**Review article**

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# Mechanisms of synaptic vesicle recycling provide a platform to explore mechanisms of neurodegeneration

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**Abstract:** The synaptic vesicle (SV) cycle, a trafficking pathway by which SV fuses with the plasma membrane to release neurotransmitters at the neuronal synapse, resides at the heart of neurotransmission. SV fusion consumes vesicle membrane and proteins, whose availability is limited, and these components must be recycled quickly to prevent synaptic fatigue. Biochemical, genetic and physiological approaches over the past five decades have led to a discovery of a large directory of proteins and lipids central to the SV cycle and several models on how these constituents account for the synapse function. The complexity of the SV cycle is starting to be comprehended, which opens new perspectives for our understanding of neuronal physiology and provides mechanistic explanations for several neurological and neurodegenerative diseases. Here, selected classic and recent insights into the mechanisms of two key SV trafficking steps (exocytosis and endocytosis) are reviewed, as well as their links to selected brain pathologies.

**Keywords:** drug targets; endocytosis; exocytosis; lipids; neurodegeneration; neurological diseases; PI(4,5)P2; synaptic vesicle cycle; vesicle acidification.

**Zusammenfassung:** Der synaptische Vesikelzyklus ist ein Transportweg, in dessen Verlauf die synaptischen Vesikel (SV) mit der Plasmamembran verschmelzen und Neurotransmitter an der neuronalen Synapse freisetzen. Er steht im Zentrum der Neurotransmission. Die SV-Fusion verbraucht die Vesikelmembran und Proteine, deren Verfügbarkeit begrenzt ist, so dass diese Komponenten schnell recycelt werden müssen, um eine synaptische Ermüdung/Erschöpfung zu verhindern. Biochemische, genetische und physiologische Ansätze der Grundlagenforschung haben in den letzten fünf Jahrzehnten die zentrale Bedeutung des SV-Zyklus für die Synapsenfunktion dokumentiert und zu einer Reihe von Modellen wie am Zyklus beteiligte Proteine dazu beitragen geführt. Die Komplexität des Zyklus wird allmählich verstanden, was neue Perspektiven für unser Verständnis der neuronalen Physiologie eröffnet und mechanistische Erklärungen für verschiedene neurologische und neurodegenerative Krankheiten liefert. Hier werden klassische und neuere Erkenntnisse über die Mechanismen von zwei wichtigen Phasen des SV-Zyklus, Exozytose und Endozytose, sowie ihre Verbindungen zu ausgewählten Hirnpathologien untersucht.

**Schlüsselwörter:** Endozytose; Exozytose; Identifizierung potentieller Wirkstoffe; Lipide; Neurodegeneration; neurologische Erkrankungen; PI(4,5)P2; synaptischer Vesikelzyklus; Vesikel Azidifizierung.

## Introduction and objectives

As a result of membrane-centred organization of life, cells have acquired routes by which biomolecules are shuffled between compartments or released to the extracellular space by exocytosis. For neuronal cells, which externalize signalling molecules (neurotransmitters and peptides) quickly and on demand, this process is typically initiated by an elevation in the intracellular calcium concentrations. Neurotransmission capitalizes on the existence of synaptic vesicles (SVs) that store neurotransmitters and define the properties of neuronal synapses, like quantal release, signal directionality and modulation. Since small presynaptic boutons can store limited amounts of SVs, proteins and membranes used during exocytosis must be swiftly recycled: accumulated SVs would be quickly consumed without the existence of robust mechanisms for the local (re)formation of new vesicles (Milosevic, 2018; Saheki and De Camilli 2012). For continuous synaptic function, it is critical that the activity-enforced demands are matched precisely by the local membrane recycling mechanisms.

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It is now recognized that the SV cycle is much more complex than previously thought (Chanaday et al., 2019). However, basic curiosity-driven research over the past 50 years has led to immense breakthroughs towards understanding synaptic physiology and functions of lipids and proteins central to the SV cycle. The body of knowledge that even subtle imbalances in neurotransmitter release or alterations in SV recycling result in disorders is increasing (e.g. Hussain et al., 2019; Lauwers et al., 2016; Li and Kavalali, 2017; Soukup et al., 2018; Verhage and Sørensen, 2020). With this, the opportunities to build on the basic mechanisms of synapse function to tackle complex brain diseases in novel ways are also rising and suggest a dawn of a new era. Dr Armin Schram once stated 'Where funding is scarce, progress and thus the well-being of people is best served in the long term by funding basic research'. This visionary perspective is likely true for many fields of research, but it resonates particularly well with the present stage of synaptic transmission research where we start capitalizing on basic knowledge to bring major benefits to medicine.

