al., 2005; Fioravante et al., 2011). Moreover, an increase in quantal size (the postsynaptic current induced by the release of neurotransmitter from a single SV) during PTP has been reported (He et al., 2009; Xue and Wu, 2010; Fioravante et al., 2011), although other groups did not detect this effect (Habets and Borst, 2005; Korogod et al., 2005). The increase in RRP size and P_r, but not in quantal size, depend on the activation of the calcium-dependent protein kinase C (PKC) subunits PKCα, PKCβ and PKCγ (Korogod et al., 2007; Xue and Wu, 2010; Fioravante et al., 2011; Chu et al., 2014) of which PKCβ acts as a calcium sensor (Fioravante et al., 2014). Additionally, the mechanisms by which PKC mediates PTP have been reported to be developmentally regulated. PKCα and PKCβ are responsible for ∼80% of PTP at the more mature calyx of Held (P11–14) primarily by an increase in RRP size (Fioravante et al., 2011). At the juvenile calyx of Held (P8–10) however, PKCγ and PKCβ mediate PTP by increasing the P_r of individual SVs (Chu et al., 2014). Nevertheless, PKC is not the only protein contributing to PTP as calyces of Held lacking PKCα and PKCβ still express 20% of PTP (Fioravante et al., 2011). These alternative mechanisms of PTP may involve MLCK, which has also been shown to be involved in the induction of PTP at the calyx of Held (Lee et al., 2008). Of note, PKC can also be activated by the continuous activation of presynaptic GlyRs, likewise increasing RRP size and P_r (Chu et al., 2012). The mechanism by which PKC mediates PTP has recently been shown to involve the phosphorylation of Munc18-1, which then increases the vesicular P_r in a yet unknown manner (Genc et al., 2014). Once the tetanic stimulus is over and the intracellular calcium concentration returns to baseline, PKC is deactivated and the phosphorylation of Munc18-1 is removed by protein phosphatases 1 and 2A, thereby terminating PTP (Genc et al., 2014).

CONCLUSIONS

The P_r of SVs to be released upon the arrival of an AP, constituting the successful transfer of information across the synapse, is one of the crucial parameters underlying brain function. However, the mechanisms and molecular machines involved in its regulation are still not fully understood. One of the problems in understanding P_r arises from the large variety of processes that impact on it, as reviewed here (**Figure 3**). At some point, most of the presynaptic machinery will affect P_r in one way or the other, may it be the availability of release-ready SVs, regulation of VGCCs or some other mechanism. An additional layer of complexity in understanding P_r derives from the fact that P_r is highly variable between synapses, different types of synapses as well as synapses originating from the same axon (Atwood and Karunanithi, 2002; Branco and Staras, 2009). Further studies will therefore focus on elucidating the regulatory mechanisms that set the P_r of SVs at a given synapse according to the current demands of the circuit.

AUTHOR CONTRIBUTIONS

CK and TK wrote the article.

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Co-existence of Functionally Different Vesicular Neurotransmitter Transporters

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The vesicular transmitter transporters VGLUT, VGAT, VMAT2 and VAChT, define phenotype and physiological properties of neuronal subtypes. VGLUTs concentrate the excitatory amino acid glutamate, VGAT the inhibitory amino acid GABA, VMAT2 monoamines, and VAChT acetylcholine (ACh) into synaptic vesicle (SV). Following membrane depolarization SV release their content into the synaptic cleft. A strict segregation of vesicular transporters is mandatory for the precise functioning of synaptic communication and of neuronal circuits. In the last years, evidence accumulates that subsets of neurons express more than one of these transporters leading to synaptic co-release of different and functionally opposing transmitters and modulation of synaptic plasticity. Synaptic co-existence of transporters may change during pathological scenarios in order to ameliorate misbalances in neuronal activity. In addition, evidence increases that transporters also co-exist on the same vesicle providing another layer of regulation. Generally, vesicular transmitter loading relies on an electrochemical gradient $\Delta\mu H^+$ driven by the proton ATPase rendering the lumen of the vesicle with respect to the cytosol positive ($\Delta\psi$) and acidic (ΔpH). While the activity of VGLUT mainly depends on the $\Delta\psi$ component, VMAT, VGAT and VAChT work best at a high ΔpH. Thus, a vesicular synergy of transporters depending on the combination may increase or decrease the filling of SV with the principal transmitter. We provide an overview on synaptic co-existence of vesicular transmitter transporters including changes in the excitatory/inhibitory balance under pathological conditions. Additionally, we discuss functional aspects of vesicular synergy of transmitter transporters.

Keywords: vesicular transmitter transporters, synaptic co-existence, vesicular synergy, glutamate, GABA

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Abbreviations: ACh, acetylcholine; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxacole propionic acid; ChAT, choline acetyltransferase; cKO, conditionally knocked out; CNS, central nervous system; EPSC, excitatory postsynaptic current; GAD65/67, glutamate decarboxylase; GAT, plasma membrane GABA transporter; GlyT, plasma membrane glycine transporter; IPN, interpeduncular nucleus; MF/MFT, mossy fiber/mossy fiber terminal; mGluR, metabolic glutamate receptor; Na^+/Pi, transport sodium-phosphate transport; NMDA, N-methyl-D-aspartate; POMC, pro-opiomelanocortin; SLC, solute carrier; SV, synaptic vesicle; VNT, vesicular neurotransmitter transporter; VAChT, vesicular acetylcholine transporter; VGAT/VIAAT, vesicular GABA/inhibitory amino acid transporter; VGLUT, vesicular glutamate transporter; VMAT, vesicular monoamine transporter; $\Delta\mu H^+$, electrochemical gradient; $\Delta\psi$, electrical gradient; ΔpH, proton gradient.

INTRODUCTION

Synaptic vesicles (SV) are key organelles for neuronal communication. They concentrate neurotransmitters produced in neural cytoplasm. Following presynaptic depolarization, cytosolic Ca^{2+} increases and SV release their content by exocytotic membrane fusion into the synaptic cleft. Fused SV are retrieved by endocytosis mainly following the clathrin-mediated pathway. In neurons, specific sets of secondary active solute transporters are responsible for filling SV with neurotransmitters. These vesicular neurotransmitter transporters (VNTs) differ from transporters in the plasma membrane with respect to energy coupling, substrate specificity and affinity. Three VNT gene families (solute carrier) comprise vesicular transporter for amines (SLC18; Schuldiner et al., 1995), for inhibitory (SLC32; McIntire et al., 1997; Sagné et al., 1997) and for excitatory amino acids (SLC17; Bellocchio et al., 2000; Takamori et al., 2000). Members account for the vesicular transport of monoamines (VMAT1 and VMAT2; SLC18A1, SLC18A2, respectively), acetylcholine (VAChT, SLC18A3), GABA/glycine (VGAT also known as VIAAT, SLC32A1), and glutamate (VGLUT1-3; SLC17A7, SLC17A6, SLC17A8, respectively). The VNTs specify the quality and quantity of SV transmitter content and thus the synaptic dynamics of neurotransmission.

With the avenue of the molecular characterization of the various VNTs, their distribution in brain areas at the cellular and subcellular level has been extensively investigated. Evidence accumulated that different types of VNTs can be expressed in the same axon terminal resulting in synaptic co-release of transmitters of opposing function like glutamate and GABA. In addition, subpopulations of SV harbor not only one type of VNT. Both, synaptic co-existence and vesicular synergy of VNTs differently affect synaptic plasticity under healthy conditions and if misbalanced may lead to various pathologies.

SYNAPTIC CO-EXISTENCE OF NEUROTRANSMITTERS AND VNTs

Dale's principle that one neuron stores and releases only a single type of neurotransmitter can no longer be maintained in its strict sense. Before, neuropeptides as well as ATP have been shown to released together with acetylcholine (ACh), GABA, and monoamines (for review, see Edwards, 2007). First direct functional evidence against this principle was the demonstration of co-release of GABA and glycine from same spinal cord synapses (Jonas et al., 1998). In this system, however, both neurotransmitters share the same transporter VGAT/VIAAT.

Studies during the last decades then demonstrated that especially glutamate could be released together with other classical neurotransmitters. Glutamate released from cholinergic spinal cord motor neurons stimulates Renshaw cells and other motor neurons (Nishimaru et al., 2005). The co-release of excitatory glutamate and inhibitory GABA neurotransmitters has been controversially discussed (Gutiérrez et al., 2003; Uchigashima et al., 2007). However, a variety of investigations including the differential synapse-specific co-existence of VGLUT1 and VGLUT2 with VGAT (Zander et al., 2010 see below) indicate that co-release of glutamate and GABA is more widespread than previously anticipated (Seal and Edwards, 2006; Chaudhry et al., 2008; El Mestikawy et al., 2011). Such co-release may not necessarily result in a direct antagonism but represents a new layer of fine-tuning synaptic networks. Following scenarios may apply to synaptic co-existence and co-release : (1) The VNTs reside on distinct populations of SVs in the axon terminal, indicating the potential for different release at distinct sites, which then activate subsets of postsynaptic receptors (**Figure 1**). This principle may primarily apply to giant terminals containing many transmitter release sites such as the mossy fiber terminals (MFTs; Boulland et al., 2009). (2) A spatial selective distribution of neurotransmitter receptors and their different affinities as well as variations in the type of synapses modulate the controlled activation of pre- and postsynaptic receptors (Rubio and Wenthold, 1997) depending on different transmitters released by a common site (**Figure 1**).

Three isoforms of VGLUTs (VGLUT1, VGLUT2 and VGLUT3) mediate the vesicular uptake of glutamate in central nervous system (CNS) neurons thereby defining glutamatergic neurotransmission. VGLUTs share salient structural and functional characteristics but differ in cellular localization and trafficking, suggesting isoform-specific physiological roles beyond their primary role of concentrating glutamate into SV (Fremeau et al., 2004; Chaudhry et al., 2008). In CNS, VGLUT1 and VGLUT2 show complementary patterns of expression mainly in subset of glutamatergic neurons (Fremeau et al., 2001; Varoqui et al., 2002). VGLUT3, by contrast, is mainly expressed in non-glutamatergic neurons characterized by other neurotransmitters (Fremeau et al., 2002; Gras et al., 2002; Takamori et al., 2002). In addition, VGLUT1 and VGLUT2 have been shown to be co-expressed in various subsets of non-glutamatergic neurons (Trudeau, 2004; Danik et al., 2005; Kawano et al., 2006), further supporting the idea that glutamate also acts as a co-transmitter.

For the inhibitory transmitters GABA and glycine only one transporter VIAAT/VGAT has been identified (McIntire et al., 1997; Sagné et al., 1997) which transports the two transmitters into SV with comparable uptake characteristics (Christensen and Fonnum, 1991). The genetic deletion of VGAT in mice finally proved, that VGAT is the only transporter for both inhibitory transmitters (Wojcik et al., 2006). VGAT is co-expressed with either the GABA-synthesizing enzymes glutamate decarboxylase (GAD65 and 67), as well as the plasma membrane GABA transporter GAT1 or the plasma membrane glycine transporter GlyT2, (Sagné et al., 1997; Chaudhry et al., 1998; Dumoulin et al., 1999; Dalby, 2003; Dufour et al., 2010), respectively. Thereby the GABAergic (Wojcik et al., 2006) or glycinergic (Gomeza et al., 2003) phenotype of neurons is defined.

Glutamate is the main excitatory neurotransmitter released from hippocampal MFTs (Crawford and Connor, 1973; Storm-Mathisen et al., 1983; Terrian et al., 1988; Bramham et al., 1990). For long, it has been known that also the inhibitory GABA transmitter is present in MFTs

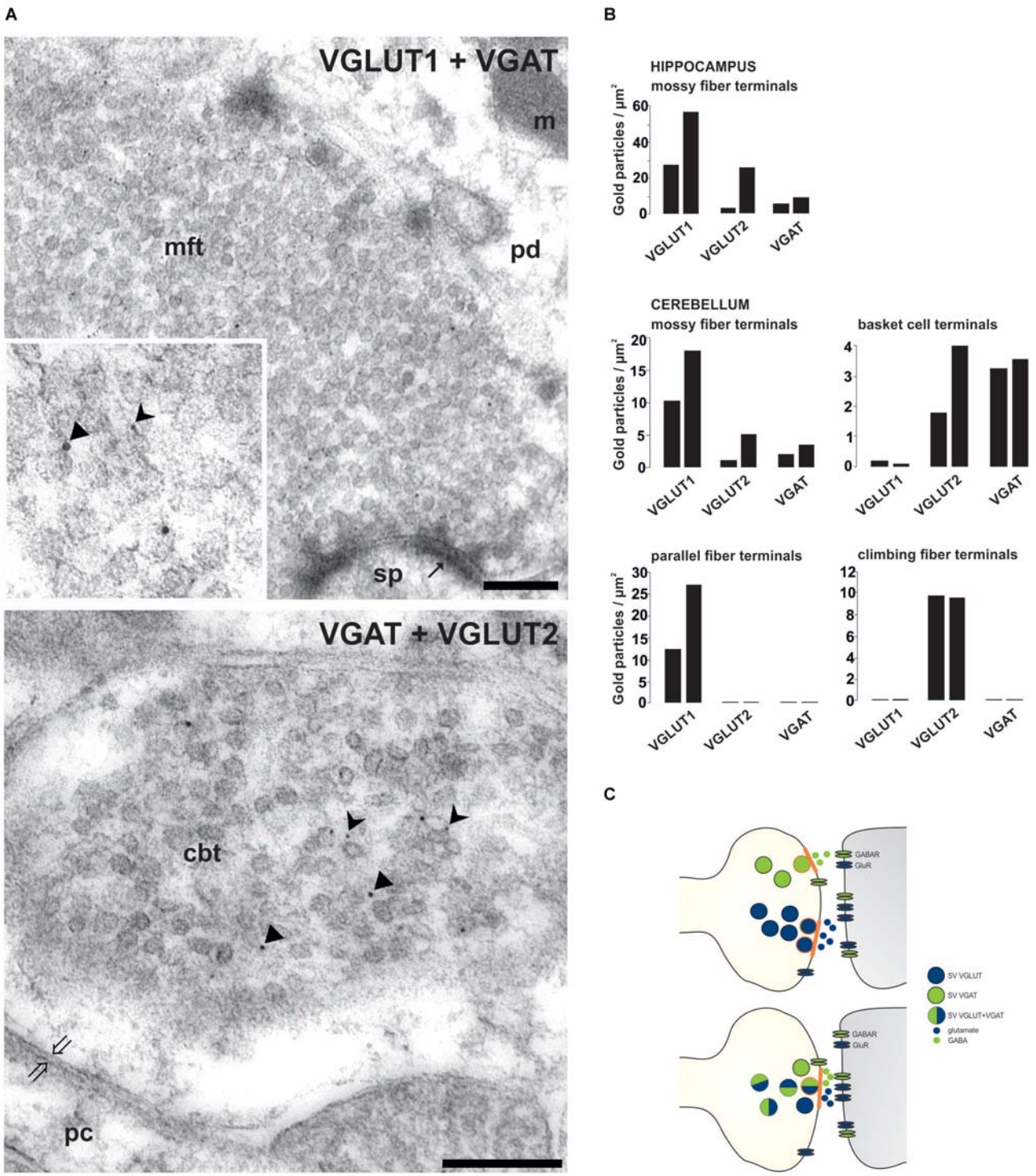

FIGURE 1 | Synapse-specific co-existence of VGLUT and VGAT. (A) Hippocampal mossy fiber terminals (mft) of the CA3 area or cerebellar basket cell terminals (cbt) were double immunolabeled with antisera against either VGLUT1 (rabbit 5 nm gold) and VGAT (guinea pig, 10 nm gold particles) or against VGAT (guinea pig, 10 nm gold particles) and against VGLUT2 (rabbit, 5 nm gold particles), respectively (Zander et al., 2010). Distinct SV associated gold particles either indicating VGLUT are marked in insert by forked arrowheads or indicating VGAT by triangles. **(B)** Quantification of the number of gold particles staining for VGLUT1, VGLUT2 and VGAT in glutamatergic and GABAergic terminals of the hippocampal CA3 area and of the cerebellum (see Zander et al., 2010). **(C)** Scheme depicting the variations involved in synaptic co-existence of VNTs of opposing function (blue-VGLUT, green-VGAT) in the same vesicle and in different vesicle populations with distinct (above) or combined (below) release sites (orange labeled) within axon terminal. pd, pyramidal dendrite; sp, spine; pc, purkinje cell body; m, mitochondria; thin arrows indicate asymmetric contacts, thick open arrows indicate symmetric contact. Bars given in **(A)** represent 200 nm.

(Sandler and Smith, 1991; Sloviter et al., 1996). A final confirmation demands that GABA: (1) occurs in SVs of MFTs; (2) is released in response to stimulation; and (3) produces specific receptor responses. To support the idea of the dual glutamatergic-GABAergic phenotype of MFTs, we performed quantitative postembedding immunogold labeling with antibodies specific to VGLUTs and VGAT at the electron microscopic (EM) level. Postembedding immunogold EM is the method of choice to label precisely subcellular organelles, such as SV (Bergersen et al., 2008; Grønborg et al., 2010; Zander et al., 2010; Ormel et al., 2012). By this method, a synaptic co-localization of VGLUTs and VGAT in glutamatergic hippocampal and cerebellar MFTs could be demonstrated (**Figure 1**). The specificity of the co-existence of VGAT with VGLUT in MFTs (**Figure 1**) is underscored by the complete absence of VGAT immunoreactivity in other glutamatergic terminals such as cerebellar parallel (VGLUT1-positive) and climbing (VGLUT2-positive) fiber terminals identified in the same section (**Figure 1**). These findings provide a strong morphological evidence for synaptic co-existence of glutamate and GABA in adult glutamatergic MFTs under healthy conditions. In a similar approach, it was shown, that GABA is present together with glutamate in large MFTs and that both amino acids were associated with distinct SV (Bergersen et al., 2003, 2008). However, GABAergic synaptic transmission at MFTs requires the presence of postsynaptic GABA receptors. Indeed, $GABA_A$ receptors appear to co-localizes, with glutamate alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, in postsynaptic densities in close apposition to MFTs (Bergersen et al., 2003). Their additional presynaptic contributing add to subthreshold electrical signaling in hippocampal MF (Alle and Geiger, 2007). This finding strongly suggests that MFs may convey not only a glutamatergic but also a GABAergic signal to their targets (Gutiérrez, 2005). Consistent with this hypothesis, monosynaptic GABAergic currents have been recorded in CA3 principal cells upon granule cell stimulation in the dentate gyrus in acute hippocampal slices from new-born (Safiulina et al., 2006) or juvenile animals (Walker et al., 2001; Gutiérrez et al., 2003). The evoked responses fulfil the criteria for identification of GABAergic MF inputs: strong, paired pulse facilitation, short-term frequency dependent facilitation, and sensitivity to group II and III mGluR agonists (Safiulina et al., 2006).

Several lines of evidence indicate that overshooting glutamatergic transmission play a key role in the ethology and progression of temporal lobe epilepsy (During and Spencer, 1993; Petroff et al., 2002; Cavus et al., 2005; El-Hassar et al., 2007). An increased expression of VGLUT may be important in the initiation and/or maintenance of seizures as shown in different animal models for epilepsy. Remarkably, treatment with antiepileptic drugs reduced VGLUT1 overexpression and seizure activity (Kang et al., 2005, 2006; Kim et al., 2005). In addition, a change in the balance between glutamatergic (VGLUT) and GABAergic (VGAT) expression in MFTs of the hippocampus appears to be crucial during the development of overexitation (Gomez-Lira et al., 2005).

In recent years, optogentic studies using the targeted expression of channel rhodopsin and modifications thereof yielded new insight in the mechanisms how different transmitters released from the same terminal may affect neurotransmission and behavior (Jiang et al., 2014; Trudeau et al., 2014). For example, optogenetic stimulation of arcuate hypothalamic neurons expressing diverse neuropeptides derived from pro-opiomelanocortin (POMC) leads to release of either glutamate or GABA causing fast postsynaptic actions (Dicken et al., 2012). Optogenetically labeled mesolimbic dopaminergic neurons were shown to release glutamate in the nucleus accumbens (Tecuapetla et al., 2010; Chuhma et al., 2014). In turn, habenula-derived cholinergic neurons release glutamate during brief optogenetic stimulation of their axons, and release ACh during stronger and more prolonged stimulation (Ren et al., 2011). Further examples are the regulation of spontaneous locomotor activities likely modulated by glutamate released from cholinergic interneurons in the striatum (Guzman et al., 2011). Using an optogenetic approach the relative importance of ACh released alone vs. ACh plus a co-released additional transmitter could be also distinguished. In this context, it has been shown that amacrine cells in the retina co-release ACh and GABA by distinct SV populations (Lee et al., 2010).