The objective of this review is to summarize the selected features of presynaptic release and SV recycling components, both lipidaceous and proteinaceous, and provide examples where the mechanistic insights into this field may be of use to tackle pathologies of selected neurological and neurodegenerative diseases.

## Exocytosis at the presynaptic bouton – a protein view

At the synapse, exocytosis comprises directed translocation of SVs to the active zone of the presynaptic bouton, the contact with the plasma membrane (termed 'docking'), the preparation of SVs for the fusion (termed 'priming') and the calcium-triggered fusion of membranes, resulting in the release of neurotransmitters (Sudhof, 2013). This fast and coordinated process builds on dozens of proteins and lipids, yet the main role is played by the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, a minimal machinery for the fusion of lipid bilayers (Milosevic and Sørensen, 2015; Sudhof, 2013). Synchronized by the calcium influx through voltage-gated channels, SNARE complexes made between vesicular Vesicle Associated Membrane Protein 2 (VAMP2)/synaptobrevin-2 and the plasma membrane–resident synaptosomal-associated protein 25 (SNAP-25) and syntaxin-1 pull the respective membranes into a close contact and lead to 'zippering' of their SNARE domains (Sørensen et al., 2006). Due to the need for high spatial and temporal coordination of synaptic activity, SNARE proteins are controlled at several steps of their generation and function (Milosevic and Sørensen, 2015). This includes the formation of the SNARE complex by activating syntaxin-1 bound to Sec1/Munc18 (SM)-like proteins (de Wit et al., 2009; Lai et al., 2017; Rizo and Sudhof, 2012; Shen et al., 2007). Next, the Munc13 proteins mediate the opening of syntaxin-1 and sequential binding of synaptobrevin-2/VAMP2 and SNAP-25 (Lai et al., 2017). A half-zippered (intermediate) SNARE complex is a characteristic of the primed SV (Sørensen et al., 2006), ready to be released when the final stage of the SNARE assembly is initiated by a calcium sensor from the synaptotagmin family, following the influx of calcium and promoting fusion of the SV with the plasma membrane (Sudhof, 2012, 2013).

SNARE-regulating proteins are vital for the exquisite regulation of exocytosis at the synapse. Hence, it is not surprising that the pathogenic mutations in the SNARE regulators have been described (Verhage and Sørensen, 2020). Notably, some of these proteins function exclusively as inhibitors of exocytosis, e.g. tomosyns/STXBP5 and amisyn/STXBP6. Tomosyn-1, a WD40-repeat protein, is thought to suppress synaptic transmission by inhibiting SV docking and priming (Ben-Simon et al., 2015; Fujita et al., 1998; Gracheva et al., 2006; Yizhar et al., 2004). Amisyn interferes with the priming of secretory vesicles, likely as a vertebrate-specific competitor of synaptobrevin-2/VAMP-2 (Constable et al., 2005; Kondratiuk et al., 2020). A recent study has shown that amisyn contains an N-terminal pleckstrin-homology domain that mediates its transient association with the plasma membrane by binding to lipid phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$; Kondratiuk et al., 2020). The importance of the negative regulation of exocytosis exerted by both amisyn and tomosyn-1 is underscored by their association to neurodevelopment and neurological diseases, including autism-spectrum disorder (ASD; Castermans et al., 2008; Cukier et al., 2014; Davis et al., 2009). Understanding the mechanisms by which such negative regulators interfere with neuronal exocytosis will advance our comprehension of synaptic physiology during different stages of brain development as well as pathological processes and may open new avenues for diagnostics and treatments of disorders such as ASD.