VESICULAR SYNERGY OF VNTs

Vesicular co-existence at the synaptic level also includes vesicular synergy. Vesicular synergy involves the presence of VNTs, which differ in their dependance on the electrochemical gradient including the co-storage of transmitters with opposing function (El Mestikawy et al., 2011). Generally, all VNTs rely on the vesicular VoATPase that pumps H^+ ions into the SV and builds up an electrochemical gradient ($\Delta\mu H^+$) rendering the vesicular lumen with respect to the cytosol positive ($\Delta\psi$) and acidic (ΔpH). The various VNTs differ in their preference for either ΔpH or $\Delta\psi$. VAChT and VMAT strongly depend on the ΔpH component. In contrast, VGLUT and VNUT (vesicular nucleotide transporter) appear to work best at a high $\Delta\psi$. Using purified VGLUT1 reconstituted with an exogenous proton pump in liposomes, liposome-fused and native SV, the $\Delta\psi$-dependance, chloride transport and H^+/K^+ exchange activity of VGLUT have been demonstrated (Schenck et al., 2009; Preobraschenski et al., 2014). For VGAT there is presently no agreement about the transport mechanism by which the inhibitory transmitters GABA and glycine are concentrated into SV, with a proton exchange and chloride-cotransport mechanism being discussed (Burger et al., 1991; Juge et al., 2009). The presence of different VNTs on a single SV implies that the loading of the basic transmitter is modulated by the additional transport of a second transmitter, which improves either ΔpH or $\Delta\psi$ depending on the combination. In this respect, the most frequent combinations involve VGLUT (one of the three isoforms) in either monoaminergic, cholinergic or GABAergic neurons. VGLUT depends on $\Delta\psi$ and VMAT/VAChT or VGAT exclusively or partially rely on ΔpH, respectively.

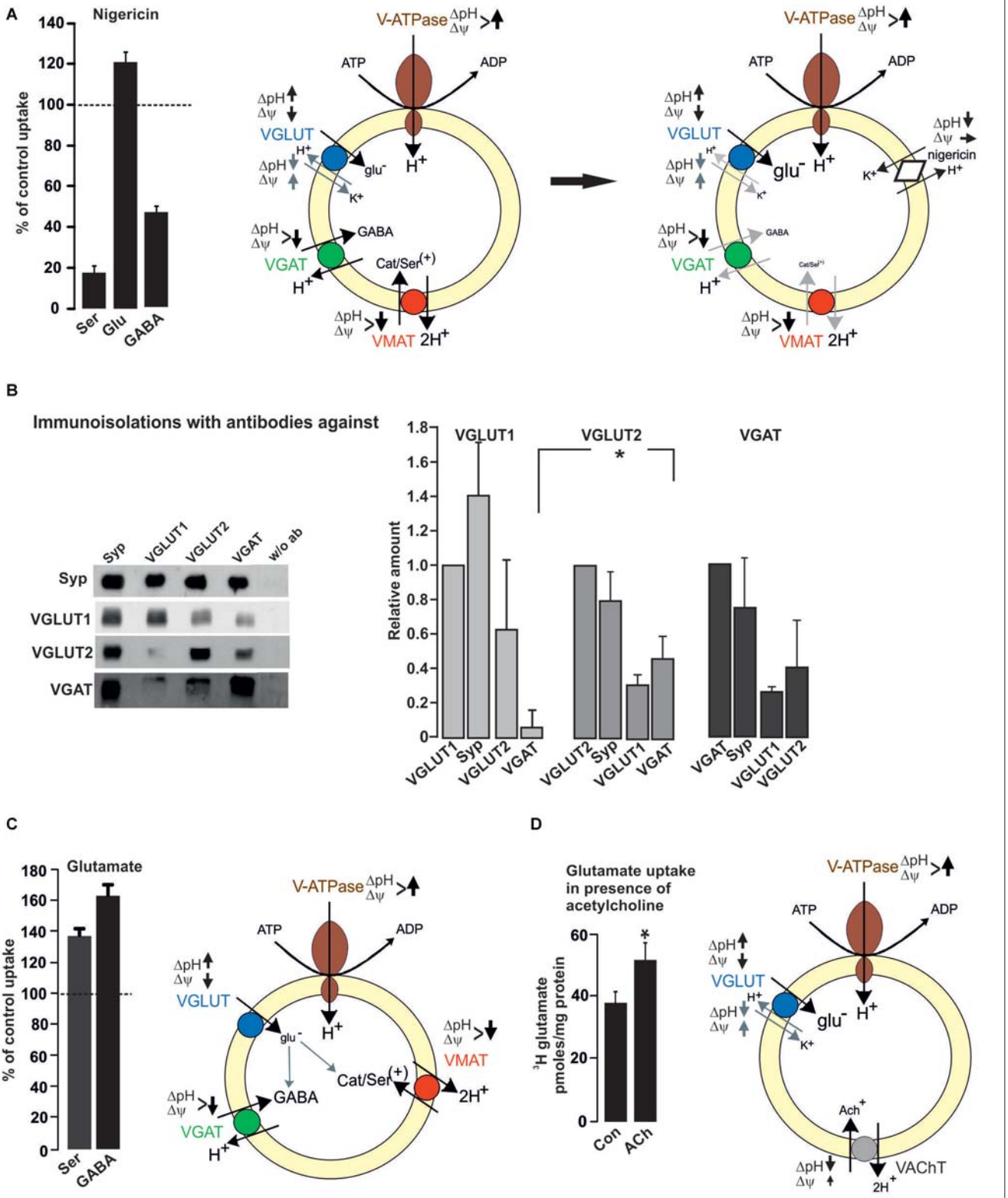

FIGURE 2 | Functional impact of vesicular co-existence. (A) Nigericin (500 nM) increases glutamate uptake into rat brain SV, while the uptake of serotonin or GABA is inhibited. Values are presented as percent of uptake in the absence of nigericin (control) and represent mean and SD of six samples obtained in two different experiments (see also Preobraschenski et al., 2014). Nigericin promotes H^+/K^+ exchange thereby decreasing ΔpH with no or small effects on $\Delta\psi$. This favors VGLUT activity while both VGAT and VMAT activity are reduced or dissipated, respectively. **(B)** Immunoisolation of SV populations was performed from whole

(*Continued*)

FIGURE 2 | Continued
rat brain with or without the antibodies indicated. A standard curve from the starting material (LS0) was run in parallel to calculate the respective protein amounts in the individual immunoisolates (Zander et al., 2010). **(C)** Glutamate increases the vesicular uptake of either serotonin or GABA into rat brain SV. Uptake in the absence of glutamate was set to 100%. Values represent the mean and SD of four samples obtained in two experiments (modified from Zander et al., 2010). Each transported glutamate molecule increases ΔpH thereby improving VGAT and VMAT activity. **(D)** Glutamate uptake into SV prepared from rat interpeduncular nucleus (IPN) is increased in the presence of acetylcholine (ACh). Values are expressed as pmoles/mg protein and were obtained from four different experiments (Frahm et al., 2015). The positively charged ACh like NH_4^+, decreases ΔpH and slightly increases $\Delta\psi$, thereby improving VGLUT activity (see also Preobraschenski et al., 2014). $^*p < 0.05$ Student's *t*-test.

The opposing effects of ΔpH for transmitter filling becomes obvious when monoamine, glutamate or GABA uptakes are performed in the presence of nigericin. Nigericin exchanges H^+ against K^+ thereby dissipating ΔpH with negligible effects on $\Delta\psi$. Consequently, nigericin almost abolishes serotonin and partially inhibits GABA uptake but promotes vesicular filling with glutamate (Preobraschenski et al., 2014; **Figure 2**).

Using immunoisolation the vesicular synergy of VGLUT2 and VMAT2 has been identified in SV preparations from rat striatum (Hnasko et al., 2010). This vesicular synergy improves the monoamine filling in the presence of glutamate since each transported glutamate has to be compensated by a proton, which increases ΔpH and supports VMAT activity (Hnasko et al., 2010; Zander et al., 2010; **Figure 2**). Consequently, tissue levels of dopamine are decreased in the ventral striatum following deletion of VGLUT2. At the behavioral level, VGLUT2 deletion in striatal dopaminergic neurons reduces motor activity following cocaine treatment (Hnasko et al., 2010). VGLUT3 appears to occur preferentially in 5-HT neurons. These neurons also rely on VMAT2 for concentrating serotonin into SV. Comparable to the VGLUT2/VMAT2 combination in the striatum the absence of VGLUT3 causes an anxiety-related behavior in the mutants probably due to the reduced filling in subsets of serotonergic SV (Amilhon et al., 2010; see also **Figure 2** and Zander et al., 2010).

All three VGLUT isoforms appear to be associated with various subpopulations of GABAergic SV. In rat cerebral cortex, immunoisolation revealed VGLUT1 but not VGLUT2 as co-existing on VGAT SV (Fattorini et al., 2009). Overall brain, vesicular synergy appears to involve VGLUT2 or VGLUT3 as dominant partners for VGAT SV (Seal et al., 2008; Grønborg et al., 2010). Immunoisolations from whole brain post nuclear fractions using either VGLUT1, VGLUT2 or VGAT antibodies support this notion with a preference of VGAT on VGLUT2 over VGLUT1 SV and *vice versa* (Zander et al., 2010; **Figure 2**). Irrespective of the VGLUT isoform, VGLUT/VGAT synergy may functionally improve vesicular filling with GABA in GABAergic vesicles and fine-tune the excitatory/inhibitory balance. Indeed vesicular GABA filling is enhanced in the presence of glutamate (**Figure 2**; Zander et al., 2010). As with VMAT, the negatively charged glutamate increases ΔpH thereby promoting VGAT activity. The functional impact for vesicular GABA loading is evident in VGLUT3 deletion mutants, which besides their deafness also suffer from rare seizures. As an additional explanation, the lack of glutamate co-release from subpopulations of GABAergic neurons may cause a hyper-synchronization of GABAergic interneurons (Ahnert-Hilger and Jahn, 2008). In the same line, the vesicular synergy of VGLUT1 on VGAT SV has been reported to fine tune an excitation/inhibition balance in cortical microcircuits (Fattorini et al., 2015). VGLUT, in particular VGLUT3 in the striatum and raphe nucleus of adult is targeted to cholinergic and serotonergic vesicles where it synergizes the action of vesicular transporters for ACh and serotonin, increasing both rate and degree of vesicle filling (Gras et al., 2008; Amilhon et al., 2010; for review El Mestikawy et al., 2011). Deletion of VGLUT3 besides the above mentioned effects in GABAergic neurons (Seal et al., 2008) causes motor hyperactivity and an increased sensitivity to cocaine explained by the reduced vesicular storage of ACh in striatal cholinergic SV (Gras et al., 2008).

A synaptic and vesicular coexistence between VGLUT1 and VAChT has been also identified in cholinergic neurons projecting from the median habenula to the interpeduncular nucleus (IPN). These neurons exhibit robust glutamatergic transmission involving AMPA and NMDA-receptor EPSCs in addition to slow nicotinic ACh-mediated currents (Ren et al., 2011). While this appears to extend the promoting effects of VGLUT on ACh filling mediated by ΔpH-dependent VAChT (see above), it was recently reported that in these neurons ACh promotes vesicular glutamate filling. Using conditionally-knocked out (cKO) mice with choline acetyltransferase (ChAT) being selectively deleted in median habenula neurons it turned out that EPSCs are reduced while the median habenula/IPN projections are perfectly equipped with VGLUT1/VGLUT2 and VAChT. The cKo–ChAT mice show reduced EPSCs and are completely insensitive to nicotine-conditioned reward and withdrawal. The findings are best explained by the lack in production and vesicular filling of ACh in the highly VGLUT expressing vesicles. ACh is a weak base, can transiently increase the luminal pH (which decreases the ΔpH) and promotes glutamate uptake (Frahm et al., 2015; **Figure 2**) comparable to NH_4^+ (Preobraschenski et al., 2014).

Taken together vesicular synergy represents the modulation of vesicular filling with one transmitter by changing either ΔpH or $\Delta\psi$ by a second transmitter (see above). As an exception, the broad co-existence between VGLUT1 and VGLUT2 (Herzog et al., 2006; Grønborg et al., 2010; Zander et al., 2010) probably does not change vesicular glutamate filling with both isoforms exhibiting similar kinetic properties. However, VGLUT1 but not VGLUT2 interacts with endophilin. This interaction changes the sorting behavior of VGLUT1 expressing SV (Voglmaier et al., 2006) and modulates their release probability (Weston et al., 2011). Thus, co-existence of VGLUT1 and VGLUT2 on the same vesicle may differentially affect endocytic retrieval modulating the persistence of VGLUTs at the plasma membrane where the transporters may be involved in Na^+/Pi transport. Such changes

in plasma membrane vs. SV associations appear to be relevant in context of a day/night cycle (Yelamanchili et al., 2006; Darna et al., 2009).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

VNTs define the transmitter phenotype of SV and neurons and their overall expression is strictly segregated. However, there are layers of modulation at the synaptic or SV level: (1) Some neurons equip their nerve terminals with VNTs transporting transmitter of opposing or different function. This synaptic co-existence increases the functional diversity in between aminergic, GABAergic and glutamatergic neuron types. (2) Different VNTs populate the same SV in some terminals thereby modulating synaptic activity elicited by the main principal transmitter of these neurons. Examples are the vesicular synergy of VGLUT with either VGAT, VAChT, or VMAT, respectively.

Additional layers of modulation refer to the putative GABA transport by VMAT in dopaminergic neurons (Tritsch et al., 2012). Accordingly, the different endocytic retrieval of VGLUT isoforms and the transient expression of VGLUT1 at the plasma membrane also differentially shapes synaptic activity in a diurnal context. Besides glutamatergic neurons, this may also apply to neurons where VGLUT1 coexists at either the synaptic or the SV level.

AUTHOR CONTRIBUTIONS

AM-W, J-FZ and KR performed experiments. GA-H and AM-W wrote manuscript.

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The Roles of Microtubule-Based Transport at Presynaptic Nerve Terminals

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Targeted intracellular movement of presynaptic proteins plays important roles during synapse formation and, later, in the homeostatic maintenance of mature synapses. Movement of these proteins, often as vesicular packages, is mediated by motor complexes travelling along intracellular cytoskeletal networks. Presynaptic protein transport by kinesin motors in particular plays important roles during synaptogenesis to bring newly synthesized proteins to establish nascent synaptic sites. Conversely, movement of proteins away from presynaptic sites by Dynein motors enables synapse-nuclear signaling and allows for synaptic renewal through degradation of unwanted or damaged proteins. Remarkably, recent data has indicated that synaptic and protein trafficking machineries can modulate each other's functions. Here, we survey the mechanisms involved in moving presynaptic components to and away from synapses and how this process supports presynaptic function.

Keywords: presynapse, intracellular trafficking, kinesin, Dynein, signaling pathways, synaptogenesis, autophagy

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INTRODUCTION

Neurons possess the capacity to modify their synapses throughout life (Holtmaat and Svoboda, 2009). Being often located significant distances away from the neuronal cell body, biomolecules destined to function at synapses must usually be transported over extended distances before reaching their destinations. Likewise, biomolecules earmarked for degradation or involved in retrograde signaling must be returned to the cell body. Both processes are critically dependent on the intracellular trafficking machinery that is powered by cytoskeletal-based molecular motors. This review will examine the roles of the microtubule-based transport machinery as an integral set of nanomachines supporting the function of presynaptic terminals.

MOLECULAR COMPLEXITY OF SYNAPSES AND CHALLENGES FOR SYNAPTIC TRAFFICKING

Presynaptic terminals play critical roles in mediating neurotransmitter release during synaptic transmission (**Figure 1**). To fulfill these roles, mature terminals possess hundreds of different proteins that are intricately assembled into molecular nanomachines involved in various aspects of presynaptic function (Morciano et al., 2009; Südhof, 2012; Boyken et al., 2013;

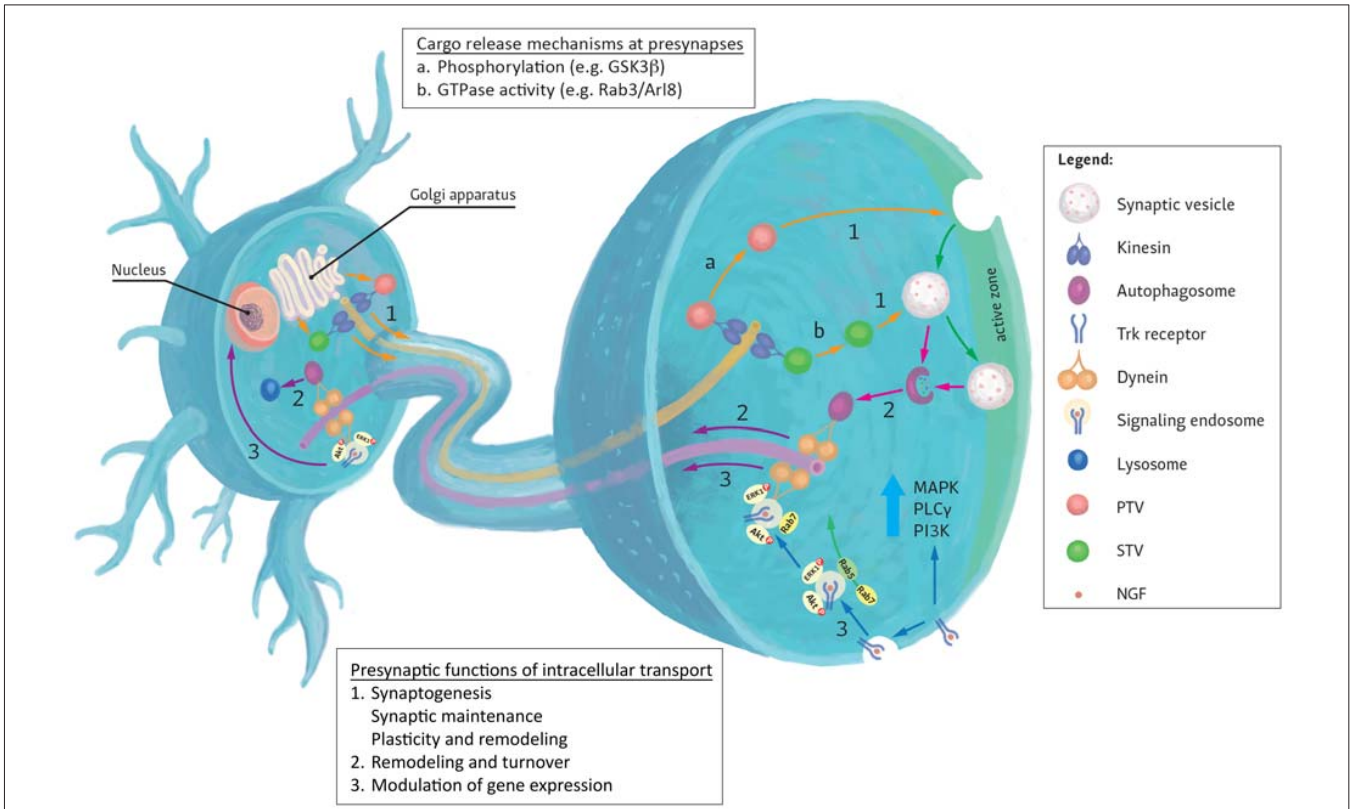

FIGURE 1 | Generalized representation of trafficking events to and from the presynapse. (1) Most presynaptic proteins are synthesized in the cell body and transit the Golgi apparatus where they are sorted to specific transporting vesicles (for example, synaptic vesicle precursor transport vesicles (STVs) or Piccolo-Bassoon transport vesicles (PTVs)) and delivered to presynapses by anterograde kinesin motors. Upon reaching their destinations, several mechanisms are known to mediate cargo unloading including: (a) post-translational modifications (e.g., phosphorylation) of kinesin motors, adaptors and cargoes or (b) via the action of GTPases and their effectors. Released cargoes can then be used to build new presynaptic sites (synaptogenesis), maintain existing synapses or contribute to synaptic plasticity. (2) Degradation and turnover of excess or damaged proteins by the autophagy pathway also contributes to presynaptic homeostatic maintenance. Autophagosomes formed here are returned to the cell body by retrograde Dynein motors. (3) Retrograde transport can also be used to convey extracellular signals received at presynaptic terminals to the nucleus where they modulate gene expression. These signals impact the growth and survival of neurons and mediate their response to injury. An example of such responses can be evoked by the binding of neurotrophins (for example NGF) to presynaptic Trk receptors. Activation of these receptors evokes local signaling response at presynapse. Alternatively, receptor-ligand complexes are endocytosed and form signaling endosomes containing other activated signaling molecules. These endosomes are then transported by Dynein to the cell body to induce changes in gene expression or directly regulate signaling cascades. Sizes of molecules and vesicles are not drawn to scale.

Wilhelm et al., 2014). In particular, synaptic vesicles (SVs) and active zones (AZs) are key components involved in this process. Elegant proteomic analysis of SV composition by Takamori et al. (2006) demonstrated that, despite its small size, SVs exhibit remarkable complexity and diversity in their protein content. Proteins directly involved in vesicle exocytosis such as the SNARE proteins (syntaxins, synaptobrevins and SNAPs), the calcium sensor synaptotagmin, V-ATPase and vesicular neurotransmitter transporters (VGLUT and VGAT) were all detected. Similarly, AZs that demarcate sites of neurotransmitter release by recruiting SVs and voltage-gated Ca^{2+} channels also comprise of a large ensemble of proteins including AZ proteins such as RIM, RIM-BP, Munc13, ELKS/CAST/ERC and α-liprin that participate in SV docking and priming (Südhof, 2012; Chua, 2014). While this complexity and diversity of proteins in the presynapse has long been appreciated, we are only starting to unravel the principles underlying the movement of these cargoes to and away from these sites.

MICROTUBULE-BASED MOTORS AS NANOMACHINES TO TRANSPORT PRESYNAPTIC CARGOES

Diffusion can be effective for migration of small cargoes within very short distances (Encalada and Goldstein, 2014). However, the process becomes highly inefficient with increasing cargo size and distance to be travelled. The substantial gap separating most synapses and the neuronal cell body necessitates the employment of the intracellular trafficking machinery to shuttle presynaptic proteins between these sites. In general, Kinesin and Dynein motor protein families are involved in delivering and removing vesicular content to and from presynaptic sites, respectively

(Hirokawa et al., 2010; Kevenaar and Hoogenraad, 2015). Both motor families utilize microtubule cytoskeletal networks to move cargo. Microtubules are inherently polar and the plus ends of microtubule tracks point away from the cell body in axons (Kapitein and Hoogenraad, 2011). Thus, in axonal transport, Kinesin motors are primarily responsible for moving cargoes from sites of synthesis to presynaptic sites, while Dynein motors mediate movement of biomolecules from synapses towards the cell body. In comparison, Myosin motors are generally regarded to be involved in short-range actin-based transport and will not be covered here (Cingolani and Goda, 2008; Hirokawa et al., 2010).

ANTEROGRADE TRANSPORT TO THE PRESYNAPSE INVOLVES KINESIN MOTORS

Many synaptic proteins traffic through the Golgi apparatus en route to synapses after their synthesis in the cell body (**Figure 1**; Sytnyk et al., 2004; Sann et al., 2009). Kinesin motors play important roles not only during the Golgi export of these proteins but also during subsequent trafficking to their destinations (Akhmanova and Hammer, 2010). Presynaptic proteins transported by Kinesin motors include SV and AZ proteins. The precise mechanisms governing the sorting and loading of cargoes onto their respective motors remain unclear but phosphorylation of cargoes, adapters and motors has been implicated as an important regulatory step in this process (Sato-Yoshitake et al., 1992; Lee and Hollenbeck, 1995; Morfini et al., 2002; Chua et al., 2012).

The trafficking of two broad categories of presynaptic cargoes has been studied. Synaptic vesicle precursor transport vesicles (STVs) carrying SV proteins were shown to be delivered to presynaptic sites by Kinesin-3 motors (KIF1A and KIF1Bβ; Yonekawa et al., 1998; Ahmari et al., 2000; Zhao et al., 2001; Sabo et al., 2006). Loss of KIF1A and KIF1Bβ in neurons caused significant reductions in SVs and their proteins in presynaptic terminals. On the other hand, vesicular transport of presynaptic membrane proteins, such as SNAP-25 and syntaxin-1, are mediated by Kinesin-1 motors (Diefenbach et al., 2002; Su et al., 2004; Chua et al., 2012). AZ proteins including Bassoon, Piccolo and RIM are also co-transported with SNAP-25 and synataxin-1 as Piccolo-Bassoon transport vesicles (PTVs; Shapira et al., 2003; Cai et al., 2007). Interestingly, emerging evidence suggests that presynaptic cargoes are probably not as discretely grouped and an appreciable cargo overlap during transport does exist (Goldstein et al., 2008; Maas et al., 2012; Wu et al., 2013).

DELIVERING CARGOES TO SPECIFIC SYNAPSES

Site-specific release of cargoes is one of the least understood steps in trafficking of presynaptic components. Given that an average mammalian neuron possesses thousands of synapses (Beaulieu and Colonnier, 1985), the transport machinery has to discriminate between the varying needs of different presynaptic sites and deliver the appropriate cargoes to these sites accordingly. The simplest explanation could be that cargoes are released upon reaching the ends of microtubule tracks at the presynapse (Rizzoli, 2014). These cargoes would be then redistributed between neighboring presynaptic sites by diffusion, retrograde transport on the same microtubule track or other mechanism (Darcy et al., 2006; Opazo et al., 2010; Staras et al., 2010). Nevertheless, release of cargo caused exclusively by discontinuity of microtubules cannot fully explain specificity of targeted delivery of presynaptic proteins. Local activation of signaling cascades involved in the arrest of anterograde transport can contribute to this process. One such pathway relates to the small GTPase Rab3 which is a marker of mature SVs and is co-transported with STVs to the presynapse (Fischer von Mollard et al., 1990; Schlüter et al., 2004). DENN/MADD is a Rab3 guanine nucleotide exchange factor (GEF) previously shown to regulate neurotransmitter release (Yamaguchi et al., 2002). DENN/MADD was shown to bind both KIF1A and KIF1Bβ and promote anterograde transport of vesicles associated with Rab3 in the GTP-bound state (Niwa et al., 2008). Either locking Rab3 in the GTP-bound state or deletion of the DENN domain containing the putative enzymatic GEF activity interferes with the transport of these vesicles to distal axons. These results suggest that local factors that induce GTP hydrolysis and cycling of Rab3 between its nucleotide bound states could act as a trigger to release cargoes at distal presynaptic sites.

Phosphorylation of motors and cargoes has been implicated in synaptic cargo transport (Sato-Yoshitake et al., 1992; Vagnoni et al., 2011; Chua et al., 2012). Similarly, phosphorylation-dephosphorylation cycles of these proteins are also likely to affect synapse specific delivery of cargoes. One possible candidate for this is GSK3β. Phosphorylation of kinesin light chains by GSK3β does not affect the binding of kinesin to microtubules but instead releases membrane cargoes from the motor (Morfini et al., 2004). GSK3β is a general inhibitor of anterograde trafficking but is selectively active in growth cones (Morfini et al., 2004; Polleux and Snider, 2010; Weaver et al., 2013). Local activation GSK3β can therefore contribute to the release of kinesin cargoes and formation of synapses *de novo* along the leading edge of growing axon.

During synaptogenesis, both PTVs and STVs move bi-directionally along axonal processes and coordinated trafficking of a portion of STVs and PTVs to specific sites along axons has been documented (Ahmari et al., 2000; Shapira et al., 2003; Sabo et al., 2006; Bury and Sabo, 2011). Pausing of STVs and PTVs coincide with cargo deposition in response to a hierarchy of cues from synaptogenic molecules (such as trans-synaptic adhesion molecules) and initiates the formation of nascent presynaptic sites (Lucido et al., 2009; Siddiqui and Craig, 2011; Chia et al., 2013). Coordination between the small GTPase Arl8 and the Mitogen-activated protein (MAP) kinase signaling pathway has been implicated in facilitating site-specific unloading of these cargoes to developing presynaptic sites in *C. elegans* (Wu et al., 2013). In this regard, Arl8 has been proposed to prevent undesirable premature assembly of presynaptic proteins during axonal transport while signaling

through JKK-1/JNK-1 promotes capturing and integration of these proteins to the presynapse. It remains to be determined if Arl8 also serves a similar function during synaptogenesis in mammalian neurons.

In addition to the involvement of specific signaling pathways, recent studies suggest that some aspects of synaptic delivery could be inherently stochastic in nature. In mature synapses, a fraction of recycling SVs is distributed between mature synapses—a phenomenon that occurs in the scale of minutes (Darcy et al., 2006). Remarkably, this exchange occurs not only between neighboring boutons but also between remote synapses. Activity-dependent regulation of synapsin-1 phosphorylation at position Ser-551 by Cdk5 modulates clustering and dispersion of SVs between different presynaptic sites (Verstegen et al., 2014). This would indicate that redistribution of some synaptic components can occur following their delivery to synapses by Kinesin motors. In *Drosophila*, dense core vesicles destined for presynaptic terminals are initially observed to oscillate between proximal and distal axonal regions, with movement mediated by both Kinesin and Dynein motors (Wong et al., 2012). While undergoing retrograde movement to the neuronal soma, a portion of these vesicles becomes recruited to presynaptic terminals along the axons. Remarkably, these retrograde moving vesicles are preferentially captured by distal as compared to proximal presynaptic sites. Similar observations have also been made for SV precursor transport vesicles in *C. elegans* (Maeder et al., 2014). Whether or not an undefined mechanism is responsible for mediating the gradient decline in synaptic capture remains to be determined.

RETROGRADE TRAFFICKING IS MEDIATED BY DYNEIN

In addition to delivering cargoes, transport of biomolecules away from presynaptic sites towards the cell body also plays important regulatory as well as homeostatic roles for presynaptic function (**Figure 1**). Retrograde movement of specific messengers in response to synaptic activity can trigger transcription of genes in the nucleus whose products contribute to long-term modifications of synaptic behavior or as a response to injury (Panayotis et al., 2015). Furthermore, sustained and optimum presynaptic function requires removal and degradation of worn out or damaged presynaptic proteins or organelles for turnover and degradation (Bezprozvanny and Hiesinger, 2013). In both instances, Dynein mediates the retrograde movement of these cargoes (Hirokawa et al., 2010; Roberts et al., 2013).

SYNAPSE-NUCLEAR SIGNALING

Communication between synaptic termini and cell body is vital for modulation of synaptogenesis, long-term potentiation, axonal growth and responses to mechanical damage (Panayotis et al., 2015). Synapse-to-soma signaling can be divided into fast response component, that involves propagation of Ca^{2+} ion waves through neurites as a signal, and slow response component, which utilizes retrograde transport of signaling molecules to the cell body. Ca^{2+} signaling from the postsynapse to soma is well studied and has been demonstrated to be involved in modulation of gene expression (Bading et al., 1993; Greer and Greenberg, 2008; Hagenston and Bading, 2011). This method of signaling may also play a significant role following injury (Ziv and Spira, 1995; Wolf et al., 2001) and during axon growth (Yamane et al., 2012). Calcium waves can be propagated to soma, for example, through axon-specific ER-like structures (Merianda et al., 2009), or locally induce other signaling pathways (Ghosh-Roy et al., 2010) to evoke responses in the cell body.

In comparison to the fast response, which is most efficient for synapses located closer to the cell body, the slow signaling component involves retrograde transport of activated signaling molecules (for example kinases or receptors) to the nucleus using Dynein motors (Schmieg et al., 2014; Panayotis et al., 2015). Among these, the best studied are the neurotrophins, a group of extracellular signaling proteins including NGF, BDNF and NT-3/4 (Huang and Reichardt, 2001). At the presynapse, binding of neurotrophins to their receptors (either tropomyosin receptor kinase (Trk) family or $p75^{NTR}$ receptors) induces their autophosphorylation and activates downstream signaling pathways through MAPK, PLCγ and PI3K (Delcroix et al., 2003; Howe and Mobley, 2005; Schmieg et al., 2014). Interestingly, activation of Trk receptors depends on the type of neurotrophin and synaptic terminus it is applied to Kuruvilla et al. (2004). Stimulation of axonal terminus by NT-3 in sympathetic neurons promotes axonal growth by local activation of TrkA independent of TrkA internalization. In contrast, NGF-mediated activation of TrkA induces its endocytosis and co-transport with downstream signaling molecules pERK1/2 and pAkt to the soma (Kuruvilla et al., 2004). Multiple signaling molecules can be transported to the soma on common vesicles. This phenomenon led to the hypothesis of the signaling endosome (Schmieg et al., 2014), a dynamic organelle that co-transports numerous signaling molecules from the axon periphery to the cell body. Transporting multiple signaling molecules on a single vesicle fulfills two functions. Firstly, it potentially allows interactions to occur between components of the different signaling pathways carried by the vesicle that can result in either enhancement or inhibition of the eventual response. Second, vesicular transport of kinases or receptors preserves them in the active, often phosphorylated, state along the way. A large amount of data demonstrates that interruption of Dynein-mediated retrograde transport of neurotrophins leads to ablation of their signaling (Purves, 1976; Salehi et al., 2006; Sharma et al., 2010). For instance, disruption of BDNF-induced TrkB signaling by perturbing snapin-Dynein interaction, which prevents their retrograde transport to the nucleus, was recently shown to reduce dendritic outgrowth (Zhou et al., 2012). Signaling endosomes are assumed to carry a range of signaling molecules and markers that define its fate during transport to the soma. In agreement with this, early endosomal markers Rab5 and EEA1 colocalize with TrkA at distal tips of neurites (Howe et al., 2001; Delcroix et al., 2003). Upon maturation, signaling endosomes exchange Rab5 for Rab7, which is a late endosome marker.

This event is crucial for targeting TrkA to the cell body as overexpression of dominant negative Rab7 in PC12 cells disrupts transport of neurotrophin receptors complexes (Deinhardt et al., 2006).

In the cell body, signals derived from presynaptic sites are converted into specific responses, such as changes in gene expression. For instance, the downstream target of NGF, ERK5 can activate transcription factor MEF2D, which in turn induces expression of anti-apoptotic protein bcl-w, promoting survival of the developing sensory neurons (Pazyra-Murphy et al., 2009). Transcription factors can also be transported to nucleus directly from distal axonal regions. In support of this, several studies indicate that local translation of importins, proteins indispensable for nuclear transport of cytoplasmic proteins, can take place in injured axons (Hanz et al., 2003; Yudin et al., 2008; Perry et al., 2012). Knockout of importin β1 in axons led to a substantial change in the transcriptome of neurons inflicted with axonal injury (Perry et al., 2012). Studies of retrograde signaling from postsynapse revealed that importins bind to transcription factors and mediate their transport to the soma using Dynein motors (Meffert et al., 2003).

SYNAPSE HOMEOSTASIS AND TURNOVER OF PRESYNAPTIC PROTEINS

Presynaptic proteins are subjected to turnover during homeostatic and activity-dependent plasticity (Lazarevic et al., 2011; Cohen et al., 2013). Apart from proteasome-dependent degradation, autophagic degradation of presynaptic components is emerging as an important strategy to deal with unwanted biomolecules (Yamamoto and Yue, 2014; Menzies et al., 2015). Presynaptic components targeted for degradation by autophagy include SVs and α-synuclein (Friedman et al., 2012; Binotti et al., 2015). However, details concerning how these components are directed to sites of degradation remain unclear. Efficient clearance of autophagosomes is challenging in neurons since many of these vesicles are formed at the distal axons located far from lysosomes that are largely present in the soma (Yamamoto and Yue, 2014; Menzies et al., 2015). Retrograde transport of autophagosomes is an essential step towards the fusion of both types of vesicles thereby enabling completion of the degradation process. An important question is to understand how neurons employ particular sorting mechanisms to achieve this.

A recent study reported that retrograde movement of autophagosomes at distal sites in neuronal axons involves binding of JIP1, a Kinesin-1 activator, to LC3 present on autophagosomes (Verhey and Hammond, 2009; Fu et al., 2014). This interaction prevents JIP1 from activating Kinesin-1. Moreover, JIP1 recruits the Dynein motor complex by binding p150(Glued), a component of the dynactin complex which is an integral Dynein subunit shown to be important for initiating retrograde trafficking from synaptic sites (Allan, 2011; Lloyd et al., 2012; Fu and Holzbaur, 2013). Intriguingly, abrogation of JIP1 binding to LC3 by deleting the LC3-interaction region on JIP1 inhibits fusion of the autophagosomes with lysosomes—an essential step towards the eventual degradation of autophagosomal content. Because this deletion also impairs retrograde trafficking of autophagosomes, it remains to be clarified if the inhibition is related to the transport defect or if indeed that JIP1 does participate in a yet understood role in the autophagy pathway.

An alternative route for the recruitment of Dynein to autophagosomes appears to be facilitated by snapin. Previous data indicated that a complex of Dynein and snapin resides in late endosomes and is responsible for driving their retrograde transport (Cai et al., 2010). A proportion of autophagosomes was observed to fuse with late endosomes to form amphisomes, a known intermediate step in the autophagic pathway (Lamb et al., 2013; Yamamoto and Yue, 2014; Cheng et al., 2015). In doing so, these autophagosomes acquire the ability to move in a retrograde fashion via the Dynein-snapin complex present on late endosomes. Thus, the study provides additional insights into trafficking routes taken by autophagsomes en route for degradation.

PART OF A TEAM: COOPERATION BETWEEN KINESIN AND DYNEIN MOTORS IN MOVING PRESYNAPTIC PROTEINS

Although protein transport has been conventionally viewed to be unidirectional, Kinesin and Dynein motors are known to bind simultaneously to most synaptic cargoes (Encalada and Goldstein, 2014). Simultaneous binding of cargoes to motors of opposing directionality confers several advantages. First of all, retrograde movement of degenerated synaptic components or endocytosed material would be only possible if Dynein is available at the distal parts of the axon. This would necessitate Dynein travelling with cargoes in anterograde direction to enable distal localization (Yamada et al., 2010). Secondly, microtubule tracks along axons are not contiguous. Microtubule breaks or clogging of the axon can happen along the path of cargo transport and switching between motors of opposite polarity may allow the cargo to bypass the hindrance. Finally, many studies indicate that opposite-polarity motors activate one another and such an interaction is essential for efficient cargo transport in either direction (Barkus et al., 2008; Ally et al., 2009; Uchida et al., 2009). Nevertheless, it remains unclear what determines which motor becomes dominant in a given environment. Specific regulatory mechanisms are likely to determine the directionality of cargo movement. For instance, adaptor protein Huntingtin (Htt) together with huntingtin-associated protein-1 (HAP1) links BDNF-containing vesicles to Dynein motor. Upon IGF signaling and consequent activation of the Akt pathway, Htt becomes phosphorylated at serine 421 which in turn recruits KHC to the cargo and favors its anterograde transport (Colin et al., 2008).

PRESYNAPTIC PLASTICITY AND INTRACELLULAR TRAFFICKING

Synapses undergo constant remodeling (plasticity changes) in accordance to their history of activity which is a basis for learning and memory (Holtmaat and Svoboda, 2009). Such remodeling can occur as subtle changes in synaptic composition or overt modifications to synaptic morphology (Lazarevic et al., 2011; Weyhersmüller et al., 2011). Recent studies indicate that in addition to constitutive transport to synapses, the trafficking machinery can itself also respond to changes in synaptic activity and contribute to synaptic plasticity and remodeling. In particular, activity-enhanced trafficking of ionotropic glutamate receptors to post-synaptic density contributes to synaptic plasticity (Yin et al., 2011; Hoerndli et al., 2013, 2015).

Similarly, activity-mediated bidirectional modulation of synaptic behavior and trafficking has also been observed at the presynapse. Repetitive stimulation of cultured neurons increases formation of new presynaptic terminals that is dependent on trafficking of presynaptic components by Kinesin-1 (Cai et al., 2007). Likewise, higher levels of the Kinesin-3 motor KIF1A corresponding with increased trafficking of presynaptic cargoes by Kinesin-3 motors has also been observed in mice placed in an enriched environment in a learning paradigm (Kondo et al., 2012). Direct morphological changes have also been described. Prolonged activation of photoreceptor synapses causes loss of T-bars at presynaptic sites—a phenomenon associated with the removal of AZ proteins liprin-α, Bruchpilot by the Kinesin-3 motor Imac (Sugie et al., 2015).