## Exocytosis at the presynaptic boutons – a lipid view

In parallel with the discovery of the proteinaceous machinery, the lipid requirements for the SNARE-mediated

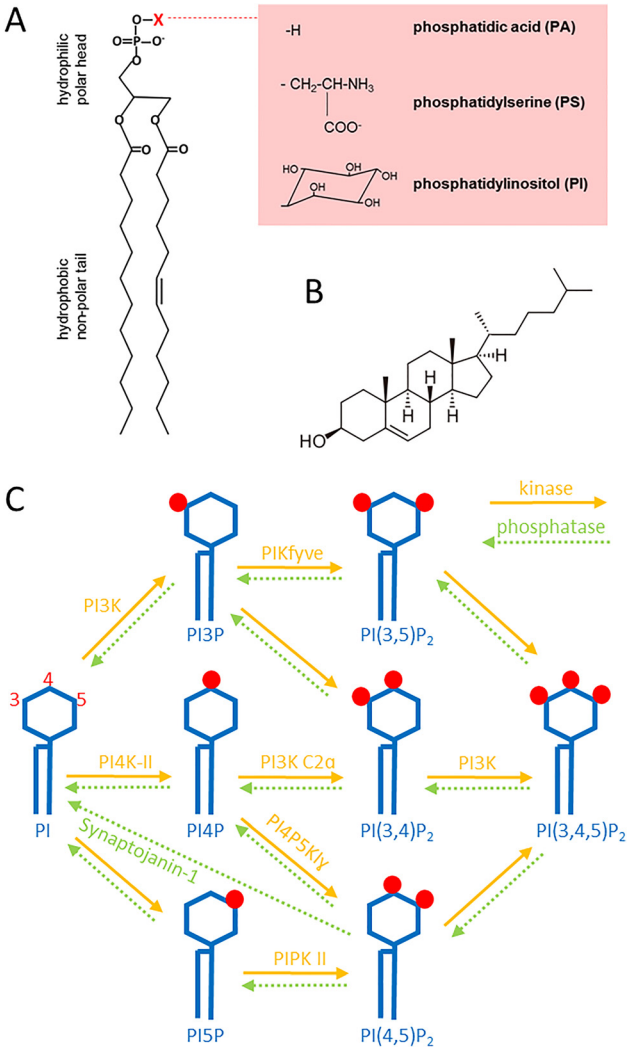


**Figure 1:** Structure and metabolism of key lipids involved in synaptic vesicle recycling. (A and B) Structure of lipids detailed in this study, i.e. glycerophospholipids (A) and cholesterol (B). (C) The phosphatidylinositide cycle, depicting the biosynthesis of phosphatidylinositides: phosphatidylinositol (PI) is the precursor of all phosphatidylinositides, the head groups of which have a different number of phosphate groups. The individual phosphatidylinositides are maintained at steady-state levels by continuous phosphorylation and dephosphorylation reactions performed by kinases and phosphatases.

exocytosis were emerging. Lipids are the core components of the fusing membranes; thus, changes in their composition, abundance or localization promptly modify the intrinsic fusogenic properties of membranes (Figure 1A and B). Lipids also activate and recruit proteins to the local environments where exocytosis happens (Chasserot-Golaz et al., 2010).

The discovery of Hokin and Hokin (1953) that the secretory cell stimulation leads to an increased production of phosphatidylinositides (PIs) has triggered the characterization of PI roles in exocytosis. The research is still ongoing, in part due to the PI versatility and rapid turnover (Milosevic and Sørensen, 2015). $PI(4,5)P_2$ is the main PI with a role in regulated exocytosis (Figure 1C) (Aikawa and Martin, 2003; Milosevic et al., 2005). Several-decade-long work on this topic resulted in a model in which PIs are delivered to the vesicle membrane via phosphatidylinositol transfer proteins (PITPs), phosphorylated to PI(4)P by vesicular protein phosphatidylinositol-4-kinase (PI4K-II), and then converted to $PI(4,5)P_2$ by phosphatidylinositol-4-phosphate-5-kinase Iγ (PI4P5KIγ) recruited from the cytoplasm (Figure 1C) (Hay et al., 1993; Martin, 2012; Milosevic and Sørensen, 2015). The production of $PI(4,5)P_2$ by PI4P5KIγ is tightly regulated by calcium, phosphorylation, a small GTPase Arf6 and phosphatidic acid (PA; Figure 1A), a product of phospholipase D activity (Aikawa and Martin, 2003; Jang et al., 2012; Martin, 2012). Further, an increase in the plasmalemmal $PI(4,5)P_2$ level led to a larger pool of primed vesicles and potentiated exocytosis, whereas reduction in $PI(4,5)P_2$ reduced exocytosis, demonstrating that the balance between the plasmalemmal $PI(4,5)P_2$ generation and degradation rates regulates vesicle priming (Milosevic et al., 2005). The mechanisms of how $PI(4,5)P_2$ drives the recruitment of secretory vesicles have been reported (Honigmann et al., 2013). Also, many exocytic proteins are known to interact with this phospholipid: $PI(4,5)P_2$ was also associated with vesicle docking and fusion based on these interactions. Namely, $PI(4,5)P_2$ binds to calcium-dependent activator protein for secretion (CAPS) and synaptotagmin-1 (Bai et al., 2004; Loyet et al., 1998), as well as Rab3 effector rabphilin 3 (Chung et al., 1998) and Mints (Okamoto and Sudhof, 1997). This dynamic interplay between $PI(4,5)P_2$ and exocytic proteins creates opportunities for therapeutic interventions to tackle exocytic defects.

Besides $PI(4,5)P_2$, its hydrolysis products and other PIs have a role in exocytosis and may function as diagnostic markers for selective brain disorders. Phospholipase C–driven hydrolysis of $PI(4,5)P_2$ results in diacylglycerol production needed for secretory vesicle priming through Munc13's activation and an opening of syntaxin-1 (Bauer et al., 2007). A FYVE finger-containing phosphoinositide kinase PIKfyve generates $PI(3,5)P_2$ from PI(3)P to inhibit exocytosis (Figure 1C) (Osborne et al., 2008), while PI(3)P itself seems to promote exocytosis (Meunier et al., 2005). Lastly, $PI(3,4,5)P_3$ also influences syntaxin-1 clustering and neurotransmission at the synapse (Khuong et al., 2013).

Cholesterol and phosphatidylserine (PS) are considered to play a role in spatial organization of exocytic sites

(Figure 1A and B) (Ammar et al., 2013). PS, mainly present in the inner plasma membrane leaflet, is needed for binding of synpatotagmin-1 and hence for triggering fusion (Ory et al., 2013). Depletion of cholesterol inhibits exocytosis and alters SNARE protein clusters (Lang et al., 2001). In addition to the aforementioned PI4P5KI$\gamma$ regulation, PA produced by phospholipase D1 under the secretory vesicle recruits syntaxin-1 and contributes to fusion (Jang et al., 2012; Zeniou-Meyer et al., 2007). Finally, fatty acids, like omega-3, omega-6 and arachidonic acid, seem to stimulate the SNARE complex formation (Darios et al., 2007), while arachidonic acid also supports docking (Garcia-Martinez et al., 2013).

Due to fast turnover, various lipid species have a potential to rapidly adapt synaptic functions. As the knowledge on the roles of lipids in brain pathologies is emerging (e.g. Hussain et al., 2019; Lauwers et al., 2016), the interplay between exocytosis and lipids will likely attract more attention in the future, in the context of neurological and neurodegenerative diseases. This is another example of how the mechanisms unveiled by 'basic science' are starting to be employed to explain, diagnose or treat pathologies.

## Endocytosis at the presynaptic bouton – a protein view

Almost five decades after a suggestion that the SVs are formed and recycled locally at the synapse (Heuser and Reese, 1973), the mechanisms of endocytosis are still debated (Watanabe and Boucrot 2017). Several mechanisms for SV endocytosis are likely at play: clathrin-mediated endocytosis, ultrafast endocytosis, bulk endocytosis in the case of high synaptic activity and, possibly, a brief and transient SV contact the plasma membrane through a fusion pore (called 'kiss-and-run') (Chanaday et al., 2019; Watanabe and Boucrot 2017). Even in the case of ultrafast and bulk endocytic forms, the subsequent clathrin-mediated endocytosis is considered to be key for the formation of homogeneously sized SVs with defined protein and lipid compositions (Watanabe and Boucrot 2017). The machinery involved in clathrin-mediated endocytosis is complex but well studied (Milosevic, 2018), often with a focus on clathrin and adaptor proteins (e.g. AP-2, AP180), as reviewed by Kononenko and Haucke in this issue, or dynamin protein family needed for vesicle scission (Ferguson and De Camilli, 2012). The endocytic process also depends on the action of the Bin/Amphiphysin/RVS (BAR) superfamily members involved in lipid bilayer deformation and reshaping (Milosevic, 2018). These proteins induce membrane curvature, stabilize curvature generated by other forces and/or recruit cytoplasmic proteins to membranes of a particular shape. Hallmarks of the BAR superfamily are endophilins-A, a family of evolutionarily conserved proteins for sensing and generating membrane curvature (Saheki and De Camilli, 2012). Endophilins-A recruit the phospholipid phosphatase synaptojanin-1 to the bud necks prior to fission by the GTPase dynamin (which also interacts with endophilin-A, but can act independently of it; Milosevic et al., 2011). Actions of endophilin and synaptojanin-1 are needed to promote vesicle uncoating by recruiting DnaJ protein auxilin, as detailed below (Milosevic et al., 2011).