FUTURE PERSPECTIVES

In the fast changing environment of synaptic communication, neurons must cope with the varying demands of different synapses. Emerging evidence indicates that the intracellular trafficking machinery does not only respond passively to replenish or remove biomolecules from synapses but can also pro-actively respond to the needs of individual synapses. Key questions that remain to be clarified include: (1) What mechanisms govern the loading of cargoes to their motors and, upon reaching their destinations, unloading the cargoes; (2) How heterogeneous is the composition of different cargo subsets and is the composition of these subsets regulated over the course of the lifespan of the neuron; and (3) What are the mechanisms regulating specificity of targeting to different synapses? Noteworthy, impairment of intracellular trafficking has been identified in a range of neurodegenerative disorders such as Alzheimer's and ALS where synapse loss presents itself as an early feature during disease onset (Millecamps and Julien, 2013). A better understanding of mechanisms governing the specificity of targeting to different synapses and the precise contribution of synaptic protein trafficking to synapse health will be important questions to address. Recent advances in optogenetics and super-resolution microscopy techniques, coupled with characterization of the molecular identities of cargoes carried by different motors will be important to bring us one step closer towards unraveling these intriguing questions (Ballister et al., 2015; Hell et al., 2015; van Bergeijk et al., 2015).

AUTHOR CONTRIBUTIONS

The manuscript was conceived by JJEC and written by JJEC, OY and TKD. OY and TKD contributed equally to the work.

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frontiers in Synaptic Neuroscience

MINI REVIEW
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Molecular Machines Determining the Fate of Endocytosed Synaptic Vesicles in Nerve Terminals

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The cycle of a synaptic vesicle (SV) within the nerve terminal is a step-by-step journey with the final goal of ensuring the proper synaptic strength under changing environmental conditions. The SV cycle is a precisely regulated membrane traffic event in cells and, because of this, a plethora of membrane-bound and cytosolic proteins are devoted to assist SVs in each step of the journey. The cycling fate of endocytosed SVs determines both the availability for subsequent rounds of release and the lifetime of SVs in the terminal and is therefore crucial for synaptic function and plasticity. Molecular players that determine the destiny of SVs in nerve terminals after a round of exo-endocytosis are largely unknown. Here we review the functional role in SV fate of phosphorylation/dephosphorylation of SV proteins and of small GTPases acting on membrane trafficking at the synapse, as they are emerging as key molecules in determining the recycling route of SVs within the nerve terminal. In particular, we focus on: (i) the cyclin-dependent kinase-5 (cdk5) and calcineurin (CN) control of the recycling pool of SVs; (ii) the role of small GTPases of the Rab and ADP-ribosylation factor (Arf) families in defining the route followed by SV in their nerve terminal cycle. These regulatory proteins together with their synaptic regulators and effectors, are molecular nanomachines mediating homeostatic responses in synaptic plasticity and potential targets of drugs modulating the efficiency of synaptic transmission.

Keywords: presynapse, synaptic vesicle, cdk5, calcineurin, recycling, small GTPases, Rab, Arf

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INTRODUCTION

The synaptic vesicle (SV) cycle is the most highly regulated membrane traffic event in cells and the proteins involved, as well as the arrays of protein-protein interactions that guarantee the fidelity of the process, are getting increasingly clear.

Kinases and phosphatases, as well as small GTPases and their effectors, are emerging as molecular machines acting at the synapse to regulate synaptic function and plasticity by rapidly modulating several synaptic targets and adapting the synapse to the needs of the network.

The high tunability of synaptic strength is obtained presynaptically from changes in quantal size, SV availability for release and number of active synapses, and all these parameters are directly or indirectly controlled by the cycling fate of SVs.

Here we provide an update on two molecular machines recently reported to act on the fate of recycling SVs in the nerve terminal: the cyclin-dependent kinase 5 (cdk5)/calcineurin (CN)

system with its multiple synaptic substrates and the small GTPase Rab and ADP ribosylation factor (Arf) systems with their relative regulators and effectors.

THE cdk5/CN SYSTEM

Kinases and phosphatases post-translationally regulate the function of a plethora of proteins and play a major role in regulating cellular functions. Phosphoproteins are highly expressed at the synaptic terminal together with dedicated kinases and phosphatases, and their activity regulates many aspects of SV cycling. The role of phosphoproteins in SV cycling has been extensively reviewed elsewhere (Valtorta and Benfenati, 1994). We here focus on one of the presynaptically expressed kinases, cdk5, and the cognate phosphatase, CN, as they act as key molecules in regulating the strength of neurotransmission by acting on SVs availability for release and endocytic recovery of SV at the plasma membrane after exocytosis.

The first evidence for a central role of the cdk5/CN system at the presynapse derives from the observation that several proteins involved in various steps of the SV cycle are specific substrates for cdk5/CN, namely munc18-1, septin5, Pictaire, synapsin, amphiphysin, dynamin and synaptojanin (reviewed in Su and Tsai, 2011). From a functional point of view, it is possible to distinguish between effects on exocytosis and endocytosis, keeping in mind that the two processes are intimately connected and both regulated by calcium waves in the terminal (Wu et al., 2014; Leitz and Kavalali, 2015). SV exocytosis is regulated by cdk5 phosphorylation of Munc18 that allows syntaxin1 to participate in the SNARE complex and SV fusion to occur (Shuang et al., 1998; Fletcher et al., 1999). A similar effect has been described for the phosphorylation of the cytoskeletal protein septin5 by cdk5, which decreases binding of septin5 to the SNARE protein syntaxin1 and regulates neurotransmitter release (Taniguchi et al., 2007; Amin et al., 2008). The cdk5 substrate Pictaire has also been proposed to regulate exocytosis by phosphorylating N-Ethylmaleimide-Sensitive Factor (NSF) and regulating the ability of NSF to oligomerize (Liu et al., 2006). Additional roles for cdk5/CN in the process of exocytosis came from the observation, made both *in vitro* and *in vivo*, that N-type calcium channels (Cav2.2) are substrates for the two enzymes (Su et al., 2012; Kim and Ryan, 2013). Although the impact of phosphorylation on the properties of the Cav2.2 channel is still controversial, it has been nicely demonstrated that the cdk5/CN balance regulates the final steps of exocytosis by potently controlling the action potential-driven calcium influx and therefore the probability of release (Kim and Ryan, 2013).

On the endocytosis side, an array of CN substrates involved in the SV retrieval process are known among the main molecular actors of SV cycling (Marks and McMahon, 1998). These proteins have been collectively named dephosphins, as their dephosphorylation is induced by calcium increase during stimulation (Cousin and Robinson, 2001). Both their dephosphorylation and subsequent phosphorylation by cdk5 is required for SV retrieval in central nerve terminals (Clayton et al., 2007). Different mechanisms of SV endocytosis operate at central synapses, namely fast endocytosis, clathrin-dependent endocytosis and bulk endocytosis (Kononenko and Haucke, 2015). However, how the various modes of endocytosis are interconnected and differently regulated is still a matter of investigation. The cdk5/CN activity on dephosphins has been reported to selectively regulate slow, activity-dependent bulk endocytosis, with no effect on fast endocytosis. The slow form of endocytosis predominantly occurs during sustained activity, requires traffic of SVs via the endosomal compartment and repopulates the recycling pool, which is only released during intense stimulation after the complete depletion of the readily releasable pool (Evans and Cousin, 2007; Cheung and Cousin, 2013). Interestingly, cdk5/CN activities concomitantly control which fraction of SVs partitions into the recycling or the release-reluctant resting pool (Kim and Ryan, 2010; Marra et al., 2012) and the SV protein Synapsin I has been identified as the main cdk5/CN substrate in mediating this effect (Verstegen et al., 2014). In particular, cdk5-phopshorylated Synapsin I sequesters recycling SVs in the release-reluctant resting pool by clustering SVs and increasing their association with actin filaments. The dual effect on bulk endocytosis and SV pool partitioning suggests that the system is involved in determining the fate of endocytosed SVs; in particular, it seems that during sustained activity bulk endocytosis proceeds via sequential CN/cdk5 activation, resulting in endosomal recycling of SVs and in the capture of the newly formed SVs into the release-reluctant pool (**Figure 1**). Moreover, the balance of cdk5/CN activities broadly varies between synapses, setting both the tone of N-type calcium channels and the ratio of recycling vs. reluctant SVs and resulting in synapse heterogeneity from silent to strongly active synapses (Kim and Ryan, 2010, 2013; Verstegen et al., 2014).

As a result of the multiple targets at the presynapse, the cdk5/CN nanomachine represents a master regulator of synaptic homeostasis and participates in the scaling of synaptic strength to compensate for the effects of sustained hypo- or hyperactivity (Seeburg et al., 2008; Kim and Ryan, 2010; Mitra et al., 2011; Peng et al., 2013). Chronic silencing of firing strongly downregulates nerve terminal cdk5 with the result of recruiting release reluctant SVs to the recycling pool. This process, which appears to be mediated by changes in the phosphorylation state of Synapsin I, can activate previously silent synapses and change the release potential of already active synapses (Kim and Ryan, 2010; Verstegen et al., 2014). Alterations of cdk5 activity are associated with a broad range of neurological disorders (see for reviews Cheung and Ip, 2012; McLinden et al., 2012) and in particular the cdk5-mediated homeostatic synaptic response has been recently involved in early Alzheimer's like synaptic pathology (Sheng et al., 2015).

Rab AND Arf SYSTEMS

Rab and Arf family proteins are master regulators of membrane trafficking and are involved in all steps of vesicular transport. As all GTPases, these proteins function as molecular switches by cycling between active guanosine triphosphate (GTP)-bound

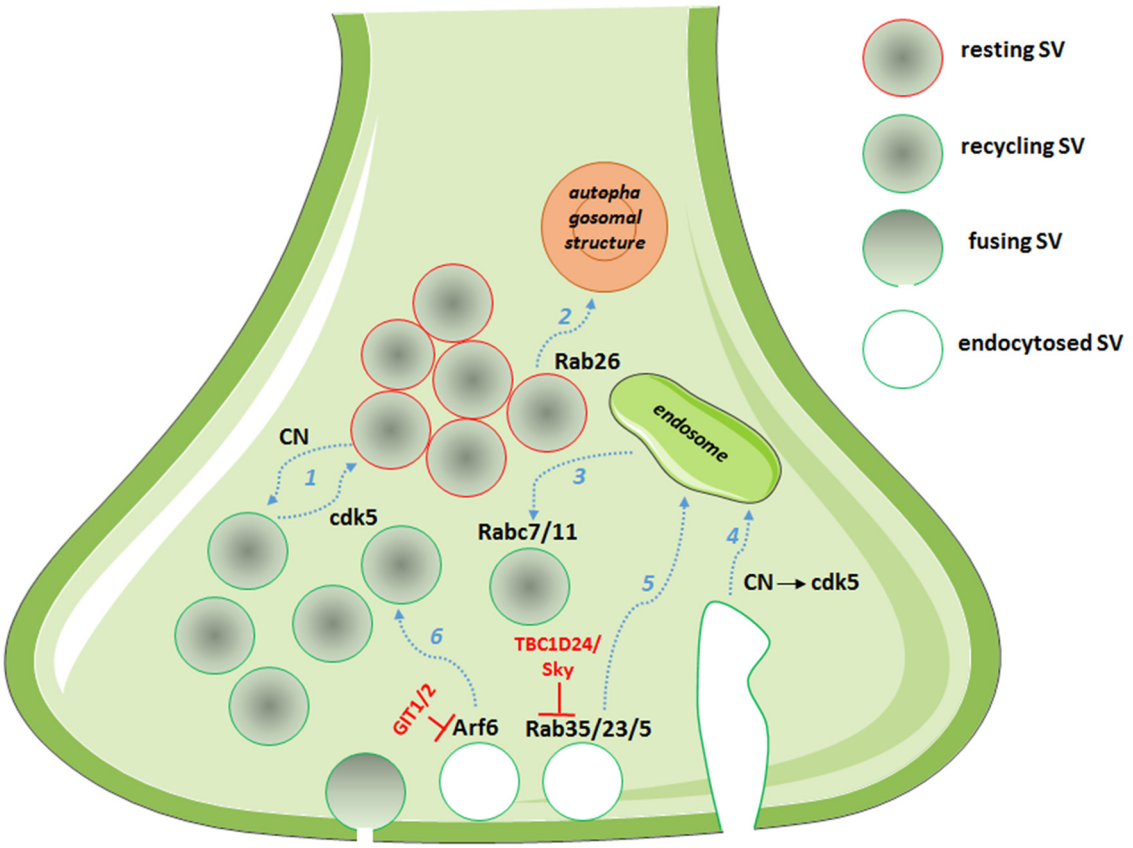

FIGURE 1 | Picture showing the multiple roles of cyclin-dependent kinase 5 (cdk5)/calcineurin (CN) and Rab/ADP-ribosylation factor (Arf) system in defining the route for endocytosed SVs. Dashed arrows in light blue represent synaptic vesicle (SV) routes: (1) partitioning into pools; (2) degradation; (3) post-endosomal trafficking; (4) endosomal sorting after bulk endocytosis; (5) endosomal trafficking; and (6) direct recycling. In red, the GTPase activating proteins (GAPs) with the described functions in controlling the SV fate.

and inactive guanosine diphosphate (GDP)-bound states. Their cycling is regulated by two families of regulatory proteins, namely guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs function as activators by facilitating the conversion from a GDP-bound form to a GTP bound form, whereas GAPs function as repressors by enhancing GTP hydrolysis. The vast number of Rab and Arf proteins and the multiple GAPs and GEFs for each isoform make the small GTPase family an ideal spatial and temporal regulator of virtually every aspect of membrane trafficking (Stenmark, 2009). As SV cycling is the prototype of an intensely regulated membrane traffic event, Rab and Arf proteins, together with their regulators and effectors, are emerging as molecular nanomachines regulating specific steps of the cycle.

More than 30 distinct Rabs were indeed identified by proteomic analysis in highly purified SV fractions (Takamori et al., 2006) and the three more abundant Rabs (Rab3, 7 and 5) were quantified in isolated terminals and found to represent 0.776, 0.135 and 0.025% of total synaptic protein, a percentage comparable with other fundamental protein for presynaptic physiology such as, for example, the calcium sensor synaptotagmin1 (Wilhelm et al., 2014). The role of the Rabs involved in exocytosis, such as the secretory Rabs Rab3a/3b/3c and Rab27b, and the role of some of the Rabs involved in SV recycling, such as Rab4, 5, 10, 11b and 14, has been reviewed and recently commented elsewhere (Sudhof, 2004; Pavlos et al., 2010; Pavlos and Jahn, 2011; Giorgini and Steinert, 2013; Rizzoli, 2014). Here we focus on additional small GTPases recently described to participate in SV cycling, particularly in the steps defining fate of SVs after recovery.

Rab35

A role for Rab35 in SV cycling originated from the identification of TBC1D24, an epilepsy gene involved in neuronal development and protein partner of the small GTPase Arf6 (Corbett et al., 2010; Falace et al., 2010, 2014). Studying the *Drosophila* mutants for TBC1D24/Skywalker, Uytterhoeven et al. (2011) revealed a strong presynaptic phenotype with a larger readily releasable pool of SVs and a dramatic increase in basal neurotransmitter release at neuromuscular junction (NMJ) synapses. They reported TBC1D24 to act as a GAP for Rab35 and proposed that active GTP-bound Rab35 favors endosomal sorting of SV proteins and replacement of dysfunctional SV components. Rab5 and

TABLE 1 | Small GTPases playing a role at the presynaptic terminal.

Small GTPases	Presynaptic role	Presynaptic GAP and GEF	Reference
Rab3a,b,c	Docking/priming	RAb3GAP, Rab3GEP...	Geppert et al. (1994, 1997), Yamaguchi et al. (2002), Giovedì et al. (2004a,b), Schlüter et al. (2004), Sakane et al. (2006), Pavlos et al. (2010), and Pavlos and Jahn (2011)
Rab4	SV recycling/sorting	To be determined	Pavlos and Jahn (2011)
Rab5a,b	SV recycling/sorting	To be determined	de Hoop et al. (1994), Fischer von Mollard et al. (1994), Shimizu et al. (2003), Wucherpfennig et al. (2003), Star et al. (2005), Hoopmann et al. (2010), Pavlos and Jahn (2011), and Uytterhoeven et al. (2011)
Rab7	SV recycling/sorting	To be determined	Pavlos and Jahn (2011) and Uytterhoeven et al. (2011)
Rab10	SV recycling/sorting	To be determined	Pavlos and Jahn (2011)
Rab11b	SV recycling/sorting	To be determined	Pavlos and Jahn (2011), Steinert et al. (2012), and Giorgini and Steinert (2013)
Rab14	SV recycling/sorting	To be determined	Pavlos and Jahn (2011)
Rab23	SV recycling/sorting	To be determined	Uytterhoeven et al. (2011)
Rab26	SV degradation	To be determined	Binotti et al. (2015)
Rab27b	Docking/priming	To be determined	Pavlos et al. (2010) and Pavlos and Jahn (2011)
Rab35	SV recycling/sorting	TBC1D24, Connecden1...	Allaire et al. (2006) and Uytterhoeven et al. (2011)
Arf1	SV budding	Arf1GAP...	Faúndez et al. (1997)
Arf6	SV recycling/sorting	GIT, Centaurin...	Ashery et al. (1999), Krauss et al. (2003), Homma et al. (2014), Podufall et al. (2014), Montesinos et al. (2015), and Tagliatti et al. (2016)
Arl8	SV component transport	To be determined	Klassen et al. (2010) and Wu et al. (2013)

Rab23 were also described to exert similar effects on endosomal trafficking of SVs, although TBC1D24 seems to act as a selective GAP for Rab35. This is the first description of a synaptic machinery dedicated to the control of the quality of proteins in SV cycling (**Figure 1**, Uytterhoeven et al., 2011). Moreover, using a functional screen to assess the impact of a battery of constitutively active Rabs on SV cycling, the same authors identified Rab7 and Rab11 as putative regulators of post-endosomal trafficking of SVs (**Figure 1**, Uytterhoeven et al., 2011). Whether a similar mechanism also operates at mammalian central synapses remains to be investigated.

Rab26

By investigating the molecular mechanisms leading to synapse elimination, Binotti et al. (2015) recently described a role for the small GTPase Rab26 in directing SVs into pre-autophagosomal structures, thus proposing a novel pathway for the degradation of SVs. The small GTPase was found in a subset of presynaptic terminals associated with marked clustering of SVs and is believed to promote SV clustering via a still unknown mechanism. Rab26, in its active GTP-bound state, was also reported to recruit Atg16L1 as an effector, representing a link between SV cycling and autophagy (**Figure 1**, Binotti et al., 2015).

Arf

The Arf proteins are a family of six small, ubiquitously expressed GTP-binding proteins (Donaldson and Jackson, 2011) that can be divided into three classes, based on sequence identity. Class I Arf proteins (Arf1, Arf2 and Arf3) regulate the assembly of various types of "coat" complexes onto budding vesicles along the secretory pathway and activate lipid-modifying enzymes (Bonifacino, 2004); Class II Arf proteins (Arf4 and Arf5), whose function is still unclear; and Arf6, which is the sole member of class III Arf proteins known to regulate endosomal membrane traffic and structural organization at the cell surface (D'Souza-Schorey and Chavrier, 2006). Other proteins that structurally resemble Arf proteins are the Arf-like (Arl) proteins, the Ras-related protein-1 (SAR1p) and the Arf-related protein ARFRP1.

Although Arf proteins are less abundant in purified SVs or isolated nerve terminals as compared to other small GTPases, the Arf analogs Arl10b and Arl10c and the Arf-interacting protein arfaptin 2 have been identified as components of purified SVs (Takamori et al., 2006). Some of the Arf proteins have been described to play a role in the definition of SV fate at the synapse.

The class III Arf protein Arf6, involved in constitutive trafficking between the plasma membrane and early endosomes and actin dynamics, has been reported to increase basal synaptic transmission at the *Xenopus* NMJ (Ashery et al., 1999), to regulate the assembly of the clathrin-coat complex during SV endocytosis (Krauss et al., 2003) and to play a role in the SV recycling pathway (Tagliatti et al., 2016). Knockdown of Arf6 in rat hippocampal neurons results in a strong presynaptic phenotype, with decreased SV density, accumulation of endosomal structures in the terminal and increased functional releasable SVs docked to the plasma membrane. Arf6 appears to act as a molecular determinant in the formation of the

readily releasable pool of SVs and in the sorting of endocytosed SVs to direct recycling, rather than through the endosomal compartment (Tagliatti et al., 2016, **Figure 1**). Interestingly, the observed Arf6-knockdown phenotype is reminiscent of the synaptic phenotype for the constitutively active Rab35 at the *Drosophila* NMJ (Uytterhoeven et al., 2011), suggestive of a functional interplay of the two small GTPases at the presynaptic compartment, as reported in different cellular systems (Allaire et al., 2013; Miyamoto et al., 2014). Although no precise synaptic role for Arf1 has been proposed, the protein in its active GTP-bound form has been described to regulate vesicle budding in PC12 cells (Faúndez et al., 1997), suggesting that also the prototype class I Arf may act in SV cycling pathway.

In support for a presynaptic role for Arf proteins, the Arf-like small G protein, Arl-8, has been identified as a critical regulator of presynaptic patterning and axonal transport in *C. elegans* (Klassen et al., 2010) and active GTP-bound Arl-8 were reported to act as an effector of the anterograde motor UNC-104/KIF1A (Wu et al., 2013). The Arl-8 GTP/GDP cycle is therefore proposed as a switch to control the association/dissociation of SV precursors from microtubule motor proteins, thus ensuring the proper delivery of novel presynaptic components at nerve terminals (Wu et al., 2013).

GAPs and GEFs

Considering the prominent roles of Rab and Arf proteins at the synaptic terminal, it is noteworthy the lack of data on synaptic GAPs and GEFs that dynamically regulate their activity and potentially represent targets to finely modulate neurotransmission in both homeostatic and hebbian plasticity (**Table 1**). In addition to the above mentioned role for the Rab35-GAP TBC1D24 (Uytterhoeven et al., 2011), the specific GAP for Arf6, G-protein coupled receptor kinase 2 interacting protein (GIT) has been involved in the organization of the cytomatrix of the active zone (Kim et al., 2003) and recently reported to play multiple roles at the presynapse (Podufall et al., 2014; Montesinos et al., 2015). Podufall et al. (2014) analyzed the localization of GIT at hippocampal glutamatergic synapses and at the *Drosophila* NMJ by employing SD-dSTORM high-resolution microscopy and revealed that the protein localizes at the periphery of the active zone. Indeed, GIT interacts with the endocytic adaptor stoninB and regulates the localization and function of stoninB at the presynaptic site. A *Drosophila* GIT mutant showed accumulation of endosomal structures and vacuoles and marked defects in post-stimulus SV endocytosis and/or re-acidification (Podufall et al., 2014). Although confirming a clear presynaptic function for the GAP of Arf6, studies in mammalian synapses revealed different roles for GIT1 and the other mammalian isoform GIT2. GIT1 and GIT1/GIT2 knocked out calyx of Held synapses showed a markedly increased initial release probability that was not associated with changes in the size of the readily releasable pool or in voltage-dependent calcium channel activity. Although some discrepancies in the reported results exist, and the precise mechanisms of the synaptic actions of GIT are still to be clarified (Montesinos et al., 2015), the small GTPase Arf6 and the two Arf6 regulators, TBC1D24 and GIT, are emerging as synaptic nanomachines operating at the presynaptic site to define both release probability and SV recycling pathways.

The ArfGAP1, acting as a GAP for Arf1, has been found to interact and regulate the activity of Leucine-rich repeat kinase 2 (LRRK2, Xiong et al., 2012), whose gene is mutated in Parkinson's disease patients, and known to play multiple roles in the presynaptic compartment (see for a review Belluzzi et al., 2012). LRRK2 kinase activity was also recently described to function as a Rab5b GAP, negatively regulating Rab5b signaling (Yun et al., 2015). In addition to LRRK2, huntingtin protein, dysfunctional in Huntigton's disease, has been proposed to function as a Rab regulator. Indeed, huntingtin was found in a complex acting as a GEF for Rab11 (Li et al., 2008), and Rab11 overexpression rescued the synaptic dysfunction associated with both huntingtin and synuclein mutations in *Drosophila* (Steinert et al., 2012; Breda et al., 2015).

CONCLUDING REMARKS

The cdk5/CN system is a master regulator of synaptic strength and synaptic functional heterogeneity by modulating calcium signaling and SV distribution in the nerve terminal. The system is highly regulated by homeostatic plasticity and represents a target for research on the molecular markers of synaptic dysfunctions and on the design of novel drugs (Shah and Lahiri, 2014; Sheng et al., 2015).

For the Rab/Arf system, we expect that additional GAP and GEF functions at the synapse will be clarified in the near future, together with the role of specific GTPase effectors in synaptic function. The synaptic targeting and/or the activity modulation of GAP and GEF will emerge as main factors in synaptic plasticity processes and in the pathogenesis of synaptic dysfunctions. Interestingly, several mutations in genes encoding proteins belonging to the Rab and Arf small GTPases families, or proteins regulating their GTP-binding cycle, have been recently described as causative for inherited neurological diseases (Falace et al., 2010; Shoubridge et al., 2010; Rauch et al., 2012; Seixas et al., 2013; D'Adamo et al., 2014; Fine et al., 2015; Kalscheuer et al., 2016), making the exploitation of their function at the synapse a prerequisite for the design of effective therapeutic strategies.

AUTHOR CONTRIBUTIONS

AF drafted the article and prepared the figure. MF assisted in drafting the article. FB drafted and revised the article.

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REVIEW
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The iTRAPs: Guardians of Synaptic Vesicle Cargo Retrieval During Endocytosis

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The reformation of synaptic vesicles (SVs) during endocytosis is essential for the maintenance of neurotransmission in central nerve terminals. Newly formed SVs must be generated with the correct protein cargo in the correct stoichiometry to be functional for exocytosis. Classical clathrin adaptor protein complexes play a key role in sorting and clustering synaptic vesicle cargo in this regard. However it is becoming increasingly apparent that additional "fail-safe" mechanisms exist to ensure the accurate retrieval of essential cargo molecules. For example, the monomeric adaptor proteins AP180/CALM and stonin-2 are required for the efficient retrieval of synaptobrevin II (sybII) and synaptotagmin-1 respectively. Furthermore, recent studies have revealed that sybII and synaptotagmin-1 interact with other SV cargoes to ensure a high fidelity of retrieval. These cargoes are synaptophysin (for sybII) and SV2A (for synaptotagmin-1). In this review, we summarize current knowledge regarding the retrieval mechanisms for both sybII and synaptotagmin-1 during endocytosis. We also define and set criteria for a new functional group of SV molecules that facilitate the retrieval of their interaction partners. We have termed these molecules *i*ntrinsic *tra*fficking *p*artners (iTRAPs) and we discuss how the function of this group impacts on presynaptic performance in both health and disease.

Keywords: endocytosis, vesicle, clathrin, presynapse, synaptobrevin, synaptotagmin, synaptophysin, SV2A

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INTRODUCTION

Efficient sustained neurotransmitter release is dependent on the correct reformation of synaptic vesicles (SVs) after stimulation by endocytosis. During this reformation process it is critical that the appropriate cargo molecules are incorporated into SVs with the correct stoichiometry to render SVs functional for the next cycle of neurotransmitter release. During normal physiological stimulation patterns, the majority of cargo are incorporated into SVs during clathrin-mediated endocytosis (CME; Granseth et al., 2006). The selection and clustering of protein cargo during endocytosis is a tightly regulated process, generating SVs that are highly

Abbreviations: iTRAP, intrinsic trafficking partner; SNARE, soluble NSF attachment protein receptor; SV, synaptic vesicle; CME, clathrin-mediated endocytosis; AP, adaptor protein; sybII, synaptobrevin II; CALM, clathrin assembly lymphoid myeloid leukemia; ANTH, AP-180 N-terminal homology; SV2A, synaptic vesicle protein 2A; vGLUT, vesicular glutamate transporter; AD, Alzheimer's disease.

homogenous in both their physical properties and the stoichiometry of their protein content (Takamori et al., 2006; Wilhelm et al., 2014; but see also Mutch et al., 2011). Clathrin adaptor proteins (APs) are central to this process and act as a hub for both SV cargo selection and the recruitment of other accessory endocytosis molecules (Kelly and Owen, 2011). However, when the expression of the classical adaptor molecule AP-2 is reduced using siRNA, or ablated using genomic knockout strategies, relatively minor effects on SV endocytosis at the plasma membrane are observed (Kim and Ryan, 2009; Willox and Royle, 2012; Kononenko et al., 2014; Jung et al., 2015). This suggests that other molecules are required to ensure efficient SV endocytosis and cargo retrieval. In agreement with this, cargo-specific monomeric adaptor molecules have been identified that facilitate the retrieval of cargo that are essential for SV fusion (Rao et al., 2012). Even more intriguing was the revelation that interactions between SV cargo themselves are critical for their accurate and efficient retrieval. This review will summarize progress in this field with particular emphasis on interactions between SV cargoes that facilitate the retrieval of the integral membrane proteins synaptobrevin II (sybII; also known as vesicle-associated membrane protein 2, VAMP2) and synaptotagmin-1. Both sybII and synaptotagmin-1 are indispensable SV cargo molecules, since their genomic deletion results in both the absence of synchronous evoked neurotransmission and early post-natal lethality (Geppert et al., 1994; Schoch et al., 2001). We have termed the SV cargo that interact with these essential proteins as *i*ntrinsic *tra*fficking *p*artners (iTRAPs) and will outline how these trafficking molecules are critical for higher brain function.

SYNAPTOPHYSIN IS AN iTRAP FOR SYBII

Synaptophysin and SybII are Interaction Partners

SybII is an essential fusogenic component of the molecular machinery of the SV. It is a vesicular soluble NSF attachment protein receptor (v-SNARE) protein, which, through its association with the plasma membrane target SNAREs syntaxin and SNAP-25 (synaptosomal-associated protein, 25 kDa), renders SVs competent for fusion and drives exocytosis (Jahn and Fasshauer, 2012; Sudhof, 2013). In keeping with its essential role, the correct targeting and localization of sybII to SVs is vital for evoked neurotransmitter release (Schoch et al., 2001). SybII is a single-pass transmembrane SV protein, with a short intraluminal C-terminus and a cytoplasmic N-terminal region that contains a highly conserved SNARE motif (Sudhof et al., 1989), with the extreme N-terminus being involved in protein-protein interactions (Martincic et al., 1997; Burré et al., 2010). It is the most abundant cargo on the SV with approximately 70 copies (Takamori et al., 2006; Wilhelm et al., 2014), however it is estimated that only 1–3 sybII molecules are required for membrane fusion (Domanska et al., 2009; Mohrmann et al., 2010; van den Bogaart et al., 2010; Sinha et al., 2011). Synaptophysin is the second most abundant cargo on the SV, with approximately 30 copies present (Takamori et al., 2006; Wilhelm et al., 2014). It contains four transmembrane domain regions and cytoplasmic N- and C-termini, the latter being the major site for protein—protein interactions (Daly and Ziff, 2002; Wheeler et al., 2002; Felkl and Leube, 2008). Synaptophysin shares an interaction with sybII (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995), thus the two most abundant cargoes on the SV also interact together (**Figure 1**).

The exact domains that participate in the synaptophysin-sybII interaction remain to be elucidated despite the fact that this association was one of the first to be identified at the presynapse. Several studies have suggested that the proteins interact through their transmembrane domains (Becher et al., 1999; Yelamanchili et al., 2005; Bonanomi et al., 2007), with the C-terminal portion of the sybII transmembrane domain being key for this interaction (Yelamanchili et al., 2005). Single particle electron microscopy analysis of the native mammalian synaptophysin—sybII complex revealed that the transmembrane domains of synaptophysin and sybII interact in a 6:12 ratio, with synaptophysin forming a hexameric core (Thomas et al., 1988; Arthur and Stowell, 2007) that binds six sybII dimers (Adams et al., 2015). Thus it is possible that the synaptophysin—sybII complex is a multimeric structure that clusters 12 sybII molecules per functional unit.

While the core interaction site for sybII and synaptophysin is likely to be the transmembrane domains of the proteins, other factors may regulate their association. For example, specific membrane lipids are proposed to contribute to the strength of synaptophysin-sybII interaction, with increases in cholesterol content favoring the binding of synaptophysin to sybII (Mitter et al., 2003; Adams et al., 2015). The synaptophysin-sybII interaction is also upregulated during mammalian development, and addition of adult cytosolic extract to SVs isolated from embryos increases the prevalence of the synaptophysin-sybII complex, suggesting a cytosolic protein regulates the interaction (Becher et al., 1999). In agreement, the addition of a peptide

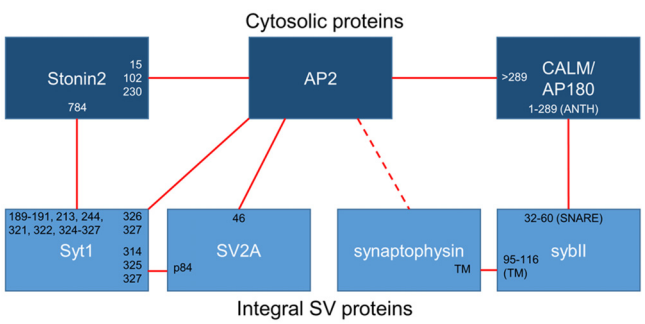

FIGURE 1 | Binding interactions between essential synaptic vesicle cargo (sybII and synaptotagmin-1; Syt1), iTRAPs (synaptophysin and SV2A), monomeric adaptors (CALM/AP180 and stonin2) and the classical adaptor AP-2. Cytosolic proteins are dark blue, integral SV proteins are light blue. Solid lines indicate confirmed interactions, dotted lines are putative interactors. The residues (numbers) or domains (ANTH, SNARE or transmembrane; TM) that have been suggested to mediate interactions between these proteins have been noted at the links between partners.

encompassing the N-terminal 32 amino acids of sybII dissociates the synaptophysin-sybII complex *in vitro* (Washbourne et al., 1995). Thus the abundant SV cargoes synaptophysin and sybII directly interact to form a large multimeric structure, which may be regulated by the local membrane and cytosolic environment.

The functional role of the synaptophysin-sybII interaction has been a source of intense scrutiny. SybII binds in a mutually exclusive manner to either synaptophysin or the plasma membrane t-SNAREs (Edelmann et al., 1995; Siddiqui et al., 2007), suggesting that it controls access of sybII to the SNARE complex. In support, an activity-dependent dissociation of the synaptophysin-sybII complex occurs following incubation with α-latrotoxin by both forster resonance energy transfer (FRET) analysis of exogenously expressed proteins in neurons (Pennuto et al., 2003) and immunoprecipitation from isolated nerve terminals (Reisinger et al., 2004). Interestingly calcium is required, though not sufficient, for this dissociation in intact nerve terminals (Chapman et al., 1995; Prekeris and Terrian, 1997; Daly and Ziff, 2002; Reisinger et al., 2004). However, synaptophysin readily dissociates from sybII in the presence of the t-SNAREs (Siddiqui et al., 2007), arguing against the proposed role as modulator of SNARE complex formation. Moreover, the synaptophysin-sybII interaction is upregulated following membrane fusion (Khvotchev and Sudhof, 2004), suggesting a functional role post-fusion for this interaction between the two major SV proteins.

Synaptophysin is Essential for Accurate SybII Retrieval during Endocytosis

The majority of the studies discussed above examined the formation and dissociation of the synaptophysin-sybII complex in isolation from its location within the nerve terminal. Fluorescence microscopy studies using genetically-encoded reporters have provided key insights into the physiological role of the synaptophysin-sybII interaction as a regulator of sybII localization *in situ*. When exogenous sybII was overexpressed in either heterologous expression systems or cultured neurons, it exhibited a predominantly plasma membrane localization (Pennuto et al., 2003; Bonanomi et al., 2007), suggesting it was being inefficiently targeted to vesicular compartments. The targeting of SV cargo occurs principally during CME in central nerve terminals, with AP-2 performing a key role (Kelly and Owen, 2011). However sybII lacks classical recognition motifs for AP-2, suggesting that it was targeted to SVs via a non-canonical mechanism. Interestingly when synaptophysin is co-expressed with sybII in similar overexpression studies, the latter is efficiently redirected to SVs (Pennuto et al., 2003; Bonanomi et al., 2007).

Recent studies in cultured neurons from synaptophysin knockout mice have confirmed that synaptophysin is critical for sybII targeting to SVs. In these studies endogenous sybII was mislocalized from nerve terminals, and exogenous sybII accumulated at the plasma membrane (Gordon et al., 2011). Crucially, this mistargeting is a direct result of a specific deficit in the activity-dependent retrieval of sybII during compensatory endocytosis, with sybII retrieval kinetics being severely slowed in synaptophysin knockout neurons. The efficient retrieval of sybII was fully rescued by the re-addition of wild-type synaptophysin back into these null neurons (Gordon et al., 2011). Confirmation of a key role for synaptophysin in sybII retrieval came from analysis of a series of synaptophysin mutants identified in X-linked intellectual disability. Every synaptophysin mutant tested failed to restore normal sybII retrieval in this knockout model system (Gordon and Cousin, 2013). Notably, all but one of these mutations are predicted to ablate or interfere with the transmembrane synaptophysin-sybII interaction (Adams et al., 2015). The final mutation causes substantial changes in the cytosolic tail of synaptophysin which, as outlined above, may regulate the strength of the interaction. Together, this data establishes that synaptophysin is essential for the accurate retrieval of sybII during endocytosis and as such is the founding member is the iTRAP group of SV proteins.

Monomeric Adaptor Protein AP180/CALM Facilitates SybII Retrieval

Synaptophysin is necessary for the accurate retrieval of sybII during endocytosis, however there is an equal requirement for other endocytosis molecules. As stated above, sybII has no canonical recognition motifs for AP-2; therefore another molecule must be present to recruit sybII to the endocytosis machinery. Synaptophysin may contribute to this role, since it contains a series of tyrosine-based repeats that may be recognized by the μ2 subunit of AP-2 (Sudhof et al., 1987). The likely molecular bridge between sybII and AP-2 however is the monomeric clathrin adaptor protein AP180 and the related protein clathrin assembly lymphoid myeloid leukemia (CALM; **Figure 1**). Both AP180 and CALM are highly conserved throughout evolution; they bind to both AP-2 and clathrin to facilitate endocytosis, and they both interact with sybII to mediate its retrieval (Koo et al., 2011b).

AP180/CALM was originally identified as a regulator of sybII targeting to synapses in *Caenorhabditis elegans*. Mutations in *unc11* (the *C. elegans* homolog of AP180 and CALM) resulted in reduced targeting of sybII to the neuropil and a broad distribution of sybII throughout the neuron (Nonet et al., 1999). Further work revealed that sybII mislocalization was a common feature of several AP180/CALM knockout systems. For example sybII is mislocalized along the axon in *lap* (*Drosophila* homolog of AP180) mutant flies (Bao et al., 2005) and in CALM/AP180-depleted mammalian neuronal cultures (Koo et al., 2011a). In addition sybII is both stranded on the plasma membrane and inefficiently retrieved during endocytosis in AP180 knockout neurons (Koo et al., 2015). Knockdown of CALM in AP180 knockout neurons exacerbates the surface stranding of sybII (Koo et al., 2015). In mammalian systems this mislocalization phenotype is specific to sybII, however in either *lap* null flies (Bao et al., 2005) or in flies where *lap* was acutely inactivated by fluorescein-assisted light inactivation (Vanlandingham et al., 2014), several other presynaptic proteins (including synaptotagmin, vGLUT

and cysteine-string protein) were also mislocalized along axons in conjunction with sybII. Further work is therefore required to delineate the specificity of AP180/CALM as a modulator of sybII retrieval vs. its actions as a regulator of CME.

The interaction site between AP180/CALM and sybII has been mapped, with the ANTH (AP180 N-terminal homology) domains of either AP180 or CALM binding to the SNARE domain of sybII (Koo et al., 2011a; Miller et al., 2011). When this interaction was perturbed in primary neurons by either point mutations in the SNARE motif of sybII or in the ANTH domain of AP180, exogenously expressed sybII is mislocalized to the cell surface (Koo et al., 2011a, 2015), confirming the requisite nature of this interaction in sybII retrieval. Thus in addition to the iTRAP synaptophysin, AP180/CALM is also essential for the accurate retrieval of sybII during SV endocytosis.