Despite the fact that the coupling between endocytosis and exocytosis has been evolutionary perfected (Gundelfinger et al., 2003), two processes are studied mainly independently. However, it is essential that newly added SV proteins and lipids are rapidly removed from the fusion sites so that the new SV can dock and fuse there (Hosoi et al., 2009). Hence, exocytosis and endocytosis should be studied concomitantly whenever feasible.

## Endocytosis at the presynaptic bouton – a lipid view

Similar to their important role in exocytosis, PIs have an essential role in endocytosis. Many endocytic proteins have domains that recognize particular PIs, and the changes in PI levels profoundly affect many cellular activities, including endocytosis (Falkenburger et al., 2010). For example, adaptor proteins (e.g. epsin, AP-2, AP180, Hip1) are recruited in part by $PI(4,5)P_2$. Besides being needed for exocytosis, $PI(4,5)P_2$ also has a central role in endocytosis (Posor et al., 2015). Specifically, $PI(4,5)P_2$ participates at the several steps of the clathrin-mediated endocytosis: cargo sorting and coat recruitment (through adaptor proteins), endocytic pit fission (through dynamins) and vesicle uncoating (through endophilins and synaptojanin-1) (Cremona and De Camilli, 2001; Posor et al., 2015; Saheki and De Camilli, 2012). This dual role of $PI(4,5)P_2$ in exocytosis and endocytosis has been a basis for a model in which a PI cycle underlies the SV cycle (Figure 2; Cremona and De Camilli, 2001).

Another PI besides $PI(4,5)P_2$, phosphatidylinositol-3,4-bisphosphate [$PI(3,4)P_2$], formed by phosphatidylinositol-3-kinase C2$\alpha$, is employed in clathrin-mediated endocytosis (Figure 1C) (Posor et al., 2013). Timed generation of $PI(3,4)P_2$ is a base for an enrichment of sorting

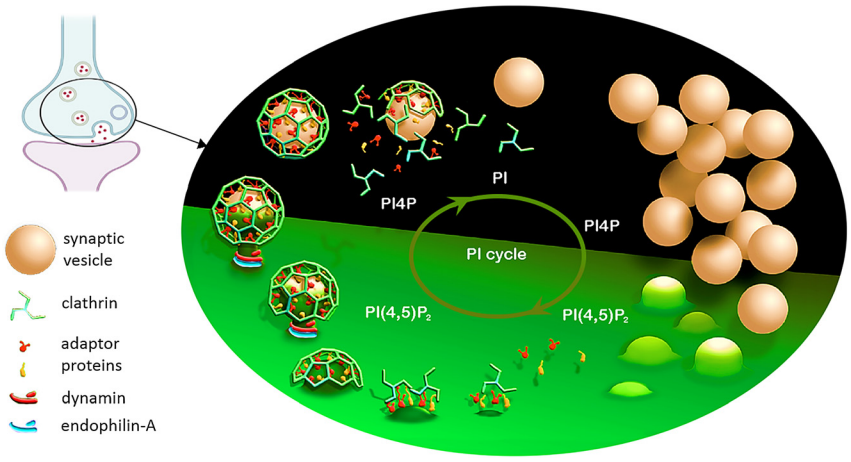


**Figure 2:** The model of a phosphoinositide cycle nested within the synaptic vesicle cycle. Links between membrane traffic at the synapse and PI(4,5)$P_2$ synthesis and dephosphorylation.

nexin 9 (SNX9), a BAR protein, and clathrin-coated pit progression during endocytosis (Posor et al., 2013, 2015). Lately, the interplay between endocytosis and lipids is raising attention in the context of neurological and neurodegenerative diseases (Hussain et al., 2019).