As the studies above indicate, the trafficking of sybII during SV turnover requires exquisite precision, and if this process is disrupted a range of deleterious effects emerge that impact on neuronal function. The dual requirement for both synaptophysin and AP180/CALM indicates that both are essential for accurate sybII trafficking, however it is still unclear how and whether they cooperate to do so. Both interact with sybII at mutually exclusive sites (**Figure 1**), and we have proposed that synaptophysin may restrict the entry of sybII into futile plasma membrane cis-SNARE complexes to allow AP180 access to its SNARE motif (Gordon and Cousin, 2014; **Figure 2**). Future experiments examining how ablation of the expression of both synaptophysin and AP180/CALM impacts on sybII trafficking should provide valuable insights into how these molecules co-operate at the synapse in this key role.

SV2A IS A PHOSPHO-DEPENDENT iTRAP FOR SYNAPTOTAGMIN-1

SV2A Binds to Synaptotagmin-1 in a Phosphorylation-Dependent Manner

The demonstration that synaptophysin is required for the accurate retrieval of sybII suggests that other potential SV cargo interactions may nucleate and facilitate their own retrieval. In agreement a second iTRAP relationship has been identified, with synaptic vesicle protein 2A (SV2A) being essential for the accurate retrieval of the calcium sensor synaptotagmin-1.

SV2A is ubiquitously expressed in the central nervous system, whereas other members of the larger gene family, are either brain-specific (SV2B) or a minor isoform (SV2C; Buckley and Kelly, 1985; Bajjalieh et al., 1994). SV2A has a requisite role in postnatal brain function, since knockout mice fail to grow and experience severe seizures before premature death after 3 weeks (Crowder et al., 1999; Janz et al., 1999a). The presynaptic role of SV2A is still unclear however, with most research focussing on a potential function as a calcium-dependent effector of SV exocytosis (Mendoza-Torreblanca et al., 2013). These potential roles include the control of short term synaptic plasticity either via regulation of residual intracellular calcium (Janz et al., 1999a;

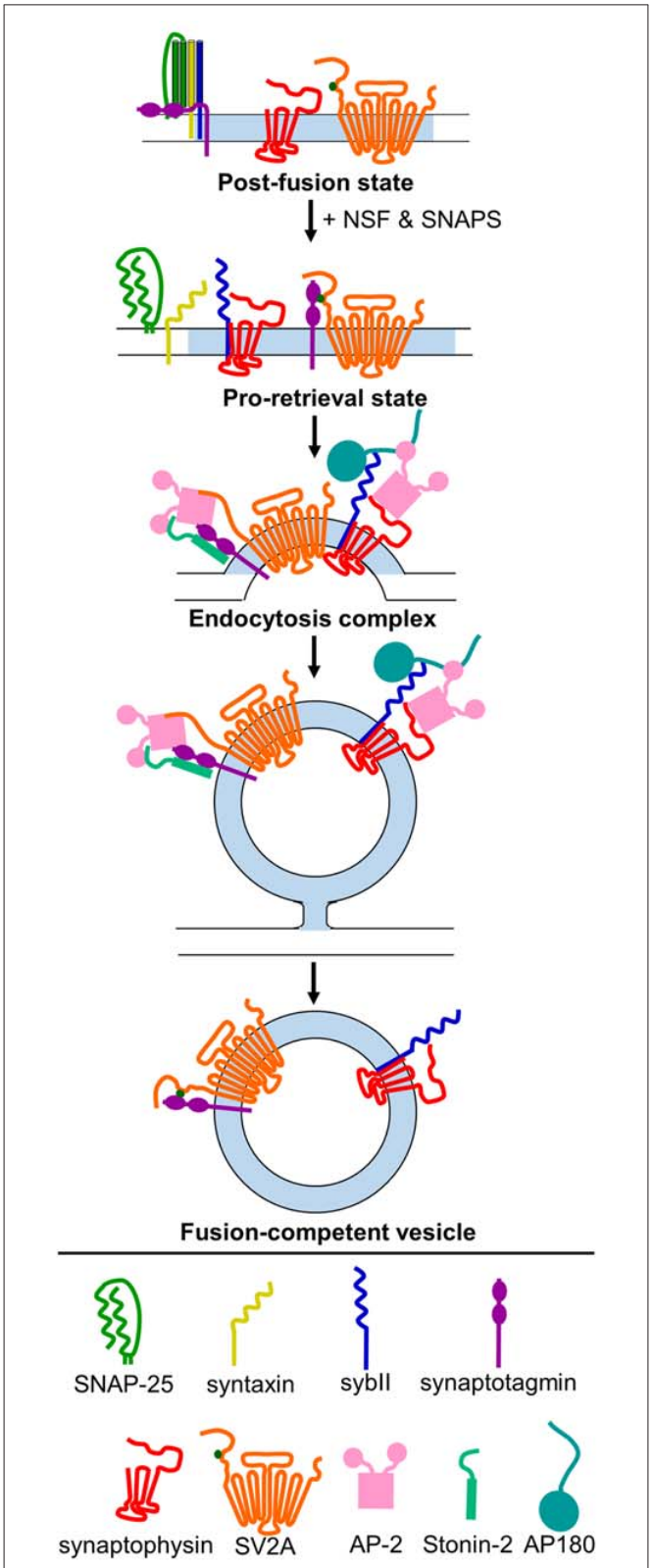

FIGURE 2 | Model for mechanism of retrieval of essential SV cargo. Following exocytosis (post-fusion state), sybII (navy blue) is trapped in a cis-SNARE complex with SNAP-25 (green) and syntaxin (yellow), and the C2A (*Continued*)

FIGURE 2 | Continued
and C2B domains of synaptotagmin-1 (purple) are embedded in the membrane. N-ethylmaleimide-sensitive factor (NSF) and soluble NSF-attachment proteins (SNAPs) act to dissociate the cis-SNARE complex, freeing sybII in the plasma membrane. Synaptophysin (red) binds to sybII to protect it from re-entering into futile cis-SNARE complexes, and T84-phosphorylated (dark green dot) SV2A (orange) binds to synaptotagmin-1 to create a pro-retrieval state. This allows AP180/CALM (dark teal) to bind to the SNARE-domain of sybII. AP-2 (pink) binds to AP180/CALM, and may also interact with synaptophysin. Simultaneously, SV2A (now dephosphorylated) dissociates from synaptotagmin-1 and binds to AP-2, which strengthens the binding of AP-2 to synaptotagmin-1. Stonin-2 (light teal) also binds to the C2 domains of synaptotagmin-1 and to AP-2 to strengthen the interaction. Together, this promotes the clustering of these cargoes for endocytosis. Following endocytosis, the monomeric adaptors AP180/CALM and stonin 2, and the classical clathrin adaptor AP-2, dissociate from their vesicle binding partners. Synaptophysin remains bound to sybII, and rephosphoryated SV2A binds to synaptotagmin-1 to produce a fusion-competent vesicle.

Chang and Südhof, 2009; Wan et al., 2010) or the size of the readily releasable pool (Custer et al., 2006). In contrast, the role for synaptotagmin-1 in presynaptic function is well established. It is essential for coupling calcium influx to evoked synchronous neurotransmitter release by binding calcium via two low affinity C2 domains (Brose et al., 1992; Geppert et al., 1994). Calcium binding neutralizes local negative charge on the C2 domains, allowing hydrophobic residues to enter the plasma membrane and evoke SV fusion (Bai et al., 2002; Martens et al., 2007; Hui et al., 2009).

The sites for the SV2A—synaptotagmin-1 interaction have been mapped, with the N-terminus of SV2A required for binding to the C2B domain of synaptotagmin-1 (Schivell et al., 1996, 2005). Phosphorylation of the N-terminus of SV2A by casein kinase 1 family kinases strongly potentiates this interaction (Pyle et al., 2000; Zhang et al., 2015). The *in vivo* phosphorylation sites on SV2A were recently identified and phosphorylation of one specific residue within the N-terminus (Thr84) permitted binding to synaptotagmin-1. The interaction site for phospho-SV2A was a basic patch of amino acids on the C2B domain (Zhang et al., 2015; **Figure 1**) which overlaps with interaction sites for a series of other molecules including phosphoinositides (Schiavo et al., 1996), calcium channels (Leveque et al., 1992), t-SNARE dimers (Bhalla et al., 2006) and even other synaptotagmins (Chapman et al., 1996). This suggests that competition may exist for binding to this region within the C2B domain, even though the combination of lysines bound by phospho-SV2A is thus far unique. It will therefore be of high importance to delineate how other molecules compete with SV2A for synaptotagmin-1 binding during SV endocytosis.

SV2A Acts as an iTRAP to Direct Synaptotagmin-1 Retrieval to SVs

SV2A not only binds synaptotagmin-1, but functions as an iTRAP to direct synaptotagmin-1 targeting to SVs. Endogenous or exogenously expressed synaptotagmin-1 accumulates at the plasma membrane in either SV2A knockout neurons (Yao et al., 2010) or in neurons depleted of SV2A (Kaempf et al., 2015; Zhang et al., 2015). This effect was SV2A-dependent, since normal plasma membrane synaptotagmin-1 levels were restored by expression of the wild-type protein in either knockout or knockdown neurons (Yao et al., 2010; Kaempf et al., 2015; Zhang et al., 2015). This effect is also specific for synaptotagmin-1, since other SV cargoes do not accumulate under the same experimental conditions (Kaempf et al., 2015). Importantly, synaptotagmin-1 remained stranded on the plasma membrane when knockout/knockdown neurons were rescued with mutant SV2A that was deficient for either SV2A internalization (Yao et al., 2010) or synaptotagmin-1 binding (Zhang et al., 2015). This indicates that an interaction with SV2A was required for the efficient retrieval of synaptotagmin-1 from the plasma membrane. In support the number of synaptotagmin-1 molecules that visited the plasma membrane during an action potential train (i.e., that were present on a SV) was reduced in the absence of SV2A (Kaempf et al., 2015).

In addition to plasma membrane accumulation, synaptotagmin-1 retrieval during SV endocytosis was accelerated in the absence of SV2A (Kaempf et al., 2015; Zhang et al., 2015). This was not a general acceleration of endocytosis, since other SV cargo were not affected in a similar manner (Yao et al., 2010; Zhang et al., 2015; but see Kaempf et al., 2015). Therefore the absence of SV2A results in synaptotagmin-1 accumulation at the plasma membrane, but also an acceleration of its retrieval. This was an unusual finding, since increased SV cargo stranding at the plasma membrane is usually a surrogate for perturbed retrieval by endocytosis (Voglmaier et al., 2006; Kim and Ryan, 2009; Yao et al., 2010; Koo et al., 2011a; Willox and Royle, 2012; Foss et al., 2013; Kononenko et al., 2014) such as described above for sybII in synaptophysin or AP180 knockout neurons (Gordon et al., 2011; Koo et al., 2015). There are a number of potential explanations for this phenotype. First, the lack of an SV2A interaction may result in synaptotagmin-1 being retrieved by a parallel endocytic mode with faster retrieval kinetics than classical CME. Second, it may be that retrieval of synaptotagmin-1 during spontaneous SV recycling is reduced (reflected in increased surface accumulation in resting neurons), but activity-dependent retrieval is facilitated. Arguing against this is the finding that normal plasma membrane synaptotagmin-1 levels are restored when neuronal activity is silenced in SV2A knockdown neurons (Kaempf et al., 2015). It will be critical to resolve these seemingly contradictory observations to determine the molecular role for this interaction in synaptotagmin-1 retrieval.

Stonin-2 and AP-2 are also Required for Efficient Synaptotagmin-1 Retrieval

The iTRAP SV2A is not the only molecule required for accurate synaptotagmin-1 retrieval during SV endocytosis. The C2B domain of synaptotagmin-1 has a well-established interaction with the μ2 subunit of AP-2 within the same patch of basic residues required for SV2A binding (Zhang et al., 1994; Chapman et al., 1998; Haucke et al., 2000; **Figure 1**). C2B domain multimerization may also increase synaptotagmin-1 affinity for AP-2 (Grass et al., 2004). Interestingly short peptides from SV2A (that contain a canonical tyrosine-based endocytosis motif)

increase the amount of AP-2 extracted from brain cytosol by the C2B domain of synaptotagmin-1 (Haucke and De Camilli, 1999; Haucke et al., 2000; Grass et al., 2004), suggesting that SV2A binding alters AP-2 in such a way that it allosterically promotes its binding to synaptotagmin-1. In support of this concept, the interaction site for this canonical motif within the μ2 subunit of AP-2 is distinct from the region that interacts with the C2B domain, explaining its ability to increase affinity (Haucke et al., 2000). Co-immunoprecipitation studies have also shown the presence of a tripartite complex of SV2A, AP-2 and synaptotagmin-1, suggesting they form a retrieval complex in mammalian brain (Haucke and De Camilli, 1999). Thus a potential iTRAP function of SV2A may be to facilitate synaptotagmin-1 interactions with the key endocytosis adaptor complex AP-2.

Efficient synaptotagmin-1 retrieval also requires the monomeric adaptor protein stonin-2. The interaction with stonin-2 is distributed over both C2 domains of synaptotagmin-1, however stonin-2 primarily interacts with the C2A domain (Jung et al., 2007; **Figure 1**). Very close parallels exist between stonin-2 and SV2A with respect to synaptotagmin-1 trafficking. In stonin-2 knockout neurons synaptotagmin-1 accumulates at the plasma membrane and displays an accelerated retrieval during SV endocytosis, in an identical manner to that seen in the absence of SV2A (Kononenko et al., 2013). However stonin-2 knockout neurons also display an acceleration of SV endocytosis, something not observed in neurons lacking SV2A. The similar phenotype in neurons lacking either SV2A or stonin-2 suggests that these molecules may be functionally redundant for synaptotagmin-1 trafficking. This was recently tested by silencing SV2A expression in stonin-2 knockout neurons either by genomic knockout or siRNA (Kaempf et al., 2015). This work revealed an exacerbation of plasma membrane synaptotagmin-1 accumulation and a further acceleration of its retrieval, suggesting both stonin-2 and SV2A are required for efficient synaptotagmin-1 trafficking. In addition, this suggests that SV2A and stonin-2 either act at discrete parallel steps or alternatively perform parallel additive roles in synaptotagmin-1 retrieval.

At least three molecules are required for efficient synaptotagmin-1 retrieval, however how do SV2A, stonin-2 and AP-2 coordinate this event during neuronal activity? The key may be the fact that almost all members share interactions with each other (**Figure 1**), ensuring maximum efficiency when all are present and redundancy when one is absent or mutated. In support of a potential redundancy of action, the retrieval kinetics of synaptotagmin-1 that has either C2A or C2B deleted are indistinguishable from wild-type, however these mutants did display defective targeting to nerve terminals (Yao et al., 2012a). Both stonin-2 and SV2A can interact with AP-2 via canonical tyrosine-based internalization motifs (Diril et al., 2006; Yao et al., 2010). As stated above, the binding of these canonical peptide motifs to AP-2 increases the affinity of the synaptotagmin-1 C2B domain for AP-2 (Haucke and De Camilli, 1999). In addition, both stonin-2, and either SV2A or AP-2, should be able to access the C2A and C2B domains of synaptotagmin-1 simultaneously, based on the interaction sites between the proteins (**Figure 1**). One key regulatory step may be the phosphorylation of SV2A, since this may determine priority of access for the C2B domain between itself and AP-2 (for model, see **Figure 2**). It may be that phosphorylated SV2A binds to the C2B domain of synaptotagmin-1 and acts as a target for AP-2 recruitment. Upon binding to AP-2, SV2A dissociates from synaptotagmin-1 (perhaps facilitated by dephosphorylation of T84) and potentiates the binding of AP-2 to the C2B domain. This interaction also increases the association of AP-2 with the membrane (Haucke and De Camilli, 1999) and facilitates endocytosis. Stonin-2 continues to interact with synaptotagmin-1 through the C2A and C2B domains, as well as binding to AP-2, to further assist in synaptotagmin-1 retrieval and endocytosis. It will therefore be of great future interest to determine where (on SVs and/or plasma membrane) and when (before, during or after action potential stimulation) SV2A is phosphorylated by casein kinase1 family kinases or dephosphorylated by an as yet unidentified phosphatase.

COMMON THEMES IN SV CARGO RETRIEVAL: DO OTHER iTRAPs EXIST?

Evidence is emerging that SV cargo retrieval during endocytosis contains a number of "fail-safe" mechanisms that ensure SVs are formed with the correct molecules with the required stoichiometry. Two of the most important cargoes on SVs are sybII and synaptotagmin-1, which are both essential for synchronous calcium-dependent SV fusion. It is becoming apparent that mammalian nerve terminals contain a high level of functional redundancy to ensure both are retrieved with high fidelity.

There are large similarities between the properties and retrieval mechanisms for both sybII and synaptotagmin-1. First, both of these essential cargoes are single-pass transmembrane proteins. Second, their SV interaction partners, the iTRAPs (synaptophysin and SV2A), are multiple pass transmembrane proteins that have arisen relatively late in evolution, only being present in vertebrate species at a functional level. Third, both sybII and synaptotagmin-1 require a monomeric adaptor protein (AP180/CALM for sybII and stonin-2 for synaptotagmin-1) for efficient retrieval. Finally the classical adaptor protein complex AP-2 is also required. Removal of any of these components results in perturbed cargo retrieval. In addition both synaptophysin and SV2A are heavily glycosylated, which is important for their targeting to SVs (Kwon and Chapman, 2012). We predict that this will also impact on the retrieval of their specific retrieval partner, however this remains to be tested experimentally.

Another similarity between sybII and synaptotagmin-1 is that they are both proposed to be required for efficient SV endocytosis in their own right. For example, SV endocytosis is slowed when sybII is depleted from neurons either at the genomic level (Deák et al., 2004), using knockdown with shRNA (Zhang et al., 2013) or after incubation with the clostridial

neurotoxin tetanus toxin (Hosoi et al., 2009; Xu et al., 2013). Similarly, multiple studies have shown that reduction or ablation of synaptotagmin-1 expression retards SV retrieval kinetics in both invertebrate and mammalian systems (Jorgensen et al., 1995; Poskanzer et al., 2003; Yao et al., 2012a,b). It will be critical to determine whether these potential roles in endocytosis are functionally distinct from their interaction with iTRAPs, monomeric adaptors and AP-2.

The identification of cognate iTRAPs that control the accurate retrieval of both sybII and synaptotagmin-1 lead to possibility that other key interactions may occur between SV cargo to facilitate their SV targeting and retrieval. The similarities between the iTRAPS, and their later appearance through evolution, suggest that common rules drive their function. Other candidate proteins that fit at least some of the criteria above include the synaptophysin-related tetraspanin proteins, synaptoporin and synaptogyrin. Synaptoporin is an N-glycosylated protein (Fykse et al., 1993) that can also bind sybII (Edelmann et al., 1995; Becher et al., 1999), however the functional role of this interaction has not yet been investigated. Unlike synaptophysin, synaptoporin has a restricted expression in the mammalian central nervous system (Marquèze-Pouey et al., 1991; Fykse et al., 1993) and displays a different expression pattern during development (Marquèze-Pouey et al., 1991), suggesting that it may play a role in specific neuronal populations. Intriguingly, the phenotype in synaptophysin null neurons that also naturally lack synaptoporin is more severe than in other neuronal groups (Spiwoks-Becker et al., 2001); whether deficient sybII retrieval underlies this phenotype is unknown. In synaptogyrin mutant *C. elegans*, sybII displayed a more diffuse localization, with wider puncta than in wild-type worms, suggesting a deficit in sybII recruitment to nerve terminals (Abraham et al., 2011). Synaptogyrin is the major vesicular tetraspanin in the *C. elegans* nervous system (Abraham et al., 2006) and may thus be acting to functionally replace synaptophysin in worms. In addition, mice lacking both synaptophysin and synaptogyrin displayed synaptic dysfunction that was not evident in single knockout mice, with deficits in both paired-pulse facilitation and long-term potentiation (Janz et al., 1999b). Together this data provides tantalizing evidence that synaptoporin and synaptogyrin may play neuronal subtype- or species- specific roles as iTRAPs, to either support or reproduce the function of synaptophysin in sybII retrieval, however further studies are required to definitively address this.