## Synaptic vesicle recycling and the aetiology of Parkinson's disease

For decades, defects in synaptic transmission have been implicated in neurodegeneration. Neurodegenerative diseases impose a significant burden on families and societies and will become of greater concern as life expectancy increases and populations around the world continue to age. Notably, ageing is the primary risk factor for most neurodegenerative diseases, including Parkinson's disease (PD) used here as an example. PD is a debilitating, progressive, age-related disorder affecting ~2% of the population over 65 years (this number is expected to double by 2030; Pilsl and Winklhofer, 2012). The pathology of PD is characterized by degeneration and death of dopaminergic neurons in the relevant brain region, i.e. the substantia nigra, and by formation of $\alpha$-synuclein and ubiquitin-positive Lewy body aggregates, causing neurological impairments (Poewe et al., 2017). Exactly what causes the degeneration and loss of these neurons is unknown. Emerging consensus points that at least some forms of PD are mediated by ubiquitin-proteasome pathway disruption and mitochondrial dysfunction (Youle and van der Bliek, 2012), yet these have not lent themselves to straightforward characterization of specific mechanisms thus far.

Curiously, several reports link endocytosis to PD and neurodegeneration (e.g. Cao et al., 2017; Edvardson et al., 2012; Krebs et al., 2013; Matta et al., 2012; Milosevic et al., 2011; Murdoch et al., 2016; Shi et al., 2009; Trempe et al., 2009). Several case studies described mutations in two clathrin uncoating factors, synaptojanin-1 and auxilin, as a cause of early-onset PD (Edvardson et al., 2012; Krebs et al., 2013). Auxilin is recruited to the clathrin-coated vesicles directly after action of synaptojanin-1, a phospholipid phosphatase with an identified PD-causing mutation (Figure 1C), which is found to impair clathrin uncoating and trigger dystrophic changes in dopaminergic axons (Cao et al., 2017). Synaptojanin-1 is brought to the neck of clathrin-coated pits by endophilin-A, an endocytic adaptor with membrane curvature–sensing and curvature-generating properties (Milosevic et al., 2011). Interestingly, endophilin-A itself is linked to PD and neurodegeneration: its levels are altered in the cortex of PD patients and associated with PD progression (Shi et al., 2009), and it directly interacts with two hallmark PD proteins, the E3 ubiquitin ligase Parkin (Trempe et al., 2009) and the leucine-rich repeat kinase LRRK2 (Matta et al., 2012), the most commonly disrupted gene in familial PD. A partial loss of endophilin in mice results in progressive neurodegeneration, ataxia and premature death (Milosevic et al., 2011). It also alters neurotransmission, SV recycling and SV acidification; elevates Parkin levels; induces the FoxO3a-Fbxo32 network in the brain and causes dysregulation of autophagy and the ubiquitin-proteasome system (Cao et al., 2014; Milosevic et al., 2011; Murdoch et al., 2016). Altogether, it is striking that several genes and proteins directly or indirectly related to endocytosis provide a mechanistic clarification to the pathology underlying PD that is expected to be further explored in the coming years.

One of the greatest challenges at present is to identify markers for neurodegenerative disease stages, which would allow PD and other neurodegenerative

disease–modifying therapies to be started earlier (Lauwers et al., 2016; Poewe et al., 2017). The presynaptic lipidaceous and proteinaceous components are appealing putative diagnostic and/or therapeutic targets since they allow for dynamic adjustments of neurotransmission, either by potentiation or inhibition. The goal in the latter case is not to completely inhibit but rather introduce subtle modifications of the system that counter disease mechanisms without interfering with the postsynaptic receptor signalling. Like this, the synaptic transmission accuracy could still be maintained at the postsynaptic side. SV cycle components may also present targets for drug screens and/or designs in a way that synaptic transmission is selectively altered in subtle ways that alleviate disorder symptoms but have limited side effects. Such approaches will likely intensify in the near future, building on the extensive knowledge already available.

In conclusion, our advanced understanding of SV cycle, synaptic transmission mechanisms and their emerging links to pathogenesis of several neurodegenerative and neurological disorders made it timely to build on the 'basic science' findings to explore new diagnostic markers and treatments. Reciprocally, studies on how pathological mutations modify presynaptic functions will also advance molecular characterizations of the SV cycle. Dr Schram's view that the support of 'basic science' will lead to the greater good is starting to pay off.

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## Bionote

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