It is possible that other potential iTRAPs conform to only a subset of the relatively stringent criteria we have outlined above for the trafficking of both sybII and synaptotagmin-1. In agreement, depletion of the SV glutamate transporter (vGLUT) has disparate effects on the retrieval of a subset of SV cargo, including sybII, SV2A and synaptophysin, but not synaptotagmin-1 (Pan et al., 2015). The retrieval of these cargoes was proposed to be coordinated by a C-terminal proline-rich motif on vGLUT. However, previous work examining C-terminal interaction partners did not demonstrate binding to SV cargo, but instead identified interactions with a subset of src-homology 3 domain containing proteins and E3 ubiquitin ligases (Santos et al., 2014). Furthermore phospho-mimetic or -null substitutions on the vGLUT C-terminal tail altered AP-2 interactions (Santos et al., 2014), suggesting that this region is important for directing its own retrieval to SVs. Therefore vGLUT is a third putative member of the iTRAP group, however further work is required to confirm its interaction with SV proteins, and determine the molecular mechanisms underlying its potential coordination of SV cargo retrieval.

DYSFUNCTION OF THE iTRAPs AND HUMAN DISEASE

Maintenance of robust, efficient retrieval of core SV protein cargo within the brain is central to human health. The iTRAPs therefore play a crucial role in correct brain functioning throughout an individual's lifetime. As evidence for this, alterations in either iTRAP levels or functionality have been implicated in a spectrum of neurological conditions, from congenital, early-onset neurodevelopmental disorders through to neurodegenerative diseases that arise in later life.

Dysfunctional iTRAP Trafficking of SybII in Human Disease

Mutations in the iTRAP synaptophysin have been identified in individuals with familial forms of intellectual disability (Tarpey et al., 2009). Concordantly, synaptophysin KO mice have mild deficits in learning and memory (Schmitt et al., 2009). Importantly, each of the intellectual disability-associated variants of synaptophysin have a reduced ability to coordinate sybII retrieval during endocytosis (Gordon and Cousin, 2013), suggesting that is it specifically this process that is perturbed in individuals harboring mutations in synaptophysin. Synaptophysin has also been implicated in schizophrenia, with multiple rare variants identified that were absent in control patients (Shen et al., 2012). Dysfunctional sybII retrieval may also alter excitatory/inhibitory balance (see below) and in agreement, the interaction between synaptophysin and sybII is upregulated following epileptic seizures in the kindling rat model of epilepsy (Hinz et al., 2001). Therefore the perturbation or loss of synaptophysin function has been linked to series of neurodevelopmental disorders that are associated with deficits in higher brain function. This suggests that loss of fine control of sybII retrieval impacts on the complex circuitry required for correct cognitive function in humans.

Further evidence that dysfunctional sybII trafficking impacts on excitatory/inhibitory balance originate from AP180 knockout mice, which die prematurely as a result of seizure activity (Koo et al., 2015). Interestingly, a more severe sybII trafficking defect was observed in inhibitory interneurons from these mice. Silencing activity in these neurons restored sybII localization to wild-type levels, suggesting the higher background activity of inhibitory interneurons (Bartos et al., 2007) exacerbated sybII retrieval defects and potentially precipitated seizure activity (Koo et al., 2015).

In addition to neurodevelopmental disorders, synaptophysin dysfunction is also linked to neurodegenerative conditions such as Alzheimer's disease (AD). Intriguingly, synaptophysin levels were reduced in the earliest stages of AD (mild AD) whilst there was no alteration in other presynaptic molecules such as synaptotagmin-1 or GAP-43, suggesting that early in disease progression there may be a selective reduction in synaptophysin (Masliah et al., 2001). A reduction in sybII levels mimics the decline in synaptophysin expression in AD, again without an appreciable loss of synaptotagmin-1 (Shimohama et al., 1997) suggesting these two SV molecules are functionally linked. This raises questions regarding the mechanisms by which synaptophysin and sybII are selectively affected in AD, and at which stage in an individual's life these levels are altered. Interestingly, one of the peptides linked to AD pathogenesis, Aβ42, binds to synaptophysin, and modulates the interaction between synaptophysin and sybII (Russell et al., 2012). Soluble Aβ levels correlate with pathogenic markers of AD progression (McLean et al., 1999), and impair synaptic function (Selkoe, 2008; Mucke and Selkoe, 2012). Disruption of the synaptophysin-sybII interaction by Aβ42 may therefore result in perturbed sybII trafficking in AD, and perhaps underlie early synaptic dysfunction and ultimately lead to synapse and neuronal loss later in disease progression.

AP180/CALM have also been linked to AD through genome-wide association studies (Harold et al., 2009). In addition CALM is abnormally cleaved in AD and found associated with neurofibrillary tangles, which are intraneuronal aggregates of hyperphosphorylated tau (Ando et al., 2013). Interestingly, CALM also regulates tau clearance through v-SNARE dependent modulation of autophagy (Moreau et al., 2014), with AP180 regulating generation of toxic Aβ peptides (Wu et al., 2009). How any of these proposed pathological functions relates to sybII trafficking and retrieval is unknown, and requires further investigation.

Dysfunctional iTRAP Trafficking of Synaptotagmin-1 in Human Disease

Dysfunction of the other iTRAP SV2A has been strongly linked to epilepsy. SV2A knockout mice have severe epileptic seizures, fail to grow, and die by 3 weeks old (Crowder et al., 1999; Janz et al., 1999a). Remarkably, SV2A is also the binding partner for the leading anti-epileptic drug, levetiracetam (Lynch et al., 2004). The mechanism by which levetiracetam exerts its effect is still under debate, but its potency directly relates to SV2A binding; the binding affinity of SV2A ligands is proportional to their protective effects in multiple models of epilepsy (Lynch et al., 2004; Kaminski et al., 2008). Furthermore mice heterozygous for SV2A knockout have higher incidence of spontaneous seizures compared to wild-type mice (Crowder et al., 1999), a reduced threshold for induced seizure activity, and a reduced responsiveness to levetiracetam (Kaminski et al., 2009). It is still unclear whether levetiracetam modulates SV2A function or whether it simply uses SV2A as an activity-dependent carrier to gain access to the presynapse. The latter possibility is supported by studies showing that levetiracetam binds to a series of residues in the transmembrane domains of SV2A (Correa-Basurto et al., 2015), with D670 in the tenth transmembrane domain being identified as a key residue (Lee et al., 2015). Furthermore functional studies have shown that the time for levetiracetam to reduce evoked SV recycling and neurotransmitter release is shortened greatly by prior neuronal activity (Meehan et al., 2011, 2012). Evidence also exists that levetiracetam could modulate the role of SV2A as an iTRAP, since it corrected synaptotagmin-1 levels in an SV2A overexpression system (Nowack et al., 2011). Adding further credence to a direct role for SV2A in epilepsy pathogenesis is the recent identification of a homozygous mutation (R383Q) in a highly conserved residue of SV2A in an individual with intractable epilepsy, developmental and growth delay (Serajee and Huq, 2015). It will be of great interest to determine how this mutation impacts on SV2A function and potentially synaptotagmin-1 trafficking.

Mice with a genetic ablation of both SV2A/B and stonin-2 display a worse seizure phenotype, and more prominent lethality than SV2A/B knockout mice alone (Kaempf et al., 2015) suggesting stonin-2 dysfunction may increase susceptibility to epilepsy. However stonin-2 knockout mice do not undergo seizures (Kononenko et al., 2013). Two exonic single-nucleotide polymorphisms in stonin-2 were associated with schizophrenia (Luan et al., 2011), however this finding could not be replicated in a separate study (Xiang et al., 2013). Instead, one of these polymorphisms (Ser307Pro) correlated with a larger cortical surface area on right inferior temporal hemisphere in individuals with schizophrenia (Xiang et al., 2013). The mechanisms by which this variant of stonin-2 causes this, as well as the functional relevance, remains unknown.

Thus, studies of human disease have provided tantalizing evidence that the potential dysfunction of the iTRAPs synaptophysin and SV2A have key roles in mammalian brain function. The fact that their roles become apparent in disorders that involve a fine balance between excitation and inhibition in complex neuronal circuits correlates with their emergence later in evolution, when fine control of neurotransmission is required to produce cognition, learning and behavior.

THE iTRAPs—IMPLICATIONS FOR NEURONAL FUNCTION

The identification of the iTRAPs and their key role in ensuring the accurate retrieval of both sybII and synaptotagmin-1 during neuronal activity have revealed a hitherto unappreciated level of complexity in cargo selection and packing into SVs. The iTRAPS have appeared relatively late in evolution, with no close functionally-related homologs present in invertebrate species (Janz et al., 1998; Abraham et al., 2006; Yanay et al., 2008). This is in stark contrast to the monomeric adaptors, which are evolutionarily conserved from *C. elegans* and *Drosophila* through to higher order mammalian species. This suggests that as neuronal circuitry became more complex, additional mechanisms were required to ensure the efficient targeting and retrieval of essential SV cargo. The association of human neurological disorders with iTRAP dysfunction, as outlined

above, adds further credence to the theory that the monomeric adaptors alone are not capable of maintaining the efficient retrieval of core SV proteins in complex mammalian circuits. The specificity of the iTRAPs in terms of their retrieval targets (sybII or synaptotagmin-1) is a key determinant of their function, with only very minor effects on SV turnover observed in either synaptophysin or SV2A knockout mice (Yao et al., 2010; Gordon et al., 2011; Kwon and Chapman, 2011). In contrast knockout systems for AP180/CALM and stonin-2 display more global defects in SV recycling. For example stonin-2 knockout neurons display alterations in both the kinetics and extent of CME and activity-dependent bulk endocytosis (Kononenko et al., 2013). Furthermore AP180 null organisms display an increase in SV diameter (Zhang et al., 1998; Nonet et al., 1999; Koo et al., 2011a, 2015; Petralia et al., 2013), a reduction in SV number (Bao et al., 2005; Petralia et al., 2013; Koo et al., 2015) and accumulation of endosomal intermediates in their nerve terminals (Koo et al., 2015).

The discovery of the iTRAP function of both synaptophysin and SV2A leads to the obvious question of whether any more iTRAPs exist. One potential candidate, as outlined above, is vGLUT, and it is a matter of urgency to determine whether it, or any other SV cargo (such as synaptoporin or synaptogyrin) perform similar functions. A critical area to address will be the relationship between the iTRAPs and the monomeric adaptor proteins that assist in retrieval of both sybII and synaptotagmin-1. The fact that depletion of both SV2A and stonin-2 resulted in an exacerbation of synaptotagmin-1 trafficking defects (Kaempf et al., 2015) suggests that they have complementary, rather than sequential functions. It will be of great interest to determine whether a similar relationship exists between synaptophysin and AP180/CALM. Another key question to determine is how the iTRAPs control the assembly of the retrieval complexes of either sybII or synaptotagmin-1 both temporally and spatially. This is of particular interest for SV2A, since a number of molecules share overlapping interaction sites for the C2B domain of synaptotagmin-1 (Leveque et al., 1992; Chapman et al., 1996, 1998; Schiavo et al., 1996; Bhalla et al., 2006). The phosphorylation-dependent control of the SV2A-synaptotagmin-1 interaction may be central to coordinating the hierarchy of association during endocytosis in this regard. Synaptophysin is the major vesicular phospho-tyrosine protein (Pang et al., 1988), and it will important to determine whether its binding to sybII is similarly regulated by phosphorylation.

The identification of the iTRAPs have revealed an additional layer of complexity in the retrieval of SV cargo during endocytosis. The specific roles of these proteins in coordinating the traffic of essential SV molecules, and their association with human neurological disorders, highlights the importance of having the correct complement of both sybII and synaptotagmin-1 on SVs.

AUTHOR CONTRIBUTIONS

SLG and MAC both devised and wrote this review article.

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SNAP-25, a Known Presynaptic Protein with Emerging Postsynaptic Functions

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A hallmark of synaptic specializations is their dependence on highly organized complexes of proteins that interact with each other. The loss or modification of key synaptic proteins directly affects the properties of such networks, ultimately impacting synaptic function. SNAP-25 is a component of the SNARE complex, which is central to synaptic vesicle exocytosis, and, by directly interacting with different calcium channels subunits, it negatively modulates neuronal voltage-gated calcium channels, thus regulating intracellular calcium dynamics. The SNAP-25 gene has been associated with distinct brain diseases, including Attention Deficit Hyperactivity Disorder (ADHD), schizophrenia and bipolar disorder, indicating that the protein may act as a shared biological substrate among different "synaptopathies". The mechanisms by which alterations in SNAP-25 may concur to these psychiatric diseases are still undefined, although alterations in neurotransmitter release have been indicated as potential causative processes. This review summarizes recent work showing that SNAP-25 not only controls exo/endocytic processes at the presynaptic terminal, but also regulates postsynaptic receptor trafficking, spine morphogenesis, and plasticity, thus opening the possibility that SNAP-25 defects may contribute to psychiatric diseases by impacting not only presynaptic but also postsynaptic functions.

Keywords: SNAP-25, synaptopathies, presynaptic role, postsynaptic role, brain diseases

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SNAP-25 is a component of the SNARE protein complex, which is involved in the exocytotic release of neurotransmitters during synaptic transmission. Through the coiled-coil assembly with syntaxin-1 and synaptobrevin, SNAP-25 mediates synaptic vesicle apposition to the presynaptic membrane permitting their Ca^{2+} triggered fusion. Consistently, the genetic ablation of this protein results in a complete block of synaptic transmission. SNAP-25 is present in two isoforms, a and b, resulting from alternative splicing of the exon 5 of the *Snap-25* gene, which are differentially expressed during development. SNAP-25a is expressed at the embryonic stage, while SNAP-25b becomes the major isoform during postnatal life (Bark, 1993; Bark and Wilson, 1994; Bark et al., 1995), a developmental trend which has been confirmed in humans (Prescott and Chamberlain, 2011).

In line with its central role in neuronal function, the *Snap-25* gene has been associated with several human neurological syndromes, including attention-deficit/hyperactivity disorder (ADHD), schizophrenia (Barr et al., 2000; Brophy et al., 2002; Kustanovich et al., 2003), and bipolar

disorder (Etain et al., 2010). The protein appears therefore to represent a shared biological element among different psychiatric diseases.

Recently, several groups started to investigate the cellular and molecular mechanisms underpinning the SNAP-25 contribution to the onset of such pathologies, or, more likely, to the manifestations of specific traits typical of these diseases. A challenging scenario is now emerging, i.e., that some of the defects in diseases involving SNAP-25 might not exclusively result from the presynaptic role of the protein. Indeed, initially recognized as a presynaptic SNARE protein, the protein has been later shown to play additional non-SNARE roles and, very recently, even postsynaptic functions. The results of these lines of research are summarized in this review (see **Table 1A**).

TABLE 1 | (A) Functions of SNAP-25 protein, (B) *Snap-25* polymorphisms discussed along the text.

(A) SNAP-25 known function	*In vitro*	*Ex vivo*	*In vivo*	Human	References
Neurotransmitter release	•	•	•	•	Oyler et al., 1989; Söllner et al., 1993a,b; Chapman et al., 1994; Poirier et al., 1998; Raciborska et al., 1998; Sutton et al., 1998; Washbourne et al., 2002; Sørensen et al., 2003; Jeans et al., 2007; Mohrmann et al., 2010; Shen et al., 2014
Modulation of VGCCs	•	•			Bennett et al., 1992; Yoshida et al., 1992; Lévêque et al., 1994; Martin-Moutot et al., 1996; Rettig et al., 1996; Zhong et al., 1999; Jarvis and Zamponi, 2001; Verderio et al., 2004; Pozzi et al., 2008; Condliffe et al., 2010; Condliffe and Matteoli, 2011; Weiss et al., 2012
Slow, clathrin-dependent endocytosis	•				Okamoto et al., 1999; Xu et al., 2013; Zhang et al., 2013
Postsynaptic receptor trafficking	•	•			Selak et al., 2009; Lau et al., 2010; Jurado et al., 2013
Short term plasticity	•	•			Pozzi et al., 2008; Antonucci et al., 2013
Long term plasticity	•	•			Jurado et al., 2013; Fossati et al., 2015
Dendritic spine morphogenesis	•		•		Tomasoni et al., 2013; Fossati et al., 2015
Cognitive ability, learning, and memory			•	•	Gosso et al., 2006, 2008; Corradini et al., 2014; Braida et al., 2015
Network excitability and epileptiform activity	•	•	•	•	Hess et al., 1992, 1995; Zhang et al., 2004; Rohena et al., 2013; Corradini et al., 2014; Shen et al., 2014

(B) Polymorphysm	Position in the gene	Traits	Effects on mRNA/protein	References
rs6039769	Promoter	Early onset bipolar disorder	Higher SNAP-25 levels in homozygous "CC" individuals	Etain et al., 2010
rs363039	Intron 1	Association with variation in IQ in normal population; verbal performances in women; working memory capacity; cognitive traits in autistic children	Transcription binding site	Gosso et al., 2006; Cagliani et al., 2012; Söderqvist et al., 2010; Braida et al., 2015
rs363050	Intron 1	Association with variation in IQ in normal population; association with intellectual disabilities; association with Alzheimer's disease and mild cognitive impairment; cognitive traits in autistic children	Transcription binding site; reduced protein expression	Gosso et al., 2006; Rizzi et al., 2012; Guerini et al., 2014; Braida et al., 2015
rs363043	Intron 1	Association with variation in IQ in normal population; hyperactivity in autistic children; association with Alzheimer's disease and mild cognitive impairment;	Transcription binding site	Gosso et al., 2008; Guerini et al., 2011, 2014
rs353016	Intron 1	Association with variation in IQ in normal population	Transcription binding site	Gosso et al., 2008
rs6108461	Intron 3	ADHD—regulation of attention and inhibition	Decreased expression of SNAP-25	Hawi et al., 2013
rs362549	Intron 4	ADHD—inattentive trait, hyperactivity trait		Zhang et al., 2011
rs362990	Intron 4	ADHD—regulation of attention and inhibition	Decreased expression of SNAP-25	Hawi et al., 2013
rs363006	Intron 7	Early onset bipolar disorder; ADHD	N/D	Etain et al., 2010; Zhang et al., 2011
rs3746544	3′untranslated	ADHD traits, especially when associated to norepinephrine transporter NET1 (rs2242447); increased risk of schizophrenia and major depressive disorder	N/D	Carroll et al., 2009; Pazvantoğlu et al., 2013; Dai et al., 2014; Wang et al., 2015
rs1051312	3′untranslated	ADHD; cognitive dysfunction in schizophrenia; impultivity trait in healthy population when in haplotype with rs3746544	N/D	Brophy et al., 2002; Spellmann et al., 2008; Németh et al., 2013

Only a selection of papers describing the role of SNAP-25 in the control of neurotransmitter release is reported owing to space limitations (see text for details). The position in the gene, traits associated with the genetic variant and effect on mRNA or protein levels are listed.

ROLE OF SNAP25 AT THE PRESYNAPSE: SYNAPTIC VESICLES EXOCYTOSIS AND SHORT TERM PLASTICITY

SNAP-25 (synaptosomal-associated protein of 25 kDa) is a soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) protein that participates together with syntaxin-1 and synaptobrevin/VAMP (Jahn et al., 2003; Sudhof, 2004; Montecucco et al., 2005) in the regulation of synaptic vesicle exocytosis (Washbourne et al., 2002; reviewed in Milovanovic and Jahn, 2015). In the absence of SNAP-25, vesicle docking at the presynaptic active zones persists, but the pool of vesicles primed for release is empty, and fast calcium-triggered exocytosis is abolished (Sørensen et al., 2003). Furthermore, by calcium-dependent interaction with synaptotagmin, SNAP25 has a role in vesicle docking and priming as well as in triggering fast exocytosis (Mohrmann et al., 2010). Indeed the proteolytic cleavage of SNAP-25 by botulinum neurotoxins (BoNTs, serotypes A, C, and E) blocks exocytosis and neurotransmitter release (Schiavo et al., 2000; Ahnert-Hilger et al., 2013; Pantano and Montecucco, 2014), leading to the neuroparalysis characteristic of botulism (Aoki and Guyer, 2001).

Besides its well characterized role in exocytosis, SNAP-25 also modulates various voltage-gated calcium channels (VGCCs) (Atlas et al., 2001; Zamponi, 2003; Catterall and Few, 2008), by interacting with N-type (Sheng et al., 1996), P/Q-type (Martin-Moutot et al., 1996; Rettig et al., 1996), L-type (Wiser et al., 1999), and T-type channels (Weiss et al., 2012). SNAP-25 has been shown to negatively control neuronal calcium responsiveness to depolarization (Verderio et al., 2004) through voltage-gated calcium channel inhibition (Pozzi et al., 2008). Consistently, silencing endogenous SNAP-25 in glutamatergic neurons results in increased VGCC activity (Condliffe et al., 2010; Condliffe and Matteoli, 2011; see **Figure 1**).

SNAP-25 also participates in slow, clathrin-dependent endocytosis at hippocampal synapses, possibly contributing to the coupling between exocytosis and endocytosis (Zhang et al., 2013). Given that SNARE proteins mediate exocytosis at all nerve terminals, their dual role in exo- and endocytosis is likely a general principle. Although how exactly SNARE proteins are involved in endocytosis remains unclear, the following binding studies provide some indications. Synaptobrevin/VAMP2 binds to the AP180 N-terminal homology (ANTH) domain of endocytic adaptors AP180 and Clathrin Assembly Lymphoid Myeloid leukemia (CALM) protein (Koo et al., 2011; Miller et al., 2011); also, stonin 2, facilitates clathrin/AP-2-dependent internalization of synaptotagmin and targets it to a recycling vesicle pool in living neurons (Diril et al., 2006).

SNAP-25 binds to the endocytic protein intersectin (Okamoto et al., 1999); syntaxin binds to dynamin (Galas et al., 2000). Based on these evidence it was proposed that the exocytosis machinery, including SNARE proteins (synaptobrevin, SNAP-25, and syntaxin), is needed in the initiating step of endocytosis and likely controls the amount of endocytosis (for a review see Wu et al., 2014).

Therefore, SNAP-25 represents a multifunctional protein involved in the control of secretion by multiple interactions. In line with the multiple roles of the protein, different neuronal processes are affected, in an unexpected way, in conditions characterized by SNAP-25 reduction. Indeed halved SNAP-25 levels in 13–14 DIV neuronal cultures not only failed to impair synaptic transmission, as expected by the SNARE role of SNAP-25, but instead enhanced evoked glutamatergic neurotransmission (Antonucci et al., 2013). This effect was dependent on presynaptic voltage-gated calcium channel activity and was not accompanied by changes in spontaneous quantal events or in the pool of readily releasable synaptic vesicles (Antonucci et al., 2013). Notably, synapses of 13–14 DIV neurons with reduced SNAP-25 expression showed paired-pulse depression as opposed to paired-pulse facilitation occurring in their wild-type counterparts (Antonucci et al., 2013). These data suggest that the more sensitive phenotype for reduced SNAP-25 levels may be the regulation of calcium channels, not the role of SNAP-25 in transmitter release. Based on these results, a dual role of SNAP-25 not only as a carrier but also as a "guardian of synaptic transmission" was proposed: in particular, reduced SNAP-25 expression, although sufficient to sustain SNARE-mediated synaptic vesicle fusion, partially releases VGCCs from SNAP-25-mediated inhibition, thus resulting in elevated calcium influx and facilitated neurotransmission (Kochlamazashvili and Haucke, 2013).

AN UNEXPECTED ROLE OF SNAP-25 AT THE POST-SYNAPSE: SPINE MORPHOGENESIS AND PLASTICITY

In the last years, different evidence indicated an unexpected postsynaptic role for SNAP-25 (see **Figure 1**). The protein was indeed shown to control NMDA and kainate-type receptors trafficking (Selak et al., 2009; Lau et al., 2010). In particular the interaction of SNAP-25 with the GluK5 subunit of KARs and PICK1 reduces the GluK5 stability on the membrane, thus favoring KAR internalization (Selak et al., 2009), whereas the PKC-mediated phosphorylation of SNAP-25 on serine 187, promotes NMDAR delivery to the cell surface via SNARE-dependent exocytosis (Lau et al., 2010). In the latter study the authors elegantly demonstrated that introduction of the constitutively active form of PKC via the recording pipette to neurons rapidly potentiated NMDA currents in cells treated with inactive BoNT/A whereas treatment of neurons with active BoNT/A abolished PKC potentiation of NMDA currents without altering basal NMDA currents, thus unveiling SNAP-25 involvement in the potentiation of the synapse. Given that LTP-inducing protocols can induce SNAP-25 phosphorylation (Genoud et al., 1999), high frequency stimulation protocols may act via phosphorylation of SNAP-25 to promote insertion of NMDARs and elicit LTP. Indeed acute SNAP-25 downregulation resulted in LTP impairment (Jurado et al., 2013). These data opened the possibility that, besides a presynaptic impact, reductions of SNAP-25 levels may affect the structure, and/or the function of the postsynaptic compartment, which would provide a logical frame for the protein involvement in psychiatric diseases, such as schizophrenia or intellectual disability, which

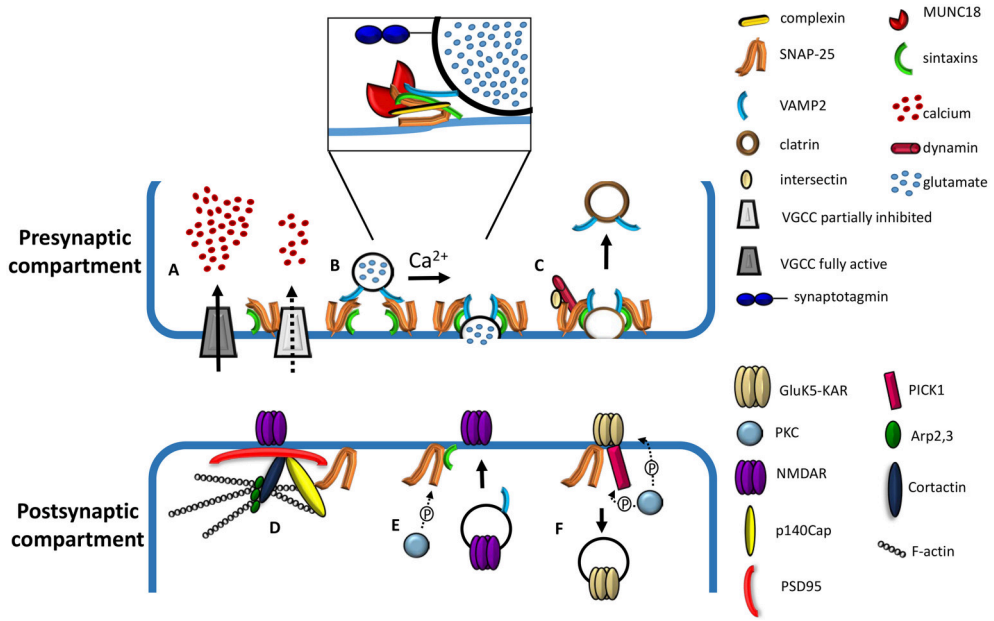

FIGURE 1 | Cartoon depicting presynaptic and postsynaptic roles of SNAP-25. (A) Effect of presynaptic SNAP-25 on VGCCs. Calcium influx in the nerve terminal is negatively regulated by the complex formation between SNAP-25 and VGCCs; removal of the clamping role of SNAP-25, occurring upon reduction of the protein expression, results in elevated calcium influx through VGCCs (adapted from Kochlamazashvili and Haucke, 2013). **(B)** Involvement of SNAP-25 in the molecular machinery mediating Ca^{2+}-triggered vesicle fusion. A docked synaptic vesicle is shown on the left. The core fusion machine is composed of synaptobrevin/VAMP2, syntaxin-1, and SNAP-25 (adapted from Kochlamazashvili and Haucke, 2013). The diagram in the box depicts a partially assembled SNARE complex including, besides synaptobrevin/VAMP2, syntaxin-1, and SNAP-25, complexins and MUNC18. The calcium sensor, synaptotagmin, is also depicted (adapted from Sudhof, 2012). **(C)** Role of SNAP-25 in slow clathrin-mediated synaptic vesicle endocytosis. SNAP-25 binds to the endocytic protein intersectin, while syntaxin binds dynamin, a GTPase mediating vesicle fission. The interactions between Synaptobrevin/VAMP2 and the ANTH domain of endocytic adaptors AP180 and CALM have been omitted for clarity. **(D)** Role of SNAP-25 in the organization of the postsynaptic density protein network. SNAP-25 interacts with p140Cap, which in turn forms a complex with PSD95, cortactin, Arp2,3, and F-actin (filamentous actin). NMDA receptors are depicted as interacting with PSD95 (adapted from Fossati et al., 2015). **(E)** Phosphorylation of SNAP-25 by PKC promotes the insertion of NMDA channels at the cell surface through the delivery of postsynaptic vesicles and their fusion with the plasma membrane, possibly via the formation of a SNARE complex (adapted from Lau et al., 2010). **(F)** Role of SNAP-25 in the removal of GluK5-contaning kainate receptors (KAR). KARs associate with SNAP-25 and the PKC-interacting protein PICK1. The PKC phosphorylation of the GluK5-C terminus may induce a conformational change facilitating the association with SNAP-25 and simultaneously decreasing GRIP binding affinity (adapted from Selak et al., 2009).

are known to be also characterized by defects at the postsynaptic compartment (Fernández et al., 2009; Penzes et al., 2011).

Despite the evidence pointing to a postsynaptic role of SNAP-25, a clear demonstration of whether SNAP-25 localizes in the dendritic spines of the postsynaptic neuron is still lacking. Some recent studies attempted to locate SNAP-25 in the postsynaptic terminal either by immunofluorescence (Selak et al., 2009), or ground state depletion (GSD) microscopy, which allows protein localization with a precision up to 20 nm (Tomasoni et al., 2013). Also by coimmunoprecipitation, bimolecular fluorescence complementation (BiFC) and biochemical fractionation, a molecular complex of SNAP-25 with postsynaptic proteins was detected (Selak et al., 2009; Tomasoni et al., 2013; Fossati et al., 2015). Nevertheless this is still a controversial topic, since other studies showed an exclusively presynaptic location of SNAP-25 through immunogold labeling of synaptic boutons (Holderith et al., 2012; Kerti et al., 2012). Certainly, the SNAP-25 expression levels in the postsynaptic compartment are quantitatively much lower than at the presynaptic one (Tao-Cheng et al., 2000; Sharma et al., 2012) and this could account for its difficult detection in dendritic spines.

In recent years, the postsynaptic role of SNAP-25 has been supported by evidence showing a structural modification of the postsynaptic compartment upon SNAP-25 reduction. In particular, acute reduction of SNAP-25 expression in primary hippocampal cultures led to an immature phenotype of dendritic spines, while overexpression of the protein resulted in an increase in the density of mature, PSD-95-positive spines (Tomasoni et al., 2013). The effect was shown to be truly postsynaptic, and not secondary to altered presynaptic function as demonstrated by co-culturing of SNAP25 heterozygous and GFP-expressing wild type neurons. SNAP-25 reductions were also shown to affect the localization of PSD95, with acute downregulation of SNAP-25 resulting in a significant reduction of PSD95-positive puncta (Fossati et al., 2015). Correspondingly, acute down-regulation of SNAP-25 in CA1 hippocampal region by lentiviral expression reduced spine density and resulted in immature spine morphology, thus recapitulating *in vivo* the spine abnormalities observed in cultures upon acute SNAP-25 silencing (Fossati et al., 2015).

Which could be the mechanism by which SNAP-25 controls dendritic spine morphology and PSD95 mobility? The cleavage

of SNAP-25 by BoNT/E, which prevents the protein to enter the fusion complex, did not reduce spine density or PSD95 size, thus excluding that SNAP-25 controls PSD95 recruitment through its SNARE function and suggesting instead a protein scaffolding role at the spine level (Fossati et al., 2015). This hypothesis was supported by the finding that p140Cap, a scaffold protein located into dendritic spines with a crucial role in regulating actin cytoskeleton, spine formation (Jaworski et al., 2009), and learning processes (Repetto et al., 2014), is a key member of the molecular complex which includes SNAP-25 and PSD95 (Tomasoni et al., 2013; Fossati et al., 2015).

The correct formation of this molecular complex preserves the proper organization of the dendritic spine. Maintaining spine integrity could further facilitate the formation of the protein complexes which contain also SNAP-25 and that regulate receptor trafficking (Selak et al., 2009; Lau et al., 2010). Based on these results, it is conceivable that postsynaptic SNAP-25 may be important for orchestrating a dynamic equilibrium among the glutamate receptors at a given synapse, thereby regulating synapse efficacy also at the postsynaptic side.

SNAP-25, A SHARED BIOLOGICAL PATHWAY AMONG DIFFERENT PSYCHIATRIC DISEASES

The defective formation of the SNARE complex for vesicle fusion and the aberrant regulation of voltage-gated calcium channels are the processes generally taken into account to explain the involvement of the protein in those psychiatric diseases which have been linked to the *Snap-25* gene. However, the recent data indicating a postsynaptic role for the protein raise the possibility that SNAP-25 defects may contribute, in these disorders, also through alterations of postsynaptic receptors trafficking or spine morphogenesis.

Several reports have shown the presence of polymorphisms in the *Snap-25* gene, which have been associated with ADHD (Barr et al., 2000; Faraone et al., 2005; Zhang et al., 2011; Hawi et al., 2013; Pazvantoğlu et al., 2013), schizophrenia (Thompson et al., 2003), and early-onset bipolar disorders (Etain et al., 2010; see **Table 1B**). Notably, some of these polymorphisms were found to control not only specific traits of the disease, but even behavioral tracts in healthy individuals. As an example, several single nucleotide polymorphisms (i.e., rs363043, rs353016, rs363039, rs363050) of the *Snap-25* gene have been associated with Intelligence Quotient (IQ) phenotypes in healthy individuals (Gosso et al., 2006, 2008). Also, although autism spectrum disorder (ASD) has not been directly linked to the *Snap-25* gene, polymorphisms analyzed in a cohort of children affected by ASD revealed a significant association between *Snap-25* SNP rs363043 and hyperactivity traits (Guerini et al., 2011), while rs363050 and rs363039 polymorphisms were shown to correlate with cognitive deficits in ASD children (Braida et al., 2015). Notably, a first analysis of transcriptional activity through luciferase reporter gene revealed that SNP rs363050, which is localized in the intron 1 of the *Snap-25* gene, leads to reduced protein expression (Braida et al., 2015). Therefore, the possibility that reduced SNAP-25 levels may contribute to specific behavioral traits, such as hyperactivity or cognitive performances in healthy individuals or in different psychiatric diseases, including those to which the gene has not been directly associated, like in the case of ASD, remains a challenging possibility to be tested in the future.

Notably, even in schizophrenia, where the SNAP-25 levels are significantly lower in the hippocampus (Young et al., 1998; Fatemi et al., 2001; Thompson et al., 2003) and in the frontal lobe Broadman's area 10 (Thompson et al., 1998), an association between the rs1051312 polymorphism of the *Snap-25* gene and cognitive dysfunctions was reported (Spellmann et al., 2008). Furthermore, and consistent with the observations already reported in SNAP-25 heterozigous mice (Antonucci et al., 2013), even in schizophrenic patients the reduction of SNAP-25 levels does not seem to correlate with an impairment in the SNARE complex formation (Ramos-Miguel et al., 2015). Of interest, and in line with the possible relevance of SNAP-25 expression levels in different psychiatric diseases, a SNAP-25 variant located in the promoter region (rs6039769) and associated with early-onset bipolar disorder was found to correlate with a significantly higher SNAP-25b expression in prefrontal cortex (Etain et al., 2010). Higher levels of the SNARE in dorsolateral prefrontal cortex of patients affected by bipolar disorder were already reported by Scarr et al. (2006).

As a support to the functional impact of the protein levels in cognitive or motor functions, genetic mice models showed that the chronic reduction of SNAP-25 affects mouse behavior. The coloboma mouse model, characterized by halved SNAP-25 levels (Hess et al., 1992), displays indeed a hyperactive phenotype (Hess et al., 1992), associated with abnormal thalamic spike-wave discharges (Hess et al., 1995; Zhang et al., 2004; Faraone et al., 2005; Russell, 2007). Similarly, juvenile SNAP-25 heterozygous mice displays a moderate hyperactivity, which disappears in the adult animals, and impaired associative learning and memory, which persist instead in the adults. Electroencephalographic recordings revealed the occurrence of frequent spikes, suggesting a diffuse network hyperexcitability, accompanied by a higher susceptibility to kainate-induced seizures, and degeneration of hilar neurons. Notably, both EEG alterations and cognitive defects were improved by antiepileptic drugs, in particular valproic acid (Corradini et al., 2014; Braida et al., 2015). A defective negative control of voltage gated calcium channels resulting from the reduced SNAP-25 levels could be at the origin of the network hyperexcitability (Corradini et al., 2014). Although, the demonstration of a direct causal link between altered SNAP-25 expression and psychiatric diseases is still lacking, evidences obtained in the coloboma mouse suggest that reduction of SNAP-25 expression may be directly involved in some psychiatric traits, rather than simply represent an epiphenomenon; indeed, when a transgene expressing SNAP-25 was bred into the coloboma strain in order to complement Snap-25 depletion, the hyperactivity displayed by the mutant mice was rescued (Hess et al., 1995 J Neurosci).

Recently a *de novo* variant was identified in the *Snap-25* exon 4 (Phe48Val), in a 15-years-old girl with intractable epilepsy and severe encephalopathy, but no neuromuscular symptoms

(Rohena et al., 2013). Later on, exome sequencing identified a *de novo* dominant mutation of a conserved residue in exon 5 of *Snap-25b* in an 11-years-old patient displaying congenital myasthenia, cortical hyperexcitability, cerebellar ataxia, and intellectual disability (Shen et al., 2014). The Ile67Asn variant was reported to be pathogenic because, by disrupting the hydrophobic alpha-helical coiled-coil structure of the SNARE complex, it inhibits synaptic vesicle exocytosis (Shen et al., 2014). Of note, a heterozygous Ile67Thr missense mutation in *Snap-25b* gene was observed in the so-called blind-drunk (1/Bdr) mouse, which shows a mild ataxic gait around age 4 weeks, impaired sensorimotor gating and increased anxiety (Jeans et al., 2007; Oliver and Davies, 2009). This mutation is located in a highly conserved codon and parallels Ile67Asn mutation observed in the 11 years-old patient (Shen et al., 2014). In the case of the Ile67Asn mutation, Shen and colleagues propose that the substitution of a hydrophobic residue with a hydrophilic one destabilizes the coiled-coil SNARE complex structure, thus hindering vesicle fusion (Shen et al., 2014); however, it is also possible that the Ile67Asn mutation causes a distortion of the coiled coil structure in such a way as to affect the interaction of the SNARE complex with its protein partners. This appears to be the case in the blind-drunk mutation which results in the enhancement of the affinity of SNAP-25 for its binding partners and is therefore likely to cause an increase in association of the SNARE complex (Jeans et al., 2007). No information about the impact of Val48Phe variant on SNAP-25 structure and function is still available.

Additional genetic mouse models underlined the role of *Snap-25* mutations in specific traits of psychiatric diseases. Single nucleotide substitution resulting in a missense Ser187Ala mutation at the site of phosphorylation of SNAP-25 by PKC has been associated with increased anxiety, decreased dopamine and serotonine release (Kataoka et al., 2011), impaired PPI of the startle response, a typical parameter of schizophrenia, deficits in working memory, immature features of dentate granule cells (Ohira et al., 2013), and epileptic seizures (Watanabe et al., 2015). Interestingly, Ser187 phosphorylation of SNAP-25 is development- and activity-dependent both *in vitro* and *in vivo* (Kataoka et al., 2006; Pozzi et al., 2008); it is associated with synaptic vesicles availability (Nagy et al., 2002; Houeland et al., 2007) and it is necessary for the negative control of voltage-gated calcium channels (Pozzi et al., 2008).

CONCLUSIONS

The recent discovery of SNAP-25 role in the control of receptor trafficking and spine morphogenesis, which points to the protein role as a postsynaptic structural hub, opens new avenues for the comprehension of the physiological role of the protein at the synapse and offers new mechanistic insights as to SNAP-25 involvement in synaptopathies that go beyond the protein's established roles in presynaptic function. The finding that the activity-driven spine remodeling is defective in neuronal networks constitutively developing in the presence of reduced levels of SNAP-25, makes a provocative link to human pathologies, such as schizophrenia, where both a reduction of SNAP-25 expression and a decrease in dendritic spine density have been described.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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